New Horizons in Biotechnology

Edited by
S. Roussos, C. R. Soccol, A. Pandey and C. Augur

Kluwer Academic Publishers

NEW HORIZONS IN BIOTECHNOLOGY

New Horizons in Biotechnology

Edited by

S. Roussos

Laboratoire de Microbiologie IRD, IFR-BAIM,
Université de Provence, Marseille, France

C.R. Soccol

Laboratorio de Processos Biotecnologicos,
Departamento Engenharia Quimica,
Universidade Federal do Parana (UFPR),
Curitiba-PR, Brazil

A. Pandey

Regional Research Laboratories, CSIR,
Biotechnology Division, Trivandrum, India

and

C. Augur

Laboratoire de Microbiologie IRD, IFR-BAIM,
Université de Provence, Marseille, France

KLUWER ACADEMIC PUBLISHERS
DORDRECHT / BOSTON / LONDON

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN 1-4020-1718-9

Published by Kluwer Academic Publishers,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

Sold and distributed in North, Central and South America
by Kluwer Academic Publishers,
101 Philip Drive, Norwell, MA 02061, U.S.A.

In all other countries, sold and distributed
by Kluwer Academic Publishers,
P.O. Box 322, 3300 AH Dordrecht, The Netherlands.

Printed on acid-free paper

All Rights Reserved
© 2003 Kluwer Academic Publishers
No part of this work may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission from the Publisher, with the exception of any material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work.

Printed in the Netherlands.

CONTENTS

Organizing Institutions ix

Organizing Committee x

Foreword xi

Preface xiii

List of contributors xv

Section 1: Industrial Biotechnology

1 Industrial production of enzymes for the feed industry 1-16
K. Filer

2 Biochemical engineering of solid-state fermentation 17-25
F.J. Weber, J. Oostra, F.J.I. Nagel, M.A.I. Schutyser, M.J. Hoogschagen, Y.S.P. Rahardjo, Y. Zhu, R.M. Boom, J. Tramper, A. Rinzema

3 Phytase production under solid-state fermentation 27-34
A. Sabu, K.M. Nampoothiri, P. Latha, V. Kannan, G. Szakacs, A. Pandey

4 Valorization of moroccan olive cake using solid-state fermentation 35-41
M. Ismaili-Alaoui, M. Kamal, A. Kademi, A. Morin, S. Roussos, A. Houde

5 Growth kinetics and esterase/lipase production by thermophilic fungi grown in solid-state fermentation on olive cake 43-55
A. Kademi, A. Morin, M. Ismaili-Alaoui, S. Roussos, J. Baratti, A. Houde

6 Growth of *Trichoderma reesei* on static and dynamic solid substrate fermentation under electromagnetic fields 57-62
S.M. Más Diego, A. González Morlá, M. Campos Sofía, D. Cabeza Pullés

7 Production of enzyme and protein hydrolysate from fish processing waste 63-71
P. Prasertsan, S. Jitbunjerdkul, Trairatananukoon, T. Prachumratana

8 New potentialities of uses of coffee industry residues in Brazil 73-88
C.R. Soccol

9 Screening of edible mushrooms for polyphenol degradation and tannase production from coffee pulp and coffee husk 89-95
M. Sobal, D. Martínez-Carrera, B. Rio, S. Roussos

10 Biocatalysis in non-conventional media by entrapment of the enzyme in water-restricted microenvironment 97-112
D. Madamwar, K. Soni

11 Organic solvent resistant lipase produced by thermoresistant bacteria 113-122

S. Torres, G.R. Castro

12 Prebleaching of kraft pulps with xylanase enzyme for paper manufacture 123-132
V.S. Bisaria, S. Mishra, V. Sahai, J.K. Jain, R.M. Mathur

13 Titration biosensors for biotechnological process control 133-151
A. Rozzi, E. Ficara, E. Remigi

14 Enhanced loading and stability of urease covalently attached to polypyrrole microspheres for application to urea biosensor 153-161
A. Gambhir, A. Kumar, B.D. Malhotra, B. Miksa, S. Slomkowski

15 True and apparent yields and maintenance coefficient and their significance on fermentation kinetics 163-172
J.A. Rodríguez-León, G. Bueno, D.E. Rodríguez, G. Delgado, P. Serrano, M.A. Brizuela

16 Bioprocessing engineering considerations in the production of secondary metabolites by plant cell suspension cultures 173-191
A.K. Srivastava

17 Genetic manipulation of filamentous fungi 193-198
A.C. Banerjee, A. Kundu, S.K. Ghosh

18 Molecular biology and biochemistry of lipoxygenases and related pathways 199-214
K. Yokota, S. Lu, I. Takata, A. Kishimoto, K. Maeta, K. Nishimura, T. Nagaya, M. Jisaka

Section 2: Environmental Biotechnology

19 Bioremediation and biobeneficiation of metals 215-220
A.D. Agate

20 Developments in enhanced biological phosphorus removal (EBPR) 221-230
R. Bhamidimarri, P.O. Bickers, N. Thayalakumaran, Z.B. Hu

21 Role of membrane bioreactors in environmental engineering applications 231-239
C. Visvanathan, D. Pokhrel

22 Application of bio-hydrolizer for the treatment of urea bearing wastewater 241-250
P.P. Pathe, T. Nandy, S.N. Kaul, C.V. Deshpande, L. Szpyrkowicz

23 Biosorption and elution of Cr(VI): A detoxification strategy 251-265
T. Srinath, S.K. Garg, P.W. Ramteke

24 Detoxification of pollutants from municipal wastewater using organic soil as a biofilter 267-270
Z. Stepniewska, U. Kotowska

25	Biochemical removal of nitrogen from industrial effluents: A full scale plant performance and stability L. Szpyrkowicz, S.N. Kaul	271-284
26	Kinetics of dissolution of the carbonate minerals of phosphate ores by dilute acetic acid solutions in the presence of carbonic anhydrase II from bovine erythrocytes E.M. Papamichael, E.D. Economou, T.C. Vaimakis	285-295

Section 3: Food Biotechnology & Health Care

27	New trends in kefir yeast technology A.A. Koutinas	297-309
28	Formation of kefir granules and their efficiency in whey fermentation I. Athanasiadis, C. Psarianos, A. Bekatorou, M. Iconomopoulou, C. Lindner, T. Paschalis, S. Voliotis	311-319
29	Isolation and identification of lactic acid bacteria from mature coffee cherries: potential application in coffee husk ensiling M.G.B. Pagnoncelli, D. Brand, S. Roussos, I. Gaime-Perraud, C. Augur, C.R. Soccol	321-333
30	Lactic acid fermented products as vehicles for probiotics J.L. Parada, M.E. Sambucetti, A. Zuleta, M.E. Rio	335-351
31	Development of a new beverage with probiotic activity M.C.R. Santos, C.R. Soccol, N. Waszczynskyj, M. Raimbault	353-364
32	Genetic engineering of fatty acid composition of Indian mustard oil S.K. Ghosh, A. Bhattacharjee, M.K. Maiti, A. Basu, D. Ghosh, S. Ghosh, S.K. Sen	365-370
33	*Pleurotus ostreatus* volatile aroma compounds identified from fruit-body and from mycelium grown in submerged and solid-state cultures W. Kabbaj, S. Breheret, J. Guimberteau, T. Talou, J.M. Olivier, M. Bensoussan, S. Roussos	371-377
34	Standardization of ELISA (Enzyme Linked Immunosorbent Assay) and indirect fluorescent antibody test (IFAT) techniques for canine cutaneous Leishmaniasis E.A. Castro, V. Thomaz-Soccol, C. Augur	379-385
35	Leishmania species in two regions of Parana, Brazil: Biochemical characterization by isoenzyme electrophoresis V. Thomaz-Soccol, E.A. Castro, E. Luz, J. Dereure, F. Pratlong, N. Membrive, J.P. Dedet	387-395

Section 4: Agricultural Biotechnology

36	Inoculants for silage in subtropical climate	397-402

Z.G. Weinberg

37 Production of a biocompost by solid-state fermentation against coffee nematodes 403-412
D. Brand, S. Roussos, F.A. Prochmann, J. Pohl, C.R. Soccol

38 Cadmium and nicotine correlation in reference to tobacco plant varieties and to sludge/soil applied ratios 413-422
D. Douma, I. Papagiannis, S. Divanidis, S. Halivopoulos, G. Manos, V. Kalfakakou

39 Applicability of fly ash is sound for rhizosphere, biomass yield, enhanced banana productivity and sustainable eco-system 423-436
N.V. Phirke, S.B. Chincholkar, R.M. Kothari

40 Impacts on vesicular-arbuscular mycorrhizal fungi diversity associated with degradation of soils in humid tropical forests 437-444
S.C. Tiwari, S.S. Singh

Authors index 445-446

Subject Index 447-449

Organizing Institutions

Regional Research Laboratory (CSIR)
Thiruvananthapuram-695 019
India
http://www.csir.res.in

Institut de Recherche pour le Développement
75480 Paris cedex 10
France
http://www.ird.fr

University of Ioannina
45110 Ioannina
Greece
http://www.uoi.gr

University of Venice
30 123 Venice
Italy
http://www.unive.it

Universidade Federal do Parana
81531-970 Curitiba-PR
Brazil
http://www.ufpr.br

Organizing Committee

Chairman : Vijay Nair, RRL, Trivandrum
Convener : Ashok Pandey, RRL, Trivandrum
Co-ordinators : H. Veeramani, Indian Institute of Technology, Mumbai, India ; S. Roussos, Institut de Recherche pour le Développement, Marseille, France ; E.M. Papamichael, University of Ioannina, Ioannina, Greece ; L. Szpyrkowicz, University of Venice, Venice, Italy ; C.R. Soccol, Universidade Federal do Parana, Curitiba, Brazil

International Organizing Committee

C. Augur, IRD, France
M.R. Das, RGCB, Trivandrum, India
E.J. DaSilva, UNESCO, France
A. Ghosh, IMT, Chandigarh, India
C.M. Gupta, CDRI, Lucknow, India
P. Nigam, Univ. Ulster, UK
E.M. Papamichael, Univ. Ioannina, Greece
S. Roussos, IRD, France
I. Russell, Labatt Lab., Canada
K.P. Sadasivan, RRL, Trivandrum, India
R.N. Singh, NEERI, Nagpur, India
J. Scriven, GBF, Germany
C.R. Soccol, Univ. Fed. Parana, Brazil
G. Szakacs, TU, Budapest, Hungary
H. Veeramani, IIT, Mumbai, India
K. Yokota, Shimane Univ., Japan

International Scientific Committee

R. Banerjee, IIT, Kharagpur, India
V.S. Bisaria, IIT, Delhi, India
R. Boopathy, BU, Coimbatore, India
S. Furusaki, Kyushu Univ., Japan
P. Gunasekaran, MKU, Madurai, India
Y. Hadar, Hebrew Univ. Jerusalem, Israel
M. Labat, IRD, France
D. Marques, CONICET, Argentina
J.M. Meyer, Univ. Louis Pasteur, France
V.T. Soccol, Univ. Fed Parana, Brazil
L. Szpyrkowicz, Univ. Venice, Italy
I.K.P. Tan, Univ. Malaya, Malaysia
V. Thankamoni, Univ. Kerala, Trivandrum, India
K.P. Tiwari, Univ. Allahabad, India
A.K. Tripathi, BHU, Varanasi, India
D.R. Walt, Tufts Univ., USA
C. Webb, UMIST, UK

Foreword

The practice of biotechnology, though different in style, scale and substance in globalizing science for development involves all countries. Investment in biotechnology in the industrialised, the developing, and the least developed countries, is now amongst the widely accepted avenues being used for economic development. The simple utilization of kefir technology, the detoxification of injurious chemical pesticides e.g. parathion, the genetic tailoring of new crops, and the production of a first of a kind of biopharmaceuticals illustrate the global scope and content of biotechnology research endeavour and effort. In the developing and least developed nations, and in which the 9 most populous countries are encountered, problems concerning management of the environment, food security, conservation of human health resources and capacity-building are important factors that influence the path to sustainable development.

Long-term use of biotechnology in the agricultural, food, energy and health sectors is expected to yield a windfall of economic, environmental and social benefits. Already the prototypes of new medicines and of prescription fruit vaccines are available. Gene-based agriculture and medicine is increasingly being adopted and accepted. Emerging trends and practices are reflected in the designing of more efficient bioprocesses, and in new research in enzyme and fermentation technology, in the bioconversion of agro-industrial residues into bio-utility products, in animal healthcare, and in the bioremediation and medical biotechnologies. Indeed, with each new day, new horizons in biotechnology beckon.

Some of these advances have been captured in this volume that is testimony to the presentations made in the international conference on *New Horizons in Biotechnology* that was held at Trivandrum, India, April 18-21, 2001. The research findings shared between over 350 researchers, academicians, biotechnologists, industrialists, policy planners from over 20 countries augurs well discussed for increased and meaningful collaboration in future. The geographic origin of the contributors and editors is testimony again to the availability of opportunities for co-operation in the field of biotechnology; and in deploying it as the motor of development for economic and sustainable development. In that context, this volume in stimulating further research horizons could also serve as a reference source for researchers dealing with agricultural and environmental biotechnology, food and healthcare; and industrial biotechnology.

Finally, the prestige and the wealth of organizing institutions and sponsorship reflected in National and international agencies and institutions such as: the Regional Research Laboratory, Council of Scientific and Industrial Research, Trivandrum, the Institute of Research for Development, France, the University of Ioannina, Greece, the University of Venice, Italy, Federal University of Parana, Brazil; and the Department of Biotechnology, New Delhi, Govt. of India, Department of Science and Technology,

New Delhi, Council of Scientific and Industrial Research, New Delhi, UNESCO, and the Third World Academy of Science, amongst others, is indicative of the need of such stock-taking conferences in charting future avenues of research endeavour and international co-operation in biotechnology with one eye on current developments in fermentation and bioprocess technologies, and with the other eye on dynamically new related fields such as bioinformatics, bioceramics, and biometrics.

Edgar J. DaSilva

Preface

Biotechnology, one of the three defining technologies, has made significant advances in recent years and emerged as a frontline area of research and development, with an overwhelming impact on the society. The developments are very fast globally and new dimensions are being added every day. With the constant increase in the world population and simultaneous decrease in the availability of fresh water and agriculture land per capita, the human race looks with great hopes at Biotechnology to meet its two most important and basic needs of life- health care and production and protection of crops. Revolutionizing developments in information technology in recent years have made the concept of "Global Village" as true. With these views in background, it was thought worth and need of time to organize an international meeting of experts to discuss global developments in these areas so as to create a network of researchers for share and exchange of knowledge and ideas. This led to the organization of the International Conference on New Horizons in Biotechnology [NHBT-2001], which was held at Trivandrum (India) during April 18-21, 2001.

NHBT-2001 was organized jointly by the Regional Research Laboratory (CSIR), Trivandrum, India, Institut de Recherche pour le Développement (IRD), Marseille, France, University of Ioannina, Ioannina, Greece and University of Venice, Venice, Italy and was hosted by the Regional Research Laboratory, CSIR, Trivandrum. This brought together a multinational body of scientist, engineers and experts to deliberate on global developments in the fields of industrial, food and environmental biotechnology and health care products. The scientific programme consisted of 90 invited speakers for the plenary and technical sessions, besides 250 contributory papers for oral and poster presentations. The technical papers focused on a variety of topics such as bioprocesses, fermentation, enzyme technology, biopesticides, bioremediation, tissue culture, antibiotics, vaccines, animal health care, waste management, pollution control etc.

The present book is the outcome of the proceedings of the NHBT-2001. It comprises 40 chapters divided into four sections, industrial biotechnology, environmental biotechnology, food and health care and agricultural biotechnology. First section on Industrial Biotechnology consists of eighteen chapters dealing with topics on solid-state fermentation, enzymes kinetics, production and applications, biosensors, biotechnological potential of agro-industrial residues, plant cell culture in bioreactors, genetics of fungi and molecular biology, etc. Second section is on Environmental Biotechnology containing eight chapters, which present and discuss most important developments on the topics such as treatment of municipal and industrial effluents, biological removal of phosphorus and nitrogen from wastewaters, bioremediation and biobeneficiation of metals, use of various kinds of bioreactors for the wastewaters, etc. The third section, which is on Food Biotechnology and Health Care also comprises nine chapters. The topics under Food Biotechnology include kefir yeast, lactic acid bacteria, probiotics, fatty acids

in edible oil, and food grade aroma compounds from mushrooms. Two chapters under this section discuss issues related with Leishmaniasis. Fourth and the last section of the book contains five chapters on Agricultural biotechnology and describes topics such as silage, biocomposting, tobacco plants, banana cultivation and vesicular-arbuscular mycorrhizal (VAM) fungi diversity. All these chapters have been presented in a dignified manner, providing state-of-art information on the topics and have been written by the internationally known experts.

NHBT-2001 was participated and supported by Alfa Laval India Limited, Pune, India, Asiatech Publishers Inc., New Delhi, India, Biocon India Private Limited, Bangalore, India, Biofin, Italy, Borosil Glass Works, Chennai, India, Council of Scientific and Industrial Research, New Delhi, India, Department of Biotechnology, Govt. of India, New Delhi, India, Durga Jewellery, Hyderabad, India, East India Pharmaceutical Works, Calcutta, India, Escotel Mobile Communications, Trivandrum, India, Industrial Development Bank of India, Mumbai, India, Institut de Recherche pour le Developpement, Marseille, France, John Wiley & Sons, UK, National Institute of Science Communication, New Delhi, India, Nature, India, PL Worldways, Trivandrum, India, Regional Research Laboratory, CSIR, Trivandrum, India, Scigenics, Chennai, India, Springer-Verlag, New Delhi, India, State Bank of Travancore, Trivandrum, India, Third World Academy of Sciences, Italy, United Nation's Educational, Scientific and Cultural Organization, France, Universidade Federal do Parana, Curitiba, Brazil, University of Ioannina, Ioannina, Greece, University of Venice, Department of Environmental Science, Venice, Italy. On behalf of the Organizers of the event, we gratefully acknowledge the support extended by these agencies and institutions. We would like to take this opportunity to thank international co-ordinators of NHBT-2001, chairpersons of various sessions, judges of poster award committee, members of international organizing committee and international scientific committee for their help, support and co-operation. A special mention needs to be made in this regard for Dr EM Papamichael, Dr L Szpyrkowicz and Professor H Veeramani. In addition, we wish to acknowledge Dr Isabelle Gaime-Perraud from IRD for her valuable technical assistance and expertise. The book would not have found its final form without her determination and dedication.

We hope that readers of his book will find it useful.

July 2002 The Editors

List of contributors

AGATE A.D.
United Western Society
7, Narmada Apts. Navsahyadri P.O.
Pune-411 052, India
Email :

AUGUR Christopher
IRD, Equipe de Mycologie et de Fermentation en Milieu Solide, UR 119, IFR-BAIM
Universités de Provence et Méditerranée
Case 925, 163 Avenue de Luminy
F-13288 Marseille Cedex 9 - France
Email : caugur@esil.univ-mrs.fr

BANERJEE Ambika C.
East India Pharmaceutical Works Limited
Biotechnology and Molecular Biology Division
119, Biren Roy Road West, Sarsuna
Calcutta-700 061, India.
Email: ambika2@vsnl.com

BENSOUSSAN Maurice
ENSBANA Université de Bourgogne
Laboratoire de Microbiologie
UMR INRA 1082
F-21000 Dijon, France
Email : bensouss@u-bourgogne.fr

BHAMIDIMARRI Rao
Massey University
Department of Process and Environmental Technology
Palmerston North, New Zealand
Email : B.Rao@massey.ac.nz

BISARIA V.S.
Indian Institute of Technology–Delhi
Department of Biochemical Engineering and Biotechnology
New Delhi-110 016, India
Email: vbisaria@dbeb.iitd.erent.in

BRAND Débora
UFPR Universidade Federal do Parana
Laboratorio de Processos Biotecnologicos
Departamento Engenharia Quimica
81531-970 Curitiba-PR, Brazil.
Email: dbrand@uol.com.br

CASTRO Edilene A.
UFPR Universidade Federal do Paraná
Departamento de Patologia Básica
Laboratório de Parasitologia Molecular
81531-970 Curitiba-PR, Brasil.
Email: edilene@bio.ufpr.br

CASTRO Guillermo R.
PROIMI Biotechnology
Avenida Belgrano y Pasaje Caseros
Tucuman 4000, Argentina
Email: grcastro@proimi.edu.ar

DOUMA D.
Technological Educational Institute of Epirus
Plant Production Department
Arta, Greece
Email : ddouma@teiep.gr

FILER Keith A.
Alltech Inc.,
3031 Catnip Hill Pike
Lexington, KY 40515, USA
Email: kfiler@alltech-bio.com

GAMBHIR Anamika
BECPRG Biomolecular Electronics and Conducting Polymer Research Group
National Physical Laboratory
K.S. Krishnan Marg
New Delhi-110 012, India
E-mail: agulati@csnpl.ren.nic.in

HOUDE Alain
Agriculture and Agri-Food Canada
Food Research and Development Centre
3600 Casavant Blvd West
St-Hyacinthe, Quebec, J2S 8E3, Canada
Email : houdea@agr.gc.ca

ISMAILI-ALAOUI Mustapha
Institut Agronomique et Vétérinaire Hassan II
Laboratoire de Bioconversions

B.P. 6202 Rabat Instituts Maroc
Rabat, Morocco
Email : m.ismaili@iav.ac.ma

KADEMI Ali
Agriculture and Agri-Food Canada
Food Research and Development Centre
3600 Casavant Blvd West
St-Hyacinthe, Quebec, J2S 8E3, Canada
Email : kademia@agr.gc.ca

KAUL S.N.
National Environmental Engineering Research Institute
Nehru Marg
Nagpur-440 020, India
Email: root@csneeri.ren.nic.in

KOTHARI R.M.
North Maharashtra University
School of Life Sciences
Jalgaon-425 001, India.
Email: rmkothari@rediffmail.com

KOUTINAS A.A.
University of Patras
Food Biotechnology Group
Department of Chemistry
Patras, Greece
Email: A.A.Koutinas@upatras.gr

MADAMWAR Datta
Sardar Patel University
Department of Biosciences
Vallabh Vidyanagar-388 120, India
Email : vish_dm@yahoo.com

MAS-DIEGO Siannah Maria
CNEA National Center of Applied Electromagnetism
Universidad de Oriente, A.P. 4078
90400 Santiago de Cuba 4, Cuba
Email : siannah@cnea.uo.edu.cu

PAGNONCELLI M.G.B
UFPR, Universidade Federal do Parana
Laboratório de Processos Biotecnológicos
Departamento de Engenharia Química
81531-970 Curitiba-PR, Brasil
Email : giobinder@hotmail.com

PANDEY Ashok
CSIR, Regional Research Laboratory
Biotechnology Division
Industrial estate PO
Trivandrum-695 019, India
Email : ashokpandey56@yahoo.co.in

PAPAMICHAEL Emmanuel M.
University of Ioannina
Department of Chemistry
P.O. Box 1186
45110 Ioannina, Greece
Email: epapamic@cc.uoi.gr

PARADA Jose Luis
Universidade de Buenos Aires
Facultad de Ciencias Exactas y Naturales
Laboratorio de Microbiologia de Alimentos
Ciudad de Buenos Aires 1428, Argentina
Email : jlparada@qo.fcen.uba.ar

PRASERTSAN P.
Department of Industrial Biotechnology
Department of Food Technology
Faculty of Agro-Industry
Prince of Songkla University
Hat Yai 90110, Thailand
Email : ppoonsuk@ratree.psu.ac.th

PSARIANOS Costas
University of Patras
Food Biotechnology Group
Department of Chemistry
26500 Patras, Greece,
E-mail: cps@upatras.gr

RAMTEKE P.W.
Allahabad Agricultural Deemed University
Department of Chemistry
Allahabad-211 007, India
Email : pwramteke@123india.com

RODRIGUEZ-LEON Jose A.
ICIDCA, Instituto Cubano de Investigaciones de Derivados de la Caña de Azúcar
Vía Blanca 804 y Carretera Central
P.O. Box 4026
La Habana, Cuba
E-mail: rodleon@icidca.edu.cu

ROUSSOS Sevastianos
IRD, Equipe de Mycologie et de Fermentation en Milieu Solide, UR 119, IFR-BAIM
Universités de Provence et de la Méditerranée
Case 925, 163 Avenue de Luminy
F-13288 Marseille Cedex 9, France
Email : roussos@esil.univ-mrs.fr

ROZZI Alberto
DIIAR-Sez. Amb. Politecnico di Milano
Department of Hydraulic Environmental Engineering and Survey
Piazza L. da Vinci 32
20133 Milan, Italy
Email : rozzi@amb1.amb.polimi.it.

SABU Abdulhameed
Biotechnology Division
Regional Research Laboratory (CSIR)
Trivandrum-695 019, India
Email : sabuahameed@yahoo.com

SANTOS Maria Carolina R.
UFPR, Universidade Federal do Parana
Laboratório de Processos Biotecnológicos
Departamento de Engenharia Química.
81531-990 Curitiba-PR, Brazil
Email: mariacarolinars@yahoo.com.br

SEN S.K.
IIT-BREF Biotek
Indian Institute of Technology
Kharagpur-721 302, India.
Email : soumitra@hijli.ittkgp.ernet.in

SOCCOL Carlos R.
UFPR, Universidade Federal do Parana
Laboratorio de Processos Biotecnologicos
Departamento Engenharia Quimica
81531-970 Curitiba-PR, Brazil.
Email : soccol@ufpr.br

SOBAL Mercedes
Mushroom Biotechnology
Colegio de Postgraduados,
A.P. 701
Puebla 72001, Mexico
Email : msobal@colpos.mx

SRIVASTAVA Ashok K.
Indian Institute of Technology Delhi
Department of Biochemical Engineering and Biotechnology
New Delhi-110 016, India
Email : ashokiitd@hotmail.com

STEPNIEWSKA Z.
Catholic University of Lublin Department of Environmental Biochemistry and Chemistry
Poland
E-mail: stepz@demeter.ipan.lublin.pl

SZPYRKOWICZ Lidia
University of Venice
Department of Environmental Sciences
Dorsoduro 2137
30123 Venice, Italy
Email : lidia@unive.it

THOMAZ-SOCCOL Vanette
UFPR Universidade Federal do Paraná
Departamento de Patologia Básica.
Laboratório de Parasitologia Molecular
81531-990 Curitiba-PR - Brasil.
Email: vasoccol@bio.ufpr.br

TIWARI S.C.
NERIST, North Eastern Regional Institute of Science and Technology
Department of Forestry
Nirjuli-791 109, India
Email : sct@nerist.ernet.in

VISVANATHAN C.
Asian Institute of Technology
Urban Environmental Engineering and Management Program
Po Box 4, Klong Luang
Pathumthani-12120, Thailand
Email: visu@ait.ac.th

WEBER Frans J.
Wageningen Centre for Food Sciences
P.O. Box 557
6700 AN Wageningen, Netherlands
E-mail: frans.weber@algemeen.pk.wau.nl

WEINBERG Zwi G.
The Volcani Center
Agricultural Research Organization
Forage Preservation and By-Products Research Unit

Department of Stored Products
50250 Bet Dagan, Israel.
Email: zgw@volcani.agri.gov.il

YOKOTA Kazushige
Shimane University
Department of Life Science and
Biotechnology
1060 Nishikawatsu-cho, Matsue
Shimane 690-8504, Japan
Email : yokotaka@life.shimane-u.ac.jp

Chapter 1

INDUSTRIAL PRODUCTION OF ENZYMES FOR THE FEED INDUSTRY

K. FILER

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Abstract

Enzymes are used commercially in the animal feed industry to improve animal performance and the fuel ethanol industry for optimization of ethanol production. Most of the enzymes are produced from microbial sources using submerged liquid fermentation (SLF) technologies. Enzymes produced using solid state fermentation technology have advantages in the animal feed and fuel ethanol industries. The medium used consists of an unrefined agricultural product that can be dried ground and feed to the animal. The nature of the substrate also results in production of a number of additional side activities that provide improved performance for enzyme preparation produced using SSF technologies. Another positive aspect of SSF has been the production of organisms. The microorganisms used tend to be non-genetically modified fungal organisms. These benefits have resulted in the development of SSF facilities for the industrial production of enzymes. The commercial production of enzymes using SSF technologies will be discussed.

Introduction: industrial enzyme applications

Virtually all chemical reactions in biological systems are catalysed by macromolecules called enzymes. A catalyst is a substance that increases the rate of a chemical reaction without undergoing a permanent chemical change; and enzymes exhibit enormous catalytic power. Chemical reactions *in vivo* rarely proceed at perceptible rates in the absence of enzymes while reaction rates increase as much as a million times when enzymes are present. Nearly all known enzymes are proteins; and they are among the most remarkable biomolecules known.

The name 'enzyme', meaning 'in yeast', was not used until 1877; however much earlier it was suspected that biological catalysts were involved in the fermentation of sugar to form alcohol. Previously these catalysts were termed ferments (**1**). Eduard Buchner extracted the enzymes catalyzing alcoholic fermentation in 1897. This demonstrated that enzymes could function independently of cell structure. Previously (in 1860) Louis Pasteur had postulated that enzymes are linked with the structure of the yeast cell. The ability of enzymes to function outside of a cell has greatly increased their use in a large variety of commercial products and reactions.

A wide range of industries use commercial enzymes. The world annual sales of industrial enzymes was recently valued at $1 billion (**2**). Three quarters of the

New Horizons in Biotechnology, 1-16, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

market is for enzymes involved in the hydrolysis of natural polymers, of which about two-thirds are proteolytic enzymes used in the detergent, dairy and leather industries; and one third are carbohydrases used in the animal feed, baking, brewing, distilling, starch and textile industries. Detergent manufacturers use 45% of all industrial enzymes produced in spot remover and detergent products containing proteases and lipases. This industry is expected to have a 10% annual growth rate for the next five years. Food processing enzymes including α-amylases, glucose isomerase and pectinases account for about 45% of enzyme usage. The starch processing industry uses half of the enzymes in the food industry, approximately 25% are used by the dairy industry and 10% by the brewers, fruit juice and wine producers. The textile and paper industry (6%) uses primarily amylases and hemicellulases and the leather industry (2%) uses proteases. Enzyme supplements for animal feeds account for about 1% (**3**). Table 1 lists some industrially important enzymes and their application.

The acceptance of enzymes by the animal feed industry has become widespread in the last decade. As the understanding of enzymes and their properties has grown, so have both their use and their effectiveness as feed supplements. The purpose of using enzymes in monogastric animals is to improve availability of nutrients in feedstuffs. The result is improved feed utilization and a reduced impact of anti-nutritional components.

Methods for production of commercial enzymes

Industrial enzymes are produced by plants, animals, and microbes. By far the largest group exploited for the use of industrial enzymes has been the microbial population. Short generation times and high yields, together with the fact that microorganisms produce extracellular enzymes, which are easy to harvest, make microbes the enzyme source of choice. Production of enzymes by microorganisms has also expanded because of the vast amounts of genetic information now available. Several industrially important microbial genomes have been sequenced; and the understanding of gene expression systems in microorganisms is much more advanced when compared to other gene expression systems. This knowledge has made it possible to select a variety of microbial organisms suitable for enzyme production with traditional submerged liquid fermentation (**5**). An alternative fermentation method for enzyme production is solid state fermentation (**6**).

Table 1. Commercial applications of enzymes.*

Industry	Application	Enzyme	Source
Baking and milling	Reduction of dough viscosity, acceleration of fermentation, increase in loaf volume,	Amylase	Fungal
	Improvement of dough texture, reduction in mixing time	Protease	Fungal/ bacterial
Brewing	Mashing	Amylase	Fungal/ bacterial

	Chillproofing	Protease	Fungal/ bacterial
	Improvement of fine filtration	ß-glucanase	Fungal/ bacterial
Coffee	Coffee bean fermentation	Pectinase	Fungal
	Preparation of coffee concentrates	Pectinase, hemicellulase	Fungal
Confectionary	Manufacture of coffee concentrates	Invertase, Pectinase	Fungal/ bacterial
Corn Syrup	Manufacture of low dextrose equivalent syrups	Amylase	Bacterial
	Production of glucose from corn syrup	Amyloglucosidase	Fungal
Feed	Reduce antinutritional factors	Xylanase, β-glucans, phytase	Fungal
	Improve feed utilization	Amylase, cellulase, protease	Fungal/ bacterial
Laundry	Detergents	Protease, lipase	Bacterial
Soft drinks	Stabilization	Glucose oxidase, catalase	Fungal

*Modified from Stanbury *et al.*, 1995 (**4**).

Submerged liquid fermentation

Submerged liquid fermentations are traditionally used in the United States for the production of microbially derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth. A research team led by Chaim Weizmann in Great Britain developed a process for production of acetone by submerged liquid fermentation using *Clostridium acetobutylicum,* which eventually led to the first large-scale aseptic fermentation vessel (**4**). The first large-scale aerobic fermenters were used in central Europe in the 1930s for production of compressed yeast (**7**). In 1943, the British government decided that solid substrate fermentation was inadequate for the production of penicillin. This decision forced the development of liquid fermenters that are aseptic and contain adequate aeration and agitation. Construction of the first large-scale plant to produce penicillin by liquid fermentation began in 1943 (**8**).

Organisms used in submerged fermentations

Fermentation using Bacillus species accounts for about half of the world's production of industrial enzymes. The main classes of Bacillus enzymes and the strains used to produce them are listed in Table 2.

Table 2. Industrial enzymes produced by *Bacillus* species.*

Enzyme	Producer strains
α-amylase	*B. amyloliquefaciens, B. circulans, B. licheniformis,*
ß-amylase	*B. polymyxa, B. cerus, B. megaterium*
Alkaline phosphatase	*B. licheniformis*
Cyclodextran glucotransferase	*B. macerans, B. megaterium*
ß-galactosidase	*B. stearothermophilus*

Metalloprotease	*B. lentus, B. polymyxa, B. subtilis*
Serine protease	*B.amyloliquefaciens, B.amylosaccharicus, B. subtilis*
Urease	*Bacillus* sp.
Uricase	*Bacillus* sp.

Bron *et al.*, 1999 (**2**).

Two enzymes dominate the industrial market: alkaline protease and α-amylase. Alkaline protease used in detergents is the single largest enzyme market. Although Bacillus species are the primary enzyme-producing organisms, other microbes are also used. Through genetic modifications the bacteria *Escherichia coli* is able to produce insulin and human growth hormones. *Penicillum chrysogenum* is used to produce penicillin. Other microorganisms used on an industrial scale include *Saccharomyces cerevisiae* for ethanol production and the fungi *Aspergillus* and *Trichoderma* for carbohydrase production.

Selection of microorganisms for the fermentation industries in the past involved a hit-and-miss screening approach. With the development of genetic engineering techniques, organisms can be engineered to produce high yields of a great variety of products. Most of the information has been developed in bacterial systems and has resulted in very efficient systems for enzyme production. Genetic manipulation of organisms can increase yield 100 fold or more over wild type strains (**4**). The genetic manipulation of genomes is common for organisms used in submerged liquid fermentations.

Fermenter design

The main function of a fermenter is to provide a controlled environment for growth of microorganisms in order to obtain a desired product. Two important criteria for a submerged liquid fermenter include the ability to operate aseptically for a number of days and provide adequate aeration and agitation to meet the metabolic requirements of the microorganism. Many different types of fermenters have been described in the literature, but very few proved satisfactory for industrial aerobic fermentations. The most common designs are based on a stirred upright cylinder with sparger aeration (**4**). Fermenter sizes can range from flasks used in the laboratory to production fermenters of 8,000 liters or more.

Fermenter operation

The success of fermentation depends upon the existence of defined environmental conditions for biomass and product formation. The temperature, pH, degree of agitation, oxygen concentration in the medium and other factors may have to be kept constant during the process. Carefully monitoring of the fermentation is performed to regulate these parameters. Table 3 lists the variety of process sensors included in a submerged liquid fermentation.

Many biochemical processes involve batch growth of cell populations. After seeding a liquid medium with an inoculum of living cells, only gas is added or removed from the culture as growth proceeds. Typically in such a batch reactor, the concentrations of nutrients, cells and products vary with time as growth proceeds.

In addition, it is often desirable to add liquid streams to a batch bioreactor as the reaction process occurs. This can be done to add precursors for desired products, to add regulating compounds such as inducers at a point in the batch operation, to maintain low nutrient levels to minimize catabolite repression or to extend the stationary phase by nutrient addition (**5**). When the fermenter is used in this manner it is known as a 'fed-batch' fermentation.

Table 3. Process sensors and their possible control functions.*

Category	Sensor	Possible control function
Physical	Temperature	Heat/cool
	Pressure, Rpm	
	Agitator shaft power	
	Foam	Foam control
	Weight	Change flow rate
	Flow rate	Change flow rate
Chemical	pH	Acid or alkali addition, carbon source feed rate
	Redox	Additives to change redox potential
	Oxygen	Change feed rate
	Exit-gas analysis	Change feed rate
	Medium analysis	Change in medium composition

*Stanbury *et al.*, 1995 (**4**).

Solid substrate fermentation

In addition to submerged liquid fermentation, an ancient fermentation technology known as solid-substrate fermentation is also used to produce enzymes. Solid-substrate fermentations are generally characterized by growth of microorganisms on water-insoluble substrates in the presence of varying amounts of free water (**6**). This process will also be referred to as solid state fermentation (SSF). Table 4 shows differences between the SSF and submerged liquid fermentation.

The origin of SSF can be traced back to bread-making in ancient Egypt. Solid state fermentations include a number of well known microbial processes such as soil growth, composting, silage production, wood rotting and mushroom cultivation. In addition, many familiar western foods such as mold-ripened cheese, bread, sausage and many Oriental foods including miso, tempeh and soy sauce are produced using SSF. Beverages derived from SSF processes include ontjom in Indonesia, shao-hsing wine and kaoliang (sorghum) liquor in China and sake in Japan (**9**). Table 5 gives examples of foods that involve an SSF process at some point in production.

In 1896, Takamine produced a digestive enzyme, Takadiastase, by SSF employing *Aspergillus niger* on wheat bran (**10**). This led to the application of SSF in other food and beverage industries. The most profitable applications of SSF are in the

Oriental and African countries where SSF processes have been perfected over long periods. In western countries, traditional applications of SSF are scarce. SSF has been largely neglected since the 1940s; and negligible research and development efforts have been made. The selection of submerged liquid instead of SSF in western countries was not based on economic comparisons of submerged liquid and SSF techniques; the choice was linked to slow growth of the microbial cultivation industries across the world (**11,12**).

Table 4. Differences between solid-substrate fermentation and submerged liquid cultures.*

Solid-substrate culture	Submerged liquid culture
Culture medium is not free flowing	Culture medium is always free-flowing
Depth of medium is usually shallow except	Medium depth varies for a few bioreactors
Single water insoluble substrate provides carbon, nitrogen, minerals and energy	Different water-soluble sources of nutrients are used
Gradients in nutrient concentration are common	Nutrients are uniformly distributed throughout the fermentation
Water availability is just sufficient to sustain optimum growth of the culture	Water availability is abundant
Culture systems involves three phases, solid, liquid, and gaseous	Two phases, liquid and gaseous
Culture system is not aseptic beyond medium cooking	Whole system is always under aseptic conditions
Rigorous control of parameters is not required except for heat removal, oxygen supply and moisture	Rigorous control of all parameters during fermentation is essential
Inoculum ratio is always larger	Inoculum ratio is usually low
System may or may not involve agitation	Agitation is often essential
Fungal growth involves penetration of the hyphae deep into solid substrate particles	Fungal mycelial cells grow in the form of individual mycelium or mycelial pellets
Bacterial and yeast cells grow by adhering to solid particles	Bacterial and yeast cells are uniformly distributed throughout the liquid

*Mitchell and Lonsane, 1992.(**6**)

While commercial use of SSF is not widespread in North America, industrial enzyme production by SSF has occurred for a number of years (**10,13**). After World War II, Underkofler *et al.* (**14**) and Terui *et al.* (**15**) used heaped bed cultures with forced aeration to produce enzymes and citric acid. Tempeh production has been established on a small scale in the US (**16**) because it has been accepted as a meat substitute by vegetarians. Mushrooms are cultivated in western countries; and soy sauce production has become highly industrialized and is widely used in both western and oriental countries. Kikkoman Foods has built a state-of-the-art facility completed in 1998 for soy sauce production in Folsom, California.

Table 5. Examples of foods produced by solid substrate fermentation.

Product	Microorganism	Materials
Natto	*Bacillus natto*	Soybean
Tempeh	*Rhizopus oligosporus*	Soybean
Tape	*Amylomyces rouxii, Rhizobium chinensis*	Rice, cassava, maize
Ontjum	*Neurospora sitophila*	Peanut meal
Cheese	*Penicillium roqueforti*	Wheat powder
Bread dough	*Saccharomyces cerevisiae, Lactobacillus*	Wheat powder
Sake	*Aspergillus oryzae, A. kawachii*	Rice, barley
Miso	*A. oryzae*	Soybean, rice
Soy sauce	*A. sojae*	Soybean, wheat

Mitchell and Lonsane, 1992. (**6**)

General features of solid substrate fermentation

The single most important feature of SFF is the low moisture content of the medium, which makes SSF very different from submerged liquid cultures. Water is essential for microbial growth; and the limited water in SSF has several consequences. It is adsorbed and to some extent held tightly; and there may even be some free water in the interior and on the surface. Water activity can be below 0.99 in SSF, where free water is virtually absent. These conditions favor filamentous fungi, many of which grow well between water activities of 0.93 and 0.98 (**17**). Bacteria and yeast grow above a water activity of 0.99.

Heat transfer is restricted in SSF, which can lead to overheating problems in large scale fermentations (**18**). Evaporative cooling is the most effective cooling method, although this will reduce water availability (**19**). Proper temperature conditions during the fermentation are a balance between the need for heat removal and the necessity of keeping the substrate sufficiently moist to support growth.

Solid substrates used in SSF are composite and heterogeneous products from agriculture or by-products of agro-industries. For many processes, substrates are chosen because they are readily available and therefore inexpensive. All substrates have a common macromolecular structure. The macromolecular portion can provide a structural component for the substrate as well as serve as the carbon and energy source (e.g. cellulose) for the microorganism. If the macromolecule serves as a structural source only, the carbon and energy source is provided by a non-structural macromolecule such as starch or a smaller, soluble compound (e.g. soluble sugar).

Differences in enzymes produced by SSF and submerged liquid fermentation

Evidence has been accumulating to support the view that SSF processes are qualitatively different than submerged liquid fermentations. The data suggest that

microbial physiology and regulation within the cell are influenced by the fermentation environment (**20**). Ayers *et al.* (**21**) reported that pectinases produced by SSF had noticeable biochemical differences from those produced by submerged fermentation. A glucosidase produced *Aspergillus phoenicis* in SSF was more thermotolerant than when produced in submerged liquid fermentation (**22**). Alazard and Raimbault (**23**) showed that amylases produced by *A. niger* using SSF were more resistant to heat denaturation than those produced in submerged liquid fermentation by the same strain. Other differences have also been reported (**24,25**) and reinforced by the observation that the induction and repression patterns of pectinase production by *A. niger* are different for each fermentation technique (**26**).

Exoenzyme production results in increased amounts of some enzymatic activities not produced by cultures in liquid fermentation. In a comparison of phytases produced by SSF (Allzyme Phytase, Alltech Inc.) and a commercially-available phytase produced using submerged liquid fermentation, carbohydrase and protease side activities were found in the SSF, but not the liquid fermentation product (Table 6).

Table 6. Comparison of enzyme activities of two commercially available phytases.

Enzyme Assayed	Production method of enzyme	
	Submerged liquid	SSF
Phytase	1,500 PU/gram	1,500 PU/gram
Fungal α amylase	Below detectable level	240 FAU/gram
β-glucanase	Below detectable level	2,160 BGU/gram
Cellulase	Below detectable level	310 CMCU/gram
Fungal Protease	Below detectable level	7,380 HUT/gram

The complex nature of feedstuffs makes these side activities beneficial to the animal industry (**27**). *In vitro* comparisons have shown increased rates of reducing sugar and amino nitrogen, and an associated increase in phophate release by an SSF phytase product (Allzyme Phytase) (**28**).

Microorganisms for solid state fermentation

Bacteria, yeast and fungi can grow on solid substrates and have applications in SSF processes. Filamentous fungi are the best-adapted species for SSF and dominate in the research and practical applications around the world. Bacterial SSF fermentations are rarely used for large-scale enzyme production, but are very important in nature and in the fermented food industry. In composting, moist solid organic wastes are decomposed by a succession of naturally occurring microorganisms. Ensiling processes are dominated by lactobacilli producing lactic acid. Natto is a fermented food involving *Bacillus subtilis*. The fermentative yeast *Endomycopsis burtonii* is involved in the production of a traditional Indonesian fermented food, tape (**29**).

Commercial use of unicellular organisms to produce enzymes has limitations. Colonies of non-motile unicellular organisms have a uniform density and this cannot be regulated in response to nutrient concentration (**30**). Cells are separate, and expansion of the colony edge does not occur because no translocation system exists. The center of the colony may be inhibited by metabolic end products (**30**). Nutrients diffuse to the colony so that the edge expands into regions with fewer nutrients. This causes expansion to slow and eventually stop. The result is that the entire substrate is not colonized (**31**). In addition, unicellular microorganisms cannot anchor themselves to facilitate penetration by mechanical pressure, and tend to spread across the surface without penetrating.

Filamentous fungi are the most important group of microorganisms for enzyme production in SSF. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and the utilization of available nutrients. The filamentous fungi have the power to penetrate solid substrates. Hydrolytic enzymes are excreted at the hyphal tip, without large dilution. This makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. This is critical for the growth of the fungi. Fungi cannot transport macromolecular substrates across the cell wall, so the macromolecule must be hydrolysed externally into soluble units that can be transported into the cell (**32**).

Many submerged liquid fermentations are performed using pure cultures. The substrate is sterilized and then inoculated with a single culture. In the case of SSF a range of culture types are commonly used:

Single organism culture processes:	Many non-traditional processes are inoculated with a single microbial species. Strict aseptic conditions are not followed; and selective pressures such as water content and inoculation rate are used to control contamination.
Defined mixed culture:	A defined mixed culture involves inoculation of the substrate with more than one pure culture.
Sequential culture:	a second organism is inoculated after growth of the first microorganism has stopped. This is a modification of the mixed culture.
Undefined mixed culture:	The substrate is inoculated with a mixture of cultures that have not been identified: either the natural microflora of the substrate itself, or inocula consisting of natural resources of mixed populations of microorganisms. Traditional SSF methods are prepared in this way.

Most applications involve one of these inoculation schemes using fungi. Among the filamentous fungi, three classes have gained importance: Phycomycetes such as the genera *Mucor* and *Rhizopus*, the Ascomycetes with the genera *Aspergillus* and *Penicillium*, and *Basidomycetes* (**33**).

Advantages and disadvantages of using solid state fermentations

Solid-state fermentation systems have a number of advantages (**9,34**):

a) The medium is often simple, consisting of unrefined agricultural product, which may contain all the nutrients for microbial growth. Examples of substrates are cereal grains, wheat bran, and wheat straw.

b) Substrates require less pretreatment compared to liquid fermentation. Pretreatment for SSF must increase the accessibility of nutrients, while pretreatment for liquid fermentation must achieve extraction of the nutrients into the bulk liquid phase.

c) The restricted availability of water helps to select against undesirable contaminants, especially bacteria and yeast.

d) Forced aeration is often easier in solid-state cultures than in liquid cultures because the interparticle spaces allow transfer of fresh air to thin films of water at the substrate surfaces.

e) Downstream processing and waste disposal is often simplified or minimized. If drying is required, less water is present to be removed.

Solid state fermentation as an enzyme production technique is not without difficulties that must be overcome. A number of disadvantages must be addressed to make a successful product (**9,34**):

a) Restricted to microorganisms that grow at reduced moisture levels. The majority of commercially profitable processes involve fungi, however, and this problem is avoided.

b) Removal of metabolic heat generated can be a problem in large scale fermentations. Depending on the organism, heat can drastically influence end product production. This problem can be lessened by using organisms that are heat tolerant.

c) The solid nature of the substrate presents problems in monitoring process parameters. Changes in pH are not easily identified and controlled in SSF; and the control of moisture content and substrate concentrations is extremely difficult. Heat production, oxygen consumption and carbon dioxide are parameters that can be measured.

d) Many important basic scientific and engineering aspects are poorly understood. Little is known about the mode of growth of fungi within substrate masses composed of irregularly shaped solid particles.

e) Cultivation times are often longer.

Bioreactor design

Reactor design is important in developing an efficient SSF process. The design of solid state reactors has to date been mostly imperical. Three basic types of reactors

can be distinguished based on the mixing regime and the aeration mode. These include tray bioreactors, packed bed reactors, and agitated bioreactors.

The simplest SSF reactor is the tray. In a tray bioreactor a relatively thin layer of substrate is spread over a large horizontal area. There is no forced aeration, although the base of the tray may be perforated and air forced around the tray. Mixing, if done is by simple automatic devices or manually. Internal temperature may vary with ambient temperature; or the tray may be placed in a temperature-controlled room.

Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial and commercial scale (**16,35**). Although the design of the reactor is simple extensive mechanization and automation have been reported in Japan (**36**).

Packed bed bioreactors are characterized by having a static substrate supported on a perforated base plate through which forced aeration is applied. Many variations of this basic design are possible (**36**). A tall, thin cylindrical column is the typical design. Most commonly the forced air is applied to the bottom. The humidity of the incoming air is kept high to minimize water loss from the substrate. The temperature of the incoming air can be changed to aid in temperature regulation of the substrate (**37**). The advantage of packed bed reactors is that they remain simple while allowing better process control than trays.

Two general types of agitated fermenters have been designed. The first is a rotating drum reactor consisting of a horizontal or inclined cylinder that rotates around a central axis and causes a tumbling motion of the substrate. Aeration is supplied in the headspace. Mixing is gentle, although problems can arise if microorganisms are sensitive to the agitation. Temperature control is difficult because the reactor is difficult to water jacket (**36**). The second type of agitated fermenter, a stirred reactor, has the reactor placed either on a horizontal or a vertical axis. Horizontal reactors are similar to rotating drums except the mixing is provided by an internal scraper or paddles, rather than rotation of the reactor. Vertical stirred reactors are subjected to forced aeration and are agitated continuously or intermittently.

Process methodology

The steps involved in solid state fermentation process consist of (**36**):

- The preparation of a solid substrate, often with pretreatment to decrease the particle size or increase the availability of nutrients in the substrate;
- A cooking step which sterilizes or at least pasteurizes the substrate and causes the absorption of water into the substrate particles;
- Growth of a suitable inoculum;
- Inoculation of the moist solids;
- Incubation in appropriate culture vessels;
- Maintenance of optimal conditions to the extent possible;

- Harvest of the solids;
- Drying or extraction of the product from the solids;
- Downstream processing.

The Alltech solid state fermentation program

The many advantages of enzyme production by SSF have convinced Alltech that it is a valuable technology for the production of enzymes. As a result of this commitment an entire SSF program has been developed. The program includes small-scale lab fermentations up to production size facilities. The development from idea to commercial production had followed the following steps:

- Culture isolation, screening and selection
- Standardization of the process at small scale
- Scale-up studies
- Design and construction of the plant
- Operation of the plant.

The development of a SSF fermentation program at Alltech is intended to produce a more useful enzyme employing a procedure with economics that make it practical for the animal feed and fuel ethanol industries. With the completion of a production facility in Serdan, Puebla, Mexico the idea became a reality.

Culture isolation, screening and selection: non-GMO organisms

The SSF program starts with the cultures. Two isolates have gone through a rigorous screening and selection process and are currently used extensively in the program. An *Aspergillus niger* has been naturally selected for overproduction of phytase. The reason for using an aspergilli strain is that the incidence of phytase production is highest in these strains. Of all the organisms surveyed, *A. niger* produces the most active extracellular phytase reported (**38**). Through numerous rounds of screening and selection, overproduction by about 400 fold has been achieved. A point that is becoming more and more critical for many consumers is that the organism is a non-genetically modified organism (non-GMO) able to produce phytase at a significant level for commercial production. Use of non-GMO organisms in SSF processes is common. The SSF growth environment is conducive to overproduction of a number of different enzymes; and genetic engineering is not required for production of large amounts of enzymes. In contrast, submerged liquid systems generally use GMOs designed for overproduction of a particular enzyme.

The second organism currently being studied extensively is *Rhizopus oryzae*, which produces glucoamylase. Glucoamylase sequentially cleaves glucose molecules from the nonreducing end of a starch molecule and is used extensively in the ethanol industry. The organism is not genetically modified and has been naturally selected for overproduction of glucoamylase. Solid substrates consist of complex

macromolecules including starch. Amylases are necessary for the utilization of starch and many filamentous fungi produce amylases. *Rhizopus* sp. have been reported to produce amylases (**39**). This organism is currently being used in lab scale and pilot scale tray fermentations and deep bed lab scale fermentations. Completion of these studies will allow the organism to be used at a production level.

Alltech is continually screening and selecting organisms for a variety of enzymes; and the search will not be limited to fungal organisms. With the great variety of environments that bacteria can be isolated in, these organisms hold enormous potential for future enzyme production. Any number of organisms are at varying stages within the SSF program. The goal is to identify organisms that will produce enzymes on a commercial level that can be used in the feed industry. This includes new enzymes and improvement of current enzymes.

Standardization of the process at small scale

The selection of strains involves SSF on a small scale. Small-scale fermentations occur in 500 ml flasks. The substrate used for the majority of fermentations is wheat bran. In these flasks the typical moisture, temperature and extraction conditions as well as the length of fermentation and inoculation rate have been determined and maximized. The information from these systems is important, although certain limitations do exist. Since these systems are extremely shallow, heat removal is not as critical and no accurate estimate of heat production can be made.

In addition to thin layer tray systems, deep bed systems of 30–50 cm will be used in production. In order to determine if organisms are suitable for growth in deep layer fermentations, lab scale deep layer fermenters have been designed. Conditioned air is blown in through the bottom with an exit port on the top. The substrate sits on top of a perforated screen. The substrate can be added to a depth of 25 cm and the diameter is about 16 cm in the lab scale fermenter. The vessel has ports for thermocouples to monitor temperature. The airflow, relative humidity of the air, and oxygen content within the chamber can all be measured. These parameters are monitored and the data collected and sent to a computer. The entire fermenter is placed in a temperature and humidity controlled room. Condensation on the inside of the vessel can be a problem so insulation is added around the outside. With this system we are able to generate data that reflect the heat produced during the fermentation and determine the amount of air required to maintain temperature in the target range. The substrate cannot be agitated during the fermentation and water is not easily added. These systems can produce data on the success of the organism in deep layers and the amount of heat produced during the fermentation.

Scale-up studies

The scale-up step is a crucial linkage in the process since it determines whether the process will operate at a commercial scale. The scale-up should result in the same overall performance at large scale that can be achieved in the laboratory. This is rarely the case since a number of additional parameters influence the fermentation. Heat removal must be addressed; and there are no simple solutions since thermodynamic and kinetic properties become more complex.

Alltech is interested in production of enzymes in deep layers as well as thin layer tray systems. In order to reach this goal a pilot scale deep bed fermentation system was constructed. The reactor is a vertical stirred reactor based on a modified lauter tun design. The diameter is 1.5 m and the system is designed to be used at a maximum depth of about 50 cm. The agitation system consists of vertical blades that act like knives to separate the substrate if needed. Attached to the bottom are blades that cut through the substrate when rotated in one direction and will push the substrate out of the reactor following the fermentation when turned in the other direction. This system is useful when heat produced by the culture cannot be removed through the conditioned air forced from the bottom of the reactor. The agitation system can be lifted out of the substrate during the fermentation. An inoculation port is present at the top of the reactor and a door near the bottom allows removal of substrate. The entire unit sits on load cells to measure weight loss during the fermentation. The substrate is sterilized by a rotary mixer that can be pressurized and heated. The mixer will also be used to inoculate the substrate. During the fermentation, temperature, oxygen, carbon dioxide, airflow and relative humidity within the system can be monitored. The reactor will enable studies to be performed to determine if organisms can perform in deep layers at pilot scale levels and develop an understanding of heat production.

Design and construction of the plant

There is no information available in the literature on SSF with respect to the theoretical and experimental comparisons of different kinds of bioreactors, methods for controlling cultivation parameters, automation, design and scale up criteria or downstream processing options. The design process was based on information obtained in lab scale and pilot scale studies as well as experience gained in the areas of fermentation and downstream processing.

From the data generated and the scant literature available it became apparent that for initial production a tray fermentation system would reach optimum production levels in the shortest amount of time. The data generated from the lab and pilot tray systems was used in the development of a production tray fermentation system. The facility has been built in Serdan, Pueblo, Mexico. The facility houses pretreatment, inoculation facilities, fermentation facilities, and down stream processing facilities. A separate building at the sight contains a laboratory for initial strain manipulation. The initial phase of the design was intended for construction of a facility that will contain about 10,000 trays.

Future potential

Most profitable applications of SSF are confined to Oriental and African countries and are scare elsewhere. A resurgence of interest has occurred in western and European countries in response to the ever-increasing demand for economy in processes. The facility in Serdan is believed to be the first commercial enzyme production facility in North America that uses SSF technology. The success of the SSF facility will lead to expansion. The future of the SSF program at Alltech includes the development of new strains for enzyme production, to enhance current enzyme systems and development of new enzyme applications. The nature of SSF will also lead us in the direction of new substrates. The use of a variety of waste products will be investigated as well as the potential for using inert supports for fermentation. The SSF technology also has the potential to be used for purposes other than enzyme production. Other metabolites such as ethanol, flavors, and other microbial by-products can be produced. SSF can be used for upgrading agro-industrial by-products that can be used in animal feed applications. Knowledge gained about the SSF process will allow construction of fermentation systems that better monitor and control fermentation parameters and utilize a wider range of substrates, as well as microorganisms.

References

1. Lehninger A.L. (1975). Biochemistry: The Molecular Basis of Cell Structure and Function. 2nd edition. Worth Publishers, Inc. New York, N.Y.
2. Bron S., Meima R., Van Dijl J.M., Wipat A., Harwood C.R. (1999) *In*: Manual of Industrial Microbiology and Biotechnology (A.L. Demain and J.E. Davies, eds.) ASM Press, Washington, D.C. pp. 392-416.
3. Amado R. (1993) *In*: Proceedings of the 1st symposium. Kartause Ittingen, Switzerland. (C. Wenk and M. Boessinger, eds.) pp. 5–15.
4. Stanbury P.F., Whitaker A., Hall S.J. (1995). An Introduction to Fermentation Processes in Principles of Fermentation Technology, Second Edition. Pergamon Publishers, Great Britain.
5. Bailey J.E., Ollis D.F. (1986) Biochemical Engineering Fundamentals. Second Edition. McGraw Hill Publishing Company. New York. p. 984.
6. Mitchell D.A., Lonsane B.K. (1992) *In*: Solid Substrate Cultivation. H.W. Doelle, D.A. Mitchell and C.E. Rolz (eds.) Elsevier Applied Biotechnology Series, London, pp 1-16.
7. De Becze G., Liebmann A.J. (1944) *Ind. Eng. Chem.* **36**:882-890.
8. Callahan J.R. (1944) *Chem. Metal. Eng.* **51**:94–98.
9. Mudgett R.E. (1986) *In*: Manual of Industrial Microbiology and Biotechnology (A.L. Demain and H.A. Solomon, eds). American Society for Microbiology, Washington, D.C. pp. 66-83.
10. Takamine J. (1914). *Ind. Eng. Chem.* **6**: 824-828.
11. Ralph B.J. (1976) *Food Technol. Australia* **28**:247-251.
12. Hesseltine C.W. (1976) *Biotechnol. Bioeng.* **14**:517–532.
13. Underkofler L.A., Barton R.P., Rennert S.S. (1958) *Appl. Microbiol.* **6**:212-221.
14. Underkofler L.A., Steverson G.M., Goering K.J., Christensen L.M. (1947) *Cereal Chem.* **24**:1–22.
15. Terui G., Shibazaki I., Mochizuki T. (1957) *Hakkokogaku* **35**:105-116.

16. Hesseltine C.W. (1987) *Int. Biodeterioration.* **23**:79-89.
17. Corry J.E.L. (1973) *Progr. Ind. Microbiol.* **12**:73-108.
18. Laukevics J.J., Apsite A.F., Viestures U.S., Tengerdy R.P. (1984) *Biotechnol. Bioeng.* **26**:1465-1474.
19. Trevelyan W.E. (1974) *Tropical Science* **16**:179–194.
20. Viniegra-Gonzalez G. (1997) *In*: Advances in Solid State Fermentation. (S. Roussos, B.K. Lonsane, M. Raimbault and G. Viniegra-Gonzalez, eds.). Kluwer Publishers, Dordrecht, pp. 5–22.
21. Ayers A., Dingle J., Phipps A., Reids W.W., Solomons CL. (1952) *Nature London* **170**:834-836.
22. Deschamps F., Huet M.C. (1984) *Biotechnol. Lett.* **6**:451-456.
23. Alazard D., Raimbault M. (1981) *Eur. J. Appl. Microbiol.* **12**:113-117.
24. Romero S., Acuna M.E., Viniegra-Gonzalez G. (1993) *Biotechnologia* (Mexico) **3**(1-2):FS65-FS69.
25. Villegas E., Aubague S., Alcantara L., Auria R., Vega C., Revah S. (1993) *Biotech. Adv.* **11**:387-397.
26. Solis-Pereira S., Favela-Torres E., Viniegra-Gonzalez G., Gutierrez-Rojas M. (1993) *Appl. Microbiol. Biotech.* **39**:36-41.
27. Classen H.L. (1996) *Feed Mix* **4**:22-28.
28. Filer K., Evans J., Newman K., Spring P. (1999) *Poultry Sci.* **78** (Suppl 1):74.
29. Steinkraus K.H. (1983) *Biotechnol. Adv.* **1**:31–46.
30. Bull A.T., Trinci A.P.J. (1977) *Adv. Microbial Physiol.* **15**:1–84.
31. Oliver S.G., Trinci A.P.J. (1985) *In*: Comprehensive Biotechnology, vol. 1 (M. Moo-Young, eds). New York Pergan Press, pp. 159 –187.
32. Knapp J.S., Howell J.A. (1980) *In*: Topics in Enzyme and Fermentation Biotechnology, vol. 4. A. Wiseman. Ellis Horwood Ltd, Chichest, England, pp. 85-143.
33. Moo-Young M., Moreira A.R., Tengerdy R.P. (1983) *In*: the Filamentous Fungi, vol. 4. (J.E. Smith, D.R. Berry and B. Kristiansen, eds). Edward Arnold, London, pp. 117-144.
34. Cannel E., Moo-Young M. (1980) *Proc. Biochem.* **15**:2-7.
35. Ahmed S.Y., Lonsane B.K., Ghildyal N.P. , Ramakrishna S.V. (1987) *Biotechnol. Tech.* **1**:97-102.
36. Lonsane B.K., Ghildyal N.P., Budiatman S., Ramakrishna S.V. (1985) *Enzym. Microbiol. Technol.* **7**:258-265.
37. Narahara H., Koyama Y., Yoshida T., Pichangkura S., Taguchi H. (1984) *J. Ferment. Technol.* **62**:453-459.
38. Wodzinski R.J., Ullah A.H.J. (1996) *Advances in Applied Microbiology*, **42**, 263–302.
39. Raimbault M. (1998) *EJB*, **1** No.3.

Chapter 2

BIOCHEMICAL ENGINEERING OF SOLID-STATE FERMENTATION

F.J. WEBER[1,2], J. OOSTRA[2], F.J.I. NAGEL[2,3], M.A.I. SCHUTYSER[1,2], M.J. HOOGSCHAGEN[1,4], Y.S.P. RAHARDJO[1,2], Y. ZHU[1,4], R.M. BOOM[2], J. TRAMPER[2], A. RINZEMA[1,2]

[1] Wageningen Centre for Food Sciences, P.O. Box 557, 6700 AN Wageningen, Netherlands, (frans.weber@algemeen.pk.wau.nl); [2] Food and Bioprocess Engineering Group, Wageningen University, Wageningen, Netherlands ; [3] current address Unilever Research Vlaardingen, Vlaardingen, Netherlands; [4] TNO Voeding, Zeist, Netherlands

Abstract

Important progress has been made recently in improving our understanding of SSF. We have developed a model-based scale-up strategy for packed-bed reactors. This strategy has been experimentally validated for the fungus *C. minitans*, an effective biological agent for pest control in many food crops, and is currently extended to *A. oryzae*, an important food fermentation organism. Mixed fermenters allow improved simultaneous control of temperature and moisture content. We have successfully cultivated several fungi in continuously mixed fermenters. For slow-growing fungi wall cooling offers sufficient perspectives for scale-up; for fast growers evaporative cooling and water addition are indispensable. A model was used to estimate dry weight, intracellular water and extracellular water in the solid substrate from on-line measurements, and to calculate the required water addition. Behaviour of solid-state fermentation at the particle level is still poorly understood. We present experimental evidence for oxygen concentration gradients in fungal mats growing on solid substrates and show the consequences. Aerial hyphae turned out to be very important for respiration of *A. oryzae* in SSF. Gradients in water content and glucose concentration in solid substrates were determined using NMR.

Introduction

A well-known problem in solid-state fermentation (SSF) is simultaneous control of temperature and moisture content of the solid substrate (**1**): In large industrial fermenters, only evaporative cooling can control temperature, which results in a decrease in moisture content of the solids over time. The moisture loss is aggravated by the uptake of water in new microbial cells (**1**). Simultaneously, extracellular microbial enzymes cause accumulation of *e.g.* sugars (**1**). The water loss and solute accumulation will result in a decrease in water activity, which can have beneficial effects on metabolism, but can also result in process failure (**2**). These changes in time, combined with gradients in the substrate bed and inside

New Horizons in Biotechnology, 17-25, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

substrate particles, make optimal design and process control of SSF relatively complex. Nevertheless, important progress has been made in the latest decade, which has significantly improved our understanding of the behaviour of SSF and our ability to design the process rationally. In this paper, an overview of results that were recently obtained with static packed beds and mixed beds will be presented, as well as progress made in studies of intra-particle gradients.

The workhorse of industrial SSF is the packed bed, which is used in several European countries for selective cultivation of mushroom mycelium (**3**) and in Asia for koji production (**4**). Packed beds used for mushroom mycelium cultivation typically have a holding capacity of ca. $2x10^5$ kg wet substrate and are not mixed (**3**). Koji beds typically have a capacity of 10^4 kg wet substrate and are mixed once every 24 hours. At the laboratory scale, several more intensively mixed systems have been developed that allow better process control. Besides the degree of mixing in the bed, fermenters can be divided based on the mode of aeration (over or through the bed) and cooling (conductive or evaporative). We discuss un-mixed packed beds with aeration through the bed and with continuously mixed beds, which are aerated mainly over the bed. Conductive and evaporative cooling will be discussed for both systems.

Packed bed fermenters – conductive cooling

In laboratory scale packed-bed reactors, adequate temperature control can be achieved by wall cooling. This cooling mechanism can only be used industrially at the expense of a lot of cooling area. Oostra *et al.* (**5**) developed a mathematical model to predict radial temperature profiles during SSF in packed beds with conductive cooling only. Simulations of the radial temperature profiles at various reactor diameters during cultivation of the slow-growing biocontrol fungus *Coniothyrium minitans* showed that at small column radii (< 0.1 m) acceptable temperature gradients can be maintained, but at a column radius of 1 m *C. minitans* will be inactive in large parts of the column. This means that in spite of the low heat generation rate of this slow-growing fungus, conductive cooling cannot be used for temperature control during cultivation of *C. minitans* in a large packed-bed reactor.

An example of a system that uses conductive cooling is the Zymotis fermenter (**6**). The Zymotis system combines conductive cooling with evaporative cooling and is therefore expected to offer more scale-up capacity. The drawback of combining both cooling mechanisms is, however, that more gradients will develop in the system.

Packed bed fermenters – evaporative cooling

Forced aeration is generally recognised as an effective cooling mechanism in large-scale packed-bed bioreactors. It results, however, in evaporation of water and desiccation of the substrate. Desiccation of the substrate can either lead to shrinkage of the substrate particles with subsequent channelling in the bed and poor aeration

of the bed, or an unfavourable water activity resulting in poor microbial activity. Weber *et al.* (**7**) developed a model-based strategy that can be used to evaluate whether cultivation in a large-scale packed-bed reactor is feasible, and used this strategy to evaluate the cultivation of *C. minitans* on different solid substrates (**7**).

The model predicted that axial temperature gradients could be controlled by moderate aeration rates. However, during cultivation up to 2.6 kg water kg^{-1} dry support would be lost. As *C. minitans* is very sensitive to reduced water activities (spore formation was completely inhibited at a_w 0.97), stringent control of water activity in the bed is of utmost importance. Replenishment of the evaporated water can only be achieved by addition of liquid water. In non-mixed beds, however, homogeneous water addition is impossible. Lack of water during the cultivation can therefore only be prevented when the initial water content of the support is sufficiently high. Hemp provided the best spore yield and control of water activity, due to its extremely high water holding capacity. In a well-insulated sterile packed-bed reactor with a total volume of 15 L, a spore yield of $9x10^{14}$ conidia per m^3 was achieved in 18 days, using hemp impregnated with a solution containing 100 g dm^{-3} glucose and 20 g dm^{-3} yeast extract. A surplus of water was still available after 18 days.

This well-insulated system was used to validate a scale-up model for packed-bed solid-state fermenters, which neglects radial gradients. Effects of temperature on respiration and growth kinetics of *C. minitans* determined in independent experiments were incorporated in material and energy balances for the packed-bed. Predicted temperatures corresponded well with experimental results (Figure 1).

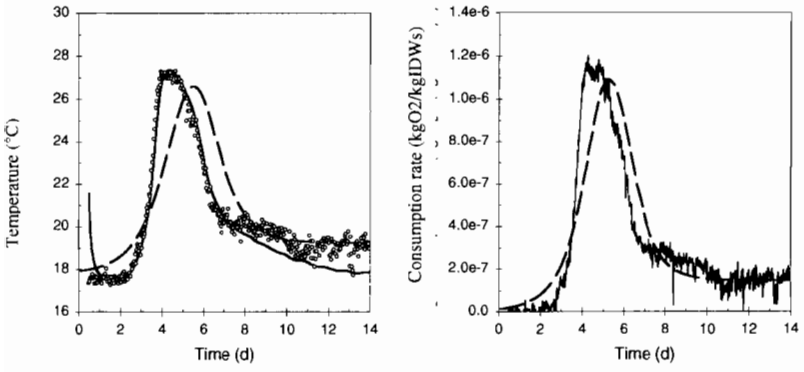


Figure 1: measurement and predicted temperature at the outlet of the reactor (left) and oxygen consumption rate (right) in an aerated packed-bed reactor during cultivation of *Coniothyrium minitans* on hemp impregnated with glucose medium (airflow 0,027 kg m^{-3} s^{-1}; temperature inlet air 17,7°C).

(- -) measurements, (---) predictions using independant isothermal O_2 consumption measurements, (°) temperature predicted using O_2 consumption measured in the reactor.

As predicted, large amounts of water were lost due to evaporative cooling. With hemp as support no shrinkage was observed and temperatures could be adequately controlled, both with *C. minitans* and *Aspergillus oryzae*. In experiments with grains, however, strong shrinkage of the grains was predicted and observed. Contrary to our expectations, cultivation of *C. minitans* on oats succeeded, because this fungus did not form a tight hyphal network between the grains. However, cultivation of *A. oryzae* failed, because shrinkage combined with the strong hyphal network formed by this fungus resulted in channelling, local overheating of the bed and very inhomogeneous growth of the fungus. The current model has various advantages compared to previously presented scale-up strategies. First, it has been validated against measurements in a pilot-scale reactor, whereas the validation of other models is poor (**8**). Second, a water balance has been incorporated, which was ignored in other models (**9**). The model is currently improved by incorporation of (i) gas-solids water and heat transfer kinetics to account for deviations from equilibrium observed with *A. oryzae*, and (ii) the dynamic response of the fungus to changes in temperature, which were neglected in the isothermal kinetic experiments.

Mixed-bed fermenters

Especially for substrates that shrink or develop unfavourable water activity upon drying, evaporative cooling should be combined with spraying of water onto the solid substrate. The use of mixed bioreactors is then necessary to ensure homogeneous water addition. A continuously mixed, aseptic scraped drum fermenter was used successfully for production of spores of *C. minitans* and other biocontrol fungi (**5**). A continuously mixed, aseptic paddle mixer was successfully used for solid-state fermentation (SSF) with *A. oryzae* on whole-wheat kernels (**2**). Sporulation and respiration rates found in these systems were comparable to those in small, isothermal unmixed beds, which show that continuous mixing did not cause serious damage to the fungi or the grain kernels. Continuous mixing improves heat transport to the bioreactor wall, which reduces the need for evaporative cooling and thus may help to prevent the desiccation problems that hamper large-scale SSF. However, scale-up calculations indicated that wall cooling becomes insufficient at a scale of only 2 m^3 for a rapid-growing fungus like *A. oryzae*. Consequently, evaporative cooling will remain important in a large-scale mixed system.

Experiments showed that water addition will be necessary when evaporative cooling is applied, in order to maintain a sufficiently high water activity of the solid substrate (**2**). We developed a stoichiometric model, which estimates the extracellular (non-fungal) and overall water contents of wheat grain during solid-state fermentation (SSF) with *A. oryzae*, using on-line measurements of oxygen, carbon dioxide and water vapour in the gas phase. The model uses elemental balances to predict substrate dry matter losses, metabolic water production, water used in starch hydrolysis, and water incorporated in new biomass. Water losses caused by evaporation are calculated from water vapour measurements. Model

parameters were determined using an experimental membrane-based model system, which mimicked the growth of *A. oryzae* on the wheat grains and permitted direct measurement of the fungal biomass dry weight and wet weight. The model accurately predicted the measured overall water content of the fermenting solid substrate during fermentations performed in a 1.5-L scraped drum reactor and in a 35-L horizontal paddle mixer. It can be used to calculate the water addition that is required to control the extracellular water content in a mixed solid-state bioreactor for cultivation of *A. oryzae* on wheat and can easily be extended to other fungi and substrates by means of some simple lab experiments.

Using this model, we achieved simultaneous control of temperature and extracellular (non-fungal) water content during cultivation of *A. oryzae* on wheat in the continuously mixed paddle bioreactor (Nagel *et al.*, submitted). The extracellular water content was controlled by adding a fine mist of water droplets onto the mixed solid substrate, using the stoichiometric model (**1**) to calculate the required addition. This resulted in an improved biomass production compared to similar fermentations with temperature control only (Fig. 2).

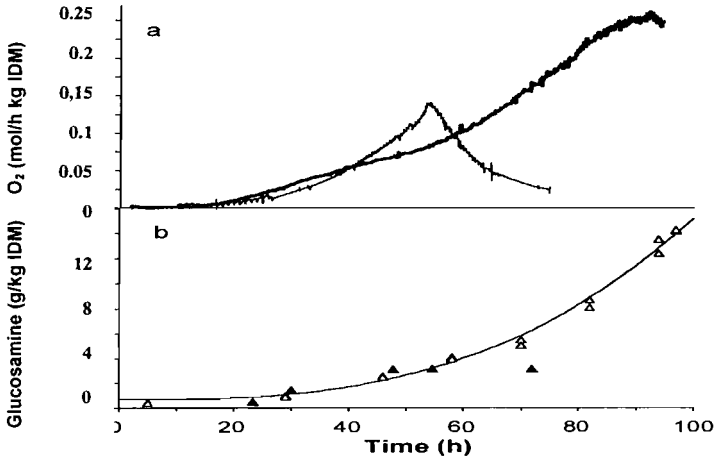


Figure 2: a. Oxygen consumption rates during cultivation of *Aspergillus oryzae* in the horizontal paddle mixer with evaporative cooling only and during cultivation with evaporative cooling plus water addition. b. Glucosamine measurements for the fermentation with evaporative cooling only (empty triangles) and with evaporative cooling plus water addition (full triangles).

Control of the extracellular water content resulted in a sufficiently high water activity to assure good growth of *A. oryzae* on wheat grain, but it was not possible to maintain a constant water activity because glucose accumulated within the solid substrate. Control aimed at constant extracellular water content was shown to be superior to control aimed at constant overall water content of the fermented

solids.Currently, our group aims at extending the possibilities to use mixed-bed SSF, by developing granular dynamics simulation models that predict forces between substrate particles and the resulting movement. Furthermore, we are currently developing a scaled-up mixed-bed fermenter and study mixed SSF for more vulnerable fungi.

Intra-particle gradients

Oxygen transfer is a major concern in scale-up and process control in industrial application of aerobic fungal SSF, for two reasons: (1) heat production is proportional to oxygen uptake and it is well known that heat removal is one of the main problems in scaled-up fermenters, and (2) oxygen supply to the mycelium on the surface of or inside the substrate particles may be hampered by diffusion limitation. Gradients inside substrate particles cannot be prevented in SSF. These gradients can have a strong effect on the physiology of the microorganisms, but have hitherto received little attention in experimental studies.

Oxygen limitation in SSF has been the topic of several modelling studies, but there has been no experimental elucidation of oxygen transfer limitation at the particle level so far. We studied intra-particle oxygen transfer in SSF cultures of *Rhizopus oligosporus*, *C. minitans* and *A. oryzae*. Direct measurements of the oxygen concentration in the fungal mat during cultivation, using oxygen microelectrodes, showed no oxygen depletion in the upper layer of aerial hyphae and steep oxygen concentration gradients in the wet bottom layer (Fig. 3).

Oxygen was depleted at ca. 100 μm below the gas-liquid interface after 35-100 hours, depending on the growth rate of the fungus. Oxygen concentration profiles in the wet fungal layer were consistent with a reaction-diffusion model. Comparison of the overall oxygen consumption rate from the gas phase to the oxygen flux at the gas-liquid interface calculated from the micro-electrode measurements, showed that oxygen consumption occurred mainly in the wet part of the fungal mat and the contribution of the aerial hyphae to overall oxygen consumption was negligible for *R. oligosporus* and *C. minitans*. However, in cultures of *A. oryzae*, aerial hyphae contributed up to 75% to the oxygen uptake rate of the fungus. This is due to the fact that *A. oryzae* forms very abundant aerial mycelium and diffusion of oxygen in the gas-filled pores of the aerial hyphae layer is rapid. It means that diffusion limitation in the densely packed mycelium layer with liquid-filled pores that is formed closer to the substrate surface, is much less important for *A. oryzae* than was found for *R. oligosporus* and *C. minitans*.

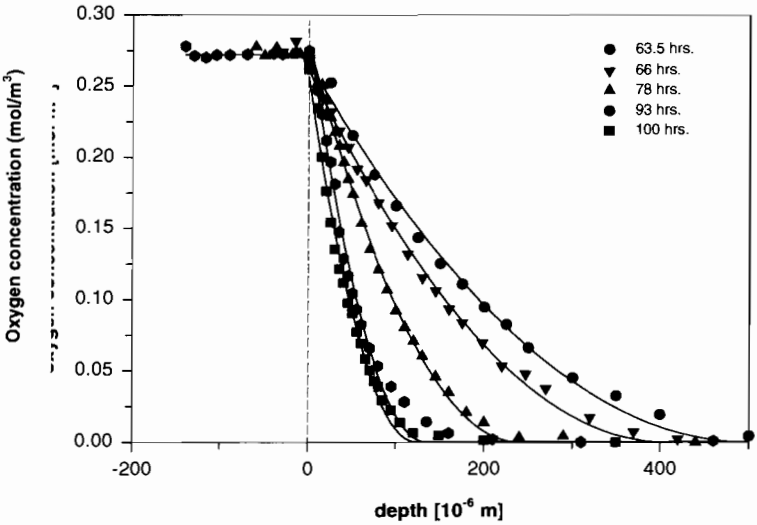


Figure 3. Oxygen concentration profiles as function of incubation time in a fungal layer of *Coniothyrium minitans* grown on agar medium containing ground oats (150 kg m^{-3}). The symbols indicate the measured profiles, and the solid lines profiles fitted with a reaction-diffusion model. The vertical line (--) is the gas-liquid interface in the biolayer.

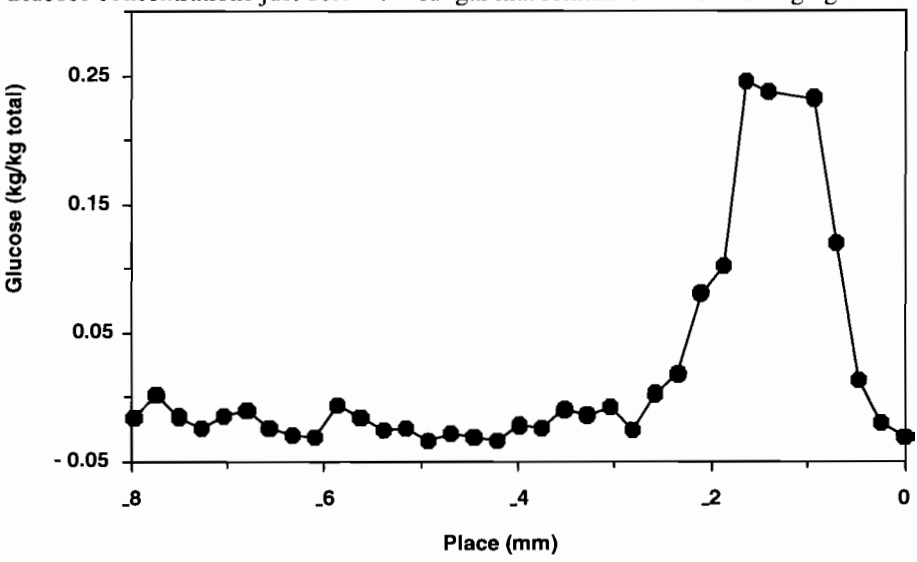
For process control in SSF, the rapid respiration of aerial hyphae presents a serious problem, due to its proportionality to heat production. The intriguing question is of course what the physiological functions of aerial hyphae are, whether they make interesting metabolites or hydrolytic enzymes or perhaps supply oxygen to mycelium in the wet layer, i.e. act as the 'lungs' of fungal mycelium in SSF. If the aerial hyphae do not produce interesting enzymes or metabolites or facilitate production in the wet layer, it might be better to suppress their growth in order to reduce heat production. This can be achieved by using a mixed fermenter (**2**), in which there no aerial hyphae were observed. If the aerial hyphae turn out to be important for production, it would be worthwhile to set up and validate models for aerial mycelium formation.

The microorganisms grow primarily near the outer surface of the substrate particle, due to oxygen supply limitations. Water uptake in new biomass and evaporation are thus both localized at the particle surface, which will cause a moisture gradient in the particles. Furthermore, inhomogeneous distribution of microbial enzymes and uptake of sugars by the microorganisms will cause solute gradients. Intra-particle solute and moisture gradients have hitherto not been taken into account in physiological studies of SSF, because it is difficult to measure them. We have used NMR imaging to obtain spatially resolved moisture and glucose content measurements at the particle level during cultivation of *A. oryzae* on wheat dough slices. We found that moisture gradients in the solid substrate remain small when

evaporation is minimized. This is corroborated by predictions of a diffusion model. In contrast, strong glucose gradients developed (Fig. 4).

Figure 4. Glucose profile during cultivation of *Aspergillus oryzae* on wheat dough, as a function of depth in the wheat dough. The profile was calculated from *in vivo* NMR measurements after 64 hours incubation. Position 0 indicates the position of the membrane.

Glucose concentrations just below the fungal mat remained low due to high glucose



uptake rates, but deeper in the matrix glucose accumulated to very high levels. Integration of the glucose profile gave an average concentration close to the measured average content. Based on published data, we expect that the glucose levels in the matrix cause a strong decrease in water activity.

Conclusions

During the past decade, several research groups have made significant progress in studies of SSF; some examples from our own work are given above. Nevertheless, our understanding of SSF and our ability to control the process in an optimal manner is still incomplete compared to submerged fermentation (SmF). We can never reach the stringent control over for example carbon source levels that is customary in fed-batch SmF, because gradients inside the solid particles cannot be prevented. Apparently, spatial or temporal variation in process conditions can give better results, but as long as we do not understand why, we cannot expect widespread application of SSF. Especially in the areas of physiology and molecular biology, coupled to gradients in the solid particles, our knowledge is too limited. Our institute has initiated research in these areas, in order to improve our understanding of the response of fungi to this non-conventional fermentation

environment. On the fermenter level, the emphasis is still on maintaining a constant temperature and moisture level. Models of mixing behaviour are developed, which need to be coupled to the biological response, for example with respect to mixing damage. In the coming years, the emphasis is expected to shift to process control based on advanced insight in physiology and intra-particle gradients.

References

1. Nagel F.J.I., Tramper J., Bakker M.S.N., Rinzema A. (2001) *Biotechnol. Bioeng.* **72**:231-243.
2. Nagel F.J.I, Tramper J., Bakker M.S.N., Rinzema A. (2001) *Biotechnol. Bioeng.* **72**:219-230.
3. Verhagen F.J.M. (2000) Mushroom Experimental Station, Horst, the Netherlands. Personal communication.
4. Steinkraus K.H. (1995) Handbook of indigenous fermented foods, 2nd edition. New York: Marcel Dekker Inc.
5. Oostra J., Tramper J., Rinzema A. (2000) *Enzyme Microbial Technol.* **27** : 652–663.
6. Roussos S., Raimbault M., Prebois J.P., Lonsane B.K. (1993) *Appl. Biochem. Biotechnol.* **42**:37-52.
7. Weber F.J., Tramper J., Rinzema A. (1999) *Biotech. Bioeng.* **65**:447-458.
8. Mitchell D.A., Krieger N., Stuart D.M., Pandey A. (2000) *Process Biochem.* **35**: 1211-1225.
9. Sangsurasak P., Mitchell D.A. (1998) *Biotechnol. Bioeng.* **60**:739-749.
10. Ishida H., Hata Y., Ichikawa E., Kawato A., Suginami K., Imayasu S. (1998) *J. Ferm. Bioeng.* **86**:301-307.

Chapter 3

PHYTASE PRODUCTION UNDER SOLID-STATE FERMENTATION

A. SABU[1], K.M. NAMPOOTHIRI[1], P. LATHA[1], V. KANNAN[1], G. SZAKACS[2], A. PANDEY[1]

[1]Biotechnology Division, Regional Research Laboratory (CSIR), Trivandrum-695 019, India; [2]Department of Agricultural Chemical Technology, Technical University of Budapest, Gellert ter 4, 1111 Budapest, Hungary

Abstract

Phytases or myo-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8) are a sub-family of high-molecular weight histidine acid phosphatases, which hydrolyse phytic acid, a major antinutrient factor present in most cereals and legumes. The hydrolysis of phytic acid by phytase results in myo-inositol and phosphoric acid and is an important metabolic process in many biological systems. While phytase can be obtained from a number of sources from Nature, commercially it is produced using microorganisms, which employ two kinds of techniques, solid-state fermentation (SSF) and submerged fermentation (SmF). In this paper we attempt to explain application of SSF for phytase production, with work carried out by our group.

Introduction

Phytase or myo-inositol-hexakisphosphate phosphohydrolase (EC.3.1.3.8) was first discovered by Suzuki *et al.* in the course of rice bran hydrolyzing studies. Phytase is capable of utilizing phytic acid or phytate as a substrate and catalyses the hydrolysis of phytic acid (Fig. 1). The principal end products of phytase action are phosphoric acid and myo-inositol. Most cereals and legumes are rich in protein and fat but they have antinutritional factors like phytic acid (myo-inositol hexakisphosphate), which discourages their use in food. The phytic acid acts as an antinutrient due to its chelation of various metals and binding of protein. This diminishes the bioavailability of proteins and nutritionally important minerals (**1,2,3**).

New Horizons in Biotechnology, 27-34, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

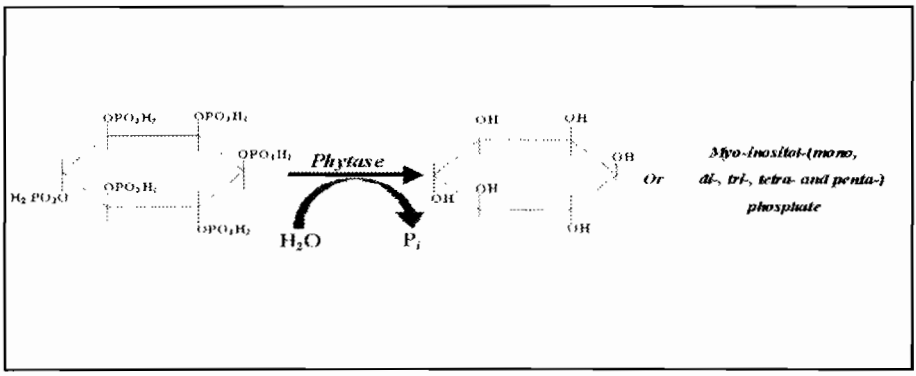


Figure 1: Hydrolysis of phytate by phytase.

Phytic acid is the main storage form of phosphorus in many seeds and cereals. In some cereals, about 80% phosphorus is found in the form of phytic acid, which if not hydrolyzed, binds to multivalent cations such as Zn^{2+}, Ca^{2+}, Fe^{3+}, etc, reducing their bioavailability. Through complex formation with proteins, it obstructs their enzymatic degradation. It has also been reported that phytic acid inhibits enzymes such as α-amylase, trypsin, pepsin, etc (**4,5**). Upon hydrolysis, phosphorus is freed and can be used in metabolic processes. The hydrolysis of phytic acid to myo-inositol and phosphoric acid is mainly affected by the enzyme phytase. Inositol is generated as intermediates or in some cases as end products. Myo-inositol and its isomers as biochemical derivatives are broadly distributed in higher plants, microorganisms and mammalian tissues and cells; in which they provide important biological functions.

Phytases belong to the family of histidine acid phosphatases. These are monomeric proteins of 20-40 kDa in molecular mass. Salt form of phytic acid is called phytate. Optimal degradation of phytate occurs around pH 4.5-6.0 and temperature of 45-60°C. Presently, phytase is commercially produced for its use in animal feed and for its role in reducing environmental pollution by the accumulation of phosphorus (**3**). Many feed stuffs such as soybean meal, conola meal etc contain high level of phytate. Monogastric animals such as pigs and poultry are not able to utilize phytic acid phosphorus, since they have only low levels of phytase activity in their digestive tracts and since phytic acid cannot be resorbed. Thus, nearly all of the dietary phytate phosphorus ingested by these species is excreted in to the environment resulting in phosphorus pollution in areas of intense animal production. Therefore, pig and poultry feed commonly is supplemented with either inorganic phosphate or a phytase of fungal origin. This addition causes a higher cost for feed processing (**6,7**).

Apart from this, the presence of phytic acid leads to the deficiency of these minerals. This affects proper skeletal formation that requires an adequate supply of

Ca, P, etc. By using phytase as a feed additive, the amount of phosphate in animal waste can be reduced, which eventually reduces pollution. Phytase also reduces the amount of phosphorus in manure. This results in the metabolizing of the bound phytin phosphorus. The elevated phosphorus concentration in lakes leads to the over growth of aquatic plants and algae. This over-growth, termed eutrophication results in the depletion of water oxygen levels and subsequent fish death. This can be reduced by the addition of phytase enzyme (**8**).

Solid-state fermentation

Solid-state fermentation, which is generally defined as the growth of micro-organisms on (moist) solid substrates in the absence or near absence of free water (**3,9,10,11**) is considered a useful technique for the production of industrial enzymes (**12**). During SSF large amounts of heat is generated which is directly proportional to the metabolic activities of the microorganisms. In the early phase of SSF, the temperature and concentration of oxygen remains uniform throughout the substrate bed but as the fermentation progresses, oxygen transfer takes place resulting in the generation of heat. In recent years, SSF has shown much promise in the development of several bioprocess and products. Over the last decade there has been a significant improvement in understanding how to design, operate and scale up SSF bioreactors (**11,13**). Last decade has witnessed an unprecedented increase in interest in SSF for the development of bioprocess, bioremediation and degradation of hazardous compounds (**9,10,11**). SSF offers potential advantages in bioremediation and biological detoxification of hazardous and toxic compounds.

Tropical agro-industrial crops such as cassava, soy bean, sugar beet, sweet potato, potato and sweet sorghum and crop residues such as bran and straw of wheat and rice, bagasse of cassava and sugar cane, residues of coffee processing industries such as coffee pulp, coffee husk, residues of fruit processing industries such as pomace of apple and grape, waste of pine apple, banana waste, waste of oil processing mills such as coconut oil cake, soybean cake, peanut cake, canola meal and palm oil waste have been mostly used as substrates in SSF processes for the production of bulk chemicals/value added products such as ethanol, single cell protein (SCP), mushrooms, enzymes, organic acids, biologically active secondary metabolites, etc (**12,14,15**).

Production of phytase

As mentioned earlier, phytase can be produced using different methods and the choice of the method could depend up on the substrate and microorganism to be used. Production of phytase from fungi could be carried out using solid-state fermentation, semi-solid fermentation and submerged fermentation.

SSF for the production of phytase

Several microorganisms including bacteria, yeast and many species of fungi have phytase producing ability. Most of the reports on bacteria include *Bacillus amyloliquifaciens, B. subtilis, E. coli, Pseudomonas* sp., *Klebsiella* sp, *Enterobacter, Streptococcus, Lactobacillus amylovorans* etc. With the exception of *Enterobacter* and *B. subtilis,* all these produce intra-cellular phytase enzymes. Among fungi *Aspergillus niger, A. ficuum, A. Flavus, A. carbonarius, A. fumigatus, Mucor* sp, *Penicillium* sp, *Rhizopus oligosporus, R. oryzae* etc are the most important. Fungi produce phytases as extra-cellular enzymes and among these *A. niger* and *A. ficuum* are the most efficient producers of active phytase (**3**). *Saccharomyces cerevisiae* and *Schwaniomyces castelli* are the two yeasts that produce phytase (**3,16**). We used several fungal cultures for the production of phytase (Table 1) under SSF.

Table 1. Microorganisms and Substrates used for the production of phytase in SSF.

Microorganism	Substrate	Reference
Aspergillus ficuum TUB F-1165	Wheat Bran	Sarita *et al.* (**17**)
Rhizopus oligosporus TUB F-1166	Coconut oil cake	Sabu *et al.* (**1**)
Aspergillus ficuum TUB F-1165	Polystyrene beads	Gautam *et al.* (**2**)
Rhizopus oligosporus TUB F-1166	Polystyrene beads	Gautam *et al.* (**2**)
Rhizopus oryzae	Coconut oil cake	Latha, (**18**)
Rhizopus oryzae	Coconut oil cake and sesamum oil cake	Latha, (**18**)
Thermoascus aurantiacus TUB F-43	Ground nut oil cake	Kannan, (**19**)

Phytase production in SSF using coconut oil cake by *Rhizopus oligosporus* showed that this could be effectively used as substrate. Maximal enzyme production (14.29 U/gds) occurred at 30°C with substrate moisture and pH 54.5% and 5.3, respectively after 96 hours of fermentation. It was interesting to note that coconut oil cake did not require any additional nutrients, i.e. inorganic or organic nitrogen source or trace minerals as when supplemented with these even at low concentrations, resulted in inhibition of product formation. Results from this study projected coconut oil cake as a novel substrate for the production of phytase.

Use of a strain of *R. oryzae* with coconut cake was very effective for phytase production. Multifold enhancement in production of phytase was obtained under SSF using coconut oil cake alone or as mixed with sesamum oil cake. Maximal phytase yield of 105.53 U/gds was obtained after optimization of the various physico-chemical and nutrient parameters. Supplementation of additional carbon sources (monosaccharide, disaccharide, polysaccharide) resulted in mixed impact on enzyme synthesis by the fungal culture.

In another study *A. ficuum* TUB F-1165 was used for the production of phytase under SSF using wheat bran. After optimizing the bioprocess conditions *A. ficuum* yielded 49.16 U/gds, which was comparatively higher than many other reports with

A. ficuum. Addition of various nitrogen sources played a key role in the enhancement in enzyme yield. Fig. 2 describes the time course of phytase from *A. ficuum* TUB F-1165 under SSF.

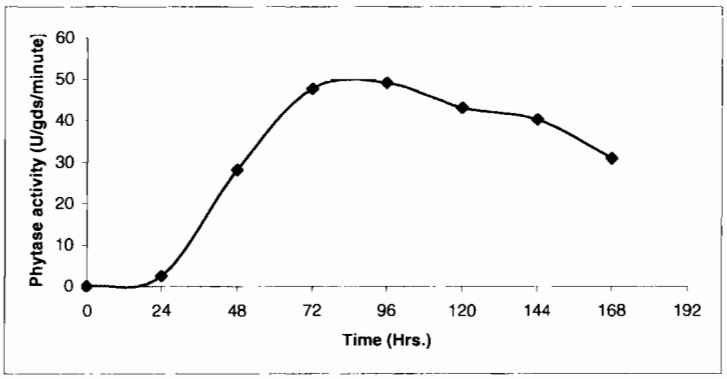


Figure 2: Time course study of phytase production by *Aspergillus ficuum* tub f-116 under SSF.

SSF on inert supports provides many advantages including enzyme with comparatively lesser impurities, which makes down stream processing easier. We used polystyrene beads as inert support for SSF and cultivated *Rhizopus oligosporus* TUB F-1166 and *Aspergillus ficuum* TUB F-1165, which produced 4.52 and 10.07 U/gram dry substrate extra-cellular phytase, respectively. SSF conditions were: substrate pH and initial moisture 6.0 and 58.3%, respectively, incubation temperature 30°C, inoculum size of $1.3x10^7$ spores/5g substrate, incubation period 72 h for *A. ficuum* and substrate pH and initial moisture 7.0 and 58.3%, respectively, incubation temperature 30°C, inoculum size of $1x10^6$ spores/5g substrate, incubation period 96 h for *R. oligosporus*. Fig 3 shows the enzyme production pattern by *A. ficuum* on polystyrene beads over a period of 168 h.

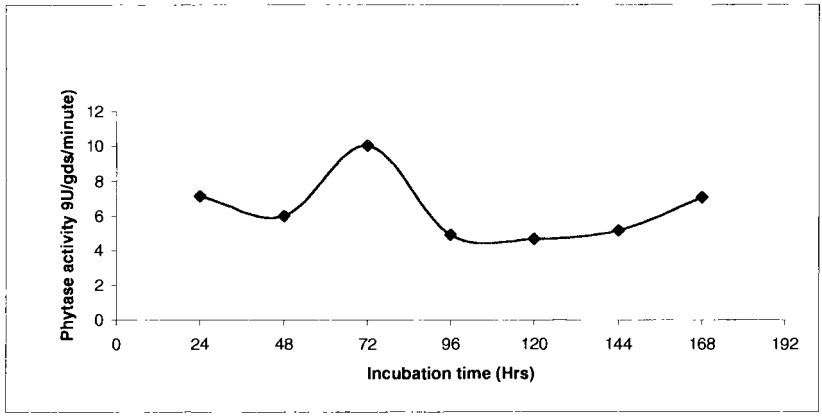


Figure 3: Effect of incubation time on phytase production on inert support by *Aspergillus ficuum* under SSF.

Two thermophilic fungal cultures, *viz. Thermoascus aurantiacus* and an unidentified strain designated as TUB-F-1117 were also used for phytase production under SSF. In these studies wheat bran and groundnut oil cake were used as substrates. An incubation time of 120 h, temperature of 45°C and 64% substrate initial moisture were observed as optimal conditions for maximal enzyme production by TUB-F-1117 using wheat bran. An incubation period of 96 h, temperature of 45°C, 64% substrate initial moisture were observed as optimal conditions for maximal enzyme production by *T. aurantiacus* using ground nut oil cake. Addition of starch as carbon source was found to enhance phytase production in TUB-F-1117 but the same was ineffective for *T. aurantiacus*. It was interesting to note that groundnut oil cake did not require any kind external nitrogen source as all the organic and inorganic N sources used in this study were either ineffective or harmful for the fungal activity of enzyme synthesis (Fig. 4).

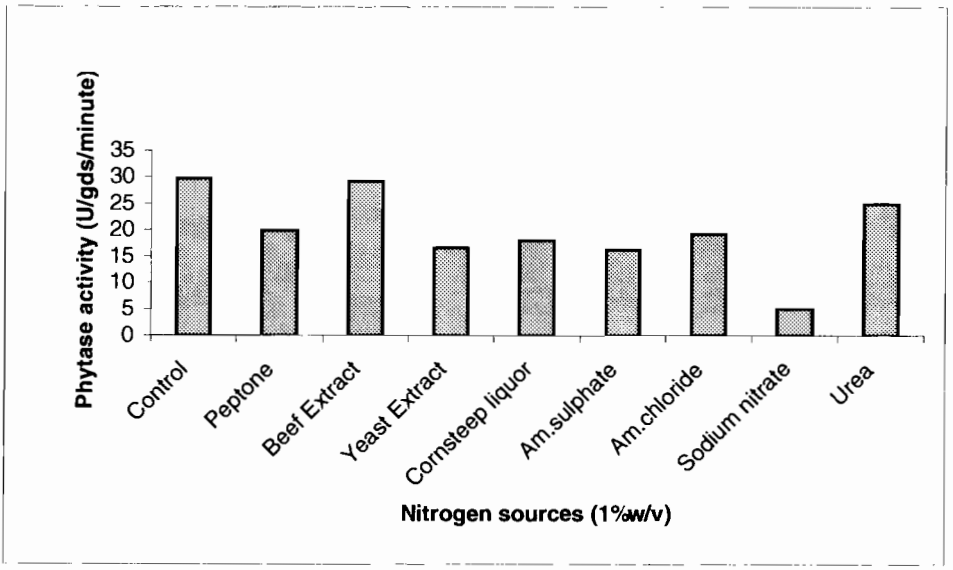


Figure 4: Effect of supplementation of N sources on phytase production by *T. aurantiacus*.

Conclusions

Utilization of agro-industrial residues and other industrial byproducts for production of enzymes such as phytase through solid-state fermentation offers the dual advantage of providing value added feed materials to the growing poultry and swine farms and prevention of phosphorous pollution from farm excreta. Since most of the fungal strains used in the study are non-pathogenic, fermented feed material, which is enriched with protein, could be effectively used as such for feeding. As many industrial feed processing plants run at elevated temperature, phytase from thermophilic strains are also important.

References

1. Sabu A., Sarita S., Pandey A., Bogar B., Szakacs G., Soccol C.R. (2002) *Applied Biochemistry and Biotechnology*, **102**, 251-260.
2. Gautam P., Sabu A., Pandey A., Szakacs G., Soccol, C.R (2002) *Bioresource Technology*, **83** (3), 229-233.
3. Pandey A., Szakacs G., Soccol C.R., Rodriguez A., Soccol V.T. (2001). *Bioresource Technology*, **77**, 203-214.

4. Martinez C., Ros G., Periago M.J., Lopez G., Ortuno J., Rincon (1996). *Food Science and Technology International*, **2**(4), 201-209.

5. Cheriyan M. (1980). *CRC Crit. Rev. Food Sci. Nutr.* **13**, 297-335.

6. Ahmad T., Rassol S., Sarwar M., Haq A.U., Hasan Z.U. (2000). *Animal Feed Science and Technology*, **83**(2), 103-114.

7. Alasheh S., Duvnjak Z. (1995) *World Journal of Microbiology and Biotechnology*, **11**(2), 228-231.

8. Yoon S.J., Choi Y.J., Min H.K., Cho K.K., Kim J.W., Lee S.C., Jung Y.H. (1996) *Enzyme and Microbial Technology*, **18**(6), 449-454.

9. Pandey A. (1992) *Process Biochemistry*, **27**, 109-116

10. Pandey A. (1994) *In*: Pandey A (Ed.), Solid State Fermentation, Wiley Eastern Limited, New Delhi, pp.3-10.

11. Pandey A., Soccol C.R., Mitchell D.A. (2000) *Process Biochemistry*, **35**, 1158-1169.

12. Pandey A., Selvakumar P., Nigam P., Soccol C.R. (1999) *Current Science*, **77**, 149-162.

13. Mitchel D. A., Krieger N., Deidre M., Sturat, Pandey A. (2000) *Process Biochemistry* **35** 1211-1225.

14. Pandey A., Selvakumar P., Soccol C.R., Soccol V.T., Krieger N., Fontana J.D. (1999) *Applied Biochemistry and Biotechnology*, **81**, 35-52.

15. Pandey A., Soccol C.R., Benjamin S., Krieger N., Soccol V.T (1999) *Biotechnology and Applied Biochemistry*, **29**,119-132.

16. Lambrechts C., Boze H., Moulin G., Galzy P. (1992) *Biotechnology Letters*, **14**(1), 61-66.

17. Sarita S., Sabu A., Pandey A., Szakacs G., Soccol C.R. (2001) Extracellular phytase of *Aspergillus ficuum* under solid state fermentation. Presented in the International conference on "New Horizons in Biotechnology"Trivandrum, April 18 – 21. 2001.

18. Latha P. (2001) Extracellular Phytase Production by *Rhizopus oryzae*. MSc Thesis. Guru Ghasidas University. Bilaspur. India.

19. Kannan V. (2001) Biosynthesis of phytase using thermophilic fungi. Msc Thesis. Cochin University of Science and Technology. Cochin. India.

Chapter 4

VALORIZATION OF MOROCCAN OLIVE CAKE USING SOLID STATE FERMENTATION

M. ISMAILI-ALAOUI[1], M. KAMAL[2], A. KADEMI[3], A. MORIN[3*], S. ROUSSOS[4], A. HOUDE[3]

*[1] I.A.V. Hassan II, Laboratoire de Biotransformation, B.P. 6202, Rabat, Maroc; [2] Département des Sciences Biologiques et Agronomiques, Faculté des Sciences et Techniques Beni Mellal, B.P. 523, Maroc ; [3] Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Blvd West, St-Hyacinthe, Quebec, Canada, J2S 8E3; [4] Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119, IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925, 163, Avenue de Luminy, F-13288 Marseille Cedex 9, France; *Present address: Imperial Tobacco Canada Limited., 3810 St-Antoine street, Montreal, Quebec, Canada, H4C 1B5*

Abstract

Three kinds of olive cakes from Moroccan area were used as substrates for solid state fermentation: 1) Crude Maasra Cake (CMaC) from a traditional unit of olive oil extraction (maasra), 2) Crude Exhausted Cake (CEC) from a semi-traditional unit, 3) Crude Mill Cake (CMC) collected from an industrial unit of olive oil extraction. Biochemical characterization of these olive cakes showed that these raw materials were rich in fibers (cellulose, hemi-cellulose and lignin), 46.8-55.8%. Fat content in CEC was lower (6.9%) than in CMaC (18.4%) and CMC (17.0%). Sixteen fungal strains were isolated from samples collected from olive cakes mainly from CMaC (11 strains). Solid state fermentation was used as a tool to screen these strains in terms of protein enrichment and fiber degradation. The best performances were obtained by the strain *Aspergillus niger* S13. After 48h of fermentation, the maximal real proteins increase (RPI), actual loss in cellulose, actual loss in hemi-cellulose and actual loss in lignin were 9.3%, 19.4%, 22.1% and 4.3%, respectively.

Keywords: Solid state fermentation, olive cake, fungal strains, *Aspergillus niger*, protein enrichment.

Introduction

Solid state fermentation is defined as any fermentation that takes place on solid or semi solid substrate or that occurs in a nutritionally inert solid support which provides to the microorganism access to nutrients, and other microbial products (**1**). Such processes have been employed in the production of useful metabolites such as enzymes (**2**), aroma (**3**), preservation of food (**4**), and proteins (**5**). Protein enrichment of agro-industrial residues makes them suitable for animal feed. Many

New Horizons in Biotechnology, 35-41, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

studies were reported on protein enrichment of different agro-industrial residues, which were well documented by Pandey (**6**).

Morocco has an olive oil industry that generates important quantities of olive cake rich in organic matter. The by-products of the olive industry are estimated to be 200,000 tons/year (**7**). A large part of these raw materials are scarcely exploited if at all, and their elimination in nature is a threat to the environment.

Cordova *et al.* (**7**) studied the lipase production by thermophilic fungal strains grown on olive cake. To our knowledge, protein enrichment of this raw matter has not been yet studied.

In this work, we report on the characterization of three kinds of olive cakes, the isolation of fungal strains from natural biotopes and their culture on olive cakes for protein enrichment.

Materials and Methods

Substrates

Three kinds of olive cakes from Moroccan area were used as substrates for solid state fermentation: 1) Crude maasra cake (CMaC) from a traditional unit of olive extraction (maasra). 2) Crude exhausted cake (CEC) from a semi-traditional unit. 3) Crude mill cake (CMC) collected from an industrial unit of olive oil extraction.

Biochemical characterization

Biochemical characterization of olive cakes was determined according to Cordova *et al.* (**7**). The dry matter and relative humidity were determined by incubating the substrate at 105°C for 24 hours. The pH value of ten-fold diluted samples in distilled water was assayed by a digital pH model 215 (Denver Instrument Company Ltd, Norfolk, UK). The Kjeldhal method according to AFNOR Norms (**8**) was used for the determination of total and mineral nitrogen, respectively. For the chemical and physical characterization, the olive cake samples were crushed and sieved to a particle size lower than 1mm. Ashes were determined according to the AOAC method (**9**). Fat was determined according to Soxhlet method (**10**). The parietal constituents, particularly the total fibers (NDF), the ligno-cellulosical fibers (ADF) and the lignin (ADL) were determined by the Van Soest method (**11**). Each analysis was done in triplicate.

Isolation of fungal strains

The isolation of filamentous fungi strains was done from three kinds of olive cakes. Ten grams of each type of olive cake were diluted in 90 ml distilled water with two drops of Tween 80. The solution was shaken for 10 minutes. Then, 0.1 ml was spread on a Petri plate containing Potato Dextrose Agar (PDA) medium (Difco Laboratories, Detroit, USA) supplemented with chloramphenicol (1g/l of medium). Plates were incubated at 25°C. Mycelium morphology and spores color were observed directly under optical microscope. Strains that presented pure mycelia

were isolated in PDA medium. The isolated strains were stored as spore suspensions on PDA medium or on sand.

Culture medium and solid state fermentation

The culture medium used contained in g/l Na_2HPO_4, 3.59, KH_2PO_4, 3.59, $(NH4)_2SO_4$, 6.66, Mg SO_4, 2.9, $CaCl_2$, 2.9 and was sterilized at 121°C for 15 min. Inoculum was produced from purified strains in Petri dishes containing 30 ml of PDA medium and incubated at 28°C for 7 to 10 days. The spores were harvested using a platinum loop in 10 ml sterile distilled water containing 2 drops of tween 80. After dilution, spores were counted using Malassez cell. Solid state fermentation was performed in laboratory columns (4 cm in diameter and 20cm in length with an effective reaction volume of 250 mL) as described by Raimbault (**12**). Columns contained two different compartments, one serving as a humidifying chamber and one reactor column. The substrate previously crushed and sieved was supplemented with culture medium allowing 35% as moisture content and sterilization was done at 121°C for 15 min. The moisture content was then adjusted to 65-70 % by addition of spore suspension containing $2x10^7$ spores/g dry weight of substrate. The inoculated substrate was transferred (30 g per unit) into sterile reactor columns. The whole device was immersed in a steam bath adjusted at the incubating temperature of 27°C. The air-flow was 15 ml/min/column. Respiratory activity was assessed by estimating O_2 consumption and CO_2 production using a chrompack CP 9001 equipped with an electrovalve and a catharometric detector. It was used as an indirect measure of the growth of different strains.

Performance indexes

Four performance indexes were used to evaluate olive cake fermentation: real protein increase (RPI), the actual loss in cellulose, the actual loss in hemi-cellulose and the actual loss in lignin.

Real protein increase (RPI) was calculated according to Durand and Chereau (**13**). This method takes weight loss into account during the culture. The RPI expresses the amount of protein (g) produced per 100 g of initial dry mater.

$$RPI = \frac{M(t) \times Pn(t) - M(i)Pn(i) \times 100}{Mi}$$

On the other hand, the actual loss in cellulose, the actual loss in hemi-cellulose and actual loss in lignin were determined by the gravimetric method of Van Soest (**11**) taking into the count the weight loss during the culture.

$$\text{actual loss in hemi-cellulose} = \frac{M(i)HC(i) - M(t) \times HC(t) \times 100}{Mi \times HC(i)}$$

$$\text{actual loss in lignin} = \frac{M(i)ADL(i) - M(t) \times ADL(t) \times 100}{Mi \times ADL(i)}$$

Where: M(i) is the weight (kg of dry mater) at the beginning of culture; Pn(i) is the initial protein content (% of dry matter); M(t) is the weight (kg of dry matter) at

time t ; Pn(t) is the protein content at time t (% of dry matter); HC(i) is the initial hemi-cellulose content (% of dry matter); HC(t) is the hemi-cellulose content (% of dry matter) at time t ; ADL(i) is the initial lignin content (% of dry matter); ADL (t) is the lignin content (% of dry matter) at time t.

Results and discussion

Biochemical characterization of the olive cakes

Biochemical composition of olive cakes was studied before the fermentation to assess their potential as fermentation substrates and to check the difference between the three kinds of cakes. Dry, organic and nitrogenous matter, crude ashes, fat, hemi-cellulose, cellulose and lignin contents were determined.

All the cakes tested contained about 91% of organic matter and 5% of nitrogenous matters (Table 1). Lignin content in all cakes was high (24-29%). This result showed that olive cake is a high lignin by-product. Fat content of Maasra cake (CMaC) and Mill cake (CMC) were 18.4% and 17% respectively while Exhausted cake (CEC) contained only 7%. This low value is explained by the fact that Exhausted cake is obtained after a second oil extraction from Maasra cake with organic solvent (usually hexane). Thus, the high fat content of CMaC and CMC makes these cakes suitable substrates for the culture of lipase/esterase producing microorganisms.

Table 1. Biochemical composition of the three kinds of olive cakes.

Parameters	Maasra cake CMaC	Mill cake CMC	Exhausted cake CEC
Dry matter	94.70	93.02	97.10
Components		(%) Dry Matter	
Crude ashes	9.05	7.97	8.90
Organic matter	90.95	92.03	91.10
Nitrogenous matter	5.25	5.02	5.18
Fat	18.41	16.98	6.88
Hemi-cellulose	14.53	15.74	17.08
Cellulose	8	10.58	9.78
Lignin	24.25	25.48	28.93

In a previous work, Cordova *et al.* (**7**) showed the potent role of olive cake as a substrate for lipase production. High content of fibers showed that, like many agricultural residues (**14**), olive cake is not attractive as an animal feed.

Isolation of fungal strains

To isolate fungal strains, samples from all cakes were diluted in sterile water containing Tween 80 and the spores collected were spread on Petri PDA medium containing Chloramphenicol to prevent a bacterial growth.

Sixteen non-identified strains were isolated from 30 samples tested. Eleven strains (S3, S4, S10, S11, S12, S13, S14, S15, S16, S17, S18) were isolated from maasra cake and five other (S5, S6, S7, S8, S9) were isolated from the exhausted cake. However, no fungal strain was isolated from the mill cake. The results suggested that the maasra cake was more favorable to contamination by microorganisms than the other olive cakes, probably due to the outdoor storage of this kind of cake.

Olive cake fermentation

The cakes were soaked with culture medium and inoculated with spores from the isolated strains. The inoculated cakes were transferred into columns and incubated at 27°C for 48 hours. Dry matter and pH were measured before and at the end of fermentation and the performance indexes were calculated.

Dry matter evolution

The loss in dry weight at the end of fermentation depended on the strains and the substrate tested. When the strain S13 was grown on the maasra cake dry weight decreased from 94.7 to 85.1 %. The growth of strain S10 on exhausted cakes resulted in the lowest dry weight loss (from 97.1 to 93.6%).

pH evolution

The pH decreased from 6.0 to 4.0 at the end of fermentation for all the strains tested. This acidification may be explained by the production of organic acids during the fermentation (**15**).

Real protein increase (RPI)

The RPI (real protein increase) represents the protein quantity produced in grams per 100 g of substrates. This parameter takes into account the initial protein content and the loss in dry matter during fermentation. The RPI obtained on all cakes tested ranged from 0.9% (strain S16 on CEC) to 9.3% (strain S13 on CmaC) (Fig. 1A). RPI values obtained on all cakes tested were slightly different except for the strains S10, S17 and S18 whose RPI values obtained on CMaC were much higher than those on CEC and CMC. The RPI obtained by the strain S13 was similar to that obtained by *Aspergillus terreus* grown on sugarcane trash (**5**) and that obtained when *Rhizopus oryzae* was grown on cassava (**16**). *Neurospora sitophila* increased the protein content of wheat bran from 13% to 30% (**17**).

Among all the strains tested, 13 strains showed a loss in hemi-cellulose lower or equal to 10% (Fig. 1B). Strains S8, S13 and S18 exhibited high values of hemi-cellulose loss i.e. 20.3%, 22.1% and 23.2% respectively on CEC.

The highest loss in cellulose observed was obtained by strains S8 and S13 on CMaC, 17.1% and 19.4%, respectively (Fig. 1C).

The results suggested that these strains produced an appreciable amount of cellulolytic enzymes. A good degradation of lignin was observed only in cultures of strains S8 and S13: 3.1% and 4.3%, respectively (Fig. 1D). For all other strains, the loss in lignin was very low. Probably these strains did not produce ligninolytic

enzymes. The performance of strains S8 and S13 was lower than that of strain *Polyporus* sp. which degraded 25% of lignin content in bagasse (**18**). In conclusion, the present work clearly indicated that olive cake was a suitable substrate for protein enrichment. Also, cellulose and hemi-cellulose contents in this substrate were reduced by the culture of strains S8 and S13.

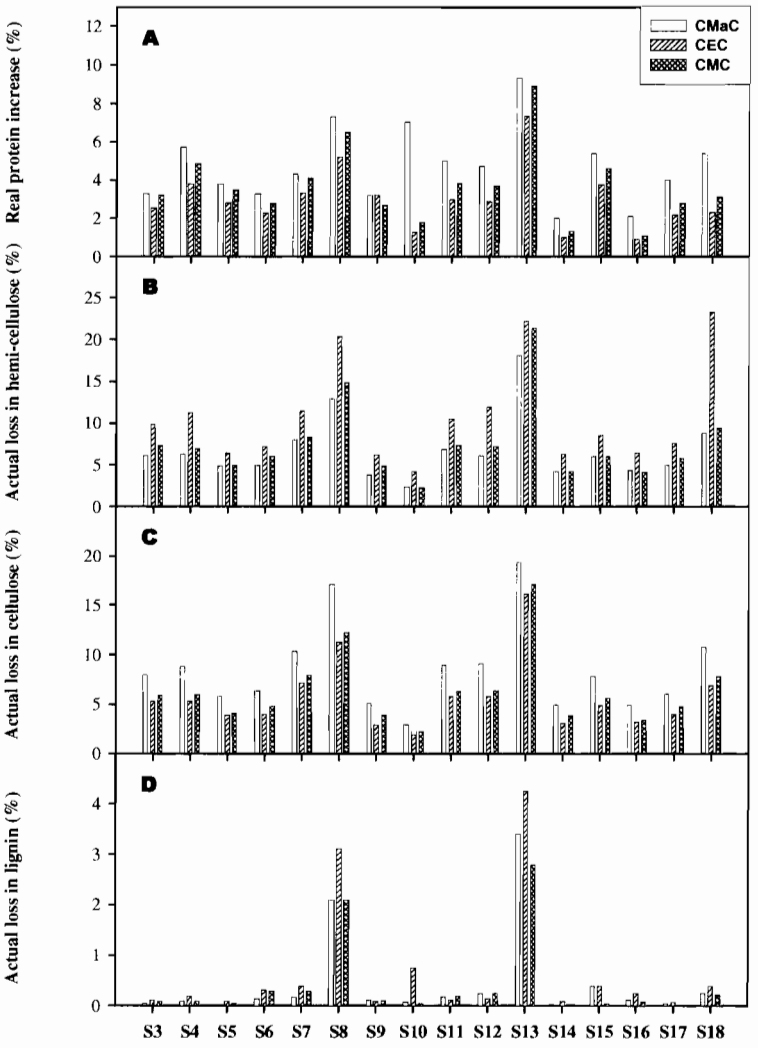


Figure 1. Real Protein Increase (A), actual loss in hemi-cellulose (B), actual loss in cellulose (C) and actual loss in lignin (D) obtained by fungal strains grown on olive cakes.

The strain S13 was identified as *Aspergillus niger*. Digestibility estimated *in sacco* incubation in the rumen of sheep was very high (unpublished data). The results suggested that olive cake enriched with microbial proteins could be a good alternative for production of animal feed. It would therefore be interesting to optimize the protein enrichment of olive cake and to use the potential of the S13 strain to produce other secondary metabolites like aroma compounds and organic acids.

Acknowledgements

Financial support from the International Development Research Center (Canada) and AUPELF-UREF are greatly acknowledged.

References

1. Aidoo K.E, Hendry R., Wood B.J.B. (1982) *Adv. Appl. Microbiol.* **25**, 201-230.
2. Pandey A., Selvakumar P., Soccol C.R., Nigam P. (1999) *Current Science* **77**, 149-162.
3. Christen P., Meza J.C., Revah S. (1997) *in* Advance in solid state Fermentation, Roussos S., Lonsane B.K., Raimbault M. and Viniegra-Gonzalez G. (eds)., Kluwer Academic Publishers, Dordrecht, pp.367-377.
4. Perraud-Gaime I., Roussos S. (1997) *in* Advance in solid state Fermentation, Roussos S., Lonsane B.K., Raimbault M., Viniegra-Gonzalez G. (eds.), Kluwer Academic Publishers, Dordrecht, pp.193-208.
5. Gonzalez-Blanco P., Saucedo-Castaneda G., Viniegra-Gonzalez G. (1990) *J. Ferment. Bioeng.* **70**, 351-354.
6. Pandey A. (1992) *Process Biochem.* **27**, 109-117.
7. Cordova J., Nemmaoui M., Ismaili-Alaoui M., Morin A., Roussos S., Raimbault M., Benjilali B. (1998) *J. Mol. Catal. B: Enzym.* **5**, 75-78.
8. Norme AFNOR (1981) T90-110
9. Association of Official Agricultural Chemists (AOAC) (1984) Official methods of Analysis, (14th ed). AOAC, Washington D.C., USA.
10. Soxhlet A. (1968) Normes Afnores, NF-V-04-403.
11. Van Soest P.J. (1982) *in*: Nutritional Ecology of the Ruminant, Van Soest P.J. and Books Inc, Oregon. 97330
12. Raimbault M. (1980) Thèse d'état. Université Paul Sabatier, Toulouse, France. 291p.
13. Durand A., Chereau D. (1988) *Biotechnol. Bioeng.* **31**, 476-486.
14. Haddadin M.S., Abdulrahim S.M., Al-Khawaldeh G.Y., Robinson R.K. (1999) *J. Chem. Technol. Biotechnol.* **74**, 613-618.
15. Raimbault M., Alazard D. (1980) *Eur. J. Appl. Microbiol.* **9**, 199-209.
16. Daubresse P., Ntibashirwa S., Gheysen A., Meyer J.A. (1987) *Biotechnol. Bioeng.*, **29**, 962-968.
17. Shojaosadati S.A., Faraidouni R., Madadi-Nouei A., Mohamadpour I. (1999) *Resour. Conserv. Recycl.* **27**, 73-87.
18. Nigam P. (1990) *Enzyme Microb. Technol.* **12**, 808-811.

Chapter 5

GROWTH KINETICS AND ESTERASE/LIPASE PRODUCTION BY THERMOPHILIC FUNGI GROWN IN SOLID STATE FERMENTATION ON OLIVE CAKE

A.KADEMI[1], A. MORIN[1]*, M. ISMAILI-ALAOUI[2], S. ROUSSOS[3], J. BARATTI[4], A. HOUDE[1]

[1] Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Blvd West, St-Hyacinthe, Quebec, Canada, J2S 8E3; E-mail: houdea@em.agr.ca ; [2] I.A.V. Hassan II, Laboratoire de Biotransformation, B.P. 6202, Rabat, Morocco ; [3] Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119, IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925, 163, Avenue de Luminy, F-13288 Marseille Cedex 9, France ; [4] Laboratoire de Biocatalyse et Chimie Fine, Faculté des Sciences de Luminy, Case 901, 13288 Marseille cedex 9, France. ; [v] Present address: Imperial Tobacco Canada Limited, 3810 St-Antoine street, Montreal, Quebec, Canada, H4C 1B5

Abstract

Three thermophilic fungal strains *Rhizopus homothallicus* 13a, *Rhizomucor pusillus* S5 and *Rhizomucor pusillus* S8 were grown in solid state fermentation on sugar cane bagasse-olive cake mixture (50% each) at 47°C for 48 h. Humidity and pH increased during fermentation, from 5.1-5.2 to 5.5-6.1 and from 64% to 69% respectively. For all strains, O_2 uptake rates reached a maximum of 1.7-2.2 ml/h/g IDM (initial dry matter) at an early stage of cultivation (7.5-9 h) and high respiratory activities (μr) were obtained (1.2-1.4 h^{-1}). The maximal O_2 uptake at 48h of culture was 28.1, 31 and 33.2 ml/g IDM for strains 13a, S5 and S8 respectively. All strains produced lipolytic activities, which varied from 15 U/g IDM for strain S8 to 47 U/g IDM for strain 13a when olive oil was used as enzyme substrate. The lipolytic activity of crude enzyme extracts from strain S8 was specific of mid-chain length fatty acid esters. There was evidence of lipase activity (active on long chain fatty acid esters) and esterase activity (active on short chain fatty acid esters) for strain 13a. The latter one was present at 24h but not at 48h of culture. The specificity pattern of strain S5 extract looked similar to that of strain 13a. After incubation in *n*-heptane for 8 days at 37°C, the enzyme extracts retained 65%, 66% and 84% of initial activity for strains 13a, S5 and S8 respectively.

Keywords: *Rhizopus homothallicus, Rhizomucor pusillus*, thermophilic fungi, solid state fermentation, lipase, esterase, organic solvent.

New Horizons in Biotechnology, 43-55, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

Solid state fermentation (SSF) processes involve the growth and metabolism of microorganisms on moist solid substrates in the absence or near absence of free-flowing water (**1**). These processes offer many advantages over submerged cultures (SMC) mainly simplicity of fermentation medium, reduced energy demand, easier scale-up of processes, greater product yields and easier control of contamination due to the low moisture level in the system (**2**).

Filamentous fungi have a strong capability to grow in the absence of free water (**1**) since these conditions are similar to their natural habitat (**3**). Therefore, they are often most suited to SSF for the production of several valuable metabolites. Many kinds of enzymes are produced in SSF: pectic enzymes (**4,5**), amylases and glucoamylases (**6,7**) and cellulases (**8,9**).

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are enzymes with great potential for biotechnological applications such as esters synthesis as flavors for the food industry (**10**), the modification of physico-chemical properties of triglycerides in the fat and oil industry (**11**) and for the preparation of optically pure bioactive molecules for the pharmaceutical industry (**12,13**).

Despite these potential applications, few studies have been reported about esterases and lipases production in SSF using filamentous fungi (**14 -20**).

In a previous work, Cordova et *al.* (**21**) reported the production of lipase by the thermophile fungi *Rhizomucor pusillus* A16 and *Rhizopus rhizopodiformis* 13a grown on sugarcane bagasse-olive cake mixture.

The aim of this study was to investigate the esterase/lipase production and the respiratory activity of three filamentous fungi strains grown on a sugarcane bagasse-olive cake mixture. The specificity and the stability of the crude enzyme extracts in *n*-heptane were also investigated.

Materials and Methods

Chemicals

Bactopeptone and Potato Dextrose Agar (PDA) were purchased from Difco Laboratories (Detroit, USA). Tris and KH_2PO_4 were purchased from ACP Chemicals (Montreal, Canada), $NaNO_3$ and $CaCl_2$ were obtained from BDH Chemicals (Montreal, Canada), $MgSO_4$ was purchased from (Fisher Scientific Nepean, Canada), *p*-Nitrophenol was obtained from American Chemicals (Montreal, Canada), 2-propanol was purchased from MAT (Beauport, Canada). Pyridine was from Omega Chemical company (Levis, Canada). Cupric-acetate-1-hydrate, gum arabic, heptane, isooctane, oleic acid (99%), olive oil, *p*-nitrophenyl esters, polyvinyl alcohol, tricaproin and Tween80 were obtained from Sigma (St-Louis, USA).

Microorganisms

Three thermophilic fungal strains were used in this study: strain *Rhizopus rhizopodiformis* 13a was obtained from ORSTOM culture collection (Montpellier, France) (**21**) and identified as *Rhizopus homothallicus* by the National Fungal Identification Service NFIS (Ottawa, Canada). Strains S5 and S8 were obtained from IAV (Rabat, Morocco) and identified by the NFIS as *Rhizomucor pusillus*. The strains were maintained on PDA agar slants at 4°C and subcultured every 4 months.

Substrate pretreatment

Sugarcane bagasse was obtained from the sugar refinery Dar El Gadari (Morocco) and olive cakes were obtained from various traditional olive oil extraction plants (Maâsra in Morocco). Both substrates were milled to 2.0 and 0.71 mm particles size range. Sugarcane bagasse was washed with 15 L of distilled water and then both substrates were dried at 60°C for 24 h for olive cake and 2-3 days for sugarcane bagasse before storage at 4°C. Before use, a mixture of these substrates was prepared (50:50) and incubated at 110°C for 15-21 h to reduce contaminants. The chemical composition of these substrates was as described by Cordova *et al.* (**21**).

Culture medium and conditions

The composition of the medium used was (/L): Bactopeptone 50 g, glucose 20 g, KH_2PO_4 1 g, $NaNO_3$ 1 g and $MgSO_4$ 0.5 g. Glucose was prepared separately (11.2% (w/v) aqueous solution) and sterilized at 121°C during 15 min.

The culture medium without glucose was prepared as a 2x medium and subsequently added to substrates mixture allowing 35% as moisture content. Sterilization was done at 121°C for 15 min. After cooling, the moisture content was increased until 64-66% by addition of spores suspension, glucose solution and sterile distilled water. Spores were collected by adding Tween 80 solution (0.1% (w/v)) to a one-week-old PDA agar culture followed by filtration through 0.19 mm filter. The inoculation rate was $2x10^7$ spores/g dry matter. The initial pH was 5.1-5.2.

The inoculated solid medium was transferred (26 g per unit) to sterile glass incubators (diam. 3 cm, length 19 cm) as described by De Araujo *et al.* (*22*) with little modification. The solid state fermentation was monitored in a similar apparatus as described by Cordova *et al.* (**21**). The incubators were maintained at 47°C and aerated with 26 ml/min water-saturated air. Growth was monitored by continuous analysis of carbon dioxide and oxygen by infrared and electrochemical sensors respectively using 3600 model CO_2-O_2 analyzer (Illinois Instruments, Ingleside, USA) coupled to automatic sampling. Before injection, outer air was dried using silica gel. Non-inoculated solid medium was used as a control and incubated in the same conditions. Each culture was done in triplicates.

Enzyme Assay

Spectrophotometric assay with triglycerides as substrate. The enzyme activity was measured using the formation of copper soaps for detection of free fatty acids according to Kwon and Rhee (**23**) with some modifications: The substrate solution contained olive oil or tricaproin (0.164 g/mL) emulsified with domestic blender during 15 min at 4°C in Tris-HCl 50 mM pH 8.7 containing 10% (w/v) gum arabic and 9.1 mM $CaCl_2$. Cupric-acetate-1-hydrate aqueous solution (50 mg/mL) adjusted to pH 6.1 with pyridine, was used as copper reagent.

The reaction was started by adding fermented substrates (0.25-0.5 g) to 10 ml of substrate emulsion preheated at 47°C. Enzyme reaction was carried out under shaking for 10-15 min and the reaction was stopped with 12N HCl. Fatty acids were extracted with 5 ml of isooctane and vigorous vortexing for 1 min 30 s. After a 10 min incubation in boiling water, phase separation was achieved by centrifugation (7810 g, 30 min). The organic phase was collected and added to 1 ml of copper reagent. After vigorous vortexing for 1 min 30 s., the absorption was measured at 715 nm versus a blank containing an autoclaved fermented substrate. Each measure was done in triplicate.

For calibration, pure oleic acid dissolved in isooctane to get final concentrations from 0 µmol/ml to 10 µmol/ml and added to copper reagent. One unit was defined as the amount of enzyme, which caused the release of 1 µmol oleic acid per minute under the given conditions.

Spectrophotometric assay with p-nitrophenyl esters as substrates. Activity was assayed by measuring the rate of hydrolysis of p-nitrophenyl esters at 37°C. One volume of 16.5 mM of substrate in 2-propanol was mixed with 9 volumes of 50 mM Tris-HCl pH 8.0, containing 0.25% (w/v) polyvinyl alcohol and 0.174% (w/v) $CaCl_2$. To 180 µL reaction mixture equilibrated at 37°C in a microplate well were added 20 µL of the enzyme solution. The absorbance was read at 405 nm using a microplate reader PowerWave X (BIO-TEK INSTRUMENTS, Vermont, USA) against a blank without enzyme and monitored continuously at 405 nm. The concentration of liberated *p*-nitrophenol (pNP) was calculated using an extinction coefficient of 8.6 10^6 cm^2/mol. This value was determined using standard solutions of pNP. For each assay, three different enzyme quantities were tested and activity was calculated from the slope of the curve of absorbance variation per min against enzyme amount. One enzyme unit was the amount of enzyme liberating one µmole of pNP per min in the conditions used. Fatty acid specificity was studied by the measure of hydrolysis of *p*-nitrophenyl esters from pNPC2 to pNPC16 as described above. This experiment was done in duplicate.

Assay techniques

The relative humidity and dry matter were determined by drying samples of solid medium (5 g) at 110°C for 24 h. The pH value of 10 fold diluted samples in distilled water was assayed by using a 718 Stat Titrino (Metrohm, Herisau,

Switzerland). Protein content of crude enzyme extracts was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Instructions 23225).

Crude enzyme extract preparation

At the end of the culture, the contents of three columns were pooled and diluted 5 fold in Tris-HCl 50 mM pH 8.7. The matter was ground with a domestic blender to obtain fine powders (6 cycles: 1 min of mix and 2 min of pause) and diluted 2-fold before mixing with an Ultra-Turrax T25 homogenizer (Janke and Kunkel) (6 cycles: 1 min of mix and 1 min of pause). The supernatant was collected by centrifugation (14,300 g, 30 min, 4°C) and dialyzed overnight against Tris-HCl 50 mM pH 8.7 and finally lyophilized and stored at 4°C until use.

Stability in organic solvent

Thirty mg of crude enzyme extract with known activity were mixed with 10 ml of *n*-heptane in 40 ml glass bottles. The bottles were shaken (250 rpm) at 37°C during 8 days. The powder was collected by filtration, washed with acetone at -20°C and dried with P_2O_5. Each experiment was done in triplicates. The residual activity was determined by the measure of the hydrolysis of *p*-nitrophenyl laurate in standard conditions.

Data analysis

The O_2 data of the cultures were fitted with Tablecurve program. The correlation coefficients obtained, for the mean values of all the cultures, were between 0.93 and 0.98 for O_2 uptake rates and between 0.96 and 0.98 for cumulatives O_2 uptake. The respiratory activity (μr) values were directly calculated from the crude data of each culture and the mean values were calculated with standard deviation less than 7%.

Results and discussion

Growth kinetics of strains

Strains were grown in SSF on sugar cane bagasse-olive cake mixture (50% each) at 47°C for 48h as described in the Materials and Methods section. Humidity and pH were determined at initial and final times of culture. In this study, O_2 consumption was used as an indirect measure of the growth of the strains. Oxygen values (%) obtained were used to calculate the O_2 uptake rates from which, after integration, cumulative O_2 uptake was obtained.

At the end of cultures, medium pH increased slightly from 5.1 to 5.5 in strains 13a and S8 cultures while it reached 6.1 in the culture of strain S5. Relative humidity increased slightly from 64-66% to 68-69% at 48 h of culture. The figure 1 showed the growth kinetics of the strain *Rhizopus homothallicus* 13a on bagasse-olive cake mixture. The accelerated growth phase started after a 3h lag phase.

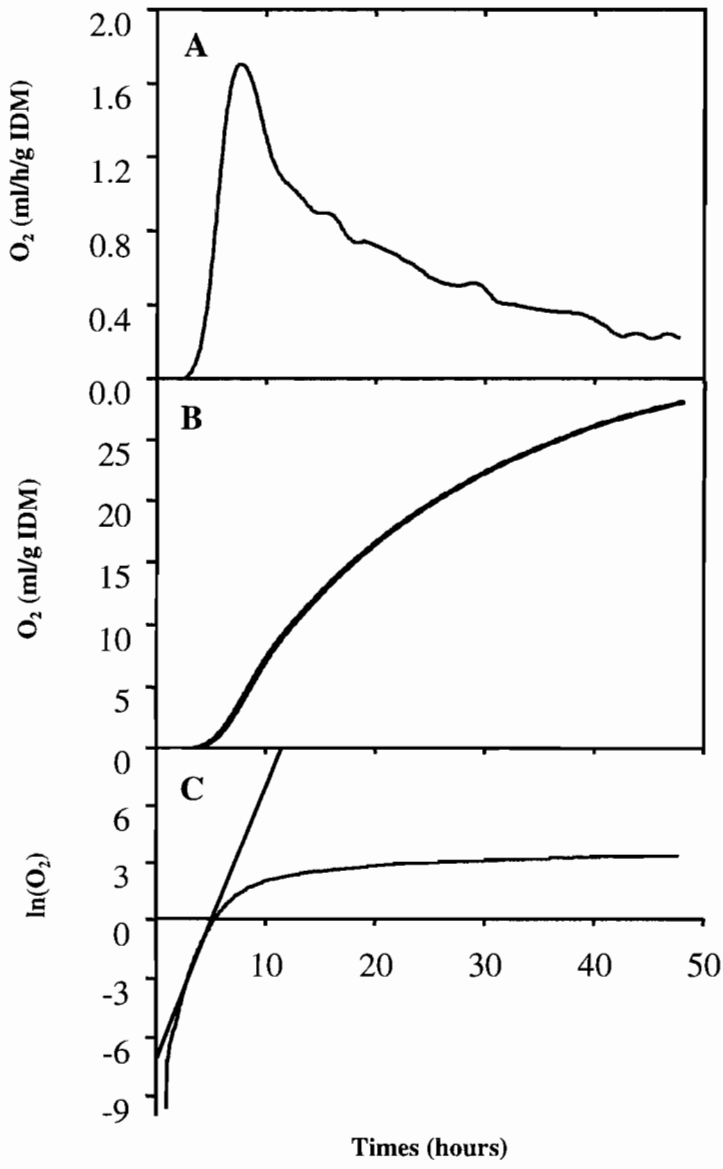


Figure 1: Growth kinetics of the strain *Rhizopus homothallicus* 13a grown in SSF on sugar cane bagasse-olive cake mixture. (**A**) O_2 uptake rate, (**B**) cumulative O_2 uptake and (**C**). Respiratory activity (μr) measurement.

Maximum O_2 uptake rate (1.7 ml/h/g IDM) was obtained at 7.5-8.0 h of incubation (Fig. 1A). At 48 h of culture, O_2 uptake reached 28.1 ml/g IDM (Fig. 1B).

Respiratory activity (μr) calculated at the beginning of incubation (0-15 h) according to Saucedo-Castaneda *et al.* (**24**) from O_2 data was 1.4 h^{-1} (fig 1C). This high value showed a fast growth of this strain in the conditions tested. This value was higher than those obtained with mesophilic strains like *Aspergillus niger* (**25**) and *Schwanniomyces castellii* CBS 2863 (**24**).

Strains S5 and S8 exhibited metabolic activities similar to strain 13a (Table 1). Maximal O_2 uptake rates were 2.0 and 2.2 ml/h/g IDM for both strains. The overall O_2 uptakes were slightly higher than that of the strain 13a. However, the respiratory activities were slightly lower than that obtained from the strain 13a.

Table 1: Growth parameters of strains S5 and S8 grown in solid state fermentation on sugar cane bagasse-olive cake mixture.

Growth parameters	Strains	
	S5	S8
Lag phase duration (H)	3.5-4.0	3-4
Maximal O_2 uptake rate (ml/h/g IDM)	2.0	2.2
Max. Time (H)*	8.0-9.0	7.5-8.0
Cumulative O_2 uptake (ml/g IDM)	31	33.2
Respiratory activity (μr)	1.3	1.2

* refers to time of maximal O_2 uptake rates.

The three strains showed the same ability to grow on a bagasse-olive cake mixture. Growth of the strains was fast despite the high temperature of culture (47°C) and the low moisture content (initial humidity was 64%).

Production of Lipolytic activities

Cordova *et al.* (**21**) reported that strain 13a, when grown on bagasse-olive cake mixture, produced a lipolytic activity assayed by the measure of olive oil hydrolysis. This result showed the occurrence of a lipase in the extracts but did not inform about esterases since they could not act on olive oil. In this study, the tricaproin (C6:0) was also used as enzyme substrate. Tricaproin is a partially soluble substrate and could be hydrolysed by both esterases and lipases. At 48h of culture, fermented matter was used as enzyme source and the activity was assayed at 47°C. The results are shown in Figure 2.

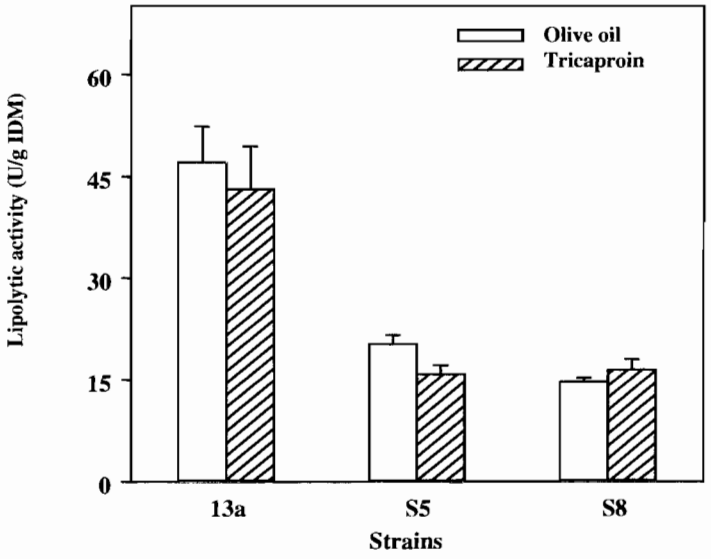


Figure 2: Lipolytic activity on olive oil and tricaproin of *Rhizopus homothallicus* 13a, *Rhizomucor pusillus* S5 and *R. pusillus* S8 grown in SSF on sugar cane bagasse-olive cake mixture at 47°C during 48h.

All strains produced lipolytic activities on bagasse-olive cake mixture. When olive oil was used as enzyme substrate, the enzyme activity was 15, 20 and 47 U/g initial dry matter (IDM) for strains S8, S5 and 13a respectively. Cordova *et al.* (**21**) reported that the lipase production by strain 13a was maximal at 24 h of culture (80 U/g IDM). When tricaproin (C6:0) was used as enzyme substrate, almost similar activities to those on olive oil were obtained. The ratios of activity on olive oil versus activity on tricaproin were 1.1, 1.3 and 0.9 for strains 13a, S5 and S8 respectively. Usually, lipase activity is maximal on long chain fatty acid esters and insoluble like olive oil (**26**) and esterase action is maximal on short chain fatty acid esters (carbon number from C2 to C5). In the present case, the similarity of activity on olive oil and tricaproin suggested the presence of several enzymes (esterases and/or lipases) responsible of the lipolytic activity of the strains or the occurrence of one enzyme with maximal activity on substrate other than olive cake and tricaproin.

The lipolytic activity obtained by strain 13a was higher than lipase production by *Aspergillus oryzae* (0.8 U/g of wet substrates) (**27**) and *Candida rugosa* on rice bran (27 U/ g) (**28**) and lower than the lipase production by *Aspergillus niger* on gingelly oil cake (363.6 U/g of dry substrate) (**19**) after 72 h of culture. *Rhizopus delemar* produced 96 U/g of Dextrin at 15 h of culture (**17**).

Substrate Specificity

Substrate specificity was studied to check whether lipases and/or esterases were responsible of lipolytic activities of the strains. Crude enzyme extracts were prepared as described in the Materials and Methods section and used as enzyme source. Instead of the triglycerides assay, activity was assayed with *p*-nitrophenyl esters because, with the former one, activity on triglycerides shorter than tricaproin (C6:0) could not be measured with a good reproducibility (**23**). A preliminary study showed a good correlation between these assays (results not shown).

Esters of different fatty acids from acetic acid (pNPC2) to palmitic acid (pNPC16) were used as enzyme substrates and the reaction rates were assayed under standard conditions.

Ratios of activity on a long chain fatty acid ester (*p*-nitrophenyl palmitate) versus activity on a short chain fatty acid ester (*p*-nitrophenyl caproate) were calculated. The values obtained were 1.2, 1.2 and 0.9 for 13a, S5 and S8 respectively. These ratios were equal or close to those calculated when the triglycerides assay was used (see paragraph 3.2). This result confirmed the correlation between the assays. The specificity results are shown in figure 3. The lipolytic activity of strain 13a (Fig. 3A) on long chain fatty acid esters (pNPC10 to pNPC16), was maximal on *p*-nitrophenyl laurate (pNPC12). On short and mid chain length of the fatty acid (pNPC2 to pNPC8) the activity was lower and the maximum was obtained on *p*-nitrophenyl caprylate (pNPC8). The relative activity on pNPC8 was 76% of the maximal activity found on pNPC12.

The lipolytic activity of the strain 13a seemed to agree with a lipase activity, which was higher on insoluble long chain fatty acids esters. When the enzyme extract obtained from a 24 h culture was used as enzyme source, activity on short chain fatty acid esters was higher than at 48h with maximal activity being on pNPC4 and pNPC6, while it remained stable on longer fatty acids esters with maximum on pNPC12 (data not shown).

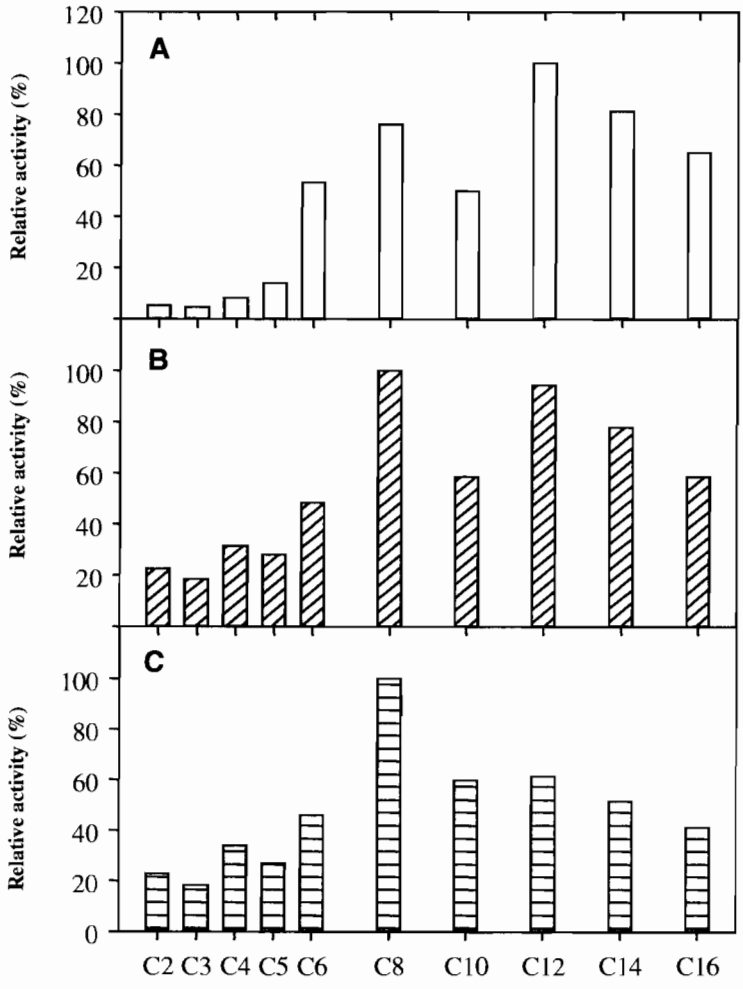


Figure 3: Effect of fatty acid chain length on specificity of lipolytic enzymes from *Rhizopus homothallicus* 13a (A), *Rhizomucor pusillus* S5 (B) and *R. pusillus* S8 (C). The initial rate of hydrolysis was assayed on substrate with fatty acid from acetic (pNPC2) to palmitic (pNPC16). The maximal activity was 0.98 U/mg protein for the strain 13a extract (on pNPC12), 0.16 U/mg protein for the strain S5 extract (on pNPC8) and 0.14 U/mg protein for the strain S8 extract (on pNPC8). Experiments were conducted in duplicate with standard deviation less than 10%.

This result showed that at least two enzymes were responsible for the lipolytic activity of the strain 13a, an esterase activity acting on short chain fatty acid esters and a lipase activity more active on insoluble fatty acid esters. The occurrence of at least two enzymes should be plausible for the lipolytic activity of the strain S5 (Fig. 3B). A specificity profile similar to that of strain 13a was observed. Activity was maximal on pNPC8 and pNPC12 (94%) and lower on short fatty acids esters (pNPC2 to pNPC6), pNPC10 and pNPC14 to pNPC16. Activity on short chain fatty acid esters was higher than that of strain 13a.

For strain S8 (Fig. 3C), the activity was maximal on pNPC8 and it decreased on shorter or longer fatty acids esters. This result suggested that at least one enzyme dominated in the S8 extract which could have a lipase activity like the lipases from *Mucor hiemalis f. hiemalis* (**29**), *Bacillus thermocatenulatus* (**30**) and *B. subtilis* (**31**) whose activities were maximal on pNPC8 or tricaprilyn. Esterases acted on short chain length fatty acids esters (C2 to C4) and few had maximal activity on mid chain length fatty acids (pNPC6 to pNPC8) like the esterase of *Bacillus acidocaldarius* (**32**) with maximal activity on pNPC6.

Stability in organic solvent

Stability of the enzyme extracts in heptane was determined to check the potential of the enzymes for biotechnological applications mainly ester synthesis. Known amounts of activity were incubated at 37°C in 10 ml heptane for 8 days with shaking. The residual activity of acetone washed extract was assayed at 37°C by the measure of hydrolysis of *p*-nitrophenyl laurate (pNPC12) in the standard conditions. The results are shown in Table 2.

Activity retention of 65%, 66% and 84% was obtained by the extracts of 13a, S5 and S8 respectively. This result showed that these enzyme extracts had a good stability in heptane. Similar result (75% of activity retention) was obtained when the mycelial lipase of *Rhizopus oryzae* was incubated for 8 days in heptane at 30°C (**33**). The lipozym of *Rhizomucor miehei* retained 90% of initial activity in 80% hexane after 1 h incubation at 25°C (**34**). Our result showed that these extracts might be useful for transesterification and ester synthesis.

Table 2 Stability of crude enzyme extracts in heptane (*).

Activity	Enzyme extracts		
Strains	13a	S5	S8
Relative activity (%)	66%	65%	84%

(*)Enzyme Extracts (30 mg) were incubated in 10 ml of heptane in shaking (250 rpm) at 37°C for 8 days. Activity was assayed by the measure of the hydrolysis of *p*-nitrophenyl laurate. Experiment was done in triplicate with standard deviation less than 5%.

Acknowledgments

Financial support from the International Development Research Centre (Canada) is greatly acknowledged.

References

1. Pandey A. (1992) *Process Biochem.* **27**, 109-117.
2. Lonsane B.K., Ghildyal N.P., Budiatman S., Ramakrishna S.V. (1985) *Enzyme Microb. Technol.* **7**, 258-265.
3. Hesseltine C.W. (1977) *Process Biochem.* **12**, 24-27.
4. Siessere V., Said S. (1989) *Biotechnol. Lett.* **11**, 343-344.
5. Fonseca M.J.V., Spadaro A.C.C., Said S. (1991) *Biotechnol. Lett.* **13**, 39-42.
6. Alazard D., Raimbault M. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* **12**, 113-117.
7. Desgranges C., Durand A. (1990) *Enzyme Microb. Technol.* **12**, 546-551.
8. Deschamps F., Giuliano C., Asther M., Huet M.C., Roussos S. (1985) *Biotechnol. Bioeng.* **27**, 1385-1388.
9. Shamala T.R., Sreekantiah K.R. (1986) *Enzyme Microb. Technol.* **8**, 178-182.
10. Langrand G., Rondot N., Triantaphylides C., Baratti J. (1990) *Biotechnol. Lett.* **12**, 581-586.
11. Mukherjee K.D. (1990) *Biocatalysis* **3**, 277-293.
12. Cambou B., Klibanov A.M. (1984) *J. Am. Chem. Soc.* **106**, 2687-2692.
13. Molinari F., Brenna O., Valenti M., Aragozzini F. (1996) *Enzyme Microb. Technol.* **19**, 551-556.
14. Rivera-Munoz G., Tinoco-Valencia J.R., Sanchez S., Farres A. (1991) *Biotechnol. Lett.* **13**, 277-280.
15. Olama Z.A., EL-Sabaeny A.H. (1993) *Microbiologia Sem.* **9**, 134-141.
16. Ortiz-Vásquez E., Granados-Baeza M., Rivera-Muñoz, G. (1993) *Biotech. Adv.* **11**, 409-416.
17. Christen P., Angeles N., Corzo G., Farres A., Revah S. (1995) *Biotech. Techniques* **9**, 597-600.
18. Benjamin, S. and Pandey, A. (1997) *Acta Biotechnol.* **17**, 241-251.
19. Kamini N.R., Mala J.G.S., Puvanakrishnan R. (1998) *Process Biochem.* **33**, 505-511.
20. Gombert A.K., Pinto A.L., Castilho L.R., Freire D.M.G. (1999) *Process Biochem.* **35**, 85-90.
21. Cordova J., Nemmaoui M., Ismaili-Alaoui M., Morin A., Roussos S., Raimbault, M., Benjilali, B. (1998) *J. Mol. Catal. B: Enzym.* **5**, 75-78.
22. De Araujo A.A., Lepilleur C., Delcourt S., Colavitti P., Roussos, S. (1997), *in* Advances in solid state fermentation, Roussos S., Lonsane B.K., Raimbault M. and Viniegra-Gonzalez G. (eds.), Kluwer Academic Publishers, Dordrecht, pp.93-111.
23. Kwon D.Y., Rhee J.S. (1986) *J. Am.Oil Chem. Soc.* **63**, 89-92.
24. Saucedo-Castañeda G., Trejo-Hernández M.R., Lonsane B.K., Navarro J.M., Roussos S., Dufour D., Raimbault M. (1994) *Process Biochem.* **29**, 13-24.
25. Pintado J., Lonsane B.K., Gaime-Perraud I., Roussos, S. (1998) *Process Biochem.* **33**, 513-518.
26. Hong M.C., Chang M.C. (1998) *Biotechnol. Lett.* **20**, 1027-1029.
27. Ohnishi K., Yoshida Y., Sekiguchi J. (1994) *J. Ferment. Bioeng.* **77**, 490-495.
28. Rao P.V., Jayaraman K., Lakshmanan C.M. (1993) *Process Biochem.* **28**, 385-389.
29. Hiol A., Jonzo M.D., Druet D., Comeau L. (1999) *Enzyme Microb. Technol.* **25**, 80-87.
30. Rúa M.L., Schmidt-Dannert C., Wahl S., Sprauer A., Schmid R.D. (1997) *J. Biotechnol.* **56**, 89-102.

31. Lesuisse E., Schanck K., Colson C. (1993) *Eur. J. Biochem.* **216**, 155-160.
32. Manco G., Adinolfi E., Pisani F.M., Ottolina G., Carrea G., Rossi M. (1998) *Biochem. J.* **332**, 203-212.
33. Essamri M., Deyris V., Comeau L. (1998) *J. Biotechnol.* **60**, 97-103.
34. Dellamora-Ortiz G.M., Martins R.C., Rocha W.L., Dias A.P. (1997) *Biotechnol. Appl. Biochem.* **26**, 31-37.

Chapter 6

GROWING OF *TRICHODERMA REESEI* ON STATIC AND DYNAMIC SOLID SUBSTRATE FERMENTATION UNDER ELECTROMAGNETIC FIELDS

S.M. MAS DIEGO, A. GONZALEZ MORLA, M. CAMPOS SOFIA, D. CABEZA PULLES

National Center of Applied Electromagnetism (CNEA), Universidad de Oriente, A.P. 4078 CP 90400 Santiago de Cuba 4, CUBA E-mail: siannah@cnea.uo.edu.cu

Abstract

Although submerged cultivation presents strong advantages from the technological point of view, including the possibility of controlling the process' parameters and to guarantee homogeneous conditions for the development of microorganisms, processes on solid substrates allow fungi to grow under conditions very similar to those found in nature, producing air conidia that are more resistant to environmental changes and producing also better yields of pure and homogeneous spores. Other advantages are: a bigger simplicity of the technique, smaller investment costs, bigger quantity of product per unit substrate and the reduction of the energy consumption with regard to the fermentation in liquid phase. In this paper Static and Dynamics Solid Substrate Fermentation of *Trichoderma reesei* with the application of electromagnetic fields were compared. It was observed that with the use of the dynamic system an increment on the production of spores of the fungus takes place with regard to the static system of fermentation. The effect of electromagnetic fields on the microorganism is manifested in an increment of the productivity of the system and in a decrease of the times of fermentation, for the given stimulation conditions. Better results were obtained with dynamic fermentation system and the use of electromagnetic fields.

Key-words: *Trichoderma reesei*, dynamic solid state fermentation, electromagnetic fields, column solid state fermentation.

Introduction

Fungal products represent a significant part of biopesticides production. Among the fungal species, those that stand out are *Paecilomyces lilacinus*, *Beauveria bassiana*, and *Verticillium lecanii*, (**1,2**). However, most of the studies published sofar refer to the genera *Trichoderma* because they attack a great variety of phytopathogenic fungi (*Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium* sp, *Colletotrichum* sp and *Fusarium oxysporum*, among others). These fungi are responsible for the most

New Horizons in Biotechnology, 57-62, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

important diseases on early stages of the cultivation of tomato, lettuce, rice, grapes and beans, which are basic food components of many cultures (**3**).

In the great majority of the cases, Solid Substrate Fermentation (SSF) processes have been developed in static systems. In spite of the advantages signaled by many authors (**1,4**), these processes are of difficult maneuverability, functionality, with a high heterogeneity of the fermentative system and of difficult control of the fundamental variables of the process. The use of agitated fermentor on solid substrate fermentation reduces to a great degree media heterogeneity and diminishes the macro gradients observed in the static systems, all of which are directly related to productivity of the fermentative system (**5**).

As opposed to submerged fermentation, for solid substrate fermentation a reactor prototype has not been established and characterized enough to be used as a model for the scale-up and modeling of this process, with a possibility to control and measure fermentative parameters. Researchers of the Cuban Institute of Sugar Cane Derivatives (ICIDCA) have done a broad bibliographical review concerning engineering aspects of these processes, mainly with regards to fermentor characteristics, remarking bioreactors types. Most used solid fermentors are the transporters, columns and rotating drum fermentors (**6**). National Center of Applied Electromagnetism (CNEA) at the Universidad de Oriente (Santiago de Cuba) has been developing, since 1993 the design and construction of solid substrate fermentation prototypes in order to satisfy the increasing demand for bio-fertilizers and bio-pesticides of the Agricultural Ministry (**7**).

Another important aspect in order to increase yields is the use of the influence of electromagnetic systems on biological samples, which have been broadly studied by many researchers. At present these investigations are centered on the study of extremely low frequency fields, and several mechanisms that can explain field interaction with cellular systems found in the literature (**8,9,10,11**). Since 1992, CNEA has been carrying out research related to this influence. Beneficial effects resulting from the influence of the electromagnetic field on growth of the yeast *Candida utilis*, include a decrease in fermentation times and an increase in produced biomass (**12, 13**).

In the case of bio-pesticides, treatment with electromagnetic fields, has been tested with seven microorganisms commonly used as biological controllers; among them are: *Bacillus thuringiensis*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Paecilomyces lilacinus*, *Trichoderma* spp. and *Bacillus megaterium*. In this study there were stimulations in the percentage of spores germination. Variations were not observed in morphologic characteristics. There were no significant variations in the kinetics of metabolite production, with the experimental conditions employed (**14**).

The objective of this work consists in studying the effect of the electromagnetic field in the cultivation of *Trichoderma reesei* on static and dynamic solid substrate fermentation in order to improve the technology by means of cellular growth stimulation and larger spore production.

Materials and Methods

Microorganism

Trichoderma reesei, A-54, was isolated by the Provincial Laboratory of Plant Sanity (LPSAV) of Santiago de Cuba.

Conservation Medium

Agar-Czapek (**15**). Medium pH adjusted to 5.4 and sterilized during 15 min. at 1 atm. and 121°C.

Experiment 1. Effect of electromagnetic fields on the growing of T. reesei in SSF.

Static solid phase : Glass columns system (**5**), charged (100 g) with an enriched substrate: bagasse (- 3 mm + 1 mm) (80 %) with powdered rice (20 %). Humidity controlled to approximately 50%.

Experiment 2. SSF of T. reesei in a dynamic system under electromagnetic fields.

Dynamic solid phase : Dynamic paddles fermentor designed and built at CNEA, (**7**) loaded with 1 kg-enriched substrate: sugar cane bagasse (80%) and powdered rice (20%). Humidity controlled to approximately 50%.

Strains were treated using a steady and non-homogeneous electromagnetic field generated by an electromagnet designed at CNEA. A 2^2 factorial experimental design was carried out in each case, considering as independent variables:

X_1: Magnetic induction (100 Gs and 600 Gs)

X_2: Time of exposure (15 min and 30 min)

Values of electromagnetic induction were chosen according to the studies found in the literature so as to guarantee that there are no negative consequences in employing our induction of electromagnetic fields to stimulate cellular growth. Related genotoxic studies were carried out in 1996 showing no mutagenic effects (**12**).

Magnetic induction values are achieved varying the output electric current of a variable resistance coupled to the electromagnet. This equipment was calibrated using Nuclear Magnetic Resonance (NMR) method.

Samples were taken at the beginning and at the end of the fermentation, determining cellular growth by the techniques described by Saucedo (**5**). All treatments were compared with a control experiment developed under the same conditions but to which electromagnetic field was not applied.

Both experiments were inoculated in a 1:10 v/w basis with a solution of $3.29 \times 10^8 \pm 7.20 \times 10^7$ spores/mL.

Results and Discussion

Results were analyzed by the computational program Statgraphics for Windows, v. 4.2 (1993), considering as the main criteria for the analysis in both cases the rate between spores production and the end of fermentation and spores production at the beginning of fermentation. This interest was geared towards the use of our product as a biocontroller.

According to the results showed in the above figure, electromagnetic fields can act in a more or less favorable way on growth of this microorganism within the range of induction used in this experiment.

Static solid phase

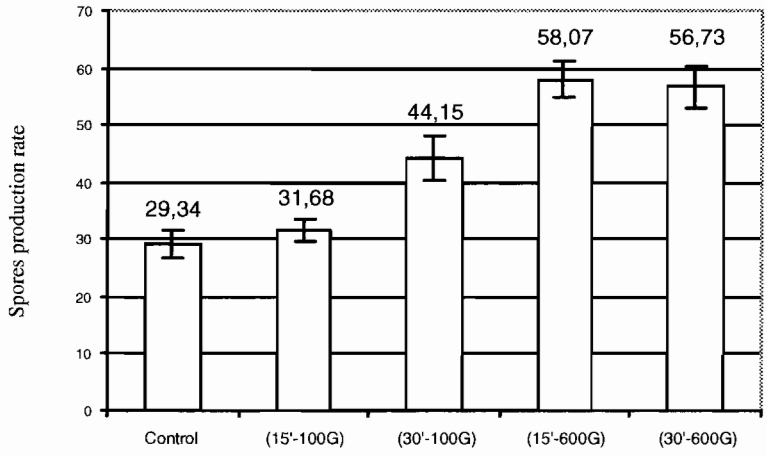


Figure 1: Influence of electromagnetic treatment on spores rate increment in glass columns fermentation.

For the experiment with 15 minutes of field exposure and 100 Gauss of magnetic induction, significant differences with the control were not observed. This could be due to the low values of both parameters, which means that the values used for the experiment are within the ranges found in nature.

Results were improved at 600 Gauss of magnetic induction and 15 minutes of field exposure with a 50.53 % increment in spores production as compared to control experiment.

Dynamic solid phase

In this case, experiments were done in order to determine the increment of spores production when employing a dynamic type fermentor and different electromagnetic fields.

It was observed that at this stage the organism increased its spore production, confirmed by the positive results with the influence of the electromagnetic field.

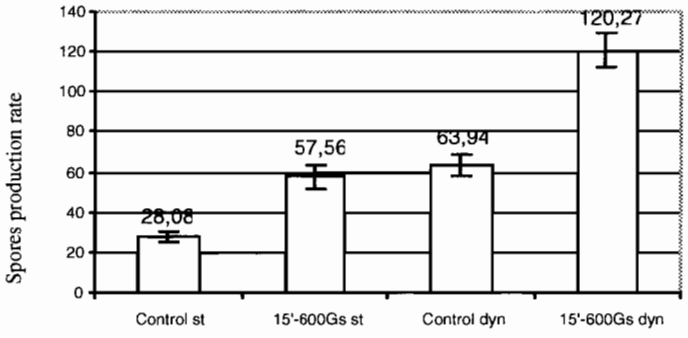


Figure 2: Comparison of *T. reesei* growth in static and dynamic solid substrate fermentation under the influence of electromagnetic fields.

With the application of electromagnetic fields to this system increments of around 50 % on spores yield was observed. It is significant that the results achieved operating the fermentor as a static system were very similar to those achieved in columns though results were not as significant. That could be explained because of the lower size of the columns, which improved the distribution of oxygen and access to nutrients.

Dynamic system can improve productivity by around 45 %, which has been explained in literature by many authors (**4,5**). When comparing the results previously analyzed with those reported in the bibliography we saw that there was a coincidence in finding stimulating effects on cellular growth in the range around 500-1000 Gs (**14**), although we did not use the same the same experimental conditions.

We explain our results considering the action of the electromagnetic field on cellular membranes, mechanism that has studied by multiple authors (**8**).

Under the action of the electromagnetic field changes are induced in the conformation of the membranes, which is translated in alterations in their physical properties, causing an alteration of the functions of the receivers of the membrane, (**9**). These effects modify in turn, the ionic flow transmembrane, causing changes in the metabolism of the affected cell. The stimulation of the cellular metabolism has a direct implication in the observed stimulating effects that are reported in our work.

Conclusions

With the application of the dynamic system, it was possible to increase by up to 45 % the production of spores with the application of a specific electromagnetic

field. Spore production reached values of 50 % over control values (without the application of the electromagnetic treatment). Significantly higher spore production will result in greater cellular growth, for the used stimulation conditions. Better effects were observed around 600 Gauss and 15 minutes of electromagnetic field exposure.

References

1. Alvarez R. M., Saura G., Rodriguez J. A., Villa P. (1996) Conference in: Diversificación 96. Congreso internacional sobre el azúcar y derivados de la caña. La Habana, pp. 5-7.
2. Bailey A. M., Herrera A., Estrella J.R., Herrera A. (1997) *Avance y Perspectiva*, **16**: 27-35.
3. Chet I. (1987) *in*: Innovative approaches to plant discase control, I. Chet (Eds.), Wiley and Sons, New York, pp. 137-143.
4. Pandey A. (1992) *Process Biochemistry*. **27**, 109-117.
5. Saucedo-Castañeda G. (1991) Contrôle du métabolisme de *Schawanniomyces castellii* cultivé sur support solide, PhD thesis, Université Montpellier II, France, 211p.
6. ICIDCA. (1995) Fermentaciones en estado sólido de procesos biotecnológicos. Reporte interno. La Habana, 24 p.
7. Mas S., González A., Campos M., Cabeza D. (1999) *Rev. Tecn. Qca.* **19** (3): 64-69.
8. Polk C., Postow E. (Eds.) (1996) Handbook of biological effects of electromagnetic fields, CRC Press. New York, 503 p.
9. Rosen A. D. (1993) *Biochemica et Biophysica Acta*. **1148**, 317-320.
10. Weaver J. C., Vaughan T. E., Adair R. K. (1997) *in* Proceedings of the Second World Congress for Electricity and Magnetism in Biology and Medicine, June 8-13, Bologna, Italy, pp. 238-240.
11. Schwan H. P. (1983) *in*: Biological effects and dosimetry of nononizing radiation. Grandolfo M., Michaelson S. M. and Rindi A. Eds. Plenum Press New York, pp. 210 -223.
12. Campos M., Camué H., González A., Pardo A. M. (1996) *Revista Tecnología Química*. **16**(2): 34-41.
13. Chacón D., Haber V., Fong A., Mas S., Serguera M., Rodriguez O. (1996) *Rev. Tecnología Química*. **16**(1): 52-60.
14. Fernández Larrea O. (1992) Efecto del campo magnético sobre microorganismos que se emplean en el control fitosanitario, Instituto de Inv. de Sanidad Vegetal, MINAGRI, La Habana, 16 p.
15. Herrera A. (1985) Manual de medios de Cultivo. Edit. Pueblo y Educ. La Habana. Cuba, 322 p.

Chapter 7

PRODUCTION OF ENZYME AND PROTEIN HYDROLYSATE FROM FISH PROCESSING WASTE

P. PRASERTSAN[1], S. JITBUNJERDKUL[2], TRAIRATANANUKOON[1], T. PRACHUMRATANA[1].

[1]*Department of Industrial Biotechnology,* [2]*Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hatyai, 90110 THAILAND*

Abstract

Thailand is the largest canned tuna producer and exporter. During the process, 5-7% (w/v) tuna viscera is generated from the whole fish. Among the three species of tuna processed in the industry, viscera of the yellowfin tuna (*Thunnus albacares*) exhibited the highest protease activity of 72.17 U/ml with the specific activity of 3.09 U/mg protein. A characterization of the enzyme extracted from the yellowfin tuna revealed that the optimum protease activity occurred at pH 10.0 and a temperature of 50°C. The enzyme was most stable at its optimum pH for activity with over 90% residual activity after 120 min. It was found to be thermostable at 60°C for 120 min. The extraction of the crude enzyme was carried out using a 50 mM carbonate-bicarbonate buffer (pH 10), which resulted in an enzyme yield of 45.67% of the total protease activity in the tuna viscera. Of the three protein sources used for the hydrolysation experiments of the crude enzyme at 37°C for 4 hours, tuna viscera was the best raw material exhibiting a 40.53% degree of hydrolysis (DH) and a 93.50% nitrogen recovery (NR), followed by the tuna viscera residue after enzyme extraction (31.30% DH and 72.82% NR) and the mackeral muscle (12.36% DH and 85.94% NR), respectively. Comparison on the crude enzyme and commercial alkaline protease Alcalase 2.4L on the hydrolysis of two substrates revealed that the Alcalase exhibited a 18.63% and 16.72% higher degree of hydrolysis on tuna viscera and casein, respectively, compared to the crude enzyme.

Introduction

Thailand is the world's largest producer and exporter of canned tuna products despite the fact that most of the raw materials are imported. There are five species of tuna used in the industry : albacore (*Thunnus alaiunga*), yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*), tonggol tuna (*Thunnus* tonggol) and frigate tuna (*Auxis thazard*). During the process of tuna canning, vast amounts

New Horizons in Biotechnology, 63-71, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

of liquid and solid wastes are generated. These include tuna condensate, viscera, head, bone, blood and dark meat. For liquid waste, tuna condensate (precooking water) is the major source and previously discharged directly to the wastewater treatment system causing the occurrence of red wastewater from the growth of photosynthetic bacteria (**1**). Besides solving this problem directly, factories turn to utilize it for the production of fish extract employing an expensive imported production technology, whereby the enzyme protease is added, thus increasing significantly production costs.

The solid wastes, on the other hand, are generally sold to a fishmeal factory at a very low price. Tuna viscera accounts for 7-8% of the whole body weight (**2**) which is similar to the amount of viscera (8%) in frigate mackerel (*Auxis rochei*) (**3**). Therefore, of the 400,000 tonnes of tuna processed in 1997, 3,200 tonnes of viscera were generated. In fact, the viscera was reported to be a good source of many commercial enzymes such as chymotrypsin from cod (*Gadus morhua*) and pepsin from cod (*Breogadus saida*) for the cheese industry (**4**) as well as trypsin from cod (*Gadus ogae*) for amino acid production (**5**). Therefore, it looks promising to use tuna viscera for the production of enzymes as well as protein hydrolysates. Marine fish digestive proteases have many applications such as recovery of protein from marine source (**6,7**), accelerating the fermentation of fish (**8**), etc. Fish protein hydrolysate (FPH) has been added as an ingredient in many food products due to its excellent functional properties such as high solubility, good emulsifying and foaming properties, and good water-binding (**9**). In addition, it was reported that the fish protein hydrolysate contained peptides possessing various bioactivities such as ACE (angiotensin coverting enzyme) inhibition activity or antioxidative property (**7**).

This research work aims at investigating the effects of various parameters on the production of enzyme and protein hydrolysate from tuna viscera.

Materials and Methods

Whole viscera of three tuna species : skipjack (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and tonggol tuna (*Thunnus tonggol*) were used in this study. They were kindly provided by two seafood-processing factories in the Hatyai District, Songkhla Province. The viscera were kept frozen at –20°C in sealed plastic bags until needed for enzyme extraction.

Mackeral muscle was obtained from deboning and skinning of the fillets. It was minced and kept at -20^0C until used.

The commercial alkaline protease Alcalase 2.4L was kindly provided by NOVO Industry (The East Asiatic (Thailand) Public Co., Ltd.).

Effect of tuna species and buffer pH on the protease activity of the crude enzyme

Whole viscera of the three tuna species (albacore, yellowfin tuna, and tonggol tuna) were washed with sterile water and weighed. Cold 50 mM buffer solution (pH 2-11) were added in the ratio viscera to buffer of 1:2 (w/v). They were prepared as

followed : pH 2.0-6.0 (citrate-phosphate buffer), pH 7.0-9.0 (Tris-HCl) and pH 10.0-11.0 (carbonate-bicarbonate buffer). The mixture was homogenized for 1-2 min before filtration through cheesecloth to remove solid residues. The fine particles left in the filtrate were removed by centrifugation at 2,800 *x g* for 30 min at 4°C. The supernatant (tuna viscera extract) was used as the crude enzyme and determined for protease activity (**10**) in which casein and hemoglobin were used as substrate and the optical density was measured at the wavelength of 275 nm after incubation for 15 min at 37°C. The best source of viscera possessing the highest protease activity would be selected for further studies.

Properties of the crude enzyme

The crude enzyme extracted from the whole viscera of the selected tuna was studied for its optimum pH (pH 2.0-11.0), pH stability (pH 2.0-11.0) optimum temperature (10-70°C), and thermal stability (incubation at 37°C-70°C for 120 min).

Determination of hydrolysis conditions

Crude enzyme was prepared by extracting the viscera of yellowfin tuna using 50 mM carbonate-bicarbonate buffer, pH 10. The protease activities of the tuna viscera (using the viscera without extraction as the enzyme source), the crude enzyme and the tuna viscera residue after enzyme extraction were determined and calculated for the enzyme yield.

Thermal stability of the extracted enzyme for longer incubation time (24 h) at 37°C and 50°C were compared. The samples were taken at 0, 4, 8, 12, 16, 20 and 24 h to analyse for residual activity. A suitable temperature would be selected for studies on the optimum time for hydrolysis of tuna viscera and its residue (after enzyme extraction). The two substrates (each containing the initial protein concentration of 5%, w/v) were homogenized with 50 mM buffer pH 10 before adding the crude enzyme (5% of the initial protein concentration), then incubated at the selected temperature for 10 h. After centrifugation, the supernatant was heated at 90°C for 15 minutes to inactivate the enzyme reaction. The mixture in which distilled water replaced the enzyme solution was used as the control. During hydrolysis process, samples were taken at 0, 0.5, 1, 1.5, 2, 4, 6 and 8 h to analyse for nitrogen recovery (NR) (**11**) and degree of hydrolysis (DH) (**12**).

$$\% \text{ Nitrogen recovery (NR)} = \frac{\text{total soluble nitrogen in the hydrolysate}}{\text{total nitrogen in the raw material.}}$$

$$\% \text{ Degree of hydrolysis (DH)} = \frac{\text{10\% TCA soluble nitrogen in hydrolysate x 100}}{\text{total nitrogen in raw material}}$$

Effect of protein source on the production of hydrolysate

Production of protein hydrolysate from three sources of protein; tuna viscera, tuna viscera residue after enzyme extraction and minced mackeral (*Auxis rochei*) muscle, were compared. The preparation of each protein source and the hydrolysis procedure were the same as described above whereby the mixture was incubated at the selected temperature and the optimum hydrolysis time. The samples were also analysed for nitrogen recovery and degree of hydrolysis.

Effect of enzyme source for hydrolysis of tuna viscera and casein

Two sources of enzyme, the crude enzyme from the tuna viscera (5.03 U/ml) and Alcalase (84.48 U/ml), were used for the hydrolysis of tuna viscera and casein. The procedure of hydrolysis was the same as described above.

Results and Discussion

Effect of tuna species and pH of buffer on the protease activity of the tuna viscera extract (crude enzyme)

Tuna species and the pH of buffer used for enzyme extraction had a substantial influence on protease activity (Figure 1). Among the three species of tuna, viscera of the yellowfin tuna exhibited the highest protease activity of 72.17 U/ml with a specific activity of 3.09 U/mg protein using a buffer of pH 10.0. The protease activities from the crude enzymes of skipjack tuna and tonggol tuna were 60.53 and 48.53 U/ml at pH 10.0 and 9.0, respectively. Their specific activities were 2.39 and 2.30 U/mg protein, respectively. The specific activities of all three crude enzymes were higher than that of chymotrypsin from caeca of rainbow trout (*Oncorhynchus mykiss*) (0.64 U/mg) (**13**) but lower than that of trypsin from the pancreas of crayfish (*Procambarus clarkii*) (**14**). All three sources of the enzymes showed their highest protease activities at alkaline pH (pH 9.0-10.0). These were in agreement with the previous report that extraction of protein in alkaline condition especially at pH 10.0 would enhance the activities of the enzyme from fish viscera and the digestive tract (**15**). Nevertheless, the highest specific activity of enzyme from gustric mucosa of Polar cod (*Boreogadus saida*) was achieved at a pH of 7.3 (**16**). Therefore, the viscera of yellowfin tuna and the buffer pH 10.0 were selected for further studies.

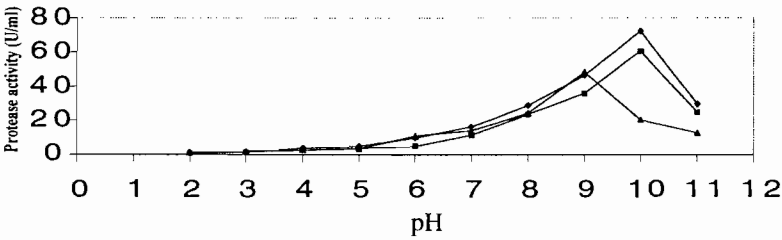


Figure 1 : Comparison on the activity of protease extracted from the viscera of three tuna species using buffer at different pH. Circles: Yellowfin tuna (*Thunnus albacares*); Squares: Skipjack tuna (*Katsuwonus pelamis*); Triangles: Tonggol tuna (*Thunnus tonggol*).

Properties of the crude enzyme

Studies on the properties of the crude enzyme extracted from yellowfin tuna viscera revealed that the optimum pH for protease activity occurred at pH 10.0 (Figure 2).

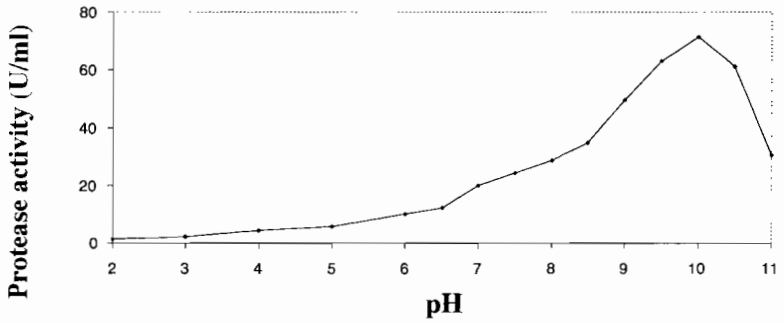


Figure 2 : Effect of pH on the activity of protease from viscera of yellowfin tuna (*Thunnus albacares*).

This was slightly higher than the optimum pH at 9.0 of chymotrypsin from caeca of rainbow trout (*Oncorhynchus mykiss*) (**13**) and much higher than that of purified trypsin from pancreas of crayfish (*Procambarus clarkii*) (pH 7.5-8.0) (**15**). The optimum pH was also found to be different depending on the substrate used as in the case of trypsin from caeca and intestine of anchovy (*Engraulis encrasicholus*) which illustrated the optimum pH at 9.5 and 9.0 when casein and BAPNA (benzamidine N-benzoyl-DL-arginine *p*-nitroanilide) were used as the substrate, respectively.

Investigations on pH stability of the crude enzyme in the range of pH 9-11 demonstrated that the enzyme was most stable at its optimum pH for protease activity (pH 10.0). The residual protease activities were 96.1 and 91.6% after incubation for 60 and 120 min, respectively (Figure 3). The pH stability of tuna viscera at pH 10 was similar to the pH stability in the range of pH 8.8-9.6 of the serine protease from cod caeca (**16**).

The optimum temperature for enzyme activity of the crude enzyme was found to be 50°C (Figure 4). The protease activities at 50°C, 60°C and 10°C were 90.61, 54 and 13 U/ml, respectively, This optimum temperature was lower than those of alkaline protease from four species of freshwater fish and 21 species of marine fish (60-65°C) (**17**). The dependence of the optimal temperature on the pH was illustrated in the case of pancreas of crayfish (*Procambarus clarkii*) whereby the optimum temperature was 60^0C at pH 6.8 and 50-60^0C at pH 8.1 (**15**).

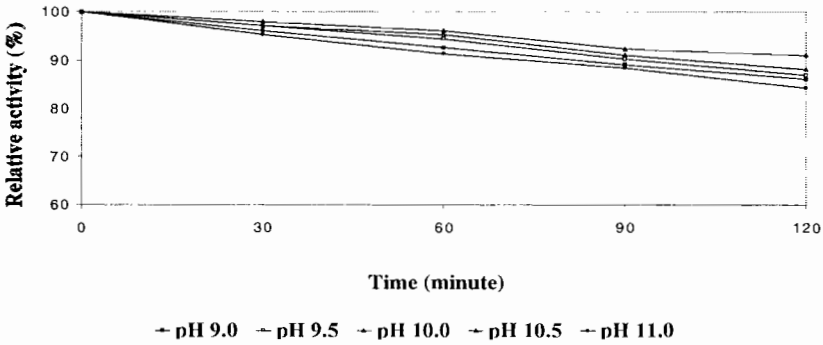


Figure 3 : pH stability of protease extracted from viscera of yellowfin tuna (*Thunnus albacares*).

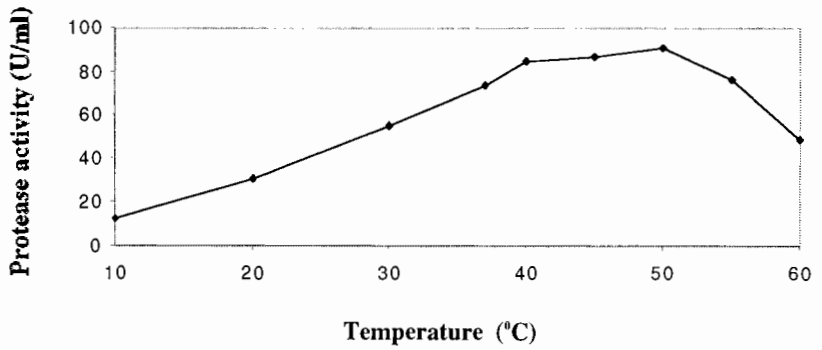


Figure 4 : Effect of temperaure on activity of protease from viscera of yellowfin tuna (*Thunnus albacares*).

Thermal stability studies at 37-70^{0}C indicated that the crude enzyme was most stable at 37^{0}C. The residual protease activities were 86.1% and 78.4% after incubation for 60 and 120 min, respectively. At 60°C the corresponding values were 65.0% and 38.57%, respectively. The half-life of the protease of the crude enzyme was found to be 75 min at 60°C. The stability of the crude enzyme at 37°C was similar to those of enzyme from Atlantic cod (*Gadus morhua*) (30°C-35°C) (**18**), or chymotrypsin from caeca and intestine of rainbow trout (*Oncorhynchus mykiss*) (<40°C) (**13**).

Determination of hydrolysis condition

In this experiment, the whole viscera of yellowfin tuna was used and found to possess protease activity of 22.03 U/g viscera. The crude enzyme and viscera residue after enzyme extraction showed the activities of 10.06 and 5.57 U/g viscera,

respectively. This gave the yield of 45.67% for the crude enzyme and 25.28% for the enzyme left in the viscera residue.

Incubating the crude enzyme at two temperatures revealed that the enzyme sample was quite stable at 37°C with no loss of enzyme activity after 4 h incubation and approximately 60% residual activity after 24 h (data not shown). At 50°C, the enzyme lost almost 40% activity after 4 h incubation while only 20% of the activity remained after 24 h. The half-life of the crude enzyme was found to be 5.6 h at 50°C. The result agreed with the report that serine protease from cod caeca was more stable at 37°C than 57°C (**16**). In addition, fish digestive proteases were known to have high activity at low temperature (**19**).

Hydrolysis of tuna viscera residue was carried out at 37°C. Results clearly demonstrated that both nitrogen recovery and degree of hydrolysis increased sharply within 1 h of incubation and increased slightly after 4 h. The hydrolysis condition at 37°C for 4 h was therefore chosen for further studies. This is similar to the hydrolysis of tuna boiled extract at 40°C for 3 h using proteinase from tuna pyloric caeca (**20**) and for 5 h in hydrolyzation of protein of *Oreochromis mossambicus* by Alcalase (**21**).

Effect of protein source on the production of hydrolysate

Among the three protein sources used for the production of protein hydrolysate by the crude enzyme, tuna viscera was the best raw material exhibiting the highest values of degree of hydrolysis (40.53% DH) and nitrogen recovery (93.5%) (Table 1).

Table 1 : Nitrogen recovery and degree of hydrolysis of hydrolysate from difference protein sources.

Protein source	Enzyme concentration (%)	Nitrogen recovery (NR)	Degree of hydrolysis (DH)
Viscera	0	86.57 ^{c}	37.47 ^{d}
	5	93.50 ^{d}	40.53 ^{e}
Viscera residue	0	71.81 ^{b}	31.92 ^{c}
	5	72.82 ^{b}	31.30 ^{c}
Flesh of fish	0	46.68 ^{a}	0 ^{a}
	5	85.94 ^{c}	12.36 ^{b}

This was due to the synergistic activities of the existing endogeneous enzymes present in the viscera and the crude enzyme added. When tuna viscera residue (after enzyme extraction) was used as substrate, the degree of hydrolysis (31.30%) was only about 9% lower while nitrogen recovery (72.82%) was 20.7% lower than those from the whole viscera. The mackerel muscle showed 12.36% DH with a nitrogen recovery of 85.94%. The results indicated that this crude enzyme or tuna viscera extract hydrolyzed the mackeral muscle protein more efficiently (12.36% DH) than the commercial alkaline proteases Flavourzyme 1,000 (7.5% DH), Corolase PN-L (6.7% DH) and Alcalase 2.4L (5.6% DH), reacted on salmon muscle mince (**9**). As a consequence, the tuna viscera extract is considered a feasible choice as an enzyme source for the production of fish protein hydrolysate.

Effect of enzyme source for hydrolysis of tuna viscera and casein

The hydrolysis of viscera protein and casein by the crude enzyme and the commercial enzyme Alcalase 2.4L were compared (Table 2). The results illustrated that for hydrolysis of tuna viscera, the Alcalase 2.4L exhibited 18.63% and 0.65% higher values of degree of hydrolysis (59.72%) and nitrogen recovery (84.39%) than those obtained from the crude enzyme (41.09% DH and 83.74% NR). For hydrolysis of casein, the commercial enzyme showed 16.72% higher value of degree of hydrolysis while there was no difference in nitrogen recovery, compared to the data from using the crude enzyme. The much lower activity of fish protease towards a standard substrate casein was also reported elsewhere (**22, 23**).

Table 2 : Nitrogen and recovery and degree of hydrolysis of hydrolysate using tuna viscera and casein as substrate.

Protein source	Enzyme conc. (%)	NR		DH	
		Crude enzyme	Alcalase	Crude enzyme	Alcalase
Viscera	0	82.31 ^{b,c}	82.31 ^{b,c}	38.67 ^{d}	38.67 ^{d}
	5	83.74 ^{b,c}	84.39 ^{c}	41.09 ^{d,c}	59.72 ^{e}
	10	79.59 ^{b}	84.01 ^{b,c}	39.05 ^{d}	43.21 ^{f}
Casein	0	50.72 ^{a}	50.72 ^{a}	2.11 ^{a}	2.11 ^{a}
	5	52.63 ^{a}	52.53 ^{a}	5.11 ^{b}	21.83 ^{b}
	10	53.40 ^{a}	51.38 ^{a}	9.01 ^{c}	23.66 ^{c}

Degree of hydrolysis of the Alcalase 2.4L reacted on the yellowfin tuna viscera (59.72%) in this work was 10 times higher than that on salmon muscle mince (5.6% DH) (*9*). This may due to the synergistic proteolytic action of Alcalase itself and the endogenous enzyme present in the tuna viscera. Nevertheless, the level of degree of hydrolysis may have to be set up according to the quality of the fish protein hydrolysate required. For application of fish hydrolysate in the food product, lower value of degree of hydrolysis and nitrogen recovery may be prefer in order to reduce the bitterness taste of the product. Morioka and his colleagues (**3**) found that the nitrogen recovery increased gradually with the increase of the initial pH in the range of 5.5-9.0 and autolysis at pH 9.0 would give more bitterness taste than that of initial pH of 7.0. In addition, Alcalase was reported to be more efficiency than Neutrase in hydrolysis of fish protein and the optimum pH of these two enzymes were 9.5 and 7.0, respectively (**24**). Source of enzyme therefore plays an important role in the hydrolysis but it is rather difficult to compare the results using different enzymes as they were varied in purity and optimization condition. Although the commercial enzyme Alcalase 2.4L gave better results than the crude enzyme (tuna viscera extract), its higher cost would make it uneconomically feasible for the proteolysis of the fish viscera. The enzyme from tuna viscera therefore would be more appropriate especially for the production of protein hydrolysate in the developing countries.

References

1. Prasertsan P., Choorit W. (1988) *Songklanakarin J. Sci. Technol.* **10**, 439-446.
2. Prasertan P., Wuttijumnong P., Sophanodora P., Choorit W. (1988) *Songklanakarin J. Sci. Technol.* **10**, 447-451.
3. Cano-Lopez A., Simpson B.K., Haard N.F. (1987) *J. Food Sci.* **52**, 503-506.
4. Morioka K., Fuji S., Itoh Y., Lin C., Obatake A. (1999) *Fisheries Sci.* **65**, 588-591.
5. Brewer P., Helbig N., Haard N. F. (1984) *Can. Inst. Food Sci. Technol. J.* **17**, 38-43.
6. Simpson B. K., Haard N. F. (1984) *J. Appl. Biochem.* **6**, 135-143.
7. Kim S.K., Jeon Y.J., Byeun H.G., Kim Y.T., Lee C.K. (1997) *Fisheries Sci.* **63**, 421-427.
8. Simpson B. K., Haard N. F. (1987) *in* Food Biotechnology. Knorr D. ed. Marcel Dekker NY.
9. Kristinsson H. G., Rasco B. A. (2000) *Process Biochem.* **36**, 131-139.
10. Hagihara B., Matsubara H., Nakai M., Okunuki K. (1958) *J. Biochem. (Tokyo)* **45**, 185-194.
11. Shihadi F., Han X.Q., Synowiecki J. (1995) *Food Chem.* **58**, 285-293.
12. Hoyle N.T., Merritt J.H. (1994) *J. Food Sci.* **59**, 76-79.
13. Kristjansson M.M., Nielson H.H. (1991) *Comp. Biochem. Physiol.* **101B**, 247-253.
14. Kim H.R., Meyers P.S., Pyeun H.J., Godber S.J. (1992) *Comp. Biochem. Physiol.* **103B**, 391-398.
15. Kim H.R. Meyers P.S., Pyeun H.J., Godbar S.J. (1994) *Comp. Biochem. Physiol.* **107B**, 197-203.
16. Meinke W.W., Rahman M.A., Marttil K.F. (1972) *J. Food Sci.* **38**, 864-866.
17. Arunchalam K., Haard N. F. (1985) *Comp. Biochem. Physiol.* **80B**, 467-473.
18. Shin D. H., Zall R.R. (1986) *Process Biochem.* **21**: 1-15.
19. Iwata K., Kobashi K., Hase J. (1974) *Bull. Jap. Soc. Sci.* **40**, 201-213.
20. Asgeirson B., Bjarnason B. J. (1991) *Comp. Biochem. Physiol.* **99B**, 327-335.
21. Haard N. F. (1992) *J. Aquat. Food Prod. Tech.* **1**, 17-35.
22. Kim S. K., Byun H. G., Jeon Y. G. (1999) *J. Korean Fisheries Soc.* **32**, 127-133.
23. Yu S. Y., Tan L. K. (1992) *ASEAN Food.* **7**, 157-158.
24. Gildberg A., Batista I., Strom E. (1989) *Appl. Biochem.* **11**, 413-23.
25. Raa J., Gildberg A. (1976) *J. Technol.* **11**, 619-628.
26. Benjakul S., Morrissey M. T. (1997) *J. Agric. Food Chem.* **45**, 3423-3430.

Chapter 8

NEW POTENTIALITIES OF USES OF COFFEE INDUSTRY RESIDUES IN BRAZIL

C.R. SOCCOL

Universidade Federal do Paraná, Departamento de Engenharia Química, Laboratório de Processos Biotecnológicos, CEP 81531-970, Curitiba-Pr, Brazil

Abstract

The market of coffee represents considerable commercial stake. Several hundred of millions of consumers, more than 1.5 billion of cups of coffee are drunken every day. Coffee is the second product in value on the word market after petroleum. It is about one of the first world markets of agricultural products with sugarcane, wheat, the bovine meat and cotton. With 4% of the world trade of the food products, it represents on the international market about 10 billions of dollars by year. Being the first non-alcoholic drink of developed countries, imports of coffee are directed toward the more industrialised countries. The first importers of coffee in the world are the United States (18 millions of 60 Kg bags), followed by Germany, France and Japan. Processing of coffee results a large quantity of coffee husk or pulp (depending up on the mode of processing), which due to its toxic contents poses serious difficulties in its disposal. Our studies that would be presented in this conference have shown that through the microbial biotechnology, new economic opportunities will be able to become a reality in a near future, especially for the valorisation of the coffee agro-industry wastes in Brazil. In the chain of the coffee industrialisation, as soon as the cherry of coffee is harvested until the cup of coffee, the residues represent 95%of the total weight. Therefore, 5% of coffee only finds again in the cup. In our studies, we have noted that in spite of the toxic component presence in the coffee husk as caffeine and tannins, coffee husk is very rich organic components and could be used like as substrate in bioprocesses to produce molecules of high value such as enzymes, aromas, plant hormones, edible mushroom, feeds, etc. The paper would discuss these findings.

Introduction

The world market of coffee has significant commercial importance- several hundreds of millions of consumers drink coffee every day (~ 1.5 billion of cups) and annually 100 millions of 60 Kg bags are produced in some 70 tropical country. After petroleum, coffee is the second product in value on the world market. Among agricultural commodities, it ranks first along with sugarcane, wheat, the bovine meat and cotton. IT occupies 4 % of the world trade of the food products and

New Horizons in Biotechnology, 73-88, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

carries business of about US$ 10 billions on the international market per year. Coffee consumption is higher in industrialized countries and United States ranks first in its import (18 millions of 60 Kg bags), followed by Germany, France and Japan. The price of coffee is controlled by several factors such as climatic conditions affecting production, political events and monetary fluctuations. Coffee could be used as raw (filer) coffee, soluble (instant) and caffeine free coffee. Soluble coffee consumption varies in different countries, e.g. 8 % of UK consumption, 50% in Japan, 35% in Canada, 26% in Spain, 13 % in France and 10 % in Germany. Caffeine-free coffee represents 10 % of the world market (4 % of the coffee consumption roasted in France) and the highest consumption is in Germany and Switzerland [**1**].

The first five producers of coffee are: Brazil, Colombia, Indonesia, Mexico and Coast of Ivory, which together account for 60 % of the world production.

Industrial processing of coffee in Brazil

In Brazil, picking of coffee cherries is done by two methods: by collecting only mature cherries and by collecting all cherries with branch (i.e. mature and green cherry mixture) [**2**]. Cherries could be processed by two processes for the obtaining of merchant grains: the humid method and the dry method (Fig. 1).

The humid method

In humid method, cherries are poured in a big container (1 to 2 tons) and are washed in the water current to remove refuse and dust. Cherries are then de-pulped, which separates the pulp of the grains. They undergo fermentation for 24-36 h and are washed strongly to eliminate remainders of the pulp. The grains are then dried in the sun on cemented areas or artificially in driers. After drying and selection (electronically or manually), the coffee is packed in bags of 60 Kg and sent for despatch to market. This process generally suits better for arabica variety. The operation consumes high quantity of water [**1**] and results a residue, cockles [**3**].

The dry method

In dry method, after picking, cherries are immediately dried in the sun by spreading about 30 Kg/m^2, moved frequently, sheltered during the night and in period of rain [**4**]. It is sometimes difficult to get dry cherries with maximum 12 % water by simple sun drying and then an artificial drying is done with the help of driers (static, rotary or vertical type).

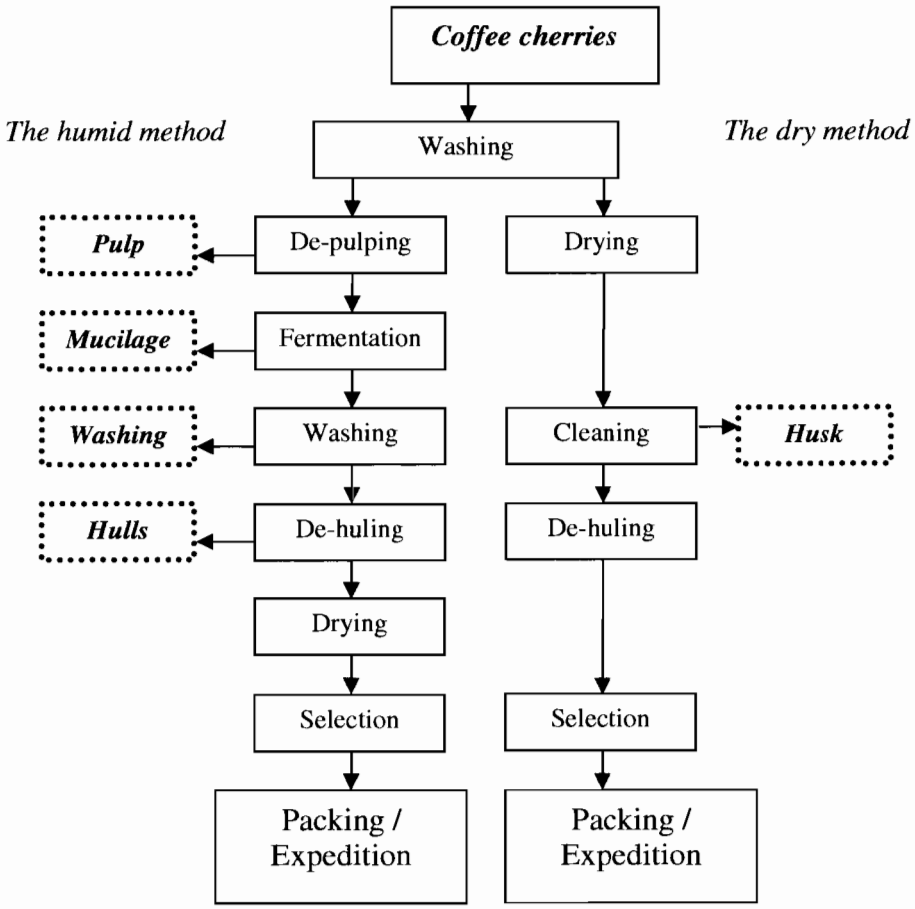


Figure 1: Different stages of the treatment of coffee cherries by the dry and humid method [2].

The most important residues of these processing are coffee pulp in the humid method and coffee husk in the dry method. About 80% of coffee produced in Brazil is by dry method, which results about 30 millions of coffee husk. Coffee husk has no potential utilization currently, because it has in toxic components such as caffeine, polyphenols and tannins. We undertook research work related with the application of coffee residues for its value-addition and application as substrate in bioprocesses [**5-39**].

Physico-chemical composition of the coffee husk

The coffee husk contains 31% fibres and 23% reducing sugars (DM). The ash content is also considerable high (11% DM). The high concentration of organic components in coffee husk makes it an important substrate for its application as animal feed or bioprocesses. However, it needs to be detoxified for such applications.

Biological detoxification of the coffee husk by filamentous fungi

Studies have been made to evaluate the capacity of certain strains of filamentous fungi to degrade the toxic components (caffeine and tannins) of the coffee husk. About fifteen strains, which were isolated locally were grown in a medium containing only an aqueous extract of coffee husk and the three strains belonging to *Rhizopus*, *Aspergillus* and *Phanerochaete* sp. were selected on the basis of their capacity of radial growth biomass production. Further studies were carried out in Erlenmeyer flasks on the effect of moisture and pH of the substrate, and temperature and period of incubation. With *R. arrizus* LPB-79, the best result were obtained after six days of culture at 28°C for the degradation of caffeine and tannins (87 and 65 %, respectively), with an initial pH of 6.0 and a humidity of 60 %. *P. chrysosporium* BK gave maximal degradation of caffeine and tannins as 70% and 45 %, respectively with 65 % of substrate humidity and an initial pH of 5.5, after 14 days of culture. Studies with *Aspergillus* sp. consisted to use a factorial design (2^{3-0}) in which three factors were analysed: pH and humidity of the substrate and the temperature of fermentation and results were submitted to the analysis of the variance (ANOVA). Data showed that for the detoxification of the coffee husk, initial pH of the substrate and temperature of incubation were significant. The best values of initial pH and temperature were respectively 4.5 and 26°C, resulting 89 and 56% degradation of caffeine and tannins.

A second phase of optimisation was made for which only two factors were varied: the initial pH of the substrate (3.0-5.0) and the temperature of incubation (26-30°C). Results were submitted to the ANOVA, which demonstrated best result at 28°C. The initial pH played an important role in the degradation of caffeine and tannins, which was 61 and 70 %, respectively at pH 4.0.

Figure 2 shows the evolution of the degradation of caffeine and tannins in relation to the time of culture with the physical and chemical conditions optimised for the strain *Aspergillus* sp. cultivated on coffee husk. It could be observed that the culture required a time of 18 h to synthesize enzymes necessary to degrade caffeine and tannins. Maximum caffeine was degraded after 72 h and tannins after 90 h of culture at 28°C.

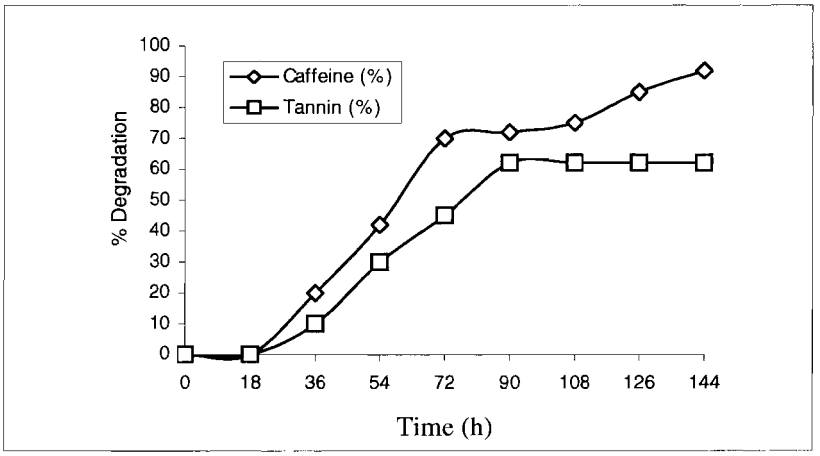


Figure 2 : Kinetics of degradation of caffeine and tannins by *Aspergillus sp.*

Further studies were carried out in a column-type bioreactor involving respirometric analysis. A balance of mass with the consumption of oxygen and the production of CO_2 in terms of flux (l/h) to the exit of the bioreactor was made. Fluxes of ventilation to the input (Fi) and to the exit (Fe) of the bioreactor were 0.045l/h g DM; we can establish equations:

$V_{O2S}e = (\%\ O_2e/100)Fe$

$V_{CO2}e = (\ \%\ CO_{2e}/100)Fe$

$V_{N2}e = ((100-\%\ O_2e-\ \%CO_2e)/100)Fe$

From the balance of mass of O_2 and N_2 one can get:

$VO_2cons = (20,9/100)Fi - (\%\ O_2S/100)\ Fe$

$VN_2i = VN_2e$

By putting the different equations together, the equation of the flux of entrance and exit of the bio-reactor can be given by :

$Fi \text{ or } Fe = ((100 - \%O_2-\%CO_2)Fe)/79.1$

To appraise the rate of consumption of oxygen (OUR) and the production of CO_2 in mmoles/h, we can consider that air is an ideal gas and that the respective volumetric fluxes (VO_2 consumption and VCO_2e) have been corrected for a temperature of 28°C.

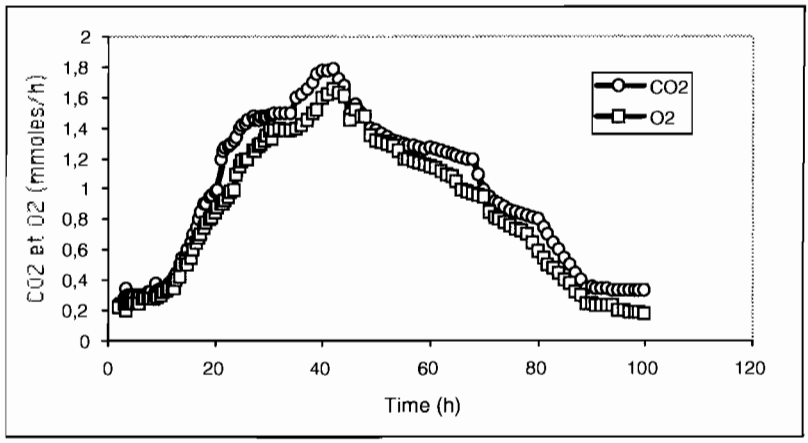


Figure 3: Consumption rate of oxygen and the production of CO_2 in mmoles/h) during the SSF of the coffee husk with *Aspergillus* sp.

Figure 3 shows the consumption of oxygen rate and the production of CO_2 during the detoxification of the coffee husk by *Aspergillus* sp. in solid-state fermentation. If we analyse the evolution of respiration coefficient (RQ) of detoxification of the coffee husk, we can note that the process has a characteristic of aerobic system until 80 h of fermentation with RQ acceptable with an average of 1.05 for this part of process. After 80 h of fermentation, RQ began to increase due to increase in CO_2 production. This increase of RQ could result in heterogeneous distribution of the air flux in the bioreactor due to the development of fungal mycelia (Fig.4).

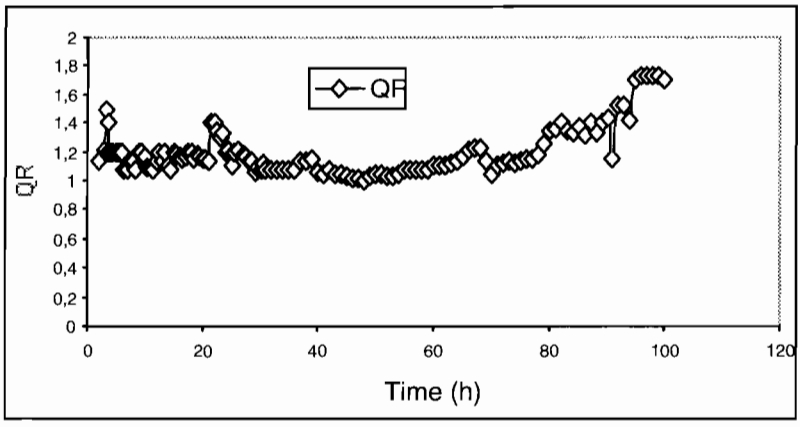


Figure 4 : Respiration coefficient (RQ) of detoxification of the coffee husk by *Aspergillus* sp.

From the calculated value for OUR and CO_2 produced, we can calculate the biotechnological parameters of the process of detoxification of coffee husk by *Aspergillus* sp. We applied these value in the equation described by [**40**].

$$Xn = (Yx/o\Delta t\ (1/2\ ((dO_2/dt)t=0 + (dO_2/dt)t=n) + \sum_{i=1}^{i=n-1} (dO2/dt)\ t=i\ + (1- a/2)Xo - a \times \sum_{i=1}^{i=n-1} xi) / (1+ a/2)$$

where: a = m(Yx/o) Δt

The process to estimate the quantity of biomass at different times (Xn) consists to make an evaluation by taking same value of the coefficient between the biomass and the oxygen consumed and the coefficient of maintenance (m) with the help of a computer program called FERSOL [41]. We took values of biomass at different times of fermentation (0, 15, 30, 45, 60, 75, 90 and 105 h). The program permitted to calculate coefficients of the equation. From values that have were experimentally for OUR and for the CO_2 production, the program calculated the yield coefficient (Yx/0) as 3.811 grams of biomass per gram of oxygen consumed with a coefficient of maintenance (m) of 0.0031 gram of O_2 per gram of biomass per hour.

Figure 5 shows results obtained with the analyses of the biomass in the laboratory. The biomass was estimated by equations (Xn) described before.

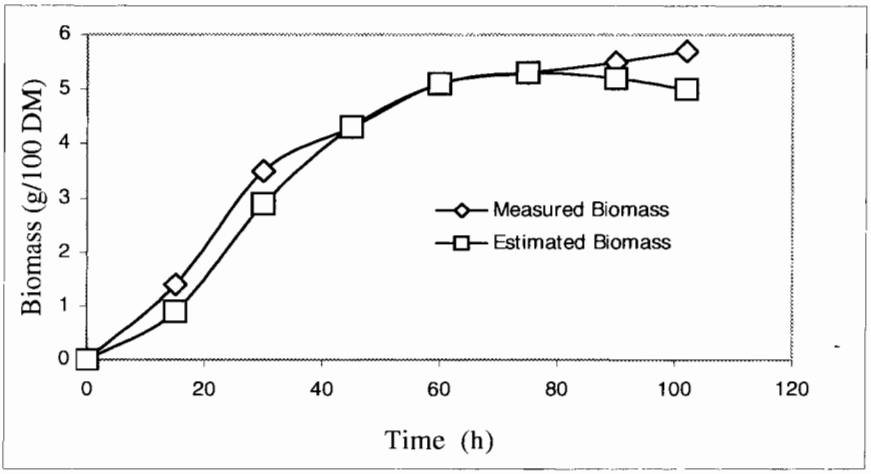


Figure 5: Comparison between the measured and estimated biomass during the detoxification of the coffee husk by *Aspergillus* sp.

There was variation of 14.26 % between 15 and 30 hours, because these values of the biomass were lower to values determined experimentally. Similar variation was

observed during 90 and 105 hours of culture. Thereafter, we calculated the specific growth rate (μmax) in the exponential phase with a regression of the Ln Xn in relation to the time of culture. The value obtained was 0.065 h^{-1}, with a regression coefficient of 0.987 for the period of 15 to 60 hours of SSF . The value Yx/0 (3.811 g of biomass produced /g of O2 oxygen consumed) appeared a little elevated, although the value of the maintenance coefficient appeared a little weak (m = 0.0031 g of O_2 consumed/gram of biomass produced/h).

Figure 6 shows the evolution of the production of CO_2 and O_2 during the detoxification of the coffee husk by *Aspergillus* sp. We can notice two completely distinct phases, an exponential growth phase that goes from 0 to 30 hours, in which the growth was very fast (rate of production of CO_2 was 8 %). From this moment, we can observe a reduction of the growth and the rate of production of CO_2 that descended below of 2 % after 90 hours of fermentation before maintaining thereafter steady to around 1.5 %.

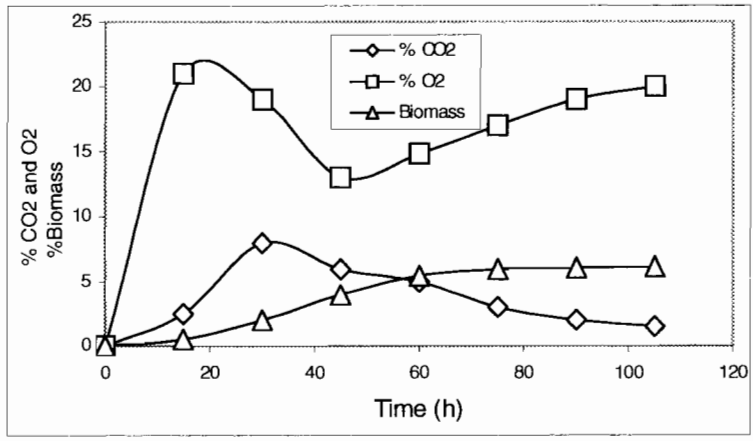


Figure 6: Evolution of the CO_2, O_2 and biomass during the detoxification of the coffee husk by *Aspergillus* sp. in SSF at 28°C.

Figures 7A and 7B showed that the reducing sugar consumption was related to the degradation of caffeine and tannins, and also with the synthesis of proteins. As no source of nitrogen was added to coffee husk, the fungal strain consumed nitrogen present in caffeine.

If we make an interrelationship between the production of CO_2 (Fig.6) and the reducing sugar consumption (Fig. 7A), we could see note that the highest production of CO_2 was at 30 h of SSF. We could also see that the toxic components were degraded until 60 h of fermentation (Fig. 7B).

There was also an increase in the content of protein in the coffee husk that reached 10.65% at the end of the detoxification. These results demonstrated the feasibility

of this process for the detoxification of the coffee husk, which could present better perspectives to be used as animal feed ingredient [**2, 5,11, 18, 25**].

Production of gibberellic acid

The gibberellic acid GA_3 ($C_{19}H_{22}O_6$), is a promoter and a regulator of plant growth and is a secondary metabolite produced *Giberrela fujikuroi* [**34**]. In spite of multiple applications of the gibberellic acid, its utilisation remain limited due to high price that varies between 1 to 3 US$/g according to its purity. Thus, it could be of great implication if the cost of production of gibberellic acid could be reduced.

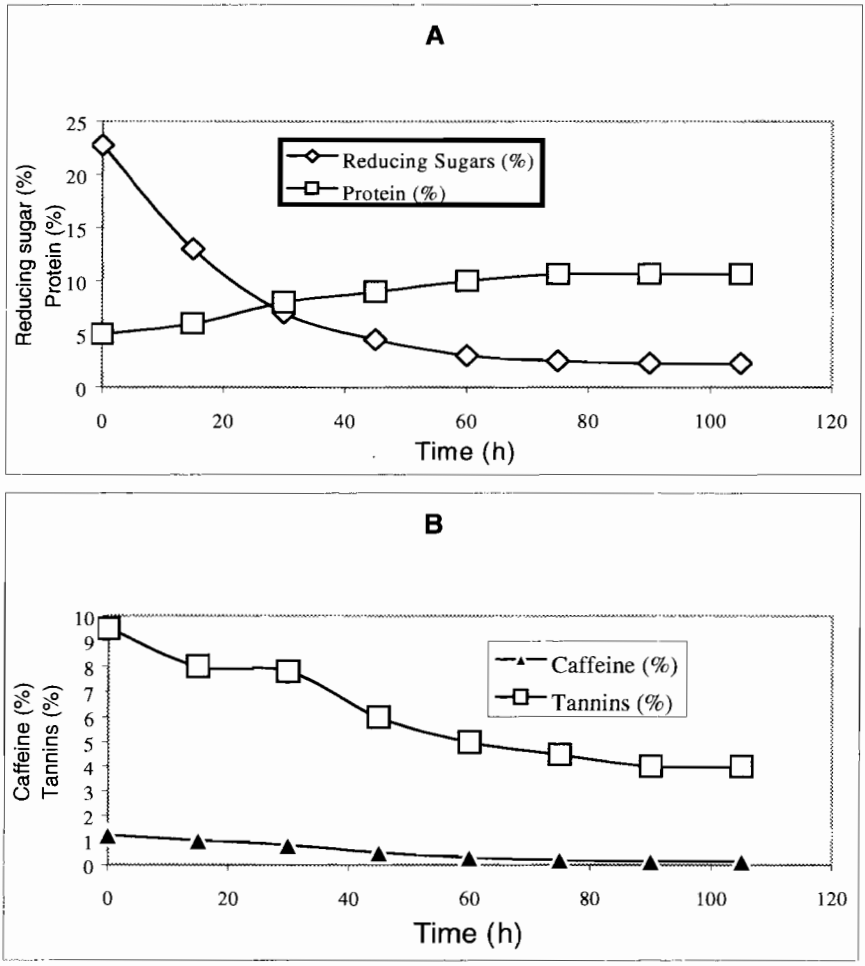


Figure 7: Detoxification coffee husk by *Aspergillus* sp. in SSF.

One of possibilities for this could be search of cheaper substrate and news techniques of fermentation. Currently, industrial production of gibberellic acid is achieved by liquid fermentation (LF) using synthetic media that are excessively expensive.

In view of these, we used coffee husk, a culture of G. *fujikuroi* and SSF for its production. Initially several strains of Giberrella genera were tested to evaluate their capacity to produce the gibberellic acid (GA_3) by SSF and by LF on coffee husk hydrolysate. The strain LPB-6 was selected for further studies. After a series of experiments to remove the toxic factors from coffee husk, the production of GA_3

reached to 112.6 mg/Kg of coffee husk. It represented an increase of 3 times the initial production of GA_3.

We also studied effect of the C/N on GA_3 production, which was reported to vary between 20 and 100 [**42**]. In coffee husk, C/N ratio was between 13-15. To increase this, cassava bagasse was added in it. Fig 8 shows the best results obtained on a mixture of coffee husk with different concentrations of cassava bagasse. Evidently 30% cassava bagasse with 70% of coffee husk gave best results (230.5 mg/Kg). This mixture had a C/N report of 43. Mixtures containing more than 45 % of cassava bagasse resulted reduction in the gibberellic acid production.

We also studied the effects of minerals ($FeSO_4$ and $(NH_4)_2SO_4$) on the production of GA_3, which under suitable concentrations resulted 389 mg of GA_3/Kg of substrate. The optimisation of other parameters of culture such as temperature of incubation (26 and 30°C), initial pH of medium (3 and 5), initial humidity (70 %) showed that these played an important role on the production of GA_3. Under optimal conditions, the production of GA_3 reached 494 mg/kg substrate. This concentration represents an increase of 13 times from the initial values [**12, 27, 34**].

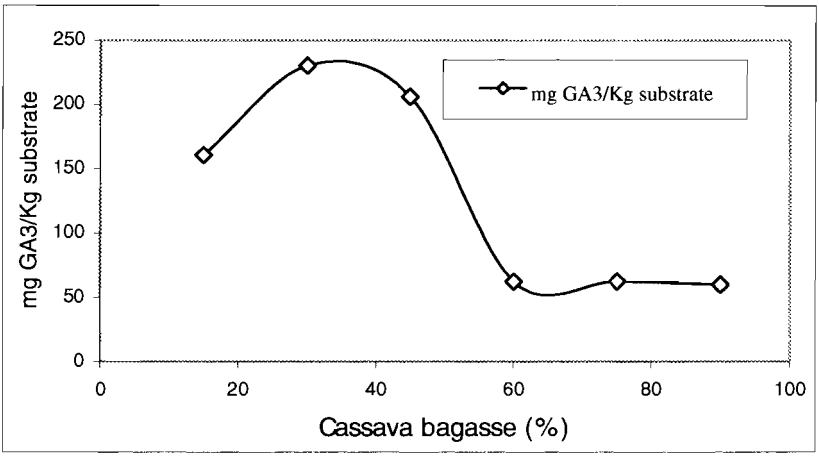


Fig 8 - Production of GA_3 on a mixture of coffee husk with different concentrations of cassava bagasse.

Edible mushrooms

Coffee husk was also evaluated to study its feasibility to produce edible mushrooms such as *Pleurotus* sp., *Lentinus edodes* and *Flamulina velutipes*. In these studies we also used other residues of coffee industry also, which included spent coffee ground and coffee leaves. Eight strains of *P. ostreatus*, 12 strains of *L. edodes* and one strain of *F. velutipeses* were tested for their capacity to grow in an agar medium containing extract of coffee husk as substrate.

Production of Pleurotus sp.

Eight strains of *Pleurotus* developed very well on agar medium. However, the strain *P. ostreatus* LPB 09 was best with dense mycelium (43.4 mg of biomass/dish) and high radial growth (9.68 mm/day) after nine days of culture. The results demonstrated that the biological efficiency of the strain cultivated on three different substrate, i.e. coffee husk, spend coffee ground and in a mixture of spend coffee ground + coffee leaves (60 :40) was 96.5, 90.4 and 76.7%, respectively after 60 days of culture. When leaves of coffee were used as substrate, the mushroom took five days to grow but there was not fructification. We observed an increase in protein content and a reduction in the fibres content in all the three substrates. Degradation of cellulose and lignin by *Pleurotus* has been reported earlier also [**43, 44**].

Production of Lentinus edodes

Based on growth in agar medium, the strain *L. edodes* LPB was selected as it showed best growth with very dense mycelium (9.68 mm/day and 43.4 mg/dish) during 12 days of culture. Untreated coffee husk resulted growth of mushroom took 20 days to grow completely but it didn't produce fruiting bodies. When coffee husk was treated with the boiling water for one hour, resulting coffee husk supported vigorous growth with a change of the colour of the substrate. The first flashes of mushroom took place after 60 days of culture and the biological efficiency was 85.8 %. When the spent coffee ground was used as substrate, the mycelium invaded the substrate after 20 days and the first flashes of mushroom appeared after 56 days of culture with a biological efficiency of 88.7 %. With the mixed substrate, the total colonization of the substrate by the mycelium took place after 25 days of incubation; the first flashes of mushroom took place after 65 days and the biological efficiency was 78.4 %.

Production of Flamulina velutipes

F. velutipes LPB 01 grew well on agar medium (7.87 mm/day and 45.78 mg/dish/10 days of culture). With the coffee husk as substrate, the substrate grew completely after 15 days of culture and the first flashes of mushroom appeared after 25 days of inoculation. The biological efficiency was 55.8%. This was first work on the culture of *F. velutipes* on the coffee husk. With the spent coffee ground as substrate for *F. velutipes,* the culture grew completely after 12 days and the first flashes of mushroom appeared after 21 days; the second flashes of mushroom took place after 45 days and the biological efficiency was 78.3 %.

Detoxification of coffee husk during the growth of the different species of edible mushroom and evolution related to caffeine and tannins in the fruiting body

In the fermented coffee husk, the reduction of caffeine and tannins was 61 and 80 %, respectively for *Pleurotus.* The concentration caffeine was 1.25 % and tannins 0.76 % in the fruiting body. The result demonstrated that the mushroom did

not degrade caffeine and tannins completely but absorbed them partially. In the fruiting body of *L. edodes*, no caffeine or tannins was found but in the fermented coffee husk, a reduction of 12% in the concentration of tannins was found. In the fruiting body of *Flamulina* also no caffeine and tannins was found, which in the fermented substrate were reduced by 10.2 and 20.4 %, respectively. These studies demonstrated that the edible mushrooms such as the *P. ostreatus, L. edodes and F.velutipes* could be cultivated on coffee husk or mixed substrates containing essentially coffee husk [**9,10,13,14,17,20,23,26,33**].

Production of food aroma compounds

Coffee husk can also be used to produce food aroma compounds. We used a strain of *C. fimbriata* for this purpose. It was found that that it was not necessary to supplement the substrate with mineral salts. We also noticed that the coffee husk treated with steam lost about 27.5 % of the mineral salts. Results of optimisation of culture conditions for the production of aromas by *C. fimbriata* demonstrated that it was necessary to supplement coffee husk treated with steam with glucose (20 to 35 %). Total volatile substances produced (TV) during fermentation were integrated using the model of Gompertz (logistical equation) [**45**]. This model describes the dynamics of the aroma production according to the time of cultures:

$TV = TVmax \exp \{-b \exp(-kt)\}$

Where :

TV = Volatile total products to the time t (mmol/l/g)

TVmax := maximal production of volatile total when the $t \longrightarrow \infty$ (mmol/l/g)

b = parameters of test, used to get the maximal production rate

($tmax = (\ln b)/k$, where k = production rate (h^{-1})

Compounds detected in the headspace of the culture depended on the quantity of glucose added in the medium of culture. When the coffee husk treated with steam was enriched with 20 and 35% of glucose, we noticed a very strong production of pineapple aroma (6.58 and 5.24 TV mmol/l respectively). The different components such as acetaldehyde, ethanol, isopropanol and ethyl acetate represented 80.5 and 75.4% of the TV. Ethyl isobutyrate, isobutyl acetate, isoamyl acetate and ethyl-3-hexanoate were identified in the headspace» of the culture. With 46 % glucose, a weak banana aroma was observed. Addition of leucine to the substrate contributed to the increase of the production of TV (8.29 mmol-l/g). In these conditions, a very strong banana aroma was detected. These results demonstrated that coffee husk could be used as a potential substrate for the production of aroma compounds by SSF [**7,12,21,22,32**].

Conclusions

Details presented as above showed that through microbial biotechnology, new economic opportunities could be made available in near future for the potential

application of residues of coffee industry, especially coffee husk in Brazil and elsewhere in the world. In the chain of the coffee processing, from the step of cherry picking until the cup of coffee, the residues represent 95 % of the total weight and only 5% of reach to the cup. Disposal of coffee industry residues in the environment, which is the current practise, causes pollution threats. In spite of the toxic component present in coffee husk as caffeine and tannins, this is very rich in sugars and proteins and could be used as substrate in bioprocesses to produce molecules of high value such as enzymes, aroma compounds, plant hormones, edible mushroom, feeds, etc. Solid-state fermentation appeared a potential technology for this purpose.

Acknowledgements

The author wishes to thank financial assistance from the European Union (INCO DC : 18* CT 970185), PNP&D-CAFÉ-Co-ordenatior EMBRAPA, Brazil (Project N° 07.199.079.01 and 07.199.079.02) and CNPq for a scholarship under the provisions of Scientific Productivity Scheme.

References

1. Café... . Brochure éditée par Le Comité Français du Café, Paris,59p.
2. Perraud-Gaime I. (1996). Cultures mixtes en milieu solide de bactéries lactiques et des champignons filamenteux pour la conservation et la décaféination de la pulpe de café. Thèse de Doctorat, Université de Montpellier II, France, 209p.
3. Coste R. (1989). Caféiers et cafés. Maison neuve et Larose et ACCT. (Eds), Paris, 373p.
4. Brand D., Pandey A., Roussos S., Soccol C.R. (2000) *Enzyme and Microbial Technology.* **26** (1-2), 127-133.
5. Pandey A., Soccol C.R., Poonan Nigan M., Brand D., Radijskumar M., Roussos S. (2000) *Biochemistry Engineering.* **6** (2), 153-162.
6. Soares M., Christen P., Pandey A., Soccol C.R. (2000) *Process Biochemistry.* **35**.(8), 857 - 861.
7. Medeiros A., Pandey A., Soccol C.R., Freitas R. J. S., Christen P. (2000) *Biochemical Engineering Journal.* **6** (1), 33-39
8. Leifa F., Pandey A., Soccol C. (2000) *Journal of Basic Microbiology.* **40** (3), 177-187.
9. Leifa F., Pandey A., R, Mohan., Soccol, C. R. (2000) *Acta Biotechnologica.* **20** (1), 41-52.
10. Brand D., Pandey J.R., Rodriguez-Leon J., Roussos S., Brand I., Soccol C.R. (2001) *Biotechnol. Prog.,* **17**, 1065-1070.
11. Soccol C.R., Leifa F., Woiciechowski A.L., Brand D., Machado C.M.M., Soares M., Christen P., Pandey A. (2000) *In:* Proceedings of III International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Iapar/IRD, p. 323-328.
12. Leifa F., Pandey A., Raimbault M., Soccol C.R., Mohan R. (2000) *In*: Proceedings of III International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR Brazil, Iapar/IRD, p. 377-380.
13. Leifa F., Pandey A., Raimbault M., Soccol C.R., Mohan R. (2000) *In*: Proceedings of III International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Iapar/IRD, p. 381-383.
14. Soares M., Christen P., Pandey A., Raimbault M., Soccol C.R.(2000) *In:* Proceedings of III International Seminar on biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil,

Iapar/IRD, p. 385-388.

15. Vandenberghe L.P.S., Pandey A., Lebeault J.M., Soccol C.R. (2000) *In*: Proceedings of III, International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Iapar/IRD, p. 389-392.
16. Leifa F., Pandey A., Raimbault M., Soccol C.R., Mohan R. (2000) *In*: Proceedings of III, International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Iapar/IRD, p.397-400.
17. Brand D., Kawata F., Pandey A., Roussos S., Rocha dos Santos M.C., Soccol C.R. (2000) *In*: Proceedings of III International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Iapar/IRD, p.401-403.
18. Leifa F., Pandey A., Soccol C.R. (1999) *In:* Proceedings 3rd. International conference on mushroom biology and mushroom products. Sydney - Australie. p.301 – 311.
19. Leifa F., Pandey A., Soccol C.R. (1999) *In*: Proceedings 3rd. International conference on mushroom biology and mushroom products. Sydney-Australie. p.293 - 300.
20. Soares M., Christen P., Soccol C.R. (1999) *In*: IV Congresso Latinoamericano de Biotecnologia y Bioingenieria. Huatulco, Oxaca, Mexico, p.480
21. Soares M., Pandey A., Christen P., Raimbault M., Soccol C.R. (2000) *In*: Coffee Biotechnology and Quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 419-425.
22. Roussos S., Augur C., Perraud-Gaime I., Pyle D.L., Saucedo Castaneda G., Soccol C.R., Ferrao I., Raimbault M. (2000). *In*: Coffee Biotechnology and Quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 377-392.
23. Woiciechowski A., Pandey A., Machado M.C., Cardoso E., Soccol C.R. (2000) *In*: Coffee Biotechnology and Quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 419-425.
24. Brand D., Pandey A., Roussos S., Brand I., Soccol C.R. (2000) *In*: Coffee biotechnology and quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 393-400.
25. Leifa F., Pandey A., Soccol C.R. (2000) *In*: Coffee biotechnology and quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 427-436.
26. Machado C.M.M., Oliveira B.H., Pandey A, Soccol C.R. (2000) *in*: Coffee biotechnology and quality, T. Sera, C.R. Soccol, A. Pandey, S. Roussos (Eds), Kluwer Dordrecht, 401-408.
27. Soccol C.R., Krieger N. (1998) *in:* Advances in Biotechnology, Ed. Educational Publishers & Distributors, New Delhi, p 25-40
28. Sera T., Soccol C.R., Pandey A., Roussos S. (2000) Coffee Biotechnology and Quality, Kluwer Academic Publisher, Dordrecht, p.625.
29. Ried C.R, Tumoru S., Soccol C.R., Roussos S. (2000) Proceedings of III International Seminar on Biotechnology in the Coffee Agroindustry. Londrina-PR-Brazil, Ed. Iapar/IRD, 513p.
30. Soccol C.R., Monteiro C.M., Heleno de Oliveira B. (2000) Production d'Acide Gibbérellique par fermentation en milieu solide avec des substrats mixtes. Patent. Br. DEINPI/PR . 0000525-8.
31. Soares M. (1998) Production d'arômes de fruits par *Pachsolium tannophilus* et *Ceratocystis fimbriata* cultivés sur coques de café en fermentation en milieu solide. Masters Thesis, UFPR, Brasil.
32. Leifa F. (1999) Production de Champignons comestibles du genre *Pleurotus* sur des résidus solides de l' agro-industrie du café. Masters Thesis, UFPR, Brasil.
33. Monteiro C.M. (2000) Production d'acide gibbérellique par fermentation en milieu solide sur des bio-résidus de l' agro-industrie du café. Masters Thesis, UFPR, Brasil.
34. Binder Pagnoncelli M.G. (2001) Physiologie de croissance et caractérisation moléculaire des bactéries lactiques isolées sur différents biotopes de café au Brésil. Masters Thesis, UFPR, Brasil
35. Zacarias Silva A.R. (2001) Production de Phytase par fermentation en milieu solide sur résidus agro-industriels (café, orange et pomme). Masters Thesis, UFPR, Brasil

36. Beux M. (2001) Isolement, caractérisation moléculaire et physiologie des levures isolées des différents biotopes du café : Développement de bio-procédés pour le traitement des déchets liquides de l'agro-industrie du café au Brésil. Masters Thesis, UFPR, Brasil
37. Tagliari Correa C.V. (2002) Extraction et purification des enzymes responsables de la détoxication des coques de café par fermentation en milieu solide Masters Thesis, UFPR, Brasil
38. Medeiros A. (2002) Développement de bio-procédés pour la production et la récuperation de bioaromes de fruits obtenus à partir des résidus solides de l'agroindustrie du café. Thesis, UFPR, Brasil,
39. Sato K., Nagatani M., Nakamuri K.J., Sato S. (1983) *J. Ferment. Technology*, **61**, 623-629.
40. Rodriguez Leon J.A., Sastre L., Echeverria J., Delgado G., Bechstedt W. (1988) *Acta Biotechnol.*, **8**, 307-310
41. Kumar P.K.R., Lonsane B.K. (1989) *Advances in Applied Microbiology.*, **34**, 26-139.
42. Zadrazil F. (1980) *European J. Appl. Microbiol. Biotechnol.*, **9**, 243-248.
43. Tsang L.J., Reid I.D., Coxworth E.C. (1987) *Appl. Environ. Microbiol.*, **53**, 1304-1306
44. Valsameda M., Martinez M.J., Martinez A.T. (1991) *Appl. Microbiol. Biotechnol.*, **35**, 817-823.
45. Christen P., Meza J.C., Revah S. (1997) *Mycol. Resear.*, **101**, 911-919.

Chapter 9

SCREENING OF EDIBLE MUSHROOMS FOR POLYPHENOL DEGRADATION AND TANNASE PRODUCTION FROM COFFEE PULP AND COFFEE HUSK

M. SOBAL[1,2], D. MARTINEZ-CARRERA[2], B. RIO[1], S. ROUSSOS[1]

[1] *Equipe de Mycologie et de Fermentations en Milieu Solide IRD, UR-119; IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925; 163 Avenue de Luminy; F-13288 Marseille Cedex 9; France; Email : msobal@esil.univ-mrs.fr ;*
[2] *Mushroom Biotechnology, College of Postgraduates; Apartado Postal 701; Puebla 72001, Puebla; Mexico; Email: dcarrera@colpos.colpos.mx*

Abstract

Edible mushrooms capable of degrading condensed tannins were selected in solid-state culture using coffee pulp and coffee husk as substrates. Eight different strains of *Pleurotus*, *Ganoderma*, and *Lentinula* were screened showing positive results. Several parameters, such as morphology of the colony, apical growth, biomass, and the pH were studied. Biochemical analyses of *P. ostreatus*, strain CP-50, growing on coffee-husk agar (CHA) have shown that this mushroom degrades the polyphenols presents in the culture medium. Three strains of *P. ostreatus* (CP-11, CP-37, CP-50), selected for further studies, degraded up to 75.4 % of condensed tannins after growing for 24 days on coffee-husk solid culture medium. This research opens up the possibility to recycle solid by-products from the coffee agro-industry as substrates in solid-state fermentation, using strains of edible mushrooms for degrading hydrolysable tannins by tannases, and for producing fruit bodies as a human food.

Key words: Coffee pulp, coffee husk, edible mushrooms, polyphenols, condensed tannins, tannases.

Introduction

The coffee pulp and coffee husk are the main solid by-products in the coffee agro-industry (**1**). These by-products are: 1) Produced in large amounts causing serious problems of environmental contamination (**2,3**); 2) Highly biodegradable due to high contents of sugars, proteins, and mineral salts (**4**); and 3) A potential animal feed (**5**). Accordingly, we carried out research work in order to stabilize fresh coffee pulp by the lactic acid silage technique during the crop season (**6**). However, recalcitrant and toxic compounds (RTC), such as hydrolysable tannins (caffeine), and condensed tannins, need to be removed as they are a serious drawback for using coffee pulp/husk as a nutritive source for feeding animals. The enzyme tannase catalyzes the breakdown of hydrolysable tannins (**7,8**), and it has many potential

New Horizons in Biotechnology, 89-95, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

applications in the food, pharmaceutical, and chemical industries (**9**). At present, many countries are trying to use coffee pulp for the cultivation of edible mushrooms for human consumption, and the production of animal feed through solid-state fermentation (**10,11**).

Coffee pulp and coffee husk are good substrates for the cultivation of edible mushrooms, such as *Pleurotus, Lentinula, Ganoderma,* and *Flammulina* (**12,13**). These mushrooms generally show a good ability to produce fruit bodies, while simultaneously reducing or degrading the toxic substances present in the substrate. Fann *et al.* (**14**) screened several strains of *P. ostreatus*, *L. edodes*, and *F. velutipes* for their ability to grow on a medium containing the extract of coffee husk and agar. Calzada *et al.* (**15**) carried out a similar study using coffee pulp as substrate for the selection of edible mushrooms. In this work, we report the degradation of condensed tannins and tannase activity in solid-state culture (SSC) by eight selected strains of edible mushrooms.

Materials and Methods

Strains

Eight strains of cultivated mushrooms were used in this study (Table 1). All strains are deposited at the culture collection from the College of Postgraduates at Puebla, Mexico (**16**).

Culture media

The mycelium was grown and maintained on potato-dextrose-agar medium (PDA), which was also used as spawn. Coffee medium for solid-state culture was prepared as follows: 200 g of coffee husk were boiled in 1 L of hot water (90°C) for 15 min. The supernatant was used to prepare one litre of coffee-husk-agar (CHA) medium, by adding 15 g of agar. This medium was sterilized at 121°C for 20 min. After sterilization, 10 ml of CHA medium were poured into Petri dishes (90 mm diameter) under aseptic conditions.

Table 1. Strains of edible mushrooms selected for this study.

Species	Code	Origin (Country/State)
Ganoderma lucidum (Leysser)Karsten	CP-158	Mexico (Morelos)
Lentinula lepideus Fr.	CP-6 (ATCC-62610)	Mexico (Veracruz)
Pleurotus ostreatoroseus Singer	CP-34	Mexico (Morelos)
	CP-44	Mexico (Morelos)
P. ostreatus (Jacq. ex Fr.) Kumm.	CP-37 (ATCC-60271)	Germany
	CP-50	Mexico
P. ostreatus f.sp. florida	CP-11	Germany
Pleurotus spp.	CP-91	Mexico (Chiapas)

ATCC= American Type Culture Collection, Manassas, Virginia, U.S.A.

Solid state culture

Substrates. The coffee husk was obtained from Damasco, Curitiba, Brazil, after processing coffee cherries by the dry process. The coffee pulp was obtained from Puebla, Mexico, by the wet process of coffee cherries. Both substrates were milled with an Ultraturrax IKA (Bioblock, USA).

Solid state culture. Coffee pulp (40 g, 70 % moisture) and coffee husk (40 g, 77% moisture) were placed within 250 ml Erlenmeyer flasks, which were sterilized at 110°C for 20 min.

Inoculation and culture conditions

Agar. Culture media were inoculated centrally with a piece of actively growing mycelia from a colony grown on PDA medium for 15 days. All plates were wrapped in Parafilm, and incubated at 25°C in the dark. Strains grown on CHA medium were incubated at 25°C for 24 days. Biomass was estimated using a sterilized cellophane disc placed on the agar surface from Petri dishes (90 mm diameter), as described by De Araujo *et al.* (**17**). The mushroom mycelium was allowed to grow over the cellophane disc, which was then lifted away, and dried at 105°C for 24 h.

Solid state culture. Substrates were also inoculated centrally with a piece of actively growing mycelia from a colony grown on PDA medium for 15 days.

Analyses

The reducing sugars were assessed by the method of Miller (**18**), while total phenols by the method of Folin and Ciocalteau (**19**). The method of Swain and Hillis (**20**) was used for condensed tannins, and the tannase assay was carried out according to Sharma *et al.* (**21**). One unit of enzyme (IU) was equivalent to the amount of enzyme able to release 1 μmole of gallic acid per min.

Results and Discussion

Screening of edible mushrooms

Growth parameters of eight selected strains of edible mushrooms growing on two different culture media are shown in Table 2. *Pleurotus ostreatus* (CP-50) showed an apical growth of 1.74 mm/day in the solid coffee-husk agar surface. A higher apical growth of 3.4 mm/day was recorded in *P. ostreatus* (CP-11).

Table 2. Screening of edible mushrooms for tannin degradation, tannase and biomass production after 21 days of mycelial growth at 25°C on coffee-husk agar medium (CHA) and coffee-husk solid culture medium.

Strains	Code	Culture media			
		Coffee-husk agar		Coffee husk solid substrate	
		Apical growth (mm/day)	Biomass (g/L/day)	Tannins (mg/g)	Tannase (IU/ml)
Ganoderma lucidum	CP-158	1.7	1.9	0	5.8
Lentinula lepideus	CP-6	0.2	0.6	1.2	71.42
Pleurotus ostreatoroseus	CP-34	2.5	1.2	2.4	9.9
	CP-44	1.4	1.2	1.7	7.8
P. ostreatus	CP-37	ND	ND	2.9	24.74
	CP-50	1.74	1.9	1.4	20.46
P. ostreatus f.sp. florida	CP-11	3.4	ND	ND	21.2
Pleurotus spp.	CP-91	1.1	1.2	1.8	16.74

ND= Not determined.

The highest quantity of biomass produced after 21 days of mycelial growth was recorded in *Ganoderma lucidum* (1.9 g/L/day), and *P. ostreatus* (CP-50: 1.9 g/L/day), followed by *P.* spp. (1.2 g/L/day), and *Lentinula lepideus* (0.6 g/L/day). However, a higher biomass production has been reported for *Trichoderma harzianum* (47 g/L/day) growing on coffee pulp juice (**22**). Brand *et al.* (**23**) reported a biomass production of 10.8 mg/20 ml/32 h by *Rhizopus* spp. grown on CHA. The strain CP-50 of *P. ostreatus* was selected for further studies.

Characterization of Pleurotus

The selected strain of *P. ostreatus* (CP-50) was grown on CHA medium for 24 days at 25°C. Colony size, biomass production, pH evolution, the consumption of reducing sugars, and total phenol degradation are shown in Table 3.

Table 3. Evolution of colony size, biomass, pH, reducing sugars and total phenols during mycelial growth of *Pleurotus ostreatus* (CP-50) on coffee-husk agar medium (CHA), after 24 days of incubation at 25°C.

Incubation time (days)	Colony diameter (mm/day)	Biomass (g/L/day)	pH	Reducing sugars (mg/ml)	Total phenols (mg/ml)
0	0	0	5.7	55.5	14.2
3	11	0.723	5.7	53.3	14.2
6	19	0.660	5.7	50.5	13.5
9	31	1.34	5.7	51.0	12.1
12	35	1.206	5.6	50.0	12.4
18	55	1.5	6.0	45.1	10.3
21	73	1.87	6.2	44.7	10.1
24	77	1.9	6.3	41.4	9.5
Utilisation (%)				25.4	33.1

A fast growth rate was recorded for the strain, producing about 1.9 g/L/day of biomass, and degrading around 33.1% of total phenols present in the culture medium (Table 3). The culture medium had an initial black colour, which changed to a yellow colour after 24 days of incubation. This change of colour indicated the action of fungal enzymes on the hydrolysis of phenols during mycelial growth (Fig. 1), as previously described for *Aspergillus* and *Penicillium* by Bradoo *et al.* (**24**) using a culture medium with tannic acid.

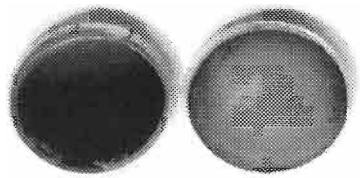


Figure 1. Petri dishes containing coffee-husk agar (CHA) medium after the inoculation of *Pleurotus ostreatus* (CP-50), which showed the hydrolysis of phenols. A: Time zero before inoculation. B: After 11 days of incubation at 25°C (the mycelium was lifted away from the agar surface).

Growth on coffee-husk solid medium

The reducing sugars consumption was 68.3 % for coffee husk in solid substrates, and the mycelial growth of *P. ostreatus* (CP-50) was vigorous. Furthermore, the degradation of phenols and condensed tannins was higher for coffee husk than for coffee pulp. Mycelial development observed on coffee husk was better than that on coffee pulp, as shown in Table 4. Degradation of total phenols and condensed tannins was very high for coffee husk substrate (58.5-75.4 %, respectively), in comparison with coffee pulp (8.3 % for total phenols). Wong and Wang (**25**) also reported degradation of tannins by *P. sajor-caju* and *L. edodes* grown on coffee grounds.

Table 4. Evolution of reducing sugars, total phenols and condensed tannins during the growth of *Pleurotus ostreatus* (CP-50) on coffee husk and coffee pulp in solid culture media after 24 days of incubation at 25°C.

Incubation time (days)	Reducing sugars (mg/g)	Total phenols (mg/g)		Condensed tannins (mg/g)	
	Coffee husk	Coffee pulp	Coffee husk	Coffee pulp	Coffee Husk
0	108.6	9.78	36.84	0.45	5.7
6	75.7	8.85	32.08	0.61	4.0
12	91.2	5.45	31.48	0.47	3.4
18	63.0	5.32	23.8	0.47	1.6
24	34.4	5.9	15.27	0.47	1.4
Utilization (%)	68.3	39.7	58.5	ND	75.4

ND= Not determined.

In general, it was established that the cellophane technique can be used successfully to allow the development of the mushroom mycelium, as well as to measure fungal biomass from edible mushrooms. The cultivation of *Pleurotus, Ganoderma* and *Lentinula* on coffee pulp and coffee husk has been reported for the production of fruit bodies (**11,12,14**). In this work, we showed that strains of these mushrooms are capable of degrading condensed tannins, and producing high levels of tannase.

Conclusions

The screening of eight strains from edible mushrooms (*Pleurotus, Ganoderma* and *Lentinus*), grown on a coffee-husk agar medium using a cellophane membrane, allowed to select *P. ostreatus* (CP-50) on the basis of its apical growth, biomass and tannase production. This strain degraded efficiently polyphenols and condensed tannins from coffee pulp and coffee husk, when used as solid substrates. Further studies are being carried out in solid-state fermentation (SSF) in order to optimise culture conditions, and to study the physiology and the metabolism of edible mushrooms for biomass and enzyme production from coffee pulp and coffee husk.

Acknowledgements

This study was supported by the European Union, through the project INCO-DC N°IC18*CT970185 BIOPULCA. M. Sobal has a Ph.D. scholarship from the National Council of Science and Technology (CONACYT), Mexico. D. Martínez-Carrera also thanks the support by CONACYT in Mexico, through the research project 36085-B.

References

1. Roussos S., Augur C., Perraud-Gaime I., Pyle L., Saucedo-Castañeda G., Soccol C.R., Pandey A., Ferrao I., Raimbault M. (2000) *In*: Coffee Biotechnology and Quality. Sera T., Soccol C.R., Pandey A. and Roussos S. (eds.), Kluwer, Dordrecht, pp. 377-392.
2. Zuluaga J. (1989) *In*: I Seminario Internacional sobre Biotecnología en la Industria Cafetalera. Roussos S., Licona-Franco R., Gutiérrez-Rojas M. (eds.), Xalapa, México, pp. 63-76.
3. Roussos S., Aquiáhualt M.A., Trejo-Hérnandez M.R., Gaime-Perraud I., Favela E., Ramakrishna M., Raimbault M., Viniegra-González G. (1995) *Appl. Microbiol. Biotechnol.* **42(5):** 756-762.
4. Bressani R. (1979) *In*: Pulpa de café: composición, tecnología y utilización, Braham J.E. and Bressani R., eds., Ottawa, Canada, International Development Research Centre, pp. 143-152.
5. Jarquin R. (1987) *In*: Utilización integral de los subsproductos del café. Memoria del Tercer Simposio Internacional. Guatemala, pp. 45-53.
6. Perraud-Gaime I., Saucedo-Castañeda G., Augur C., Roussos S. (2000) *In*: Coffee Biotechnology and Quality. Sera T., Soccol C.R., Pandey A. and Roussos S. (eds.), Kluwer, Dordrecht, pp. 437-446.
7. Van de Lagemaat J., Pyle D. L., Augur C. (1999) *In*: Proc. III SIBAC, Riede C. R., Sera T., Soccol C. R. and Roussos S. (eds.), Londrina, Brazil, pp. 409-411.
8. Aguilar C.N., Augur C., Favela-Torres E., Viniegra-González G. (2001) *Process Biochem.* **36:** 565-570.
9. Lekha P.K., Lonsane B.K. (1997) *Advances in Applied Microbiology* **44:** 215-260.
10. Martínez-Carrera D., Soto C., Guzmán G. (1985) *Rev. Mexicana Micol.* **1:** 101-108.
11. Rolz C., De Leon R., Arriola M.C. (1988) *Acta Biotechnol* **8(3):** 211-223.
12. Martínez-Carrera D., Aguilar A., Martínez W., Bonilla M., Morales P., Sobal M. (2000) *In*: Coffee Biotechnology and Quality, Sera T., Soccol C.R., Pandey A. and Roussos S. (eds.), Kluwer, Dordrecht, pp. 471-488.
13. Mata G., Gaitán-Hernández R. (1992) *Rev. Mexicana Micol.* 1992; *8:* 125-29.
14. Fan L., Pandey A., Vandenberghe L.P.S., Soccol C.R. (1999) *In*: Proc 3rd Internatl Conf Mushroom Biology & Mushroom Products. Sydney, Australia, pp. 293-300.
15. Calzada J.F., Leon R., Arriola M.C., Rolz C. (1987) *Biol. Wastes* **20(3):** 217-226.
16. Martínez-Carrera D., Bonilla M., Sobal M., Aguilar A., Martínez M., Larqué-Saavedra A. (1999) *Micol. Neotrop. Apl.* **12:** 23-40.
17. De Araujo A.A., Aquiahualt-Ramos M.A., Roussos S. (2000) *In*: Coffee Biotechnology and Quality, Sera T., Soccol C.R., Pandey A. and Roussos S. (eds.), Kluwer, Dordrecht, pp. 245-253.
18. Miller G.L. (1959) *Analytical Chemistry* **31:** 426-428.
19. Folin O., Ciocalteau V. (1927) *J. Biol. Chem.* **73:** 627-650.
20. Swain T., Hillis W.E. (1959) *J. Sci. Fd. Agric.* **10:** 63-68.
21. Sharma S., Bhat T.K., Dawrat R.K. (2000) *Anal. Biochem.* **279:** 85-89.
22. Roussos S., Lonsane B.K., Raimbault M., Viniegra-Gonzalez G. (1997) *Advances in Solid State Fermentation*, Kluwer, Dordrecht, p. 631.
23. Brand D., Pandey A., Roussos S., Soccol C.R. (2000) *Enzyme Microb. Technol.* **27:** 127-133.
24. Bradoo S., Gupta R, Saxena R.K. (1996) *J. Gen. Appl. Microbiol.* **42(4):** 325-329.
25. Wong Y.S., Wang X. (1991) *World Journal of Microbiology Biotechnolgy* **7(5):** 573-574.

Chapter 10

BIOCATALYSIS IN NON-CONVENTIONAL MEDIA BY ENTRAPMENT OF THE ENZYME IN WATER-RESTRICTED MICROENVIRONMENT

D. MADAMWAR, K. SONI

Post Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar-388 120, Gujarat, India

Abstract

Efficient functioning of enzymes in organic solvents by any means opens up new possibilities of applications in biocatalysis. Different methods have been proposed for retaining the catalytic power of enzymes and making them functional in organic solvents. One such promising approach is to investigate the properties of enzymes in water-restricted environment, which is based on enzyme entrapment in reverse micelles.

In reverse micelles enzyme molecules are solubilized in discrete hydrated micelles formed by surfactants within a continuous phase i.e. non-polar organic solvent. Under appropriate conditions these solutions are homogenous, thermodynamically stable and optically transparent. However, there are very few examples of preparative scale enzymatic synthesis in water–in-oil microemulsion. One reason for this is that despite the advantages offered by microemulsion media, product isolation and enzyme reuse from such single-phase liquid medium is more complex than in competing methodologies in which the catalyst is present as a separate solid phase. Therefore, the approach simplifying product isolation, and enzyme reuse from microemulsion based media has been the use of gelled microemulsion system. The potential scope will be discussed.

Key Words: Water restricted microenvironment, enzymes, biocatalysis, reverse micelles, microemulsion, organogel.

Introduction

The ability of enzymes to fulfill their roles in living organisms largely resides in their specificities and their power of catalysis. Moreover, even if they are isolated from their highly integrated structural network they retain their catalytic properties. These properties make them attractive as reagents in a wide variety of industrial, medical and research activities.

For several years, biological studies at molecular level started with isolation from their natural environment followed by studies in buffered aqueous solution. With

New Horizons in Biotechnology, 97-112, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

new developments in chemistry and biology and requirements in industries, large numbers of enzymatic reactions have been demonstrated to transform both natural and unnatural or synthetic substrates. The major synthetic value of enzymes as catalysts is their selectivity due to which they are able to contribute to stereospecific transformations.

As the field developed its targets and constraints have changed. The mere perception that enzymes function only in aqueous systems and that it cannot function in organic solvents is out of question today.

Water as such is a poor solvent for nearly all applications in industrial chemistry. Most organic compounds of commercial interest are very sparingly soluble and are often unstable in aqueous solutions. Chemists realized these limitations long ago and replaced water with more suitable in organic solvents. Unlike chemical processes, conventional biocatalysis was being performed in aqueous solutions. This is mainly due to the preconceived notion that nature intended enzymes to be catalytically active in water and that organic solvents serve only to destroy the catalytic power of enzymes.

There are numerous potential advantages in carrying out enzymatic reactions in organic media as opposed to aqueous solution and gave birth to non-aqueous enzymology. Thus the reactions which were impossible in aqueous media due to kinetic or thermodynamic constraints and synthetic reactions under low water condition became possible (**1,2**).

- Non-aqueous enzymology is of particular relevance when desired reactants are poorly soluble in aqueous solutions and when reversal of hydrolytic reactions are desired. One example is to employ lipase in non-aqueous media for esterification and transesterification reactions (**2,3**).
- Lower inhibition effects due to a reduced product concentration in the vicinity of the enzyme resulting from the normally better solubility of the product in the surrounding organic phase than in the microenvironment of the hydrophilic enzyme surface.
- Enhancement of thermal stability of the enzymes.
- Immobilization is often unnecessary as enzymes are insoluble in organic solvents. If immobilization is desired, adsorption onto non-aqueous surface can be done, enzymes are unable to desorb from these surface in non-aqueous media.
- Microbial contamination is eliminated.
- Recovery and reuse of the enzyme is easier.
- Fewer side reactions that involve water.

Various methods have been developed to use enzymes in organic solvents. These include immobilization, protein engineering, reactions in monophasic, biphasic and reverse micellar systems (**4-12**). One common method is the use of biphasic system consisting of water and a water immiscible organic solvent. Since the enzyme is

dissolved in water phase and the hydrophobic substrate is dissolved in the organic phase the contact between the catalyst and the substrate is a limiting factor although stirring is applied (**13**).

Another method is to suspend the enzyme as a dry powder directly in an organic solvent with the addition of minimal amount of water, which is necessary for the enzyme to be active. This method also requires stirring and relatively high amounts of enzyme have to be used. In order to improve this system the enzyme can be immobilized by adsorption on solid supports, thus creating larger contact areas and also preventing the enzyme molecules from aggregating, resulting in increased enzyme activity. The commonly used immobilization methods include deposition of the enzyme from aqueous solution, precipitation of the enzyme from water in the presence of cold water- miscible solvent, and covalent coupling of the enzyme to a support (**14**). The adsorbed enzyme may be cross-linked further with, for example glutaraldehyde. The support used for immobilization can be an inorganic material, a synthetic polymer or a polysaccharide (**14**). The immobilization techniques listed above require several preparation steps prior to the catalytic reaction.

Covalently modifying enzymes avoid the use of stirring. This is done using polyethylene glycol or surfactants making enzyme soluble in organic solvent (**15**). A widely used method is to solubilize the enzyme in spontaneously formed water-in-oil (w/o) microemulsion; which are suitable for studying enzyme catalyzed reactions in small scale (reverse micelles) (**7,16**). Both water-soluble and water insoluble substrates may be used since the system consists of small water droplets separated by a monolayer of surfactants from the continuous organic phase.

Reverse Micelles

Reverse micelles are spheroidal aggregates formed by surfactants in apolar media (Fig. 1).

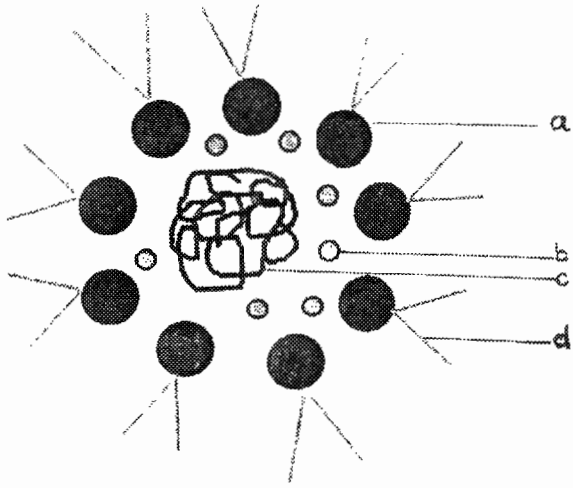


Figure 1: Schematic diagram of reverse micelles; (a) surfactant polar head; (b) water buffer molecule; (c) enzyme/protein; (d) surfactant nonpolar tail.

In contrast to normal micelles in water, the polar head groups of the surfactant molecules are directed towards the interior of the aggregate and form a polar core which can solubilize the water (the 'water pool') and the lipophilic chains are exposed to the solvent. Under appropriate conditions these are optically transparent and thermodynamically stable. These spheroidal droplets of diameter 10 to 200 nm are spontaneously generated and remain dispersed in continuous organic phase as a result of molecular self-assembly (**17,18**). Due to its mimicking of supramolecular structures, it plays an important role in different areas of basic and applied research. These reverse micelles constitute a novel environment for enzyme-catalysed reactions (**19, 20**).

Components of Reverse Micelles

Amphiphlic Compound

Amphihiles (surfactants) are characterized by having two groups that differ markedly in their solubility relationship i.e. hydrophobic lipophilic balance (HLB). For C_nH_{2n+1} COOK n=1– 8 surfactants are predominantly hydrophilic and for n=12 they are predominantly lipophilic. The tendency of the surfactant to form reverse micelles is a result of a balance between the net cohesive energy of the surfactants with the oil and water at the interface (**21**). Surfactants can be classified according to the nature of their hydrophilic part into anionic, cationic, nonionic and zwitterionic (ampholytic) surfactants.

Anionic Surfactants

Among the anionic surfactants which form water-in-oil microemulsion, the most widely used is Aerosol OT (AOT, sodium di –2- ehtylhexysulphosuccinate). No co-surfactant is required while using AOT. AOT has six water hydration per Na^+ ion, two to four per sulfonate head group and one per carbonyl group (**22**). Other anionic surfactants include SDS (sodium dodecyl sulphate), CPCl (cetylpyridinium chloride) etc.

Cationic Surfactants

One of the most studied cationic surfactant is CTAB (cetyltrimethyl ammonium bromide). Generally in this case a co-surfactant is required to obtain reverse micellar solution. The hydration number is low for cationic surfactants (**23**).

Nonionic Surfactants

Various non-ionic surfactants like Tween, Triton and Brij series have been employed to form reverse micelles. Triton X-100 is a polydisperse preparation of isooctylphenyl polyoxyethylene ether with an average chain length of 9.5 units. It enhances catalyst stability, relative to both aqueous solutions and ionic reversed micelle system. An important feature of Tween is that it is not toxic. The HLB of non- ionic surfactant shifts to lipophilic when temperature increases and hydrophilic when the temperature falls respectively.

Zwitterionic Surfactants

The first preparation of reverse micelles was done with a zwitterionic surfactant, that was a phospholipid – phosphotidylcholine (**24**).

Non- polar Solvent

High grade, non-polar solvents like n-hexane, n-heptane, dodecane, benzene, xylene, isooctane, cyclohexane, toluene are used to dissolve amphiphiles. When the substrate of enzymatic reaction is water soluble, it will be solubilized in the aqueous core of the reverse micelles. Hence no direct effect is observed on the reaction with different solvents but in the case of reactions such as lipase-catalyzed hydrolysis of olive oil, the effect of solvent on the apparent activity is probably related to the effect of solvent on substrate partitioning between the aqueous and organic phases. The partition coefficient of the substrate decreases activity of enzyme increases (**18**).

Co-surfactant

Some surfactants need a co-surfactant in order to reside in the organic phase and form water-in-oil microemulsions. Co-surfactants, which are usually long chain alcohols, usually participate in the interfacial region of the water-in-oil microemulsion (**25**). Surfactants do lower the interfacial tension but sometimes the critical micellar concentration (CMC) or the limits of solubility is reached before the interfacial tension is close to zero. Addition of a second surfactant of a

completely different nature then lowers the interfacial tension of water-in-oil microemulsion system.

Buffer/ Water

Small amount of water/buffer is necessary to stabilize the reverse micelles. Water/surfactant molar ratio (Wo) is one of the important parameters in enzyme-catalyzed reactions. The size of the reverse micelles is dependent on Wo. As Wo increases, the micellar size increases. The water molecules that are located in the core of the reverse micelles have different properties from that of bulk water (**26**).

Reverse Micelles : Structure and Dynamics

A typical reverse micellar solution is constituted by a proper composition of water, surfactant and (co-surfactant) and solvent. To find out the specific volume of each component required to make a reverse micelles, normally a triangular phase diagram is used. From a triangular diagram it is easy to calculate the volume of each component and mix them by microinjection of the aqueous buffer solution into a stirred solution of surfactant dissolved in organic solvent in order to obtain a transparent solution.

The diameter of filled and unfilled micelles is determined by using Small Angle Neutron Scattering Spectroscopy (SANS) (**27**), Small Angle X Ray Scattering (SAXS), Quasi Elastic Light Scattering Spectroscopy (QELS) and Dynamic Laser Scattering Spectroscopy (DLS) (**28**).

Wong *et al.* (**29**), used Nuclear Magnetic Resonance (NMR) Spectroscopy to study the structure of water in AOT reverse micelles. NMR also used to determine partition coefficient between the micellar core, interface and continuous phase in microemulsions. The physical properties of the reverse micelles as a function of water content have been studied by Zuluaf & Ecike (**30**), using Dynamic Light Scattering Spectroscopy.

The water added to form reverse micelles determine two important parameters Wo (water-to-surfactant molar ratio) and θ (water volume fraction), which affect reverse micelles stability and enzyme activity. The Wo, also named as R, represents the size of the reverse micelles. The θ expresses the concentration of identically sized reverse micelles at a fixed Wo and it is the percentage of water with respect to the whole volume of the system.

The physical parameters of water in the reverse micellar medium strongly depend on the value of Wo and on the nature of the surfactant. Water in the reverse micellar medium with a low Wo value has the same properties as that of immobilized water. Here the water has low dielectric constant and lower freezing point (**31**). When the amount of water in the system is increased, the water gradually starts to behave like bulk water (**32**). El Sound *et al.* (**33**), defined different domains of reverse micelles based on the presence of three differently organized water molecules.

Free water, whose properties and structure become closer to bulk water as Wo increases.

Bound water has properties that are qualitatively different to those of bulk water, because of hydrophilic interactions with the polar head groups of the surfactant.

Surfactant tails, penetrate the apolar solvent.

When the amount of water is not sufficient to hydrate the surfactant molecules, there is no free water and reverse micelles have only two domains; bound water and surfactant.

In contrast to micelles formed in aqueous solution, reverse micelles are electrically neutral in their exterior shell, so that no columbic repulsion occurs and collisions are frequent. Exchange of the contents of reverse micelles is believed to occur by means of a collision-fusion-fission process (**34**). That is upon collision of two reverse micelles, a dimer is formed with a single mixed aqueous pool. Separation of the fused micelles results in two new reverse micelles, in which the contents are redistributed.

The water pool present inside the reverse micelles provides a unique medium for the reactions of water-soluble substrates because the effective polarity, acidity and microscopic viscosity are quite different than those of bulk water (**20, 26**).

Reverse Micelles As a Host for Enzymes : Methods of Protein Solubilization

In practice, the enzyme solubilization in reverse micellar systems can be carried out by three different ways (**28, 35**): injection, dry enzyme addition and enzyme transfer from an aqueous phase to an organic phase containing the surfactant (Fig. 2). Injection and addition methods are basically used in biocatalytic applications, and the transfer method is the base of the protein liquid- liquid extraction.

In the injection method, a few microlitres of the enzyme is injected into the organic phase that contains the surfactant. In the second method, the enzyme (as a powder) is gently stirred with a certain amount of water. In both the techniques the resultant solution is clear and contains only one phase. According to the transfer method, the protein is present initially in an aqueous solution, which is covered with a layer of the reverse micellar solution. Upon gentle stirring, the protein is slowly transferred from the aqueous phase into the micellar phase. In 1988, Luisi *et al.* (**19**), first reported the transfer of proteins from an aqueous phase to an organic phase.

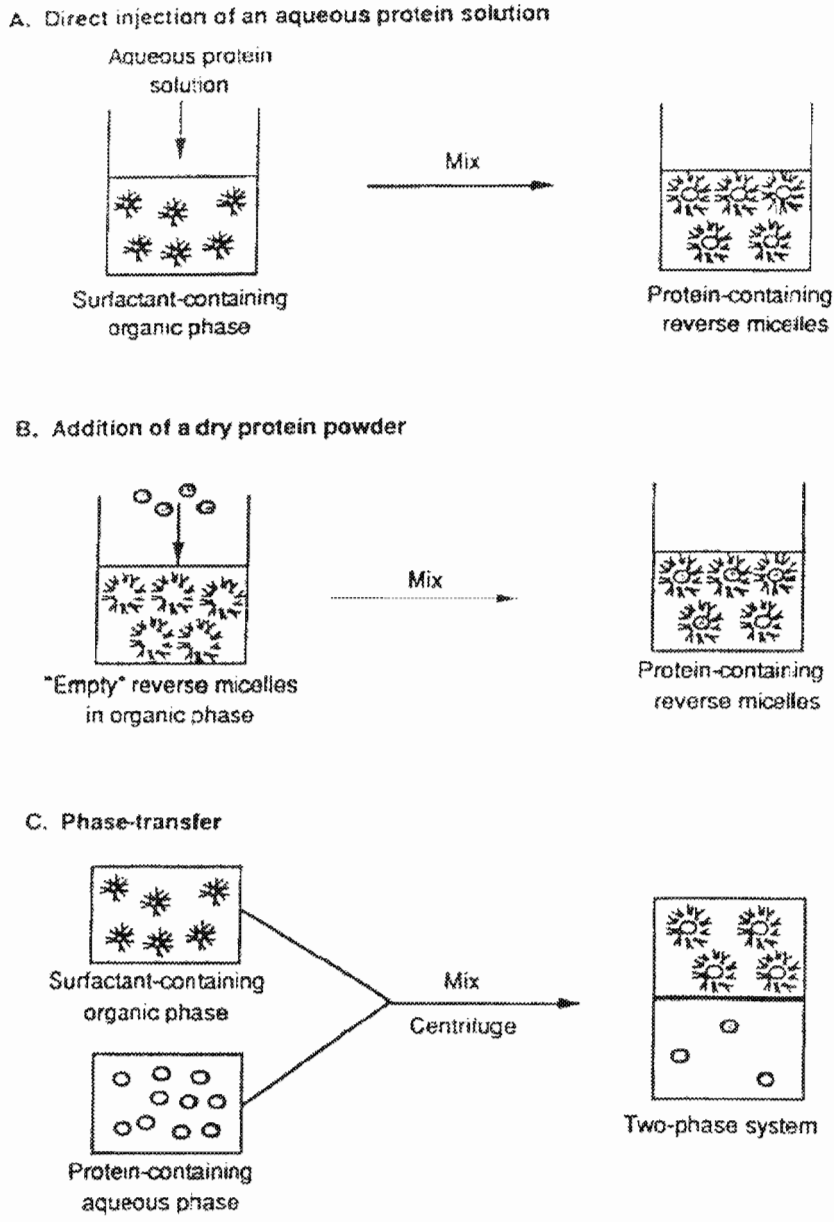


Figure 2: Methods of protein solubilization in reverse micelles.

Protein solubilization in reverse micelles is influenced by several parameters : pH, aqueous phase ionic strength, reverse micelles size and type of surfactant used.

Structural Aspects

How the enzyme is allocated inside the reverse micelles is not completely clarified yet. An important feature of reverse micelles is their flexibility, i.e. the entrapped protein molecule can itself choose the environmental optimal for its functioning.

Most of the data for hydrophilic proteins could be interpreted on the basis of the so-called water shell model according to which the protein is confined to the middle of the water pool and protected by a layer of water from the charged inner wall of the micelle and from the external organic medium. A molecule of surface-active enzyme interacts with the interface formed by surfactant molecules, where as a hydrophobic enzyme molecule makes contact with the hydrophobic region of the micelle and even, with the solvent (Fig. 3) (**35**).

Paradkar and Dordick (**36**) have proposed a new model for chymotrypsin. Results of their spectroscopic studies indicate that chymotrypsin does not exist inside a discrete water pool of reverse micelles, but is located at the aqueous/ organic interface. The major driving force behind this process is the electrostatic attraction between the negatively charged head group of AOT and the positive charge on the enzyme surface. The use of low surfactant concentrations favours the ion-paired form. If excess of AOT is present, enough surfactant molecules interact with the enzyme molecule, and the protein-AOT complex can be converted into the reverse micellar form. Simultaneous transfer of water and ions into the organic phase takes place. Hence, depending on AOT concentration the protein can reside in either a reverse micelle or as hydrophobic ion-pair.

In the same way, Lye *et al.* (**37**) suggested that it was the exposure of the solubilized enzyme to the bulk organic solvents. This was suggested through calculations of micellar protein occupancy and through the relative surface areas of protein molecules and surfactant head groups that prompted protein-surfactant complex formation. In light of their experimental result, a mechanistic model was proposed (**37**), to account for the partitioning of lysozyme and ribonuclease between aqueous and AOT/ isooctane reverse micelles. This model is based upon the competing effects of increasing the solubilized enzyme concentration and the corresponding increase in the rate of enzyme-surfactant complex formation.

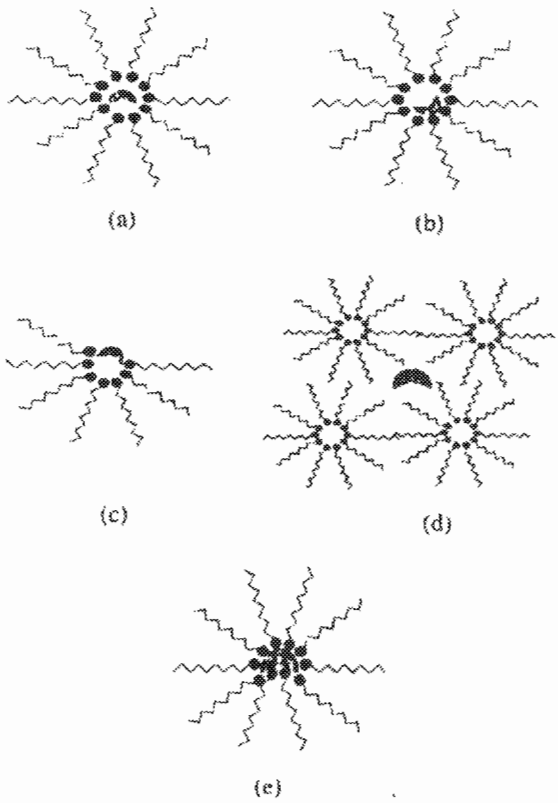


Figure 3: Possible models for a protein hosted in a reverse micelle; (a) enzyme located in the middle of the water pool; (b) enzyme adsorbed to the micellar inner wall by electrostatic effects; (c) enzyme adsorbed to the aqueous lumen of a reverse micelle; (d) membrane active proteins have the capacity to interact with multiple reverse micelles; (e) surfactant enzyme ionic pair solubilized in reverse micellar media.

Enzymatic Reactions in Reverse Micelles

Reverse micelles are discrete structures in a dynamic state. One important characteristic of reverse micelles is their coalescence upon collision. It is assumed to take place through a 'transient dimer', a collision-fusion-fission process (**34**), having a communication channel which permits rapid exchange of material. A typical reverse micelle experiences between 10^9 and 10^{10} collisions per second and about one in every thousand collisions results in dimer formation. This means that reverse micelles communicate with each other in a micro to millisecond timescale, which is faster than the time scale for enzymatic reactions.

Enzymatic reactions in reverse micelles are found to obey normal Michaelis-Menten kinetics (**38**), although interpretation of results may be difficult due to the compartmentalization of the enzyme, substrate and products between the microdomains of the reverse micelles.

Theoretical models have been proposed to explain enzymatic data obtained in reverse micellar systems, which can be divided into diffusional and non-diffusional models. Diffusional models assumes that changes in enzyme activity results from a restriction in substrate accessibility (diffusional factors) (**39, 40**) It is extremely difficult to measure the rate of exchange between reverse micelles because of their high speed (**38**), which is faster than the enzymatic rate. Non-diffusional models are based on the fact that differences in kinetic constants (V_{max} and K_m) with respect to bulk water are due to conformational changes taking place in the reverse micellar microenvironment. Bru *et al.* (**38**) have proposed a non-diffusional model based on the existence of four physicochemically different psuedophases where enzyme and substrate can be distributed in free water, bound water, surfactant tails and organic solvents. Enzyme activity is, then the sum of three Michaelis-like equations describing the activity expressed by the enzyme in each pseudophase. This model simulates and fits the behaviour of enzyme in reverse micelles including superactivity and inhibition kinetics, using adequate adjustable parameters.

Enzymatic Activity and Stability in Reverse Micelles

In reverse micellar systems enzymatic activity depends on the micellar core size (Wo) and micelle concentration. Different patterns of behaviour are found by changing Wo (Fig. 4).

Enzyme activity decreases continuously as Wo increases. As large Wo values increase 'free' water increases establishing a diffusional barrier between the enzyme and the substrate, thus decreasing the global reaction rate (**19**).

A bell shape curve is observed, explained by the existence of an optimal micellar core size adequate to accommodate the enzyme molecule with a suitable and active conformation (**41**).

A saturation curve explained by the need of the enzyme for free water in order to achieve maximal activity, having no effect on activity with higher Wo values (**42**).

'Superactivity' of the enzyme (higher than that observed in bulk water) due to a greater reactivity of the water in the micellar core and/or to the relatively restricted environment experienced by the enzyme molecule in the micellar 'cage' (**20**).

Data about enzymatic activity both in aqueous solution and in reverse micellar media at different Wo values are shown in Table 1 (**35**).

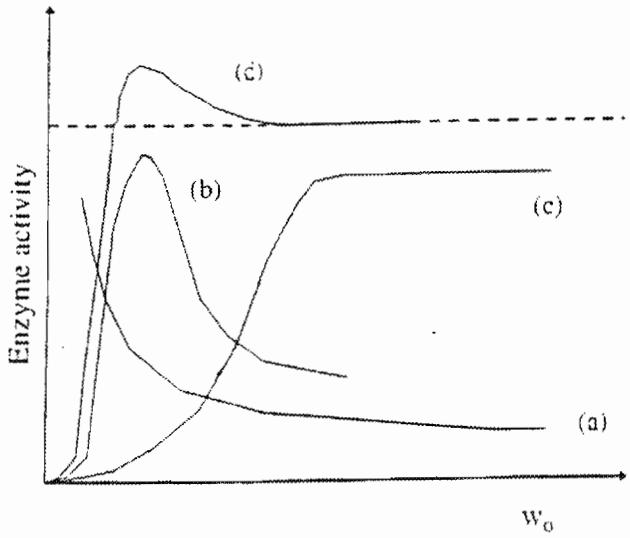


Figure 4 : Wo influence on enzyme activity.

(a) Enzyme activity decreases continuously as Wo increases. As large Wo values increases "free" water increases, establishing a diffusional barrier between the enzyme and the substrate, thus decreasing the global reaction rate.

(b) A bell shape curve, explained by the existence of an optimal micellar core size adequate to accommodate the enzyme molecule with a suitable and active conformation.

(c) A saturation curve explained by the need of the enzyme for free water in order to achieve maximal activity, having no effect on activity higher Wo values.

It is a well known fact that enzymes are usually more stable in low-water conditions because dehydration drastically hinders the thermal inactivation of the enzyme and protects its conformational rigidity (**43**). However, only a few studies have been carried out about this question in reverse micellar systems. Barbaric and Luisi (**44**), have verified that in an AOT/isooctane reverse micellar system the chymotrypsin enzymatic stability depends on the Wo value: at low Wo values the enzyme shows the greatest stability compared to what is observed in aqueous solution. In the same way, papain (**45**), exhibits an improvement in life time replacing the aqueous media for an AOT/isooctane micellar system (Wo = 21), although the enzymatic activity decreases. Martinek *et al.* (**46**), have studied the stability of several enzymes in different reverse micellar systems.

Table 1 : Enzymatic activity in reverse micellar system.

Enzyme	Aq. solution Activity (s^{-1})	Reverse micelles Activity (s^{-1})	Wo
Papain	0.025	0.005 (AOT/Isooctane)	21
α-chymotrypsin	0.0224	0.139 (AOT/isooctane)	10
Lipase (*Rhizopus arrhizus*)	-	0.3135	11.8
Xanthine oxidase	11.60	2.10 (AOT/isooctane)	10.4
Liver alcohol Lactate dehydrogenase	4.13	0.748 (AOT/Isooctane)	70
Subtilisin [a]	6.21	2.05 (Tween 85 / isopropanol / hexane)	60

[a]Activity Expressed as μmol min^{-1} mg $enzyme^{-1}$

Application of Reverse Micelles

Research in the field of protein hosted in reverse micelles has been developing rapidly over the last few years. Some of the biotechnology applications of reverse micelles are as follows:

Enzymes in Reverse Micelles as a Catalyst in Organic Media

Enzymes entrapped into reverse micelles can perform hydrolytic reactions in the reverse sense (**2,3,47**) and also use compounds poorly soluble in water as substrates (**11,48**). The ability of microemulsion to dissolve both active enzyme and apolar substrates, provides the means for the efficient conversion of substrates into useful products such as steroids and leukotrienes (**49,50**).

Solubilization of Whole Cells and Organelles

α-Amylase and invertase containing baker' yeast and Brewer's yeasts have been entrapped in reverse micelles formed by cetylpyridinium chloride (CPCI) in n-hexane and the activity measured (**51**). Also, *Acetobacter calcoaceticus*, *Escherichia coli* and *Corynebacterium*, were also solubilized to study the cellular behavior under such conditions. Organelles like mitochondria and nucleic acid are solubilized in reverse micelles (**52**). These systems can be tailored for specific purposes.

Extraction of Proteins

Reversed micellar solutions are attractive organic solvent solutions systems for use in the liquid-liquid extraction of biological products. Many biochemicals, including amino acids, proteins and nucleic acids can be solubilized within and recovered from such solutions without the loss of native function. The experimental method for reversed micellar extraction consisted of two steps, forward transfer and backward transfer. During forward transfer the enzyme enters into reversed micellar phase form an aqueous phase. While during backward transfer protein present in reversed micellar phase enter into an aqueous phase.

The use of reverse micelles to extract and separate protein has been widely reported (**53-55**). A good specificity of the separation can be achieved based on electrostatic effect (pH, pI) (**56**), even for proteins with quite similar sizes.

Cryoenzymology

Since the water entrapped in reversed micelles freezes well below zero. The reverse micellar medium has given new impetus to the progress of cryoenzymology (**26**). At this temperature, reaction is slowed down and therefore provides enough time to study the kinetics of formation and decomposition of intermediates.

Micellar Enzymology

Hydrated associates of protein with amphiphile compounds formed in organic solvents represent a rather realistic model of 'elementary' fragments of bio-membrane structures.

The basis for this conclusion lies in the fact that the protein inside the reversed micelles comes into contact (at a low water content) with structural lipid (**20**).

Microemulsion based Organogels (MBGs)

Various methodologies exist in the literature for effecting biotransformations using cell free enzymes in apolar media. One of the most intensively studied has been the technique of solubilizing enzymes in hydrated reverse micelles or water-in–oil (w/o) microemulsions. A major attraction of this procedure is that the enzyme is dispersed at the molecular level, rather that as a solid aggregate, in thermodynamically stable liquid solution which is capable of solubilizing polar, apolar, and interfacially active substrates. Studies describing the behaviour of a wide variety of enzymes solublized in w/o microemulsions have been reported, and the field has been extensively reviewed (**19,22,57**). However, there are very few examples of preparative scale regioselective or stereoselective enzymatic synthesis in w/o microemulsions. One reason for this is that despite the advantages offered by microemulsion media, product isolation and enzyme reuse from this type of single-phase liquid medium is more complex than in competing methodologies in which the catalyst is present as a separate solid phase.

One approach to simplifying product isolation and enzyme reuse from microemulsion-based media has been the use of gelled microemulsion systems; in particular, those based on gelatin and Aerosol-OT (AOT) w/o microemulsions. The formation of gelatin-containing microemulsion-based organogels (MBGs) was first described in 1986 (**58**), and their physical/structural characterization has been the subject of a number of studies (**59**). The MBG is proposed to comprise extensive rods stabilized by monolayers of surfactant, in coexistence with a population of conventional w/o micoemulsion droplets.

Gelatin-containing microemulsion based gels (MBGs) are rigid and stable in hydrophobic organic solvents and may be used for enzyme catalyzed large scale conversion of hydrophobic substrates (**60**), Rees *et al.* (**60**) demonstrated repeated

preparative scale kinetic resolutions using gelatin containing microemulsion based organogels (MBGs) in a batch reactor, yielding hundreds of gram of product. We have shown repeated use of lipase immobilized in gelatin containing Aerosol-OT MBGs, with intermittent AOT treatment (**3**).

References

1. Buckland B.C., Dunhill P., Lilly M.D. (1975) *Biotechnol. Bioeng.***17**, 815- 826.
2. Borzeix F., Monot F., Vandecasteele T.P.(1992) *Enzyme Microb. Technol.* **14**, 791- 797.
3. Soni K., Madamwar D. (2001) *Process Biochemistry.* **36** (7) 607-611
4. Dabulis K., Klibnov A.M. (1993) *Biotechnol. Bioeng.* **41**, 566- 571.
5. Dordick J.S. (1989) *Enzyme Microb. Technol.* **11**, 194- 211.
6. Janssen A.E.M., Van der Padt A., Van Sonsbeck H.M., Van't Reit K. (1993) *Biotechnol. Bioeng.* **41**, 95- 103.
7. Svensson I., Wehtje E., Adlercruetz P., Mattiason B. (1994) *Biotechnol. Bioeng.* **44**, 549 –559.
8. Claudia S., Sellapan S., Madamwar D. (1997) *Appl. Biochem. Biotechnol.* **62**, 183- 189.
9. Jaeger K.E., Reetz, M.T. (1998) *Tibtech.* **16**, 396 –403.
10. Khmelnistsky Y.L., Levashov A.V., Klyachko N.L., Martinek K. (1988) *Enzyme Microb. Technol.* **10**, 710-724.
11. Klibnov A.M. (1989) *Trends Biochem. Sci.* **14**, 141-144.
12. Soni K., Shah C., Madamwar D., (2000) *Biocatalysis Biotransformation.* **18**, 331–341.
13. Antonini E., Carrea G., Cremonesi P. (1981) *Enzyme Microb. Technol.* **3**, 291 –296.
14. Hedstrom G., Backlund S., Eriksson F., Karlsson S. (1998) *Colloids Surf. B. Biointerface*, **10**, 379 –384.
15. Kamiya N., Murakami E., Goto M., Nakashio F. (1996) *J. Ferment. Bioeng.* **1**, 37 –41.
16. Avramiotis S., Stamatis S., Kolisis F.N., Lianos P., Xenakis A. (1996) *Langmuir*, **12**, 6320–6325.
17. Fletcher P.D.I., Horsup D.I. (1992) *J. Chem. Soc. Faraday Trans*, **88**, 855- 864.
18. Yang F., Russell A.J. (1994) *Biotechnol. Bioeng.* **47**, 60- 70.
19. Luisi P.L., Giomini M.P., Robinson B.H. (1998) *Biochem. Biophys Acta.* **947**, 209–246.
20. Martinek K., Levashov A.V., Klyachko N.L., Khmelnitski Y.L., Berezin I.V. (1986) *Eur. J. Biochem.* **155**, 453–468.
21. Winsor P.A. (1968) *Chem. Rev.* **68**, 1–40.
22. Luisi P.L., Magid L.J. (1986) *Crit. Rev. Biochem.* **20**, 409 –474.
23. Lalitha J., Mulimani V.H. (1996) *Biochem. Mol. Boil. Int.* **40**, 571 –598.
24. Hanahan D.J. (1952) *J. Biol. Chem.* **195**, 199 –206.
25. Bansal V.K, Shah D.O., 0' Connell J.P. (1980) *J. Coll. Interf. Sci.*, **75**, 462 –469.
26. Douzou P. (1980) *In:* Advances in Enzymology, Ed. Meister, A., New York, **51**, 30- 69.
27. Rahman R.S., Hatton T.A. (1991) *J. Phys. Chem.* **95**, 1799 –1811.
28. Matzke S.F., Creagh A.L., Hayner C.A., Pransntiz J.M., Blanch H.W. (1992) *Biotechnol. Bioeng.*, **40**, 91 –102.
29. Wong M., Thomas J.K., Gratzel M. (1976) *J. Amer. Chem. Soc.*, **98**, 2391 – 2397.
30. Zulauf M., Eicke H.F. (1979) *J. Phys. Chem.*, **83**, 480 –486.
31. Douzou P., Keh E., Balny C. (1979) *Proc. Nat. Acad. Sci. U.S.A* **76**, 681– 685.

32. Poon P.H., Wells M.A. (1974) *Biochem. J.* **13**, 4928 –4936.
33. El Seoud O.A. (1984) *In*: Reverse Micelles: Biological and Technological Relevance of Amphilic Structures in Apolar Media, Ed. Luisi P.L. and Straub B.E. Plenum Press, New York, pp. 81– 85.
34. Fletcher P.D.I., Howe A., Robinson B.H. (1987) *J. Chem. Soc. Faraday Trans.* **83**, 985 -1006.
35. Luis G. P., Valencia-Gonzalez M. J., Diaz-Garcia E.M. (1996) *Anales De Quimica Int.* **92**, 312 - 319.
36. Paradkar V.M., Dordick J.S. (1991) *Biotechnol. Prog.* **7**, 330 –334.
37. Lye G.J., Asenjo J.A., Pyle D.L. (1995) *Biotechnol. Bioeng.* **47**, 509 –519.
38. Bru R., Sanchez –Ferrer A., Garcia-Carmona F. (1989) *Biochem. J.* **259**, 355- 361.
39. Oldfield C. (1990) *Biochem. J.* **272**, 15 –22.
40. Varhaert R.M.D., Halhorst R., Vermue M., Schaatsma T. J., Veeger G. (1990) *Eur. J.Biochem.* **187**, 59 –72.
41. Klyachko N.L., Levashov A.V., Pshezhetsky A.V., Bogdanova N.G., Berezin I.V., Martinek K. (1986) *Eur. J.Biochem.* **161**, 149 –154.
42. Bru R., Sanchez-Ferrer A., Garcia-Carmona F. (1990) *Biochem.* J. **268**, 679 –684.
43. Volkin D.B., Staubli A., Langer R., Klibanov A.M. (1991) *Biotechnol. Bioeng.* **37**, 843 – 853.
44. Barbaric S., Luisi P.L. (1981) *J. Am. Chem. Soc.* **103**, 4239 –4244.
45. Vicente L.C., Aires-Barros R., Empis J.M.A. (1994) *J. Chem. Tech. Biotechnol.* **60**, 291 –297.
46. Martinek K., Levashov A.V., Klyachko N.L., Pantin V.J., Berezin I.V. (1981) *Biochem. Biophys. Acta.* **657**, 227 –294.
47. Hayer D.G., Gulari E. (1991) *Biotechnol. Bioeng.* **38**, 507-517.
48. Vos K., Lanne C., Vanhoek A., Veeger C., Visser A. (1987) *Eur. J. Biochem.* **169**, 275-282.
49. Luisi P.L., Luthi P., Tomka I., Prenosil J., Pande A. (1984) *Ann. N.Y. Acad. Sci.* **434**, 549 –557.
50. Hilhorst R., Lanne C., Veeger C. (1983) *FEBS Lett.* **159**, 225 – 228.
51. Gajjar L., Singh A., Dubey R.S., Srivastava R.C. (1997) Appl. *Biochem. Biotechnol.* **66**, 159 –172.
52. Hochkoeppler A., Luisi P.L. (1989), *Biotechnol. Bioeng.* **33**, 1477 –1481.
53. Gordana S., Julian, C.B. (1998) *Biotechnol. Bioeng.* **38**, 374 – 379.
54. Soni K., Madamwar D. (2000) *Process Biochem.* **36**, 311 – 315.
55. Dekker M., Van'triet K., Weijers S.R., Baltussen J.W.A., Laane C., Bijsterbosh B.H. (1986) *Chem. Eng. J.*, **3**, 27 – 33.
56. Bratko D., Luzar A., Chen S.H. (1988) *Bioelectrochem. Bioeng.*, **20**, 291–296.
57. Oldfield C. (1994) *Biotechnol. Genetic. Engg. Rev.*, **12**, 255 –327.
58. Hearing G., Luisi P.L. (1986) *J. Phys. Chem.*, **90**, 5892 – 5895.
59. Atkinson P.J. (1991) Characterization of microemulsion–based organogels, Ph.D Thesis, University of East Anglia, Norwich, U.K..
60. Rees G.D., Robinson B.H., Stephenson G.R. (1995) *Biochem. Biophys. Acta*, **1259**, 73 – 81.

Chapter 11

ORGANIC SOLVENT RESISTANT LIPASE PRODUCED BY THERMORESISTANT BACTERIA

S. TORRES, G.R. CASTRO

PROIMI - Biotechnology. Avenida Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina. e-mail: grcastro@proimi.edu.ar

Abstract

A thermoresistant microbial wild-type strain designed S-86, which can be grown in presence of 3-methylbutanol in the medium, and can produce lipolytic enzyme was isolated. The S-86 strain was identified as a member of the *Bacillus* genus and belonged to the *subtilis* group. It is tentatively named *B. licheniformis*. Exocellular lipase was detected during stationary growth phase, and displayed high enzymatic activity in 50 % homogenous solvent mixtures of glycerol, ethylenglycol, propilenglycol, and methanol. Highest lipase activity was detected in 25 to 50 % glycerol-water mixtures, and optimal reaction temperature in the range of 65 to 70°C in both solvent systems.

Introduction

Biocatalysis in non-aqueous media is now a well-established academic area, and also matter of industrial interest. Advantages of non-aqueous enzymology include displacement of thermodynamic equilibrium towards synthesis, increase of substrate specificity and selectivity, suppression of water-dependent side reactions, enhanced thermostability of substrates and products, lack of microbial contaminations (**1**). However, there are some drawbacks of biocatalysis in organic solvents such as enzyme instability, and very poor enzyme solubility. Low enzyme solubility implies that most of the biocatalyst systems in non-aqueous media are heterogeneous, producing diffusional limitations by decreasing substrates- and enzyme-product interactions. To increase enzyme solubility and reaction rates, homogeneous organic-aqueous solvent mixtures is an alternative for non-conventional biocatalysis.

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are gaining relevance in organic solvent biotransformations by their enantioselectivity, substrate specificity and physicochemical properties (**2**). On the other hand, a bottleneck is the small diversity of available lipases, which restricts the catalytic properties of the enzymes. Most commercial lipases are produced from fungi or mammals, a few from Gram-negative bacteria, and only one report described the selection of lipase-producing microorganism for non-aqueous biocatalysis purposes (**3**). Additionally, scarce

New Horizons in Biotechnology, 113-122, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

information is available on lipases produced by Gram-positive bacteria, and particularly from *Bacillus* species, one of the most important enzyme producers of the microbial genera (**4**).

In several microorganisms, organic solvent tolerance was tested, but most of the assays were performed on aromatic hydrocarbons. Gram-negative microorganisms were preferred models because they are more solvent tolerant than Gram-positive bacteria according to previously established scale based on log P (or Solvent Hydrophobicity), defined as the solvent partition coefficient between 1-octanol and water (**5, 6, 7**).

Recently, effects of aliphatic alcohols, like butanol and isomers, were tested on yeast metabolism and morphology (**8, 9**). Nevertheless, only a few reports dealt with the effects of organic solvents, in particular and to our knowledge none with alkanols on Gram-positive bacteria (**10**).

3-Methylbutanol (isoamyl alcohol) is a very attractive organic solvent model due to its high cellular toxicity and strong effects on metabolism and cell differentiation process. It also presents inhibitory effects of some lipases (**9,11**). In addition, isoamyl esters are important for the production of sake and banana flavors (**12**).

The aim of the present study was the screening of 3-methylbutanol and temperature- resistant *Bacillus* strains able to produce lipolytic enzymes. Non-aqueous homogeneous medium enzymatic activity, and optimum temperature will be also tested.

Materials and Methods

Bacteria cultures

Enrichment medium (EM) containing (g/l): yeast extract, 5.0; peptone (from caseine), 3.0; NaCl, 10.0, NH_4NO_3, 1.2; and tributyrin, 10.0. For plate assays, EM was supplemented with agar 15.0 g/l.

Culture medium (CM) containing (g/l): $NaNO_3$, 1.2; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 3.0; K_2HPO_4, 6.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.05; $MnSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001; Peptone, 0.5; yeast extract, 1.0; and tributyrin, 10.0.

Enzyme production (EP) medium (g/l): $NaNO_3$, 1.2; KH_2PO_4, 3.0; K_2HPO_4, 6.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.05; $MnSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001; Peptone, 0.23; yeast extract, 0.46; and maltose, 10.0. The pH media were adjusted to a final pH of 7.5 using 250 mM phosphate buffer prior to sterilization.

Screening procedure

One gram of South American soil samples was suspended in 10 ml of sterile 145 mM NaCl. The suspension was heated for 10 minutes at 80°C, followed by centrifugation at 2000 x g for 10 minutes at room temperature. Supernatant aliquots of 200 µl were added to 125 ml Erlenmeyer flasks containing 30 ml of EM, and incubated for 72 h at 55°C and 4.0 Hz. The procedure was repeated twice for each

sample in the same experimental conditions using CM media. After 72 h culture, samples were streaked on agar plates at 55°C. Lipolytic microbial activity was detected by their ability to hydrolyze tributyrin. Colonies able to hydrolyze tributyrin were surrounded by clear halos in turbid medium. To isolate pure clones, lipase-producing single colonies were picked, cultured, and spread on tributyrin agar at 55°C twice as described before.

Enzyme production experiments

Selected colonies were grown in Erlenmeyer flasks (125 ml) containing 30 ml of EP media at 55°C supplemented or not with isoamyl alcohol (0 to 1.0 %) for 72 h on a rotatory shaker at approximately 3.3 Hz. Growth was measured as a function of optical density at 560 nm in a spectrophotometer (Metrolab 1250, Corswant, Argentina). Cell-free enzyme activities were assayed in supernatant by centrifuging the cultures at 8,000 x g for 10 minutes (4°C). Alternativily, lipase activity was assayed in partially purified extracts obtained by precipitation of crude extract with cold acetone (-20°C) at 0°C for 6h. The precipitate was dried, and resuspended in 50 mM Tris HCl buffer (pH= 7.0). All experiments were carried out twice in duplicate.

Strain characterization

Wild-type strain was characterized morphologically and biochemically according to international criteria (**13**). Antibiotic sensitivity profile was performed as previously reported (**14**). Jacquard coefficient (**15**) were calculated for taxonomic analysis considering a matrix that included 0 up to 49 % as negative and 50 up to 100 % as positive values, respectively. The matrix frequencies were derived from the data of previous workers (**13,16**). Maintenance procedures used for microorganisms were performed according to methods previously described (**13**).

Enzyme assays

Lipase activity was quantified by using p-nitrophenyl derivative of acetate (**17**). The reaction mixture contained 60 µl of 200 mM Tris HCl buffer (pH 7.0), 5 µl of 74 mM p-nitrophenyl derivative, 275 µl of solvent-water mixtures, and 60 µl of enzyme. Organic solvents (e.g. acetone, dioxane, ethanol, methanol, etc.) were used in the concentration range of 0 to 80 % of total reaction volume in the enzyme assays. Enzyme reactions were carried out at 37°C for 30 minutes, and the production of p-nitrophenol was determined spectrophotometrically at 400 nm in a 1-cm light path (Metrolab 1250, R. Corswant, Argentina). An enzymatic unit (EU) was defined as the amount of enzyme producing 1 µmol of p-nitrophenol per minute.

Determination of protein concentration

Coomassie blue G-250 reagent was used to determine sample protein content using bovine serum albumin (Fraction V) as standard as previously described (**17**).

Temperature optima

To determine the optimum temperature, the assay mixtures were incubated for 5 minutes at pH 7.0 at temperatures varying from 37 to 75°C and lipase activity determined as described in this paper.

Reagents

All reagent used in this work were of analytical or microbiological grade from Sigma (St. Louis, Mo, USA) or Merck (Darmstad, Germany).

Results and Discussion

Twelve of eighteen thermoresistant colonies displaying lipolytic activity on tributyrin agar were isolated from ten South American soil samples. Microbial solvent tolerance to 3-methylbutanol was tested on the twelve selected lipolytic colonies ranging from 0.1 to 0.6 % solvent concentrations at 55°C. In our experimental conditions, alcohol concentrations higher than 0.6 % increased microbial death, as previously reported for some yeasts (**18**).

Microbiological properties of the four wild-type most lipase-producer promising strains were investigated under microscope following Bergey's Manual of Systematic Bacteriology. Morphological strain tests showed Gram-positive rod shapes with round ends containing endospores. Based on the assays, the four strains could be classified into the *Bacillus* genus (**13**).

Different 3-methylbutanol concentrations in the four selected *Bacillus* strains were tested in batch cultures to measure microbial growth and enzyme production after 12 to 48 h culture at 55°C. Highest enzyme activity in the four strains was detected at 48 h (Table 1). Two different growth responses in media supplemented with 0.10 % of 3-methylbutanol: biomass determined as optical density of *Bacillus* sp. S-851 and S-852 strains were depleted; and at least two times cellular concentration stimulation for *Bacillus* sp. S-84 and S-86 strains were found (Table 1). However, slight to strong growth inhibition was observed using 3-metylbutanol concentrations higher than 0.20 % in all four strains (Table 1).

Microbial growth rate decreased, and changes from single cell growth pattern to complex filamentous growth forms were observed under microscope concomitantly with the increase of alcohol concentration in the medium (results not shown). Cell macrofibers and structured filamentous growth forms have been described for *B. subtilis* species previously (**19**). In addition, it has been observed that 0.5 % concentration of 3-methylbutanol induces strong changes on cell morphology conducing to aberrant cellular forms during the cell division cycle on yeasts (**18**). Filamentous multicellular bacterial growth of wild-type *Bacillus* strains cultured in presence of 3-methylbutanol at 55°C could be explained by cell separation failure after division induced by organic solvent and temperature stress factors. Also, microbial filaments could be interpreted as bacterial strategy for survival in environments under unfavorable cultural conditions because the advantages of multicellular state buffers the cells growing under environmental stress.

On the other hand, 3-methylbutanol enhanced the production of exocellular lipases in all four *Bacillus* strains (Table 1). Maximal enzyme activity in cell-free extracts was detected in all strains at 0.40% organic solvent. This fact could be explained by the interaction of 3-methylbutanol with components of cellular membrane. Previously, butanol and higher molecular weight alcohols have been reported to produce large disorder in the glycerol backbone of lipid membranes indicating that hydroxyl group of alcohols is anchored near the aqueous interface of the lipid (**20**). The integrity loss of cellular membrane could be responsible of the increased enzyme activity by the release to the medium.

Table 1. Effect of isoamyl alcohol on growth and lipase production of selected strains.

Strain	3-methylbutanol concentration (%)	Growth (560 nm) 48 h – 55°C	Lipase Activity (U/ml)
S84	0.00	0.690	0.008
	0.10	1.420	0.087
	0.25	0.522	0.233
	0.40	0.920	0.068
	0.60	N.D.	N.D.
S851	0.00	0.884	0.034
	0.10	0.700	0.101
	0.25	0.560	0.139
	0.40	0.819	0.278
	0.60	0.070	0.020
S852	0.00	1.280	0.135
	0.10	0.900	0.156
	0.25	0.937	0.109
	0.40	0.580	0.322
	0.60	0.413	0.009
S86	0.00	0.470	0.105
	0.10	1.150	0.186
	0.25	0.670	0.255
	0.40	0.840	0.434
	0.60	0.274	0.007

N.D.: not detectable.

In previous work, some representative strains of *Bacillus* species from cultures collections were tested in agar assays overlaid with different solvents to determine the growth limiting solvent hydrophobicity (log P). Growth limiting log P values ranging from 4.5 (corresponding to cyclooctane) of *B. subtilis* to 7.0 (corresponding to dodecane) of *B. circulans* were found (**5**). These results suggested that *Bacillus* species could not grow in media with more hydrophilic solvents with lower log P. However, using 3-methylbutanol (log P: 1.3) several solvent tolerant *Bacillus* clones were successfully isolated in the present work. These results are in agreement with previous work in which a novel *Bacillus* sp. was able to grow in presence of aromatic solvents such us benzene, toluene, and p-xylene with 2.1, 2.5 and 3.1 log P respectively (**21**). These discrepancies could be attributed to the experimental culture conditions since techniques using agar tests overlaid with

organic solvents present some problems related to oxygen and carbon dioxide exchange; they can be avoided using liquid media with shaking.

Bacillus sp. S-86 was selected on the basis of its higher enzyme activity, and cellular concentration in presence of 0.40% of 3-metylbutanol compared with the other selected strains. *Bacillus* sp. S-86 was identified by morphological and biochemical tests. The results are detailed as follows: spore-former with rounded ends and oval to cylindrical morphology in terminal position, no bulging, uniformly stained, no vacuoles were observed. Submerged cultures of this strain observed under microscopy showed long filamentous chains or single cells with motility. Growth occurred in presence of 20 to 146 g l^{-1} NaCl, at 30 to 55°C. Catalase activity was positive.

Positive growth and fermentation were detected in: amygdalin, L-arabinose, arbutin, esculin, casein, cellobiose, D-fructose, galactose, glycerol, glycogen, N-acetyl-glucosamine, D-glucose, α-Methyl-D-glucoside, inositol, maltose, mannitol, D-mannose, D-raffinose, ribose, salicine, sorbitol, starch, sucrose, D-tagatose, trehalose, D-turanose and D-xylose.

No growth or fermentation were observed in media supplemented with adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, ß-gentobiose, gluconate, 2-keto-gluconate, 5-keto-gluconate, inulin, D-lyxose, α-methyl-D-mannoside, melizitose, rhamnose, L-sorbose, L-xylose, xylitol, and ß-methyl-xyloside. Variable results were found in tests supplemented with lactose and melibiose as carbon sources.

The Jacquard coefficient calculated in the *Bacillus* genus employing these data allowed to identify Bacillus sp. S-86 into the *subtilis* group with values higher than 0.70 (Table 2), and it could tentatively be classified as *B. licheniformis* species by the highest S_J of 0.90.

Table 2. Strain identification of *Bacillus* sp. S-86 in the *subtilis* group using Jacquard coefficient.

Bacillus subtilis group	S_J
B. *amyloliquefaciens*	0.87
B. licheniformis	0.90
B. pumilus	0.70
B. subtilis	0.83

Batch cultures of *B. licheniformis* S-86 started to produce exocellular lipase in stationary phase showing a continuous excretion until 48 h was reached, and concomitantly with the decrease of cell concentration in the culture (Fig. 1). Probably, lipase production was related to the differentiation process of *Bacillus* genus members, sporulation, which implied synthesis *de novo* and release of lytic enzymes pool to the medium (**17**).

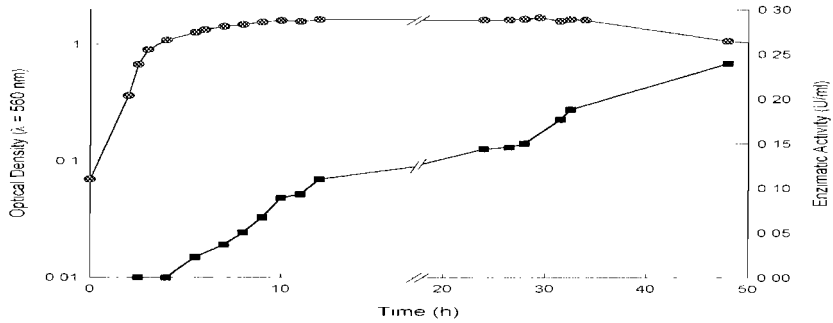


Figure 1. Time course of *Bacillus* sp. S-86 lipase production in batch culture using EP media at 55°C. Symbols: (●) Optical density at 560 nm, and (■) cell-free enzyme activity.

Dependence of enzyme activity in 50% homogeneous solvent mixtures was assayed at 37°C (Fig. 2). High lipase activity in presence of glycols solvent mixtures were found with the exception of 2,3 butanediol. Also, lipase activity was higher in reaction mixture containing glycerol and ethyleneglycol than water.

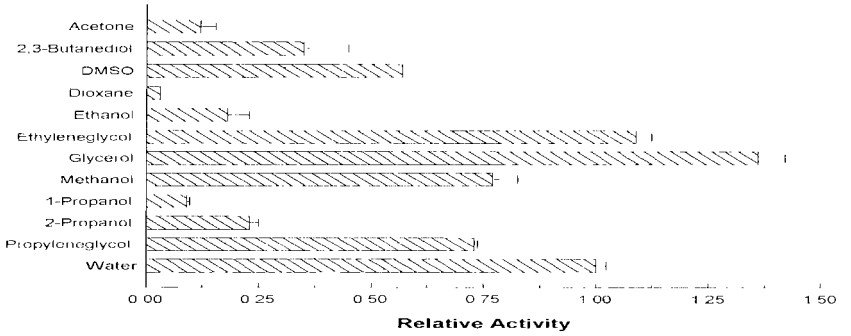


Figure 2. Dependence of lipase activity by 50% organic cosolvent homogeneous mixtures at 37°C.

Based on results displayed in Figure 2, dependence of lipase activity from glycerol concentration was tested. Higher enzyme activity was detected between 25 to 50 % glycerol concentration mixtures than water (Fig.3). Biocatalytic activities with varied results in homogeneous aqueous-organic solvent mixtures have been reported in the literature (22-24). Lipase from *Candida cylindraceae* was inhibited

by ethanol at higher concentrations than 1.3 % (22). Derivatized PEG-Lipase from *Pseudomonas cepacia* was able to work on dioxane-water mixtures, however it was denatured on other solvent water miscible solvents (**23**). Also, lipase synthesized by solvent tolerant *Pseudomonas aeruginosa* LST-03 presented low enzymatic activities in 50 % homogeneous solvent mixtures containing hydroxylic compounds (**24**).

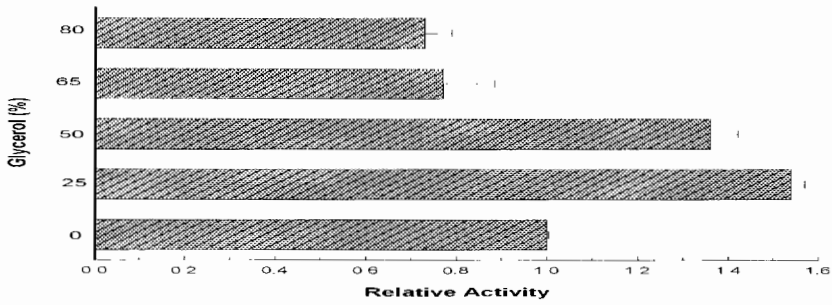


Figure 3. Influence of glycerol co-solvent concentration mixtures on lipase activity.

Temperature profiles of *B. licheniformis* S-86 lipase in water and water-glycerol (25 %) mixtures displayed similar profiles (Fig. 4). High enzyme activities were found in the range of 65 to 70 °C in water and water-glycerol mixtures. On the contrary, optimal reaction temperature from lipases of solvent tolerant *Pseudomonas aeruginosa* LST-03, and *Pseudomonas pseudoalcaligenes* F-111 were reported around 40°C in buffered systems, and immobilized lipase from *Candida cylindracea* was inactivated in organic solvent at temperatures higher than 45°C (**22, 24, 25**).

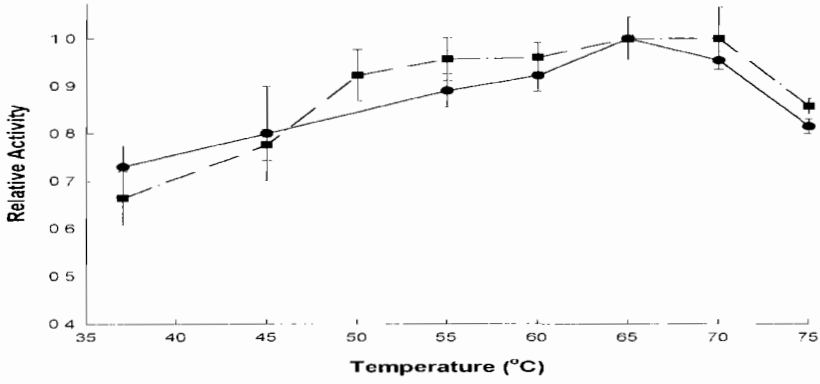


Figure 4. Effect of reaction temperature on the lipase activity in water (■), and water-25 % glycerol mixtures (●).

Concluding, lipase produced by *B. licheniformis* S-86 could be used in high organic homogeneous solvent mixtures concentrations. It may be useful for industrial biocatalysis purposes.

Acknowledgments

The authors gratefully acknowledge the financial support from Pew Charitable Trust (USA), CONICET, Agencia Nacional de Promoción Científica y Tecnológica (BID 1201/OC-AR), and Fundación Antorchas (Argentina).

References

1. Vulfson E.N., Halling P.J., Holland H.L. (2001) Enzymes in non-aqueous solvents, Humana Press, Totowa.
2. Jaeger K.E., Reetz M.T. (1998) *Tibtech* **16**, 396-403.
3. Ogino H., Miyamoto K., Ishikawa H. (1994) *Appl. Environ. Microbiol.* **60**, 3884-3886.
4. Haba E., Bresco O., Ferrer C.,Marqués A., Busquets M., Manresa A. (2000) *Enzyme Microb. Technol.* **26**, 40-44.
5. Inoue A., Horikoshi K. (1991) *J. Ferment. Bioeng.* **71**, 194-196.
6. Salter G.J., Kell D.B. (1995) *Crit. Rev. Biotechnol.* **15**, 139-177.
7. Rekker R.F. (1977) The hydrophobic fragmental constant. Elsevier, New York.
8. Dickinson J.R., Lanterman M.M., Danner D.J., Pearson B.M., Sanz P., Harrison S.J., Hewlins M.J.E. (1997) *J Biol. Chem.* **272**, 26871-26878.
9. Lorenz M.C., Cutler N.S., Heitman J. (2000) *Mol. Biol. Cell* **11**, 183-199.
10. de Bont J.A.M. (1998) *Tibtech* **16**, 493-499.

11. Krishna S.H., Manohar B., Divakar S., Prapulla S.G., Karanth N.G. (2000) *Enzyme Microb. Technol.* **26**, 131-136.

12. Fukuda K., Yamamoto N., Kiyokawa Y., Yanagiuchi T., Wakai Y., Kitamoto K., Inoue Y., Kimura A. (1998) *Appl. Envirom. Microbiol.* **64**, 4076-4078.

13. Sneath P.H.A. (1986) *in*: Bergey's Manual of Systematic Bacteriology, vol. 2, Sneath P.H.A. ed., The Williams and Wilkins Co., Baltimore, pp. 1104-1139.

14. Castro G.R., Ferrero M.A., Méndez B.S., Siñeriz F. (1991) *J. Biotechnol.* **20**, 105-108.

15. Colwell R., Austin B. (1981) *in* Manual Methods for General Bacteriology, Gerhardt P., Murray R.G.E., Costilow R.N., Nester E.W., Wood W.A., Krieg N.R. and Briggs Philips G. eds., American society of Microbiology, Washington DC, pp. 441-449.

16. Priest F.G., Goodfellow M., Todd C. (1988), *J. Gen. Microbiol.* **134**, 1847-1882.

17. Baigorí M.D., Castro G.R., Siñeriz F. (1996) *Biotechnol. Appl. Biochem.* **24**, 7-11.

18. Dickinson J.R. (1996) *Microbiol.* **142**, 1391-1397.

19. Mendelson N.H., Bourque A., Wilkening K., Anderson K.R., Watkins J.C. (1999) *J. Bacteriol.* **181**, 600-609.

20. Westerman P.W., Pope J.M., Phonphok N., Doane J.W., Dubro D.W. (1988) *Biochim. Biophys. Acta* **939**, 64-78.

21. Moriya K., Horikoshi K. (1993) *J. Ferment. Bioeng.* **76**, 397-399.

22. Carta G., Gainer J.L., Gibson M.E. (1992) *Enzyme Microb. Technol.* **14**, 904-910.

23. Secundo F., Carrea G., Vecchio G., Zambianchi F. (1999) *Biotechnol. Bioeng.* **64**, 624-629.

24. Ogino H., Nakagawa S., Shinya K., Muto T., Fujimura N., Yasuda M., Ishikawa H. (2000) *J. Biosci. Bioeng.* **89**, 451-457.

25. Lin S.F., Chiou C.M., Yeh C.M., Tsai Y.C. (1996) *Appl. Environ. Microbiol.* **62**, 1093-1095.

Chapter 12

PREBLEACHING OF KRAFT PULPS WITH XYLANASE ENZYME FOR PAPER MANUFACTURE

V.S. BISARIA[1], S. MISHRA[1], V. SAHAI[1], J.K. JAIN[2], R.M. MATHUR[2]

[1]*Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology – Delhi, Hauz Khas New Delhi-110016; email:vbisaria@dbeb.iitd.ernet.in*
[2] *Central Pulp and Paper Research Institute, Saharanpur, India*

Abstract

Wood and agricultural residues, containing cellulose, hemicellulose and lignin as three main polymers, are used in paper manufacture. Removal of lignin from these raw materials by chlorine-based bleaching agents generates dioxins and other toxic chlorinated organic compounds. Prebleaching of Kraft pulps with xylanase enzyme decreases the consumption of chlorine and thereby results in lowered generation of these environmental pollutants. The fungus, *Melanocarpus albomyces*, seems to be an effective source of xylanase enzyme for prebleaching of pulps, as it produces thermostable xylanases along with xylosidase and xylan-debranching enzymes. Application of the enzyme resulted in 15 % chlorine savings for Kraft pulp based on eucalyptus plus bamboo, and improved the brightness of the pulp by 3 percentage points to 83 % ISO. The enzyme was less effective against bagasse-based Kraft pulp.

Keywords: Enzymatic prebleaching, Xylanase, *Melanocarpus albomyces*, paper manufacture, Kraft pulp.

Introduction

Any fibrous raw material such as wood, straw, bamboo, hemp, bagasse, sisal, flax, and cotton can be used in paper manufacture. Separation of the fibers in such materials is called pulping. While any species of wood can be used, softwoods (gymnosperms) are preferred to hardwoods (angiosperms) because of their longer fibers and absence of vessels. Wood and most other fibrous materials have cellulose as their main structural component, along with hemicellulose, lignin and a large number of substances collectively called resins or extractives.

Fibers separated by dissolving out most of the lignin and much of the hemicellulose in the widely used Kraft process in which chemicals under high temperature and pressure are used, make stronger paper and are most easily bleached to high brightness. Bleaching is done by processes employing mainly chlorine and its oxides. The residual lignin is degraded and dissolved in various sequences of

New Horizons in Biotechnology, 123-132, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

bleaching and extraction steps in which chlorine, hypochlorite, chlorine dioxide, oxygen and hydrogen peroxide are used. The use of elemental chlorine and chlorinated compounds as bleaching agents is a cause for environmental concern since the process produces dioxins and other toxic chlorinated organic compounds.

Currently the most common sequence of bleaching kraft pulp is chlorine / chlorine dioxide, sodium hydroxide, chlorine dioxide, sodium hydroxide and chlorine dioxide, designated C/DEDED for softwood and is DEDED for hardwood. This means that most of the hardwood bleach sequences are already elemental chlorine free (ECF), and as the degree of substitution in softwood bleaching is also increasing and approaching 100%, the kraft mills will soon produce mainly ECF pulps. Also, the demand for total chlorine free (TCF) pulps is expected to increase. The deciding factors will be the success of environment groups in their moves against the use of chlorine and consumer demands for chlorine-free paper. Regardless of the pace of change, a market has been created for ECF and TCF pulps where either alternatives have to be found to chlorine-containing bleaching chemicals or new technologies have to be considered (**1-3**).

Substitution of chlorine, by chlorine dioxide results in lower absorbable organic halogens (AOX) but has the disadvantages of high bleaching costs and requires high investment. Oxygen delignification to reduce kappa number prior to bleaching results in lower AOX and lower bleaching costs but requires high investment. Replacing chlorine-based chemicals with hydrogen peroxide/ozone has the advantage of lower AOX but involves high bleaching costs, a risk of pulp viscosity and strength; and in the case of ozone very high investment as well (**4**). The use of xylanases after the alkaline cooking and before the bleaching is an innovative answer to chlorine-reduced (and chlorine-free) bleaching to kraft pulp and is drawing increased attention. In the enzymatic pretreatment of the pulp, the hydrolysis of hemicellulose is restricted to a minimum by using only a small amount of enzymes in order to maintain a high pulp yield and the advantageous properties of hemicellulose in pulp. Hemicellulose in pulp plays an important role in fibre morphology and quality, and also improves pulp strength.

The way in which xylanase prebleaching affects subsequent bleaching is not well understood. One possible explanation is that disruption of the xylan chain by xylanases appears to cleave lignin-carbohydrate bonds, which improves the accessibility of the bleaching chemicals to the pulp and facilitates easier removal of the solubilized lignin in bleaching. There is a significant decrease in xylan degree of polymerization (DP), and only a small amount of xylan is removed during xylanase prebleaching. The decreased chain length of xylan or its removal results in increased freedom for lignin to diffuse from the hemicellulose-lignin matrix (**5**).

Xylanase preebleaching

Xylanase producers are found both among bacteria and fungi. Several criteria are essential for choosing a microorganism to produce xylanases. In addition to giving the desired biobleaching effect, the enzyme must be produced in sufficiently high

quantity, should be completely free of cellulase activity. Any cellulase activity will have serious economic implications in terms of cellulose loss, degraded pulp quality and increased effluent treatment cost. Noncellulolyitic preparations could be produced by recombinant DNA technology, selective inactivation or bulk scale purification. High productivity could be achieved by exhaustive screening, genetic engineering and growth optimization programs.

Several factors are to be taken into account in order to use the xylanase enzymes effectively in a mill. The key factors include:

- The enzyme pH optimum generally falls between pH 4 and 8.
- The temperature optimum and operating range for enzyme treatment varies among enzymes but is between 35°C and 70°C.
- An adequate dispersion of enzyme and acid (which may be required to adjust the pH) into the pulp is extremely important for enzyme performance.
- Reaction time- a minimum of 1 to 2 h residence time is required for the enzyme treatment and there is little enzyme action on the pulp beyond 4 to 6 h.

The following mill operations also affect the performance of enzymes.

- Pulping process: This can affect the content and structure of the hemicellulose in the pulp, which, in turn, changes the extent of enzyme action that is achievable with the pulp. For example, sulfite pulping destroys most of the hemicellulose and so sulfite pulp is not suitable for enhanced bleaching by enzymatic treatment. Kraft pulping under severe conditions, such as conventional cooking of softwood to kappa number less than 23, also destroys much of the hemicellulose that is accessible to the enzyme. On the other hand, oxygen-delignified pulps with low unbleached kappa number respond well to enzyme treatment.
- Brown stock washing: The properties of brown stock black liquor vary greatly among mills. Some mills' black liquor can inhibit enzyme performance due to the presence of highly oxidizing compounds.
- Bleaching sequence: This influences the enzyme's benefit to the mill in several ways.

Enzymatic treatment of pulp

Typically the enzyme is added as an aqueous solution to the pulp at the final brown stock washer. The brown stock is highly alkaline (pH 9-12), must be neutralized with the acid to be compatible with enzyme treatment unless the enzyme can act at this high pH. The pulp is then pumped to the high-density storage tower where the enzyme acts. It has to be ensured that mixing is sufficient both after pH adjustment and enzyme addition. From there the enzyme treated pulp goes to the bleaching tower where the first contact with the oxidizing chemicals destroys the enzyme. Xylanases modify the pulp to make the lignin more accessible to removal by other bleaching chemicals. Savings in total active chlorine to the extent of 20-25% for hardwoods and 10-15% for softwoods are obtained if the pulps are pretreated with

xylanase enzyme. Enzymatically treated pulps show unchanged or improved strength properties. Also these pulps are easier to refine than the reference pulps. Improved viscosity of the pulp has been noted as a result of xylanase treatment. However, the viscosity of the pulp is adversely affected when cellulase activity is present. The capability of the enzyme to reduce the consumption of bleaching chemicals makes it possible to consider significant modification in the bleaching sequence. It is possible to completely exclude the first chlorination stage and replace it with an enzyme stage.

The benefits of xylanase treatment in pulp bleaching are, therefore, environmental (reduction in chlorine, chlorine dioxide, hypochlorite loadings), economic (decreased chlorine dioxide and/or peroxide requirements), improved pulp quality (higher brightness and final strength) and improved mill flexibility. Xylanase prebleaching belongs to the soft technologies that require very little or no capital investment to operate. Process changes (e.g. neutralization of brown stock) are minimal in most cases.

Production of xylanase enzymes

We have been working with the fungus *Melanocarpus albomyces* IIS68 (**6**), as it has been found to be a good source of thermostable xylanase along with xylosidase and xylan debranching enzymes. The debranching enzymes produced by the fungus include acetyl esterase, α-L-arabino-furanosidase and α-D-glucuronidase. Amongst various carbon sources tested, the inexpensible lignocellulose residue, wheat straw was found to be the best carbon source for production of extracellular xylanase, which also induced all the seven isoenzymes. The isoenzyme Ic was constitutive whereas the other six exhibited more specific inducibility, the magnitude of which depended on the nature of the inducer (**7**).

The purified xylanase isoenzymes of *M. albomyces* were characterized in terms of various physico-chemical and kinetic parameters. The molecular weights of the isoenzymes, as determined by SDS-PAGE, were in the range of 18,600 to 38,500 and pIs of isoenzymes Ia, Ib, Ic, IIa and IIb were in the range of 3.7 to 6.4. The pH and temperature optima were determined to lie in the range of 5.5 to 8.5 and 55° to 70°C, respectively. Two isoenzymes, IIa and IIc, showed the maximum activity in alkaline range. Three isoenzymes, Ia, Ib and Ic, showed maximum activity at 70°C. Out of the seven xylanase isoenzymes, Ia was found to be the most stable xylanase with half lives of 164.28, 16.67 and 6.56 min at 60°, 70° and 80°C, respectively. The range of K_m of xylanase isoenzymes was 0.4 to 6.9 mg/ml whereas V_{max} varied from 16.39 to 198 μmol/min.mg. The turnover number (k_{cat}) was computed to lie in the range of 10.52 to 83.82 s^{-1} and k_{cat}/K_m in the range of 1.52 to 133.62 ml/mg.s. Five out of seven xylanases were found to be specific only for xylans. Xylanases Ic and IIa showed some activity on Solka Floc and p-nitrophenyl-β-D-xylopyranoside, respectively. The effect of seven purified xylanase isoenzymes, separately and in combination, was studied on three xylans, namely birchwood, larchwood and oat spelt xylan and the extent of their hydrolysis was compared with that obtained with

crude extract of *M. albomyces*. All seven xylanase isoenzymes acted synergistically and the presence of β-xylosidase and xylan debranching enzymes in crude extract further increased the xylan hydrolysis (Table 1).

Table 1: Hydrolysis of xylan by xylanases.

Enzyme	% Saccharification		
	Birchwood xylan	Larchwood xylan	Oatspelt xylan
I. Purified xylanase isoenzymes			
I_a	3.87	9.94	6.05
I_b	5.13	9.00	8.10
I_c	3.73	10.81	9.14
II_a	11.70	10.10	6.10
II_b	2.55	1.82	2.16
II_c	1.72	0.56	1.24
II_d	2.51	0.86	1.06
Additive value of % saccharification (A)	31.21	43.09	33.85
II. Purified pooled isoenzymes (B)	33.74	47.55	37.84
% Increase in saccharification with respect to additive value of A	8.10	10.34	11.78
III. Crude extract	35.44	52.78	41.13
% Increase in Saccharification with espect to A	13.55	22.49	21.51
% Increase Saccharification with respect to A	5.04	10.10	8.69

The nature and extent of xylan hydrolysis products were also determined by individual and combined action of purified xylanases, as well as by crude extract on various xylans. The major product of xylan hydrolysis was xylose for Ib and IIa but it was not the only reaction product. Four xylanase isoenzymes, namely Ia, Ib,Ic and IIa exhibited arabinose releasing property.

Xylanase Production by Solid State Fermentation

Solid state fermentation (SSF) offers advantages over liquid cultivation, especially for fungal cultures, as there is higher productivity per unit volume, reduced energy requirements, lower capital investment, low waste water output, higher concentrations of metabolites obtained and low downstream processing cost (**8-10**). We, therefore, studied xylanase production by the fungus in SSF.

The aim of this work was to apply fractional factorial design, such as the Box-Behnken design (**11**) followed by response surface methodology to investigate and optimise six variables (inoculum age, initial moisture, Tween 80 level, urea level, yeast extract level and the harvest time) which may affect xylanase production by *Melanocarpus albomyces* IIS68 in solid state fermentation.

In order to identify the optimum conditions, Box-Behnken design was selected, as this is recommended over central composite design when the number of variables is large (more than four). This design evaluates the quadratic effects and two-way interactions among the variables and thus determines the non-linear nature of the

response, if any. Each variable is experimented at '+', '0' and '-1' levels which represent the higher, middle and the lower levels of the variable. The coefficients of the quadratic model were calculated using standard regression techniques. The software, Design-Expert™, Version 5.0 of State-Ease Inc. (USA) was used to design the experiments, calculate the coefficients and generate the response surface.

The possible variables that could be affecting the enzyme production were inoculum age, concentration of urea, concentration of yeast extract, concentration of Mg^{2+} / initial moisture content, harvest time, extraction pH and the surfactant. Initially the effect of each of these variables was independently observed keeping the other variables constant in order to strike off the variables, which did not have observable effect on the production of xylanase. Based on these preliminary experiments, it was observed that there was no noticeable effect of concentration of Mg^{2+} and the extraction pH on xylanase production. The remaining six variables (inoculum age, yeast extract concentration, urea concentration, surfactant concentration and harvest time) were optimised using the Box Behnken design. The range and levels of variables investigated in this study are given in Table 2. The 'O' level indicates the values of variables obtained in the preliminary experiments. The response of the variables was measured by assaying xylanase activity.

Table 2: The range of values of the variables used for Box-Behnken design.

Independent variable	Unit of the variable	Level		
		-1	0	+1
Yeast extract (A)	%(g per 100 g dry substrate)	0.4	0.6	0.8
Surfactant (B)	%(ml per 100 g dry substrate)	0.1	1.0	1.9
Urea (C)	% (g per 100 g dry substrate)	4.5	6.0	7.5
Initial moisture (D)	ml/g dry substrate	2.0	4.5	7.0
Inoculum age (E)	H	32	40	48
Harvest time (F)	h	84	96	108

Using the response surface methodology and the multivariant statistical approach, the optimum levels of the variables affecting xylanase production were determined. The optimum levels of the variables were 600-850 μm particle size, 43 h inoculum age, 1.37% Tween 80, 86% initial moisture content, 5.1% urea, 0.74% yeast extract and a harvest time of 96 h. Under these optimized conditions, xylanase a activity of 7760 U/g initial dry substrate was obtained which was in very good agreement with the value predicted by the quadratic model (7890 U/g initial dry substrate).

Prebleaching of kraft pulps

The effectiveness of the xylanase enzyme as prebleaching agent was assessed by conducting various sets of experiments. The results of enzymatic pretreatment of pulp (eucalyptus + bamboo and bagasse) procured from the mills followed by their bleaching using conventional CEH bleaching sequence are shown in Table 3 and 4.

The different parameters such as Kappa number pulp filtrate characteristics, viscosity, savings in bleach chemicals, strength and optical properties, and environmental impact were obtained for both categories of pulps.

Effect on Enzyme – Treated Wood pulp

- **Kappa Number**: From the results shown in Table 3a, it is evident that there was a reduction in Kappa number of unbleached Kraft wood pulp after enzyme treatment which was decreased by 1.5 points i.e. from 18.0 to 16.5 with gain in pulp brightness of 1.0 point i.e. from 24 to 25% ISO.
- **Characteristics of pulp filtrates**: The extractability of the dissolved lignin and the chromophores in pulp filtrates after xylanase treatment and the control pulp sample were studied and the solubilized lignin and chromophores were measured by UV at 280 nm and in visible range at 465 nm respectively. The filtrate after enzyme treatment was found to be more coloured as compared to the control pulp sample. Determination of lignin at 280 nm showed extraction of higher amount of lignin i.e. 1.03 kg/ton as against 0.39 kg/ton in control pulp. The chromophores released were also high i.e. 2.72 kg/ton after xylanase treatment against 0.93 kg/ton in control pulp indicating the effectivity of the xylanase enzyme for wood Kraft pulp (Table 3b). The reduction in Kappa number of the enzyme treated sample from 18.0 to 16.5 could be attributed to the release of lignin and chromophores. The brightness of the unbleached pulp could also be improved by 1.0 point i.e. from 24 to 25 % ISO due to this (Table 3a).
- **Viscosity of pulp**: There was no reduction in viscosity of the pulp after enzyme treatment when the pulp was pre-treated with optimized dose of enzyme (10 IU/g). A little increase in CED viscosity was observed which was from 690 cm^3/g (control pulp) to 740 cm^3/gm (enzyme treated pulp) (Table 3a). As expected this little increase in viscosity could be due to the fact that the xylan has a molar mass that is generally lower than that of cellulose. So its removal leaves behind cellulose that has a higher viscosity. Therefore, the conventional view that higher pulp strength is observed with higher pulp viscosity might not hold for xylanase treated pulp.

Table 3: Enzymatic Pretreatment of Wood Pulp (Eucalyptus, Bamboo) using Xylanases from *Melanocarpus albomyces*.

Table 3a: Characteristics of Unbleached Pulp, Before & After Enzyme Treatment.

Particulars	Control Pulp	Enzyme Treated Pulp
Kappa No. of Pulp	18.0	15.5
Brightness, % ISO	24.0	25.0
CED Viscosity, cm^3/g	690	740

Table 3b: Analysis of Pulp Filtrates Before & After Enzyme Treatment

Particulars	Control Pulp	Enzyme Treated Pulp
$Lignin_{280}$ (kg/t)	0.39	1.03

Reducing sugars as xylose, (kg/t)	1.21	4.08
$Colour_{465}$ PCU (kg/t)	0.93	2.72

- **Savings in bleaching chemicals**: One of the main objectives of the study was to evaluate the response of xylanases as prebleaching agent and to reduce the requirement of chlorine as bleaching chemical by maintaining targeted brightness level. This could ultimately reduce the release of AOX in bleach effluents. From the results shown in Table 4a, it was found that by xylanase treatment of wood Kraft pulp, there was reduction in chlorine requirement from 4.1 % (41 kg/t) to 3.5% (35 kg/t) after xylanase treatment. The alkali usage during extraction stage was also reduced to some extent as reflected by consumption of alkali (60.1% against 68.4% for control pulp).
- **Strength and optical properties of enzyme treated pulp**: Strength and optical properties of the untreated and treated pulp, shown in Table 4b, were found to be almost at par in terms of tensile index and burst index which were 64.0 and 62.0 Nm/g and 4.40 and 4.20 Kpa.m^2/g respectively. A little drop in tear index could be noticed after enzyme treatment, which dropped from 6.04 to 5.8 Mnm^2/g. Such a drop can be arrested by reducing the treatment time and/or enzyme dose. Final brightness of the pulp was improved by 3 points i.e. from 80 to 83% ISO (Table 4a).

Table 4 : Enzymatic Prebleaching of Wood Pulp (Eucalyptus+ Bamboo) using Xylanases from *Melanocarpus albomyces.*

Table 4a: Bleaching of Pulp Using Conventional CEH Sequence Before and After Enzyme Treatment.

Particulars	Control Pulp	Enzyme Treated Pulp
Chlorination Stage		
% Chlorine Applied	4.1	3.5
%, Chlorine Consumed	98	91
Saving in Chlorine, %	---	15
Alkali Extraction		
NaOH, %	1.5	1.5
%, NaOH Consumed	68.4	60.1
Final pH	11.0	11.2
Kappa No.	4.6	3.3
Hypo Stage		
%, Applied	2.0	2.0
%,Consumed	98	93
Final Brightness of the Pulp,% ISO	80	83

Table 4b: Strength & Optical Properties of Pulp Before & After Enzyme Treatment.

Particulars	Control Pulp	Enzyme Treated Pulp
Revolution, PFI	3000	3000
Freness, CSF	295	270
Apparent density, g/m^3	0.78	0.81
Burst Index, Kpa.m^2/g	4.40	4.20
Tensile Index, Nm/g	64.0	62.0
Tear Index Mnm^2/g	6.04	5.80

Optical Properties		
Brightness, % ISO	80	83
Opacity, %	82.1	79.0

Table 4c : Characteristics of Bleach Effluent (C+E Stage) Before & After Treatment

Particulars	Control Pulp	Enzyme Treated Pulp
COD, (kg/t)	32.0	27.0
AOX, (kg/t)	2.12	1.8
AOX Reduction. %	---	20.0

- **Environmental impact of enzyme treatment**: Characterisation of the effluents after enzyme treatment showed an improved environmental status in terms of AOX, colour and COD. The AOX level in bleach effluents was reduced by more than 20% i.e. from 2.12 kg/t to 1.8 kg/t in enzyme treated bleach effluent (Table 4c). There was an indirect improvement in terms of COD after enzyme treatment. This number was reduced from 32 kg/t to 27 kg/t.

Comparative studies were carried out with commercially available xylanase (Bleachzyme F) from Biocon. This enzyme formulation had similar pH optimum as the enzyme produced by *M. albomyces*. The results indicate that enzymes from the test organism had very similar effectivity as the commercial enzyme. Savings in chlorine were of the order of 15% *M. albomyces* enzyme and 16% for the commercial enzyme. Slight improvement in final brightness of the pulp was observed with the fungal enzyme (increase of 3 percentage points) compared to the commercial enzyme (increase of 2.5 percentage points) by 3%. The strength properties also indicate similar values for both the *Melanocarpus* as well as the commercial enzyme.

Effect on Enzyme - Treated Agro Based Pulp

The magnitude of the effect of enzymatic treatment on the Kappa number of bagasse Kraft pulps was not as pronounced as with wood Kraft pulp. However, it has been observed that the effect of xylanases varied in their ability to decrease the Kappa number of Kraft pulps. It depended on the nature and type of the pulp. The Kappa number of enzyme treated Kraft bagasse pulp was reduced by 0.5 – 1.0 percentage points i.e., from 7.7 to 7.0 for one pulp (A) and from 26.5 to 25.5 for another pulp (B).

Although small, this drop in Kappa number has consistently been observed in the pulps after enzyme treatment which could be supported from the increased brightness of unbleached pulp which was improved from 55.5% to 57.3% ISO for pulp A and from 30.0% to 31.0% ISO for pulp B. As in the case of wood Kraft pulp, the analysis of the pulp filtrates of bagasse Kraft pulp after enzyme treatment indicated it to be more coloured as compared to the control pulp.

Extensive testing of the xylanase enzyme treated pulps showed that the treated pulps needed slightly more refining energy to refine the pulp at same freeness level

than control pulp. However, it showed the strength properties almost at par with the control (untreated pulp) in terms of tensile & burst indices. Brightness of the pulp was improved by about two percentage points. Characterization of the effluents after enzyme treatment showed an improved environmental status it terms of AOX, colour and COD. The results revealed that the AOX level in bleach effluents was reduced by 25% from 4.13 kg/ton to 3.13 kg/ton in enzyme treated bleach effluent. There was an indirect improvement in terms of colour and COD also. The biodegradability of effluent was also improved as reflected by COD:BOD ratio of 1:3 as compared to 1:4 in the case of untreated pulp effluent.

Thus, the xylanase enzyme from *Melanocarpus albomyces*, was effective in prebleaching of both wood – as well as nonwood – based pulps.

References

1. Viikari L., Suurnakki A., Buchert J. (1996) *in* Enzymes for Pulp and Paper Processing, ACS Symposium 665, Jeffries T.W. and Viikari L., eds. pp.15-24.
2. Biely P. (1985) *Tr. Biotechnol.*, **3**,286-290.
3. Bisaria V.S. (1998) *in* Bioconversion of Waste Materials to Industrial Products, Martin A.M. ed., Blackie Academic & Professional, London, pp.197-246.
4. Ragauskas A.J., Poll K.M., Cesternino A.J. (1994) *Enz. Microb. Technol.*, **16**, 492-495.
5. Farell R.L., Skerker P.S. (1992) *in* Xylans and Xylanases, Visser J., Beldmen G., Kusters-van Someran M.A. and Voragen A.G.J., eds., Elsevier, Amsterdam, pp.315-324.
6. Maheshwari R., Kamalam P.T. (1985) *J. Gen. Microbiol.*, **131**, 3017-3027.
7. Saraswat V., Bisaria V.S. (1997) *J. Ferment. Bioeng.*, **83**, 352-357.
8. Nigam P., Singh D. (1994) *J. Basic Microb.*, **34**(6), 405-423.
9. Chatterjee R., Dutta A., Bannerjee R., Bhattacharya B.L. (1996) *Bioprocess Eng.*, **14**, 159-162.
10. Kumaran S., Sastuy C.A., Vikineswary S. (1997) *World J. Microb. Biotechnol.*, **13**, 43-49.
11. Box G.E.P., Behnken D.W. (1960) *Technometrics*, **2**, 455-475.
12. Narang S., Sahai V., Bisaria V.S. (2001) *J. Biosci. Bioeng.*, **91** (4) 425-427

Chapter 13

TITRATION BIOSENSORS FOR BIOTECHNOLOGICAL PROCESS CONTROL

A. ROZZI, E. FICARA, E. REMIGI

Politecnico di Milano, DIIAR, Department of Hydraulic Environmental Engineering and Survey – Environmental Section, Piazza L. da Vinci 32, 20133 Milan, Italy; rozzi@amb1.amb.polimi.it.

Abstract

This paper describes the development of titration biosensors, a relatively new family of instruments which measures the activity of various microbial populations evaluated either as consumption rates of reagents or as production rates of metabolites. Two main types of titration biosensors are described: pH-stats and DOstat. They determine the amount of converted substrate and the reaction rate of respectively, pH or DO (dissolved oxygen) affecting reactions from the amount of titrant and its addition rate, required to maintain pH or DO constant within the suspension. Their main applications are then discussed which include: assessment of nitrification activity and inhibition, evaluation of denitrification rates and of rbCOD and volatile fatty acids concentration, determination of methanogenic activity and inhibition, assessment of oxygen uptake rates of nitrifiers and heterotrophs.

Keywords: Titration biosensor, pHstat, DOstat, respirometry, activated sludge, nitrification, denitrification, methanogenesis.

Introduction

The term "biosensor" is very extensively used nowadays, as also noted by Love and Bott (**1**): "Biosensors are broadly defined as devices which produce a quantifiable response based on the action or reaction of a biological or bio-mimic element, which is integrated with or located immediately adjacent to a physical/chemical transducer detection system. Examples of biological elements include enzymes, antibodies, proteins, nucleic acids or whole cells, whereas physical/chemical transducers are electrochemical, optical or acoustical (**2,3**). In contrast, a bioassay includes a biological element (as defined above) which is detected by a physical / chemical detector that is not intimately integrated with the active biological element. Given these definitions, a respirometry system that measures dissolved oxygen changes across a 10-liter chamber containing activated sludge is a bioassay. On the other hand, a 1 cm diameter probe connected to a photomultiplier and containing an immobilized genetically engineered microorganism which emits light in response to the presence of a specific class of chemicals, is a biosensor. Rogers

New Horizons in Biotechnology,133-151, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

and Gerlach (**3**) recognized that biosensors and bioassays encompass a broad spectrum of analytical methods and, in fact, are a hybrid within a chemical-biological continuum. In many cases, a biosensor or bioassay may incorporate use of a chemical sensor (e.g., O_2 or pH electrode) to detect reactions caused by the biological element (e.g., bacterial cells)".

According to this definition, devices such as respirometers or titration instruments should be defined as bioassays, but in practice they are commonly named "biosensors" by environmental engineers dealing with wastewater treatment, as one may be easily verify from the specialised literature.

Biosensors which directly measure biological reaction rates in a reactor have distinct advantages over conventional physico-chemical sensors, for example NH_3, NO_3, DO, pH and ORP electrodes because the latter react only after the value of the variable under observation into the reactor has appreciably varied, which in some cases may take a long time because of mass inertia effects. This feature is particularly important when a toxic substance enters a reactor and slowly and insidiously starts to inhibit the biomass. Last but not least, on-line selective electrodes or spectrophotometric sensors require a careful pre-treatment of the sample and re-calibration, which can be either expensive or labour intensive while most biosensors designed to measure biological activity simply need the sample to be coarse screened.

Biosensors may be installed on the reactor influent stream for fast detection (generally within 20 minutes) of potential inhibition of the active biomass. Therefore, they enable implementation of preventive actions, such as diverting the contaminated wastewater to a calamity tank, or remedial actions such as rapid addition of suitable additives to counter or mitigate inhibiting effects with a speed that is practically impossible to achieve using most physico-chemical sensors.

This paper looks at the development of a relatively new family of instruments, titration biosensors, which measure the activity of various microbial populations evaluated either as consumption rate of reagents or as production rate of metabolites. The usefulness of these instruments has already been proven in the area of environmental protection, especially in wastewater treatment monitoring, but they could also be used for other industrial biotechnological processes.

The activity of a microbial population may be evaluated by monitoring, in a batch reactor, the concentration of one of the chemical species, either consumed or produced by the biomass under observation, and by keeping that species concentration constant by a controlled addition (titration) of an appropriate titrant.

When the H^+ species is monitored indirectly through the pH, the instrument is named pHstat. pHstats are the first family of titration biosensors designed to monitor those microorganisms which may increase or reduce acidity or alkalinity. Most titration biosensors developed up to the present day are of this type.

A second, very promising family of these new instruments includes the DOstats, i.e. titration respirometers where dissolved oxygen (DO) is the species to be kept

constant. They may be used to evaluate the activity of any aerobic heterotrophic and autotrophic microbial population.

This paper presents a state of the art on these instruments.

Principle of operation of pHstats

The principle of operation of these biosensors is rather simple and exploits the peculiar ability of some microorganisms to convert a neutral substrate into an acid or basic product, or to consume an acid or basic substrate to make a neutral product (**4**). The layout of a titration biosensor is shown schematically in Figure 1. Basically, the instrument consists of a thermostated reaction vessel (V = 0.5 ÷ 2 litres), a titration unit and a PC for pH control, data logging and data processing.

A sample of the pH-affecting microbial population to be assayed is transferred into the reaction vessel, and an aliquot of its substrate is added. The biomass starts producing acidity or alkalinity, which is immediately neutralised by the alkaline or acid solution (titrant) added by the titration unit to keep the pH at a constant pre-set value.

The biological activity of the sample is determined by measuring the flow rate of titrant required to neutralise the acidity or alkalinity being produced into the reaction vessel and taking into account the stoichiometry of the reaction. The initial concentration of the consumed substrate is derived in the same way, by measuring the total volume or the total mass of titrant which was added until the titration had been completed.

Obviously, any appreciable interfering acidifying or alkalising reaction must be stopped or carefully controlled into the reaction vessel during the titration test, otherwise accurate results cannot be obtained. In particular, attention should be paid to the production of CO_2, which affects the pH of the mixed liquor in accordance to the well-known CO_2/HCO_3^- equilibria (**5**).

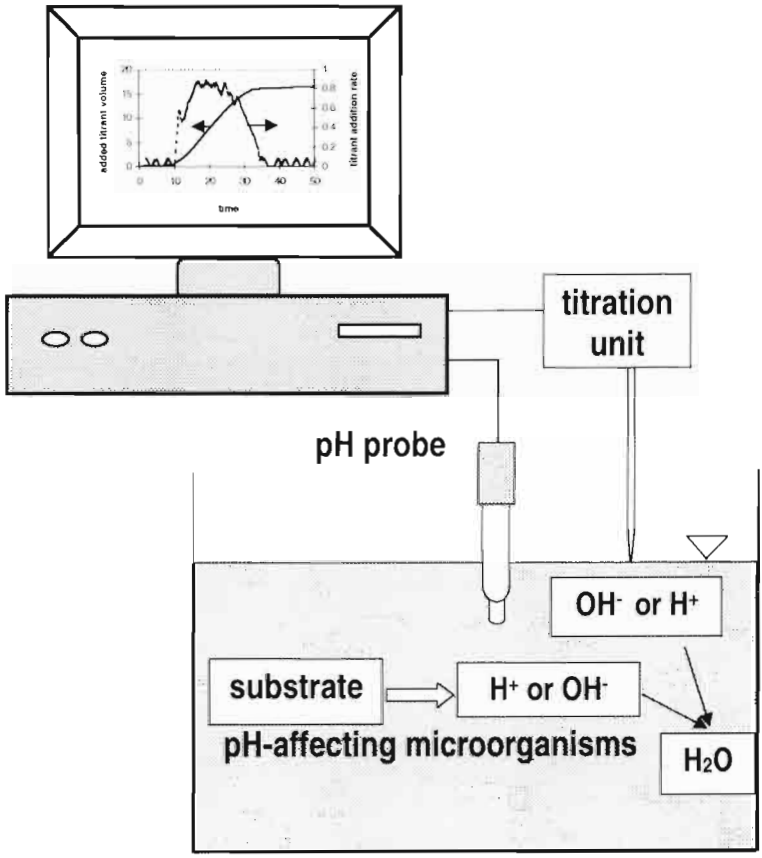


Figure 1. Lay out of a pHstat biosensor.

The ANITA$_{lab}$ biosensor for measuring nitrification

The first known titration biosensor (**6**) was developed to evaluate the activity of microorganisms responsible of the first step of nitrification:

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + NO_2^- + H_2O \quad (1)$$

The activity of ammonia oxidisers may therefore be measured as the production of acidity (protons) rather than oxygen or ammonia depletion, as proposed by the current literature at the time.

This prototype titration biosensor, which used pure oxygen, was also applied to inhibition tests on coke oven liquors (**7**). A biosensor based on a similar principle, i.e. a continuous flow reactor filled with nitrifying biomass attached on a porous glass carrier, was tested by Aivasidis *et al.* (**8**) for on-line monitoring of a wastewater treatment plant fed on a chemical industry effluent.

The titration biosensor concept was later improved to measure ammonia concentration at the same time as nitrification activity (**9,10**) and successfully patented (**11**). The new instrument was named ANITA (Ammonia and NITrification Analyser), and a detailed evaluation of the instrument's performance can be found in Massone *et al.* (**12**).

This titration method has proved to be quite simple and robust. Nevertheless, to obtain accurate and reliable activity determinations it is important to start measuring only after the equilibrium pH has been reached and to ensure that temperature, mixing and aeration remain constant during the test. If these conditions were not achieved, the shifting of CO_2 equilibriums in the system would cause extra production or consumption of acidity, which can seriously hamper the accuracy of the measurements, as elucidated in detail by Ficara (**5**). An example of the course of action followed in pHstat titration tests related to nitrification activity is given in Figure 2, where the titration curve is plotted vs. time for a sample of an activated sludge (AS).

The main output of the titration biosensor test is the volume of alkaline titrant (a 0.05M NaOH solution) added during the test to keep the pH of the AS sample constant. This is represented as the bold line in Figure 2 (the titration line). Observing this line, three phases can be distinguished. First, the biomass is aerated in endogenous conditions (no substrate from 0 to 3 minutes). In this case, the equilibrium pH of the AS sample had already been reached, which means that no acidity was being produced in the beaker and consequently no NaOH titrant was added. Then, at 3 minutes, substrate (3 mg/l N-NH_4Cl) is added, and nitrifiers take up ammonium oxidation. During this phase of substrate oxidation (from 3 to 13 minutes in Figure 2), the titration line is basically a straight line. This is because the substrate concentration is much higher than the semi-saturation constant K_s for ammonia oxidisers (which was estimated to be 0.5 mg/l in this test) and, therefore, the substrate oxidation is close to a zero-order reaction. The maximum nitrification rate is proportional to the slope of the interpolating dashed line (m). Finally, when the substrate concentration decreases to values of the order of K_s down to zero, the titration line becomes a curve that reaches an asymptotic horizontal value when the substrate is completely depleted. This latter part of the titration line can be used to derive the K_s value for ammonia oxidisers. In addition, the mass of NH_4 added depends on the alkali consumed to neutralise the acid which is produced by ammonia oxidation as indicated by equation (1). Therefore, the initial N-NH_4 concentration is proportional to the final amount of added titrant (T_f on the ordinate in Figure 2). Moreover, a simple stoichiometric conversion enables us to back-calculate the ammonium concentration profile against time from the titration line, which is the continuous, decreasing, line in Figure 2. Recovery of the spiked substrate, as measured by the biosensor in the reported test, was of the order of 90 %. This example shows the method simplicity and rapidity. In fact, no analytical determinations are needed. In general, depending on the initial concentration of substrate and on the nitrification capacity of the sludge sample, the average duration of a nitrification test using the titration biosensor is between 30 to 60 min. This

pHstat has been successfully tested also to measure the nitrifiable nitrogen in wastewater (**13**).

The ANITA biosensor has also been effectively used to determine conversion rates and substrate depletion by other acidifying microorganisms, such as phenol oxidisers. These heterotrophic bacteria produce carboxylic acids, such as oxalic acid, as metabolites, which means that their biological activity can be monitored by the acidification of the mixed liquor (**14**).

Evaluation of nitrification inhibition by $ANITA_{lab}$

The ANITA biosensor can also be used to measure inhibiting effects on nitrifiers, either to check the potential toxicity of the influent to a plant, or to carry out ecotoxicological assessments on chemicals to be released into the environment.

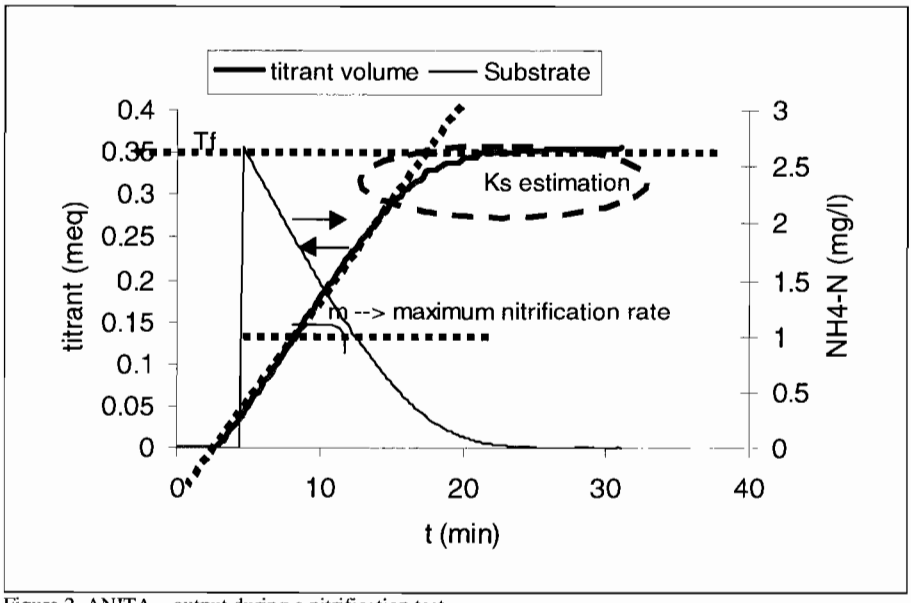


Figure 2. $ANITA_{lab}$ output during a nitrification test.

The practical usefulness of the first application was proved at a full-scale wastewater plant in Lecco, Italy, which was fed mainly by domestic sewage and some industrial wastewater (for instance, permeate from spent cutting fluids). Regular determination of nitrification activity in the activated sludge samples from the plant enabled the detection of any toxic effects from the industrial effluent on the nitrifying biomass and allowed a better dosage to be determined for these inhibiting effluents.

The eco-toxicological application is potentially very interesting because, as mentioned, nitrifiers are among the bacteria most sensitive to a great variety of toxic substances. They could, therefore, be used to provide a realistic estimate of the potential toxicity of new chemicals, especially xenobiotics.

A nitrification inhibition test on CS_2, carried out using the laboratory version of ANITA, is plotted in Figure 3. It was performed on a sample of nitrifying activated sludge drawn from a domestic wastewater treatment plant. First, ammonium was spiked at such concentration (25 mg $N\text{-}NH_4Cl/l$) that nitrification could be considered as a zero order reaction during the whole test. The maximum nitrification rate is considered as the 'blank' activity. Starting from t = 20 min, carbon sulphide was spiked several times (as indicated by the arrows in the diagram), in order to obtain progressive concentrations from 0.5 to 6.0 $mgCS_2/l$.

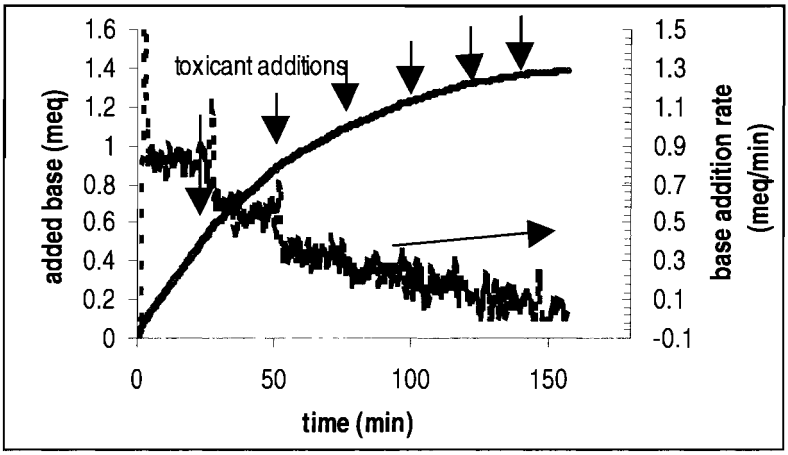


Figure 3. Progressive inhibition test on nitrifiers by $ANITA_{lab}$.

The reaction rate after each addition is compared (as a ratio) to the blank value and plotted against the corresponding CS_2 concentration (the dose-response curve). The latter graph allows the estimation of the EC_{50} concentration (50% effect concentration) for the tested toxic substance.

A standard titration procedure based on the above experimental procedure is presently being assessed by the Italian Union of Chemical Industries (UNICHIM) for determining nitrification inhibition as an alternative to the ISO 9509 standard. The latter requires a considerable number of time-consuming and expensive chemical analyses (seven determinations for each parameter: NH_4, NO_2 and NO_3), whereas the proposed biosensor methodology allows the EC_{50} to be obtained in less than four hours with no analytical determinations apart from suspended solids (**15**).

Coupling titration and respirometry: the ANITA-DO biosensor

The titrimetric technique for nitrification measurements described above can be coupled to a respirometric technique (open respirometry) using the same biosensor by providing ANITA with an oxygen electrode. This experimental set-up allows the simultaneous collection of titration and respirometric data, and has been used to compare the two techniques. The kinetic parameters of the ammonia oxidisers estimated using these two techniques appeared to compare very favourably (**16**).

Furthermore, titrimetric and respirometric data allow for the simultaneous estimation of the kinetic constants for both ammonia and nitrite oxidisers. In fact, although ammonia and nitrite oxidisers consume oxygen, only the former bacteria affect pH. This means that titration data enable subtraction of the contribution of ammonia oxidisers to the total oxygen uptake rate (OUR), evidencing the OUR of nitrite oxidisers (**16**). This procedure enables the complete characterisation of the nitrifying biomass kinetics by means of simple, short (1-1.5 h) tests without the need for analytical substrate determination.

A more sophisticated coupling of respirometry and pHstat titration may be obtained by the DOstat, which titrates oxygen to a batch reactor to maintain constant dissolved oxygen concentration, as described in a subsequent paragraph.

The $ANITA_{on\ line}$ biosensor

The ANITA titration biosensor has been automated and tested in real field conditions both in Italy and in Belgium (**17**,**18**) and an industrial prototype is currently being used at a wastewater treatment plant in the Como area in Italy. In Figure 4, the maximum nitrification velocity V and the in-reactor $N\text{-}NH_4$ concentration measured by the biosensor in the full-scale plant are plotted against time.

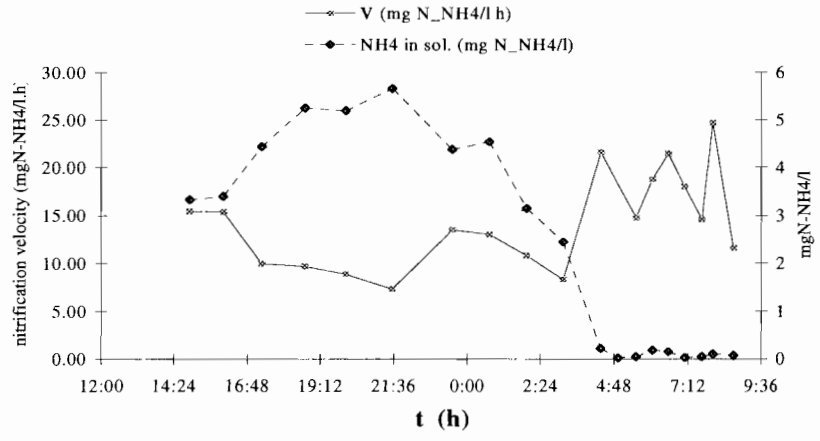


Figure 4. Nitrification rate and ammonium concentration estimated by $ANITA_{on\text{-}line}$.

It can be seen that in the early hours, when a toxic compound entered the aeration basin, the measured activity was relatively low and N-NH_4 was high. Later on, as the nitrification rate increased after the toxic substance in the mixed liquor had been diluted by new wastewater, the N-NH_4 concentration dropped below the analytical detection limit. This example confirms that ANITA could potentially be applied to the on-line detection of any toxic substances which may contaminate accidentally and unpredictably the influent of a wastewater plant.

The DENICON denitrification biosensor

It is well known that denitrifiers are alkalising bacteria. The DENICON (DENition CONtroller), is the first application of titration biosensor technology to an alkalising biomass (**10**). A wastewater-containing nitrate is added to a vessel with denitrifying activated sludge and readily biodegradable organic carbon (rbCOD) well in excess of denitrification requirements. Denitrifying activity and nitrate concentrations are respectively measured by monitoring the acid consumption rate needed to keep the pH in the vessel constant, and the acid required to neutralise the alkalinity until the reaction is completed. In this case pH control is more difficult than during nitrification because there is no aeration flow to keep the CO_2 partial pressure constant in the vessel. To overcome this problem it is advisable to sparge, at reduced flow rates, a N_2/CO_2 mixture containing some 1-5% CO_2.

Titration profiles and nitrate recovery factors obtained using the DENICON are similar to those obtained by ANITA in nitrification tests and therefore are not reported here. Details of DENICON's operation and on data processing can be found in Massone *et al* (**10**) and Rozzi *et al* (**19**).

Measurement of rbCOD and the BND concept

The above methodology, originally designed to measure nitrates, was modified to additionally evaluate rbCOD. The procedure is as follows: a sample of wastewater containing readily-biodegradable organic carbon (rbCOD) is added to the biosensor reactor, which contains a denitrifying biomass and nitrates in excess of denitrification requirements. As in the previous application denitrification produces alkalinity, which is neutralised by the addition of an acid titrant.

The biodegradation of rbCOD is therefore indirectly determined by the reduction of the nitrate fraction needed to oxidise those organics (**20**,**21**). The main problem with this application is the variable stoichiometric ratio of COD/N-NO_3, which depends on the composition of the organics to be biodegraded. In Figure 5, a denitrification test carried out on a sample of distillery slops is presented.

The amount of nitrates needed to biologically oxidise a given mass of organics in anoxic conditions has been defined as the Biochemical Nitrate Demand (BND), and this could be used to estimate the denitrification potential of a wastewater or a given chemical product (**20**).

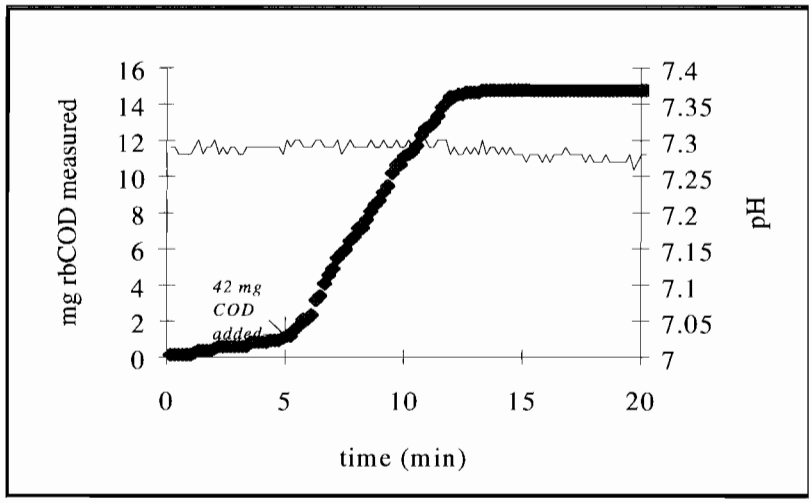


Figure 5. Measurement of the rbCOD in distillery slops, from Rozzi *et al.* (**19**).

Use of the DENICON biosensor to measure VFA as rbCOD in anaerobic digesters

Volatile Fatty Acids (VFA) are generally considered to be a crucial monitoring and control variable in anaerobic digesters, although they are not simple or cheap to measure, especially in on-line applications. As they make up the main fraction of rbCOD in anaerobic reactor mixed liquors and effluents, it could be possible to monitor them by measuring their BND. Preliminary trials with the DENICON biosensor confirmed the possibility that it could be used to monitor VFAs in digested mixed liquors (**19**).

$DENICON_{online}$ for biological process control

An automated DENICON was used to monitor the influent concentration in a pilot scale fluidised bed anaerobic reactor, as explained above. In Figure 6, the COD measured by an on line instrument is plotted (**21**).

The potential applications for this instrument appear to be considerable, although sampling problems in high concentration influents have not been completely solved, especially when gas bubbles are released.

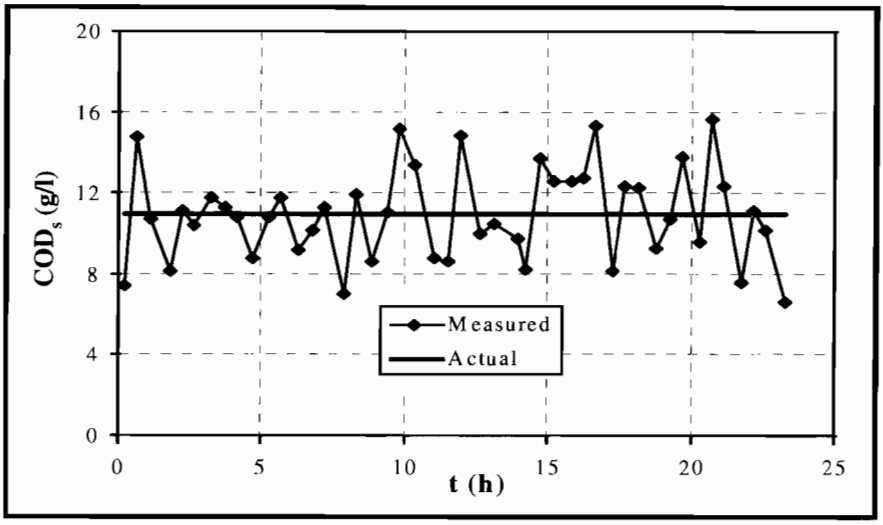


Figure 6. COD measured on line by the DENICON biosensor.

The MAIA biosensor to measure methanogenic activity

The Methanogenic Activity and Inhibition Analyser (MAIA) is a modification of the DENICON biosensor. The main difference between this and the nitrification and denitrification titration instruments is in the reaction vessel, which is a sealed conical flask instead of an open beaker.

In practice, a test on MAIA consists of a first phase in which the anaerobic sludge, poured into a flask with the temperature controlled at 34°C, is flushed with a 1:1 CO_2:CH_4 gas until its pH stabilises. Next, a certain aliquot of sodium acetate is added to the sludge to activate the methanogenic activity. During this second phase, the titration unit adds acetic acid to balance the production of alkalinity. As well as keeping the pH constant, adding acetic acid compensates for its biological consumption, allowing the substrate to be kept constant.

The anaerobic degradation of acetate to methane produces alkalinity:

$$CH_3COONa + H_2O = CH_4 + NaHCO_3 \quad (2)$$

Because of the above conversion, the concentration of bicarbonate in the sludge sample increases while, at the same time, the release of methane reduces the CO_2 concentration in the liquid phase. As a result, there is an increase in sludge pH due to a shift in CO_2/HCO_3 equilibrium. That is:

$$Ka_1 = [H^+] [HCO_3^-]/[CO_2] \quad (3)$$

In the MAIA biosensor, the pH of the mixed liquor is maintained constant by addition of acid as titrant which consumes the excess bicarbonate alkalinity:

Thus the titration unit adds the titration solution (concentrated acetic acid of the order of 0.5 ÷ 1 M), restoring the previous pH and acetate levels, while bicarbonate is protonated as:

$$CH_3COOH + NaHCO_3 = CH_3COONa + H_2O + CO_2 \quad (4)$$

It follows that one mole of methane and of carbon dioxide are released for each mole of acetic acid consumed by the acetoclastic methanogens, that is:

$$CH_3COOH = CH_4 + CO_2 \quad (5)$$

and consequently the molar fractions in the gas mixture released from the system are $X_{CH4} = X_{CO2} = 0.5$, while the environmental conditions (pH and substrate) remain constant ($\Delta S < 0.1$ meq/L, $\Delta pH < 0.01$). The methanogenic activity is then derived from the flow rate of the titration solution by a simple stoichiometric conversion.

The main advantage of the MAIA biosensor is that it provides the possibility of measuring the conversion rate of a methanogenic culture while keeping the substrate concentration and pH constant as long as required. Generally, determining methanogenic activity takes longer than determining nitrification or denitrification activity (24 to 48 hours against 0.5 to 4 hours).

Numerous tests, carried out in Italy (**22**) and South Africa (**23**), have shown that this instrument enables easier and more accurate evaluation of maximum activity, kinetics, inhibition of acetoclastic methanogens than can be obtained using existing volumetric or manometric procedures. To show the sensitivity and rapid response of the MAIA biosensor, the inhibition induced by two spikes of industrial toxic effluent to a methanogenic culture is shown in Figure 7.

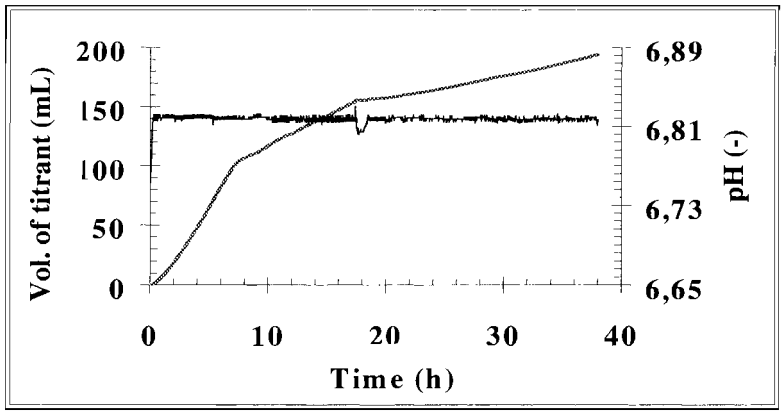


Figure 7. Methanogenic activity inhibition by toxic effluent (**22**).

The MAIA biosensor to measure ANAMMOX activity

An extremely interesting new family of anaerobic microorganisms has been recently discovered in Netherlands, the ANaerobic AMMonium Oxidation bacteria which are able to oxidize NH_4^+ by NO_2^- (**24**). Innovative processes exploiting these microorganisms might soon deeply modify the future of nitrogen removal technologies, especially for concentrated wastewater. The MAIA has been adapted to evaluate kinetics of Anammox bacteria.

The most recent conversion reaction which has been suggested for the Anammox bacteria is, according to Van de Graaf *et al.* (**25**):

$$NH_4^+ + 1{,}3\ NO_2^- + 0{,}042\ CO_2 \rightarrow$$
$$0{,}042\ CH_2O + N_2 + 0{,}22\ NO_3^- + 0{,}08\ OH^- + 1{,}87\ H_2O$$

where CH_2O is the gross composition for the synthesised biomass.

The above reaction induces a pH change because of CO_2 consumption and OH^- ions production. Even though the specific production of alkalinity, per mole of substrate consumed, is quite low compared to other alkalising bacteria such as denitrifiers (**10**) and acetoclastic methanogens (**22,23**), preliminary experiments carried out by Rozzi and Remigi (**26**) indicate that it is possible to evaluate the kinetic parameters of Anammox bacteria by a pH-stat titration procedure.

Principle of operation of DOstats

A new technique based on DO-stat titration was developed, based on the general principle of assessing the kinetics of a reaction from the flow rate of an appropriate titrant needed to keep constant a chemical species which is directly or indirectly produced or consumed by the reaction under investigation. While the above described pH-stat titrations maintain the pH constant by adding an alkaline or acid titrant and are thus applicable to any pH affecting reaction, the DO-stat titration is suitable to monitor aerobic bio-oxidations which involve the consumption of oxygen. The field of applicability of this technique is the same of that one of other already existing respirometers and it has been successfully tested to evaluate kinetics of autotrophic bacteria such as nitrifiers (**27**) and heterotrophic biomass (**28**).

The DOstat respirometer

A completely mixed thermostated vessel (V = 1-2 liters), where a DO probe is inserted, is used as bioreactor. The instrument layout is outlined in Figure 8.

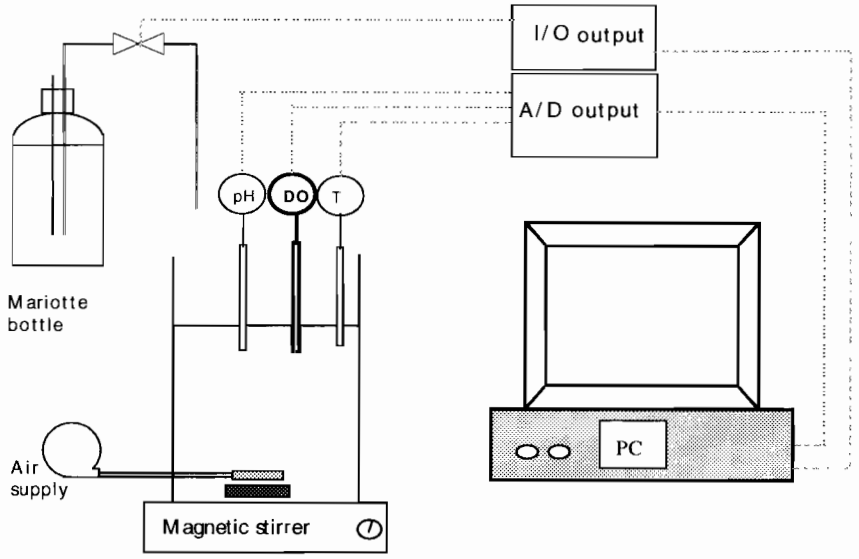


Figure 8. The layout of the DOstat unit.

DO concentration is maintained constant by titrating a solution which contains oxygen. As a rule, in DO-stat determinations the appropriate titrant should be an oxygen-oversaturated solution. To overcome technical limitations related to the use of such solutions, which do not allow to concentrations in excess of 40 mg/l, an adequately diluted hydrogen peroxide solution may be used as titrant.

Compared to classical open and closed respirometers, this instrument offers the following advantages:

- It allows to work at a constant DO level which can be freely chosen by the operator, the only limitation being the necessity to avoid significant oxygen transfer between liquid and gas phases. Since tests are performed at a constant DO level which can be selected well above the semi-saturation constant for DO, there is no interference due to kinetic limitations by DO depletion during the test. As DO can be freely chosen, tests can be performed at the most appropriate DO concentration (e.g. at the DO level at which the biomass was grown or the DO level maintained in the aeration tank). Moreover, by performing several respiration tests at various DO level, the semi-saturation constant for oxygen can be easily and accurately assessed.
- There is no need to estimate the gas transfer coefficient (K_La), differently from gas flowing systems, since oxygen is provided by a liquid flow;
- No re-aeration cycles are required, thus continuous measurements can be performed, differently from gas static systems, since oxygen is supplied whenever it is consumed;

- Accurate calibration of the DO probe, required for both gas flowing and gas static systems, is unnecessary, since the probe is only employed to sense oxygen variations within a given tolerance range. Moreover, the response dynamic of DO probe does not significantly affect r_o estimation since the probe delay only influences the maximum range of DO oscillations around the set point value.
- Experimental data processing is very simple since H_2O_2 addition rate is directly proportional to the oxygen uptake rate and the volume of H_2O_2 required to keep DO at the set point value is directly proportional to the substrate oxidation rate (Figure 9).

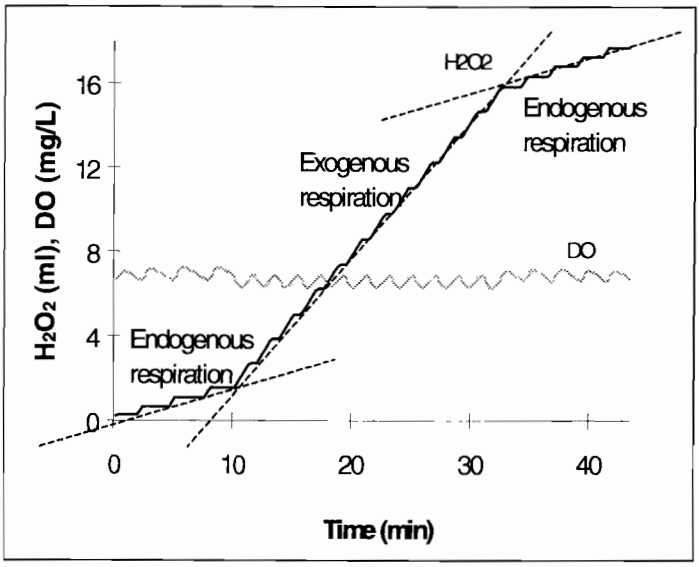


Figure 9. Typical DOstat test output.

Apart from the above advantages, this procedure may lead to underestimation of the biomass respiration during long duration tests using low biomass concentration levels (< 2÷3 g TSS/L). This toxic effect could limit the applicability of the DO-stat respirometer for respiration experiments on diluted biomass. However, because of its simplicity, this procedure is suitable for on-line process control. In such an application, H_2O_2 toxicity effects are unlikely to occur since a new biomass sample can be drawn from the aeration basin for each respirometric determination.

The DOstat to monitor autotrophic and heterotrophic activity

The potential of the DOstat has been verified on a reactor where an accurately controlled addition of a reducing chemical (sulfite) was titrated by hydrogen peroxide titration. An excellent correspondence was found among oxidation rate estimated by the open respirometry, closed respirometry and DOstat titration as indicated in Figure 10 (**28**).

This bioassay was also used to monitor the activity of both ammonia and nitrite oxidizers as described in Ficara *et al.* (**27**) and to evaluate the metabolism of heterotrophic biomass (**28**).

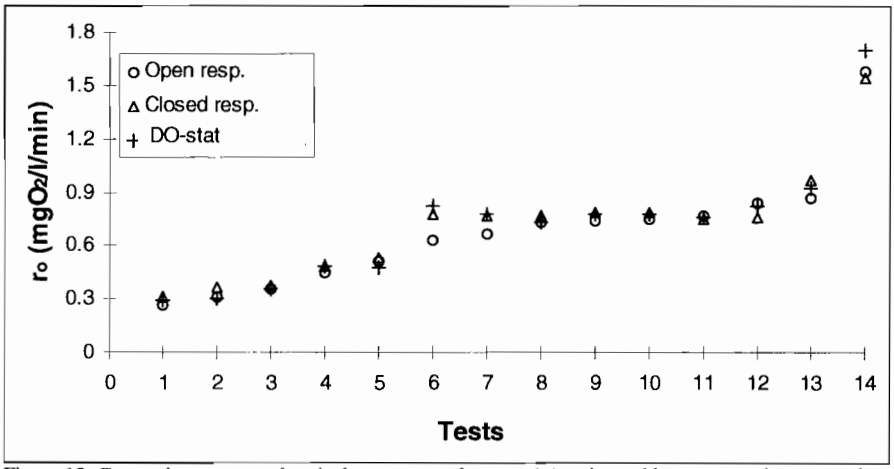


Figure 10. Comparison among chemical oxygen uptake rates (r_o) estimated by open respirometry, closed respirometry and DOstat titration.

Combination of pHstat and DOstat techniques

Similarly to the ANITADO system, the DO-stat and the pH-stat techniques were simultaneously applied to assess ammonium oxidisers kinetics and combined to enable the simultaneous assessment of both ammonium and nitrite oxidations kinetics leading to very reproducible estimates of both ammonium and nitrite oxidisers kinetic parameters (**27**).

The pH-stat and DO-stat techniques can be also coupled to assess organic substrates oxidation by both, pH-stat titration of the evolving CO_2 and by DO-stat titration of the DO consumed. Preliminary short term biodegradation tests on simple substrates (**5**) indicated that the two techniques did not lead in all the cases to the results theoretically expected from the stoichiometry of substrate complete mineralisation suggesting that the in some cases only partial oxidation takes place. Results therefore suggest that, by coupling pH-stat and DO-stat titration, more information can be obtained about substrate biodegradation pathway since the process is monitored by reagent (O_2) disappearance and by product (CO_2) formation.

Outlook on future developments and conclusions

Titration biosensors are a fairly new analytical technology within the wide family of instruments available for operation and control of wastewater treatment plants. Following a couple of preliminary investigations in the early 1980s, their development actually began in 1995 as part of a co-operative research between the Politecnico di Milano (I) and the University of Gent (B) which subsequently followed independent research approaches.

A first generation of titration biosensors has been developed which are particularly suited for laboratory applications related to measuring the activity biomass (nitrifiers, denitrifiers, methanogens) in a wastewater treatment plants. Because they are specific to a given trophic group, it is possible to obtain accurate kinetic evaluations compared to competing methodologies.

Some of the instruments, and MAIA and pH-DOstat in particular, are still being investigated and improved as prototypes for laboratory use. This means they cannot yet be compared, in terms of performance and diffusion, to 'mature' instruments such as conventional respirometers, which have been used for years in wastewater plants, both for laboratory and on-line applications.

A second generation of titration biosensors is being developed to monitor on-line the metabolic state of activated sludge in biological wastewater treatment plants and the concentration of the related substrate. This simultaneous monitoring of biological activity and substrate concentration allows in principle a better process control by titration biosensors rather than by P/C sensors, in particular an earlier detection of incoming toxic substances. In practice, especially for the pH-stats, the on-line applications appear to be more complex than initially estimated, mainly because of unpredictable interferences due to contemporary acidifying or alkalising reactions.

In conclusion, within a few years titration biosensors will probably emerge and establish themselves as a reliable and relatively cheap technology to monitor and control wastewater treatment plants. Their development will undoubtedly be boosted by the seemingly endless advances in hardware such as more sensitive and specific electrochemical probes for organic and inorganic compounds, more sensitive potentiometers and amperometers, cheaper input/output interfacing cards, and more user-friendly software. Last but not least, these instruments could also be used for industrial biotechnology applications.

Acknowledgements

The authors gratefully thank the International Water Association (IWA) for permission to include into this "State of the art" parts of the paper " Titration biosensors for wastewater treatment process control" published on the April 2000 Issue of the IWA magazine Water 21.

References

1. Love N.G., Bott C.B. (2000) Upset Early Warning Technologies For Biological Treatment Systems: Evaluation And Research Recommendations Project 99-WWF-2 funded by the Water Environment Research Federation. Alexandria, USA
2. Sharma A., Rogers K. (1994) *Measurement Science & Technology* **5**: 461-472.
3. Rogers K., Gerlach C. (1996) *Environmental Science and Technology* **30**: 486-491
4. Rozzi A., Ficara E., Massone A., Verstraete W. (2000) *Water*, **21**, 50-55..
5. Ficara E. (2000) pHstat and DOstat titration techniques for activated sludge control, Ph.D. Thesis, Politecnico di Milano, Italy.
6. Ramadori R., Rozzi A., Tandoi W. (1980) *Water Research* **14**, 1555-57.
7. Beccari M., Passino R., Ramadori R., Tandoi V. (1980) *Environment Technology Letters* **1**(5), 245-252.
8. Aivasidis A., Hochscherf H., Rottmann G., Hagen T., Mertens M.T., Reiners G., Wandrey C. (1992) *Awt abwassertechnik.* **5**(43), 48-55.
9. Massone A., Gernaey K., Rozzi A., Willelms P., Verstraete W. (1995) *In* Proceedings 9th Forum of Applied Biotechnology, September 27-29, Gent (Belgium). Mededelingen Faculteit Landbouwkundige, University of Gent, 60, 2361-2368.
10. Massone A., Antonelli M., Rozzi A. (1996) The DENICON: a novel biosensor to control denitrification in biological wastewater treatment plants. Mededelingen Faculteit Landbouwkundige, University of Gent, 1709-1714.
11. Rozzi A., Massone A., Verstraete W. (1999) European patent 'Method to monitor in liquids the concentration of substances which are degraded by acidifying or alkalising microorganisms and related instrumentation'. N. 0757017 (granted 20.10.99)
12. Massone A., Gernaey K., Rozzi A., Verstraete W. (1998) *WEF Research Journal* **70**, (3), 343-350.
13. Yuan Z.G., Bogaert H. (2001) *Water Res.* , **35** (1), 180-188.
14. Rozzi A., Bonomo L., Massone A., Pollice A. (1997) Evaluation of phenol biodegradation kinetics using a biosensor. *In*: Proceedings ECCE-1, the first European Congress on chemical engineering, Florence (Italy) May 4-7
15. Ficara E., Rozzi A. (2001) *Journal of Env. Eng. ASCE*, **127** (8), 698-704.
16. Ficara E., Musumeci A., Rozzi A. (2000) *Water SA* **26** (2), 217-224.
17. Massone A., Rozzi R. (1997) Nitrification and toxicity on line control in an industrial wastewater treatment plant. *In* Proceedings Instrumentation Control and Automation, IAWQ Brighton, 6-9 June
18. Gernaey K., Bogaert H., Vanrolleghem P., Massone A., Rozzi A., Verstraete W. (1998) *Water Science Technology*, **37** (12), 103-110.
19. Rozzi A., Massone A., Antonelli M. (1997) *Water Science and Technology*, **36**, 6-7, 183-189.
20. Rozzi A., Massone A., Alessandrini A. (1997) Measurement of rbCOD as biological nitrate demand using a biosensor: preliminary results. 3rd International Symposium Environmental Biotechnology, Oostende, Belgium, April 21-23.
21. Rozzi A., Buffière P., Steyer J.P., Massone A. (1998) Monitoring readily biodegradable COD load in winery wastewater and distillery slops by a titration biosensor. *In*: Proceedings 2c International special. Conference on winery wastewaters, Bordeaux, 5-7 May.
22. Rozzi A., Remigi E., Buckley C. (2000) Methanogenic activity measurements by the MAIA biosensor: instructions guide. VI Latin American Workshop and Seminar on Anaerobic Digestion, p. 408-415. Recife (Brasil) 5-9 november.
23. Murugen L.H., Govender M., Sacks J., Buckley C.A., Rozzi A., Frestel S. (1999) Measuring methanogenic activity using a titrating biosensor. African international environmental protection Symposium, Pietermaritzburg (ZA), 4-8 July.

24. Van de Graaf A., Mulder A., de Bruijn P., Robertson L., Jetten M., Kuenen J. (1995) *Applied and Environmental Microbiology*, **61**, (4), 1246-1251

25. Van de Graaf A., de Bruijn P., Robertson L., Jetten M., Kuenen J. (1997) *Microbiology*, **143**, 2412-2415

26. Rozzi A., Remigi E. (2001) Kinetics of the ANAMMOX bacteria by a pH-stat bioassay. Poster paper accepted at the AD2001 Conference Antwerpen, 2-5 September.

27. Ficara E., Rocco A., Rozzi A. (2000) *Water Science and Technology*, **41**, (12), 121-128.

28. Rozzi A., Ficara E., Rocco A. (2001) A novel respirometer based on DO-stat titration: principle of operation and preliminary results. Submitted for publication.

Chapter 14

ENHANCED LOADING AND STABILITY OF UREASE COVALENTLY ATTACHED TO POLYPYRROLE MICROSPHERES FOR APPLICATION TO UREA BIOSENSOR

A. GAMBHIR[1], A. KUMAR[1], B.D. MALHOTRA[1], B. MIKSA[2], S. SLOMKOWSKI[2]

[1]Biomolecular Electronics and Conducting Polymer Research Group, National Physical Laboratory, K. S. Krishnan Marg, New Delhi 110012, INDIA E-mail: agulati@csnpl.ren.nic.in ; [2]Center of Molecular and Macromolecular Studies Sienkiewicza 112, 90-363 Lodz, Poland E-mail: staslomk@bilbo.cbmm.lodz.pl

Abstract

An electrode for measurement of urea, based on urease covalently attached to polypyrrole (PPY) microspheres immobilized on conducting polypyrrole polyvinyl sulphonate (PPY-PVS) films has been described. Conducting polypyrrole polyvinyl sulphonate films were prepared using indium-tin-oxide (ITO) glass plates as working electrode and standard calomel electrode as the reference electrode. The films were deposited on ITO glass plates for 5-7 minutes at a constant current of 2mA (surface area 1x1cm^2). Urease immobilized onto polypyrrole microspheres (by reaction of protein amino groups with microsphere surface aldehyde groups of the surface of microspheres) was entrapped/adsorbed on electrochemically prepared conducting polypyrrole polyvinyl sulphonate films deposited on ITO. Potentiometric measurements were taken in different concentrations of urea using an ammonium ion analyser.

Key words: Urea biosensor, Urease, Polypyrrole microspheres.

Introduction

Determination of serum urea (BUN) is highly relevant for the determination of state of kidney functions. Though laboratory methods, both colorimetric as well as kinetic are available, they are tedious and time consuming. Besides this, they can only be performed in serum thereby requiring a considerable volume of blood sample. Owing to their reliable, fast and accurate results and lack of interference from color and other analytes routinely found in blood, biosensors are presently gaining interest for the determination of various analytes including BUN in whole blood. A number of biosensors based on conductometry (**1**), potentiometry (**2-3**), amperometry (**4-5**) have been described for urea determination but none of these has yet come into routine clinical application. Recently ENFET (enzyme-field-effect-transistor) based on ion-sensitive-field-effect transistor (ISFET) depending on detection of pH changes due to

New Horizons in Biotechnology, 153-161, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

the enzymatic reaction or detection of ammonium ions with an ammonium ISFET has been described (**6-7**). Urea sensor based on the integration of both ammonium based urea enzyme-field-effect-transistor in a single chip has also been described.

An essential prerequisite for the development of a biosensor is immobilization of its biological element in such a way that it retains its activity and is quite stable in its matrix. The technique of immobilization of enzymes has experienced a phenomenal growth in the recent past as it overcomes various limitations in the analytical uses of enzymes in aqueous solutions. The free enzyme can be immobilized by trapping it in an inert matrix such that the immobilized enzyme retains its catalytic properties for a much longer duration, is stable over a wider temperature and pH range as compared to the free enzyme and can be re-used for several analyses over a period of time. Use of polypyrrole microspheres for immobilization of urease on polypyrrole-polyvinyl sulphonate matrix is significant since it has been shown that electropolymerized inactive PPY on Pt shows a nerntian response to pH changes (**8**). In this context, several urea biosensors have been reported using PPY film and urease which hydrolyses urea causing change in pH (**9-11**). Use of microspheres enhances the loading of an enzyme since the surface area available for immobilization can be increased many fold as compared to plane and smooth surfaces. Specific surface of typical microspheres is very large (1g of microspheres with diameters equal to 100 nm has surface area close to 60 m^2) and thus, allows for adsorption and/or covalent immobilization of large amount of bioactive material. Besides, electrodes modified by attachment of polymer films or gels with physically entrapped enzymes usually suffer from enzyme leaking. These disadvantages could be avoided using films with embedded polymer microspheres bearing at their surfaces the covalently attached enzymes. Due to bulkiness the microspheres can remain permanently entrapped within the film layer. Many types of microspheres are known onto which proteins could be immobilized with sufficient extent of retention of their biological activity. However, for each type of polymer film one has to select microspheres taking into account their expected compatibility with the film matrix. Hence for PPY-PVS film based matrix PPY microspheres were used.

Polymer micropheres were extensively studied as carriers of bioactive compounds, in particular as carriers of proteins and oligonucleotides (**12-22**). In many instances the particles bearing antibodies (or antigens) were used for diagnostic purposes, most often for simple aggregation tests (**23-30**). However, properties of polymer microspheres indicate that these particles could be considered as interesting and valuable materials for more complex diagnostic devices, including biosensors. In last few years, methods have been developed which are suitable for immobilization of microsphere monolayers on quartz and glass supports which could be used as elements of sensors for optical detection (**31,32**). PPY-PVS microspheres could be conveniently used for preparation of electrodes for amperometric and/or potentiometric biosensors by adsorption or entrapment on ITO glass plates coated with electrochemically prepared PPY-PVS films.

In the present communication an electrode for measurement of urea based on urease covalently attached to polypyrrole microspheres immobilized on conducting polypyrrole polyvinyl sulphonate (PPY-PVS) films has been devised.

Material and Methods

Synthesis of polypyrrole microspheres with aldehyde groups.

Polypyrrole microspheres with aldehyde groups on their surface layer suitable for covalent immobilization of proteins by the Schiff linkage formation in reaction between aldehyde groups of microspheres with protein amino groups were synthesized (**33**). Polyvinyl (N-pyrrolidone) (1.6 g, K30, Aldrich) was dissolved in 400 ml of three times distilled water. The solution was put into the flask and deaerated by bubbling nitrogen. Thereafter, freshly distilled pyrrole (8 g, Aldrich) and $FeCl_3 \cdot 6H_2O$ (70 g) were added to this solution. The mixture was stirred for about 24 hours under nitrogen at room temperature. Next, the synthesized microspheres were isolated by centrifugation and redispersed in pure water. The centrifugation and redispersion sequence was repeated at least three times. The final suspension (80 ml) contained 6.5 wt/vol % of polypyrrole microspheres. These microspheres were equipped with aldehyde groups by building the polyacrolein adlayer at each of them. For this purpose 0.2 ml of freshly distilled acrolein (Aldrich) and 0.05 g of $K_2S_2O_8$ were added to 100 ml of microsphere suspension containing 1.2 g of solid. The mixture was purged with nitrogen and thereafter polymerization of acrolein was carried out in stirred mixture at 65°C for about 30 hours. The functionalized microspheres were purified by four times repeated centrifugation and redispersion in three times distilled water.

Surface concentration of aldehyde groups on functionalized microspheres available for reaction with amine groups bearing compounds was evaluated by depletion of 1-aminopyrene from ethanol solution incubated with microspheres. Typically, 2 ml ethanol solution with 1-aminopyrene ($1.6 \cdot 10^{-3}$ mol/l) was mixed with 2 ml of microsphere suspension containing $1.11 \cdot 10^{-2}$ g of microspheres. Concentration of 1-aminopyrene in initial solution and in supernatant, after microsphere removal by centrifugation, was determined spectrophotometrically ($\varepsilon_{360} = 15800\ l \cdot mol^{-1} \cdot cm^{-1}$). For synthesized polypyrrole microspheres with polyacrolein adlayer, concentration of aldehyde groups available for reaction with 1-aminopyrene was found to be $7.93 \cdot 10^{-7}$ mol per 1 g of microspheres.

Immobilization of urease onto microspheres

The enzyme was immobilized onto microspheres (0.31 g) by incubating them with urease (Type II from Jack beans, 16000 units/g solid, Sigma) in 2.5 ml of phosphate buffer with pH = 7.4. Initial concentration of the enzyme in the mixture was varied from $2.2 \cdot 10^{-4}$ g·ml^{-1} to $4.3 \cdot 10^{-3}$ g·ml^{-1}. Incubation was carried out for about 24 hours at room temperature. Amount of attached urease was evaluated from the difference of the enzyme concentration in initial solution and in supernatant after removal of microspheres by centrifugation. Concentration of the enzyme was determined

spectrophotometrically by measuring absorption at 278 nm arising due to tryptophan and tyrosine residues in protein.

Immobilization of urease-microspheres on PPY-PVS film

Conducting polypyrrole polyvinyl sulphonate films were electrochemically deposited on ITO glass plates serving as working electrode (WE), platinum (Pt) as counter and standard colomel electrode (SCE) as the reference electrode from a solution comprising of pre-distilled pyrrole (0.1M) and PVS (0.1M) at a constant current (2 mA). Electropolymerization was carried out for about 15 minutes to get a thick film. Immobilization of urease-microsphere onto PPY-PVS films was accomplished by electrochemical entrapment for about 12 minutes after the film deposition started. Urease immobilized onto polypyrrole microspheres (by reaction of protein amino groups with microsphere surface aldehyde groups; [Urease] = 1.15 mg per 1 m^2 of the surface of microspheres) was also physically adsorbed on electrochemically prepared conducting polypyrrole polyvinyl sulphonate films deposited on ITO. Potentiometric measurements on these electrodes were conducted as a function of urea. Urease covalently attached to polypyrrole microspheres was also characterized using UV-visible spectroscopy.

Results and Discussion

Figure 1 illustrates dependence of surface concentration of urease (Γ_{Urs}) attached onto microspheres on initial concentration of the enzyme in solution (urease). The plot is typical for adsorption processes in which adsorbed molecules occupy certain space and reduce surface available for adsorption of the new ones.

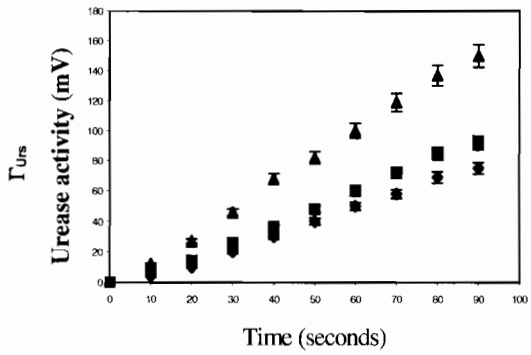


Figure 1: Dependence of surface concentration urease (Γ_{Urs}) immobilized onto the surface of microspheres on concentration of urease in solution.

Scanning electron microscope (JEOL 550 LV) and quasielastic light scattering apparatus (Zetasizer 3000 Hsa, Malvern) were used for characterization of microphercs before and after functionalization. Number average diameters of microspheres (Dn) measured by SEM before and after functionalization were estimated as 92 nm and 88 nm respectively. Polydispersity factors (Dw/Dn, ratio of the weight and number average diameters) for particles were determined as 1.073 before and 1.045 after functionalization. It is worth noting that for identical particles Dw/Dn = 1. SEM measurements indicate that within the experimental error, diameters of microspheres did not change as a result of functionalization. Quasielastic light scattering studies indicate that in suspension the microspheres were aggregated. Hydrodynamic radius of these aggregates equals 233. After functionalization microspheres aggregated forming two sub-populations, one containing aggregates with diameters 355 nm and the second one containing aggregates with diameters 1490 nm.

Response Characteristics and Stability

Effect of pH on the activity of free urease and urease-PPY microspheres immobilized on PPY-PVS films is presented in Fig. 2. Lesser shift in optimum pH in case of urease -PPY microspheres (pH optimum, 7.2) (as compared to free enzyme in solution, pH optimum, 7.0) was observed than urease directly adsorbed on PPY-PVS films (pH optimum, 7.5). It is interesting to see that the rise and fall in urease activity was more gradual on both sides of pH optimum in immobilized state which is due to proven higher stability in this state (**7**). Since displacements of the pH/activity profiles are known to depend upon partitioning of protons effected by the presence of ionized groups on the polymer matrix as well as due to substrate diffusion limitation, therefore, lower shift of pH optimum in the case of immobilized urease-PPY microspheres as compared to free enzyme entrapped in polymer matrix was observed (**34**) The effect of negative charge due to PVS as well as partitioning effects due to multilayers of polymers are negated in case of urease-PPY microspheres as the surface concentration of enzyme increases many folds and also some of the enzyme attached to the distal surface of entrapped microsphere are not in direct contact with the polymer film.

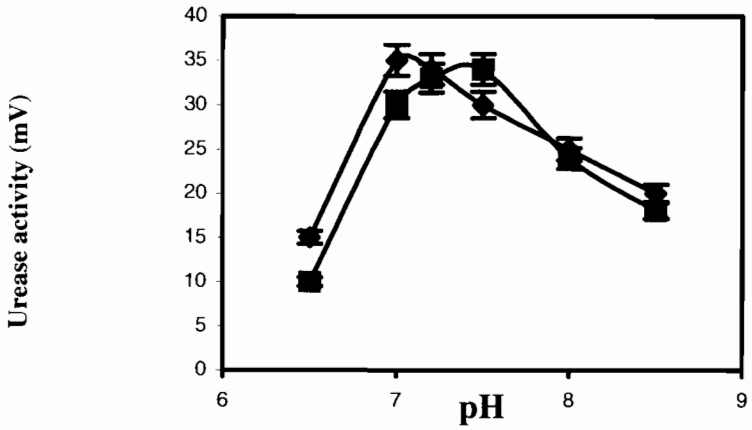


Figure 2: Effect of pH on the activity of urease in (◆) Free and (■) Immobilized on polypyrrole microspheres.

A good linear correlation between potential sensed by an ammonium ion selective electrode and urea concentration was obtained in the range from 5mM to 50mM when this electrode was used. Two linear ranges were obtained viz. 0-4mM and 5-60 mM (Fig. 3).

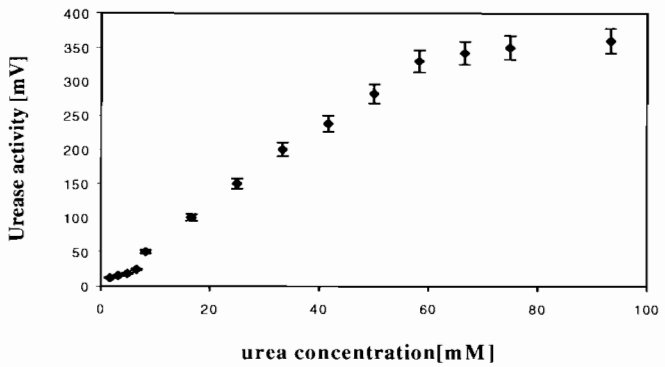


Figure 3: Variation of potential (mV) as a measure of urease activity associated with polypyrrole microsphere based urease electrode with increasing concentration of urea (0-100mM) in phosphate buffer (pH 7.2), linear range 5-50mM.

Km^{app} for the higher range was found to be less than that for free enzyme in solution showing the apparent ease with which enzyme and substrate can come in contact with each other owing to higher concentration of urease per unit area of electrode. When urease activity in this electrode was measured with respect to time it was found that

saturation value is attained within about 80 seconds (Fig. 4) from the start of reaction showing increased activity due to enhanced loading from the start of the reaction.

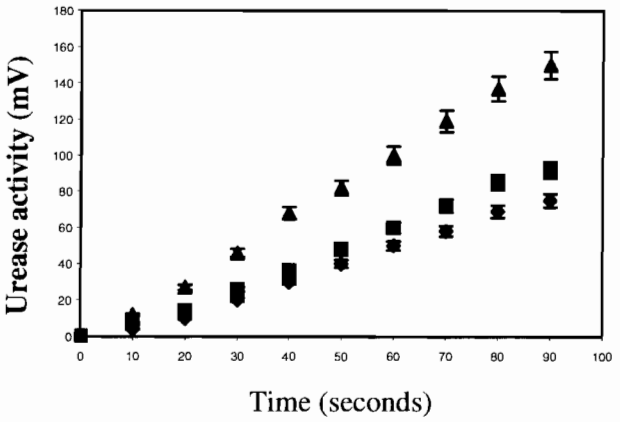


Figure 4: Variation of potential (mV) as a measure of urease activity associated with polypyrrole microsphere based urease electrode as a function of time with different concentrations of urea ;10mM(◆), 20mM (■) , 50mM (▲).

The leaching was found to be about 5-10% in the case of entrapped enzyme over a period of one week in solution whereas in case of urease-PPY microspheres it was reduced to about 5% over a period of one month in solution. This enhanced retention in polymer film is likely to be due to the bulkiness of the microspheres. Half-life of this electrode was found to be about 40 days. These electrodes were thermally stable between 25-50^{0}C, although reduced activity was observed above 42^{0}C.

The response time of this electrode was 40 seconds as compared to urease entrapped in PPY-PVS electrode where the response time was 60 seconds. This reduced response time could be attributed to enhanced loading of urease in this electrode. The detection limit and the sensitivity for this electrode were 10mg/dl and 12mV/decade, respectively. The same electrode can be used for as many as 10 experiments.

Conclusions

Enhanced loading of urease on PPY-PVS films could be carried out by immobilization of covalently linked pyrrole microspheres as evident from results on activity measurements with respect to urea concentration and temperature. It has been shown that this electrode shows a reduction in response time and has a half-life of about 40 days. The electrode also shows considerable stability upto 50°C.

Acknowledgements

The authors are grateful to Dr. K. Lal, Director, National Physical Laboratory for his encouragement and support. AG is grateful to CSIR for a Research Associateship.

Grant from DST sponsored Indo-Polish project (INT/POL/015.00) is duly acknowledged.

References

1. Ho W.O., Krause S., McNeil C.J., Pritchard J.A., Armstrong R.D., Athey D., Rawson K. (1999) *Anal. Chem.*, **71**, 1940-1946
2. Guilbault G.G., Nagy G. (1973) *Anal. Chem.*, **45,** 417
3. Campanella L., Sammartino M. P., Tomassetti M. (1990) *Analyst*, **115,** 827-830.
4. Cho W.J., Huang H.J. (1998) *Anal. Chem.*, **70,** 3946-3951.
5. Bertocchi P., Compagnone D. (1996) *Biosens. & Bioelectron.*, **11,**1-10.
6. Koncki R., Radomska A., Glab S. (2000) *Talanta*, **52,** 13-17.
7. Senillou A., Jaffrezic-Renault N., Martelet C., Cosnier S. (1999) *Talanta,* **50,** 219-226.
8. Osaka T., Komaba S., Fujino Y., Matsuda T., Satoh I. (1999) *J. Electrochem. Soc.*, **146,** 615-619.
9. Komaba S., Seyama M., Momma T., Osaka J. (1997) *Electrochim. Acta,* **42,** 383.
10. Osaka J., Komaba S., Seyama M., Tanabi K. (1996) *Sens.Actuat. B,* **463,** 35-36.
11. Gambhir A., Gerard M., Mulchandani A.K, Malhotra B.D. *Applied Biochemistry and Biotechnology* (accepted)
12. Slomkowski S. (1998) *Progr.Polym.Sci.,* **23**, 815-874.
13. Elgersma., Zsom R.,Norde W., Lyklema J.(1987) *J. Colloid Interface Sci.*, **138**, 145-156.
14. Oenick D.B., Warshawsky A. (1991) *Colloid Polym. Sci.*, **269**, 139-145.
15. Caldwell K.D., Li J., Li J.T., Dalgleish D.G. (1992) *J. Chromatography,* **604**, 63-71.
16. Betton F., Theretz A., Elaissari A., Pichot C. (1993) *Colloids Surfaces B: Biointerfaces*, **1**, 97-106.
17. Tuncel A., Denizli A., Purvis D., Lowe C.R., Piskin E. (1993) *J.Chromatogr.*, **634**, 161-168.
18. Elaissari A., Cros P., Pichot C., Laurent V., Mandrand B. (1994) *Colloids Surfaces A: Physicochem. Eng. Aspects*, **83**, 25-31.
19. Basinska T., Kowalczyk D., Miksa B., Slomkowski S. (1995) *Polym.Adv.Technologies*, **6**, 526-533.
20. Norde W., Gonzalez F.G., Haynes C.A. (1995) Polymers *Adv. Technol.*, **6**, 518.
21. Fritz H., Maier M., Bayer E. (1997) *J. Colloid Interface Sci.*, **195**, 272-288.
22. Lacasse F. X., Filion M.C., Phillips N.C., Escher E., McMullen J.N., Hildgen P. (1998) *Pharm. Res.*, **15**, 312-317.
23. Bolin V. S., Sue Chase B., Alsever J.B. (1968) Am. *J. Clinical Pathology,* **49**, 635-646.
24. Hipp S., Berns D.S., Tompkins V., Buckley H. (1970) *Sebouraudia,* **8**, 237-241.
25. Heymer B., Schachenmayr W., Bultmann B., Spanel R., Haferkamp O., Schmidt W. (1973) *J. Immunology,* **111**, 478-484.
26. Lim P.L., Ko K.G.H. (1990) J. *Immunological Methods*, **135**, 9-14.
27. Galanti L.M., Cornu C., Masson P.L., Robert A.R., Becheanu D., Lamy M.E., Cambiaso C.L. (1991) *J. Virological Methods*, **32**, 221-231.
28. Borque L., Rus A., Ruiz R. (1991) *J. Clinical Laboratory Analysis*, **5**, 175-179.
29. Miksa B., Wilczynska M., Cierniewski C., Basinska T., Slomkowski S. (1995) *J. Biomater. Sci. Polymer Edn*, **7**, 503-513.
30. Molina-Bolivar J.A., Galisteo-Gonzalez F., Hidalgo-Alvarez R. (1998) *J. Biomater Sci., Polym. Edn.*, **9**, 1089-1101.
31. Slomkowski S., Kowalczyk D., Trznadel M. (1995) *Trends in Polym. Sci.* **3**, 297-304.
32. Slomkowski S., Kowalczyk D., Trznadel M., Kryszewski M. (1996) *ACS Symp. Ser.* Ottenbrite R.,

Huang S. and Park K (eds), ACS, Washington, DC. **627**, 172-186

33. Miksa B., Slomkowski S. (1995) *Colloid and Polymer Sci.*, **273**, 47-52.
34. Trevan M.D. (1980) Immobilized enzymes (ed.) John Wiley and Sons. 11-53.

Chapter 15

TRUE AND APPARENT YIELDS AND MAINTENANCE COEFFICIENT AND THEIR SIGNIFICANCE ON FERMENTATION KINETICS

J. A. RODRIGUEZ-LEON*, G. BUENO, D. E. RODRIGUEZ, G. DELGADO , P. SERRANO , M. A. BRIZUELA

Instituto Cubano de Investigaciones de Derivados de la Caña de Azúcar (ICIDCA). Vía Blanca 804 y Carretera Central. La Habana. Cuba; e-mail: rodleon@icidca.edu.cu

Abstract

Yield based on substrate ($Y_{x/s}$) or oxygen consumption ($Y_{x/o}$) is a very important parameter. This parameter indicates how efficient a fermentation is. At the same time it is very closely related with the maintenance coefficient (m). By means of yield and maintenance coefficient it is possible to cstimate the proportion of energy that cells consume in biomass and metabolites synthesis and the proportion of energy that allows the cells to maintain their capability for their biological performance. Unfortunately the maintenance coefficient is not normally considered due to difficulties in its estimation and therefore the assessment yield could be disturbed. A method for the estimation of the former parameters is proposed in this work. The method was applied to a submerged fermentation with *Lactobacillus rhamnosus* and compared with results previously obtained for solid state fermentation of sugar cane and citrus residues with *Candida utilis* and *Aspergillus niger* respectively.

Key words: *Lactobacillus rhamnosus*, fermentation, sugar consumption balance, yield, maintenance coefficient.

Introduction

Carbon source or substrate in a heterotrophic fermentation process is the main basis for energy and elements that growing cells require. At the same time the measurement of substrate utilization is a very important index that characterizes the development of the process. Therefore, yield based on substrate consumption is one of the most common parameters that indicates how efficient the process is.

Yield based ($Y_{x/s}$) on substrate consumption is defined as:

$$Y_{x/s} = dX/dS \qquad (E1)$$

Where: X: biomass concentration (g/l) ; S: substrate concentration (g/l)

New Horizons in Biotechnology, 163-172, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

In Equation (E1) there are no distinctions concerning how the substrate is employed and with what purpose. It is common to postulate that the substrate is consumed in order to allow cell multiplication, often called "cell growth", and for cell maintenance (**1**). A substrate consumption balance that considered the former implications was therefore proposed as:

$$dS/dt = (1/Y_{x/s})\, dX/dt + m\, X \qquad (E2)$$

Where: m : maintenance coefficient (g substrate/g biomass h)

The maintenance coefficient represents the energy that the individual cell requires to maintain its biological performance without considering the energy required for cell duplication. It is defined through the concept of specific substrate consumption as:

$$m = 1/X\, ds/dt \qquad (E3)$$

In the case of an aerobic fermentation and when O_2 consumption is considered,

Equation (E2) can be written as:

$$dO_2/dt = (1/Y_{x/O2})\, dX/dt + mX \qquad (E4)$$

Where: $Y_{x/O2}$: yield based on O_2 consumption ; m : maintenance coefficient (g O_2 consumed /g biomass h)

Equation (E4) keeps the same considerations as in Equation (E2) or in other words, it represents an O_2 consumption balance considering whole cell performance. This equation was resolved by numerical methods (**2**) in order to estimate the synthesized biomass in a solid fermentation process. In this method Equation (E4) was solved for a particular fermentation considering values of $Y_{x/O2}$ and m that were estimated from former data available. At the same time, it was proposed that the parameters included in the equation could be estimated by a trial and error procedure (parameter optimization procedure) (**3**). Then Equation (E4) would estimate not only the synthesized biomass but also the parameters corresponding to a particular fermentation without the need of previous values that correspond to other processes. This procedure was applied previously in several solid state fermentations (**4-6**).

The former equations and their solutions and definitions enable the obtaining of a more precise analysis of the meaning of some parameters that characterize the development of a fermentation process.

The present work proposes a procedure to analyse a *Lactobacillus rhamnosus* fermentation, growing in a submerge culture, to estimate the yield based on sugar consumption and the maintenance coefficient when sugar cane molasses are employed as carbon source.

Materials and Methods

Strain.

The *Lactobacillus rhamnosus* strain employed belongs to ICIDCA Microorganism Collection. This strain is maintained at 4°C in MRS broth (Difco Laboratories, Detroit, Michigan). Additionally it is conserved as a lyophilized stock.

Fermentation and media.

MRS media was modified by substituting the glucose content by cane molasses for a final content of 20 g/l. Yeast extract was employed as nitrogen source to a final content of 2g/l of N.

Fermentations were carried in a 5 l fermenter (Marubishi) with pH and temperature control at 6.5 and 37°C respectively. Produced biomass was measured by optical density calibrated with a lineal regression model.

Reduced sugar determinations.

The reduced sugars were determined by the modified Eynon-Lane procedure (**7**).

Results and Discussion

Results obtained during the fermentation of sugar cane molasses in a submerged fermentation of *Lactobacillus rhamnosus* are reported in Table 1.

A lag phase of 1h and a log phase that lasted eight hours were deduced from Table 1. A specific growth rate (μ) of 0,239 h^{-1} was obtained from the log phase. The yield based on sugar consumption ($Y_{x/s}$) was calculated as 24.4%, considering data corresponding to the log phase. Other authors reported this yield considering the sugar consumed from the beginning of the fermentation until the end of the log phase. In this case the yield was 24.21% too. In this fermentation it can be seen that there were no practical differences between both estimations. This may due to the fact that the lag phase lasted only 1 hour and the accelerated growth phase was practically non-existent. Considerations for the estimation of this overall yield must also be kept in mind.

Table 1. Variation of sugar and biomass content during a fermentation of sugar cane molasses by Lactobacillus rhamnosus at an initial concentration of 20 g/l.

Time (h)	Sugar concentration (g/l)	Biomass dry matter (g/l	Sugar consumed (g/l)	Consumed Sugar rate R_s (g/l/h)
0	20.4	0.62	0	0
1	19.5	0.81	0.9	0.9
2	18.8	1.03	1.6	0.7
3	17.2	1.23	3.2	1.6
4	15.3	1.63	5.1	1.9
5	14.4	1.96	6	0.9
6	8.35	2.63	12.05	6.05
8	5.2	4.30	15.2	1.575
10	3.67	4.49	16.73	0.765
12	1.8	4.84	18.6	0.935

Based on sugar consumption balance when solving Equation (E4) with the data reported in Table 1, the yield based on sugar consumption ($Y_{x/s}$) and the maintenance coefficient (m) corresponding to this data could be estimated.

Equation (E2) was written as:

$$\mu S=(1/Y_{x/s})\,\mu X+mX\,\mu\, t \qquad (E5)$$

Applying the solution proposed for Equation (E5) and substituting R_o by R_b and $Y_{x/o}$ by $Y_{x/s}$ in the original equation (**2**), the following equation was obtained:

$$X_n=\left(Y_{x/s}\Delta t\left(\frac{1}{2}\left(\left(\frac{dR_i}{dt}\right)_{t=0}+\left(\frac{dR_i}{dt}\right)_{t=n}\right)+\sum_{i=1}^{i=n-1}\left(\frac{dR_i}{dt}\right)_{t=i}\right)+\left(1-\frac{a}{2}\right)X_o-a\sum_{i=1}^{i=n-1}X_i\right)\Big/\left(1+\frac{a}{2}\right) \qquad (E6)$$

Where for simplicity, it was considered that:

$$a = mY_{x/s}\Delta t \qquad (E7)$$

In order to solve Equation (E6) an initial biomass concentration of 0.62 g/l and a final concentration of 4.84 and the rate for sugar consumed at different times, were considered. The values for the parameters estimated by the trial and error procedure (parameter optimization) were obtained so that the final concentration predicted by the Equation (E6) was approximately the same as in the original data. The results achieved for the parameters were: $Y_{x/s}$ = 0.239 and m = 0.0945 g substrate/g biomass/h. The predicted biomass value for the fermentation with *Lactobacillus rhamnosus* at each time was obtained by applying the values determined for $Y_{x/s}$ and m to Equation (E6).

Biomass variation from the data in Table 1 and from the values estimated from the Equation (E6) was reported. As observed, there was very good correlation between real and predicted values. The differences between the values were less than 8% except for two points.

There was also good similarity, when comparing the yield based on sugar consumption (24.21%) taken from the data in Table 1 and the value estimated (23.9%) from the Equation (E6). One must take into account the values obtained for $Y_{x/s}$ when solving Equation (E6).

From the final values obtained it was possible to estimate sugar quantity employed in cell duplication or growth and the sugar employed for maintenance. However it seemed necessary to think about the feasibility of considering the values of $Y_{x/s}$ and m as constant during the fermentation particularly when the evaluation for the whole balance was made considering the data reported in Table 1. It would therefore be useful to consider the balance of sugar consumed for each growth phase.

The lag phase balance

The lag phase is characterized by the fact that there is no cell duplication or growth. That means that the specific growth rate (μ) was zero and therefore it was feasible to assume that $\Delta X = 0$. Then, the expression for Equation (E5) that corresponded to this phase was reduced to:

$$m = \Delta S/X \Delta t \qquad \text{(E8)}$$

The maintenance coefficient corresponding to the lag phase as 1.452g sugar/g biomass/h was estimated from Equation (E8). Comparing this value with the value obtained in Equation (E6), it was not possible to consider the last (0.0945g substrate/g biomass/h) constant during the whole process. In this case the increase of the maintenance coefficient was in the order of 15. At the same time, the yield based on sugar consumption was zero. This fact indicated that parameter validity must always be checked very closely during the different stages of the process.

After these considerations it seemed obvious that it was very important to establish fermentation conditions that avoid long lag phases or at least the shortest lag phase could be obtained. This fact may seem trivial but could result in a loss of material, energy and time in any fermentation process.

The log or exponential phase balance

This phase is characterized by a constant specific growth rate.

Taking into account the definition of growth rate:

$$\mu = (1/X)\, dX/dt \qquad \text{(E9)}$$

and from Equations (E2) and (E9) and rearranging, the following was obtained:

$$dS = (1/Y_{x/s} + m/\mu)\, dx \qquad \text{(E10)}$$

Equation (E10) is very useful when considering the log phase because it represents the complete relation between sugar consumption and biomass production and maintenance in a very simple manner. Expressing this equation as finite differentials was achieved by:

$$\Delta S = (1/Y_{x/s} + m/\mu)\, \Delta X \qquad \text{(E11)}$$

The value for sugar consumption (Δ S) through Equation (E11) was estimated considering $Y_{x/s}$=0.239, m=0.0945 g substrate /g biomass / h, μ=0.238 h^{-1} and ΔX = 3.03 g. The predicted consumed substrate by Equation (E11) during the log phase was 13.9 g. From Table 1 data, the value obtained for sugar consumption in the log phase was 14.3 g.

The proportion of sugar that was employed in growth and maintenance could be calculated forthwith considering the first and the second term of the right side in Equation (E11). They were 14.6 and 1.4 g respectively, which meant that 8,7% of sugar consumption was employed in maintenance.

When considering the proportion of sugar employed for growth and maintenance one should not forget that in the present case the fermentation with *Lactobacillus rhamnosus* was a homolactic fermentation in which lactic acid was produced as result of a main metabolic pathway characteristic of this strain (**8**). The lactic acid produced was associated with growth such that for each synthesized cell an exact quantity of acid was produced. That implied that sugar related to the acid synthesis was already considered during microbial growth. This was why, in this process, the yield based on sugar consumption was lower when compared to those processes in which only produced biomass and CO_2 were present. Meanwhile, taking into account these considerations it was necessary to report the yield of lactic acid produced on basis of biomass synthesized and not on yield based on sugar consumed.

If considered in Equation (E5), the proportion of energy or substrate employed for lactic acid synthesized could be re-written as:

$$\Delta S = (1/Y_{x/s}) \Delta X + mX \Delta t + \Phi \qquad (E12)$$

Where Φ is the fraction of sugar consumed for a particular metabolite synthesis as g substrate consumed.

Defining the specific metabolite production as:

$$\Phi = (1/X) \Delta P/\Delta t \qquad (E13)$$

Where: Φ : g metabolite/g biomass h ; ΔP : grams of the particular metabolite produced in the time interval analyzed.

Considering the yield of product based on sugar consumption ($Y_{p/s}$) as:

$$Y_{p/s} = \Delta P/\Delta S \qquad (E14)$$

Equation (E12) could be written as:

$$\Delta S = (1/Y_{x/s}) \Delta X + (m + \Phi/Y_{p/s})X \Delta t \qquad (E15)$$

The term ($m + \Phi/Y_{p/s}$) in Equation (E15) is a constant that considers not only the maintenance coefficient, but the specific metabolite production too. So it can be postulated that:

$$m^I = m + \Phi/Y_{p/s} \qquad (E16)$$

As a matter of fact, the previous value obtained for m (0.0945g substrate/g biomass/h) was actually an estimation of the value of m^I.

From Equation (E13) and the definition for the maintenance coefficient in Equation (E3), the relation between m and Φ was obtained as:

$$\Phi/m = Y_{p/s} \quad \text{(E17)}$$

Then Equation (E16) was reduced to:

$$m^1 = 2m \quad \text{(E18)}$$

Taking into account the value obtained for m^1 as the value for m reported previously, the real value for m could be estimated as 0.0473g substrate/g biomass /h if lactic acid produced is taken into account.

The value for specific metabolite production of lactic acid was already obtained from Equation (E17) taking into account the value of $Y_{p/s}$. Unfortunately the production of lactic acid was not reported in the data analyzed.

The method here proposed could be generalized. If more metabolites are considered, the Equation (E12) could be written as:

$$\Delta S=(1/Y_{x/s})\,\Delta X+mX\Delta t+\Phi_1+\Phi_2+.....\Phi_n \quad \text{(E19)}$$

Specific metabolite production for the different metabolites considered were defined as:

$$\Phi_n = (1/X)\,\Delta P_n/\Delta t \quad \text{(E20)}$$

Then Equation (E15) was written as:

$$\Delta S = (1/Y_{x/s})\,\Delta X + (m + \Phi_1/Y_{p1/s} + \Phi_2/Y_{p2/s} + \ldots \Phi_n/Y_{pn/s})\,X\Delta t \quad \text{(E21)}$$

The particular relation between specific metabolite production of each metabolite with the maintenance coefficient was already obtained as:

$$\Phi_n/m = Y_{pn/s} \quad \text{(E22)}$$

Then Equation (E18) could be postulated as:

$$m^1 = (n+1)\,m \quad \text{(E23)}$$

where n is the number of metabolites considered in the process. It must be kept in mind that m^1 was calculated as a first approximation considering only the biomass produced.

From the results achieved with Equation (E23) it could be postulated that the maintenance coefficient was an index not only of the substrate consumed for maintenance of individual cells and the metabolites synthesis but a measure of the metabolites that are synthesized and need to be considered. However, if production of any metabolite were partially associated with growth, then the maintenance coefficient and the different yields may be no longer constant during the log phase and should be considered.

The negative accelerated growth phase balance

Negative accelerated growth phase occurs immediately after the log or exponential phase. In this phase the specific growth rate is lower than the specific growth rate attained at the log phase and changes with time. Although this is a phase in which

specific growth is not a constant, it may be assumed that the yield based on sugar consumption and the maintenance coefficient are not constant either. However, Equation E6 was employed to give a first estimate for these values considering a period time between 8 and 12 h. The values obtained are reported in Table 2.

The values for X predicted by Equation (6) for 8-12 interval time considering the corresponding values of $Y_{x/s}$ and m the values differ by less than 1% in relation to the biomass reported. An overall specific growth rate (constant) for this phase was estimated as 0.026 h^{-1} taking these values as reference.

Table 2. Values estimated trough Equation 6 for different time intervals.

Time interval (h)	$Y_{x/s}$	maintenance coefficient : m (g sugar/g biomass/h)
2-8	0.239	0.0945
8-12	0.207	0.0714

To determine how far this assumption was from reality, an estimation of the specific growth rate employing Equation (E9) was made for specific time intervals namely 8-10 and 10-12 h using data reported in Table 1. The values obtained for both intervals were 0.022 and 0.036 h^{-1} respectively. These values indicate a variation that seemed to be under the influence of the measurement employed. A smaller time interval should therefore be considered. However, the decline in the specific growth phase which is characteristic of this phase, was obvious when compared with values obtained from the specific growth rate at log or exponential phase.

When considering this phase it was very important to determine whether other metabolites not associated with growth were synthesized because this phase was closely related with secondary metabolites. This is not the case of the present fermentation.

The values of the maintenance coefficient for *Lactobacillus rhamnosus* and the values obtained in solid state fermentation of *Candida utilis* grown in a molasses medium and *Aspergillus niger* growing on citrus waste (**9**) were compared. The values of the maintenance coefficient reported for *Candida utilis* and *Aspergillus niger* were corrected because the original units for maintenance coefficient were g oxygen/g biomass/h. Taking into account that for each mole of glucose (180g) consumed in an aerobic fermentation 6 moles of O_2 (192g) are necessary, the correction factor employed was 0.94.

In Table 3 the values obtained in the case of *Candida utilis* and *Aspergillus niger* are reported. A two time interval was considered to determine whether the maintenance coefficient could be considered constant during the whole process. In the case of *Aspergillus niger* such an assumption is not valid. This was attributed to the fact that inducible enzymes (pectinases and cellulases) were synthesized during consumption of the initial sugar content. When comparing the value obtained for *Lactobacillus rhamnosus* with the highest values reported for *Aspergillus niger* and

for *Candida utilis*, an increase of 350% was observed, indicating, according to the procedure here analyzed, that production of metabolites was important, an element that was not taken into consideration when the data was evaluated.

Table 3. Comparison of maintenance coefficient (g substrate/g of Biomass/h) values obtained for different fermentations.

Microorganism	Fermentation type	*Substrate*	Maintenance coefficient (m)
Lactobacillus rhamnosus	Submerge	molasses	0.0945
Candida utilis	Solid	citric residues	0.0202*
			0.0245*
Aspergillus niger	Solid	citric residues	0.0064*
			0.0181*

*values corrected and estimated at two different time interval

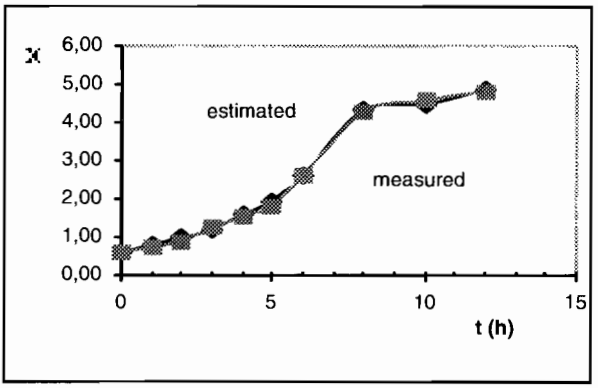


Figure 1. Comparison between the biomass measured during the fermentation of sugar cane molasses at 20 g/l with a strain of *Lactobacillus rhamnosus* and the biomass predicted by Equation (E6) with the values of $Y_{x/s} = 0.239$ and m = 0.0945 g substrate/g biomass/h.

Conclusions

In the case of sugar cane molasses fermentation with *Lactobacillus rhamnosus*, the value obtained for the maintenance coefficient indicated that around of 10% of the sugar consumed during the process was employed in cell maintenance and metabolites synthesis.

The procedure hereby employed confirmed that the fermentation with *Lactobacillus rhamnosus* was not as efficient in relation to biomass synthesis. In the latter case there was a five-fold increase of the value for the maintenance coefficient, as compared to the other processes considered.

The value obtained for m when considering the production of lactic acid was still high indicating that other metabolites must be considered, referring to the possibility that the fermentation was heterolactic.

The procedure here reported allowed the prediction of the maintenance coefficient and gave more accurate calculations for the yield based on sugar consumption and specific metabolite production.

Acknowledgements

The present work was partially financed by the National Technical and Scientific Program from CITMA, Agriculture Branch. Projects No.0.03.00.004 and 0.08.0070

References

1. Pirt S.J. (1965) *Proc. R. Soc. Lond.* **163B**, 224-231.
2. Sato K., Nagatani M., Nakamura K., Sato S. (1983) *J. Ferment. Technol.* **61** (6) 623-629
3. Rodríguez León J.A, Sastre L., Echevarría J., Delgado G., Bechstedt W. (1988) *Acta Biotechnologica* **8** (4): 299-302
4. Soccol C., León J.R., Martin B., Roussos S., Raimbault M. (1993) *Biotechnology Techniques,* **7** (8): 563-568
5. Rodríguez León J.A., Domenech F., León M., Rodríguez D.E. (1999) *Brazilian Archives of Biology and Technology* **42** (1) 69-76
6. Stertz S.C., Soccol C.R., Raimbault M., Rodríguez León J.A. (1999) *J. Chem. Technol. Biotechnol.* **74**:580-586
7. Biart J. R. (1975). Analytical Manual ICIDCA.
8. Brizuela Ma. A. (1999) M.Sc. thesis. Havana University, Cuba.
9. Rodríguez León J. A. (1966) *in* Anais do IV Sem. Hidrólise Enzimática de Biomassas Eds. G. M. Zanin and F. F. Do Morais, Maringa, Brasil. pp 41-52

Chapter 16

BIOPROCESSING ENGINEERING CONSIDERATIONS IN THE PRODUCTION OF SECONDARY METABOLITES BY PLANT CELL SUSPENSION CULTURES

A.K. SRIVASTAVA

Dept. of Biochemical Eng. & Biotechnology; Indian Institute of Technology Delhi, Hauz Khas, New Delhi (India)

Abstract

Plant cell cultivation technique serves as an alternative source of a variety of chemicals of diverse nature. However, major bottlenecks in large-scale cultivation of plant cells are low growth rate, shear sensitivity due to their fragile nature, adequate mixing, oxygen transfer and maintenance of aseptic conditions. To complicate the situation further, plant cell products are intracellular and concentrations are generally very low. These problems have been addressed to some extent by optimizing biological and/or technological parameters. Biological parameters include proper selection of medium composition with respect to its C and N source C/N ratio, phosphate ions, and precursor and plant growth regulators. Also light temperature and aeration can be altered to improve yield. The major technological parameter is mixing. Adequate mixing and homogenous conditions in reactor are particularly difficult to achieve due to shear sensitive nature of plant cells. The problem is further compounded when at high cell concentration the changes in fluid rheology result in Non Newtonian behaviour leading to clumping of cells and enhanced sedimentation. This phenomenon restricts effective mass and heat transfer inside cell clumps and therefore has an unpredictive effect on growth and product formation. Plant cells therefore, require balanced aeration as high aeration may remove CO_2 (possible nutrient) from culture broth. Another major challenge in growth of plant cells is the sticky nature of late exponential phase cells due to excretion of polysaccharides. This results in problems relating to nutrient and oxygen diffusion to cell. Optimum concentration of calcium ions &/or addition of pectinase/cellulase could be used to minimize clumping to the desirable level.

There are several cases where plant cell culture has produced high amounts of secondary metabolites than the whole plant. One such example of our laboratory is *Holarrhina antidysentrica*, which produces antidysentric drug, conessine. Modification of MS medium by addition of growth factors, sucrose and optimal inoculum resulted in increased yield of the alkaloid. The same suspension culture was propagated in the bioreactor with precursor, cholesterol feeding, which led to 160 times higher productivity than that of the native plant. Salient features of this

New Horizons in Biotechnology, 173-191, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

and other plant cell suspension systems will be discussed with respect to bioprocessing strategies for production of various phytochemicals.

Efforts are being made to save valuable and rare plants of medicinal value from extinction. Another example within our laboratory is the development of plant cell culture of *Podophyllum hexandrum* which produces the precursor podophyllotoxin for anticancer drugs, etoposide and teniposide. MS and B5 media along with different growth factors have been studied to develop suspension culture in shake flask. B5 medium along with 2 % coconut milk produced 4 g/L biomass (dry basis). Plackett Burman design yielded the sensitive environmental parameters as inoculum level, glucose concentration and pH for culture growth and podophyllotoxin production. The exact response equation by manipulating the above three parameters was found out by Central Composite Design. The response of cell culture in a bioreactor having special low shear Setric impeller will also be presented.

Introduction

Plants are a rich source of pharmaceuticals, fragrances natural flavour, agrochemical and fine chemicals. Continuous harvesting these chemicals from natural or cultivated sources will lead to several ecological and/or extinction problems. Besides, the yield of the compound is variable due to climatic variations. Due to above problems and increasing demand for plant based products, the application of cell culture technology for production of important pharmaceuticals in vitro is seen as a promising alternative as opposed to harvesting chemicals from whole plants.

It has been suggested that plant cells are totipotent (**1**). All of the necessary genetic and physiological potential for natural product formation should be present in an isolated cell and according to this theory cultured cells obtained from any part of plant might be expected to yield secondary compounds similar to those of the plants grown *in vivo*. In practice a wide range of secondary metabolites have been successfully produced in plant cell cultures in only a few cases has production of the desired compounds been comparable to that of the parent plant (**1**).

As productivity is dependent on product yield organism growth rate and accumulated biomass levels, it is essential that there be a role of both biologists and engineers in improving system performance (**2**).

Plant cells, like microbial cells require nutrients, oxygen, adequate mixing and controlled physiological environment for growth and metabolite production. The basic difference between plant cell and microbial cell results in differences in their cultivation. Plant cells are 10-100 times larger than bacterial/fungal cell. They are therefore capable of withstanding tensile strain but are sensitive to shear stress. Their metabolism is slower and so they have slow growth rates. Culture must be maintained asceptically for longer period of time. The tendency of plant cells to grow in clumps results in sedimentation, insufficient mixing and diffusion limited

biochemical reactions. Oxygen requirements of plant cells are low in comparison with the oxygen requirements of microbial cells.

The *in vitro* culture of plant cell by definition provides artificial conditions for plant growth and it is possible that the imposition of such condition may induce stresses, which contribute to changes in the level of secondary metabolite formation. Manipulation of culture medium can be employed to enhance the yield of metabolites for plant cell cultures. Composition of standard media, e.g,, M.S., Gamborg, White, Nitsch and Nitsch and Limasmair and Skoog are available in literature, which adequately provide inorganic salts, trace elements, organic carbohydrate and growth regulators. Suitability of a particular media depends on nature of plant species, Chemical nature of product and relationship between growth and product formation during cell culture. Concentration of C, N, P growth regulator precursors are generally manipulated to enhance the yield of secondary metabolite.

Effect of C source

Biomass production is dependent solely on supply of carbohydrate its nature and concentration. Although sucrose is most commonly used (**3**) carbon source, a variety of other sugars have been investigated for their ability to support growth and influence biosynthetic pathways. These sources range from glucose, fructose, galactose, mono and di-saccharides to complex chemically indefined growth factors that are contained in molasses cereal and potato starch coconut milk and other plant extract (**1**). Sucrose and glucose were by far the best as effective carbon sources as reflected in high growth rate and biomass yield, leading to higher level of product synthesis.

Effect of N source

Oxidized/ reduced organic and inorganic sources are responsible for the most pronounced effect on growth of cultured cell. Media for plant cell suspension usually provide nitrogen as a mixture of NH_4^+ and NO_3^- Ammonium ions as a sole N source are unsuitable probably because pH of the medium has a tendency to fall below 5.0 during culture growth. Addition of KNO_3 along with Ammonium prevents the extreme fluctuations of pH, the nitrogen supply has been found to affect the biomass yield as well as growth rate of cells. The effect of nitrogen source was most dramatic in case of *Lithospermum erythrorhizon* (**4**). Shikonin production was stable in cell culture when nitrate was the sole nitrogen source; whereas ammonia as the nitrogen source completely inhibited the production of shikonin.

Effect of Phosphate

The concentration of inorganic phosphate which ranges between 0.05 to 3 mM in medium has profound effect on growth and metabolite production Deregulation of secondary metabolism by phosphate limitation is well established in microbial systems, but its regulatory affect in plant cell culture is not well defined. This is

because phosphate is rapidly taken up by the cell culture during early stages of growth. Studies with ^{31}P - NMR in *Nicotiana tabacum* culture showed that the cells take up phosphate with in 2 days of inoculation and phosphate is stored in vacuoles from which it is depleted during growth. As long as phosphate is present in the vacuoles there is no product formation, suggesting a regulatory role of phosphate. Accumulation of secondary metabolites by controlling phosphate concentration has been reported for Anthocyanin by *Daucus carota* (**3**).

Effect of Trace elements

The trace elements included in plant tissue culture media are Fe, Mn, B, Zn, Mo, Cu, Co and I. The effect of all these elements on growth has been well documented. However their role on secondary metabolite production has been demonstrated only in few cases. Increase in Cu^{2+} concentration by a factor of 30 enhanced shikonin production but had no effect on cell growth, where as increase in sulfate concentration in medium increased shikonin production but inhibited growth.

Effect of Plant growth regulators

Both natural and synthetic plant growth regulators (auxins and cytokinins) play an important role on ability of various plant cells to grow as suspension and also on metabolite production (**5**) 2,4D and kinetin have been used in cell suspension culture.

Technological Barriers

Complex changes of the cells during the cultivation result in technological problems leading to suboptimal utilization of the reactor (**6**). These could be summarized as :

Mixing

Mixing maintain homogenous conditions in the reactor and accelerates oxygen transfer by ensuring the transfer of nutrients and oxygen from liquid and gaseous phase. This promotes growth and product formation. High agitation rates, however, cannot be employed as it is deleterious to the rather shear sensitive plant cells. Low agitation rates, Airlift reactor and special agitator types are therefore applied to ensure proper low shear mixing. At high concentrations plant cell suspension cultures are viscous and their behavior is like non-newtonian fluids, which limit mass and heat transfer in the reactor. The results of inadequate mixing of plant cell suspension culture is clumping which results from excretion of polysaccharides at later stages of cell growth. This phenomenon results in limited availability of nutrients, which may lead to unpredictable effect on the cell growth and product formation.

Oxygen transfer

The oxygen requirements of plant cells are rather limited. Besides high oxygen sparging may flush out the carbon dioxide, which acts as nutrients to plant cell from

the broth and may have a negative effect on cell growth. Normal mechanically agitated vessel over aerates plant cell cultures. Airlift reactor, bubble free aeration surface aeration are some of the means to achieve efficient oxygen transfer and better growth. K_L a volumetric mass transfer coefficient, provides an indication of effective aeration and agitation in the bioreactor. High K_La value bioreactor will feature better oxygen transfer but poor cell growth. Similarly increase in viscosity decrease K_L a and signals the need for intensive agitation. Carefully designed experiments are therefore needed to study the effect of K_L a in growth and product formation.

Cell adhesion

Plant cells become sticky because of increased excretion of polysaccharide in bioreactor. This leads to wall growth and formation of large aggregates. Big clumps of cell create complex mixing problems and choke the sample line and also get deposited over the sensors, thereby limiting the measurement and control of the reactor. However, aggregates have shown favorable effects on cell physiology and biochemistry as it promotes cell-cell contact, which is essential for secondary metabolite production.

Calcium ions increase clumping and cell adhesion. Addition of ethylene diamine tetraacetate (which chelates the cations and release them shortly) cellulose and pectinase also control aggregation. Polyhydroxy propylene glycol ether has also been replaced to prevent cell adhesion and clumping. Wall growth may be minimized by using novel reactor designs. Rotary Drum reactor has major advantages over other membrane/immobilized and pulse column reactors. It may also be useful in preventing cell adhesion and clumping.

Plant cell reactors

A variety of reactors have been used to grow large-scale plant cell suspension cultures, major types being Stirred tank, Bubble column, Air lift and Rotating Drum Reactors (**6**).

Stirred Tank Reactors (STR)

STR with flat blade turbine impeller and modified impeller have been used for cultivation of plant cell suspension cultures. A large flat bladed turbine impeller with a high width to diameter ratio (7.6 dia and 14 cm width for a 5 liter reactor) has been found to be optimal for growth and phenolics production. These types of impellers produce well distributed mixing patterms with out dead zones at low shear. It has been observed that cells were more susceptible to shear damage during decelerating log phase of growth. It may be worthwhile to study different agitation speed for different phase of growth.

Air Lift Reactors

Airlift bioreactors are new type of bioreactors, which provide several advantages over conventional agitated tank reactors. Even though aeration capacity of airlift

bioreactors are less compared to agitated reactors, they provide required aeration and agitation during fermentation with low power consumption on account of absence of mechanical agitation. It is very simple in operation and absolute sterility can be maintained due to absence of shaft seals. Lower levels of hydrodynamic stress generated during agitation and aeration makes them extremely suitable for plant and animal cell cultivation.

There are however some problems encountered in providing adequate mixing for cultures at high cell concentrations and it features dead zones inside the reactor.

Rotating Drum Reactors

This consists of a horizontally rotating drum on rollers connected to a motor. The rotating motion of drum facilitates the mixing of gas and nutrient containing liquid inside the reactor. Baffles in the inner walls of the drum help to increase oxygen supply. It promotes high oxygen transfer to high cell density cultures and it prevents wall growth.

Bubble Column Reactors

It consists of a cylindrical vessel aerated at bottom, where it contacts gas and liquid. To minimize the mechanical shear due to bubble, sintered plates having low gas flow rates have been suggested. It features high mass and heat transfer areas with out input of mechanical energy.

Case Study Conessine

Holarrhena antidysenterica wall is deciduous laticiferous shrub or small tree of about 10 meter in height belonging to the family of Apocynaceae. It occurs throughout India but is especially abundant in sub-Himalyan tract. It is an important plant in Indian medicine and its stem bark is listed in Indian Pharmacopea. The ayurvedic texts describe Kutaja (H. antidysenterica) as the best drug for causing astringent effect and alleviatig "Kapha" (phlegm), Pitta (heat or bile) and curing diarrhoea and dysentery (**7**). The stem bark of the plant is an item of commerce and is commonly known as "Kurchi" "Kora" or "Kura Chal" in the drug market. The bark of the plant contains the most important *Holarrhena* alkaloid Connessine, the principal medicine for the treatment of dysentery. Connessine has particularly been tried in different forms for antidysentric purposes. Apart from this property of Holarrhena alkaloids they have also been reported to be antimicrobial. Their use in treatment of leprosy, piles cancer and also an antifertility drug has been reported. Due to its importance in medicine and fear of its extinction, this plant has been categorized as one of the endangered species by the Indian Government.

Plant cell cultivation of Connessine has been studied in our laboratories and following conclusions were drawn (**8**).

- The callus culture was derived from auxillary bud of the plant in MS medium supplemented with 1 mg/L 2,4 D and 0.5 mg/L kinetin and 30 g/L sucrose.

After 4-5 subcultures, friable callus was obtained which was used for development of suspension culture.

- The suspension culture was homogenous in early stages of growth during which the cells were found to be present in aggregates of about 65 μm. The cells grew well in dark. The medium for growth was MS medium with 30 g/L sucrose, 1 mg/L 2,4 D, 0.5 mg/L kinetin and 2 g/L inoculum (dry weight basis). The cell growth was complete in 10days giving maximum alkaloid on 8^{th} day.In callus culture maximum alkaloid formation was 300 mg/100 gm dry cell weight after 40 days and 130 mg/100 g dry weight in suspension culture after 8 days (**9**).
- HPLC analysis showed the presence of Connessine, the principal alkaloid in Holarrhenna bark, which constituted about 90 % of the total alkaloids.
- Optimal medium (**10**) for maximum synthesis of alkaloids was NH_4^+ :NO_3^- = 5:1 at total nitrogen of 60 mM, 0.25 mM KH_2PO_4, 0.5 mg/L 2,4 D, 1.0 mg/L kinetin, 40 g/L sucrose and 3 g/L of inoculum. Maximum cell mass of 15 g/L and maximum alkaloids of 100 mg/L are produced in 8 days in the optimal medium.
- Chloesterol served as a precursor of alkaloid biosynthesis (**11**). In suspension culture, a maximum of 50 mg/L cholesterol was transferred to 43 mg/L alkaloids (93 % conversion). Using optimized medium and optimal cholesterol feeding total alkaloid concentration of 143 mg/L was achieved on 8^{th} day (7.3 times higher than that of original MS medium).
- For maximum synthesis of alkaloids, the optimal requirement of phosphate was 0.25 mM.
- To achieve large-scale production of alkaloids plant cells were grown in the bioreactor using the optimal medium at 26^0C, pH 5.8 and agitation speed of 100 rpm, 0.5 v.v.m airflow rate. The fermentation profile is shown in Figure 1. The maximum biomass of 12 g/L and maximum alkaloid of 64 mg/L were produced on 8^{th} day.
- With the optimal medium and similar conditions as above but with cholesterol feeding policy, it was possible to get the maximum alkaloid formation of 106 mg/L in 8 days with a cell mass of 12 g/L. However wall growth was more pronounced during the later stages of the fermentation.

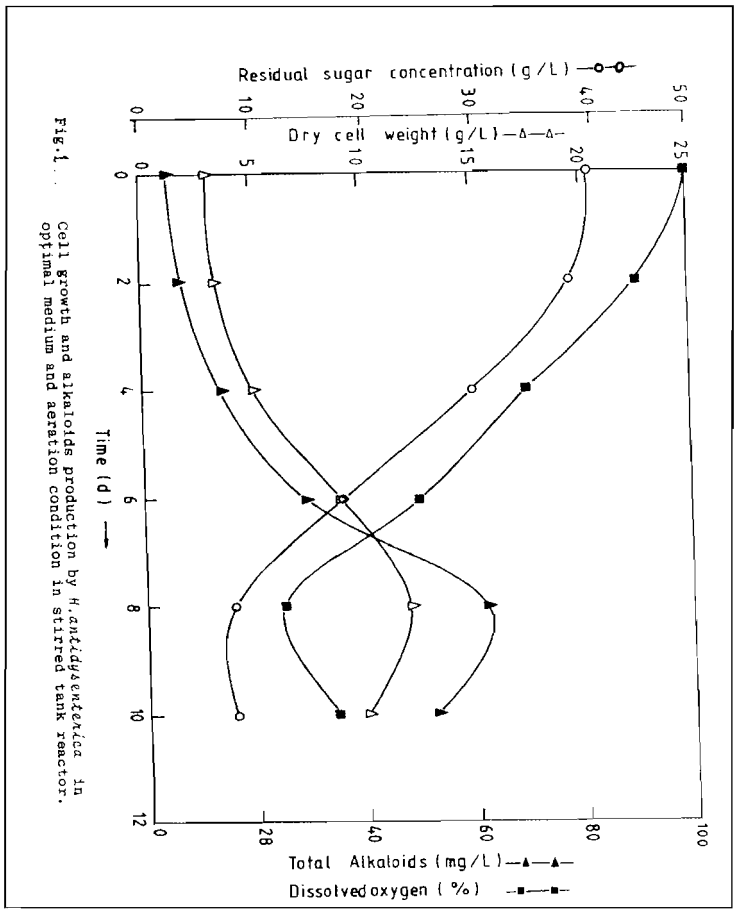


Fig.1. Cell growth and alkaloids production by *H.antidysenterica* in optimal medium and aeration condition in stirred tank reactor.

Case Study Podophyllotoxin

Podophyllum hexandrum Royle (Himalayan May Apple) is a typical Indian medicinal plant growing between 2500 and 3500 meter altitude in temperate Himalayan regions. The rhizomes and roots of the plant form the source of a medicinal resin. The resin podophyllin and its active principle, podophyllotoxin, have received considerable attention for their anti cancerous properties. The Indian *Podophyllum* species contain higher the amount of anticancer agent, podophyllotoxin, as compared to its American counterpart *P. peltatum*. Furthermore Indian *Podophyllum* contains less or no α- and β-peltatins, making the process of isolation of Podophyllotoxin simpler than that from the American *Podophyllum*.

Podophyllotoxin is used as a starting material for the chemical synthesis of anticancer agents like Etoposide (VP-16-213) and Teniposide (VM-26). Their cytotoxic action is based on the inhibition of Topoisomerase II, while Podophyllotoxin acts as an inhibitor of the microtubule assembly.

The production of secondary metabolites such as Podophyllotoxin via plant cell suspension has many advantages over conventional isolation from the intact plant. These include stable supply, freedom from disease and variation in climates, closer relationship between supply and demand and growth of large amount of plant tissue in minimal space.

Establishment of callus culture of *P. hexandrum* has been reported by a number of investigators (**12,13**). Propagation of *P. hexandrum* cells at the callus level, however, would not be able to meet the demand of the drug. A few studies have also been reported on cultivation of *P. hexandrum* in suspension culture (**12,14,15**). An advantage of suspension culture is its ability to mass propagate at a larger scale in bioreactors.

The present study deals with the optimization of growth of *P. hexandrum* and production of podophyllotoxin by plant cell suspension culture. Plackett-Burman design was applied to shortlist the most significant parameters, which influence growth and podophyllotoxin production and finally the concentration of screened variables were optimized by Response Surface Methodology using Central Composite Design methodology. The statistically optimized medium was eventually used for mass production of Podophyllotoxin in 3 liter bioreactor.

Materials and Methods

Initiation of Callus and Suspension Culture

Seeds of *Podophyllum hexandrum* were surface sterilized in 0.1% mercuric chloride for 5 min and rinsed with distilled water. Decoated seeds were again treated with 0.05% mercuric chloride for 3 min followed by rinsing with distilled water. The sterilized seeds were transferred to MS (**16**) and B5 (**17**) medium supplemented with selected growth regulators for germination. The root explants of the germinated seeds were then inoculated on MS and B5 media, supplemented with selected growth regulators for initiation of callus. The cultures were incubated at 20°C, with 16/8 hr day/night regime. The suspension culture was initiated by transferring the callus into MS and B5 media (without agar) supplemented with selected phytohormones. The suspension cultures were incubated on gyratory shaker (Orbitek, Sciengenics, India) at 125 rpm and 20°C under 16/8 hr day/night regime and also in the dark and subcultured after every three weeks.

Initiation of Suspension Culture using Pectinase and Polyvinylpyrrollidone (PVP)

The callus was transferred into MS and B5 media (without agar) supplemented with phytohormones, 0.005% pectinase (prepared in MES buffer, pH 6.0) and

1% polyvinylpyrrollidone (PVP). The cultures were incubated on the gyratory shaker at 125 rpm and 20°C under complete darkness. After three weeks the cells were subcultured (Inoculum level 2g dry weight per liter) into fresh medium containing 0.5% PVP without pectinase.

Cultivation of P. hexandrum in 3 Liter bioreactor

A three week old suspension culture was used as inoculum (consisting of clumps of different size) for the cultivation of *P. hexandrum* cells in a laboratory scale 3 L bioreactor. Cells of *P. hexandrum* were cultivated in 'MS + PVP (0.05 %) + Glc (3 %) medium at 20 ^{0}C, 125 rpm. The pH of the medium was controlled at 5.8 by 0.1 M NaOH and 0.1 N HCl. The impeller used was a specially designed one (Setric impeller) for the cultivation of plant cells in bioreactor, so that the cells are not damaged by shear stress produced by the impeller.

Determination of Growth Parameters

The suspension-grown cells were harvested at different time intervals during the growth cycle. The cell-suspension (15ml) was centrifuged in conical centrifuge tubes at 3000 rpm for 15 min. After centrifugation, the cells were washed with distilled water and then weighed to get the fresh weight (FW). The packed cell volume (PCV) was also determined by measuring the volume of the cells pelleted at the bottom of the centrifuge tube. The cells were then dried at 60°C for 16 hours to determine the dry weight (DW).

Extraction of Podophyllotoxin

The dried cell mass (20-200 mg) was powdered and heated with ethanol (1.5 ml) at 60°C for 30 min with constant stirring on a magnetic stirrer followed by sonication (Soniprep, U.S.A.) for 15 min. Constant stirring helped in leaching out the lignans from the cells. The supernatant was then removed after centrifugation at 12,000 rpm for 10 min and evaporated to dryness under vacuum. The lignan extract was redissolved in analytical grade ethanol prior to analysis.

TLC Analysis

The crude extract (20 µl) and podophyllotoxin (Sigma P-4405) as a reference compound (0.1 mg/ml; 20 µl) were chromatographed on silica gel 60-F-254 (Merck) plate using chloroform:methanol (25:1, v/v) over a distance of 10 cm in a saturated chamber. The spots were detected under UV light (254 nm) and developed with methanol:sulphuric acid (100:5, v/v), followed by heating at 110° C for 10 min.

HPLC Analysis

HPLC analysis of the extract was carried out using Nova Pak C18 (Waters, USA) column (250 × 4.6 mm) in HPLC system (Waters, U.S.A.) connected with UV-detector. Acetonitrile : water : methanol (37:58:5) was used as a mobile phase with flow rate of 1.5 ml/min. The crude extract (20µl) was injected into the HPLC

system for analysis. Podophyllotoxin was detected at 280 nm. Podophyllotoxin (Sigma P-4405, 0.1 mg/ml) was used as a standard for calculating podophyllotoxin content in the samples on the basis of total area under the peak obtained from integrator.

Experimental Design

Plackett – Burman Design (18)

Six variables (as shown in Table 1) were selected on the basis of existing literature reports and previous experiments to investigate their influence on growth of *P. hexandrum* cells in suspension and production of podophyllotoxin using the methodology of Placekett – Burman. Each independent variable was tested at two levels, a high (+) and a low (-) level as shown in Table 1.

Table 1: Range of variables selected to screen the most important parameters by Plackett–Burman design.

Level	IAA (mg/L)	NH_4^+:NO_3^- Ratio (Total N = 60 mM)	PO_4^{3-} (mM)	Glucose (g/L)	Inoculum (g/L)	pH
Level –1	0.0	1:5	0.0	20.0	2.0	5.0
Level +1	5.0	5:1	2.5	100.0	10.0	6.0

Eight experiments were formulated using six parameters. The experimental design protocol was developed using Design-Expert version 5.0.9 (Stat-Ease Corporation, Minneapolis, MN 55413, USA) software and is represented in Table 2. The inoculum (2 g/L, DW) used for the experiments was three-week dark-grown culture of *P. hexandrum*. The cultures were incubated on gyratory shaker (125 rpm) at 20°C under complete darkness. The flasks were harvested at the end of 30 days. The cell dry weight and podophyllotoxin content were analyzed for each flask.

Table 2: Design of the experiments by Plackett-Burman methodology using Design-Expert software and the responses.

Run Order	IAA (mg/L)	NH_4^+ NO_3^-	PO_4^{3-} (mM)	Glucose (g/L)	Inoculum (g/L)	pH	Response: Dry Weight (DW) (g/L)	Response: Podophyllotoxin (%, DW basis)
1	5.00	0.20	0.00	20.00	2.00	6.00	6.8	0.078
2	0.00	5.00	2.50	20.00	2.00	6.00	8.8	0.043
3	0.00	0.20	2.50	100.00	2.00	5.00	11.2	0.021
4	0.00	0.20	0.00	100.00	10.00	6.00	30.4	0.015
5	5.00	5.00	0.00	100.00	2.00	5.00	5.2	0.01
6	5.00	5.00	2.50	100.00	10.00	6.00	21.6	0.021
7	5.00	0.20	2.50	20.00	10.00	5.00	16.0	0.027
8	0.00	5.00	0.00	20.00	10.00	5.00	16.0	0.055

*Central Composite Design (**19**)*

Once the effective parameters were selected from the above Plackett-Burman design, Central Composite Design (CCD) was used to determine the actual level of the parameters for optimal growth and podophyllotoxin production. A 2^4-factorial central composite experimental design, leading to a total of 30 sets per experiment was formulated to optimize the initial inoculum level, glucose concentration, initial pH and IAA level for growth of *P. hexandrum* cells in suspension culture and podophyllotoxin production. The CCD experimental protocol was also developed using Design-Expert version 5.0.9 (Stat-Ease Corporation, Minneapolis, MN 55413, USA) software and is represented in Table 3. Inoculum level and incubation conditions were same for these experiments as that of Placekett-Burman design.

Table 3: Regression coefficients and t-values of the selected variables .

Factors	Growth		Podophyllotoxin Production	
	Coefficient	t-value	Coefficient	t-value
IAA	-2.1	-0.63	0.00025	0.026
$NH_4^+ : NO_3^-$	-1.6	-0.48	-.00015	-0.15
PO_4^{3-}	-0.1	-0.03	-0.005	-1.05
Glucose	2.6	0.78	-0.017	-3.10
Inoculum	6.5	3.57	-0.004	-0.46
PH	2.4	0.78	0.005	0.60

Suspension Culture Studies:

The suspension culture was initiated on MS and B5 media supplemented with different growth regulators either alone or in combination. Among the different media combinations tested, 'B5 + Coconut milk (5%)' produced a maximum of 4 g/L dry weight (DW) of biomass with an inoculum level of 2 g/L on dry weight basis. A few problems were associated with the growth of the cells in the suspension culture: (i) clumping of cells, (ii) browning of the culture media and (iii) drop in pH during culture growth. These problems were addressed with the inclusion of pectinase and PVP in the media. Use of pectinase helped in releasing the cells from the clumps into the suspension and polyvinylpyrrollidone (PVP) helped in preventing the browning presumably by arresting the oxidation of phenolic compounds. The use of PVP in *Cinchona ledgeriana* suspension culture has also been reported to prevent phenolic oxidation by Scragg *et al.* (**20**). This improved the magnitude of growth of *P. hexandrum* cells in suspension culture. The best growth response was observed in 'MS + IAA (2 mg/L) + PVP (1%)'. Pectinase was used at a very low conc. (0.005%) for initiation of suspension culture from the callus and was withdrawn in subsequent subcultures. PVP level was reduced to 0.5% during subsequent subcultures. It was also established that glucose was a better substitute than sucrose for the suspension culture of *P. hexandrum*. The highest cell mass of 8 g/L (DW) was observed in 'MS + PVP (0.5%) + Glucose (3%)' medium without any growth regulator.

Plackett-Burman Design:

Regression Coefficient and t-values:

Table 3 presents the results of the data analysis for the effect of six selected environmental parameters on growth of *P. hexandrum* and production of podophyllotoxin through regression coefficients and t-values. The regression coefficient and the t-values were calculated using Design-Expert version 5.0.9 software.

From the data on coefficients and t-values it can be concluded that IAA, ammonium:nitrate ratio and phosphate showed negative effects on growth of *P. hexandrum* cells whereas initial inoculum level, glucose concentration and initial pH of the media indicated positive response towards the growth of *P. hexandrum*. On the other hand, production of podophyllotoxin was mainly influenced by IAA level and initial pH of the culture media. Inoculum level indicated highest positive influence on growth of *P. hexandrum*, this was followed by glucose concentration and pH had the least effect on the growth. Negative values of coefficients and t-values of IAA, NH_4^+: NO_3^- ratio and PO_4^{3-} concentration established that they do not have any effect on growth. Similarly positive values of coefficient and t-values of initial pH and concentration of IAA indicated that pH has highest effect on production of podophyllotoxin followed by some effect of concentration of IAA.

Central Composite Design:

The above four parameters (initial inoculum level, glucose concentration, initial pH & IAA) were finally identified from the original list of six for determination of their exact optimum levels by using Central Composite Design. The experimental design matrix was formulated using Design-Expert software as shown in Table 4. The responses in the form of growth of *P. hexandrum* and production of podophyllotoxin were substituted in the above experimental design protocols (Table 5).

Table: 4: Design matrix of independent variables by CCD using Design-Expert software and the responses (values in the parentheses are the coded values of the parameters).

Run Order	Inoculum (A) (g/L)	Glucose (B) (g/L)	PH (C)	IAA (D) (mg/L)	Response: Dry Weight (DW, g/L)	Response: Podophyllotoxin (% DW basis)
1	2.00 (-2)	60.00 (0)	6.00 (0)	2.50 (0)	9.41	0.074
2	6.00 (0)	20.00 (-2)	6.00 (0)	2.50 (0)	6.89	0.045
3	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16	0.072
4	6.00 (0)	60.00 (0)	5.00 (-2)	2.50 (0)	16	0.096
5	8.00 (+1)	80.00 (+1)	6.50 (+1)	1.25 (-1)	23.2	0.1
6	6.00 (0)	60.00 (0)	7.00 (+2)	2.50 (0)	17.1	0.043
7	4.00 (-1)	80.00 (+1)	6.50 (+1)	1.25 (-1)	19.26	0.048

8	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	17.3	0.08
9	4.00 (-1)	40.00 (-1)	5.50 (-1)	1.25 (-1)	15.4	0.054
10	6.00 (0)	60.00 (0)	6.00 (0)	5.00 (+2)	17.9	0.13
11	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
12	4.00 (-1)	80.00 (+1)	5.50 (-1)	3.75 (+1)	11.85	0.073
13	6.00 (0)	60.00 (0)	6.00 (0)	0.00 (-2)	18.21	0.02
14	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
15	8.00 (+1)	80.00 (+1)	5.50 (-1)	3.75 (+1)	19.1	0.06
16	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
17	8.00 (+1)	80.00 (+1)	6.50 (+1)	3.75 (+1)	24.4	0.011
18	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
19	8.00 (+1)	40.00 (-1)	6.50 (+1)	1.25 (-1)	11.45	0.068
20	8.00 (+1)	80.00 (+1)	5.50 (-1)	1.25 (-1)	24.9	0.082
21	4.00 (-1)	80.00 (+1)	5.50 (-1)	1.25 (-1)	16.3	0.06
22	8.00 (+1)	40.00 (-1)	6.50 (+1)	3.75 (+1)	17.9	0.032
23	4.00 (+1)	40.00 (-1)	6.50 (+1)	3.75 (+1)	9.78	0.031
24	6.00 (0)	100.00 (+2)	6.00 (0)	2.50 (0)	14.62	0.027
25	8.00 (+1)	40.00 (-1)	5.50 (-1)	1.25 (-1)	18.79	0.03
26	4.00 (-1)	40.00 (-1)	5.50 (-1)	3.75 (+1)	12.74	0.025
27	10.00 (+2)	60.00 (0)	6.00 (0)	2.50 (0)	24	0.041
28	4.00 (-1)	80.00 (0)	6.50 (+1)	3.75 (+1)	8.3	0.01
29	8.00 (+1)	40.00 (0)	5.50 (-1)	3.75 (+1)	15.85	0.032
30	4.00 (-1)	40.00 (0)	6.50 (+1)	1.25 (-1)	8.6	0.022

Table 5: Regression coefficients and t-values of the selected variables and their interactions.

Factors	**Growth**		**Production**	
	Coefficient	**t-value**	**Coefficient**	**t-value**
Inoculum (A)	3.44	8.85	0.001	0.18
Glucose (B)	2.18	5.60	0.0047	0.77
pH (C)	-0.41	-1.05	-0.008	-1.36
IAA (D)	-0.78	-1.99	0.0012	0.2
A^2	0.14	0.38	-0.0068	-1.19
B2	-1.35	-3.71	-0.012	-2.13

C2	0.1	0.27	-0.0038	-0.67
D2	0.48	1.31	-0.0025	-0.43
AB	1.15	2.42	0.002	0.27
AC	0.54	1.14	0.0067	0.9
AD	0.99	2.07	-0.0062	-0.83
BC	1.13	2.37	-0.0074	-0.98
BD	-1.38	-2.89	-0.0051	-0.68
CD	0.86	1.80	-0.0073	-0.98

Proposed Model for Growth and Podophyllotoxin Production

The growth of *P. hexandrum* could be expressed in terms of the four selected variables (in actual factor level) by the following quadratic equation,

$$\text{Growth} = 100.24 - 4.66 \times A - 0.20 \times B - 19.05 \times C - 9.44 \times D + 0.035 \times A^2\ 0.0033 \times B^2 + 0.40 \times C^2 + 0.30 \times C^2 + 0.029 \times A \times B + 0.54 \times A \times C + 0.39 \times A \times D + 0.11 \times B \times C - 0.0055 \times B \times D + 1.37 \times C \times D$$

The production of podophyllotoxin could also be expressed in terms of the four selected variables (in actual factor level) by the followoing equation,

$$\text{Product} = -0.83 - 0.016 \times A + 0.0085 \times B + 0.20 \times C + 0.11 \times D - 0.0017 \times A^2 - 0.003 \times B^2 - 0.015 \times C^2 - 0.0016 \times D^2 + 0.005 \times A \times B + 0.0067 \times A \times C - 0.0025 \times A \times D - 0.0074 \times B \times C - 0.002 \times B \times D - 0.012 \times C \times D$$

The model parameters of the above model equations were identified with the help of thirty experimental responses with respect to growth and production as shown in Table 4 using Design-Expert computer program.

To determine the most adequate operating conditions and analyze the process of the growth and product formation, the response surfaces were studied in detail for all the possible contributions by keeping two parameters constant at a time. Simultaneous analysis of so many plots is complex task, the predicted responses of the above equations were therefore studied by a particular programming feature of the Design-Expert software, which allows to study the responses of above equations by independent variation of one parameter at a time. Also it has been practically observed that podophyllotoxin production was better at pH 6.0 and when the concentration of IAA was 1.25 mg/L. the same was also predicted by the above model equations. Therefore, pH and concentration of IAA were kept constant at 6.0 and 1.25 mg/L respectively and contour plots were made for growth (Fig. 2) and product formation (Fig. 3) to critically study the interactions between the glucose concentration and inoculum level. It was observed that glucose concentration of 72 g/L and inoculum level of 8 g/L produced best results in terms of growth of *P. hexandrum* and production of podophyllotoxin.

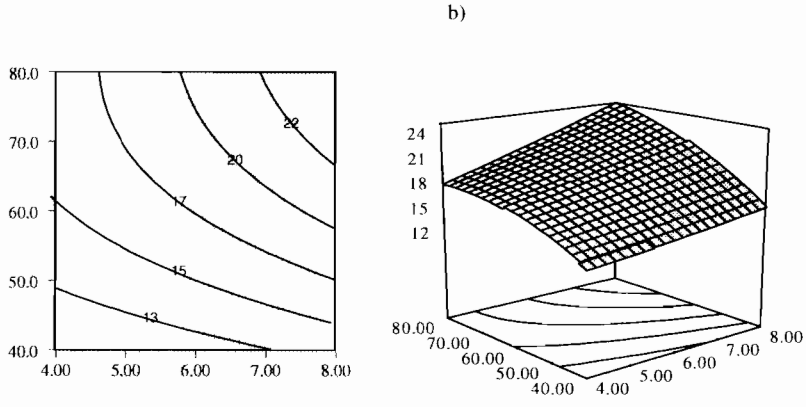


Figure 2 : Isoresponse contour of growth of *P. hexandrum* between inoculum level and glucose concentration (IAA: 1.25 mg/L, pH: 6.00). a) 2D ; b) 3D.

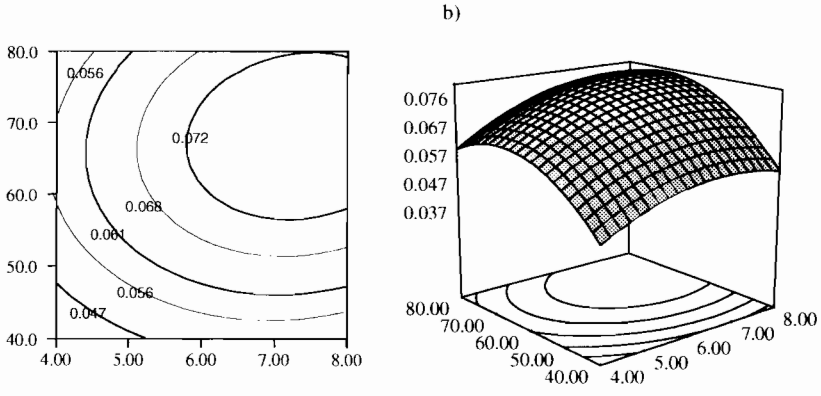


Figure 3 : Isoresponse contour of production of podophyllotoxin between inoculum level and glucose concentration (IAA: 1.25 mg/L, pH: 6.00). a) 2D ; b) 3D.

Using the above optimized values of pH (6.0), IAA (1.25 mg/L),Glucose (72 g/L), Inoculum level of 8 g/L along with the usual MS medium the suspension culture was grown in 3 Liter bioreactor. The kinetics of fermentation with respect to Dryweight (g/L), Podophyllotoxin (g/100 g DW) is shown in Figure 4. The trends with respect to Residual sugar and Dissolved oxygen are shown in Figure 4.

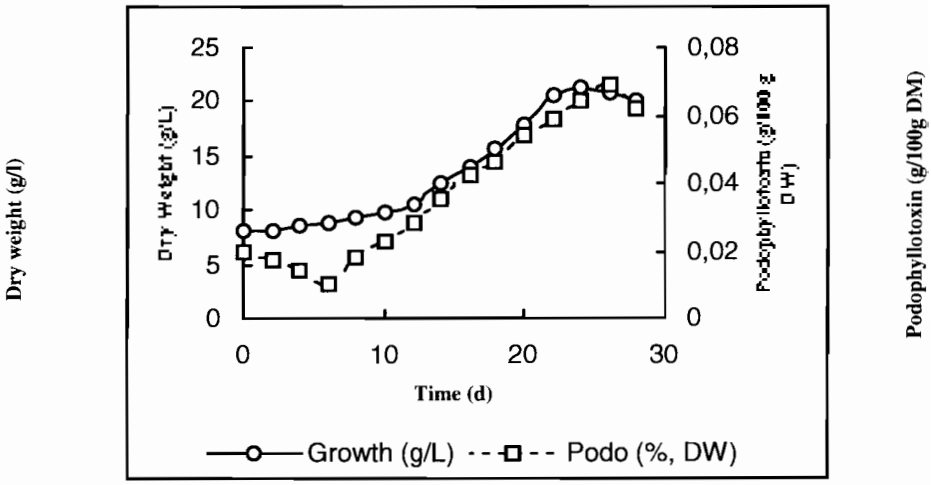


Figure 4 : Growth and product profile of *P. hexandrum* growth in RSM optimised medium bioreactor.

As expected from the CCD prediction (22.73 g/L) the biomass concentration was (21.4 g/L). Podophyllotoxin production was 0.069 g/ 100 g DW as compared to the predicted values of 0.075 g/100 g DW. The success of the fermentation was further demonstrated when it was possible to accumulate such a huge amount of biomass (20 g/L) in almost the same time as that with shake flask fermentation. This indirectly indicated overall high production of podophyllotoxin due to large amount of volume being processed in the fermenter. Another notable feature of the fermentation was the growth associated production of podophyllotoxin from day 6 till almost the end of fermentation 26 days. The peak biomass accumulation was observed on 22nd day and the peak in podophyllotoxin production was at 26th day indicating that some amount of product was produced even after termination of the growth. This established that product formation is a net effect of growth and nongrowth activities of the culture. The fermentation featured high residual accumulation of glucose (30 g/L) at the end of the fermentation (~ 20 days) indicating only around 45 g/L glucose was consumed in the fermentation. The cessation of the growth and no further uptake of the sugar was also reflected in the dissolved oxygen concentration after day 20. The total accumulation of the product at the end of fermentation was estimated to be 14.35 mg/L which resulted in a productivity of 0.55 mg/L/d.

Conclusion

Plant cell cultures require careful selection of the biological and technological parameters for optimal growth and production of the product. Callus culture was derived from the auxilliary bud of *H. antidysenterica* in MS medium supplemented

with 1 mg/L 2,4 D and 0.5mg/L kinetin and 30 g/L sucrose. After 4-5 subcultures friable callus was obtained which was used for development of suspension culture.

The *H. antidysenterica* cells grew well in the dark. The medium for growth was MS medium with 30 g/L glucose, 1 mg/L 2,4 D, 0.5 mg/L kinetin and 2 g/L inoculum (dry weight basis). The cell growth was complete in 10 days giving maximum alkaloid on 8th day. In callus culture maximum alkaloid was 300 mg/100 gm dry weight after 40 days and 130 mg/100 g dry weight in suspension culture after 8 days.

Optimal medium for maximum synthesis of alkaloids was NH_4^+:NO_3^- = 5:1 at total nitrogen of 60 mM, 0.25 mM KH_2PO_4, 0.5 mg/L 2,4 D, 1.0 mg/L kinetin, 40 g/L sucrose and 3 g/L of inoculum. Maximum cell mass of 15 g/L and maximum alkaloids of 100 mg/L were produced in 8 days in the optimal medium.

Chloesterol served as a precursor of alkaloid biosynthesis. Using optimized medium and optimal cholesterol feeding total alkaloid concentration of 143 mg/L was achieved on 8th day (7.3 times higher than that of original MS medium).

Large-scale production of alkaloids was done in the bioreactor using the optimal medium at 26 ^{0}C, pH 5.8 and agitation speed of 100 rpm, 0.5 v.v.m airflow rate. Maximum biomass of 12 g/L and maximum alkaloid of 64 mg/L were produced on 8th day.

With the optimal medium and similar conditions as above but with cholesterol feeding policy, it was possible to get the maximum alkaloid formation of 106 mg/L in 8 days with a cell mass of 12 g/L.

For *Podophyllum hexandrum* different media combinations were tested, 'B5 + Coconut milk (5%)' produced a maximum of 4 g/L dry weight (DW) of biomass with an inoculum level of 2 g/L on dry weight basis.

A few problems were associated with the growth of the cells in the suspension culture these were clumping of cells and browning of the culture media and drop in pH during culture growth. These problems were addressed with the inclusion of pectinase and PVP in the media.

Initial inoculum level, glucose concentration, initial pH & IAA were identified by Plackett Burmen design for determination of their exact optimum levels by using Central Composite Design protocol.

Optimal concentrations obtained from CCD were glucose concentration of 72 g/L, inoculum level of 8 g/L, pH 6.0 and IAA 1.25 mg/L in terms of growth of *P. hexandrum* and production of podophyllotoxin.

Using the optimal concentrations of different parameters in the bioreactor the total accumulation of the product at the end of fermentation was estimated to be 14.35 mg/L which resulted in a productivity of 0.55 mg/L/d.

References

1. Fowler M.W. (1983) *In*: Plant Biotechnology (Mentall, S.H. and Smith, H. (Eds.) Cambridge

University Press, Cambridge U.K. p 3.

2. Panda A.K., Mishra S., Bisaria V.S., Bhojwani S.S. (1989) *Enzyme Microbial Technol.*, **11**,386-397.
3. Dougall D.K. (1980) *In* : Plant Tissue Culture as a source of Biochemicals (Staba, E.J. Ed.) CRC Press, Boca Raton, Florida, p 21.
4. Fugita Y. Hara Y., Ogino T., Suga C. (1981) *Plant Cell Rep.* **1**, 61.
5. Mentall S.H., Smith H. (1983) *In*: Plant Biotechnology (Mentall. S.H. and Smith, H. Ed.) Cambridge University Press, Cambridge p 75.
6. Bisaria V.S., Panda A.K. (1991) *Current Opinion in Biotechnology*, **2**(3), 370-374.
7. Koul M.K., Atal C.K. (1983) *J. Enthopharmacol.*, **8**, 349.
8. Panda A.K (1990) Growth and Product formation in Plant cell culture of *Holarrhena anti dysenterica* Phd. Thesis, IIT Delhi.
9. Panda A.K., Bisaria V.S.,Mishra S., Bhojwani S.S. (1991) *Phytochemistry* **19**(3), 833-836.
10. Panda A.K., Mishra S., Bisaria V.S. (1992) *Biotechnol. Bioeng.* **39**, 1043-1051.
11. Panda A.K., Mishra S., Bisaria V.S. (1992) *Biotechnol. Bioeng.* **39**, 1052-1057.
12. Van Uden W., Pras N., Visser J.F., Malingre M. (1989) *Plant Cell Reports*, **8**: 165-168.
13. Heyenga A.G., Lucas J.A., Dewick P.M. (1990) *Plant Cell Reports*, **9**: 382-385
14. Van Uden W., Pras N., Malingre T.M. (1990) *Plant Cell, Tissue, Organ Culture* **23**: 217-224.
15. Woerdenbag H.J., van Uden W., Frijlink H.W., Lerk F.C., Pras N., Malingre T.M. (1990) *Plant Cell Reports* **9:** 97-100.
16. Murashige T., Skoog F. (1962) *Physiol. Plant.*, **15**: 473-497.
17. Gamborg O.L., Miller R.A., Ojima V. (1968) *Exp. Cell Res.*, **50**: 151-158.
18. Plackett R.L., Burman J.P. (1946) *Biometrika*, **33**: 305-325
19. Deming S.N. (1990) *Chemtech*, **20** (2), 118-126.
20. Scragg A.H., Allan E.J., Morris P. (1988) *J. Plant. Physiol.*, **132**: 184-189.

Chapter 17

GENETIC MANIPULATION OF FILAMENTOUS FUNGI

A.C. BANERJEE, A. KUNDU, S.K. GHOSH

Biotechnology & Molecular Biology Division; East India Pharmaceutical Works Limited, 119, Biren Roy Road (West), Calcutta 700061, India. Email : eisf@vsnl.com

Abstract

Biotechnology, a multidisciplinary science, has many applications. A major application in chemical and pharmaceutical industries involves the use of enzymes, mostly from microbial sources, in the production process of many useful products. Various compounds including many proteins and enzymes are produced through microbial fermentation. Filamentous fungi, with their many virtues and long history of industrial use, have lately become targets for gene manipulation for producing strains with improved yield and better expression-secretion system for homologous and heterologous eukaryotic gene products. To genetically modify filamentous fungi for enhanced expression of a desired gene and secretion of its product, it requires a suitably designed gene-construct with correctly chosen and aligned sequences of the gene of interest and its controlling elements, as well as an efficient gene-transfer system for transforming the host fungus. An important feature of the transformation of filamentous fungi is that the transforming DNA stably integrates into the host genome. Substantial research for understanding the molecular aspects of filamentous fungal expression systems is necessary for its biotechnological application in an industrial context.

Introduction

Filamentous fungi belong to a diverse group of lower eukaryotic microorganisms and some of those are used in a variety of industrial processes including the production of fermented foods, primary and secondary metabolites (e.g., organic acids, antibiotics, alkaloids, gibberellins) and a broad range of extracellular enzymes viz., amylase, protease, cellulase, xylanase pectinase, lipase, etc. These microbial fermentation processes are relatively inexpensive when compared to production with higher eukaryotic cell cultures. Filamentous fungi of industrial interest include *Aspergillus* species such as *A. awamori*, *A. niger*, *A. oryzae* and *A. nidulans*, *Mucor* species (e.g. *M. miehie*) and *Trichoderma* species (e.g. *T. reesei*). Some fungi, for instance, *A. niger* and *A. oryzae* have been used for many years for the production of enzymes in the food processing industry and have the GRAS (generally regarded as safe) status (**1**).

New Horizons in Biotechnology, 193-198, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

In view of the economic importance and the ecofriendly nature of these fungal enzymes, demands have been felt not only for better yields by improving the enzyme production process, but also for enhancing the value added quality of the product as well as its yield by genetic modification of the producer fungi. Filamentous fungi, with their many virtues and long history of industrial use, have lately become targets for directed gene manipulation for developing these fungi as host organisms for the production of foreign (heterologous) protein as well (**2-5**). We shall review here some characteristics of fungal systems and their genetic manipulation for expression of homologous and heterologous proteins.

For a number of reasons filamentous fungi appear suitable for heterologous protein production. Probably the most attractive feature is their excellent natural capacity of secretion. Many fungal species secrete large amounts of various proteases in culture media. Many aspergillus fungi while growing in starch, secrete mixtures of enzymes for hydrolysis of starch finally to glucose; these enzymes (glucoamylase, alpha- and beta-amylases and amyloglucosidase) are upregulated by starch and down regulated by glucose (**6**). Cellobiohydrolase, endoglucanase and β-glucosidase, which are expressed in *Trichoderma reesei* for completely degrading cellulose to glucose, are induced in the presence of cellulose, but are repressed by glucose (**7**).

Most of the secreted enzymes from fungi are inducible. Those are expressed under the control of promoter and undergo post-translational modifications before secretion. Expression systems which secrete the recombinant protein product extracellularly, are generally preferred to those accumulating intracellularly; this is important not only for easier downstream processing of the product, but also because many of the post-translational modifications occur during the secretion process in eukaryotes. Many enzymes and pharmaceutically important proteins like human cytokines and hormones are secreted; their post-translational processing events, viz., glycosylation, phosphorylation, proteolytic processing or disulfide formation are often essential for the stability and most importantly for the biological activity of these products. To utilize filamentous fungus for producing heterologous or homologous gene products in desired amounts requires suitable modification of the expression-secretion system by genetic manipulation. Traditional strain improvement approaches (**8**) and the recombinant DNA technology (**1**) are two complementary strategies that are exploited to improve the yield of secreted proteins under bioreactor conditions, for the purpose of commercial production.

Expression-secretion system

In developing a suitable expression-secretion system for producing a protein in a homologous or heterologous host, two basic components are important: one is the gene of interest, properly placed in a DNA vector or 'expression cassette' containing all the necessary information for its transcription, translation and secretion; the other is an appropriate host strain for optimal production of the desired protein (**9**). Expression cassettes designed for the efficient production of

extracellular proteins generally include the following sequences: (a) a region comprising all 5' transcription control sequences; (b) a translation initiation region; (c) secretion-facilitating (signal) sequences; (d) DNA sequences encoding the protein of interest; and (e) a transcription termination and polyadenylation control region. The expression system is obtained by inserting the expression cassette into the host cell by a gene-transfer system (involving protoplasting or electroporation), developed for transformation of various biotechnologically important filamentous fungi (**10, 11**). Recently, an alternative transformation procedure for these fungi has been developed based on *Agrobacterium tumefaciens* mediated transfer (**12**). This approach enabled the introduction of fungal sequences into the genome of *A. awamori* at a predetermined locus without concomitant introduction of non-fungal sequences (**13**).

For heterologous gene expression, it is essential that the transcription control sequences are functional in the host organism, and as such, these sequences should be from either the host organism itself, or, a related species. Two types of transcription control sequences may be used: i) Control sequences from fungal housekeeping genes, which are efficiently and constitutively expressed. ii) Control sequences from genes that are subject to specific metabolic regulation (inducible or de-repressible) and are expressed efficiently only under specific growth conditions, through regulation at the level of transcription. The latter type, which offers the possibility of inducing protein synthesis at a specific stage during fermentation, can be advantageous, when the desired protein product happens to be unstable or detrimental to the host (**2,4,5,14**). Exploiting specific fungal transcriptional control sequences in heterologous gene expression, nevertheless, requires a detailed knowledge of the organization and regulation of these sequences.

Most protocols for selecting and analysing genes of interest start by first isolating the DNA segment from the genomic DNA, through the use of a variety of restriction enzymes of different specificities, followed by gel electrophoretic separation. Hybridization with specific nucleic acid probe allows the detection of the fragment(s) containing the desired gene, which is subsequently put into a suitable plasmid vector at chosen restriction sites and cloned in *E. coli.* This makes it easier to determine the nucleic acid sequence of each fragment and thus the whole gene. For eukaryotic DNA, which has also noncoding sequences or introns that bacteria can not recognize and hence cannot express, the usual method is to isolate the mRNA of the protein, then use reverse transcriptase to make complementary DNA (cDNA) copies of the mRNA and subsequently clone the cDNA, which represents the coding region of the protein of interest.

Fungal expression vectors are generally constructed in *E. coli* for convenience; plasmids and markers are employed for selection in the host strains. For the purpose of selection, nutritional marker genes and dominant selection system are preferred (e.g., fungal acetamidase gene, fungal benomyl resistance gene and bacterial hygromycin resistant gene), since they require no specific mutant genotype in the recipient (**10, 15**). Transformants can also be obtained by co-transformation when the selectable marker and the gene of interest are on separate plasmids. Since co-

transformation occurs quite efficiently (50-80% using most selection markers) without the necessity for often time-consuming vector construction, it represents a convenient method for the introduction of multiple copies of a given expression cassette (**5, 16**).

An important feature of the transformation of filamentous fungi is that the transforming DNA integrates into the host genome. Such integration occurs by either homologous or nonhomologous integration events. Also, the number of integrated copies varies considerably and depends on the host strain as well as the method of selection (**17**). Often the integration occurs at random sites. However, in a few systems, predominantly multicopy transformants carrying tandemly repeated copies of the vector are obtained. Multicopy transformants can also be obtained by co-transformation. Although by common belief, apparently a higher copy number of an expression-cassette should result in a higher protein production, interestingly, however, a comparative analysis of fungal transformation showed that in most cases there was no strict correlation between the two. A more or less linear dose/response relation was observed with strains with a relatively small number of gene copies (< 5) but not with higher copy numbers (**18**). In general, with higher copy numbers, the production level is often lower than what is expected from the copy number. Also, transformants with a comparable number of gene copies show a considerable variation in the production level. These are probably due to different sites of integration, which may also affect the expression of the genes introduced (**19**). The multiple gene copies may as well cause the transcription factors become limiting. However, a study on the production of a heterologous protein thaumatin (a sweet protein) in *A. awamori* reported a good correlation between the copy number (ranging from 5 to 14) of the expression cassette and the extracellular production of thaumatin (**20**).

Host factors

Besides an optimal design of the expression vector and an optimally high copy number of the expression cassette in the transformants, host characteristics too, may be important for optimal gene expression and protein secretion. Genetic modification of the host can lead to further improvements in production yields. One approach for strain improvement involves random mutagenesis and screening. The yield of bovine chymosin, produced by *A. awamori*, was optimized through several rounds of mutagenesis and screening and thus, a classical strain-improvement strategy finally brought the production yield to an economically attractive level (**21**). Another approach is to directly eliminate factors and circumstances responsible for limiting protein production. Efficient production of heterologous proteins in many filamentous fungi may be hampered due to proteases, whether intracellular, or when secreted extracellularly (**22**). Efforts to overcome such problem of proteolysis of the desired product have been directed towards making protease-deficient host strains (**23,24**). A study on the molecular basis of glucoamylase overproduction by an industrially used mutagenized strain of *A. niger*

(**25**) has concluded that *A. niger* strain improvement by mutagenesis and screening for glucoamylase overproduction led to a multiplication of the glucoamylase encoding gene (*glaA*) and an expression defect in the gene encoding aspergillopepsin (*pepA*), an extracellular protease. In another study, antisense expression of a portion of the gene encoding the major carbon catabolite repressor CREA in *A. nidulans* (**26**) resulted in a substantial increase in the levels of glucose-repressible enzymes, both endogenous and heterologous. However, proprietary regulations often preclude the reporting of results from further optimized production processes.

Conclusion

Genetic manipulation of filamentous fungi has a major prospect in the production of eukaryotic proteins of choice for enzymatic, food or pharmaceutical use. Successful development of improved strains requires knowledge of physiology, metabolic control and selective screening procedure. Nonetheless, each new strain may require optimization of the fermentation process with expertise of engineering know-how, media optimization and fine-tuning of process control (**27**). Regulatory issues, however, play an important role in determining the methodology employed in genetic manipulation, and large-scale production process involving genetically modified organism. Authentic glycosylation of secreted proteins is also important for the stability as well as the biological activity of pharmacologically active proteins, and is probably a prerequisite for obtaining the permission for their commercial production.

To successfully create and exploit genetically modified filamentous fungi for producing commercially viable quantities of eukaryotic proteins (whether homologous, or, heterologous in origin), a better understanding of the fungal system is essential. Substantial effort in fundamental research is necessary, particularly for knowledge about the molecular details of transcription control, mRNA stability and processing, translation efficiency, secretion and glycosylation pathways, proteolysis, and also the structural elements of fungal proteins that allow them to be secreted more efficiently. Therefore, in an industrial context, the long-term future of filamentous fungal expression systems depends upon a concerted molecular analysis of those areas.

References

1. Leong S.A., Berka R.M. eds (1991) Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi. Dekker, New York.
2. Cullen D., Gray G.L., Wilson L.J., Hayenga K.J., Lamsa M.H, Rey M.W., Norton S., Berka R.M. (1987) *BioTechnology*, **5**, 369-376.
3. Gwynne D.I., Buxton F.P., Williams S.A., Garven S., Davies R.W. (1987) *BioTechnology*, **5**, 713-719.
4. Upshall A., Kumar A.A., Bailey M.C., Parker M.D., Favreau M.A., Lewison K.P.L., Joseph M.L., Maraganore J.M., McKnight G.L. (1987) *BioTechnology*, **5**, 1301-1304.

5. Christensen T., Woldike H., Boel E., Mortensen S.B., Hjorshoej K., Thim L., Hansen M.T. (1988) *BioTechnology*, **6**, 1419-1422.
6. Sakaguchi K., Takagi M., Horiuchi H., Gomi K. (1992) *in* Applied Molecular Genetics of Filamentous Fungi, Kinghorn J.R. and Turner G., eds., Blackie Academic and Professional, Glasgow pp. 54-99.
7. Seiboth B., Hakola S., Mach R. L., Suominen P. L., Kubicek C.P. (1997) *J. Bacteriol.*, **179**, 5318-5320.
8. Rowlands R.T. (1992) *in*: Biotechnology of Filamentous fungi, Finkelstein D.B. and Ball C., eds., Butterworth - Heinemann, Boston, pp. 41-64..
9. Van den Hondel C.A.M.J.J., Punt P.J., van Gorcom R.F.M. (1991) *in* More genetic Manipulation of Filamentous Fungi, Bennett I.W. and Lasure L.L. eds., Academic Press, San Diego, pp. 396-428.
10. Fincham J.R. (1989) *Microbiol. Rev.*, **53**, 148-170.
11. Timberlake W.E., Marshall M.A. (1989) *Science*, **244**, 1313-1317.
12. Groot M.J.A. de, Bundock P., Hooykaas P.J.J., Beijersbergen A.G.M. (1998) *Nat. Biotechnol.* **16**, 839-842.
13. Gouka R.J., Gerk C., Hooykaas P.J.J., Bundock P., Musters W., Verrips C.T., de Groot M.J.A. (1999) *Nat. Biotechnol.* **17**, 598-601.
14. Cantwell C.A., Beckmanan R.J., Dotzlaf J.E., Fisher D.L., Skatrud P,L. Yeh W.K., Queener S.W. (1990) *Curr. Genet.* **17**, 213-221.
15. Punt J.P., Van Den Hondel C.A.M.J.J. (1992) *Methods in Enzymol.*, **216**, 447-457.
16. Hynes M.J., Kelly J.M. (1985) *EMBO J.*, **4**, 475-479.
17. Mainwaring D.O., Wiebe M.G., Robson G.D., Goldrick M., Jeenes D.J., Archer D.B., Trinci A.P.J. (1999) *J. Biotechnol.*, **75**, 1-10.
18. Verdoes J.C., Punt P.J., van den Hondel C.A.M.J.J. (1993) *Appl. Microbiol. Biotechnol.*, **43**, 195-205.
19. Verdoes J.C., Punt P.J., Stouthamer A.H., van den Hondel C.A.M.J.J. (1994) *Gene*, **145**, 179-187.
20. Moralejo F.J., Cardoza R.E., Gutierrez S., Martin J.F. (1999) *Appl. Environ. Microbiol.*, **65**, 1168-1174.
21. Ward M., Wilson L.J., Kodama K.H., Rey M.W., Berka R.M. (1990), *BioTechnology*, **8**, 435-440.
22. Asgeirsdottir S.A., Scholtmeijer K., Wessels J.G.H. (1999) *Appl. Environ. Microbiol.*, **65**, 2250-2252.
23. Van den Homberg J.P.T.W., van de Vondervoort P.J.I., van der Heijden N.C.B.A., Visser J. (1995) *Curr. Genet.*, **28**, 299-308.
24. Van den Hombergh J.P.T.W., Vondervoort P.J.I., Fraissinet Tachet L., Visser J. (1997) *Trends Biotechnol.*, **15**, 256-263.
25. MacKenzie D. A., Jeenes D.J., Gou X., Archer D.B. (2000) *Enz. Microbial. Technol.*, **26**, 193-200.
26. Bautista L. F., Aleksenko A., Hentzer M., Santerre-Henriksen A., Nielsen J. (2000) *Appl. Environ. Microbiol.*, **66**, 4579-4581.
27. Parekh S., Vinci V. A., Strobel R. J. (2000) *Appl. Microbiol. Biotechnol.*, **54**, 287-301.

Chapter 18

MOLECULAR BIOLOGY AND BIOCHEMISTRY OF LIPOXYGENASES AND RELATED PATHWAYS

K. YOKOTA*, S. LU, I. TAKATA, A. KISHIMOTO, K. MAETA, K. NISHIMURA, T. NAGAYA, M. JISAKA

Department of Life Science and Biotechnology, Shimane University, Nishikawatsu-cho, Matsue, Shimane 690-8504, Japan; yokotaka@life.shimane-u.ac.jp

Abstract

Lipoxygenases (LOXs), a family of dioxygenases, with distinct regio-specificities have been found in both plants and mammals. The substrates for LOXs are linoleic and α-linolenic acids from plants and arachidonic acid from mammals. The LOX pathway in animals produces bioactive lipids called eicosanoids to serve as autacoids, while plants also have unique LOX pathways to form signaling molecules termed octadecanoids. The studies with gene cloning have revealed the existence of several types of isoforms that exhibit a similar reaction with distinct expression or reaction properties, postulating specific roles of individual enzymes. As one of our studies, results are presented about the cloning, expression, and characterization of cDNA encoding an LOX isoform from potato tubers. We also provide the facets of the metabolic pathways of fatty acid hydroperoxide in potato tissues. Alternatively, we provide the approaches to identify the amino acid residues to determine the positional specificity of mouse 8-LOX that is a novel LOX inducible by phorbol diesters and its newly found human homologue, 15-LOX-2. Recently, some of LOX products have been shown to cause nuclear action as a ligand through binding to nuclear receptors. This presentation also reviews the biological significance of certain LOXs in relation to the onset of life-style diseases such as obesity.

Key words: lipoxygenase, cyclooxygenase, eicosanoid, prostaglandin, hydroperoxyeicosatetraenoic acid, hydroxyeicosatetraenoic acid, nuclear factor, peroxisome proliferator-activated receptor, arachidonic acid, linoleic acid.

Abbreviations used are: COX: cyclooxygenase; LOX: lipoxygenase; HPETE: hydroperoxyeicosatetraenoic acid; HETE: hydroxyeicosatetraenoic acid; PPAR: peroxisome proliferator-activated receptor; PG: prostaglandin ; TX: thromboxane ; LT: leukotriene.

New Horizons in Biotechnology, 199-214, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

LOXs are a family of non-heme ion-containing dioxygenases that introduce molecular oxygen stereospecifically with own positional specificities into polyunsaturated fatty acids containing 1,4-*cis,cis*-pentadiene moieties. Until now, these enzymes are found widely throughout plants, fungi, and animals including slime molds, marine alga, and coral, not in bacteria and yeast which have no polyunsaturated fatty acids in membrane lipids. Historically, soybean flour containing LOXs has been used as a commercial purpose for bleaching carotenoids by its oxidation and modifying the rheological properties in dough in the baking industry. Thus, the presence of LOXs in foodstuffs can affect their attributes including taste, flavours, aromas, functionality, texture, color, and nutritional values. The pathways involving LOXs generate a variety of oxygenated fatty acids which serve as bioactive lipid mediators such as prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and lipoxins in mammals or plant signaling or regulatory molecules like jasmonic acid and traumatin. Alternatively, peroxidation reactions in biological membranes cause structural changes in red cell maturation or plant senescence. Furthermore, the oxygenation of polyunsaturated fatty acids esterified in triacylglycerols catalyzed by some plant LOX results in the mobilization of storage lipids for the use of germination process as fuel in oil seeds. Recent progress of LOXs has been reviewed (**1**).

Plant LOX Pathways

Although the physiological function of plant LOX has not been understood with certainty, one of functions of plant LOX pathways has been recently regarded as the formation of regulatory molecules in plant cells and organs. The roles include different aspects of plant growth, development, defense against pathogens, and response to wounding (**2,3**). The regiospecificity of LOXs varies from source to source. Some plant species from soybean, flaxseed, and tea leaves have been shown to have LOXs that catalyze the insertion of molecular oxygen at the C-13 position of linoleic acid or α-linolenic acid, the most abundant polyunsaturated fatty acid from plant membrane lipids. Other sources including potato, tomato, and corn have different types of enzymes which catalyze the formation of 9-hydroperoxides of the above unsaturated fatty acids. In addition, various isoforms are known to exist in certain plant species. The specific function of each isoform waits further molecular and cellular characterization.

In plant leaves, the LOX pathway recently called octadecanoid pathway to form 13-hydroperoxide of α-linolenic acid is present, after which the product is converted to either traumatin by the action of hydroperoxide lyase or jasmonic acid by allene oxide synthase (Fig. 1). As plant regulators, taumatin is involved in wound healing, whereas jasmonic acid serves as phytohormone-like activities to promote senescence and inhibition of cell growth (**3,4**). In contrast, the function of 9-LOX on plant unsaturated fatty acids remains still unclear. Studies on the understanding of plant lipoxygnase pathways have advanced considerably by using

recent biochemical, molecular, and genetic technologies (**5**). To get further insight into the molecular mechanism of the action of the LOX products, specific receptors and signal transduction mechanisms should be clarified.

Potato LOX isoforms

Higher levels of LOX activity have been reported in potato tubers (**6**). In contrast to soybean LOXs, the enzymes from potato tubers are characterized in the dioxygenation of linoleic acid and α-linolenic acid to 9-hydroperoxides. The role of this type of LOX is poorly understood. Potato tubers have been shown to contain at least three types of isoforms on the basis of the elution profile of crude enzyme preparations by anion-exchange column chromatography (**7,8**). However, it is unclear whether the separation of enzyme activities reflects the existence of distinct isoforms or the partial proteolytic degradation of some enzyme species. The LOX pathway in potato tubers is unique because this pathway leads to the formation of colneleic acid which is a characteristic divinyl ether derivative (Fig. 2) (**9**). This reaction product is only identified in potato tubers. Divinyl ethers of fatty acids were relatively unstable and subject to the conversion of aldehydes. In addition, the enzymatic action of hydroperoxide lyase on 9-hydroperoxides has been known to form aldehydes and oxoacids with 9-carbon moieties, which would contribute to odors and flavors (**4**).

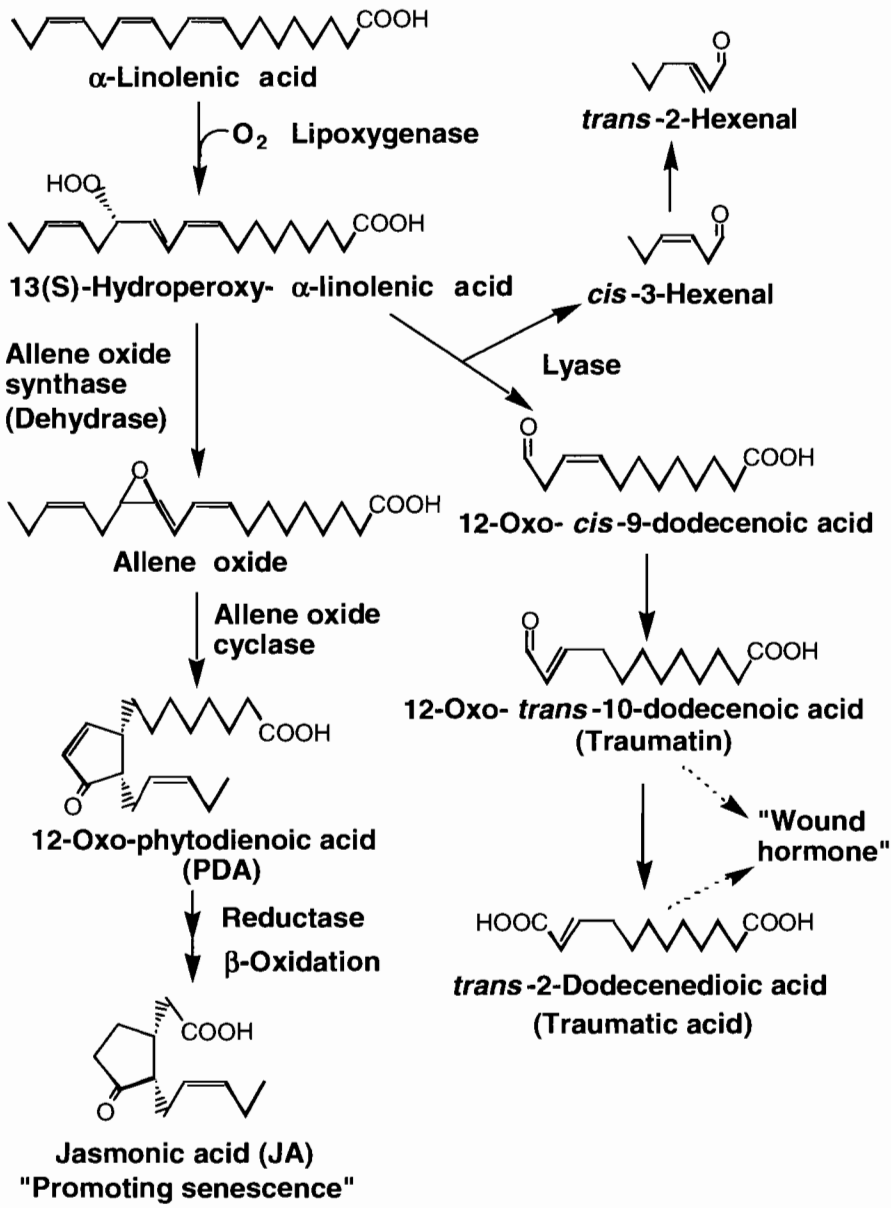


Figure 1. Plant lipoxygenase pathway to form jasmonic acid and traumatin from α-linolenic acid.

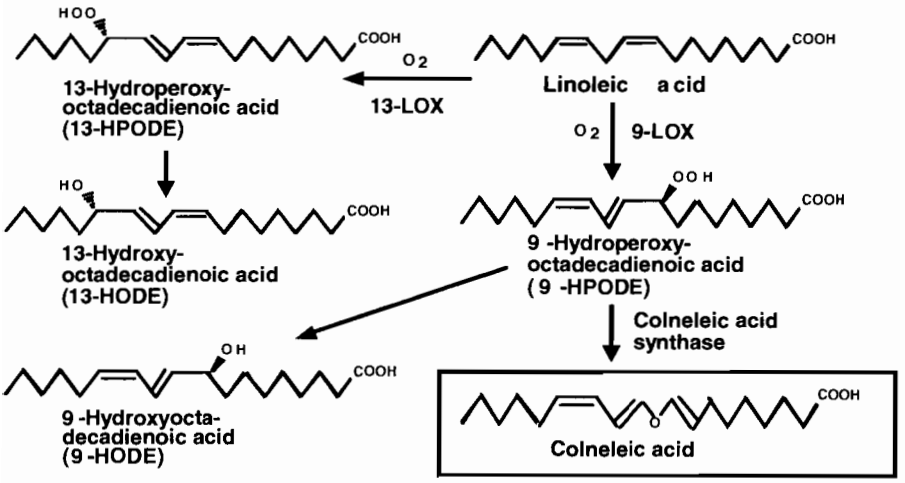


Figure 2. Potato tuber lipoxygenase pathway to form colneleic acid, a divinyl ether derivative.

Potato tuber LOXs have been reported to act on arachidonic acid, which is not found in plant cell membranes but is a component of mammalian cell membranes, to catalyze the formation of 5-hydroperoxide as well as the subsequent conversion to LTA_4, which is an intermediate for LTB_4 and related compounds (**10**). Thus, those enzymes have been used as a useful model enzyme of related mammalian 5-LOX (**7,8,11**). Our laboratory has been trying to identify a further novel cDNA clone encoding one of isoforms from potato tubers and to extend to the study on the determination of its structural basis of the specific reactivity.

To isolate cDNA clones from potato (*Solanum tuberosum* L. cv. Danshaku) tubers, we constructed a cDNA library by using the Uni-Zap XR vector after synthesis of cDNA from mRNA. On the other hand, cDNA fragments encoding potato tuber LOXs were amplified by the use of reverse-transcriptase polymerase chain reaction, namely RT-PCR with specific primers for the conserved regions between other plant LOXs.

The primers used are 5'-TGGATGAC(TC)GATGAAGAATTT-3' as an upstream primer and 5'-ATA(CT)TG(TA)CCAAA(AG)TTAAC(AG)GCTGC(AG)TG-3' as a downstream primer. The amplified cDNA product was radiolabeled with ^{32}P and used as a probe for screening positive phage clones. Finally, after getting the cDNA clones in plasmids, their DNA sequences were determined by automated sequencing on an Applied Biosystems 377 Genetic Analyzer following the reaction of fluorescence-tagged dye terminator cycle sequencing (Perkin-Elmer).

As shown in Fig. 3, we were able to determine the nucleotide sequence of a full-length cDNA clone covering the proposed initiation site to termination site. The deduced open reading frame consisted of 864 amino acids with a molecular weight of 97.4 kDa and an isoelectric point of 5.5. The resulting amino acid sequence predicted by our cDNA clone was compared with others. We found conserved residues, namely three histidine, one asparagine, and isoleucine at the carboxyl terminus presumably as ligands for iron atom. Our clone is almost similar to the potato T8 clone as reported before (**12**). We observed higher homology with *Arabidopsis* LOX-1 and one of the tomato isoforms. As proposed elsewhere, the region is involved in the specificity of the reaction. But, there is no homology between mammalian 5-LOX and potato 5-LOX in spite of the similarity of regiospecificity.

To express the cDNA sequence for a potato tuber LOX, we attempted to construct a recombinant expression vector in *E. coli*. Since the in-frame stop codon was found before the initiation codon, we amplified the equivalent region without in-frame stop codon. After ligation of two fragments, total 2.6-kb fragment could be cloned into the expression vector pET-28C in *E. coli*. The resulting clone should encode a fusion protein consisting of total 892 amino acids with a molecular weight of 100.4 kDa. This allowed us to express a fusion protein with His-tag and T7-Tag, which was useful to purify or detect the enzyme easily. We tried to express the fusion protein by using the construct at 17°C or 37°C.

As predicted, the IPTG-induced expression at 37°C led to the appearance of a dense band of about 100 kDa on a SDS-PAGE gel. However, to obtain the higher enzyme activity, the incubation at 17°C for 16 h was more favorable. Our laboratory had already obtained monoclonal antibodies against the 90-kDa protein corresponding to the potato LOX. As shown here, Western blot analysis revealed that the expressed proteins were only immunostained in the extract from *E. coli* harboring recombinant expression vector containing a cDNA insert. These observations support the idea that the expressed protein corresponded to the desired potato tuber LOX.

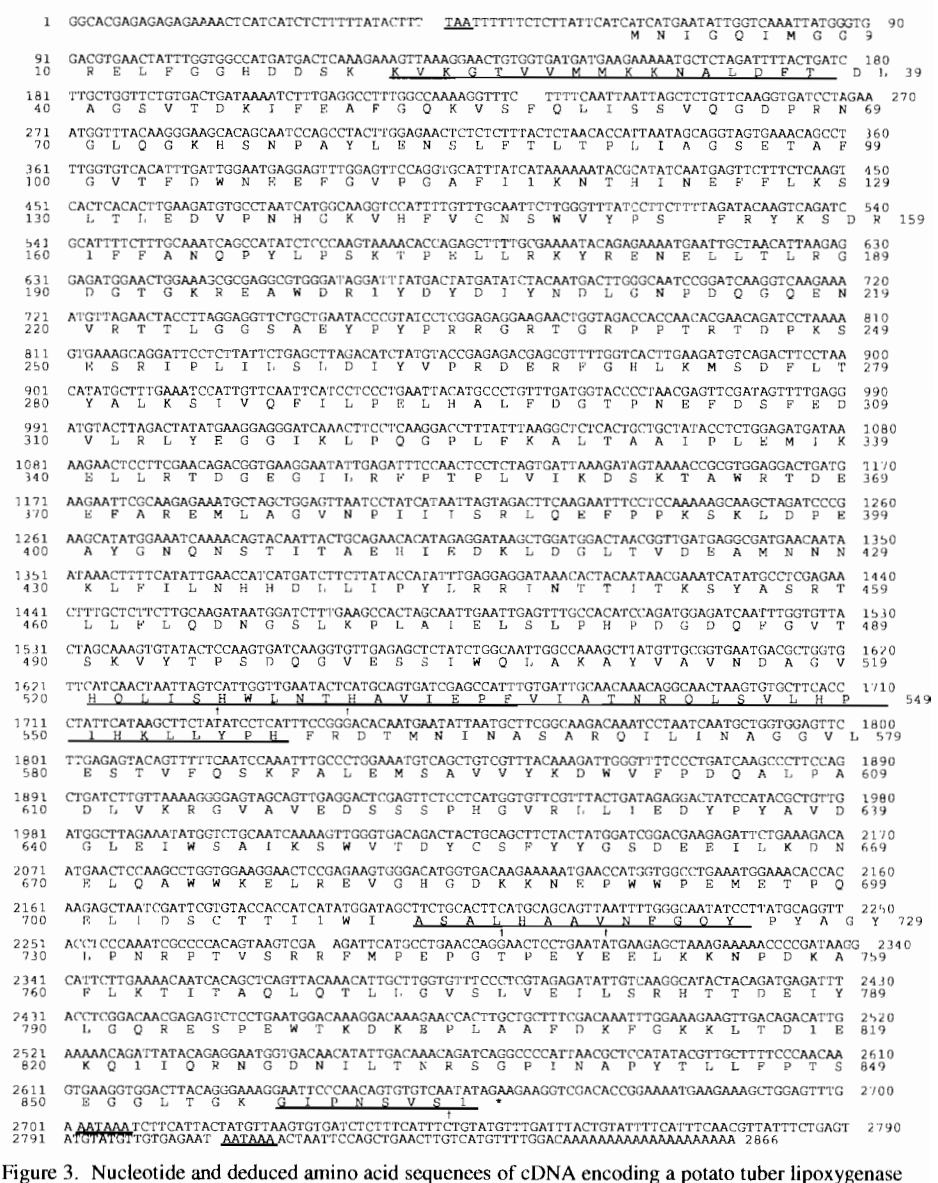


Figure 3. Nucleotide and deduced amino acid sequences of cDNA encoding a potato tuber lipoxygenase isoform. The highly conserved regions of amino acid sequences were underlined. The symbol, Å1, indicates the residues that would serve as iron ligands. In-frame stop codon, TAA, and potential polyadenylation signals were also underlined at 5'- and 3'-untranslated regions, respectively.

The enzyme activity was determined in crude extract of bacteria expressing the fusion protein by measuring the increase in the absorbance at 234 nm with linoleic acid as a substrate. When the pH optimum was compared between the recombinant enzyme expressed in bacteria and 30-50% saturated ammonium sulfate-fractionated

preparation from potato tubers, both enzymes exhibited nearly the same pH profiles at maximum of pH 6. Recombinant enzyme showed lower activity at more acidic or alkaline region than potato-derived crude enzymes. The reaction products from linoleic acid were analyzed by reverse-phase HPLC on an octadecylsilyl silica column. As presented in Fig. 4, the recombinant enzyme expressed in bacteria formed 9-hydroperoxide as a predominant product.

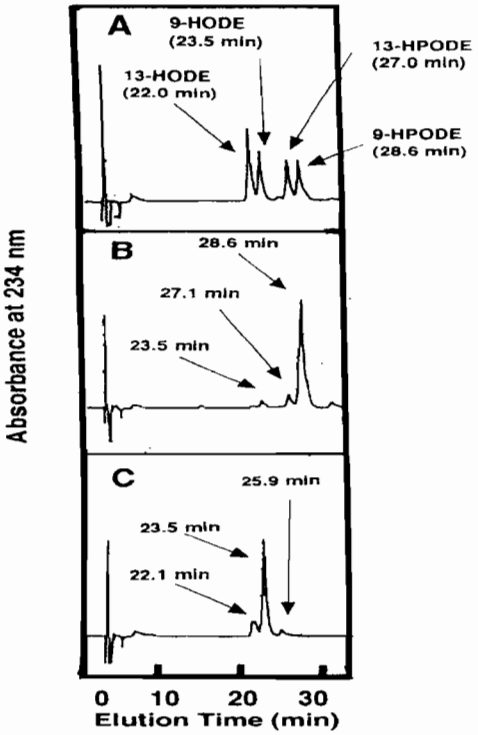


Figure 4. Reverse-phase HPLC of lipoxygenase products from linoleic acid. (A) Mixture of 9- or 13-H(P)DE. (B) Reaction products from linoleic acid by a recombinant enzyme expressed in *E. coli*. (C) $NaBH_4$-reduced reaction products of (B). HPLC was done on a L-ODS column (Kagakuhin Kensakyokai, Tokyo) with a solvent of tetrahydrofuran/acetonitrile/ water/acetic acid (200:360:440:0.5, v/v) and a flow rate of 1 ml/min.

Attempts were made to compare the substrate specificity of both enzyme sources for free polyunsaturated fatty acids (Fig. 5). The most reactive substrate was found to be linoleic acid followed by α–linolenic acid and arachidonic acid. Some differences were found between both enzyme sources in the reactivity with α–linolenic acid and arachidonic acid. The recombinant enzyme showed lower reactivity with them than the prepared enzymes from tubers. This finding suggested that potato tubers might include separate isoforms reacting more efficiently with those fatty acids. Another possibility is the presence of some activators involved in

the above reactions. Since potato tubers include galactolipids and phosholipids in which the predominant esterified fatty acid species is linoleic acid, the higher reactivity with linoleic acid rather than α–linoleic acid would be reasonable. The activation mechanism in potato tuber tissues remains to be clarified.

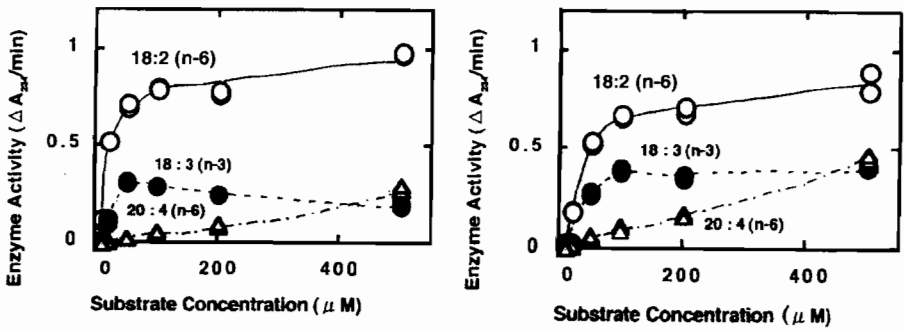


Figure 5. Substrate specificity of enzymes with polyunsaturated fatty acids. (A) Recombinant enzyme expressed in *E. coli*. (B) 30-50% saturated ammonium sulfate-precipitated enzyme preparation from potato tubers.

In the present study, a cDNA clone encoding potato tuber LOX was isolated from cDNA library. The complete sequence analysis and the expression in bacteria were successfully done. We were able to characterize the reaction features of the expressed enzyme which is one of the isoforms of potato tuber LOX. Further screening with the present cDNA clone as a probe might find additional novel isoforms in potato tubers. More recent work by Royo *et al.* (**12**) have reported the isolation of three different types of potato LOX cDNA clones including *lox1* expressed mostly in tubers and roots, *lox2* in leaves, and *lox3* in leaves and roots. The alignment of deduced amino acid sequences showed the highest homology between our result and their *lox1*. However, the sequence is not completely the same. This might account for the difference in varieties of tubers. Geerts *et al.* (**13**) in 1994 have isolated partial cDNA clones from potato, and revealed the stimulation of the expression in stored potato discs in response to methyl jasmonate. Jasmonic acid is synthesized from 13-hydroperoxide of α-linolenic acid by one of the isoforms found in leaves through the octadecanoid pathway involving allene oxide synthase. This indicates the induction of potato tuber LOX by a product of other type of LOX in leaves. The mechanism of the activation will be clarified by analyzing 5' flanking sequences of genomic sequences for the corresponding LOXs.

A more recent study has reported that potato stolons have new cyclopentenone compounds, derivatives of jasmonic acid precursor, indicating the presence of 9-LOX activity in potato stolons and roots, and the new roles of the LOX pathway in the potato plant (**14**). While arachidonic acid is not found in higher plants, this polyunsaturated fatty acid is present in the lipids of some plant-pathogenic fungi.

Arachidonic acid has been shown to be a potent elicitor of sesquiterpenoid phytoalexins in potato (**15**), whose action was thought to require the activation of 5-LOX activity to act on arachidonic acid in potato tubers (**16**). The regulatory mechanism by which a class of oxygenated fatty acids mediate the response to wound or pathogens remains to be determined.

Mammalian LOX Pathways

After the discovery of soybean LOX, the mammalian LOXs have been found and characterized within about twenty years. Mammalian membrane lipids provide arachidonic acid as a substrate to form a variety of oxygenated fatty acids which act as local mediators or autacoids because of being relatively unstable. One of the pathways is the cyclooxygenase (COX) pathway (Fig. 6) in which PG endoperoxide synthase catalyzes the rate-limiting step to produce PGH_2 through PGG_2, which are intermediates for the formation of PGs and TXs by specific synthases (**17**). Aspirin as well as other nonsteroidal anti-inflammatory drugs are well-known inhibitors of COX. Recent studies have revealed the presence of two isoforms of COX, one of which is called COX-1 and constitutively expressed in seminal vesicles and platelets. Other type COX-2 is inducible in response to inflammatory agents and mitogenesis. Since the X-ray structures of both COX isoforms have been determined, the studies on the relationship between structure and function would be promoted, and new drugs to specifically inhibit either COX isoform are being designed (**18**).

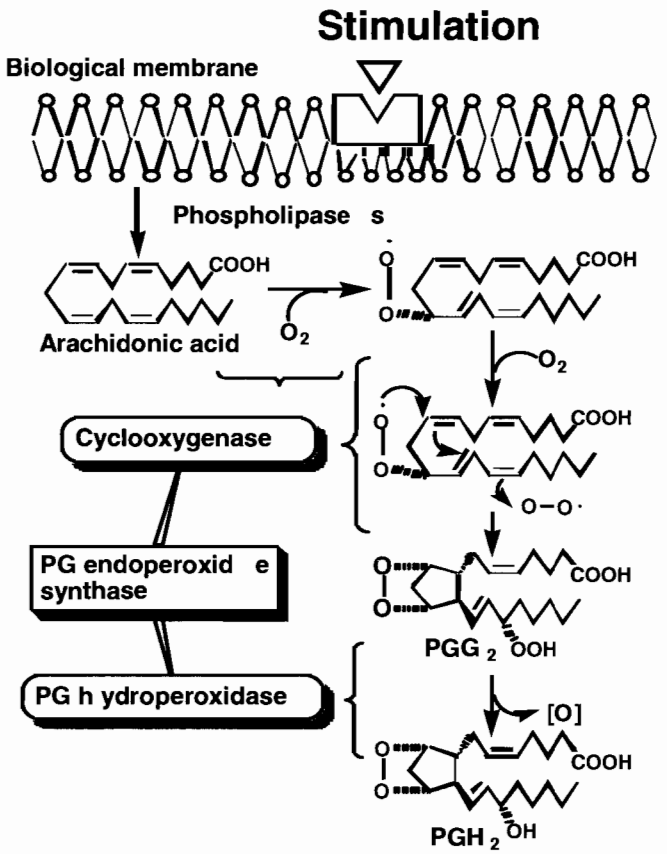


Figure 6. The reactions of PG endoperoxide synthase including cyclooxygenase and PG hydroperoxidase.

The alternative pathway is the so-called "linear pathway" to produce eicosatetraenoic acids (HPETEs) from arachidonic acid through the action of different types of mammalian LOXs (Fig. 7). Along with the finding of TXs, 12-LOX was found first in human and bovine platelets. Later, distinct isoforms such as leukocyte and epidermal types were identified. Rabbit reticulocytes are known to have 15-LOX-1, responsible for the degradation of mitochondria by programmed structural changes through the peroxidation of membrane lipids. 5-LOX catalyzes the formation of 5-HPETE and subsequent conversion of leukotriene A_4 which is an intermediate for the biologically active LTB_4, a potent chemotactic agent, and LTC_4, one of slow reacting substances of anaphylaxis in asthma. More recently,

novel 8-LOX and 15-LOX-2 have been found from mouse and human skins, respectively. Recent molecular cloning of cDNA encoding LOX isoforms have established a phylogenetic tree in plant and animal enzymes (**1**).

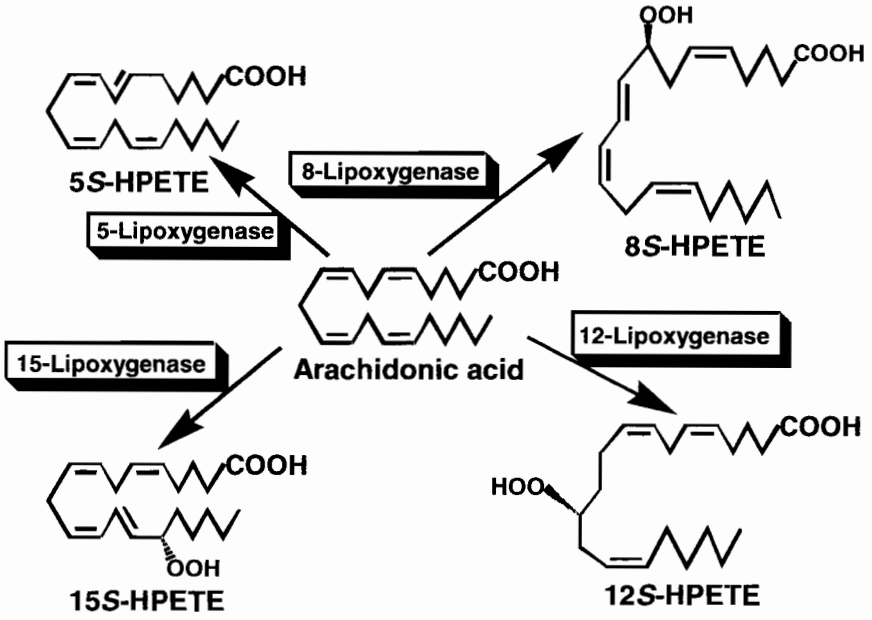


Figure 7. Mammalian lipoxygenase pathway to form HPETEs from arachidonic acid.

Recent Progress in Mammalian LOX Pathways

More recently, novel mammalian LOXs as well as related isoforms have been found by using molecular cloning of cDNA of the enzymes. Brash and his colleagues have been successful in cloning a second type of 15S-lipoxygnase-2 from human skin (**19**) and a phorbol ester-inducible 8S-LOX from mouse skin (**20**). Although the regiospecificity of the reactions of both enzymes are quite different, these enzymes shared 78% identity in their primary sequences of amino acids. By contrast, the amino acid identity between human 15-LOX-1 and -2 is only 35%. These findings suggest that the similarity is necessarily accounted for by the positional specificity for the substrate. Jisaka *et al.* (**21**) have just recently identified amino acid determinants to confer the positional specificity of mouse 8S-LOX and human 15S-LOX-2 by using *in situ* chimeragenesis in bacteria followed by site-

directed mutagenesis. The change in the reactivity between 8 and 15 lipoxygenation by mutation of the corresponding amino acid residues led them to propose the mechanism of switch in the head to tail binding of arachidonic acid in the same active site of those enzymes to make 8S or 15S oxygenation. X-ray structural analysis of these novel LOXs will provide more detailed information on the mechanistic actions.

Until recently, mammalian LOX products were considered as almost exclusively S enantiomers. Later, the LOXs to form R configuration have been found in marine invertebrates and other organisms. Earlier, the areas of epidermis in psoriasis had increased levels of 12-HETE. Chiral analysis revealed that the predominant enantiomer was 12R. More recently, a cDNA encoding 12R-LOX was cloned from human psoriatic skin and keratinocytes, indicating the existence of R-LOX in mammals (**22**). Molecular cloning of human 12R-LOX has also been accomplished from the B cell line CCL-156 (**23**). Further studies will provide therapeutic treatments in psoriasis. Roles of 12R-LOX in normal tissues or cells remain to be determined. No characteristic sequences or motifs to account for the opposite stereospecificity are identified, suggesting subtle conformational changes in the active site of the enzymes.

Molecular cloning technologies have identified several types and subtypes of prostanoid receptors from various species. Moreover, the signal transduction mechanism of the corresponding receptors has been studied. As for the LOX pathway, the receptors for LTB_4 and peptide LTs have been cloned. Molecular basis of the LOX metabolites remains unclear. Recently, the nuclear action of eicosanoids received more attention because peroxisome proliferator-activated receptors (PPARs), which are a ligand-activated transcription factors or nuclear receptors, have been shown to bind some of polyunsaturated fatty acids and eicosanoids as endogenous ligands. Among these, 15-deoxy-$\Delta^{12,14}$-PGJ_2, a COX product through PGD_2, was demonstrated to bind directly to PPARγ and activate its function (Fig. 8) (**24,25**). PPARγ is found primarily in adipose tissues, where it plays a critical role in the differentiation of preadipocytes to adipocytes. In addition, the activation of PPARγ with the above PGJ_2 agonist that caused programmed cell death, apoptosis, in breast cancer cells (**26**) or in endothelial cells from blood vessels (**27**). However, the means of production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 *in vivo* has not been established. It is unclear whether the PGJ_2 derivatives are synthesized in the target cells expressing PPARγ or transported from other cells. It remains possible that the activated synthesis of PGD_2 and PGJ_2 derivatives takes places either locally or transiently during the process of the differentiation in adipose tissues and related tissues in response to some bioactive signals.

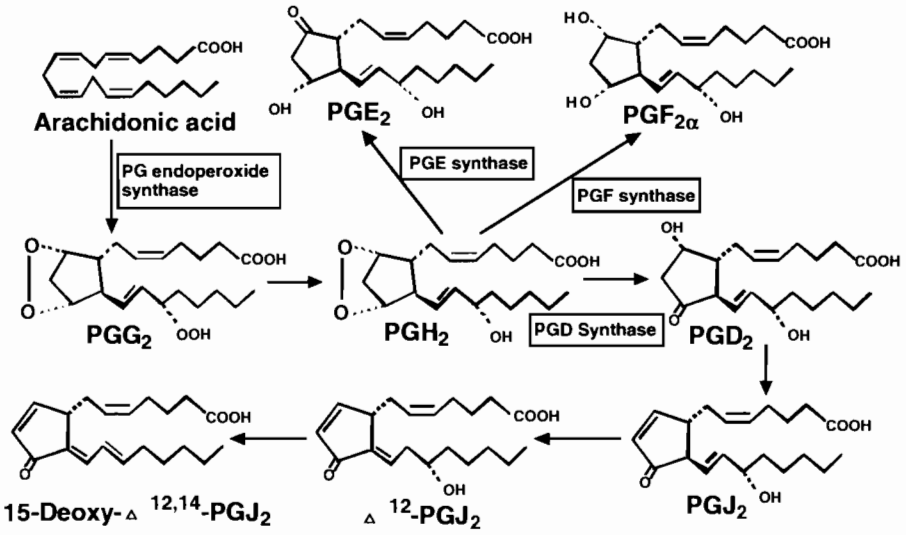


Figure 8. The metabolic pathway leading to the generation of 15-deoxy-$\Delta^{12,14}$-PGJ_2, an activator of PPARγ, through the formation of PGD_2.

As for the LOX metabolites, the nuclear actions have also been found. Mouse 8S-LOX produces 8S-hydroxyeicosatetraenoic acid (HETE), which has an ability to stereoselectively activate another PPARα and was reported to induce the differentiation of cultured 3T3-L1 preadipocytes (**28**). Oxidized low-density lipoprotein (LDL) is known to be taken up by macrophages through scavenger receptors whose expression needs the activation of PPARγ. From components of oxidized LDL, 13-hydroxyoctadecadienoic acid from linoleic acid and 15-HETE from arachidonic acid were identified to be effective in the activation of the above nuclear receptor which is linked with the formation of foam cells leading to the onset of atherosclerosis and related pathogenesis. As means of providing PPARγ ligands, interleukin-4, a cytokine from T cells, has been shown to upregulate a 12/15-LOX in macrophages, and also to induce the expression of PPARγ (**29**). The ligand-binding pocket of PPARγ appears larger because both COX and LOX metabolites can bind to the same receptor. To establish the physiological relevance of these compounds as regulators *in vivo*, further studies would be required in macrophage development and function. Interestingly, the promoter region of inducible type of COX-2 has a PPAR responsive element that is responsible for the increase in COX-2 expression (**30**). In addition, as ligands of PPARs, a variety of substances that are substrates, products, and inhibitors of the COX enzyme activity have been shown to enhance the gene expression of COX-2. These findings indicate diverse mechanisms for the biological actions of polyunsaturated fatty acids, eicosanoids, and non-steroidal anti-inflammatory drugs.

Acknowledgments

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

References

1. Brash A.R. (1999) *J. Biol. Chem.* **274**, 23679-23682.
2. Gardner H.W. (1991) *Biochim. Biophys. Acta* **1084**, 221-239.
3. Siedow J.N. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 145-188.
4. Vick B.A. (1993) *in* Lipid Metabolism in Plants, Moore T.S. ed., CRC Press, Boca Raton, Florida, pp.167-191.
5. Shibata D., Axelrod B. (1995) *J. Lipid Mediators Cell Signalling* **12**, 213-228.
6. Galliard T., Phillips D.R. (1971) *Biochem. J.*, **124**, 431-438.
7. Mulliez E., Leblanc J.P., Girerd J.J., Rigaud M., Chottard J.C. (1987) *Biochim. Biophys. Acta*, **916**,13-23.
8. Reddanna P., Whelan J., Maddipati K.R., Reddy C.C. (1990) *Methods Enzymol.*, **187**, 268-277.
9. Galliard T., Phillips D.R. (1972) *Biochem. J.* **129**, 743-753.
10. Shimizu T., Radmark O., Samuelsson B. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 689-693.
11. Shimizu T., Honda Z.I., Miki I., Seyama Y., Izumi T., Radmark O., Samuelsson B. (1990) *Methods Enzymol.*, **187**, 296-306.
12. Royo J., Vancanney G., Perez A. G., Sanz C., Stormann K., Rosahl S., Sanchez-Serrano J.J. (1996) *J. Biol. Chem.*, **271**, 21012-21019.
13. Geerts A., Feltkamp D., Rosahl S. (1994) *Plant Physiol.*, **105**, 269-277.
14. Hamberg M. (2000) *in* 11th International Conference on Advances in Prostaglandin and Leukotriene Research: Basic Science and New Clinical Applications, Florence, Abstract Book, p.7.
15. Choi D., Bostock R.M. (1994) *Plant Physiol.*, **104**, 1237-1244.
16. Bostock R.M., Yamamoto H., Choi D., Ricker K.E., Ward B.L. (1992) *Plant Physiol.* **100**, 1448-1456.
17. Smith W.L., Garavito R.M., DeWitt D.L. (1996) *J. Biol. Chem.*, **271**, 33157-33160.
18. Yokota K., Morishima T., Kanaumi K., Tsuruhami K., Kishimoto T., Nagaya T., Jisaka M., Takinami K. (1998) *in* Recent Research Development in Agricultural and Biological Chemistry., Vol. **2**, Research Signpost, Trivandrum, pp. 333-344.
19. Brash A.R., Boeglin W.E., Chang M.S. (1997) *Proc. Natl. Acad. Sci., U.S.A.* **94**, 6148-6152.
20. Jisaka M., Kim R.B., Nanney L.B., Boeglin W.E., Brash A.R. (1997) *J. Biol. Chem.* **272**, 24410-24416.
21. Jisaka M., Kim R.B., Boeglin W.E., Brash A.R. (2000) *J. Biol. Chem.* **275**, 1287-1293.
22. Boeglin W.E., Kim R.B., Brash A.R. (1998) *Proc. Natl. Acad. Sci. U.S.A* **95**, 6744-6749.
23. Sun D., McDonnell M., Chen X.S., Lakkis M.M., Li, H., Isaacs S.N., Elsea S.H., Patel P.I., Funk C.D. (1998) *J. Biol. Chem.* **273**, 33540-33547.
24. Forman B.M., Tontonoz P., Chen J., Brun R.P., Spiegelman B.M., Evans R.M. (1995) *Cell* **83**, 803-812.
25. Kliewer S.A., Lenhard J.M., Willson T.M., Patel I., Morris D.C., Lehmann J.M. (1995) *Cell* **83**, 813-819.
26. Clay C.E., Namen A.M., Atsumi G., Willingham M.C., High K.P., Kute T.E., Trimboli A.J., Fonteh A.N., Dawson P.A., Chilton F.H. (1999) *Carcinogenesis*, **20**, 1905-1911.

27. Bishop-Bailey D., Hla T. (1999) *J. Biol. Chem.* **274**, 17042-17048.

28. Yu K., Bayona W., Kallen C.B., Harding H.P., Ravera C.P., McMahon G., Brown M., Lazar M.A. (1995) *J. Biol. Chem.* **270**, 23975-23983.

29. Huang J.T., Welch J.S., Ricote M., Binder C.J., Wilson T.M., Kelly C., Witztum J.L., Funk C.D., Conrad D., Glass C.K. (1999) *Nature* **400**, 378-382.

30. Meade E.A., McIntyre T.M., Zimmerman G.A., Prescott S.M. (1999) *J. Biol. Chem.* **274**, 8328-8334.

Chapter 19

BIOREMEDIATION AND BIOBENEFICIATION OF METALS

A.D. AGATE

7, Narmada Apts. United Western Sociciety, Navsahyadri P.O., Pune - 411 052; India

Abstract

Metals have gained unprecedented importance in the industrial revolution all over the world and with it, the efficient processes that deal with wastes from extraction of metals from ores as well as wastes from metal processing and finishing industries have gained high priority for their disposal, using cost effective and eco-friendly methods such as bioprocessing using newer applications of biotechnology, viz. the bioremediation and biobenefication of metals. Therefore, these are very important in preventing environmental pollution. Whereas the former process of bioremediation deals mainly with removing the harmful and toxic metals from the metal-containing wastes of finished products using bioadsorption process by living or dead cells or cell bodies such as mycelia on beads or some other inert material ("Fluidized Bed Reactor" type) the latter, i.e. biobeneficiation, works at a stage prior to making the product (metal) on the principle that, if one could remove the impurities associated with the desired metal or change / modify the metal quality, it would yield the precious metal for value addition of the process and reduce the metal content in the waste, so it is easier to dispose off by conventional techniques. Moreover, such bioprocessing techniques are eco-friendly and basically depend upon microbe-metal interactions that are present in nature.

In this review, appropriate examples of these phenomena are furnished with their principles and practices, for e.g. in steel making industry by removal of iron-oxides from pickling liquors, choice of biomass type, (living / dead) and counter current system to remove Pb, Cu, U and Cr, microbial oxidation and reduction of manganese and its applications in biobenefication and ground water clean-up. The other associated topics dealt with are molecular mechanisms of heavy metal resistance in microorganisms and the work in our laboratory dealing with these 2 aspects viz. use of fungal biomass in bioremediation and biobeneficiation of manganese ores.

Introduction

During industrialization, metals have gained unprecedented importance all over the world. Efficient processes that deal with wastes resulting after extraction of metals from ores, as well as wastes from metal processing and finishing industries have gained a high priority for their safe disposal. This can be effectively carried out by using cost effective and eco-friendly methods using the latest applications of

New Horizons in Biotechnology,215-220, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

biotechnology, viz, the bioremediation and bio beneficiation of metals. Therefore, such methods have become very important in preventing environmental pollution (**1**).

Bioremediation deals with accumulation and removal of harmful or toxic metals from metal containing wastes using a biosorption process by living or dead cells or mycelia of microorganism embedded on beads or some insert material.

Biobeneficiation on the other hand, works on the principle that if one could remove the impurities associated with desired metal or change metal quality, it would yield a value-added product i.e., metal from waste and reduce metal content in the waste, so that it could be disposed off by conventional techniques.

These bioprocessing techniques are eco-friendly and basically depend on microbe-metal interactions, present in nature and hence, cause no harm to the environment (**2**).

Bioremediation Applications

It is well known that certain algae, bacteria, yeasts and fungi can remove metal ions from dilute solutions very effectively. Many researchers have tested the possibility of detoxifying metal-containing effluent by using microbial agents and these studies were demonstrated to be cost-effective for removing metals efficiently (**3**).

Biosorption uses biomass raw materials, which are abundantly found in sea (algae) or bye-products from large-scale industrial operations (fungal mycelia). Brown marine algae such as *Ascophyllum* and *Sargassum* accumulate more than 30% of its biomass dry weight as metal. Mycelia of industrially steriod-transforming fungi *Rhizopus* and *Absidia* are excellent biosorbents for lead, cadmimum, copper, zinc and uranium, binding heavy metals up to 25% of their biomass dry weight. *Saccharomyces cerevisiae*, the common alcohol fermenting yeast, is a metal biosorbent (**4**).

In our laboratory, mycelial waste of *Penicillium chrysogenum* was used to absorb metal from solution and cadmium was shown to be adsorbed on *Streptomyces pimprina* mycelial waste biomass (**5,6**). The heavy metal biosorption by dead mycelial biomass of *Rhizopus arrhizus*, *Mucor miehei* and *P. chrysogenum* was shown to have improved by pH control and cationic activation of the biosorbent (**7**). Waste fungal biomass was considered as a new bioremedial material to control heavy metal pollution (**8**).

A biological process for removal of heavy metals from aqueous flow was described, where immobilized cells of *Citrobacter* sp. were used to precipitate the metals as cell-bound metal phosphates (**9**).

An automated bench scale counter current biosorption system was designed to remove metals from aqueous effluents, where activated sludge microorganisms were used as biosorbent and lead and copper as model metals. Lead was removed from lead nitrate solution (100 $mg.l^{-1}$ lead) to 0.1 $mg.l^{-1}$ lead (99.9 % removal) and

copper from copper chloride solution (100 $mg.l^{-1}$ copper) to 35-45 $mg.l^{-1}$ concentration (60% removal) (**10**).

Lab-scale recovery of iron oxides (Fe_2O_3 type) was also attempted through bioxidation with *Thiobacillus ferroxidans* of the ferrous sulphate contained in sulfuric picking liquors from steel industry. The process carried out produced 50 kg. Fe_2O_3 of per m^3 of waste pickling liquor with 99.8% weight iron recovery (**11**).

Mechanism of biosorption

Different metal-binding mechanisms are postulated to be active in biosorption, such as:

- chemisorption : by ion exchange, complexation, coordination, chelation
- physical adsorption, - micro precipitation
- possible oxidation – reduction reactions.

Due to complexity of biomaterials, it is quite possible that one or more mechanisms are taking place simultaneously or in varying degrees in the biosorbent (**12**).

Future outlook

Granulation of biosorbents could scale-up these technologies to industrial level, for which it is necessary to achieve fixed – bed reactor / contactor configuration. Hence, the biosorbents have to be hard enough to stand pressures, porous for metal ion - sorbate species. Microbial biomass may need drying to remove the high water content. Techniques for immobilizing microbial biomass have been developed and patented (**13**) and a need for standardizing experimental protocols has been emphasized (**14**).

The combination of biosorption along with metabolically mediated processes such as bioreduction of chromium ($Cr^{6} – Cr^{3}$) and selenium ($Se^{4} – Se^{0}$) is also possible in a novel reactor design. (**15**). Biosorption of metal-cyanide complexes from solutions was also shown to be possible using fungal biosorbent from *Cladosporium cladosporides* sp. (**16**).

Finally, what is needed is to develop a market-reliable robust, effective process design to arrive at a commercial success story.

Biobeneficiation Applications

Biobeneficiation is a kind of indirect phenomenon akin to bioleaching of metals, where instead of metals getting solubilized and extracted from the heap, dump or *in-situ* types of leaching, the impurities get leached out in the leachate and what remains is the desired metal in solid form.

A patent assigned to Kennecot Copper Corporation described a cyclic process of heap leaching, where copper, molybdenum, chromite and titanium ores were upgraded or benefited by this process, in that the metal value was enriched, instead of metal value being extracted (**17**).

Leaching of gold arsenopyrite ore, where due to the oxidation of arsenopyrite moiety, which was first reported in 1964 by Ehrlich, the gold was beneficiated and could be obtained as a value-added product (**18**). Biobeneficiating pyritic gold was patented by Livsey Goldblatt in 1983, as "Back-Fox Process", where pyrite and arsenopyrite layers are partially oxidized and uncovered for extraction and lessen the consumption of cyanide, a toxic pollutant used for extraction of the ore (**19**).

From dump leaching operations, the commercial processes have evolved into stirred-tank reactors for higher grade, high value ores and concentrates of copper and gold. Next generation technologies include pretreatment of refractory gold and uranium by using archean thermophiles such as *Sulfolobus* species for oxidation of sulfides (**18**).

In nature, manganese is found as reduced soluble or absorbed Mn II and insoluble Mn III and Mn IV forms. Manganese oxidation has been reported in several microorganisms such as bacteria, algae and fungi. The mechanism of manganese oxido – reduction could be a direct enzymatic process or an indirect interaction with organic and inorganic compounds (**20**).

The bacterial oxidation of manganese, carried out by bacteria belonging to *Hyphomicrobium* and *Arthrobacter* sp. could be fruitfully used to biobeneficiate lower oxides of manganese to their higher forms ($MnSO_4$ to MnO_2 transformation) can be demonstrated in the laboratory (**21**) The removal of soluble manganese from water by employing manganese oxidizers can be a solution to darkening of ground wastes and plugging of pipes in drinking water or industrial process waters. For removal of Fe II amd Mn II from water, various treatments employing bacteria belonging to *Gallionella* and *Leptothrix* sp are used either *in situ* or in sand filters in Germany, Finland, France, Belgium and Austria (**22**).

In case of metal oxide ores, anaerobic bacterial processes, where bacteria reduce the metal oxides and solubilize it, may be the most promising for industrial exploitation (**23**). If conditions of sterility and anarobiosis are guaranteed, keeping cost considerations in view, a two-reactor system could be ideal, keeping the first reactor axenic. Meterohtrophic, anaerobic leaching for beneficiating oxide, silicate and carbonate ores will be the technology of the future (**18**).

In this direction a few attempts were made to beneficiate bauxite ores by reducing the amount of iron anaerobically (**24**) and also removal of iron from phosphatic ores in our laboratory (**21**), but these were bench - scale attempts and commercially not tried out. However, it was shown at pilot plant level, that manganese oxidizing bacteria, belonging to *Arthrobacter* sp. could be used to clarify iron / manganese fines containing wash water at a mine site in Goa (**25**).

Mechanisms of metal resistance

The key to most of the metal-microbe interactions and processes developing thereby lies in metal resistance in microorganisms, which react with metals and not only withstand high concentrations of metals, but carry on with all metabolic activities. For a long time, it was suspected that the cellular envelope or membrane

layer plays a part in it, but the proton pump concept revealed the underlying truth. However, efforts are underway to seek a genetic explanation for the phenomenon of metal resistance by examining the chromosonal and extrachromosomal entities, that could be found, which have a direct relationship with metal resistance (**26**).

In certain plant pathogenic species of *Pseudomonas syringae*, which are exposed to high copper concentrations, the bacteria were found to accumulate Cu^{2+} ions in the periplasm and outer membrane and, at least, part of the sequentering ability is determined by copper binding proteins of the copper resistance operon (*cop*). Such copper resistance operon systems were also shown in related plant pathogen, *Xanthomonas campestris* and in *E. coli*, but here they behave function-wise differently (**27**).

In *Alcaligenes eutrophus* sp. which are adapted to high levels of heavy metal environment, it was found that it contains two mega plasmids pMOL28 and pMOL30, which carry determinants to cobalt, nickel, chromium, mercury, titanium, cadmium, copper and zinc resistance. The best-characterized operons are *cnr* (resistance to Co and Ni) on pMOL28 and *czc* operon (resistance to Co, Cd and Zn) on pMOL30 (**28**).

In case of *Thiobacillus ferrooxidans*, a small 40 kb plasmid was detected in copper tolerating strains, which remains to be characterized yet. If the metal resistance is located / associated with metal tolerance, it would be possible to transfer such a plasmid into good leaching activity showing cultures, thereby improving the process designs of a biareactor by reducing the pulp density and develop concentrate bioleaching / beneficiation in a commercially feasible manner (**29**).

Conclusions

From the brief survey of bioremediation and biobeneficiation processes, it is apparent that bioremediation can make a most significant impact in using it for removing toxic heavy metals from industrial effluents. As the scientifically based biohydrometallurgy using acidophiles has made significant strides in processing of sulfide ores, biobeneficiation techniques also would be soon applicable commercially. These are environmentally benign techniques, which would be successful in process application, if an inter-disciplinary approach is used judiciously.

References

1. Lewandowski G.A., de Filippi L.J. (1998) Biological Treatment of Hazardous Wastes, John Wiley & Sons, N.Y.
2. Bosecker K. (1986) *In*: Workshop on Biotechnology for the Mining, Metal refining and Forssil – Fuel Industries. Ehrlich H. L. and Holmes D. S. (eds.) Biotechnol Bioeng. Symp. 16, Wiley, N.Y., p. 105.
3. Brierley J.A., Goyak G. M., Brierley C.L. (1986) *In*: Immobilization of ions for biosorption. Eccles L H. and Hunt S. (eds.) Eillis Horwood, Chichester, U.K. pp. 105-117.
4. Volesky B. (1994) *FEMS Microbiol Rev.* **14**, 201-302.

5. Paknikar K.M., Palnitkar V.S., Puranik P.R. (1993) *In*: Biohydrometallurgical technologies. Torma A.E., Apel M.L. and Brierley C.L. (eds.) Vol. 2, Minerals, Metals and materials soc. USA, pp. 229-233.
6. Puranik P.R., Chabukswar N.S., Paknikar K. M. (1995) *Appl. Microbiol. Biotechnol.* **43**, 1118-1121.
7. Fourest E., Canal C., Roux J.C. (1994) *FEMS Microbiol. Rev.* **14**, 325-332.
8. Paknikar K.M., Puranik P.R., Agate A.D., Naik S.R. (1995) *In*: Bioremediation – Principles and practice, S.S. Sikdar (ed.), Technomic Publ. Co. Lancaster, PA, USA, pp. 19-51.
9. Macaskie L.E., Jeong B.C., Tolley M.R. (1994) *FEMS Microbiol. Rev.* **14**, 351-386.
10. Gourdon R., Diard P., Funtowicz N. (1994) *FEMS Microbiol. Rev.* **14**, 333-338.
11. Garcia F.J., Rubio A., Sainz E., Gonzalez P., Lopez F.A. (1994) *FEMS Microbial Rev.* **14**, 397-404.
12. Vokesky B. (1999) *In*: Intl. Biohydrometallurgy Symp. Part B, Amils R. and Ballester A. (eds.), Elsevier, N.Y. pp. 161-169.
13. Tsezos M. (1999) *In*: Intl. Biohydrometallurgy Symp. Part B, Amils R. and Ballester A. (eds.), Elsevier, N.Y. pp. 171-173.
14. Paknikar K.M., Puranik P.R., Petkar A.V. (1999) *In*: Intl. Biohydrometallurgy Symp. Part B, Amils R. and Ballester A. (eds.), Elsevier, N.Y. pp. 363-371.
15. Rajwade J.M., Salunkhe P.B., Paknikar K.M. (1999) *In*: Intl. Biohydrometallurgy Symp. Part B, Amils R. and Ballester A. (eds.), Elsevier, N.Y. pp. 105-113.
16. Patil Y.B., Paknikar K.M. (1999) *In*: Intl. Biohydrometallurgy Symp Part B, Amils R. and Ballester A. (eds.), Elsever, N.Y. pp. 707-715.
17. Zimmerky S.R., Wilson D.G., Prater J.D. (1958) Cyclic leaching process employing iron oxidizing bacteria U. S. Patent No. 2, 829, 964.
18. Ehrlich H.L. (1999) *In*: Intl. Biohydrometallurgy Symp. Part A, Amits R and Ballester A (eds.) Elsevier, N.Y. pp. 1-12.
19. Livsey-Goldblatt E., Norman P., Livsey-Goldblatt D.R. (1983) *In*: Recent Progress in Biohydrometallurgy. Rossi G. and Torma A.E. (eds.), Associazione Mineraria Sarda, Iglesias, Italy, p. 627.
20. Ehrlich H.L. (1996) Geomicrobiology, Marcel Dekker, N. Y.
21. Agate A.D. (1985) *In*: Biogeotechnology of Metals. Center for Intl. Projects, Karavaiko G.I. and Groudev S.N. (eds.), Moscow, USSR, pp. 257-277.
22. Gounot A. M. (1994) *FEMS Microbiol. Rev.* **14**, 339-350.
23. Ehrlich H.L. (1993) *In*: Soil Biochemistry, 8, Bollog J.M. and Stotzky G. (eds.) Marcel Dekker, N.Y. pp.219
24. De Sa C. (1979) Studies on microorganisms associated with iron ores, M.Sc. Thesis, Univ. of Bombay.
25. Agate A.D., Ghosh J.S., Verenkar L.B. (2000) *In*: Bioprocessing of metals, Conf. United Engineering Foundation, Forssberg E. and Hanumanth Rao K. (eds.), Lulea Univ. of Technology, Lulea. Sweden, Abstracts, p. 29.
26. Rawlings D.E. (1999) *In*: Intl. Biohydrometallurgy Symp. Part B, Amils R. and Ballester A. (eds.), Elsevier, N.Y., pp. 3-20.
27. Cooksey D.A. (1994) *FEMS Microbiol. Rev.* **14**, 381-386.
28. Collard J. M., Corbisier P., Diels L, Dong Q, Jeanthon C., Mergeay M., Taghavi S., Van der Lelie D., Wilmotte A., Wieertz S. (1994). *FEMS Microbiol. Rev.* **14**, 405-411.
29. Brierley J.A., Brierley C.L. (1999) *In*: Intl. Biohydrometallurgy Symp. Part A, Amils R. and Ballester A., (eds.), pp. 81-89.

Chapter 20

DEVELOPMENTS IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR)

R. BHAMIDIMARRI[1], P.O. BICKERS[2],
N. THAYALAKUMARAN[2], Z.B. HU[2]

[1] Office of the Principal Massey University, Palmerston North, New Zealand ; E-mail: B.Rao@massey.ac.nz ; [2] Institute of Technology & Engineering, Massey University, Palmerston North, New Zealand

Abstract

Phosphorus removal using Phosphorus Accumulating Organisms (PAOs) has attracted significant interest in recent years for controlling nutrient pollution in the aquatic environment. Enhanced Biological Phosphorus Removal (EBPR) has been known for several decades since the concept of "luxury uptake" was proposed in 1965 but the mechanisms have not been understood until recently. Biological phosphorus removal may occur through growth associated coupling or storage within the biomass as polyphosphate. The latter mechanism is exploited in the EBPR systems. Until recently, *Acinetobacter sp* alone was thought to be responsible for EBPR, but it is now known that this may not be the case, although the organism(s) responsible for polyphosphate accumulation are still unknown. There are two biochemical mechanisms proposed for PAOs under anaerobic and aerobic conditions based on the reducing power required for the synthesis of polyhyroxyalkanoate (PHA). A range of reactor configurations has been developed to accomplish EBPR essentially exploiting the anaerobic and aerobic metabolism of PAOs. The initial EBPR systems relied upon modifications to continuous flow activated sludge reactors incorporating the anaerobic stage prior to aeration basin for phosphorus removal and anaerobic and anoxic stages for nitrogen and phosphorus removal. More recently, Sequencing Batch Reactor (SBR) has been shown to be an effective reactor system for either phosphorus removal alone or for the removal of both nitrogen and phosphorus. Much of EBPR work over the last four decades has been done on low phosphorus concentration (about 15mg/L) domestic effluents. More recently, industrial effluents containing high phosphorus concentration (about 90 mg/L) have also been successfully treated by EBPR. The understanding of EBPR both in terms of mechanisms and reactor systems reached a level ensuring that EBPR is now more attractive alternative to chemical treatment for phosphorus removal.

Key words: EBPR, *Acinetobacter*, PAOs, PHA.

New Horizons in Biotechnology, 221-230, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

Modified carbon and nitrogen cycles in the environment due mainly to human activity form the basis for the macronutrient (nitrogen and phosphorus) pollution in the environment. Phosphorus has been known to be the primary element responsible for eutrophication in natural water bodies for nearly fifty years. Transformation of nitrogenous pollutants into gaseous form for easy separation from water is well established and widely practiced. However, phosphorus presents a more complex problem.

Phosphorus is present in waste streams generally in the form of ortho-phosphates, inorganic polyphosphates or organic phosphorus. Sometimes it is also present in the form of particulate phosphorus. Phosphorus can be readily removed by chemical methods (**1**) involving precipitation, coagulation, flocculation and separation. Precipitation is by the addition of calcium, iron or aluminum salts. Organic polymers are also commonly used to facilitate rapid coagulation and flocculation. Although the chemical precipitation is efficient, it is cost intensive and results in large quantities of sludge with increased metal content.

Biological phosphorus removal offers an environmentally friendly and cost effective solution to phosphorus pollution problem. Biological system also provides the potential for removal of both nitrogen and phosphorus in the same systems in addition to allowing for phosphate recovery.

Early developments

Phosphorus is an essential element for biological growth. Typically microorganisms uptake phosphorus through growth process up to about 1.5% on dry weight basis. However, Greenburg *et al.* (**2**) proposed that phosphorus removal beyond that which is required for normal growth is possible. Several other researchers investigated the phenomenon. These included Srinath (**3**) who confirmed the occurrence of excess phosphorus uptake by activated sludge. Levin and Shapiro (**4**) described this excess uptake as "luxury uptake". They also demonstrated that the excess phosphorus removal was via biological activity and that phosphorus was released under anaerobic conditions. Barnard (**5**) identified operational features of activated sludge processes with enhanced biological phosphorus removal. The operational experience at several municipal sewage treatment plants led to the conclusion that cycling sludge under alternating anaerobic and aerobic condition with substrate addition at anaerobic stage results in enhanced biological phosphorus removal (EBPR).

EBPR Mechanism

The early research into EBPR confirmed that bacteria are capable of uptake of excess amounts of phosphorus beyond that is stoichiometrically required for growth and that they absorb excess quantities of substrate under anaerobic conditions and accumulate phosphorus in the form of polyphosphates under aerobic conditions.

Such organisms are generally referred to as phosphorus accumulating organisms (PAOs) (**6**).

The microbiology of mixed culture wastewater treatment systems is complex. Fuhs and Chen (**7**) concluded that *Acinetobacter* was responsible for enhanced phosphorus removal. Subsequently, several other researchers confirmed the dominance of *Acinetobacter* in EBPR sludges (**8-11**). Brodish and Joyner (**11**) however found that organisms other than *Acinetobacter* might also be responsible for excess phosphorus uptake. Subsequently, Cloete and Steyn (**12**) showed that an EBPR sludge contained less than 10% *Acinetobacter* using a fluorescent staining technique. Auling *et al* (**13**) demonstrated that the *Acinetobacter* populations were very small in an active EBPR sludge using a polyamine biomarker. Wagner *et al* (**14**) and Kampfer *et al* (**15**) confirmed that *Acinetobacter* is not the dominant PAO in EBPR sludges using oligonucleotide probes.

Microbiology of EBPR was discussed by Jenkins and Tandoi (**16**) and concluded that the characteristics of EBPR sludges were not represented by the behaviour of pure cultures of *Acinetobacter*. The identity of culture(s) responsible for EBPR however, is not yet known.

Without the knowledge of the organisms responsible for EBPR, the biochemical models of EBPR are limited to those based on projections from practical observations. Mino *et al* (**17**) proposed that the PAO reduce the volatile fatty acids to polyhydroxyalkanoate (PHA) with the reducing power (NADH) derived from glycolysis of stored glycogen. It was suggested that the ATP required for PHA production is generated from the degradation of the stored polyphosphate and glycolysis resulting in the release of stored phosphate. During the aerobic stage, the PHA is oxidised for cell growth and glycogen and excess polyphosphate accumulation occurs at the same time. This model is summarised in Figure 1.

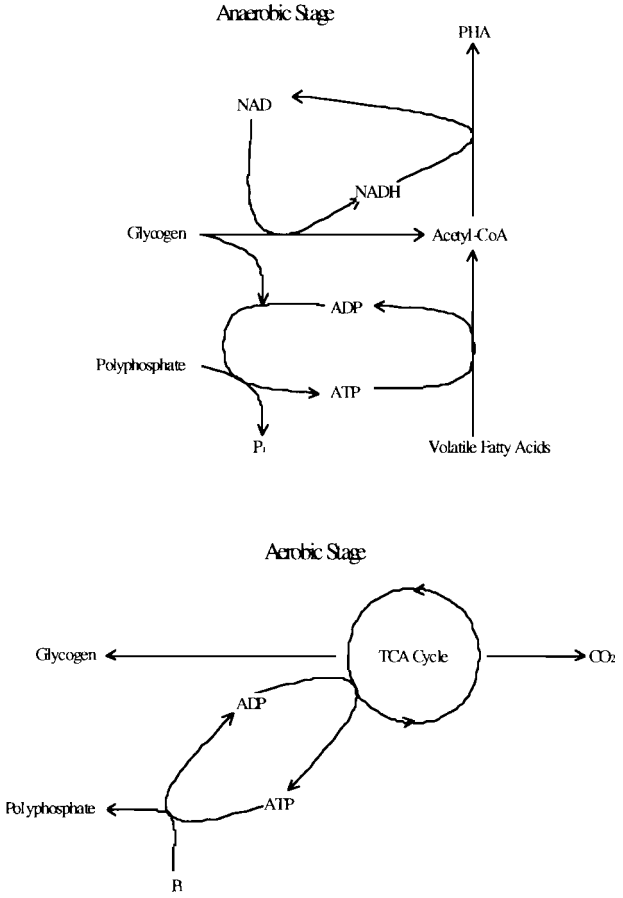


Figure 1. Biochemical model for EBPR proposed by Mino *et al* (1987).

Comeau *et al* (**18**) proposed another biochemical model using *Acinetobacter* as the typical PAO. Wentzel *et al* (**19**) subsequently compared the two models concluded that the model proposed by Mino *et al* (**17**) with Entner-Doudoroff pathway replacing EMP pathway would be more appropriate.

EBPR Processes

A number of EBPR process configurations have evolved over the last 35 years exploiting the behaviour of organisms under aerobic and anaerobic environments. Levin and Shapiro (1965) first developed the basis for EBPR by incorporating an anaerobic stage in the conventional activated sludge process. In this side stream

configuration (PhoStrip process) shown in Figure 2a, a portion of the return activated sludge is held under anaerobic conditions typically for 8 to 12 hours. Phosphorus released with the supernatant is chemically precipitated out. Once the significance of anaerobic stage in the substrate utilisation by organisms was recognized, Barnard (**20**) developed a continuous flow system placing anaerobic stage ahead of aerobic stage and this basic process was named the Phoredox process (Figure 2b).

This process was subsequently modified by inserting an anoxic stage leading to the development of A^2O process (Figure 2c). The addition of anoxic stage enhanced the denitrification capability.

Over the last twenty years, several systems have been developed for combined nitrogen and phosphorus removal leading to improved performance of EBPR. There are two key processes on which the current EBPR plants operate. These are modified Bardenpho (Figure 3a) and UCT (Figure 3b) configurations.

In modified Bardenpho process, the influent and the return sludge are contacted in the anaerobic stage to promote production of fermentation products and phosphorus release. UCT process is configured to ensure that nitrates are not recycled with return sludge into anaerobic stage.

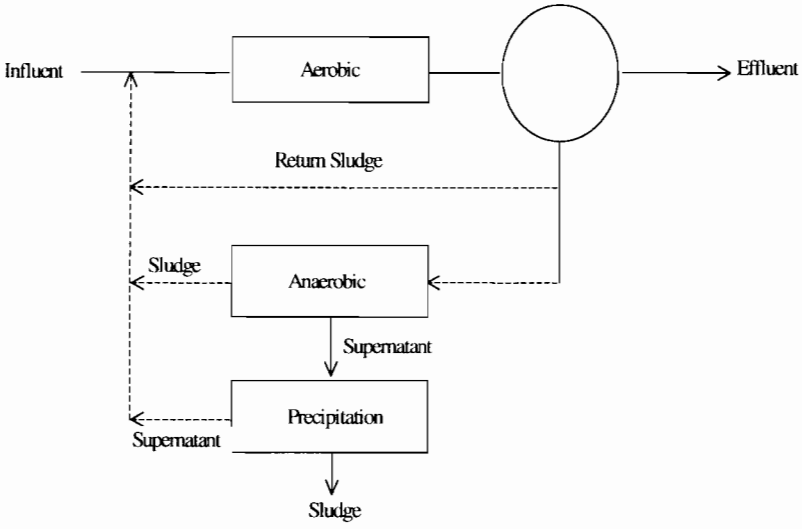


Figure 2a. PhoStrip Process.

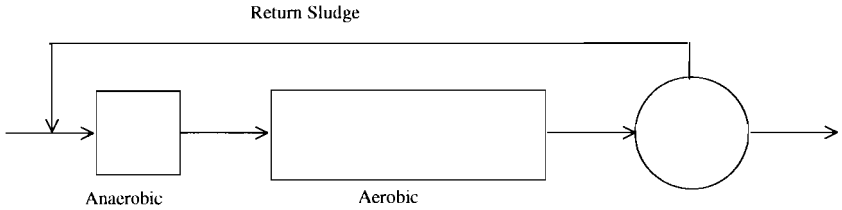


Figure 2b: Two-stage biological phosphorus removal system.

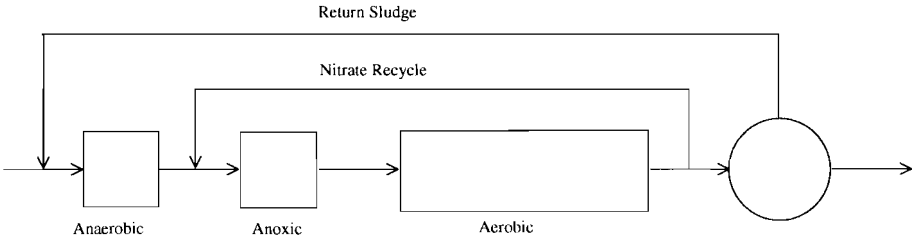


Figure 2c. Three-stage biological nutrient removal system.

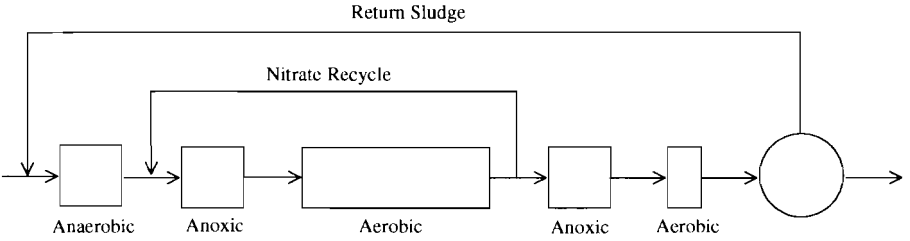


Figure 3a. Five-stage biological nutrient removal system (modified Bardenpho).

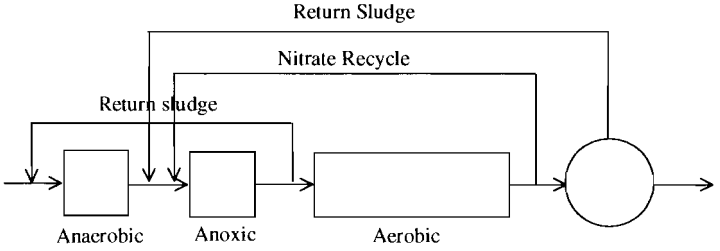


Figure 3b: University of Cape Town biological nutrient removal system.

More recently sequencing batch reactors (SBR) have been shown to offer several advantages over continuous systems for phosphorus removal (**21-24**). The configuration as shown in Figure 4 allows manipulation of interactions between anaerobic, anoxic and aerobic phases of the operating cycle in addition to providing a simple operating system.

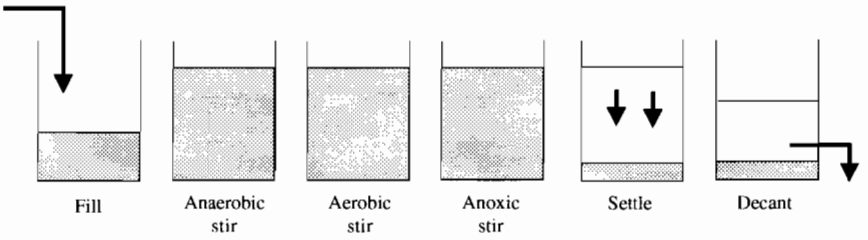


Figure 4. Sequencing batch reactor operation for carbon, nitrogen, and phosphorus removal.

Industrial applications

Much of the research and applications of EBPR have been directed to nutrient removal from domestic sewage, which contains relatively low concentrations of phosphorus. Industrial effluents such as those from dairy and other food industry contain high concentration of phosphorus. The following examples of research at the Centre for Environmental Technology and Engineering demonstrate the feasibility of application of EBPR principles to remove phosphorus from industrial effluents.

Dairy Processing Wastewater

EBPR was accomplished in both continuous and sequencing batch reactor systems to achieve a reduction of phosphorus concentration from about 90 mg/L to 60 mg/L. Figure 5a shows the performance of a continuous reactor operating in A/O configuration with 15-day solids retention time (SRT) and 2.5 d hydraulic retention time (HRT). The reactor received a pre-fermented feed with a 2L anaerobic zone and 18 L aerobic zone.

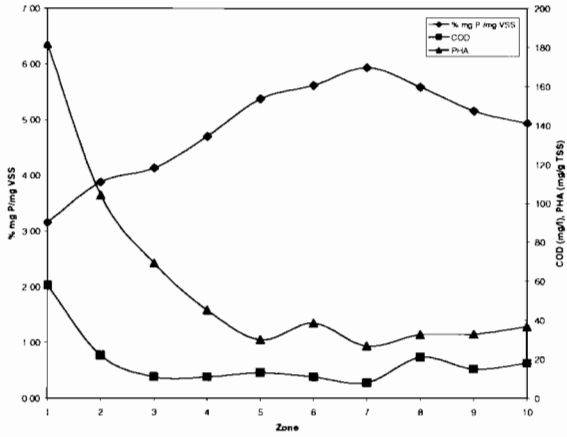


Figure 5a: EBPR in a continuous A/O reactor with prefermented feed.

The dairy effluent was also treated in a 10L sequencing batch reactor with a 12h cycle consisting of 3 h anaerobic, 3 h aerobic, 2.5 h anoxic and 2 h aerobic phases. These were followed by 1h settling and 0.5h decanting and feeding steps. The phosphorus removal from this system is shown in Figure 5b. The solids retention time was 20 days.

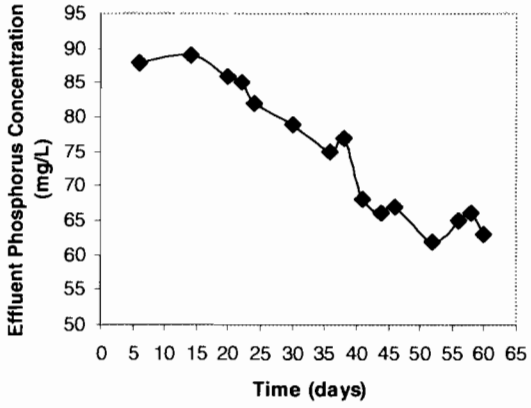


Figure 5b. EBPR in a sequencing batch reactor receiving high phosphorus wastewater.

Meat Industry Effluent

Combined COD, nitrogen and phosphorus removal from a meat industry effluent was carried out in a sequencing batch reactor. The 6h cycle consisted of 2h feeding/anoxic/anaerobic phase followed by 1h aerobic, 0.5h anoxic and 1.5h aerobic phases. The remaining 1h was used for settling and decanting. As shown in Figure 6, complete removal of phosphorus (<0.1 mg/L) was achieved.

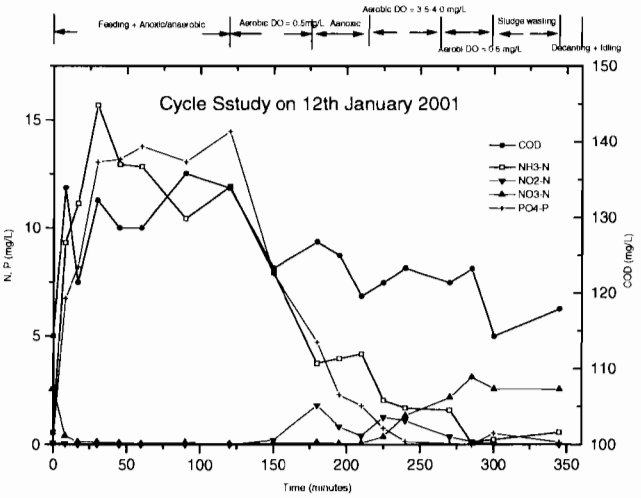


Figure 6: COD, Nitrogen and Phosphorus removed from meat industry effluents in a Sequencing Batch Reactor.

Conclusions

The practice of EBPR is widespread although the microbial ecology and biochemical mechanisms are not yet understood. Bardenpho and UCT processes form the basis for majority of EBPR systems currently in operation. The role of anaerobic environment is now recognised to be critical in achieving EBPR in the aerobic stage. Both batch and continuous systems can be applied to achieve EBPR. High phosphorus bearing effluents such as the dairy processing effluents can be treated to reduce phosphorus concentrations from over 90 mg/L to about 60 mg/L while almost complete removal of phosphorus is possible in case of low phosphorus bearing effluents such as meat industry effluents.

References

1. Eckenfelder Jr. W.W. (1989) *Industrial Waste Pollution Control.* McGraw-hill, p376.
2. Greenburg A.E., Levin G., Kaufman W.J. (1955) *Sew. Ind. Wastes.*, **27**, 227.
3. Srinath E.G., Sastry C.A., Pillai S.C. (1959) *Experientia*, **15**, 339.
4. Levin G.V., Shapiro J. (1965) *J. Water Pollut. Control Fed.*, **37**, 800.
5. Barnard J.L. (1976) *Water SA*, **2**, 3, 136.
6. Mino T., van Loosdrecht M.C.M., Heijnen J.J. (1998) *Water Res.*, **32**, 3193.
7. Fuhs G.W., Chen M. (1975) *Microbiol. Ecol.*, **2**, 119.
8. Florenz M., Hartemann P. (1984) *Environ. Technol. Lett.*, **5**, 457.
9. Suresh N., Warburg R., Timmerman M., Wells J., Coccia M., Roberts M.F., Halvorson H.O. (1985) *Wat. Sci. Technol.*, **17**, 99.
10. Buchan L. (1983) *Wat. Sci. Technol.*, **15** 87.
11. Brodish K.E.U., Joyner S.J. (1983) *Wat. Sci. Technol.*, **15**, 117.

12. Cloete T.E., Steyn P.L. (1987) Adv. Water. Pollut. Cont. R.Ramadori (ed.), pp. 335-338. Pergamon Press, U.K.
13. Auling G., Pilz F., Busse H.J., Karrasch S., Streichan M., Schon G. (1991) *Appl. Environ. Microbiol.*, **57**, 3685.
14. Wagner M., Erhart R., Manz W., Amann R., Lemmer H., Wedi D., Schleifer K.H. (1994) *Appl. Environ. Microbiol.* **60**, 792.
15. Kamfer P., Erhart R., Beimfohr C., Bohringer J., Wagner M., Amann R. (1996) *Microbiol. Ecol.*, **32**, 101.
16. Jenkins D., Tandoi V. (1991) *Water Res.*, **25**, 1471.
17. Mino T., Tsuzuki Y., Matsuo T. (1987) Adv. Water Pollut. Cont., R. Ramadori (ed.) pp 27-38. Pergamon Press, U.K.
18. Comeau Y., Hall K.J., Hancock R.E.W., Oldham W.K. (1986) *Water Res.*, **20**, 1511.
19. Wentzel M.C., Lötter L.H., Ekama G.A., Lowenthal R.E., Marais, G.V.R. (1991) *Water Sci. Tech.*, **23**, 567.
20. Barnard J.L. (1975) *Water Res.*, **9**, 485.
21. Kolarski R., Nyhuis G. (1995) *In*: 50th Purdue Ind. Waste Conf. pp 485-494.
22. Comeau Y., Lamarre D., Roberge F., Pervier M., Desjardins G., Hade C., Mayer R. (1996) *in*: Proc. 18th IAWQ Biennial Conference, June 23-28, Singapore, 140.
23. Danesh S., Oleszkiewicz J.A. (1997) *Water Sci. Tech.*, **35**, 137.
24. Stephens H.L., Stensel H.D. (1998) *Water Environ. Res.*, **70**, 362.

Chapter 21

ROLE OF MEMBRANE BIOREACTORS IN ENVIRONMENTAL ENGINEERING APPLICATIONS

C. VISVANATHAN, D. POKHREL

Urban Environmental Engineering and Management Program, Asian Institute of Technology, Po Box: 4, Klong Luang, 12120 Pathumthnai, Thailand.

Abstract

Replacement of conventional tertiary treatment components by membrane filtration was the first step of membrane application in water and wastewater treatment processes. Depletion of water resources, increasing water price and stringent regulation caused the development of various combination of membrane with other conventional treatment components. Membrane bioreactor is becoming one of such flourishing technology in water and wastewater treatment field. Researches are underway to find the most efficient and economical combination of biological and membrane processes for the purpose of water recycle and reuse. Based on the literature review and field experimental studies, submersible bioreactors are found economical compared with other combinations due to its low energy requirements and compact size. High energy saving, low F/M ratio, higher percentage of COD, BOD, nitrogen, pathogen removal are some of the advantages of membrane bioreactor.

Introduction

Rapid population growth, urbanization and industrialization have exerted enormous pressure on earth's natural resources. Water, although available in large quantities, is not uniformly distributed. Most of the available water is either unsuitable for use or difficult to extract. Over extraction of available water has caused various environmental problems of land subsidence, salt-water intrusion, leaching of pollutants into ground and surface water. For example, Bangkok is experiencing an average land subsidence of 150mm per year due to over pumping of ground water. Disposal of waste generated from various activities are adding pollutants to these water bodies making these sources unsuitable without advanced water treatment. Scarcity of water in Middle East countries has caused the introduction of advanced water treatment or desalination plants to purify water suitable for their use. On the other hand increase in potable water demand and sewage volume also creating pressure on existing potable water supply networks as well as the sewer systems and it is not that easy to implement any expansion to existing systems in densely built-up cities.

New Horizons in Biotechnology, 231-239, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

After the invention of biological treatment process as a standard wastewater treatment method in late 19th century (**1**), both aerobic and anaerobic biological treatment methods have become a common domestic and industrial wastewater treatment technology. The end products of both aerobic and anaerobic treatment processes are different. The final product of biological system, biomass, is separated from the final effluent in a settling tank.

The quality of the final effluent from conventional biological treatment systems depends on the hydrodynamic conditions in the sedimentation tank and the settling characteristics of the sludge. Consequently large volume sedimentation tanks offering resident times of several hours are required to obtain adequate solid/liquid separation (**2**), which increases the capital and operation and maintenance cost. At the same time close control of biological treatment unit is necessary to avoid the conditions, which lead to poor settleability and/or bulking of sludge.

Environmental awareness is continuously forcing governments to implement stringent effluent standards for wastewater to protect remaining water bodies. This forced the industries and municipal bodies to implement advanced wastewater treatment technologies. Physiochemical, chemical and biological treatment technologies alone are not been able to solve the problem. Scarcity of raw water sources, contaminated available sources, and stringent regulation supplemented by environmental awareness are some of the factors forcing the industries and water and wastewater treatment companies to look for a suitable advanced treatment technology for water treatment for potable use; and reuse and recycle of wastewater.

Some of the conventional advanced treatment technologies such as ion exchange, carbon adsorption etc. have their own disadvantages and cannot be considered reliable and long-term solution. In order to achieve, better effluent quality, membrane technologies are found effective and reliable technologies at present condition. The membrane offers a complete barrier to suspended solids and yields higher quality effluent. High effluent quality, compact size, easy to operate and maintain are some of the advantages. However, the disposal of retentate and fouling problem are some of the disadvantages. Presence of biodegradable organic compounds in contaminated water and wastewater has encouraged researchers to look for a technology, which can combine conventional biological processes and membrane processes.

Application of membrane separation (micro or ultra filtration) technique coupled with biological processes (known as Membrane Bioreactor-MBR) for biosolid separation can overcome the above disadvantages of the sedimentation tank and biological treatment step and problems with membrane separation processes. Although the concept (activated sludge process coupled with ultrafiltration) was commercialized in late 1960s by Dorr-Oliver (**3**) the application has only recently started to attract serious attention (Figure 1) and there has been a considerable development and application of membrane processes in combination with biological treatment over the last ten years.

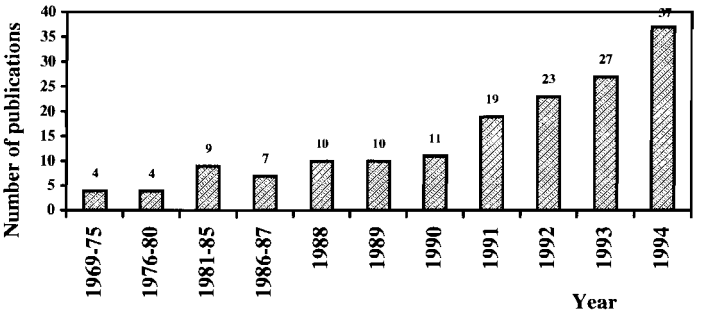


Figure 1. Number of Studies Published on MBR

Figure 1 : Number of studies published on MBR

The various advantages of MBR over conventional treatment processes are: production of excellent water quality, small foot print size of the treatment plant, reduced sludge production and the process reliability even at less attention. The objectives of this paper is to introduce the MBR concept; its development; role of MBR in Environmental Engineering applications especially water and wastewater treatment, reuse and recycle; and future research directions.

Membrane and Membrane Bioreactor Technologies

Membrane is a selective barrier that allows specific entities to pass through, while restricting the passage of others. They exhibit selective transport properties under the influence of an external driving force. Based on the pore size and its selectivity, membranes are classified as Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF) and Reverse Osmosis (RO). These membranes are used in water and wastewater treatment processes in order to attain the effluent of usable quality. Based on the requirement of final effluent quality, single, or a combination of different membranes is used in the processes. In wastewater treatment, use of microfiltration (for colloids/suspended solids removal) as a pretreatment followed by nanofiltration/reverse osmosis (for organic matter/salt removal) is normally practiced. The advantages of direct membrane separation are as follows.

- No biological systems involved, the operation can be shutdown readily and restarted when required without loss in water quality.
- Closed systems, breakthrough of odors and obnoxious smell can be eliminated.
- Process automation is easy thus the process control is much better.
- Product water quality does not suffer from feed water quality.

- Membrane technologies are very space efficient. A typical water reclamation facility consist of microfiltration and reverse osmosis could occupy as little as 400 m^2 area for a capacity of 2,400 m^3/d (**4**)

The second stage (NF/RO) operates at high pressure consuming high energy. The main objective of incorporating the second stage in the case of municipal wastewater is, removal of soluble organic matters. Combination of first stage with biological system can eliminate the second stage reducing significant amount of energy requirements. The benefits could be attained by this combination are stated bellow.

- Settleability of the sludge, which is the problem in conventional activated sludge, has absolutely no effect on the quality of the treated effluent. Consequently the system is easy to operate and maintain.
- Very long SRT can be maintained resulting in complete retention of slowly growing microorganisms, such as nitrifying bacteria, in the system.
- The overall activity level can be raised. In addition the system requires neither sedimentation nor any post treatment equipment to achieve reusable quality, so the space saving is enormous.
- Treatment efficiency is also improved by preventing leakage of undecomposed polymer substances. Dissolved organic substance with low molecular weights, which cannot be eliminated by membrane separation alone, can be taken up, broken down and gasified by microorganisms or converted into polymers as constituents of bacterial cells, thereby raising the quality of treated water.
- Removal of bacteria and viruses can be expected, so the disinfection process is ecologically sound.
- Maintaining low F/M ratio will produce less excess sludge to be handled and treated.
- No odor dispersion can occur.

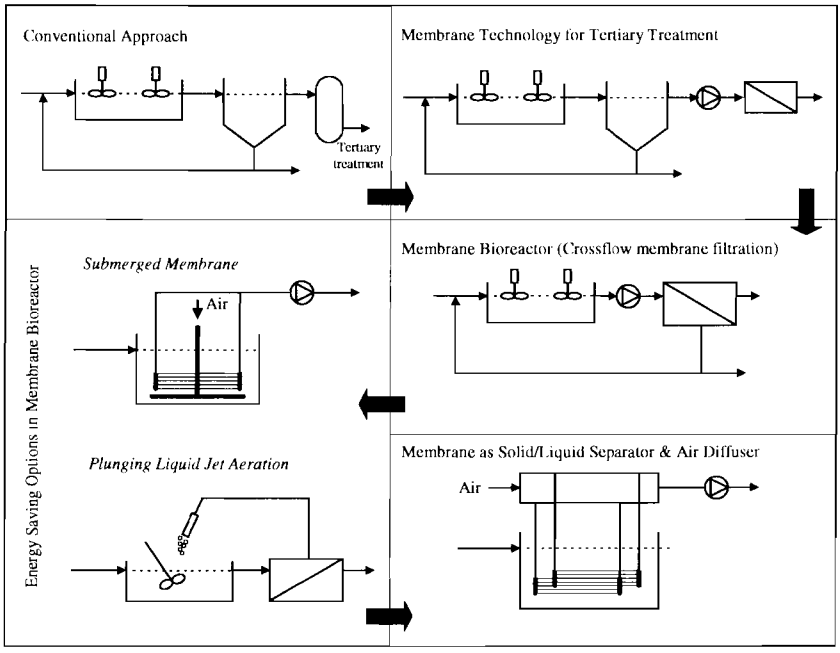
Membrane applications for wastewater treatment can be grouped into three major categories: a). biosolid separation, b). biomass aeration, and c). extraction of selected pollutants. Biosolid separation is most widely studied and has found full-scale applications in many countries.

Development of MBR:

Developments in membrane separation technology in biological wastewater treatment are schematically represented in Figure 2 (**5**). The conventional approach to get reusable quality water is by applying tertiary treatment techniques such as multimedia filtration, carbon adsorption, etc.

The first step of the development is the replacement of these tertiary treatment methods with membrane (ultra/micro) filtration, which ensures almost bacterial and viral free effluent in addition to colloids and solid removal (**6-8**).

In the effort to utilize membrane technology more effectively, the secondary sedimentation tanks in biological process were replaced with cross flow membrane filtration. These solid liquid separation bioreactors employ micro- or ultrafiltration modules for the retention of biomass. Chaize and Huyard (**9**) used this type of arrangement in a pilot plant study with completely mixed biological reactor connected to an ultrafltration module (MWCO=50,000 D) to treat domestic wastewater. In first run of the experiment (160 days, HRT=8h, SRT=100 days), reduction in COD from 250-550 mg/L to less than 30 mg/L; TKN from 65-150 mg/L to less than 10 mg/L was reported. The F/M ratio was found between 0.06 to 0.1 kg COD/kg MLSS.d. Second run of the experiment with different HRT and SRT (HRT= 8,4,2 h; SRT = 100,100, 50 days) did not produced any significant carbonaceous removal efficiency.



However higher energy cost to maintain the cross flow velocity led to the next development of submerging membranes in the reactor itself and withdrawing the treated water through membranes (**10**). The external circuit membrane can be outer- or inner skinned. However, the submerged membrane should be outer-skinned. A number of experimental studies were carried out on MBR with submerged membranes. More than 90% COD removal, 80% nitrate removal was reported by Yamamoto *et al.* (**10**) using 0.1 μm hollow fiber membrane used as submerged MBR. Most importantly, the power consumption was found to be as low as 0.007 kWh/m^3. Chiemchaisri (**11**) used 0.1 μm hollow fiber membrane module for

the treatment of low strength domestic wastewater under aerated and non aerated condition and different initial HRT of 1, 3 and 6 hours with intermittent extraction of 10:10 minutes operating time. The non-aerated bioreactor was found better over the aerated condition at an initial HRT of 3 and 6 hours due to low energy requirements to achieve similar effluent quality and process stability. The critical value of the flux was reported as 4.17 $L/m^2.h$.

Jet aeration in the bioreactor was studied in order to save more energy required for the aeration in biological systems (**12**). In this arrangement, membrane module is incorporated into the liquid circulation line for the formation of the liquid jet so that both the operation of aeration and membrane separation could be accomplished using only one pump. The jet aeration works on the principle that, a liquid jet after passing through a gas layer plunges into a liquid bath, entrain considerable amount of air. A pilot scale unit was installed and performance was investigated for 10:10 minute intermittent operation by Chiemchaisri *et al.* (**13**) using two numbers of hollow fiber membranes with pore size 0.03 μm and 9 m^2 surface area. The selected values jet aeration period was 0.5 and 1 hour, and jet aeration pattern was 15 and 30 minutes twice and once a day respectively. More than 90% nitrification was reported throughout the experiments. Some other studies conducted with jet aeration submerged bioreactors are : two hollow fiber membranes with 0.03 and 0.1 μm pore, 0.3 m^2 surface area; organic matter reduction > 85%; nitrification and denitrification >90% (**13**); hollow fiber modules for upgrading wastewater treatment plants; 96% COD and 95% total Kjeldahl nitrogen (TKN) removal (**14**).

Japanese researchers conducted study on submerged flat plate membrane "Kuboto". The system was able to remove almost 96% BOD and COD when tested with degritted sludge. The sludge production rate was reported to be 0.3 kg/kg BOD, approximately 40% of the normal ASP sludge production rate.

Use of two sets of membrane module immersed in reactor air back washing technique for membrane de-clogging was the next invention (Figure 2). In this technology, permeate is extracted through one module, the other one is supplied with compressed air for back washing. The cycle was repeated alternatively. Therefore there is continuous airflow into the aeration tank, which is sufficient enough to aerate the mixed liquor. Scott and Smith (**15**) used ceramic membrane (0.2 μm) in an external circuit for food processing industry wastewater and it was found to produce fine bubbles superior to traditional aerators. More than 95% of COD and BOD reduction was reported with influent COD and BOD of 13,33 mg/L and 6,50 mg/L respectively.

Visvsanathan *et al.* (**16**) used a 0.1 μm hollow fiber membrane module for domestic wastewater treatment and reported an improvement in flux by 370% in intermittent operation (15:15 minutes) compared to continuous operation although clogging was not completely eliminated. COD, TKN and P removal was found to be >90%, >90% and 50% respectively. Effects of TMP were also investigated and 13 kPa was reported to be the limiting pressure for all the experiments. Membrane modules are found better air diffusers than stone air diffusers by Parameshwaran *et al* (**17**) after an experiment with two hollow fiber microfiltraiton membrane (pore size 0.2 μm).

Irrespective of the operating conditions, in all experiments COD, BOD, TKN and total nitrogen removal of more than 95, 98, 95 and 80 % respectively were achieved.

Anaerobic MBR

Application of MBR in anaerobic wastewater treatment was also reported by some researchers. Removal of colloidal and suspended solids; increase in biomass concentration without increasing the reactor volumes; improvement in treatment efficiency; removal of toxins and higher methane production rates are some of the advantages of anaerobic MBR. Septic tank membrane system was studied by Grethlein (**18**). Flat sheet membrane module and helicore RO units were used in this experiment. Using 2:2 minute operating cycle, the system was able to produce effluent with very low (below detection limit) *E coli* and turbidity; 85-90% BOD removal (influent BOD=270 mg/L); 75% nitrate removal and excellent pH value (6.5 to 7.2).

Ross and Strohwald (**19**) conducted a comprehensive study on ADUF (anaerobic digestion-ultrafiltration) process for the treatment of different industrial wastewater in South Africa. The performances of AUDF plant was encouraging with around 95% COD removal. The operating parameters and some of the results of the ADUF is given in Table 1. Study on wheat starch wastewater, pulp and paper wastewater, high strength SS distillery wastewater, Brewery wastewater were also conducted by various researchers (**20-22**). In most of these studies significant amounts of COD removals (~90%) were reported.

Table 1: Mean Operating Criteria of ADUF Plants Treating Various Industrial Effluents.

Operating Criteria	Brewery	Wine Distillery	Maltin g	Egg Process	Maize Process
Volume of digester (m^3)	0.05	2.4	3.0	80	2610
Operational period (month)	3	18	5	8	36
Feed COD (kg/L)	6.7	37	3.5	8	4-15
Permeate COD (kg/L)	0.18	0.26	0.8	0.35	0.3
COD removal (%)	97	93	77	95	97
Space load rates (kg COD / m^3.d)	17.0	12.0	5.0	6.0	3.0
Sludge load rate (kgCOD/kg VSS.d)	0.7	0.58	0.5	0.33	0.24
HRT (day)	0.8	3.3	0.8	1.3	5.2
Temperature (^oC)	35	35	35	30	35
MLSS (kg/m^3)	30-50	50	10	10-30	23
Membrane area (m^2)	0.44	1.75	9.6	200	800
Flux (L/m^2.h)	10-40	40-80	20-40	15-30	10-70
Inlet pressure (kPa)	340	400	500	500	600
Crossflow velocity (m/s)	1.5	2.0	1.8	1.8	1.6
Tube diameter (mm)	9.0	12.7	9.0	12.7	9.0

From Ross and Strohwald (**19**).

Cost aspects of MBR

Membrane processes are considered to be expensive in terms of investment and operating cost. However cost of potable water and the disposal cost of wastewater supported by the stringent legislation lead to the full-scale application of MBR processes in Japan (**23**). Additionally, introduction of submerged membrane in MBR processes have contributed to reduce energy requirement significantly. Submerged membranes could reduce the pumping energy requirement merely to 0.007 kwh/m^3 of permeate (**10**) compared to more than 3 kwh/m^3 permeate required for crossflow mode. Based on the comparison made between ASP and MBR for small-scale wastewater treatment process by Visvanathan et al (**5**), MBR systems seem to be more attractive than ASP in terms of land and space requirements and energy consumptions.

Conclusion

Application of membrane bioreactors in water and wastewater treatment processes has brought a new revolution in environmental engineering. Problems associated with conventional biological and tertiary treatment systems are being solved by the application of MBR. Water treatment and wastewater reuse has become a major achievement of MBR due to its excellent effluent characteristics. Increasing production due to increasing demand has reduced the capital cost of membrane modules and low energy requirement and small land area requirements are the attraction of MBR. Continuous researches on different combination of biological processes with membrane are generating new and effective ideas of MBR combinations and it is becoming economical and easy to operate.

References:

1. Rittmann B. E. (1987) *Environmental Science and Technology* **21**(2), 128-136.
2. Fane A. G., Fell C. J. D. (1987) *Desalination* **62**, 117-136.
3. Smith C.W., Di Gregorio D., Talcott R. M. (1969) *In*: Proceedings of the 24th Annual Purdue Industrial Waste Conference, Purdue University, West Lafayette, Indiana, USA, pp. 1300-1310,
4. Johnson W. T., Phelps R. W., Beatson P. J. (1996) *in*: Proceedings of the "Water Reuse for the community and Industry - Latest Developments and Future Directions " Symposium, University of South Wales, Sydney, Australia, August 01,1996.
5. Visvanathan C., Ben Aim R., Parameshwaran K. (2000) *Critical Reviews in Environmental Science and Technology*. **30**(1), 1-48.
6. Pouet M.-F., Grasmick A., Horner G., Nauleau F., Cornier J.C. (1994) *Water Science and Technology* **30**(4), 133-139.
7. Langlais B., Denis Ph., Triballeau S., Faivre M., Bourbigot M. M. (1992). *Water Science and Technology* **25**(10), 219-230.
8. Kolega M., Gorhmann G. S., Chiew R. F., Day A. W. (1991). *Water Science and Technology* **23**, 1609-1618.
9. Chaize S., Huyard A. (1991) *Water Science and Technology* **23**, 1591-1600.
10. Yamamoto K., Hiasa H., Talat M., Matsuo T. (1989). *Water Science and Technology* **21**, 43-54.
11. Chiemchaisri C. (1990). Design Consideration of Membrane Bioreactor in Domestic Wastewater

Treatment, Master's Thesis, EV 90-97, Asian Institute of Tecnology, Bangkok, Thailand.

12. Yamagiwa K., Ohmae Y., Dahlan M. H., Ohkawa A. (1991). *Bioresource Technology* **37**, 215-222.
13. Chiemchaisri C., Yamamoto K., Vigneswaran S. (1993) *Water Science and Technology* **27**(1), 171-178.
14. Buisson H., Cote P., Praderie M., Paillard H. (1998) *Water Science and Technology*, **37** (9), 89-95.
15. Scott J. A., Smith K. L. (1997) *Water Research* **31**(1), 69-74.
16. Visvanathan C., Byung-Soo Y., Muttamara S., Maythanukhraw R. (1997) *Wat. Sci. Tech.* **36**(12), 259-266.
17. Parameshwaran K., Visvanathan C., Ben Aim R. (1998) *Journal of Environmental Engineering* (under consideration).
18. Grethlein H. E. (1978) *Water Pollution Control Federation* **50**(3), 754-763.
19. Ross B., Strohwald H. (1994). *Water Quality International*, **4**, 1994.
20. Kimura S. (1991). *Water Science and Technology* **23**, 1573-1592.
21. Nagano A., Arikaw, A., Kobayashi H. (1992). *Water Science and Technology* **26**(3-4), 887-895
22. Fakhru'l-Razi A. (1994). *Water Science and Technology* **30**(12), 321-327.
23. Aya H. (1994) *Water Quality International*, **4**.

Chapter 22

APPLICATION OF BIO-HYDROLIZER FOR THE TREATMENT OF UREA BEARING WASTEWATER

P.P. PATHE[1], T. NANDY[1], S.N. KAUL[1], C.V. DESHPANDE[1], L. SZPYRKOWICZ[2]

[1] *National Environmental Engineering Research Institute, Nagpur - 440 020, India;*
[2] *Department of Environmental Sciences, Venice University, Venice, Italy*

Abstract

Coal-based nitrogenous fertilizer industries in India produced mostly ammonia, urea, nitric acid, ammonium nitrate and ammonium bicarbonates using coal and fuel oil as raw materials. Coal is converted into carbon dioxide. Ammonia is manufacture from atmospheric nitrogen (air) and steam. Urea and ammonium bicarbonate are manufactured from carbon dioxide and ammonia. Ammonium nitrate is synthesized from nitric acid and ammonia. Therefore, the wastewater from fertilizer industry contains nitrogenous compounds viz, urea, ammonia, nitrate and bicarbonate. As urea cannot be oxidised by usual oxidation methods, microbiological hydrolysis of urea is necessary for removal of urea from wastewater. Biological stabilization of urea is a two staged process; (i) urea hydrolysis and (ii) ammonia stripping/nitrification-denitrification.

Hydrolysis of urea in the fertilizer industry is brought out by employing microbiological techniques, in which, ureolytic bacteria *Bacillus pasteurii* convert urea into ammonium carbonate as an intermediate product and then into ammonia and carbon dioxide as the end products. Ammonia thus produced is either stripped off by usual methods or after converting into nitrate using chemoautotraphic bacteria. *Nitrosomonas sp.*, which oxidizes ammonia into nitrate and further by *Nitrobacter sp.* into nitrate. On dentirification, nitrate is finally converted into nitrogen gas by means of heterotrophic bacteria.

Based on microbiological treatment technique, an additional unit of urea bio-hydrolyser at existing effluent treatment plant to treat-urea and ammonia bearing wastewater was designed, installed and operated at a fertilizer industry. In presence of ureolytic bacteria and paddy straw, urea from fertilizer industry wastewater was hydrolysed into ammonia and carbon-dioxide. Paddy straw works as a carbon source for microorganism. On air stripping of ammonia, nitrogen free treated wastewater is obtained by routing ammonia stripped wastewater along with septic tank sewage from industry and township through series of ponds cultured with algae. The final treated effluent confirmed standards prescribed by regulatory agencies for discharge of effluent into inland surface water. Evaluation of a full-scale effluent treatment plant was undertaken and modifications were suggested to

New Horizons in Biotechnology, 241-250, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

improve the quality of treated effluent. After incorporating the suggested modifications, post evaluation was carried out. The results of post evaluation studies are presented in this paper. The findings of low cost method of treating nitrogenous wastewater from fertilizer industry will be mostly useful in developing countries.

Key Words: Urea, ammonia, nitrogenous fertilizer industry, microbiological treatment, wastewater treatment, nitrification, denitrification, urea bio-hydrolysis.

Introduction

In India, the consumption of fertilizer nutrients per hectare has been steadily rising from merely 0.55 kg in 1950-51 to 68.00 kg in 1995. The production capacity of nitrogenous fertilizer in the country has increased from a modest capacity of 85, 000 tonnes in 1950-51 to 9.480 million tonnes in March, 1997, and the projected production by the end of 2004-05 is 9.940 million tonnes (**1,2**). Urea and ammonia are the major constituents of wastewater being generated from nitrogenous fertilizer industry, which manufacture ammonia and urea as the major products. The volume of wastewater from ammonia production generally varies from 4.9 to 6.7 m^3/tonne of ammonia produced with concentration of ammonia and urea varying from 4.65 to 10.0 and 15.0 to 17.5 kg/tonne of ammonia produced, respectively. Wastewater generation from urea plant varies from 8.0 to 12.0 m^3/tonne of urea produced depending on the quantity of raw material consumption, with ammonia and urea concentrations varying between 200-1500 mg/L and 340-20,000 mg/L, respectively (**3**).

Urea as such is non-toxic for human, animal and aquatic life even at high concentrations. The test fish- *Creek Chub* in the Detroit river were observed to survive even at higher dose of more than 1000 mg/L of urea (**4**). However, urea under favourable conditions hydrolyses to ammonia and carbon dioxide, and ammonia concentration of 1.2 to 3.0 mg/L is toxic to fish.

Evans *et al* (**5**) reported that 4 to 6 days are required for urea degradation at 20°C with urea concentration ranged between 1 to 15 mg/L and 14 days or more at a temperature lower than 8°C. This indicates that urea hydrolysis is a very slow process and can take place only in presence of urase, otherwise the hydrolysis may assume to be negligible.

Process of Nitrogen Removal

Nitrogen removal through microbiological treatment of wastewater containing urea involves the following unit processes:

Urea Hydrolysis

Urea hydrolysis is brought out by microorganisms mainly ureolytic bacteria *Bacillus pasteurii* as follows:

$$O=C\begin{matrix}NH_2\\NH_2\end{matrix} + H_2O \rightarrow (NH_4)_2CO_2 \rightarrow 2NH_3 + CO_2 + H_2O$$

Urea	Ammonium carbonate	Ammonia	Carbon dioxide

Ammonia and ammonium ions is aquous solution remain in equilibrium as follows:

$$NH_4^+ ======= NH_3 + H^+$$

At pH 7.0 and temperature 0 to 250°C, over 99% of ammonical nitrogen is in ammonium (NH_4^+) form. Autotrophs include the nitrifying bacteria which obtain energy from oxidizing inorganic nitrogen (chemoautotrophs) and algae, which obtain energy from sunlight (photoautotrophs).

Nitrification

Ammonia is biologically oxidised to nitrate in two stages process by two groups of chemoautotrophic bacteria, which operate in sequence. The first step is oxidising ammonia into nitrite by members of genus *Nitrosomonas sp.* Nitrite is further oxidised to nitrate by members of genus *Nitrobacter sp.* as follows

$$2NH4^+ + 3O2 \xrightarrow{\text{NitrosomonasSp.}} 2NO2 + 4H^+ + 2H2O + \text{Energy}$$

$$2NO2 + O2 \xrightarrow{\text{Nitrobacter Sp.}} 2NO3 + \text{Energy}$$

Energy released in this oxidation is used in synthesizing cell materials from carbon-di-oxide. It is evident from these equations that oxygen is required for nitrification process, which gives rise to oxygen demand in wastewater. Liberation of hydrogen ion tends acid production during nitrification, which may result in pH drop in poorly buffered wastewater, resulting in loss of process stability.

Denitrification

Denitrification of nitrate is brought out by heterotrophic bacteria first into nitrite and then to nitrogen gas. As the energy yield is lower than that from oxygen respiration, denitrification is effective only at low dissolved oxygen concentration. An external carbon source is necessary to obtain maximum reaction rate. Methanol can be used as one of the carbon source and the reaction can be represented as follows:

$$NO3 + CH3OH \rightarrow N2 + CO2 + H2O + OH^-$$

Urea bio-hydrolyser breaks urea molecule into ammonia and carbon dioxide with the help of ureolytic bacteria *Bacillus pasteurii.* After hydrolysis the ammonia produced is stripped off. The remaining ammonia nitrogen undergoes nitrification and nitrate is formed. Nitrate and ammonia are utilized by algae for growth.

Unutilised nitrate is converted into nitrogen gas after denitrification. Thus nitrogen free effluent is produced.

Brief Details of Nitrogenous Fertilizer Industry Under Study

The major products being produced at the coal based nitrogenous fertilizer industry were ammonia, urea, nitric acid, ammonium nitrate, ammonium bicarbonate, coke and tar with rated capacities of 900, 1000, 200, 35, 12, 325 and 10 tonnes/d, respectively.

The total water requirement of the fertilizer industry was around 79124 m^3/d which includes water for process, cooling and domestic purposes. Wastewater generation from the various unit processes of the industry were measured. The total wastewater generation which was in the range of 34608-37944 m^3/d. Table 1 presents the quantity of wastewater generated from the various units of the industry.

This paper deals with studies on a full-scale effluent treatment plant comprising of urea bio-hydrolyser unit and additional online lagoons in series cultured with algae to treat nitrogenous wastewater from a fertilizer industry.

Table 1: Quantity of Wastewater Generated from various Process Units.

Sr.No.	Process Unit	Quantity, m^3/day			
		Range			Average
1.	Oil Gasification	1656	-	1752	1680
2.	Rectisol	792	-	936	840
3.	DM				
	- Acidic	1344	-	1560	1440
	- Alkaline	864	-	1056	984
4.	Steam Generation	1584	-	1656	1608
5.	Compressor House	384	-	528	456
6.	Coal and handling house	384	-	576	480
7.	Urea	1800	-	1968	1896
8.	Coke Oven	1152	-	1296	1224
9.	Nitric Acid	360	-	528	432
10.	Ammonium Nitrate	288	-	432	360
11.	Ammonium Bi-carbonate	168	-	264	216
12.	Power Plant	9600	-	10800	10128
13.	Septic Tank Effluent	14322	-	14592	14424
	Combined Effluent	34608	-	37944	36168

Material and Methods

Full Scale Urea Bio-hydrolyser Plant

The nitrogenous fertilizer industry had a full-scale effluent treatment plant comprising of a urea bio-hydrolyser unit followed by lagoons in series. Urea bio-hydrolyser plant installed comprised of 16 compartments of different sizes and arranged in 8 horizontal rows. Total volume of all these compartments was 6012 m^3 with detention period of 90 hours.

Pressurised steam was introduced at the bottom of compartment Nos. 11,12, 13,14,15 and 16 for stripping of ammonia produced during urea hydrolysis. In addition the treated effluent from hydrolyser plant was routed through cascade aeration system to reduce ammonia concentration in the effluent and further treated through 10 lagoons in series. Ammonia stripped effluent was received in lagoon 1 and after routing through other lagoons was being finally discharged into lagoon 9. Lagoon 9 has a holding capacity of 1,00,000 m^3. This lagoon also received combined wastewater from other process units except steam generation and power plant. Septic tank effluent from industry and township was also routed through the ETP and was being discharged into lagoon 4. Total detention time of all these lagoons was 25 days.

Microbial culture produced during pilot plant studies was used for startup of the urea bio-hydrolyser. Chopped paddy straw of 10 to 50 mm size was used as the carbon source for microorganisms. As urea-bearing wastewater was phosphate deficient super phosphate was added in requisite doses. Algae *Chlorella* was cultured in lagoon 5,6,7 and 8 to utilize ammonia.

The performance of the urea bio-hydrolyser unit and effluent treatment plant was monitored. Wastewater samples were collected from each compartment of the urea hydrolyser plant at one-hour intervals over a period of 8 hours for 7 days. The samples were analysed for pH, TDS, SS, total ammonical nitrogen, free ammonical nitrogen, total kjeldhal nitrogen, nitrite nitrogen, nitrate nitrogen as per Standard Methods (**6**). Urea was analysed as per method described by Watt and Chrisp (**7**). Characteristics of inlet and outlet wastewater from each compartment of urea bio-hydrolyser are presented in Table 2. Hourly samples from each lagoon were also collected over a period of 8 hours for 7 days and analysed for usual parameters. Characteristics of influent and effluent from urea bio-hydrolyser and effluent characteristics from various lagoons are in Table 3.

The characteristics of combined final treated effluent from the fertilizer industry being discharged into river are presented in Table 4.

Results and Discussion

The wastewater from urea and ammonia sections having urea concentration ranging between 1048 to 1410 mg/L was reduced to 108 to 212 mg/L in the urea bio-hydrolyser unit of the ETP. Around 85 to 90 percent urea hydrolysis was achived. The reaction of urea was brisk upto compartment No. 6 reducing around 70 to 73 percent urea in first 30 hours. The remaining 15 to 17 percent urea was reduced in the remaining ten compartments of the hydrolyser plant. The treated effluent from urea bio-hydrolyser contained ammonia nitrogen ranging between 272 to 336 mg/L, removing around 61.3 to 62.9 percent of free ammonia.

Table 2: Characteristics of Influent and Effluent from each Compartment of Urea Bio-hydrolyser Plant.

Effluent Type	Parameters									
	Temp, °C	pH	TDS	SS	Total Ammo-nical-N	Free Ammo-nical-N	Total Kjeldhal-N	Urea	Nitrite (NO_2)-N	Nitrate (NO_3)-N
Compartment No.1 Influent	24 ±3	10.1 ±0.3	1726 ±120	126 ±18	975 ±55	794 ±106	1840 ±168	1230 ±182	0.9 ±0.10	2.0 ±1.0
Compartment No.1 Effluent	25 ±3	10.1 ±0.3	1694 ±104	794 ±76	984 ±64	804 ±101	1794 ±152	1200 ±176	1.28 ±0.10	2.1 ±1.1
Compartment No. 2 Effluent	25 ±3	10.1 ±03	1538 ±102	748 ±84	996 ±58	864 ±94	1678 ±152	1200 ±176	1.28 ±0.11	2.2 ±1.1
Compartment No. 3 Effluent	25 ±3	10.1 ±0.3	1420 ±96	680 ±92	1034 ±68	936 ±88	1476 ±136	854 ±122	1.36 ±0.11	2.3 ±1.1
Compartment No. 4 Effluent	25 ±2	10.2 ±0.2	1392 ±90	656 ±74	1082 ±78	996 ±92	1282 ±140	628 ±108	1.40 ±0.14	2.4 ±1.2
Compartment No. 5 Effluent	25 ±2	10.2 ±0.2	1356 ±92	632 ±68	1132 ±80	1074 ±86	1204 ±132	440 ±82	1.46 ±0.12	2.5 ±1.2
Compartment No. 6 Effluent	25 ±2	10.3 ±0.2	1318 ±91	600 ±52	1158 ±84	1092 ±90	1196 ±124	356 ±64	1.52 ±0.10	2.6 ±1.2
Compartment No. 7 Effluent	25 ±2	10.3 ±0.2	1250 ±66	580 ±48	1004 ±92	978 ±84	1042 ±116	334 ±58	1.64 ±0.11	2.7 ±1.1
Compartment No. 8 Effluent	25 ±2	10.3 ±0.2	1222 ±78	528 ±50	820 ±68	798 ±70	886 ±110	310 ±50	1.76 ±0.13	2.7 ±1.2
Compartment No. 9 Effluent	25 ±3	10.2 ±0.2	1204 ±62	462 ±38	806 ±52	728 ±46	865 ±112	294 ±56	1.88 ±0.12	2.8 ±1.2
Compartment No. 10 Effluent	25 ±3	10.2 ±0.2	1178 ±72	370 ±42	712 ±46	641 ±48	830 ±98	278 ±52	2.06 ±0.14	2.9 ±1.1
Compartment No. 11 Effluent	25 ±3	10.1 ±0.2	1144 ±64	296 ±38	604 ±50	558 ±42	782 ±84	264 ±64	2.18 ±0.18	3.0 ±1.1
Compartment No. 12 Effluent	25 ±3	10.1 ±0.2	1109 ±70	252 ±32	534 ±38	496 ±44	622 ±76	238 ±56	2.26 ±0.20	3.2 ±1.1
Compartment No. 13 Effluent	24 ±3	10.0 ±0.2	1090 ±62	224 ±36	478 ±32	442 ±40	572 ±64	222 ±60	2.56 ±0.24	3.5 ±1.2
Compartment No. 14 Effluent	24 ±3	10.0 ±0.2	1058 ±74	208 ±32	414 ±30	394 ±42	500 ±58	196 ±70	2.64 ±0.26	3.7 ±1.2
Compartment No. 15 Effluent	24 ±3	10.0 ±0.2	1028 ±68	196 ±24	382 ±34	346 ±36	442 ±56	178 ±62	2.66 ±0.28	3.8 ±1.2
Compartment No. 16 Effluent	24 ±3	10.0 ±0.2	994 ±60	138 ±16	346 ±32	304 ±32	400 ±52	160 ±52	2.70 ±0.30	4.0 ±1.2

All values except pH and temperature are in mg/L;
Hourly sample collection over a period of 8 hours for 7 days

Table 3: Characteristics of Influent and Effluent from Urea Bio-hydrolyser Plant and Effluent Characteristics of various Lagoons at ETP.

Effluent Type	Parameters														
	pH	TDS	SS	DO	COD	BOD	Total Ammonical N	Free Ammonical N	Total Kjeldahl N	Urea	Nitrite (NO_2)-N	Nitrate (NO_3)-N	Oil & grease	Total chromium (Cr)	Cyanide (CN)
Urea Bio-hydrolyser Influent	10.1 ±0.3	1726 ±120	126 ±18	ND	332 ±56	200 ±25	975 ±55	794 ±106	1840 ±168	1230 ±182	0.9 ±0.10	2 ±1	45 ±10	ND	ND
Urea Bio-hydrolyser Effluent	10.0 ±0.2	994 ±60	138 ±16	0.5 ±0.1	258 ±48	145 ±20	346 ±16	304 ±32	400 ±52	160 ±52	2.70 ±0.30	4 ±1	40 ±8	ND	ND
Lagoon 1 Effluent	9.9 ±0.2	750 ±22	100 ±8	0.6 ±0.2	216 ±32	105 ±16	316 ±20	282 ±30	382 ±48	142 ±46	4.40 ±0.8	4 ±1	34 ±9	ND	ND
Lagoon 2 Effluent	9.7 ±0.3	700 ±24	74 ±6	1.1 ±0.2	186 ±36	85 ±20	288 ±22	264 ±28	367 ±44	104 ±32	6.90 ±0.6	5 ±1	32 ±8	ND	ND
Lagoon 3 Effluent	9.6 ±0.3	644 ±16	53 ±7	1.3 ±0.3	158 ±24	58 ±13	256 ±28	240 ±24	350 ±38	56 ±16	6.80 ±0.7	5 ±1	30 ±7	ND	ND
Lagoon 4 Effluent	9.6 ±0.4	604 ±20	35 ±4	2.0 ±0.3	102 ±12	32 ±6	234 ±24	212 ±22	371 ±34	32 ±8	8.15 ±0.85	5 ±1	25 ±5	ND	ND
Lagoon 5 Effluent	9.6 ±0.3	584 ±16	25 ±3	2.5 ±0.4	62 ±10	28 ±4	208 ±22	194 ±20	320 ±30	20 ±6	9.35 ±0.85	6 ±1	19 ±3	ND	ND
Lagoon 6 Effluent	9.5 ±0.4	542 ±14	17 ±3	3.1 ±0.3	50 ±7	20 ±2	184 ±20	160 ±16	250 ±26	12 ±5	11.50 ±0.5	7 ±2	15 ±2	ND	ND
Lagoon 7 Effluent	9.5 ±0.3	504 ±10	10 ±2	3.6 ±0.3	38 ±5	15 ±2	162 ±22	124 ±16	198 ±25	8 ±4	12.20 ±0.9	9 ±2	11 ±2	ND	ND
Lagoon 8 Effluent	9.4 ±0.3	480 ±12	7 ±2	4.0 ±0.3	34 ±6	11 ±3	138 ±20	82 ±18	132 ±24	5 ±2	14.00 ±1.0	10 ±3	7 ±2	ND	ND
Lagoon 8A Effluent	9.0 ±0.3	462 ±18	5 ±2	4.9 ±0.2	30 ±5	7 ±2	112 ±20	60 ±16	108 ±20	2 ±1	16.75 ±0.75	12 ±3	5 ±1	ND	ND
Factory combined Effluent to Lagoon 9	7.6 ±0.7	952 ±96	140 ±8	1.9 ±0.2	284 ±54	130 ±18	85 ±16	42 ±12	88 ±16	1 ±1	8.95 ±0.95	45 ±8	13 ±2	0.07 ±0.02	0.10 ±0.03
Lagoon 9 Effluent	8.1 ±0.2	946 ±84	36 ±6	4.7 ±0.3	42 ±8	14 ±4	76 ±6	7 ±2	52 ±12	ND	13.10 ±2	15 ±1	7 ±0.01	ND	0.03

ND - Not Detectable

All values except pH are in mg/L

Table 4: Characteristics of Combined Treated Effluent discharged into River.

Sr.No.	Parameter	ETP Combined Treated Effluent	Standard for Treated Effluent (Inland Surface Water)	
			MEF*	SPCB#
1.	pH	7.5-8.0	5.5-9.0	6.5-8.0
2.	Total Dissolved Solids	750±72	-	-
3.	Suspended Solids	46±14	100	100
4.	DO	4.8-5.2	-	-
5.	COD	54±18	250	250
6.	BOD_5d,20°C	16±5	30	30
7.	Total Ammonical-N	40±5	50	75
8.	Free Ammonical-N	2.3±0.3	5	4
9.	Total Kjelhdal-N	52±12	100	100
10.	Nitrite (NO_2)-N	7.2±0.4	-	-
11.	Nitrate (NO_3)-N	9.0±0.8	10	10
12.	Urea	ND	-	-
13.	Total Phosphate	0.08±0.3	5	-
14.	Total Phenolics	0.3±0.1	1	-
15.	Cyanides	0.02±0.01	0.2	-
16.	Fluorids	ND	2.0	-
17.	Oil & grease	4.6±2.1	10	10
18.	Heavy Metals			
	- Iron (Fe)	0.52±0.08	3	3
	- Lead (Pb)	0.002±0.001	1	1
	- Zinc (Zn)	0.11±0.04	5	5
	- Nickel (Ni)	ND	3	3
	- Chromium (Cr)	ND	2	2

ND- Not Detectable

All values except pH are in mg/L

* Ministry of Env & Forests, Govt. of India; # State Pollution Control Board

The urea bio-hydrolyser unit was designed to accept initial urea concentration up to 6000 mg/L and to hydrolyse 90 to 95 percent urea from urea bearing wastewater. Paddy straw served as carbon source to ureolytic bacteria *Bacillus pasteurii* and algae utilized ammonia nitrogen for their growth. Urea is being hydrolysed into ammonia which stripped off by steam and also utilized by algae in lagoons. Up to compartment No.6 a constant increase in ammonia concentration and constant

decrease in urea concentration indicated brisk reaction of urea hydrolysis. However, the concentration of ammonia in these compartments showed lower values as compared to stoichiometric values, due to ammonia stripping at high wastewater pH. At pH above 8.6, ammonia is released from ammonia solution into the atmosphere. Thus, by maintaining high pH, ammonia stripping was achieved. Urea bio-hydrolyser removed about 61-63 percent ammonia nitrogen due to ammonia stripping. The reduction in dissolved solids in the effluents from different compartments indicates the release of ammonia nitrogen into atmosphere. Increase in concentration of suspended solids in the effluent was mostly due to addition of chopped rice straw which floats on water surface and escape through effluent.

The insignificant increase in nitrite and nitrate concentrations in effluent from various compartments of urea bio-hydrolyser indicate that nitrification of ammonia was not taking place. The slow build-up of nitrite and nitrate concentration in the lagoons indicated nitrification of ammonia during wastewater stabilization. The urea concentration in lagoon effluent was reduced due to the presence of ureolytic bacteria present in septic tank effluent from the industry and township being discharged into lagoon 4. Reduction of ammonia nitrogen was also obtained due to utilization of ammonia by algae *Chlorella*, which were present by virtue of addition of algal culture in these lagoons. Algae utilized ammonia and other nitrogen species for cell synthesis and growth. The concentration of COD and BOD also reduced significantly. The characteristics of final treated effluent being discharged into river conformed to the discharge standards prescribed by regulatory agencies into inland surface water.

Conclusion

Based on the studies on full scale urea bio-hydrolyser plant to treat urea bearing wastewater from a fertilizer industry it was concluded that ureolytic bacteria *Bacillus pasteurii* could hydrolyse 85 to 90 percent urea from wastewater. Urea hydrolysis process was brisk at initial period of 30 hours, hydrolysing around 70 to 73 percent urea in the first six compartments of the urea hydrolyser plant. Ammonia nitrogen produced during urea bio-hydrolysis could be stripped off at pH 10.2±0.2 without applying mechanical means. Injecting pressurised steam at the bottom of latter compartments facilitated 44 to 48 percent ammonia removal by stripping. Paddy straw, an agricultural residue could profitably be used as a carbon source for microorganisms as compared to costly chemicals like methanol and other organics. Ammonia nitrogen trapped in the form of inorganics further nitrified with help of microorganisms into nitrate via nitrite in lagoons. Algae such as *Chlorella*, present in lagoons, reduce ammonia nitrogen and nitrate nitrogen from the wastewater. Nitrate nitrogen was denitrified by microorganisms into nitrogen gas to obtain nitrogen free wastewater. BOD and COD were also reduced in the lagoons.

Urea bio-hydrolyser cum stripper reduced significantly the concentration of urea and ammonia in the wastewater from the nitrogenous fertilizer industry to conform

standards prescribed by regulatory agencies for discharge of treated effluent into inland surface water.

References

1. India (1995) "A reference annual", Published by Ministry of Information and Broadcasting, Government of India pp. 545-546.
2. Monthly Review of Indian Economy (1997), Economy Intelligence Service series, Published by Centre for Monitoring Indian Economy (CMIE) Pvt. Ltd., February 1997, pp. 58.
3. IS: 9841 (1981) "Guide for Treatment and Disposal of Fertilizer Industry", Indian Standards Institution, New Delhi.
4. Gillette L.A., Miller D.L., Redman H.E. (1952) *Sewage and Industrial Waste*, **24**, 1397.
5. Evans W.H., David E.J., Patterson S.J. (1973) *Water Res.*, **7**, 975.
6. "Standard Methods for the examination of water and wastewater" (1989) 17th Edition, APHA, AWWA, WPCF, Washington, DC.
7. Watt G.W., Chrisp J.D. (1954) *Anal. Chem.* **26**, 452.

Chapter 23

BIOSORPTION AND ELUTION OF CR(VI) : A DETOXIFICATION STRATEGY

T. SRINATH[1], S. K. GARG[2], P.W. RAMTEKE[3*]

[1] *Environmental Microbiology Section, Industrial Toxicology Research Centre, Lucknow-226001, India;* [2] *Department of Microbiology, Dr. R. M. L. Avadh University, Faizabad 224 001, India;* [3]*Dept. of Chemistry, Allahabad Agricultural Deemed University Allahabad-211007, India. E-mail: pwramteke@123india.com*

Abstract

Biosorption of Cr(VI) from solution was studied using pH-conditioned biomass of *Bacillus coagulans*. Effect of several environmental factors on metal uptake was characterised. Biosorption of Cr(VI) was found to be influenced by the pH of the solution. As the pH of metal solution decreased, a rise in the biosorption could be noticed, which peaked at pH 2.5 and declined on further pH decrease. Equilibrium metal sorption was found to fit the Langmuir adsorption model, and the 'best fit' parameter for Cr(VI) biosorption at pH 2.5 and 28°C was determined to be Q_{max} = 62.11 mg/g and b = 0.071 L/mg. The metal biosorption process was not affected by contact time and the system attained equilibrium in 60 min. Increase in incubation temperature resulted in a marginal increase in biosorption of Cr(VI). Biosorption of Cr(VI) was not affected by presence of heavy metals, except for lead, which resulted in a maximum of 11.81% decrease in the presence of 200 mg lead/L solution. A 1.0 M H_2SO_4 solution could elute chromium loaded on *B. coagulans* biomass and regenerate biomass for another cycle of Cr(VI) biosorption.

Index entries: *Bacillus coagulans*, biomass regeneration, biosorption, chromium (VI), elution, heavy metals.

Introduction

The discovery of usage of chromium for a better tanning of leather and addition of metallic chromium to steel could produce corrosion resistant steel set the stage for the usage of large quantities of chromium in many products. Hexavalent chromium [Cr(VI)], ranging from 40 to 270,000 mg/L, was introduced into aqueous systems through discharges from tanning, iron and steel factory, electroplating, metal finishing, paints and pigments, chromate preparation, dyeing, textile and fertilizer industries (**1,2**). Unregulated disposal of the chromium containing effluent of these units at developing and developed countries has led to the contamination of soil, sediment, surface and ground waters (**3,4**).

New Horizons in Biotechnology, 251-265, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

In trace amounts, chromium is considered an essential nutrient for numerous organisms but at elevated levels it is toxic and mutagenic (**5**). In India, there are around 3000 tanneries employing over 2.5 million people (**6**). Nearly 80 % of them are engaged in the chrome tanning process. Around 80 million hides and 130 million skin pieces are processed yearly and yield more than Rs. 7000 crores of foreign exchange, which is about US $ 1560 million. Around 50 L of effluent is generated per kg of skin/hide processed. In India, the leather, iron and steel industries put together discharge more than 50% of the toxic pollutant (**7**). Standard limit for discharge of Cr(VI) in the inland surface waters is 0.1 mg/L in India (IS: 2296 and IS: 2490) (**8**) and 0.05 mg/L in United States (**9**).

The methods developed for detoxification of Cr(VI) ions include reduction and precipitation, ion exchange and adsorption on activated carbon. However, in developing countries, none of these methods have achieved economic viability. Few industrial setups treat chromium-rich wastewater by conventional aerobic or anaerobic treatment plants but during the last step of the treatment, it is difficult to conciliate the desired achievement of low metal concentration with economic and technical constraints. Furthermore, disposal of heavily metal-laden sludge disposal itself is a problem. To meet these requirements, new processes are under development. It has been suggested that the biomass could be used to detoxify metal-bearing wastewater and to concentrate metals (**10,11**). A broad range of biosorbent materials like cyanobacteria and microalgae (**12**), marine algae (**13**), several bacterial species (**14,15**), fungi (**16,17**), yeast and filamentous bacteria (**18**) were studied for their potential to remove the heavy metals from the solutions and found to be optimistic for treatment of wastewater/effluents, being selective in nature (**19**).

The objective of the present work was to describe the biosorption performance of the pH-conditioned biomass of *Bacillus coagulans* in batch scale experiments at various environmental conditions as a function of biosorption of Cr(VI). As effluents contain several heavy metals, effect of various heavy metals on Cr(VI) uptake was carried out. Another objective of this work was to examine the elution of bound Cr(VI) to make the detoxification process economical by conservation of this metal as well as regeneration of biomass for another cycle of Cr(VI) biosorption.

Materials and Methods

Growth and preparation of biomass

Bacillus coagulans, one of the isolates from tannery effluent (**20**) was maintained on nutrient agar slants. Biomass of *B. coagulans* was added to 100 mL of peptone water (peptone 1.0%; NaCl 0.5%) (HiMedia, Mumbai, India) in 500 mL conical flasks and incubated on a shaker at 150 rpm for 24 h at 28°C. The cells grown to late exponential phase were harvested by centrifugation (C 24, REMI, Mumbai, India) at 10,000 rpm for 30 min at 4°C and washed thrice with deionized water. The

biomass obtained was pH-conditioned with repeated washing with deionized water (acidified to desired pH by H_2SO_4) until the pH of the washed water showed no change. This pretreatment minimizes the change in the solution pH after biomass addition. Biomass was then dried in an oven (80°C) and later crushed in a blender and sieved through a 14-16 British standard size mesh (1.2-1.6 mm).

Metal solutions

A standard stock solution containing 10,000 mg/L of Cr(VI) was made in deionised water by dissolving an appropriate amount of $K_2Cr_2O_7$ (AR grade; Qualigens, Mumbai, India). The following metal salts (AR grade; Qualigens, Mumbai, India) were used to prepare their aqueous stock solutions (1000 mg/L): $CdCl_2$, $CuSO_4.5H_2O$, $Ni(NO_3)_2.6H_2O$, $Pb(NO_3)_2$ and $ZnSO_4$. Stock solutions were diluted with deionised water to obtain working solutions. Solutions were adjusted to desired pH values with 0.1M NaOH and 0.1M HNO_3.

Chromium(VI) uptake studies

A batch equilibrium method was used to determine the biosorption of Cr(VI) by pH-conditioned biomass of *B. coagulans*. A set of 150 mL Erlenmeyer flasks containing 25 mL of Cr(VI) was used in experiments. The biomass (50 mg) was contacted with Cr(VI) working solution at 150 rpm on an orbital shaker at 28°C for 1 h. The experiment was run in duplicate with two types of controls, metal-free and biomass-free solutions. The biomass was separated by centrifugation at 10,000 rpm for 10 min at 4°C and washed twice with deionized water. The biomass thus obtained was dried at 80°C in an oven and weighed. The biomass and supernatant were digested with a mixture of concentrated nitric (six parts) and perchloric (one part) acid and analysed on an atomic absorption spectrophotometer (Perkin Elmer-5000, Norwalk, USA) to quantify the biosorbed and residual chromium content, respectively.

Adsorption isotherms and other calculations

In order to obtain sorption kinetics data, the chromium uptake value was calculated using the following equation:

$$Q_{eq} = V (C_i - C_{eq})/M$$

Where, Q_{eq} is the biosorption capacity (mg/g biomass), V is the volume of solution (L), C_i is the initial concentration of metal in solution (mg/L), C_{eq} is the residual concentration of metal in solution (mg/L), and M is the weight of biomass (g).

The Q_{eq} value thus obtained was used to plot Freundlich and Langmuir isotherms. The Freundlich isotherm, used to describe the adsorption of solutes from solutions, can be given by:

$$\log Q_{eq} = \log K + (1/n) \log C_{eq}$$

where, K (biosorption capacity) and 1/n (adsorption intensity) are Freundlich constants. This equilibrium equation should be linearised plotting $\log C_{eq}$ vs $\log Q_{eq}$ to determine the Freundlich constants from slope (1/n) and intercept (log K).

Unlike the empirical Freundlich isotherm, the Langmuir isotherm has a theoretical basis, which relies on a postulated chemical or physical interaction between solute and vacant sites on the adsorbent surface. The Langmuir isotherm model has the following equation:

$$C_{eq}/Q_{eq} = 1/(Q_{max} \times b) + C_{eq}\,(1/Q_{max})$$

where, Q_{max} is the maximum amount of metal biosorbed per weight of biosorbent (mg/g) and b is a ratio of adsorption rate constant to desorption constant (L/mg), related to the energy of adsorption and is also indicator of affinity of metal to binding sites on biosorbent. This equilibrium equation also can be linearised by plotting C_{eq} vs C_{eq}/Q_{eq} to determine the Langmuir constants from the slope ($1/Q_{max}$) and the intercept ($1/Q_{max} \times b$).

The specific surface area (S_s) of the biosorbent was calculated using following expression *(**21**)*:

$$S_s = (Q_{max} \times N \times A_m) \times 10^{-20} / MW$$

Where S_s is the specific surface area of the biosorbent (m^2/g), Q_{max} is the value obtained from Langmuir isotherm (g/g), N is the Avogadro's constant (6.02×10^{23}), A_m is the ionic cross-sectional area of solute ($(Å)^2$) and MW is the molecular weight of the solute. For $HCrO_4^-$, MW= 117 and $A_m = 18.4\ (Å)^2$ *(**21**)*.

The essential feature of the Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor (or equilibrium parameter, R), which is defined by the following relationship *(**22**)*:

$$R = 1/[1+(b \times C_i)]$$

Where b is the Langmuir constant and C_i is the initial concentration of Cr(VI) (mg/L). If $0<R<1$, then the separation factor is favourable for Cr(VI) removal.

Effect of pH

To check the effect of pH on biosorption, the biomass of *B. coagulans* was conditioned to different pH (ranging between 1.0 to 5.5) prior to addition into Cr(VI) solution at the respective pH.

Effect of initial metal concentration

Metal solution (25 mL) of varying concentrations of Cr(VI) (ranging from 10 to 500 mg/L) adjusted to pH values 2.0, 2.5 and 3.0, were treated with biomass (50 mg). Data obtained with respect to the effect of initial metal concentration on metal biosorption were applied to the widely used Langmuir and Fredulich isotherms.

Effect of biomass concentration

The effect of biomass concentration (2 to 10 g/L) was checked by exposing biomass (50 to 250 mg) to 25 ml of Cr(VI) solutions (100 mg/L) at the optimum pH 2.5.

Effect of temperature

The pH-conditioned biomass (50 mg) of *B. coagulans* was exposed to 25 ml of Cr(VI) solution (100 mg/L) in flasks incubated at different temperatures (20 to 40 °C) in an incubator shaker at a speed of 150 rpm for 60 min. Metal biosorption at different temperatures was determined by estimating the residual concentration of Cr(VI) in solution.

Effect of contact time

To examine Cr(VI) biosorption with respect to contact time, 100 mg of biomass was contacted with 50 mL of Cr(VI) solution in 100 mL Erlenmeyer flasks. Experiments were performed in duplicate along with appropriate controls run simultaneously. Flasks were incubated on a rotary shaker at room temperature. Samples of metal solutions withdrawn from each flask at different time intervals (5-240 min) and analysed for residual chromium concentration.

Effect of other heavy metals

The effect of other heavy metals on Cr(VI) biosorption by biomass of *B. coagulans* was checked in binary as well as multiple metal systems. Biomass (50 mg) was exposed to 25 ml binary/multimetal solutions containing 100 mg Cr(VI)/L and cadmium, copper, lead, nickel, zinc and iron (25 to 200 mg/L) at pH 2.5. Samples of metal solutions were withdrawn after 60 min and analysed for residual chromium concentration.

Biosorption-elution studies

Samples (2 g) of *B. coagulans* biomass loaded with chromium (39.89 mg/g) were used in these experiments. The eluants (50 mL) employed were 0.1 and 1.0 M HNO_3 and H_2SO_4. After 24 h, 25 mL of the eluants were separated from the biomass and the chromium content eluted was analysed by atomic adsorption spectroscopy. Following the elution, the biomass was washed with 0.1 M $CaCl_2$ + 0.1 M $MgSO_4$ for 15 min (**11**). Then the biomass was reconditioned by repeated washing with deionised water, acidified to pH 2.5 by H_2SO_4. This biomass was reused in subsequent biosorption-elution cycles.

Results and Discussion

Effect of pH on Cr(VI) biosorption

To find the optimum conditions, biosorption of Cr(VI) by *B. coagulans* biomass was studied at solutions of varying pH levels. As shown in Fig. 1, the pH of solution had a significant effect on Cr(VI) biosorption.

As shown in Fig. 1, the pH of solution had a significant effect on Cr(VI) biosorption.

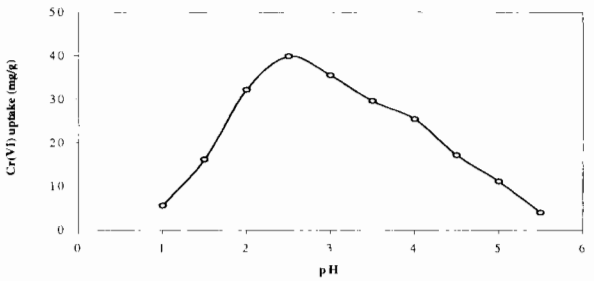


Figure 1: Effect of pH on biosorption of Cr(VI) from solution by *B. coagulans* biomass (50 mg biomass was contacted with 25 mL solution containing 100 mg/L Cr(VI) of desired pH for 1h at 28°C).

As the solution pH decreased from 5.5 to 2.5, Cr(VI) uptake was enhanced. The optimal pH for maximal chromium uptake was found to be 2.5. Further decrease in solution pH reduced the chromium uptake. Similar behaviour of Cr(VI) biosorption has been observed in a variety of biosorbents including *Sargassum* sp., peat moss, sugarcane bagasse, beet pulp, maize cob and also by activated carbon (**13,21,23-25**). It is suggested that at lower pH, $HCrO_4^-$ ions start oxidising the biomass thereby producing Cr(III) ions. These Cr(III) ions then compete for binding sites on biomass with protons via cation exchange reaction (**13**). Thus, the optimum pH for biosorption of Cr(VI) occurs when the reduction potential of $HCrO_4^-$ is equal.

Effect of initial metal concentration

A curvilinear adsorption isotherm was obtained from the Cr(VI) biosorption data. The Cr(VI) uptake increased with increase in initial metal concentration (Fig. 2). Observed enhancement in metal biosorption could be due to increased electrostatic interactions (relative to covalent interactions), involving sites of progressively lower affinity for metal ion (**26**). Equilibrium sorption isotherm studies revealed that metal uptake by biomass was chemically equilibrated and involved saturable mechanism. Thus, there was an increase in metal uptake as long as binding sites were free. To ensure the efficiency and to understand the pattern of metal biosorption, the experimental data were fitted into Freundlich and Langmuir isotherms (Figs. 3, 4).

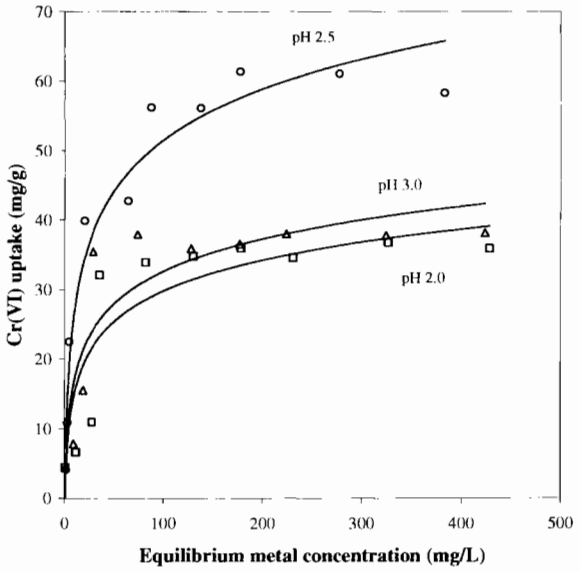


Figure 2 : Effect of initial metal concentration on biosorption of Cr(VI) by *B. coagulans* biomass (25 mL of Cr(VI) solutions (pH 2.0 (squares), 2.5 (circles) and 3.0 (triangles)) containing 10-500 mg/L of Cr(VI) for 1h at 28°C).

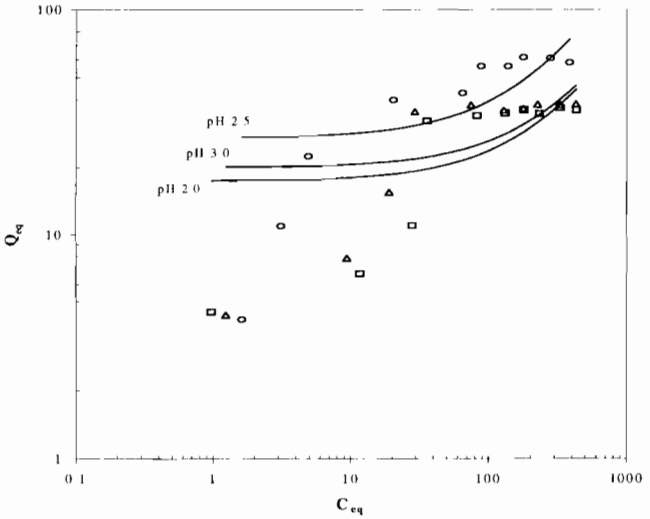


Figure 3 : Fredulich isotherm for Cr(VI) biosorption by *B. coagulans* (pH 2.0 (squares), 2.5 (circles) and 3.0 (triangles)).

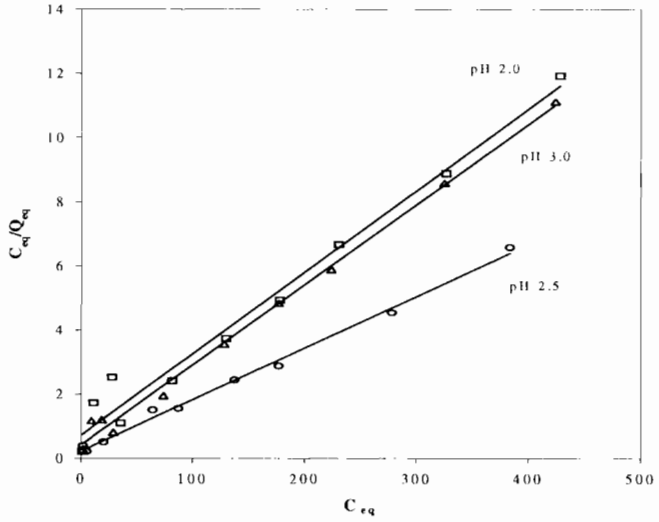


Figure 4 : Langmuir isotherm for Cr(VI) biosorption by *B. coagulans* (pH 2.0 (squares), 2.5 (circles) and 3.0 (triangles)).

The regression coefficient (r^2) calculated, as shown in Table 1, indicated that Langmuir relationship (Fig. 4) gave 'better fit' to the experimental data than Freundlich (Fig. 3).

Table 1: Comparison of Freundlich and Langmuir isotherm constants for Cr(VI) biosorption by *B. coagulans* biomass at various pH [a].

pH	Freundlich			Langmuir			S_s (m^2/g)	R[b]
	r^2	Log K	1/n	r^2	Q_{max}	b		
2.0	0.463	17.517	0.629	0.9	39.	0.0	37.27	0.054
2.5	0.547	27.184	0.122	0.9	62.	0.0	58.80	0.027
3.0	0.420	20.150	0.061	0.9	40.	0.0	37.86	0.032

[a] The biomass (50 mg) was contacted with 25 mL solution containing 10-500 mg Cr(VI)/L.
[b] At initial concentration of 500 mg/L of Cr(VI).

The calculated values of specific surface areas (S_s) at different pH values are shown in Table 1. For a good biosorbent, Q_{max} should be high with a low b value. The separation factor (R) showed that Cr(VI) uptake was favourable for *B. coagulans* biomass. These data showed that Q_{max} value was 62.11 mg/g biomass; b value was 0.071 L/mg at pH 2.5 with 58.80 m^2/g of specific surface area. The following data confirmed that at higher metal concentrations, the lower affinity sites contributed in higher uptake of Cr(VI).

Effect of biomass concentration

Figure 5 showed Cr(VI) uptake values obtained at various biomass concentrations. There was a decrease in the Cr(VI) uptake with increasing biomass concentration.

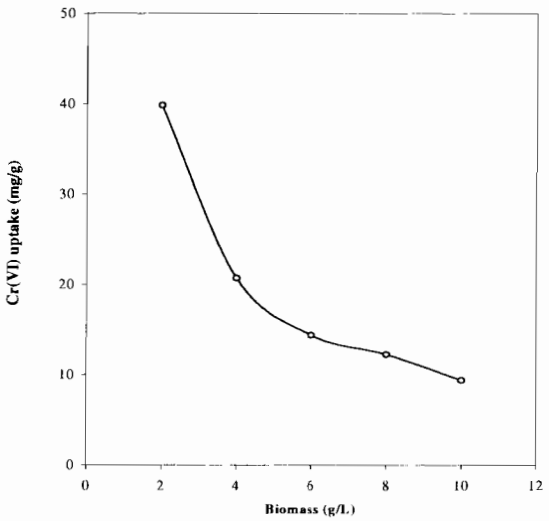


Figure 5: Effect of biomass concentration on Cr(VI) biosorption by *B. coagulans* (25 mL solution containing 100 mg/L Cr(VI) at pH 2.5 for 1h at 28°C).

These results were in accordance with several other earlier observations **(16,24)** on biosorption of Cr(VI). Increase in biomass concentration lead to interference between binding sites leading to decreased biosorption. As electrostatic interactions were the main factors for metal adsorption, an increase in biomass concentration lead to a decreased electrostatic interaction between metal ions and binding site **(27)**. The decrease in metal biosorption could also be attributed to insufficiency of metal ions in solution with respect to the available binding sites **(28)**. Antuner *et al.* **(29)** observed 5% decrease in gold uptake efficiency by dried biomass of *Azolla filiculoides* when biomass concentration was increased from 1 mg/L to 9 mg/L. Lower uptake of metal at higher biomass concentrations could be attributed to the decrease in metal-to-biosorbent ratio **(30)**.

Effect of temperature

Results of metal biosorption experiments at various temperatures (20 to 40°C) are depicted in Figure 6.

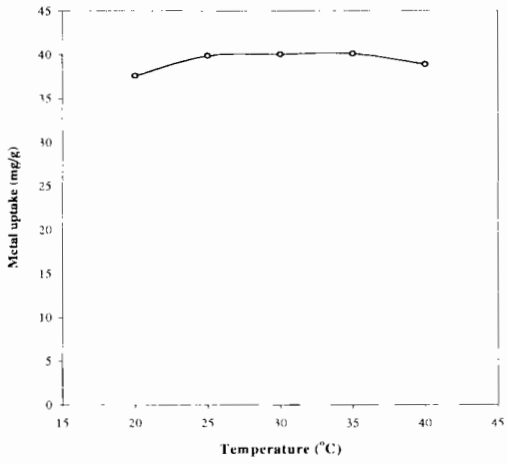


Figure 6 : Effect of temperature on biosorption of Cr(VI) by *B. coagulans* biomass (50 mg biomass was contacted with 25 mL Cr(VI) solution (100mg/L) for 1h at pH 2.5).

There was no significant difference in Cr(VI) uptake value. As 25 to 40°C is the most prevalent temperature range in tropical countries, under such practical situations Cr(VI) biosorption by *B. coagulans* will be independent of temperature. Marques *et al.* **(14)** showed that uranium uptake by *Pseudomonas* sp. was independent of temperature. Sag and Kutsal **(31)** reported an increase in rate of Cr(VI) biosorption by *Rhizopus arrhizus* biomass with temperature rise. However, effect of temperature on metal uptake capacity was not mentioned. Al-Asheh and Duvnjak **(26)** observed an increase in metal uptake when the temperature was increased from 4 to 25°C but a decrease when further increased to 50°C during studying biosorption of copper by *Aspergillus carbonarius*. They attributed enhancement in metal biosorption to the increase in energy of the system that facilitated copper attachment to the cell surface whereas, the decrease in metal biosorption to distortion of some of the sites of the cell surface available for metal biosorption. It is also possible that increase in metal uptake at increased temperature was due to either higher affinity of sites for metal or an increase in binding sites on relevant biomass **(14)**.

Effect of contact time

Metal uptake data as a function of time was depicted in Fig. 7. It was noticed that biosorption was a rapid phenomenon, attaining equilibrium in 60 min and further incubation (240 min) increased biosorption by 4% only.

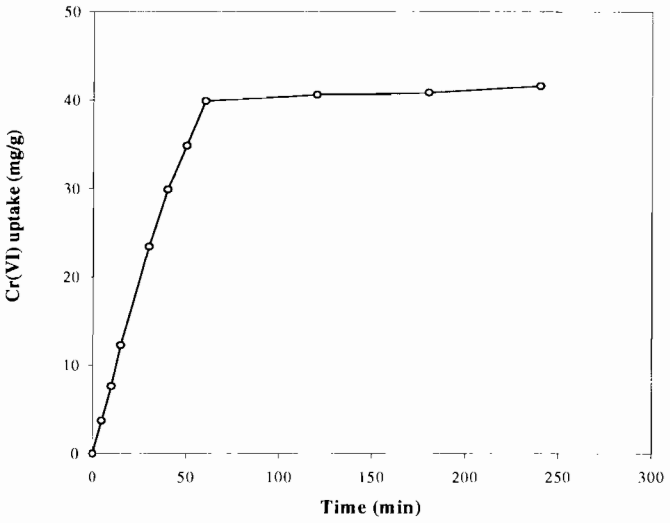


Figure 7: Effect of incubation time on biosorption of Cr(VI) from solution by *B. coagulans* biomass (50 mg biomass was contacted with 25 mL Cr(VI) solution (100mg/L) at pH 2.5 for 1h at 28 °C).

Various steps of the metal transfer from bulk solution to binding sites have been reported in literature *(**32**)*. The first step, viz. bulk transport of metal ions in solution phase, was usually rapid because of mixing and advective flow *(**33**)*. The second step, i.e., film transport, involves diffusion of metal through a hydrodynamic boundary layer around the biosorbent surface. The third step, i.e., actual adsorption of metal ions by active sites of the biomass, was considered to be rapid, equivalent to an equilibrium reaction *(**32**)*. In the case of Cr(VI) biosorption by *B. coagulans*, experimental conditions allowing good mixing of solutes and biomass in the system might have suppressed kinetic limitations due to the first and second steps. Therefore, it was likely that Cr(VI) biosorption process was influenced only by the step of metal transfer from solution to the binding sites (i.e., third step). Prakasham et al. *(**17**)* observed biosorption of Cr(VI) by *R. arrhius* to occur in two phases, probably the first and second steps of kinetics were not suppressed.

Effect of other heavy metals

The effect of various other heavy metals on uptake of Cr(VI) by *B. coagulans* at pH 2.5 was investigated (Fig. 8).

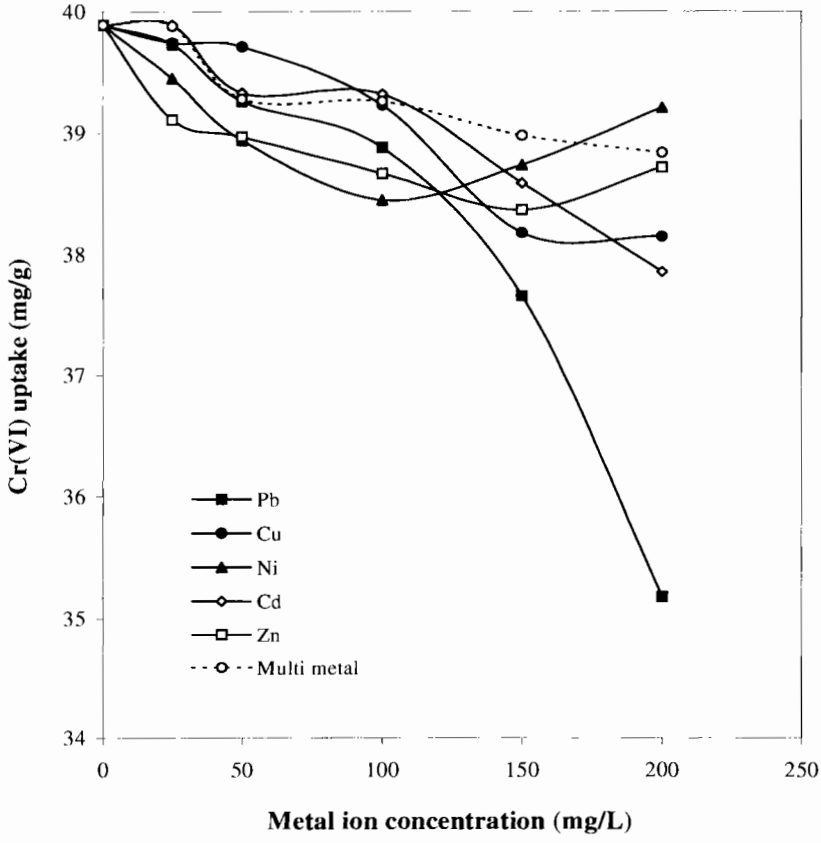


Figure 8 : Effect of different heavy metals (25-200 mg/L) on biosorption of Cr(VI) by *B. coagulans* from solution containing Cr(VI) (100mg/L) (50 mg biomass was contacted with 25 mL solution at pH 2.5 for 1h at 28 °C).

In the present study, there was no enhancement in Cr(VI) uptake in the presence of any of these cations. The data obtained showed that Pb did not affect Cr(VI) uptake up to 150 mg/L concentration, while other cations had little effect. A maximum of 11.81% decrease in Cr(VI) biosorption was seen in presence of 200 mg lead/L. However, the biosorption of Cr(VI) was less affected in multimetal studies. The less influence of these cations on biosorption of Cr(VI) can be explained by the theory of acid-base equilibrium that in the pH range 2.5-5.0, the binding of cationic heavy metals is determined primarily by the state of dissociation of the weakly acidic groups. Furthermore, this could also be due to competition for metal binding sites between metal ions and hydrogen (H^+) and hydronium (H_3O^+) ions *(**29,33**)* and

electrostatic repulsive force between protonated biomass and cations. As biosorption of many of these cations could be done specifically by several other types of biomass at selective pH *(**10,34**)*, recovery of Cr(VI) could be achieved by biomass of *B. coagulans* at the pH 2.5.

Biosorption and elution studies

Potential of mineral acids to elute bound Cr(VI) were examined (Table 2). Sulphuric acid (1.0 M) was found to be the most efficient eluent. It seems that H_2SO_4 causes low stress than HNO_3 to elute chromium from biomass.

Table 2: The biosorption/elution of chromium using *B. coagulans* biomass [a].

Eluting agent (50 mL)		Elution efficiency (%) 1st cycle	Biosorption (mg/g) 2nd cycle	Elution efficiency (%) 2nd cycle
H_2SO_4	1.0 M	86.11	35.21	61.47
	0.1 M	72.59	36.54	68.90
HNO_3	1.0 M	78.07	34.37	47.92
	0.1 M	67.24	32.79	43.85

[a] The biomass (2 g) loaded with chromium (39.89 mg/g) was contacted with eluting agent for 24 h.

During the second cycle of biosorption the loading of chromium decreased, possibly due to loss of weight during elution process. Similar to our results, 100% elution of chromium was not achieved by *Sargassum* biomass *(**13**)*, probably due to slow reduction of Cr(VI) bound by biomass to Cr(III). They found higher amount of chromium recovery from biomass equilibrated to higher concentrations. This was attributed to the elution of chromium adsorbed to lower affinity sites, which bound to biomass after higher affinity sites were occupied.

Conclusion

The present investigation revealed that biosorption process was highly influenced by pH of the solution and was maximal at pH 2.5. Specific uptake of Cr(VI) increased with increase in initial metal concentration, while it reduced at higher biomass density. The ratio of metal concentration to the amount of biomass was an important factor in the biosorptive uptake of Cr(VI). These studies also indicate possibility of selective removal of Cr(VI) from multimetal solution, unaffected by temperature and Cr(VI) can be eluted with biomass regeneration. Work described so far highlights the commercial significance of metal biosorption technology employing *B. coagulans* biomass.

Acknowledgement

The authors would like to thank Dr. P. K. Seth, Director, Industrial Toxicology Research Centre, Lucknow, for his constant encouragement. T. Srinath is thankful to Department of Biotechnology, Government of India, for financial assistance.

References

1. Ganguli A., Tripathi A.K. (1999) *Lett. Appl. Microbiol.* **28**, 76-80.
2. Patterson J.W. (1977) *Waste Water Treatment*, Science Publishers, New York.
3. Xing, L., Okrent D. (1993) *J. Hazad. Mater.* **38**, 363-384.
4. Szulczewski M.D., Helmke P.A., Bleam W.F. (1997) *Environ. Sci. Technol.* **31**, 2954-2959.
5. Shen H., Wang, Y.T. (1993) *Appl. Environ. Microbiol.* **59**, 3771-3777.
6. Rajamani S., Ramasami T., Langerwerf J.S.A., Schappman J.E. (1995) *in* Proceedings of 3rd International Conference on appropriate waste management technologies for developing countries, Nagpur, India, pp. 965-973.
7. Pandey R., Ghosh S. (2000) *in* Estimating industrial pollution in India: Implications for an effluent change, National Institute of Public Finance and Policy, New Delhi.
8. Bhide J.V., Dhakephalkar P.K., Paknikar K.M. (1996) *Biotechnol. Lett.* **18**, 667-672.
9. Environmental Protection Agency (1979) *in* USEPA technology transfer report, EPA 625/5-79-016, June.
10. Gadd G.M., White C. (1993) *Trends Biotechnol.* **11**, 353-359.
11. Akhtar M.N., Mohan P.M. (1995) *Curr. Sci.* **69**, 1028-1030.
12. Garnham G.W., Codd G.A., Gadd G.M. (1993) *Appl. Microbiol. Biotechnol.* **39**, 666-672.
13. Kratochvil D., Pimentel P., Volesky B. (1998) *Environ. Sci. Technol.* **32**, 2693-2698.
14. Marques A.M., Roca X., Simon-Pujol M.D., Fuste M.C., Congregado F. (1991) *Appl. Microbiol. Biotechnol.* **35**, 406-410.
15. Andres Y., Thouand G., Boualam M., Mergeay M. (2000) *Appl. Microbiol. Biotechnol.* **54**, 262-267.
16. Merrin J.S., Sheela R., Saswathi N., Prakasham R.S., Ramakrishna S.V. (1998) *Indian J. Exptl. Biol.* **36**, 1052-1055.
17. Prakasham R.S., Merrie J.S., Sheela R., Saswathi N., Ramakrishna S.V. (1999) *Environ. Pollut.* **104**, 421-427.
18. Mattuschka B., Junghans K., Straube G. (1993) *in* Biohydrometallurgical Technologies, vol. 2, Torma A.E., Apel M.L. and Brierley C.L., eds., The Mineral, Metals and Materials Society, Warrendale, PA, pp. 125-132.
19. Niu H., Xu X.S., Wang J.H., Volesky B. (1993) *Biotechnol. Bioeng.* **42**, 785-777.
20. Verma T., Srinath T., Gadpayale R., Ramteke P.W., Hans R.K., Garg S.K. (2001) *Biores. Technol.* **78**, 31-35.
21. Sharma D.C., Forster C.F. (1994) *Biores. Technol.* **47**, 257-264.
22. Poots V.J.P., McKay G., Healy J.J. (1978) *J. Water Pollut. Control* **50**, 926-934.
23. Sharma D.C., Forster C.F. (1993) *Water Res.* **27**, 1201-1208.
24. Sharma D.C., Forster C.F. (1994) *Biores. Technol.* **49**, 31-40.
25. Huang C.P., Wu M.H. (1977) *Water Res.* **11**, 673-679.
26. Al-Asheh S., Dunjak Z. (1995) *Biotechnol. Prog.* **11**, 638-642.
27. De Rome L., Gadd G.M. (1987) *Appl. Microbiol. Biotechnol.* **26**, 84-90.
28. Fourest E., Roux J.C. (1992) *Appl. Microbiol. Biotechnol.* **37**, 399-403.
29. Antuner A.P.M., Watkins G.M., Duncan J.R. (2001) *Biotechnol. Lett.* **23**, 249-251.
30. Puranik P.R., Paknikar K.M. (1999) *Biotechnol. Prog.* **15**, 228-237.
31. Sag Y., Kutsal T. (1996) *Process Biochem.* **31**, 573-585.

32. Weber W.J.Jr. (1985) *in* Adsorption technology: A step-by-step approach to process evaluation and application, Slejko, F.L., ed, Marcel Dekker, New York, pp. 1-35.
33. Gadd G.M. (1988) *in* Biotechnology, Rehm, H.J., ed., Weinheim, Germany, pp. 401-433.
34. Ahn D.H., Chung Y.C., Pak D. (1998) *Appl. Biochem. Biotechnol.* **73**, 43-50.

Chapter 24

DETOXIFICATION OF POLLUTANTS FROM MUNICIPAL WASTEWATER USING ORGANIC SOIL AS A BIOFILTER

Z. STEPNIEWSKA[1,2], U. KOTOWSKA[2].

[1] *Department of Environmental Biochemistry and Chemistry, Catholic University of Lublin, Poland, stepz@demeter.ipan.lublin.p ;* [2] *Institute of Agrophysics Polish Academy of Sciences, Poland*

Abstract

The following studies were performed on Eutric Histosol fields (9 fields, each 0.35 ha in area) with different plant covers (*Salix americana, Populus alba* and grass mixture) and drain system at a depth of 100 cm. The fields were irrigated 10 times per vegetation season by surface flooding technique with municipal wastewater after two-step (mechanical and biological) treatment. The experimental fields were equipped with porous cups for extraction of soil solution. Probes were installed at depths of 10, 20, 50, 70 and 100 cm. Three irrigation treatments were applied for each plant cover. The control treatment (A) received only water from precipitation (about 600 mm per year). The single dose treatment received 60 mm (B) and the double treatment (C) - 120 mm of wastewater each time. Wastewater introduced to the fields contained about 25 g $N\text{-}NO_3$ m^{-3}, 5 g P m^{-3} and some amounts of heavy metals among which Zn and Cd dominated. It was found that nitrate content in the recharge water was reduced to 7-14 g m^{-3} and that of total P – down to 0.8 g P m^{-3}.

Keywords: Irrigation fields, municipal wastewater, nitrogen, phosphorus, treatment wetlands.

Introduction

Land application and irrigation of municipal wastewaters is common practice, around the globe. The techique has been used successfully to treat various wastewaters, such as selenium containing wastewaters (**1**), municipal wastewaters (**2**) or industrial wastewaters (**3**). A control of the fate of the components present needs to be thoroughly carried out in order to prevent further pollution problems. In the present study, irrigation of soil with municipal wastewater after mechanical and biological cleaning was applied as a third step of the wastewater treatment (**4**). Usually, mineral soils are utilised for the wastewater treatment on irrigation fields.

The aim of the study was to investigate the rate of purification of pre-treated municipal wastewater on organic soil (Eutric Histosol) in the presence of cultivated plants.

New Horizons in Biotechnology, 267-270, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Material and Methods

The experimental fields were located in the valley of the Bystrzyca River, with a muck peat soil (Eutric Histosol). The fields of a surface 0.35 ha each were prepared in order to obtain three treatments with different plant covers viz. *Salix americana*, *Populus alba*, and meadow (with *Alopecurus pratensis*, *Phalaris arundinacea*, and *Festuca pratensis* as dominating grass species). The fields were flood irrigated 10 times during the vegetation season with 600 and 1200 mm of wastewater per year. A single water dose was 60 and 120 mm for lower and higher irrigation level, respectively. The irrigation wastewater contained on average 25 g NO_3-N m^{-3}, and 30 g of total N m^{-3} while the average content of total phosphorus was 5 g m^{-3} of which 4.8 g m^{-3} constituted phosphate P.

Table 1. Physicochemical parameters of the input wastewater, after Kotowski *et al.* (**5**).

Parameter	Unit	Range
PH	-	6.47 - 8.41
ChOD	gO_2/m^3	30.1 – 56.3
BOD_5	gO_2/m^3	8.3 – 22.6
NH^+_4-N	gN/m^3	1.1 - 7.1
NO^-_3-N	gN/m^3	20.2 – 38.4
N-tot	gN/m^3	22.3 – 43.6
PO_4-P	gP/m^3	3.1 - 6.8
P-tot	gP/m^3	3.7 - 7.0
Na^+	gNa/m^3	24.3 – 69.4
K^+	gK/m^3	11.8 – 27.7
Ca^{2+}	gCa/m^3	59.7 – 95.2
Mg^{2+}	gMg/m^3	12.6 – 19.7
SO^{2-}	gSO_4/m^3	43.6 – 116.3
Cl^-	gCl/m^3	67.8 – 121.6
Zn	$mgZn/m^3$	18 – 800
Cu	$mgCu/m^3$	6 – 198
Pb	$mgPb/m^3$	7 – 96

Thus the wastewater, after mechanical and biological treatment, could have been applied to the soil as the Polish threshold values for chemical oxygen demand (COD), biological oxygen demand (BOD_5) and heavy metal concentration for wastewater introduction to the soil are as follows:

COD<150 gO_2 m^{-3}, BOD_5< 30 gO_2 m^{-3}, Zn< 2.0 g m^{-3}, Cu< 0.5 g m^{-3}, Pb< 0.5 g m^{-3}, Cd< 0.1 g m^{-3}. The wastewater showed periodically somewhat elevated concentrations of total phosphorus and nitrogen.

Results and Discussion

The average chemical composition of the water recharged to the drainage system is presented in Table 2 for flooding cycles performed in 1998.

As it can be seen the degree of reduction of (total and nitrate) nitrogen forms ranged from 43 to 77% while that of phosphorus form – from about 77 to 88%.

Table 2. Average chemical composition of the drainage waters from the experimental fields with the ranges of variability.

Parameter	Unit	Mean value	Range of variability	Reduction in %
NH^+_4-N	gN/m^3	1.26	0.38-2.69	62-77
NO^-_3-N	gN/m^3	10.6	6.7-14.2	43-72
N-tot	gN/m^3	12.7	6.8-14.6	43-70
PO^-_4-P	gP/m^3	0.68	0.23-084	77-89
P-tot	gP/m^3	0.79	0.25-0.86	78-88

Average concentrations of nitrate-N at different depths are presented in Table 3. As it can be seen nitrate concentration in the control (A) fields was within the range 3-6 g m^{-3}, while in the irrigated fields it increased to 10-30 g m^{-3}.

The nitrate concentration decreased with depth and time probably due to the uptake by plant roots. Another reason might have been denitrification process (**6,7**). At a depth of drainage system i.e. 100 cm, the nitrate concentration was lower and less variable probably as a result of contact with the deeper ground water.

Concentration of total phosphorus in the recharge drainage water is presented in Table 4. As in the case of nitrate, the total phosphorus concentration increased due to wastewater application from about 0,12 g m^{-3} to around 0,65 g m^{-3} but contrary to nitrate, its concentration was much more stable with time, up to 7th day from the flood onset.

Table 3. Average nitrate–N concentrations in soil solution at different depths during and directly after flooding (± standard deviation). Nitrate N concentration in the input wastewater was 24.3 g N m^{-3}.

Treatment	Depth (cm)	Time-period from flooding		
		1 day	2 days	7 days
A	10	6.6±3.3	4.6±2.3	4.9±2.5
	30	8.1±4.2	4.0±1.5	4.1±2.0
	50	6.0±2.3	6.7±2.1	4.2±2.6
	70	4.8±1.7	6.3±2.0	4.3±2.1
	100	2.7±0.3	3.1±1.5	3.0±1.5
B	10	29.3±2.8	20.6±4.6	10.9±4.0
	30	19.2±2.1	16.9±2.7	11.4±6.4
	50	15.5±2.6	12.2±1.1	10.2±5.3
	70	14.4±1.8	11.5±0.64	8.3±4.5
	100	8.4±2.2	9.3±1.7	6.1±4.2
C	10	22.7±5.2	21.2±5.2	13.9±5.4
	30	18.6±2.6	18.2±2.1	12.9±6.2
	50	15.5±0.7	15.3±2.3	10.8±5.5
	70	16.8±0.7	14.6±3.3	8.2±3.3
	100	10.8±1.5	12.1±2.4	6.0±2.6

Table 4. Total phosphorus concentration (average values from the three plant treatments ± standard deviation) in drainage waters during the irrigation period and directly after it (autumn 98). Total phosphorus concentration in the input wastewater was 5.08 g P m^{-3}.

Treatment	Time period from flooding		
	1 day	3 days	7 days
A	0.13±0.04	0.13±0.03	0.10±0.02
B	0.63±0.16	0.64±0.06	0.52±0.05
C	0.65±0.14	0.60±0.17	0.58±0.10

Conclusions

Phosphorus (total and phosphate-P) reduction degree in the organic soil irrigation field exceeded 80% and its concentration in the drainage waters was below 0.8gm^{-3}.

Nitrate concentration in the soil profile decreased with depth and time.

Total and nitrate-N was reduced by 43-72% in the soil filter.

Ammonia reduction on the organic soil filter was by 62-77%.

Eutric Histosol appeared to be an efficient filter for nitrogen and phosphorus from municipal wastewater.

References

1. Lemly A.D., Ohlendorf H.M. (2002) *Ecotoxicology and Environmental Safety*, **52** (1): 46-56.
2. van de Graaff R.H.M, Suter H.C, Lawes S.J. (2002) *Journal of Environmental Science and Health* **37** (4): 745-757.
3. Phillips I.R. (2002) *Australian Journal of Soil Research* **40** (3): 515-532.
4. Obarska-Pempkowiak H. (1991) *in*: Ecological Engineering for Wastewater Treatment, Etnier C. and Guaterstarm B. (Eds.)., Bakskogen, Szwecja, 239-247.
5. Kotowski M., Stepniewska Z., Saczuk M., Kotowska U., Pasztelan M. (1999). *Acta Agrophysica*, **22**, 93-113.
6. Glieski J., Stepniewski W. (1985) Soil Aeration and its Role for Plants. CRC Press, Boca Raton, Florida, USA.
7. Reddy K. R., Saccon P.D., Graetz D.A., (1980) *J. Environ. Qual*, **9**, 283-288.

Chapter 25

BIOCHEMICAL REMOVAL OF NITROGEN FROM INDUSTRIAL EFFLUENTS: A FULL SCALE PLANT PERFORMANCE AND STABILITY

L. SZPYRKOWICZ[1], S. N. KAUL[2]

[1] *Department of Environmental Studies, University of Venice, Dorsoduro 2137, 30123 Venice, Italy; e-mail* ***lidia@unive.it*** *;* [2] *Wastewater Technology Department, National Environmental Engineering Research Institute, Nagpur 440020, India.*

Abstract

Biological nitrification/denitrification is a well-known cost-effective method of domestic or mixed wastewater treatment. If used to solve problems of industrial effluents, the performance of the plant is often unsuccessful, with periodic block of nitrogen removal. The present paper concerns the performance of nitrification/denitrification during a 1.5-year period in a plant treating tannery wastewater. Operational parameters are evaluated on a day-to-day basis and their role, along with pH, temperature, TKN, NH_3 concentration and other analytical parameters, which characterise wastewater quality at different points in the treatment scheme, is discussed in reference to process rates. As shown, biological nitrification/denitrification of tannery wastewater is extremely difficult to conduct in a full-scale plant and there may be many reasons for the periodic failure of the system. Only a slight deviation of the optimal pH in nitrification or denitrification basins, a drop in temperature or a high influent nitrogen content are factors whose magnitude would not affect the performance of a plant treating domestic wastewater, but during treatment of industrial effluents they can readily cause system failure. Most often however, it is the concomitant occurrence of several causes that leads to an unsuccessful performance or inhibition of one or more biochemical reactions. In such a complicated situation sludge age can be the key parameter in controlling the plant. Keeping it high, far above the value related to the specific growth velocity for a given temperature, can be a useful tool for preventing problems in plant performance, particularly when high fluctuations in the concentration of inflow solids make a proper sludge management difficult. Furthermore on-line monitoring of wastewater characteristics at key points of the plant can give useful information on the inhibition risk.

Key words: nitrification, denitrification, tannery wastewater, inhibition, full-scale plant performance

New Horizons in Biotechnology, 271-284, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

The term nitrification denotes oxidation of ammoniacal nitrogen into nitrites and nitrates by a biochemical route. It is performed by specific autotrophic microorganisms, whose behaviour is significantly different from those of the heterotrophic type. The process is conducted in two steps:

and

$$2NH_4^+ + 3O_2 \xrightarrow{Nitrosomonas} 2NO_2^- + 2H_2O + 4H^+ \qquad 1.$$

$$2NO_2^- + O_2 \xrightarrow{Nitrobacter} NO_3^- \qquad 2.$$

The nitrifying bacteria consume ammonium for their cellular synthesis and as a source of energy. The quantity of this ion used for biomass production of nitrifiers constitutes an insignificant fraction (about 2% (**1**)) of the total quantity processed, which results in this quantity generally being neglected in the mass balance for nitrogen. Since generally the conversion of ammonium into nitrite is slower than the transformation of nitrite into nitrate, it is assumed that the nitrification kinetics is governed by the first step of the process. In the absence of any inhibition, a Monod expression for biomass growth can be applied:

$$\mu = \mu_{max} \frac{[NH_4^+]}{K_N + [NH_4^+]} \frac{[DO]}{K_O + [DO]} \qquad 3.$$

where: μ - specific growth rate (T^{-1}); μ_{max} – maximum specific growth rate (T^{-1}); $[NH^+_4]$ – bulk ammonium concentration (ML^{-3}); K_N – semisaturation constant for ammonium (ML^{-3}); K_O – semisaturation constant for dissolved oxygen (ML^{-3}); [DO] - bulk concentration of dissolved oxygen (ML^{-3}).

From equation 3, the importance of dissolved oxygen in the process of nitrification is evident. The value of the semi-saturation constant for oxygen is generally assumed to be equal to 0.5 mg dm^{-3} (**2**). For concentrations higher than 2 mg dm^{-3}, dissolved oxygen is no longer considered to be a limiting factor.

Both the specific growth rates and semi saturation constants are very sensitive to temperature and pH variations. The influence of temperature on the magnitude of the specific growth rate is given by (2):

$$\mu_{max}{}^T = \mu^{20}{}_{max}\, 1.123^{(T-20)} \qquad 4.$$

A similar exponential equation describes the dependence of the semi saturation constants on temperature.

Temperature can also affect the overall process of nitrogen elimination by diminishing the efficiency of the removal of ammonium by ammonification during the biomass production (**3**) or by increasing the inhibition or toxicity effects of the constituents of the wastewater (**4, 5**).

Regarding the influence of pH on the variation of μ_{max}, it is generally assumed that it has no impact if the pH ranges between 7.2 and 8.5, but for lower values the specific growth rate diminishes, according to:

$$\mu^{pH}{}_{\max} = 7.2\Theta^{(pH-7.2)} \qquad 5.$$

where: Θ - sensitivity coefficient, equal to 2.35 (**2**).

The variation of the semi saturation constant K_N as a function of pH is not documented in the literature.

In the case of inhibition of nitrification, the Monod-type equation may remain valid, but the value of the maximum specific growth rate will be much lower. It may also happen that the hypothesis made for the specific growth rate, as being a function of the substrate concentration only, may no longer be true. In this case other types of equations, based on enzymatic kinetics, are applied. The Haldane equation, which describes the process kinetics inhibited by the substrate itself, is most commonly used in this case (**6**):

where: K_i – inhibition constant (ML^{-3}).

$$\mu = \frac{\mu_{\max}\left[NH_4{}^+\right]}{K_N + \left[NH_4{}^+\right] + \frac{\left[NH^+{}_4\right]^2}{K_i}} \qquad 6.$$

Material and Methods

The research consisted of the long-term observation (about 600 days) of the performance of a full-scale common effluent treatment plant, processing tannery wastewater with a mean flow of 30000 m^3 day^{-1}, generated by a cluster of tanneries. The plant, based on a single-sludge process, is schematically depicted in Fig. 1.

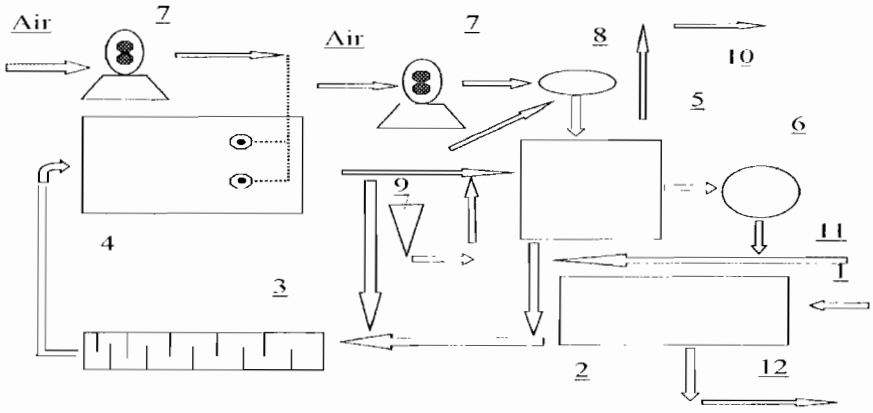


Figure 1. Scheme of the full-scale treatment plant : (1) inlet of equalised industrial effluents; (2) primary sedimentation tank; (3) denitrification basin; (4)nitrification basin; (5) flotation unit; (6) secondary sedimentation tank; (7) air compressors; (8) air saturation unit; (9) polyelectrolyte dosing; (10) biological sludge to waste; (11) waste sludge from a municipal wastewater treatment plant; (12) primary sludge to waste).

The wastewater was sampled at different points of the plant to assess the removal efficiency of various treatment stages and analysed for pH, redox potential, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), total and settable solids, chlorides, sulphates, sulphides, total chromium, nitrates, nitrites, Total Kjeldahl Nitrogen (TKN), dissolved oxygen and total volatile solids. The analyses were performed following Standard Methods (7).

Results and Discussion

The mean values of the parameters analysed at different sampling points are reported in Tab. 1. As can be seen, the wastewater was characterised by an elevated content of organic substances of high biodegradability (BOD/COD = 0.74), a high concentration of nitrogen, present mainly as ammoniacal nitrogen and a high concentration of chlorides. Suspended solids were also present in significant quantity.

Table 1. Mean values of the characteristics of the effluent at different sampling points (mg dm^{-3}, except for pH, sedimentable solids (cm^3 dm^{-3}) and redox (mV)).

Parameter	Sampling point			
	inlet into denitrification	secondary tank sedimentation	recycle into denitrification	inside basin nitrification
pH	8.0	7.85	7.5	7.2
COD	5422	185		
COD filtered	2736		228	183
BOD	2030	13		
Total solids	2582	81	6.7	6.9
Sedimentable solids	38.06	2.4		
Chloride	3558	2964		
Sulfate	1615	1817		
Cr tot	103			
Fe tot	20.0			
N-NO2-		1.8	0.6	0.3
N-NO3-		22.8	12.7	18.5
N-NH3		34.1		40.0
TKN	493.0	38.5		
Sulfide	121.2	0		
redox	-438	76		
P tot		1.0		
DO				3.1
Phenol			756	616
Alcalinity				24.5

The day-by-day variation of the characteristics of the wastewater entering the plant during the whole period of the study was very high. Fig. 2 and 3 show mean daily values of COD and that of the ratio between TKN and COD, respectively. This ratio is of particular importance for the single sludge process, considering the need to assure the organic load during denitrification at a level sufficient to accomplish biochemical conversion of nitrates to gaseous nitrogen by means of heterotrophic bacteria, which use organic matter as a source of energy and carbon.

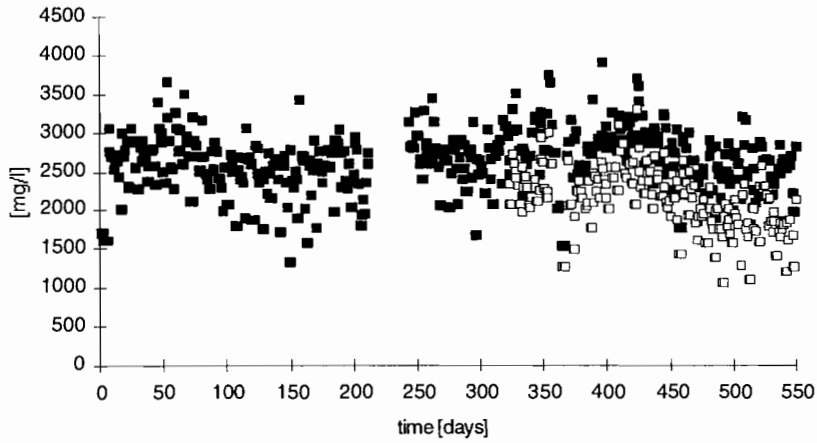


Figure 2: COD in the inlet to the biological section (raw samples-full symbols; filtered samples-empty symbols).

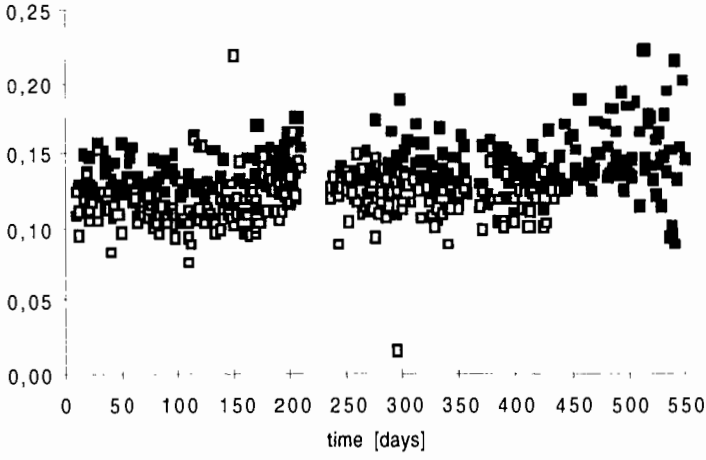


Figure 3 : TKN/COD ratio in for the raw wastewater (empty symbols) and at the inlet to the biological section (full symbols).

An example of the denitrification reaction is the following one, which employs methanol as an external carbon source:

$$6NO_3^- + 5CH_3OH \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \qquad 7.$$

Fig. 4 a and b show the concentration of different forms of nitrogen in the treated wastewater.

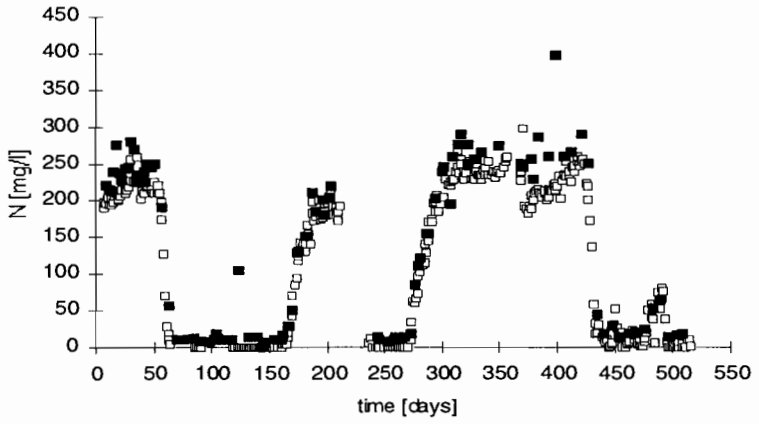


Figure 4a: TKN (full symbols) and NH4 (empty symbols) in the effluent from the secondary sedimentation .

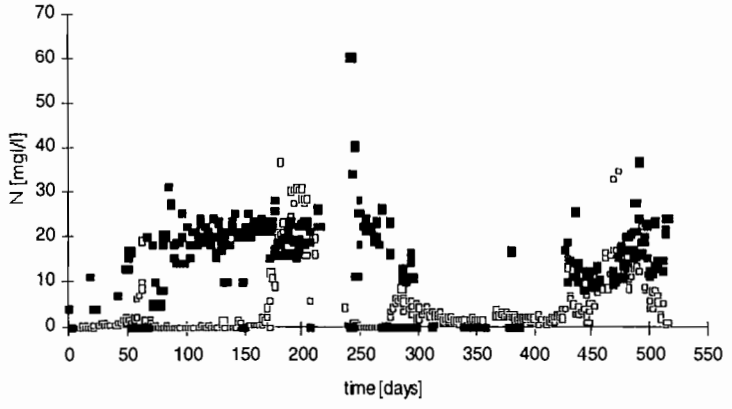


Figure 4b: NO2(full symbols) and NO3 (empty symbols) in the outlet from the secondary sedimentation.

As can be seen, the performance of the plant was very unstable, with long periods of insufficient nitrification and the consequent discharge of ammonium and/or oxidised nitrogen compounds present in the final effluent. As shown in Fig. 5, in the periods when nitrification performed well, the rate of this process was equal to 0.02 kg N kg^{-1} MLVSS day^{-1}, which is about five-fold lower than the rates observed at a pilot plant treating the same kind of wastewater (**8**).

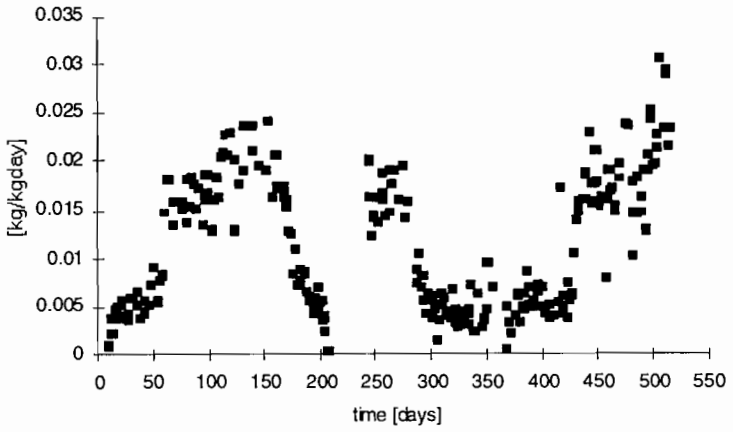


Figure 5: Nitrification rate in time.

During the periods when nitrification caused problems, the final effluent was also often characterised by increased COD, indicating a decrease in removal of the organic load (see Tab. 2). It may be that this was caused by a lack of chemical oxygen in the denitrification basin, resulting in the lower efficiency of this section.

As can be seen from Fig. 6, the plant exhibited significant fluctuations of pH in nitrification and denitrification sections. It is of a particular interest to note, comparing these data with Fig. 5, that the high pH (particularly above 7.7) can be associated with low rates of nitrification due to inhibition by the increased level of free ammonia. In fact, on the increase of the pH, the equilibrium between ammonium ions and ammonia shifts towards this last compound. At the pH of 8.0 and the temperature equal to 20° C, about 5% (**9**) of ammoniacal nitrogen is present in the form of free ammonia. This means that in wastewater bearing a TKN content as high as 300 mg dm^{-3}, the concentration of free ammonia can be approximately equal to 15 mg dm^{-3}.

Table 2. Global efficiency of nitrogen and COD elimination at the plant .

Period	Removal efficiency [%]			
	mean value		st. dev.	
	N	COD	N	COD
Problems in nitrification				
01.01.93-02.03.93	43.46	89.74	10.01	1.17
15.06.93-31.07.93	50.00	90.21	17.34	2.02
01.10.93-14.03.94	44.18	88.44	14.99	2.49
24.03.94-31.03.94	92.98	87.40	2.00	3.36
20.04.94-09.05.94	83.87	88.93	6.38	3.92
Nitrification in function				
03.03.93-14.03.93	99.80	91.07	0.74	2.11
01.09.93-30.09.93	100.00	92.46	0.00	1.14
15.03.94-23.03.94	98.34	87.52	1.32	2.15
01.04.94-19.04.94	96.75	89.53	2.18	7.04
10.05.94-23.05.94	99.32	87.63	1.52	2.97

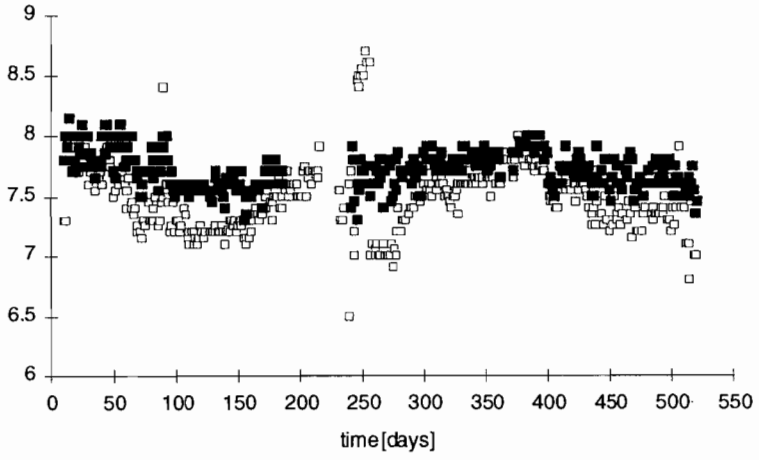


Figure 6 : Trend of pH in nitrification (empty symbols) and denitrification (full symbols) basins.

While examining possible reasons for deficiency in nitrification, dilution rate should be taken into particular consideration. To avoid a wash-out of the biomass, the plant should operate at a dilution rate lower than the critical value:

$$D = \frac{F_o}{V} < D_{crit} \qquad 8.$$

where: D – dilution rate (T^{-1}); F_o – nominal flow rate (L^3T^{-1}); V – volume of the reaction basin (L^3).

This critical dilution rate is equal to the specific growth rate μ for the plants without the sludge recycle. For the plants which operate with sludge recycle the critical dilution rate, calculated on the basis of the sludge mass balance is given by (**10**):

$$D = \frac{\mu}{1 - \frac{F_r}{F_o}\left(\frac{x_o}{x_1} - 1\right)} \qquad 9.$$

The specific growth rate being a function of several operational parameters and wastewater characteristics, it is clear the critical dilution rate also changes with variation of these parameters and an optimal value cannot be indicated. Furthermore, in the case of nitrification of industrial wastewater, the combined effect of temperature and inhibiting factors can occur. This can lead to a total or partial block of nitrogen removal, even under conditions of relatively high temperature.

In the present study, critical dilution rates were calculated assuming the μ_{max} value for nitrifiers at a temperature of 20 °C equal to 0.3 day^{-1} (**2**). This value was further corrected, considering that the temperature of the wastewater measured in the basin differed from 20°C and the actual pH often was not optimal. Equations (4.) and (5.) were used for calculation purposes. It has to be stressed that the plant should be operated at dilution rates below and not close to the values indicated as critical, considering that an eventual inhibition was not taken into consideration while determining the critical values. Inhibition, if present, would affect the specific growth rate and, as a consequence, the critical dilution rate would be lower.

Fig. 7 displays the critical dilution rates calculated for those days of the study for which data were available. The figure also shows the actual daily dilution rates at which the plant operated. As can be seen, in some periods the critical and actual dilution rates were very close or the plant even exceeded the critical value. These periods corresponded to winter temperatures, those manifested in January – February of the first year of the study and November – January of the second year (respectively the periods between 1 and 60 and 300 and 420 days on the time scale).

Under these conditions the plant nitrified poorly, which confirmed the validity of the dilution rate approach.

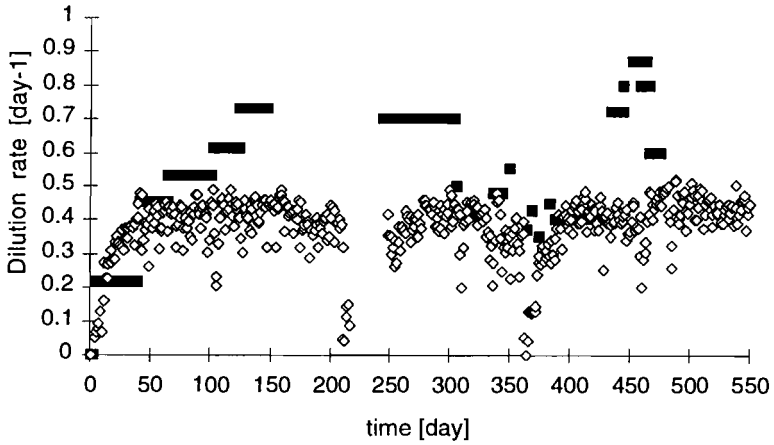


Figure 7: Critical (full symbols) and real (empty symbols) dilution rates at the nitrification basin.

There exists a vast list of different substances, which can cause an inhibition of nitrification (**9**). It includes organic compounds used in the tanning industry, e.g. surfactants or substances preventing the development of fungi (e.g. chloromethacreosol). Particularly noxious are cationic surfactants, which can block nitrification at a concentration as low as a few μg dm^{-3} (**11,12**). Anionic surfactants are less dangerous, but their inhibitory effect also manifests itself at low concentrations, in the range of few mg dm^{-3}.

Another inhibiting factor is salinity; 50% inhibition of nitrification was observed when the NaCl concentration was equal to 0.4 eq dm^{-3} (**13**).

The greatest danger of inhibition was, however, associated with the same compounds, which were used by nitrifying bacteria as substrates. In fact both steps of nitrification could be inhibited by free ammonium (threshold levels reported in the literature are 0.1 mg dm^{-3} and 10 mg dm^{-3}, respectively for Nitrobacter and Nitrosomonas (**9**)) and undissociated nitric acid (0.2 mg dm^{-3} for Nitrobacter (**14**).

If nitrification were conducted at a higher temperature, the risk of an accumulation of nitrites would arise. This effect was not caused by a partial or total block of the second step of nitrification, but derived from a variation of the kinetics of the two steps, the first one being more sensitive to the temperature rise than the second. Thus there existed a critical temperature level, above which the transformation of nitrites into nitrates was slower than their production (**4,5**). In the case of the "single sludge" process such a situation lead to the discharge of nitrite-rich final effluent.

Accumulation of nitrites in the wastewater could also influence alkalinity and pH. The decrease of pH could be observed if the "reserve" of alkalinity was insufficient and lower than its stoichiometric need, resulting from the mass balance calculated

using equations 1, 2 and 7. The decrease of pH caused a shift of the equilibrium between dissociated and undissociated forms of nitrites, leading to an increase in the quantity of free nitric acid. Since this compound was a strong inhibitor of the first step of nitrification, the whole process could become completely blocked. This was what happened between days 270 and 300 of the study.

Other possible causes of inhibition of oxidation of nitrites into nitrates include the oxygen concentration being lower than the minimum value equal to 0.5 mg dm^{-3} (**15**) (a situation which occurred on 380 – 400 day) or a presence of organic substances in the nitrification basin at a high level. This could result in the predominance of heterotrophic over autotrophic bacteria, with a consequent competition for oxygen. Under such conditions the maximum specific growth rate of nitrifying bacteria diminished and the value of the semisaturation constant for oxygen increased (**15**).

The parameter, which is of a paramount importance in biological wastewater treatment plants, is sludge age. This is calculated from the sludge mass balance, as a ratio between the quantity of the biomass in the activated sludge basin and the quantity wasted daily. Considering that the inflowing wastewater contains solids, their quantity should be subtracted from the total mass of solids present in the system. Thus the following equation was used to calculate the sludge age SRT:

$$SRT = \frac{C_{MLSS}V - C_s F_o}{Q} \qquad 10.$$

where: C_{MLSS} and C_s – concentration of solids in the activated sludge basin and in the influent, respectively (ML^{-3}); Q – quantity of MLSS wasted daily (MT^{-1}); other notations as above.

From the above equation it results, that if the quantity of the solids arriving at the biological unit was not carefully determined or considered at all, it could cause a significant discrepancy between the real and calculated value of the sludge age. As can be seen from Fig. 8, the quantity of solids arriving at the nitrification/ denitrification section of the plant was very variable and was often in the range of the wasted quantity. This made the calculation of the sludge age an impossible task, as even negative values were obtained. This difficulty indicates the importance of correct determination of the concentration of the solids, while determining the daily quantity of the sludge to be wasted and, generally, in the management of the nitrification plant.

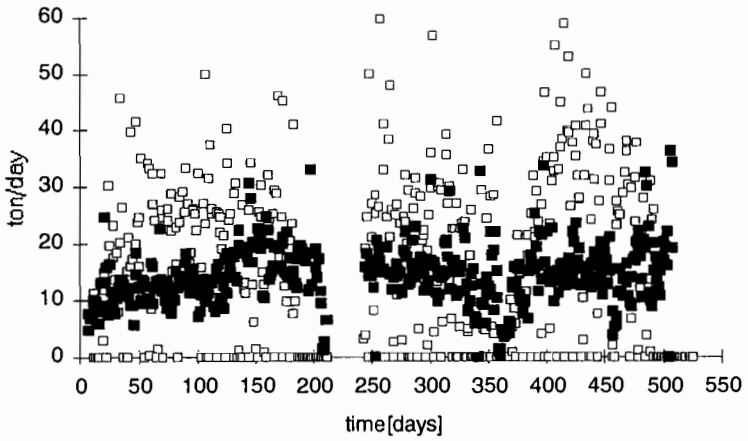


Figure 8: Daily quantities of solids arriving at the biological section (full symbols) and of wasted sludge (empty symbols).

Another way to calculate the SRT is to consider volatile instead of total solids in the mass balance. In such an approach the inflowing solids can be neglected, assuming that they represent the organic matter, which is hydrolysed and further decomposed in the biological basin; thus this quantity constitutes a "feed" for the biomass. It has to be outlined that this assumption may not be in practice totally fulfilled. The apparent SRT values calculated for the plant using this approach varied widely between 8 to 40 days. The loss in nitrification was observed for the values below 10 days (days 175 to 200 on the time scale). Considering that in this period the temperature in the basin was relatively high (above 19°C), the SRT of 10 days could have been regarded safe for nitrification of municipal wastewater, but resulted too short for industrial effluents. Thus, while treating industrial sewage, it is advisable to assure the stability of the plant performance to operate at a much higher SRT than the value estimated from the specific growth rate of nitrifiers.

Conclusions

Analysis of causes of the failure in the performance of the full-scale plant lead to the conclusion that the lack of nitrification observed at the plant could not be explained by one single cause. The whole range of factors that influenced the development of the nitrifying biomass had to be considered. Among the factors which could be cited as the most frequent were: exceeding of the critical value of the dilution rate, pH too low (below 7.0) or too high (above 8.0), resulting in generation of inhibition by nitric acid and free ammonia, respectively, and insufficient oxygenetion (dissolved oxygen concentration below 1 mg dm^{-3}). In the majority of the cases more that one cause occurred.

To ensure the stable and satisfactory performance of the plant, it is advisable to install sensors to monitor continuously ammonium, nitrites, nitrates, pH, temperature and dissolved oxygen. Caution should also be paid to sludge management practice, to ensure the possibility of defining the sludge age at which the plant operates.

In such a complicated situation sludge age can be the key parameter in controlling the plant. Keeping it high, far above the value related to the specific growth velocity for a given temperature, can be a useful tool for preventing problems in plant performance, particularly when high fluctuations in the concentration of inflow solids make a proper sludge management difficult.

References

1. Van Handel A. C., Marais G.R. (1981) Research Report W 42, Dept. of Civil Eng., Univ. of Cape Town.
2. Water Research Commission by the University of Cape Town, City Council of Johannesburg and the National Institute for Water Research of the CSIR (1984), Theory, Design and Operation of Nutrient Removal Activated Sludge Processes, Pretoria , Rep. of South Africa.
3. Oleszkiewicz J.A., Berquist S.A. (1988) *Wat. Res.*, **22**, 1163-1171.
4. Randall C.W., Buth D. (1984) *J. WPCF*, **56**, 1039-1044.
5. Randall C.W., Buth D. (1984), *J. WPCF*, **56**, 1045-1049.
6. Rozich A.F., Castens D. J. (1986) *J. WPCF*, **58**, 220-226.
7. APHA (1989). Standard Methods for examination of water and wastewater, 17th ed., American Public Health Assoc. Washington D.C.
8. Szpyrkowicz L., Rigoni-Stern S., Zilio Grandi F. (1991) *Wat. Res.*, **25**, 1351 – 1356.
9. Sedlak R. (1991) Phosphorus and Nitrogen Removal from Municipal Wastewater, 2nd ed., Lewis Publishers, New York.
10. Bailey J., Ollis D. (1986) Biochemical Engineering Fundamentals, Mc Graw-Hill, Singapore.
11. Munaò F., Di Pietro A., Scoglio M.E., Grillo O.C., Anzalone C., Minolfi P. (1990) *ACQUA-ARIA*, 769-774.
12. Munaò F., Di Pietro A., Scoglio M.E., Grillo O.C., Anzalone C., Minolfi P. (1991) *ACQUA-ARIA*, 465-470.
13. Dollan J.F., O'Neill M.J., Horan N.J. (1990) *J. IWEM*, **4**, 457-468.
14. Anthonisen A.C. (1978) *J.W.P.C.F.*, **48**, 835-842.
15. Hanaki K., Wantawin C., Ohgaki S. (1990) *Wat. Res.*, **24**, 297-302.

Chapter 26

KINETICS OF DISSOLUTION OF THE CARBONATE MINERALS OF PHOSPHATE ORES BY DILUTE ACETIC ACID SOLUTIONS IN THE PRESENCE OF CARBONIC ANHYDRASE II, FROM BOVINE ERYTHROCYTES

E.M. PAPAMICHAEL, E.D. ECONOMOU, T.C. VAIMAKIS

University of Ioannina, Department of Chemistry, P.O. Box 1186, 45110 Ioannina, Greece. E-mail: epapamic@cc.uoi.gr

Abstract

We report here, in detail, on the effect of carbonic anhydrase II from bovine erythrocytes, on the dissolution process of carbonate minerals of phosphate ores (Epirus area, Greece) in dilute acetic acid, by recording the pH value of the acid solution as a function of time in the pH range from 2.37 to 6.40. Two different reaction systems and two model-equations fitted perfectly the corresponding experimental data, and were used to evaluate all rate constants and parameters. Then, based on these constants and parameters we proposed the possible mechanisms of action for this enzyme from pH 2.37 - 6.40.

Key words: Carbonic anhydrase II, mechanism of action, dissolution of carbonates.

Abbreviations

CA II : Carbonic anhydrase (carbonate hydro-lyase) from bovine erythrocytes EC 4.2.1.1

D : the operator $\frac{d}{dt}$ **D^2 :** the operator $\frac{d^2}{dt^2}$

t : the time in seconds **$p[H^+]$:** $p[H_3^+O]_{ob_{(\text{time of orig})}}$

k_c : $\frac{k'_2(k_5 + 2k_4)}{k_4}$

Introduction

The selective dissolution of ores is an important chemical procedure in which several elementary steps are involved, as mass transport of reactants, adsorption of solutes, and chemical reactions (**1**). The dissolution of pure calcite under acidic conditions, by channel-flow techniques at pH<4, has been examined exhaustively

New Horizons in Biotechnology,285-295, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

by Compton and co-workers (**2-4**). We have also reported previously (**5,6**) two detailed mechanisms of the selective dissolution of calcite (from low-grade phosphate ores - Epirus area, Greece), using dilute acetic acid, and we distinguished five separate pH regions in the pH-range from 2.37 to 6.40, where two different model-equations best fitted the corresponding experimental data.

Carbonic anhydrase II is a zinc metalloenzyme, which catalyzes the reversible reaction of hydration of CO_2. The active site of carbonic anhydrase II (CAII) comprises a Zn^{2+} ion bound by three histidine-imidazoles in a distorted tetrahedral geometry; the fourth metal site is occupied by a H_2O or HO^- depending on the pH (**7-9**).

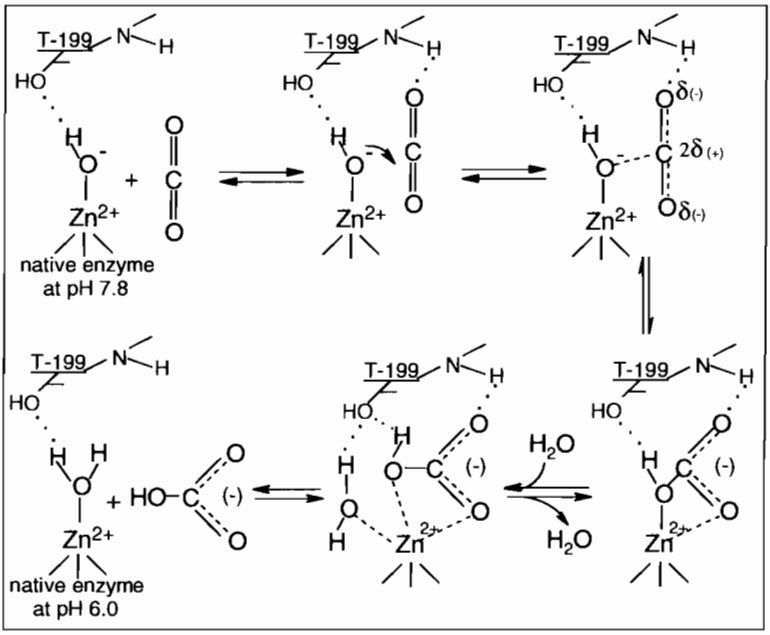
The conversion of CO_2 into HCO_3^-, and vice-versa, proceeds by a "zinc-hydroxide" mechanism, as indicated by reactions [a] - [c]. Distortions around CO_2 site, in the $\mathbf{HCO_3^-}$ complex, allow H_2O to bind near the metal ion, suggesting that $\mathbf{HCO_3^-}$ is exchanged for H_2O through a quasi-six coordinated metal-ion [Scheme 1] (**10**).

$$E\text{-}Zn^{2+}\text{-}OH^- + CO_2 \rightleftharpoons E\text{-}Zn^{2+}\text{-}HCO_3^- \qquad [a]$$

$$E\text{-}Zn^{2+}\text{-}HCO_3^- \underset{H_2O}{\overset{H_2O}{\rightleftharpoons}} E\text{-}Zn^{2+}\text{-}H_2O + HCO_3^- \qquad [b]$$

$$E\text{-}Zn^{2+}\text{-}H_2O \rightleftharpoons {}^+H\text{-}E\text{-}Zn^{2+}\text{-}OH^- \underset{{}^+H\text{-}Base}{\overset{Base}{\rightleftharpoons}} E\text{-}Zn^{2+}\text{-}OH^- + {}^+H\text{-}Base \qquad [c]$$

Other authors (**11**) have suggest that the central step in CO_2-catalyzed hydration is a nucleophilic attack of a zinc-coordinated HO^- upon CO_2 followed by the bidentate coordination to the metal ion of a newly formed $\mathbf{HCO_3^-}$, and its subsequent release into solution upon binding of a H_2O to the Zn^{2+} ion.



Scheme I

In this manuscript we report on the effect of CA II on the dissolution process of carbonate minerals of phosphate ores by dilute acetic acid solutions, in the pH range 2.37-6.40, by repeating previous experimental procedures (**5,6**), and adding also in the reaction mixture different quantities of CA II. As before (**5,6**) we distinguished five pH regions in the pH-range 2.37-6.4, two different reaction systems, and two model-equations, which fitted perfectly the corresponding experimental data, and were used to evaluate all rate constant and parameters. Accordingly, we propose the most likely mechanisms of action of CA II from pH 2.37 to 6.40.

Materials and Methods

Materials

As before (**5,6**), the material used was unaltered phosphate ore, from the Kosmira-Epirus area, in northwest Greece. The acetic acid solutions were prepared by dissolution with distilled water of glacial acetic acid (100 wt%) (Ferak Laborat GmbH Berlin). Carbonic anhydrase II (carbonate hydro-lyase EC 4.2.1.1.) from bovine erythrocytes, electrophoretically purified, was purchased from Sigma, and used without further purification.

Methods : The Dissolution Procedures

As in our earlier works (**5,6**), a fraction of 500-250 μm was yielded, by crashing and sieving natural phosphorite from the Kosmira-Epirus (Greece) area, and the dissolution process was carried out in a 500-ml open glass reactor with a spherical bottom at a rate of 200 RPM. In each experiment a 41.4-ml volume of a 1M solution of CH_3COOH was transferred into the reactor, the temperature was set at 25°C, and the pH value of the solution was measured ($pH_{t=0} = 2.37$). The reaction was initiated by the addition 5.00 g of phosphorite sample, at time $t = 0$, and its progress was followed up by recording the pH value of the acid solution up to $pH \approx 4$ (**6**) and/or $pH \approx 6.40$ (**7**), as a function of time. A PHM 83 pH meter (Radiometer Copenhagen) autocalibrated by two buffers was used, and equipped with a HAMILTON LIQ-Glass electrode valid in the pH range from 0 to 14. The calibration procedure was routinely carried out successfully before and after any series of measurements.

The above experimental procedures were repeated by adding in the reaction mixtures different quantities of the enzyme CAII. We performed successive experiments by adding 3, 6, 9, 12, and 15 mg of lyophilized enzyme powder in the reactor while the rest of the reactants and the conditions of the reaction were kept the same, as above. In all cases, additional series of experiments were performed as before (**5,6**). Thus we tested both the relationship between the process rate and the flow pattern of $\mathbf{H_3^+O}$ through reaction coordinates at 25°C, and the change in the overall process rate by performing experiments at 15°C.

Methods : Empirical Fitting and Preliminary Tests

As before (**5,6**), all experiments were recorded as functions of the pH values of the acid solutions versus time, and they were fitted empirically according to Eqs. [1], and [2], where the subscript **ob** is referred to $\mathbf{[H_3^+O]}$ (from the recorded pH values), and $z > 0$ and $0 < m < 1$ are arbitrary constants, provided that $-z = \log_{10}(m)$.

$$\log_{10}\left\{[H_3^+O]_{ob}\right\} = -z - n\log_{10}(t) \qquad [1],$$

$$[H_3^+O]_{ob} = \frac{m}{t^n} \qquad [2],$$

Likewise our experimental data were transformed according to Eq. [1], divided into five separate sets of which the different z and n values were calculated by linear regression analysis. The results were quite similar to previously reported ones (**6**). By applying three suitable tests (**12**), we identified the controlling step for each one of the five sets corresponding to five pH regions in the pH-range 2.37-6.40; we found that the controlling step in the pH region 2.37-3.96 was a chemical change, however, mass transfer significantly contributed to the rate-determining step of the reaction in the pH-range 3.96-6.40.

Results and discussion

Based on both the results from the empirical fitting and the preliminary tests, we could propose the following general reaction system, which was helpful in distinguishing the differences between pH regions. In that reaction system we maintained the previous symbolism (**5,6**), however, the k_2' in reaction [e] denotes a rate constant of a reaction whose controlling step is either a chemical change or a mass transfer.

$$4\,CH_3COOH + 4\,H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 4\,CH3COO^- + 4\,H_3^+O \qquad [d]$$

$$(CaCO_3)_s + H_3^+O \xrightarrow{k_2'} Ca^{++} + HCO_3^- + H_2O \qquad [e]$$

$$3\,HCO_3^- + 3\,H_3^+O \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 3\,H_2CO_3 + 3\,H_2O \qquad [f]$$

$$2\,H_2CO_3 \xrightarrow{k_4} 2\,(CO_2)_{gas} + 2\,H_2O \qquad [g]$$

$$(CaCO_3)_s + H_2CO_3 \xrightarrow{k_5} Ca^{++} + 2\,HCO_3^- \qquad [h]$$

Based on the same concepts, as previously, we should accept that the condition $\frac{d[HCO_3^-]}{dt} = 0$ is valid for the pH regions 2.37-3.96, and 3.96-4.95, while we may assume that $\frac{d[HCO_3^-]}{dt} \neq 0$, and $\frac{d[H_2CO_3]}{dt} = 0$ are valid in case of the pH regions 5.50-5.93 and 5.93-6.40 (**5,6**). However, both of the preceding cases are appropriate for the pH region 4.95-5.50, due to its transitional character (**6**). Therefore, $Vr = -\frac{d[H_3^+O]_{ob}}{dt}$, is regarded as the overall reaction rate, and depending on the pH region, Vr is the sum of two components; the first component $Va = -\frac{d[H_3^+O]_a}{dt}$ it is due to attack of a phosphorite particle by $[H_3^+O]$ on the particle's surface according to reaction [e], and it is valid for all fiver pH regions. The second component Vb varies considerably, and in case where the condition $\frac{d[HCO_3^-]}{dt} = 0$ is valid it is equal to $-\frac{d[H_2CO_3]}{dt}$ (Eq. [3]). In that latter case, reaction [h] should be ruled out from the reaction system. However, in cases where both $\frac{d[HCO_3^-]}{dt} \neq 0$ and

$\frac{d[H_2CO_3]}{dt} = 0$ are valid the second component seems most likely to be due to the increase of HCO_3^- (**13**), and becomes $V_b' = \frac{d[HCO_3^-]}{dt}$ (Eq. [4]).

$$V_r = -\frac{d[H_3^+O]_{ob}}{dt} = V_a + V_b = -\frac{d[H_3^+O]_a}{dt} - \frac{d[H_2CO_3]}{dt} \quad [3]$$

$$V_r = -\frac{d[H_3^+O]_{ob}}{dt} = V_a + V_b' = -\frac{d[H_3^+O]_a}{dt} + \frac{d[HCO_3^-]}{dt} \quad [4].$$

In all cases we assumed that the steady-state condition was established within a time interval considerably less than the time scale of the reaction. On the other hand and in analogy to our previous works (**5,6**), we could produce the differential equation [5], suitable for the pH regions 2.37-3.96 and 3.96-4.95, and the system outlined by reactions [d] up to [g]; the primitive of this latter differential equation is Eq. [6], where r_1, and r_2 are the roots of the quadratic polynomial in brackets to the variable **D**, and equal to $-\frac{(k_2' + k_4)}{2} \pm \frac{\sqrt{(k_2' + k_4)^2 - 8k_2'k_4}}{2}$, while C_1 and C_2 are the arbitrary integration constants.

$$[D^2 + D(k_2' + k_4) + 2k_2'k_4][H_3^+O]_{ob} = 0 \quad [5]$$

$$y = C_1 \cdot e^{r_1 \cdot t} + C_2 \cdot e^{r_2 \cdot t} \quad [6].$$

The case of the system outlined by reactions [d] up to [h], which is appropriate for the pH regions 5.50-5.93 and 5.93-6.40, can be described by Eq. [7]. However, both Eqs. [6], and [7] can be suitable for the pH region 4.95-5.50 (**6**).

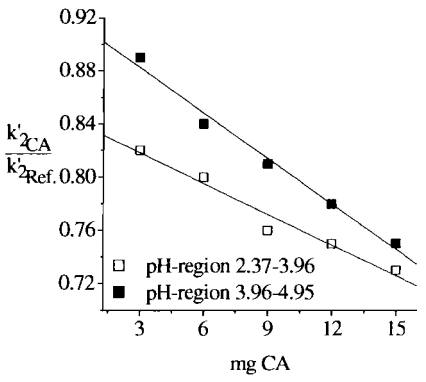
$$[H_3^+O]_{ob} = [H_3^+O]_{ob(\text{time of orig})} \exp\left[-\frac{k_2'(k_5 + 2k_4)}{k_4} t\right] \quad [7].$$

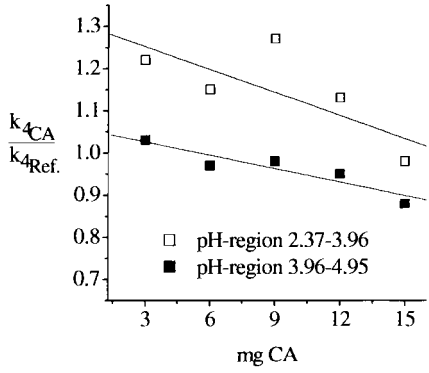
Equation [6] fitted successfully the experimental data of the pH regions 2.37-3.96, and 3.96-4.95, including these of pH region 4.95-5.50, and by taking into account the same considerations as before (**5,6**). Consequently, we assigned the estimates of the rate constants k_2' and k_4 of the slow reactions for the pH regions 2.37-3.96, 3.96-4.95, and 4.95-5.50, as before (**6**). The dependence of ratios of the estimated rate constants k_2' and k_4 of all enzymic reactions of pH regions 2.37-3.96, 3.96-4.95 and 4.95-5.50, to the corresponding rate constants from reference non-enzymic reactions followed a negative correlation, when the amount of CA II in the reactor was increased, as it is evident from Fig.1.

Equation [7] fitted successfully the experimental data of the pH regions 5.50-5.93, and 5.93-6.40; moreover, the experimental data of the pH region 4.95-5.50 were also fitted successfully by Eq. [7]. The dependence of ratios of all the estimated parameters of the enzymic reactions in case of the pH regions 4.95-5.50, 5.50-5.93, and 5.93-6.40, to the corresponding parameters of reference nonenzymic reactions

followed: (a) a negative correlation (parameter $\mathbf{p[H_3^+O]_{ob_{(time\ of\ orig)}}}$, simplified as $\mathbf{p[H^+]}$ or (b) a positive correlation (parameter $\frac{k'_2(k_5+2k_4)}{k_4}$, simplified $\mathbf{k_c}$ as it is evident from Fig. 2. These findings were less obvious for the pH region 4.95-5.50 whose transitional character had been emphasized earlier (**6**). In that latter case and on the basis of our results, we should suggest a zero correlation for all the ratios of $\mathbf{k_2'}$ and $\mathbf{k_4}$ rate constants and $\mathbf{p[H^+]}$ and $\mathbf{k_c}$ and parameters.

In agreement to our previous works (5,6), Scheme II describes more likely reaction [g] of nonenzymic reactions, for the pH regions 2.37-3.96, 3.96-4.95, and to some extent (**7**) for the pH region 4.95-5.50. Therefore, in view of the above results it seems logical to suggest that Scheme III illustrate the mechanism of action of carbonic anhydrase II for the first two pH-regions; however we may propose Scheme IV for the pH region 4.95-5.50 as a more likely one.





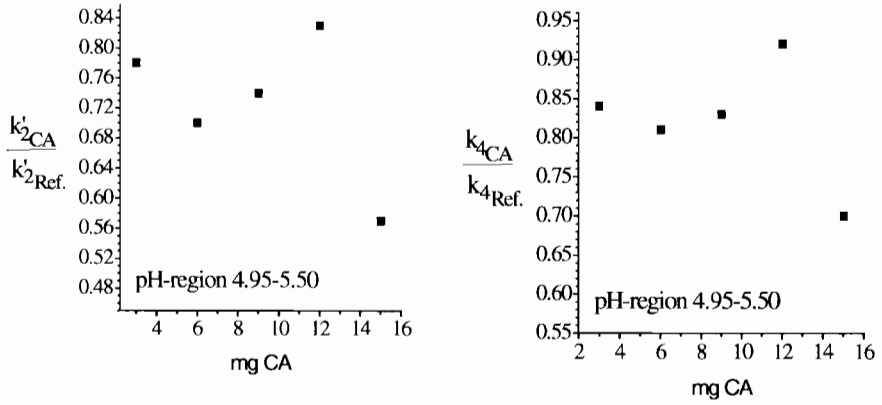
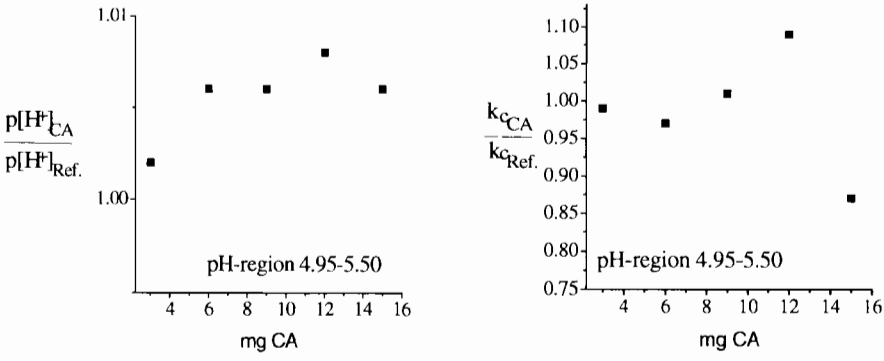


Figure 1. A linear model was found inadequate to best fit the ratios of the estimated rate constants k_2' and k_4 to the corresponding ones from the reference nonenzymic reactions (pH-regions 2.37-3.96, 3.96-4.95 and 4.95-5.50), versus the mg of CAII added in the reaction mixture. However, we observed a negative correlation between these regression variables (pH-regions 2.37-3.96, and 3.96-4.95), and an almost zero correlation between them, in case of the pH-region 4.95-5.50.



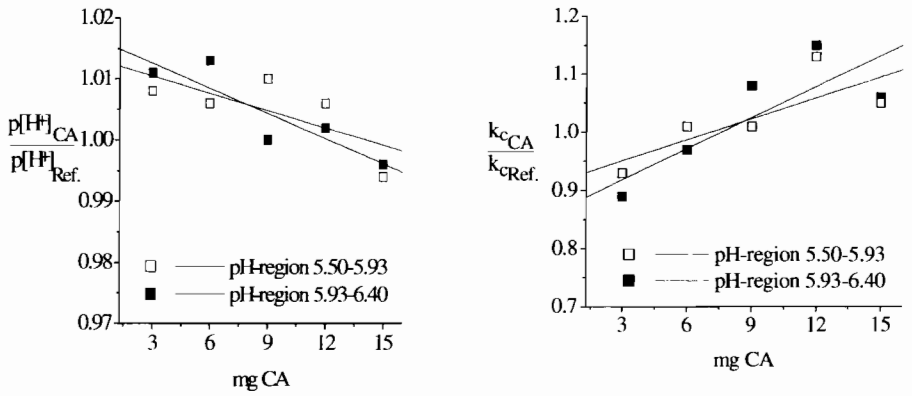
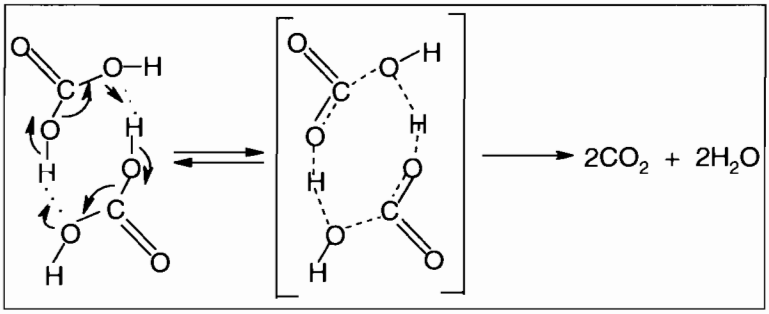
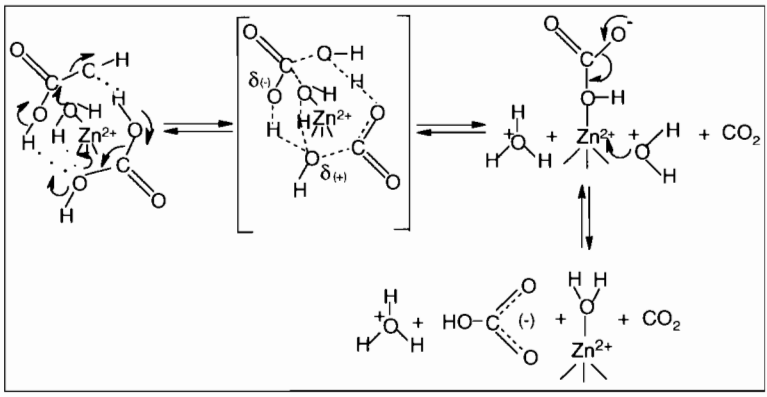


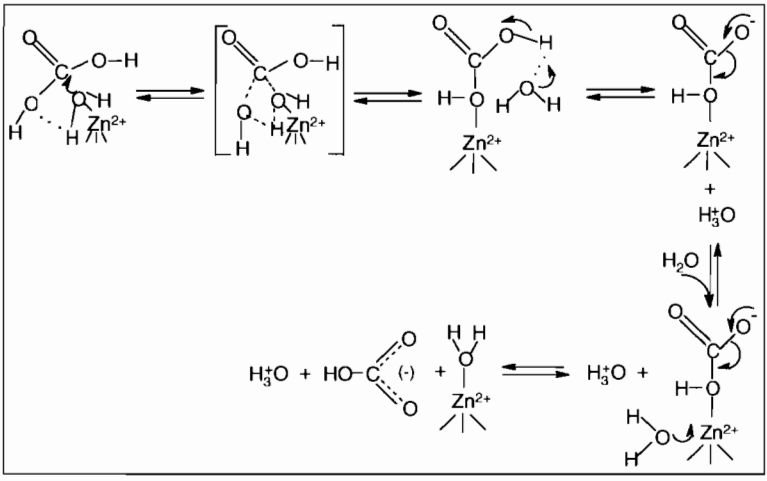
Figure 2. A linear model was found inadequate to best fit the ratios of the estimated parameters $\mathbf{p[H^+]}$ and ($\mathbf{k_c}$) to the corresponding ones from the reference nonenzymic reactions (pH-regions 4.95-5.50, 5.50-5.93 and 5.93-6.40), versus the mg of CAII added in the reaction mixture. However, we observed a negative correlation between these regression variables (pH-regions 4.95-5.50, 5.50-5.93 and 5.93-6.40), and an almost zero correlation between them, in case of the pH-region 4.95-5.50.



Scheme II



Scheme III



Scheme IV

Concerning the mcchanism of action of CA II in the rest two pH regions 5.50-5.93, and 5.93-6.40, we should take into account the ratios of the estimates of parameters $\mathbf{p[H^+]}$ and ($\mathbf{k_c}$) of the enzymic reactions to the corresponding parameters of reference nonenzymic reactions, and accordingly, we suggest that again Scheme IV illustrates the mechanism of action of CA II, in these pH regions.

References

1. Vaimakis T.C., Papamichael E.M. (2002) *Encyclopedia of Surface & Colloid Science,* 1471-1485.
2. Compton R.G., Pritchard K.L., Unwin P.R. (1989) *J. Chem. Soc. Chem. Commun.* **4**, 249-251.
3. Compton R.G., Pritchard K.L., Unwin P.R. (1989) *Freshwater Biol.* **22**, 185.
4. Compton R.G., Unwin P.R. (1990) *Philos. Trans. R. Soc. London, Ser. A* **330**, 1.

5. Economou E.D., Vaimakis T.C., Papamichael E.M. (1997) *J. Colloid Interface Sci.* **201**, 165.
6. Economou E.D., Vaimakis T.C., Papamichael E.M. (2002) *J. Colloid Interface Sci.* **245**, 133.
7. Lesburg C.A., Huang C.C., Christianson D.W., Fierke C.A. (1997) *Biochemistry* **36**, 15780.
8. Steiner H., Jonsson B.H., Lindskog S. (1975) *Eur. J. Biochem.* **59**, 253.
9. Christianson D.W., Fierke C.A. (1996) *Acc. Chem. Res.* **29**, 30.
10. Liljas A., Håkansson K., Jonsson B.H., Xue Y. (1994) *Eur. J. Biochem.* **219**, 1.
11. Ren X., Jonsson B.H., Millqvist E., Lindskog S. (1988) *Biochimica et Biophysica Acta* **953**, 79.
12. Kafarov V. (1964) "Cybernetic Methods in Chemistry and Chemical Engineering." p. 383, Mir, Moscow, [translated in English].
13. Somasundaran P., Ofori Amankonah J., Amanthapadmabhan K.P. (1985) *Colloid Surfacse* **15**, 309.

Chapter 27

NEW TRENDS IN KEFIR YEAST TECHNOLOGY

A. A. KOUTINAS

Food Biotechnology Group, Department of Chemistry, University of Patras, Patras, Greece. E-mail: A.A.Koutinas@upatras.gr

Abstract

The development of new biotechnologies and foodstuffs using kefir yeast are reviewed. The strategy adopted was to examine fermentations of synthetic media containing sugars of raw materials and wastes of food production. Kefir cell growth was also examined. An important efficiency of kefir yeast in glucose, fructose, sucrose and lactose fermentations have been proven. Fermentations were performed in comparison with immobilized biocatalysts prepared by immobilization of kefir on delignified cellulosic (DC) materials, gluten and mineral kissiris. DC material supported kefir is suitable for low-temperature alcoholic fermentation in the range of 5-15°C.

The above research supported the development of technologies and products using whey. Therefore, the production of an alcoholic drink, potable and fuel-grade alcohol and SCP production from whey are reported. Whey fermentation by free cells resulted in a fermentation time of 35h, while DC material supported cells of kefir gave less than 10h. The addition of 1% raisin extracts reduced the fermentation time in whey fermentation by free cells to 12h. Mixtures of whey with molasses were also used to increase alcohol concentration. The SCP production using whey resulted to granular biomass lead to avoidance of centrifugal separators in production plants. Kefir protein showed improved emulsifying, foaming and gel forming properties in comparison with Soya protein and fermented wort at 15°C in one day for brewing. That research was the basis for scale-up processes in a pilot plant with 100L, 1000L, 10,000L bioreactors for repeated batch and continuous processes employing kefir granules and DC materials supported kefir.

Introduction

Solid and liquid wastes containing carbohydrates are produced on a large scale by the food industry, strengthening the universal environmental issue. Those wastes contain mainly lactose, sucrose, glucose and fructose. Known wastes are whey from the dairy industry, those from childlike food production, chocolate and ice cream as well as candy processing. Solid wastes and liquid effluents will create a high capacity of raw material for the production of foodstuffs through the development of new biotechnological processes and products. However, a new material could be produced by mixing solid and liquid wastes that contain relatively high lactose concentrations. Therefore, the cost effective conversion of lactose and its mixture with glucose, sucrose and fructose will have to be achieved.

New Horizons in Biotechnology, 297-309, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands

Research efforts have been undertaken over the last two decades to obtain alcohol production employing lactose. Strains of *Kluyveromyces fragilis* (**1-3**) and *Kluyveromyces marxianus* (**4,5**) and *K. fragilis* immobilized on alginates (**6,7**) have been proposed. *K. fragilis* was also immobilized on beech wood and pellets of activated carbon (**8**). Recombinant DNA techniques (**9,10**) and co-immobilization using *Saccharomyces cerevisiae* with β-galactosidase (**11**) have also been examined. The above research shows the need for a suitable organism and immobilization support to ferment effectively, each sugar separately and mixtures of lactose, sucrose, glucose and fructose. The strategy adopted was (i) the use of kefir yeast to examine its efficiency on the fermentation of glucose, fructose, sucrose and lactose. Kefir yeast is a known culture employed in the production of the traditional Russian alcoholic drink kefir, from milk. (ii) Subsequently, to examine whey fermentation to produce an alcoholic drink, potable alcohol and SCP production. (iii) Scale-up of kefir yeast technology using whey. (iv) The creation of a new raw material by mixing whey and the above solid wastes for the development of kefir yeast technology.

Variation of carbohydrate substrate in fermentation by immobilized *Kefir* yeast

To find the most suitable form of biocatalyst, fermentations were performed by using free cells of Kefir yeast in comparison with cells immobilized on mineral kissiris and on delignified cellulosic (DC) material and the results are presented in Figure 1 (**12**).

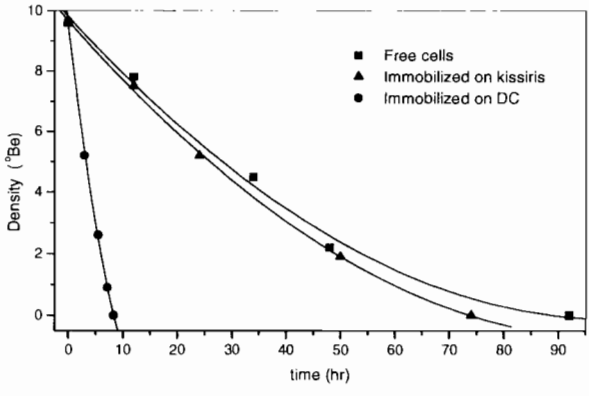


Figure 1. Kinetics of glucose fermentation by the presence of DC material supported kefir yeast compared with kissiris supported kefir and its free cells.

DC material supported biocatalyst resulted in a drastic drop of the fermentation time as compared with kissiris supported cells of Kefir and free cells. Therefore, DC material was thought to be a convenient support for immobilization of Kefir

yeast and then DC material supported biocatalyst was used to ferment sucrose, fructose, glucose and lactose. The results are presented in Figure 2 (**12**).

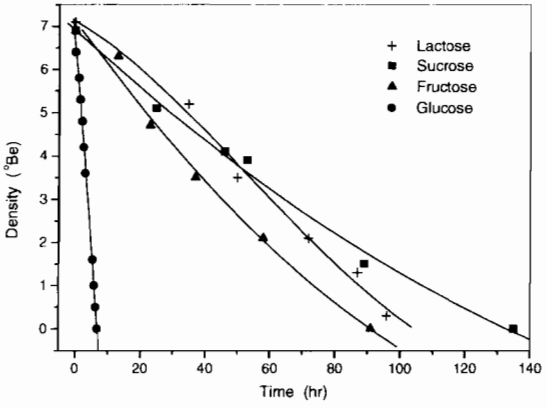


Figure 2. Effect of carbohydrates on kinetics of the alcoholic fermentation by DC material supported kefir yeast.

Glucose fermentation was faster in comparison with sucrose, lactose and fructose, while lactose was fermented faster than fructose and sucrose. Sucrose fermentation showed a longer fermentation time. The DC material used in this research and development seemed to be a convenient support for the industrialization of cell immobilization, because it satisfied prerequisites of abundance in nature, low cost, food grade purity, inability to be destroyed in the bioreactor (as are alginate beads) and ease of handling at the industrial scale due to it is light and porous material.

The above results clearly showed that it was quite interesting to study fermentations of mixtures of fructose, sucrose and glucose, as well as mixtures of whey with raisin extracts using DC material supported Kefir. This study was to be performed since industries may employ raw materials contain more than one carbohydrate. Figure 3 shows the efficiency of this biocatalyst to ferment the mixture (**12**).

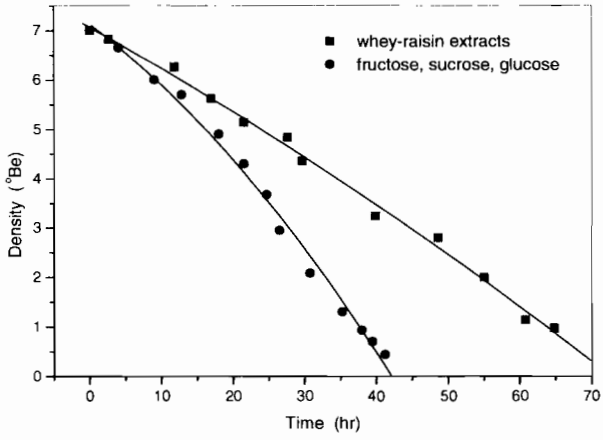


Figure 3. Fermentation kinetics observed using DC material supported kefir yeast in mixtures of fructose, sucrose and glucose as well as whey with raisin extracts.

In comparison with fructose and sucrose mixtures had higher fermentation rates. Fermented products obtained from mixtures of whey with raisin extracts had a fine aroma. This could be considered for the production of a novel drink product. Furthermore, fermented products were analysed so as to determine the most abundant volatiles present in traditional alcoholic drinks. Therefore, it was found that acetaldehyde, ethylacetate, propanol-1, iso-butyl-alcohol and amyl alcohols were formed in the fermentation of all sugars studied. Concentrations of those volatiles were in the range of traditional alcoholic drinks. That meant that *Kefir* yeast could be a convenient microorganism for alcoholic drink production.

Low temperature fermentation by immobilized Kefir

The DC material supported cells of Kefir were then used for the repeated batch fermentation of a liquid culture containing glucose. A total of 30 batches at various temperatures ranging from 5 to 30°C were performed to study the activity of the biocatalyst at low-temperature fermentations and its ability to carry out repeated batch fermentations. Results are summarized in Table 1. These results show (i) the high operational stability of the biocatalyst, (ii) the system is not easily contaminated, (iii) the possibility of low temperature fermentation, (iv) relatively high ethanol concentration was obtained, (v) productivity up to 5°C could be acceptable by the industry.

Table 1. Effect of Temperature on kinetic parameters of alcoholic fermentation of glucose by DC material supported cells of kefir yeast (**13**).

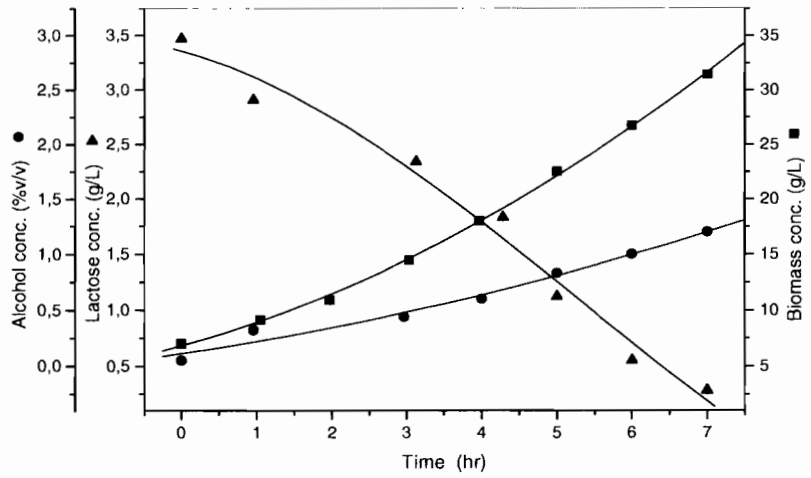
Temp. °C	Repeated batch fermentation	*Fermentation* Time (h)	*Ethanol* concent. (%v/v)	Residual Sugar (g/L)	Yield (g/g)	Ethanol Productivity (g/L/h)	Conversion (%)
30	1-5	7.7	6.4	5.8	0.31	6.8	96.6
20	11-15	13.3	9.0	1.2	0.42	5.3	99.3
15	16-20	23.5	9.1	1.5	0.43	3.1	99.1
10	21-25	64.7	8.1	4.2	0.38	1.0	97.1
5	26-28	137.7	7.9	13.9	0.40	0.4	91.9

It is important to note that the fermentation rate was comparable to that obtained by DC material supported cells of *S. cerevisiae* (**14**). Likewise, percentages of amyl alcohols on total volatiles determined were reduced, while percentages of ethyl acetate were increased as the temperature was decreased. These results indicated an improvement of the aroma of the fermentation product due to the process.

SCP production by Kefir using whey

The aerobic bioconversion of whey containing lactose was an alternative solution to produce products with added value from whey. Therefore SCP production from whey using kefir could lead to a protein-rich livestock feed for animals and may be to protein that could be used as an additive in foods to provide emulsifying, foaming and gel forming properties.

SCP production was studied firstly employing synthetic media containing lactose and then whey. Figure 4 shows cell growth of kefir in a bioreactor of 2L supplying air by an air compressor passed through a sterile filter. This result clearly showed that after 7 hr all lactose was utilized and formed 33 g/L biomass and 1-2% v/v ethanol.



Figures 4. Cell growth of kefir in a bioreactor contained 1L synthetic media consists of lactose 3.5%, 0.1% $(NH_4)_2SO_4$, 0.4 KH_2PO_4, 0.4% yeast extract and 0.5% $MgSO_4$. Aerobic fermentation using inocula of 6.5g/L was performed, pH adjustment at 5.5 during the aerobic fermentation. Temp. 30°C (**15**).

Likewise, Figure 5 showed *kefir* yeast grew also in whey with salinity, and provided about the same final biomass concentration (33-35 g/L) with whey without salt, as well. No significant differences in the rate of lactose utilization were observed.

The SCP production was examined with regards to its emulsifying, foaming and gel forming properties in foods in comparison with Soya protein. Kefir protein resulted in a three-fold increase of foam volume while the foam was more stable in comparison with that obtained by Soya. Likewise, the emulsifying property of this protein seems to be improved, since the increase of diameter of the emulsion particles during 32 days was higher for Soya protein emulsifier. Finally, the results obtained by the texture profile analysis in gels prepared with kefir SCP and Soya showed that gels from kefir had 2-3 fold higher gel rigidity, fragmentation and elasticity as compared with Soya (**16**).

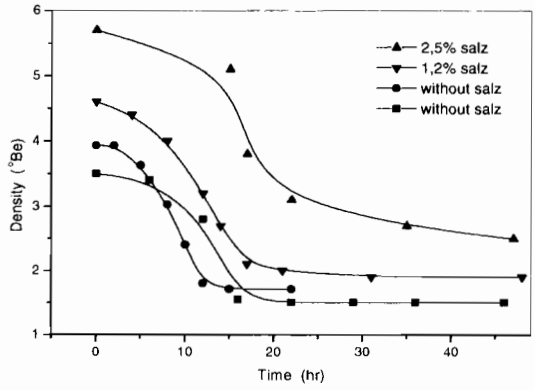


Figure 5. Kinetics of whey aerobic fermentation for biomass production at 30 °C. Inoculum 2g/L (**15**).

Kefir granules

Granular biomass is obtained by the aerobic fermentation of whey. Kefir granules have a particle size usually less 1 mm and are precipitated after stopping air supply. Synthetic media containing lactose did not form granular biomass. Those granules were used to obtain repeated batch fermentation of whey and wort in brewing without centrifugation of cells. They could be used and substitute immobilization of cells of kefir to obtain continuous processes. However, the latter needs experimental validation, by pilot scale experiments.

Fermentation of whey to produce alcoholic drink employing Kefir granules

Kefir granules were employed to ferment whey. The fermentation lasted two days. However, the addition of 1% raisin extracts calculated on whey volume dropped the fermentation time to 16 hr. Likewise, cell immobilization on DC material reduced further the fermentation time to 9 hr. Both completed fermentations, resulted in an alcohol concentration of 2.5-3% v/v and traces of residual sugar. Five-repeated batch fermentations promoted by raisin extracts were obtained without any loss of the activity and without contamination of cells.

The same was obtained by DC material supported Kefir yeast. A preliminary taste test showed an improvement of the aroma obtained in the fermented products.

Brewing using kefir granules

Since the efficiency of kefir yeast in glucose fermentation was proved, runs for fermentation of wort were organized employing kefir granules. However, the direct use of kefir granules at 15°C, which is the usual temperature of industrial brewing, resulted in a fermentation time of 400 hr to complete the fermentation of wort. Therefore, an adaptation of the temperature resulted in successful results. The adaptation was performed by gradual decrease of the temperature from batch to batch in a series of repeated batch fermentations using the same kefir granular biomass.

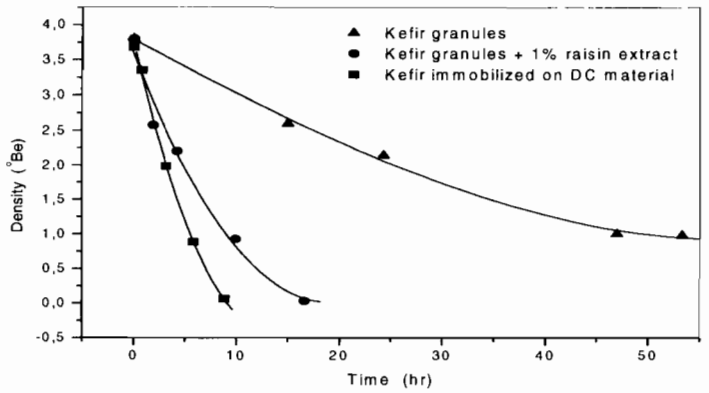


Figure 6. Whey fermentation kinetics observed by kefir granules, kefir granules and addition of 1% raisin extracts and immobilized kefir on DC material. PH was adjusted at 5.5 by the addition of Na_2CO_3 during fermentation. Biomass conc. 20g/L. Temp. 30°C .

The decrease of temperature from batch to batch was 3°C so as to catch 15°C and then 11°C. This adaptation reduced sharply the fermentation time to 24 hr while at 11°C it was 45-50 hr. Granular biomass was used without centrifugation from batch to batch and the fermentation was continued at even lower temperatures. An alcoholic degree of 6.4-8 %v/v was obtained along with fermentation of glucose

and maltose. Several repeated batch fermentations were obtained using the same biomass without loss of activity, while this granular biomass was not contaminated after 15 batches. GC analysis of beer showed the formation of volatiles during the fermentation as in brewing using *S. cerevisiae* .

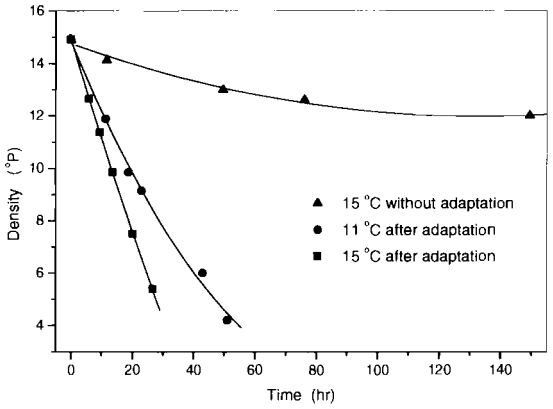


Figure 7. Fermentation kinetics of wort observed at 15°C and 11°C using kefir granules. Biomass conc. 20 g/L .

Reinforcement of whey to increase alcohol concentration

An increase in the final alcohol concentration of fermented whey is needed for potable alcohol production from molasses as well as lactina (a commercial product produced from whey containing mainly lactose). Figure 8 shows that lactina provides a higher fermentation rate than molasses even when immobilized cells on DC material are employed. Therefore the final alcohol concentration obtained was in the range of 5-6 % v/v.

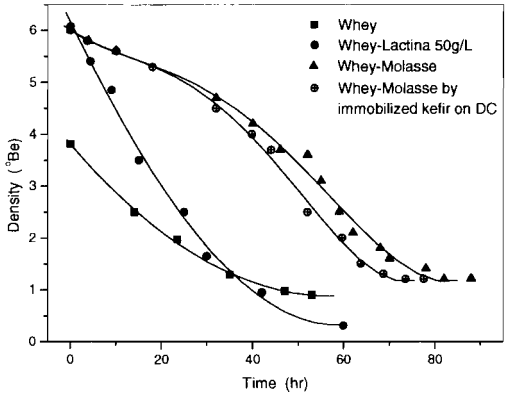


Figure 8. Fermentation kinetics observed to reinforce final alcohol concentration using kefir granules and its immobilized cells .

Technical advantages by *kefir* yeast technology

The above outline of the results obtained by the use of kefir yeast lead to the following conclusions. The formation of granular biomass may result in the avoidance of centrifugal separators in the aerobic and anaerobic fermentation of whey. This could decrease construction cost, labour cost, maintenance cost and electricity. Likewise, facilitating repeated batch fermentations and thereby contributing to industrialization of the use of kefir yeast. The promotion effect of whey fermentation by a small amount of raisin extract ensures cost-effective whey fermentation. The adaptation treatment by a gradual decrease of temperature gave the possibility for low-temperature fermentations. This in relation with the important fermentability in glucose fermentation provides the advantage of kefir yeast being efficient in brewing. The significant fermentability of glucose, lactose, sucrose and fructose made this microorganism capable of treating of wastes of dairy industry, those of childlike food production, chocolate and ice cream as well as candy processing. Furthermore, the equal fermentability of salty whey, with that without salt, in SCP production made the exploitation of whey independent from the dairy process. The improved results obtained by the DC material supported kefir yeast, increased the possibility for industrialization of immobilized cells due to the fact that DC material satisfied prerequisites for cost effective immobilization.

Scale-up

The aforementioned results encouraged us to scale-up processes. The design included (i) the production of kefir biomass to ferment 70, 1000, 7000 L whey and to examine in a preliminary base the possibility of a protein rich livestock feed for animals or the protein as food additive to improve emulsifying, foaming and gel forming ability of foods. (ii) Also, to produce an alcoholic product which could be the starting material for the production of a novel alcoholic drink. (iii) To strengthen the alcohol concentration of the fermented product that could be used as raw material for potable alcohol production in an alcohol distillery.

To do this scale-up we designed bioreactors of 100 L, 3000L, 11000 L to perform experiments by kefir granules and then by immobilized cells of kefir on DC material.

Experiments in the bioreactor of 100 L by *Kefir* granules

In a lab bioreactor of 5 L were prepared 360 g kefir and this was the starter of the bioreactor of 100 L (Fig. 9) contained 50 L whey. The aerobic bioconversion produced about 2000 g kefir biomass in 12-15 hr. The liquid contained also traces of lactose and 0.5% v/v ethanol. Most of the biomass was precipitated. About 35 L were decanted and then 60 L of whey were added followed by 0.6 L of raisin extract. The pH was adjusted to 5.5 with citric acid and during fermentation by the

addition of Na_2CO_3 solution. The fermentation lasted 10 hr and produced 2.5-3% v/v alcohol and less of 2 g/L lactose.

The bioreactor of 100 L was supplied by a compressor with air, passed through a sterile filter and spread into the whey by a vertical perforated pipe. Manual cooling and heating were provided by a circular perforated pipe, which externally wetted the bioreactor with tap or warm water.

Whey fermentation in the bioreactor of 3000 L by *Kefir* granules

Kerir biomass of 3500 g inoculated in 500 L of whey at 28°C containing 1.6 g/L $(NH_4)H_2PO_4$ and 7.5 g/L $(NH_4)_2SO_4$ and after supplying air for 12-15 hr, kefir biomass of 20-25 Kg was produced. Then the supernatant liquid of 250 L was decanted. 500 L of whey (contained 1% v/v raisin extracts) were added and allowed to ferment by anaerobic conditions. Furthermore, the bioreactor was fed upto 2000L with whey containing 1% raisin extract. An alcohol concentration of 2.5-3% v/v and less of 2 g/L residual lactose was obtained within 15-20 hr.

A compressor supplied the bioreactor of 3000 L with air passing through the sterile filter and spreading into the whey by a system similar to that of the 100 L bioreactor system. The bioreactor was joined with a plate heat exchanger for cooling or heating of the fermented whey.

Whey fermentation in the bioreactor of 11000 L by *Kefir* granules

The 11000 L bioreactor was a stainless steel tower batch and continuous system. The reactor has three packed sections of 1 m depth. Radial beams at each packed sections support the netting to prevent DC material flotation. Openings are provided to fill the bioreactor with DC material. Manual cooling and heating were provided by a circular perforated pipe, which externally wetted the bioreactor with tap or warm water. For continuous operation the door at the top was closed tightly and whey was pumped by the bottom inlet and the product received by the upper outlet. A door at every level to accommodate supports and emptying of kefir granules and windows to observe fermentation are also provided. This bioreactor was also able to operate using kefir granules as well as DC material supported cells of kefir yeast.

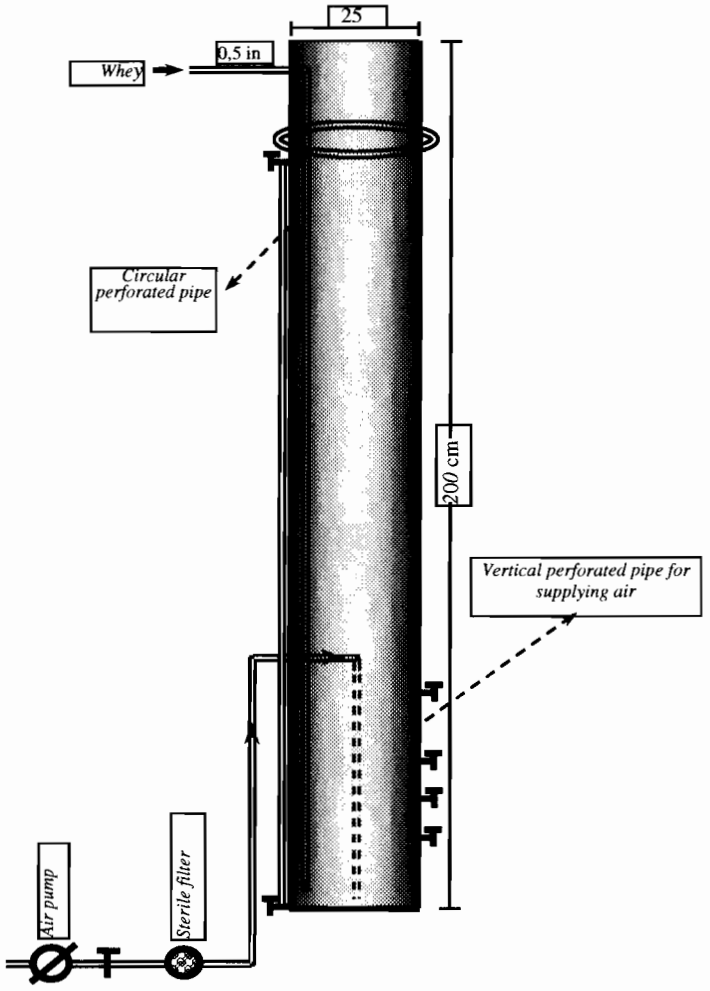


Figure 9. Bioreactor of 100L for biomass production using whey and alcoholic fermentation of whey.

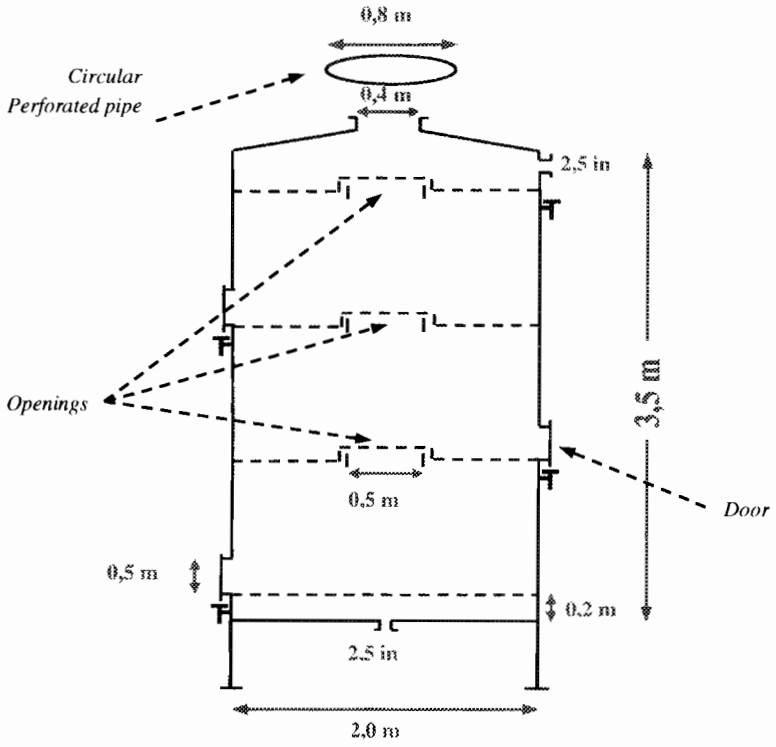


Figure 10. Bioreactor of 11,000L for whey fermentation using kefir granules and DC material supported cells of kefir yeast.

Fermentation

In the bioreactor of 100 L we prepared 3500 g kefir granules, which were pumped in 500 L of whey forced in the bioreactor of 3000 L. After an aerobic treatment at 28°C, as above described, 20-25 Kg kefir granules were obtained. Subsequently, 2000 L of whey were pumped for aerobic fermentation. An amount of 150 Kg of kefir granules was obtained.

Granules were precipitated in the bioreactor of 3000 L are pumped as a slurry in the bioreactor contains 7000 L and for anaerobic fermentation was allowed to take place (**20**).

Economic evaluation of *Kefir* yeast technology and Conclusions

Through kefir yeast technology using whey, potable alcohol, fuel-grade alcohol, kefir drink, protein rich livestock feed for animals, can be produced. Their production has low construction costs and their production is cost effective as it shown in the Table 2.

Table 2. Products and costs as well as added value are created by the new kefir yeast technology.

Product	Price (Euro/Kg)	Estimation of construction cost* in Greecc (Euro)	Estimation of production cost (Euro/Kg)	Annual production capacity (m^3)	Estimation of created added value in Greece (Euro/year)	Estimation of created added value in European Union (Euro/year)
Potable alcohol	0.74	8,000,000	0.26	21000	$12.4x10^6$	$600x10^6$
Kefir drink	0.70	8,000,000	0.25	700,000	$500x10^6$	$25x10^9$
Protein rich Livestock feed for animals	0.80	12,000,000	0.15	21,000	$100x10^6$	$5.2x10^9$
Baker's Yeast	0.80	15,000,000	0.05	63,000	$300x10^6$	$15.6x10^9$
Protein additive as emulsifier in foods	0.80	12,000,000	0.15	21.000	$100x10^6$	$5.2x10^9$

Annual production capacity in Greece is estimated to 700.000 m^3
*This cost is estimated taking into account the production capacity of whey in Greece.

References

1. O'Leary V.S., Sutton C., Bencivengo M., Sullivan B., Halsinger V. (1977) *Biotechnol. Bioeng.* **19,** 1689.
2. Gawel J., Kosikowski F. (1978) J. *Foods*, **43**, 1717-1719.
3. Moulin G., Guillaume M., Galzy P. (1980) *Biotechnol. Bioeng.* **22**, 1277.
4. Marwaha S.S., Kennedy J.F., Seghal V.K. (1988) *Process Biochem.* **24**, 17.
5. Marchant R., Nigam P., Brandy D., McHale L., McHale P. (1996) *Biotechnol. Lett.* **18**1213.
6. Hahn-Haegerdal Baerbel (1982) *Appl. Biochem. Biotechnol.* **7**, 43.
7. King V.A., Zahl R.R. (1983) *Process Biochem.* **18**, 17.
8. Gianneto A., Berruti F., Glick B., Kempton A. (1986) *Appl. Microbiol. Biotechnol.* **24**, 277.
9. Sreekrishna K., Dickson R.C. (1985) *Proc. Natl. Acad. Sci.* USA, **82**, 7909.
10. Poro D., Martegani E., Ranzi B.M., Alberghina L. (1992) *Biotechnol. Bioeng.* **39**, 799.
11. Roukas T., Lazarides H.N. (1991) *J. Inst. Microbiol.* **7**, 15.
12. Athanasiadis I., Boskou D., Kanellaki M., Koutinas A.A. (2001) *J. Agric. Food Chem,* **49** (2), 658-663.
13. Athanasiadis I., Boskou D., Kanellaki M., Koutinas A.A. (1999) *J. Agric. Food Chem.* **47**, 4474.
14. Bardi E., Koutinas A.A. (1994) *J. Agric. Food Chem.* **42**, 221.
15. Athanasiadis I., Becatorou A., Iconomopoulou M., Kanellaki M., Psarianos K., Koutinas A.A. Paper under preparation.
16. Paraskevopoulou A., Athanasiadis I., Kanellaki M., Bekatorou A., Blekas G., Kiosseoglou V. (2001) 3rd International Symposium on Industrial Proteins, Hagen. March 2001.
17. Athanasiadis I., Psarianos C., Bekatorou A., Iconomopoulou M., Lindner C., Paschalis T., Voliotis S. (2001) 3rd International Symposium on Industrial Proteins, Hagen. March 2001.
18. Lidner C., Kanellaki M., Psarianos C., Koutinas A.A. Paper under preparation.
19. Paschalis Blekas M., Kounavi C., Iconomopoulou M., Bekatorou A., Kanellaki M., Koutinas A.A., Psarianos C. Paper under preparation.
20. Koutinas A.A., Athanasiadis I., Bekatorou A., Kanellaki M., Kioseoglou V., Boskou D. Paper under preparation.

Chapter 28

FORMATION OF KEFIR GRANULES AND THEIR EFFICIENCY IN WHEY FERMENTATION

I. ATHANASIADIS, C. PSARIANOS, A. BEKATOROU, M. ICONOMOPOULOU, C. LINDNER, T. PASCHALIS, S. VOLIOTIS

Food Biotechnology Group, Department of Chemistry, University of Patras, Patras, Greece. e-mail: cps@upatras.gr

Abstract

This paper discusses the formation of kefir granules in the aerobic fermentation of whey as well as the efficiency of that biocatalyst in whey treatment. Granular kefir biomass of 85 g/L was obtained. Biomass was rapidly precipitated. This property will lead to SCP production without use of centrifugal separators and could result in use of filtration in industrial production plants. The effect of initial cell concentration (ICC) was examined and found that ICC of 20 g/L was able to ferment whey in 35 h, which is a fermentation time acceptable by the industry. Likewise, the effects of raisin extracts on whey fermentation are reported. Concentrations of 1-8 % v/v were examined and found a promotion effect on whey fermentation more of 100 %. Granular biomass was compared with delignified cellulosic (DC) material supported cells of kefir yeast with regards to their efficiency in whey fermentation. It was found that DC material supported biocatalyst fermented whey 2 fold faster. However, granular biomass seemed to be easier to manipulate in industry as compared with DC material supported cells of kefir. Finally, the formation of the main volatile by-products was examined to correlate the chemical composition and quality of an alcoholic drink produced by whey fermentation with traditional products.

Index Entries: kefir, granules, whey, alcoholic fermentation, promotion, raisin extract, cell growth.

Introduction

Whey utilization or disposal still remains a serious problem. At the same time, cheese production is increasing worldwide, which complicates much more the problems of whey utilization and disposal. Whey lactose content remains mostly unused and is often subjected to a costly waste treatment process. As an alternative, to this procedure, lactose could be converted to ethanol (**1-9**), or single cell protein (**2,5,10**), but this solution of the problem is challenged by important and variable economic constraints. One of the major problems in this case is the high

New Horizons in Biotechnology, 311-319, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

concentration of lactose in whey that inhibits microbial growth (**1,2,3,4,5,11,12**). Besides, the industry produces considerable amounts of salt whey in various concentrations, sometime up to 12%, and this is especially troublesome (**5,13,14**) because the salt decreases the ability of microorganisms to ferment lactose. However, research efforts have been made to obtain alcohol production employing lactose with specific microbial strains. Various strains of *Kluyveromyces* (**1,2,4,5,12,15,16**) and *Saccharomyces* (**9,17**) were proposed and various techniques applied (**1,2,9,12**) to cell growth in synthetic media containing lactose and whey and their fermentation.

The proper immobilization of cells is an important process in alcoholic fermentation (**7,8,18,19**). Delignified cellulosic (DC) materials have been proposed as an immobilization support of yeast strains for wine-making (**18**) and brewing (**19**).

Kefir is a fermented beverage, having its ancient origins in the Caucasian area. This light alcoholic beverage is a native dairy product like yogurt. It is prepared by inoculation of raw milk with existing kefir grains. Kefir grains consist of a polysaccharide matrix in which yeast and bacteria are embedded and live symbiotically. The microflora of kefir grains and beverages depends on their origin, the storage conditions and the mode of handling (**20-22**) and consists predominantly of yeasts of the genera *Saccharomyces, Candida, Torula,* and lactic bacteria of the genera *Lactobacillus* and *Streptococcus.* This microbial population of kefir, which ferments lactose, seems to have a potential for alcohol production using milk whey to produce potable or fuel grade alcohol. The latter can be mainly produced by mixing whey with a raw material containing higher sugar concentration to avoid whey condensation and consequently a high-energy demand. Potable alcohol production (**11,12,23**) and the production of an alcoholic drink from milk whey should be accompanied by the formation of volatile byproducts that are constituents of traditional alcoholic beverages and contribute to a typical organoleptic character. In the frame of this purpose a novel system consisting of kefir yeast immobilized on delignified cellulosic (DC) material has developed to convert quickly the lactose (**24**).

The aim of this investigation was to study the formation and use of kefir cell granules, the aggregated cells of kefir, after their aerobic incubation on whey and their utilization in alcoholic fermentation of whey.

Material and Methods

Kefir yeast, a commercial product that is employed to produce kefir drink, was used in the present investigation. It was grown in whey in which 0.1% $(NH_4)_2SO_4$, 0.1% $(NH_4)H_2PO_4$ was added. For the preparation of solid culture 2% agar was used. This medium was sterilized at 130 °C for 15 min.

Aerobic cell growth

Pressed wet weight cells were prepared at late log phase of a kefir culture. Cells were separated after the liquid was decanted and the biomass was inoculated in a bioreactor of 2.5L contained 1.5L whey, having an initial pH equal to 5.5 and nutrients as above. To create aerobic conditions a compressor supplied air and the pH adjusted during the aerobic fermentation by the addition of a solution of Na_2CO_3. After 10 hrs most amount of lactose was converted and granular biomass was formed and precipitated to the bottom. During the fermentation samples were collected for cell mass and °Be density determinations, while the final liquid was analysed for residual sugar and ethanol concentration. The liquid was decanted and the bottom biomass used for the following fermentations.

Whey used was a product of MEVGAL dairy Co and was a product without salt, with salt of 1.2% and 2.5% concentrations.

Anaerobic fermentations

Anaerobic fermentation of whey was performed at 28°C using granular biomass in a concentration of 20g/L. The pH was adjusted to 5.5 by the addition of citric acid. The promotion effect of raisin extract was also studied by its addition in 1%, 2% and 4% concentrations. Kinetics were performed by measuring the °Be density at various time intervals. Raisin extracts were obtained by hot extraction of 500g black raisin using 1L water, performed for at least 2 hours, in a constant temperature water bath adjusted at 72°C. A comparison was made between the fermentation rate of kefir yeast granular biomass with delignified cellulosic (DC) material supported cells of kefir and those with 1% raisin extract, whey with 1% raisin extract, whey with immobilized kefir on DC material and whey without them.

The preparation of DC material and the immobilization of cells of kefir yeast were performed as described in a previous paper (**18**). Repeated batch fermentations were also performed using the same granular biomass from batch to batch to show the stability of the system. The biomass was obtained after decanting the fermented whey and used for the next fermentation batch. Samples from each fermentation batch were analysed for alcohol, residual sugar and main by-products.

HPLC analysis

Ethanol and residual sugar were determined by high-performance liquid chromatography (HPLC). A Shimadzu HPLC chromatograph, Model LC-9A, connected with an integrator, C-R6A Chromatopac, column SCR-101N (packed with a cation exchange resin-sulfonated polystyrene-divinylbenzene copolymer), CTO-10A column oven, and a refractive index detector RID-6A were employed. The elution was made using water distilled and filtered three times. The determination was performed using a pressure of 78-82 atm, and the flow rate of the mobile phase was 0.8 ml/min. An oven temperature of 60°C was used. Samples of 0.5 and 2.5 ml of 1% butanol as internal standard were added in a 50 ml volumetric flask with distilled and filtered water. This solution was filtered using microfilters

of 0.45 μm hole size and injected directly into the column. Quantitative determinations of volatile by-products were made with a Shimadzu gas chromatograph GC-8A, connected with the integrator Chromatopac C-R6A. Ethanol, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols (total amount of 2-methylbutanol-1 and 3-methylbutanol-1) were determined using a stainless steel column (4 m long, 1/8 in. i.d.), packed with Escarto 5905 [consisting of squalene 5%, Carbowax 300 900%, and bis(2-ethylexyl) sebacate 5% V/V], with N_2 as the carrier gas (20 mL/min). The injection port and detector temperatures were 210°C, and the column temperature was 58°C. The internal standard was butanol, at a concentration of 0.5%V/V. Samples of 2 μL of the fermented liquid were injected directly in the column.

Results

The kinetics of kefir cell growth in whey is shown in the Figure 1. Cell growth shows a diauxic scheme, which did not appear in a synthetic medium containing lactose. The substrate consumption (residual sugar) also showed the same scheme, which enforced the aspect of the diauxic growth of kefir cells. Therefore, one can conclude that the lactose was not the only substrate present in the whey, able to be up taken by the kefir cells. The form of microbial population created during the cell growth was in the form of cell aggregates forming granules of different size. These cell granules appeared at the end of fermentation and gradually precipitated to the bottom of the fermentation vessel.

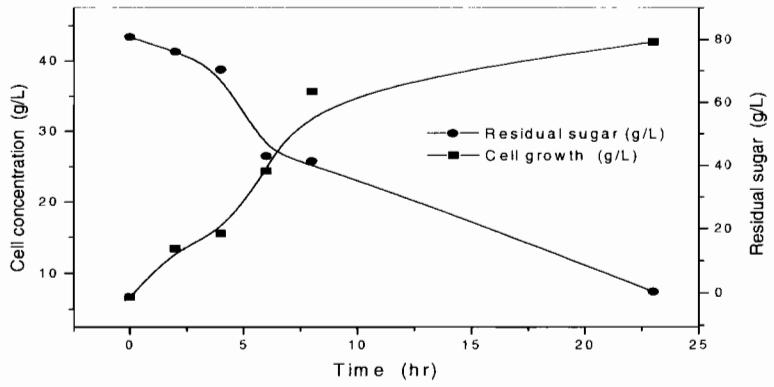


Figure 1. Cell growth of kefir cells vs. time in whey aerobic fermentation.

To find the optimum conditions of kefir mass production, experiments of cell growth were performed in different temperatures and initial cell concentrations (ICC). The results are shown in the Table 1.

The data indicate that ICC greater than 10g/1.5L and temperatures higher than 25°C do not cause substantial increment both in the amount of the produced biomass and the rate of the production. The influence of these two agents on the produced biomass was much more in a temperature of 20°C and when the inoculum was in the quantity of 3g/1.5L, which increased when the two agents were combined. Therefore, temperatures of 25-28°C and inoculum no more than 10g/1.5L were good conditions for the production of kefir cells by aerobic fermentation of whey, in relation with the amount and the rate of biomass production.

Table 1. Effect of ICC and Temperature on cell growth of kefir.

ICC (g/1.5L)	Temperature (°C)	Fermentation time (h)	Biomass (g/1.5L)	Ethanol (%v/v)	Residual sugar (g/L)
3	20	19	21	0.24	0.017
	28	14	30	0.21	0.013
10	25	7	37	0.41	0.047
	28	7	40	0.51	0.035
45	25	6.5	27	0.41	0.031
	28	6.5	40	0.32	0.029

Due to the fact that whey produced by the industry contains 1.2% or 2.5% of salt, the influence of these salt concentrations on kefir cell growth was examined. The results are presented in figure 2. The salt has a light negative influence on cell growth rate for 1.2% and 2.5% salt concentrations in respect to that of 0% salt concentration. The fermentation time for all above quoted fermentations in general is close to 20h.

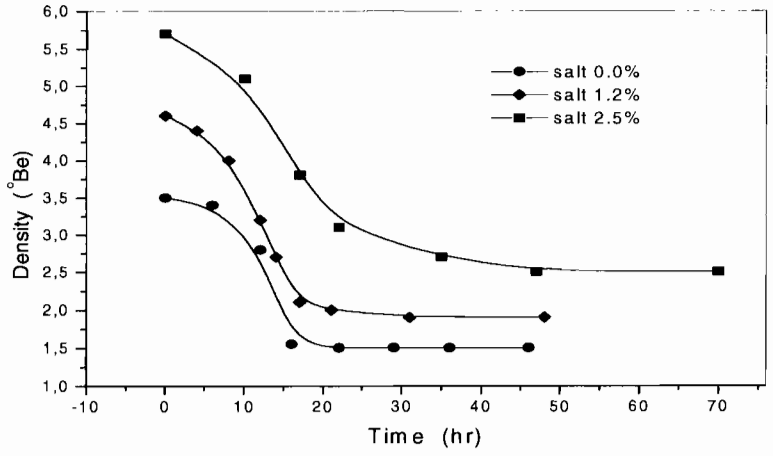


Figure.2. Effect of salinity on the kinetic of aerobic fermentation of whey using kefir for biomass production.

To clarify whether the microbial population under the form of granules was able to carry out anaerobic fermentations of whey in a satisfied mode and rate, such fermentations were compared with fermentations carried out by kefir cells immobilized on (DC) material. The use of biocatalysts is known to increase of the fermentation rate. (**25**). The kinetics of fermentation of these experiments is shown in Figure 3. Fermentations performed using cell granules did not reach high rates in comparison with those performed using immobilized kefir cells on DC. However, with the addition of a small amounts (1%) of raisin extract in the fermenting whey, the fermentation rate by the cell granules rose, almost reaching the fermentation rate obtained by the immobilized cells on DC material

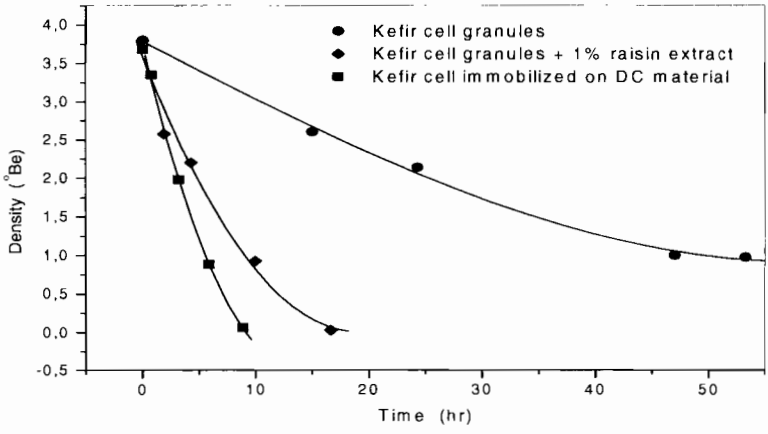


Figure 3. Fermentation Kinetics observed in whey fermentation by kefir granules, kefir granules with 1% raisin extract and DC material supported cells of kefir.

To find out whether the fermentation system of cell granules-raisin extract was also a steady state system, repeated batch fermentations of the whey were carried out in order to show the operational stability of the system. Table 2 contains the results of 5 repeated batch fermentations for two raisin extracts proportions used (1% and 4%) plus fermentations without raisin extracts (0%).

These results, concerning the fermentation time and the efficiency of this fermentation, were nearly identical over at least 5 batches. This showed the operational stability of the system for at least 5 batches of whey and meant that kefir cell vitality was high and that no contamination occurred.

Finally, to show the suitability of kefir granules-raisin extract system in anaerobic fermentation for the production of alcoholic drinks using whey, repeated batch fermentations were carried out to study the formation of main volatile byproducts

(ethylacetate, propanol-1 and iso-butyl alcohol). The results are presented in Table 3. The results showed a relative stability of the system with no substantial changes concerning the formation of byproducts for at least 5 batches.

Table 2. Repeated batch fermentations of whey using kefir granules with 1%, 4% raisin extract and without any of it.

Repeat batch	With 1% raisin extract				With 4% raisin extract				Without raisin extract			
	Initial dens.	Final dens.	Ferm. time	Alcoh. conc. (%v/v)	Initial dens.	Final dens.	Ferm. time	Alcoh. conc. (%v/v)	Initial dens.	Final dens.	Ferm. time	Alcoh. conc. (%v/v)
1	2.3	0	9	2.3	2.5	0	10	2.4	3.78	0.90	47	2.5
2	3.8	0	16	2.8	4.3	0	15	3.0	3.80	1.32	54	2.2
3	4.4	0	12.5	3.1	4.7	0	14.5	3.1	3.77	1.25	44	1.6
4	2.5	0	10	2.5	1.7	0	10	1.8	3.80	1.70	45	1.9
5	1.9	0	11	2.0	3.4	0	15	2.7	3.83	2.15	83	1.4

Table 3. Volatile formation of whey fermentation using kefir granules with the addition of small amounts of raisin extracts.

Repeated Batch fermentation	Ethylacetate (mg/L)			Propanol-1 (mg/L)			Iso-butyl alcohol (mg/L)		
	Raisin extract			Raisin extract			Raisin extract		
	0%	1%	4%	0%	1%	4%	0%	1%	4%
1	01.9	02.6	00.9	25.2	28.0		42.4	24.5	31,7
3	05.4	22.1	31.7		29.6	29.0	19.2	78.5	61.5
5	03.9	03.1	06.9	24.6	21.7	23,5	44.7	41.1	35.3

Discussion

The formation of kefir cell granules is of great biotechnological significance because the granular biomass could be supplied to industrial alcoholic fermentation, in place of immobilized cells systems, for rapid conversion of whey lactose, to produce useful products of added value such as drinkable alcohol, fuel alcohol and alcoholic drinks. The significance is strengthened by the presented results and suggestions for fermentation applications are given below. The kefir cell granules become able to perform fast anaerobic fermentations approaching those of immobilized cells on CD material after the addition of a small quantity of raisin extract, a cheap material, which is widely produced in Greece, and other Mediterranean Countries and the USA. It should be mentioned here that black raisin extracts also promoted the alcoholic fermentation using *Zymomonas mobilis* (**26**). So, cell immobilization should be avoided as it makes fermentation systems more complicated, with an increased need for effort and labor, to handle the biocatalyst at the industrial scale.

The immobilization time, the time needed for bioreactor filling, as well as emptying of the support and regeneration time after each batch of fermentation could avoided

by the use of kefir granules in combination with 1% raisin extract, added to the fermentation broth. On the other hand, the use of cell granules as opposed to the use of separated cells by centrifugal separators has the great advantage that it does not need centrifugation in order to separate the cells from the final product, a process that increases alongside the cost of production by the decrease of productivity and increase of the energy demand and investment.

However, the recovery of the granules from the fermentation products can be easily done by the use of filters that do not get blocked or clogged easily because of the size of granules. Moreover, the separation system does not need agitation. In conclusion, the kefir biomass by the form of granules, combined with the use of a small quantity of 1% raisin extract, is an excellent combination for the fermentation process of whey with respect to alcoholic drink production, which is analogous to wine making and brewing.

References

1. Gawel J., Kosikowski F.V. (1978) *J. Food Sci.* **43**, 1717.
2. Mahmoud M.M., Kosikowski. F.V. (1978) *J. Dairy Sci*, **62**, 114.
3. Burgess J., Kelly J. (1979) *J. Food Sci. Technol.* **3**, 1.
4. Moulin G., Guillaume M., Galzy P. (1980), *Biotechnol. Bioeng.* **22**, 1277
5. Mahmoud M.M., Kosikowski F.V. (1982) *J. Dairy Sci.* **65**, 2082.
6. Chen H.C., Zall R. (1982) *Process Biochem.* **17**,20.
7. King, V.A.E., Zall R.R. (1983) *Process Biochem.* **18**,17.
8. King, V.A.E., Zall R.R. (1983) *J. Gen. Appl. Microbiol.* **29**, 379.
9. Terrell S.L., Bernard A., Bailey R.B. (1984) *Appl. Envir. Microbiol.* **9**,577.
10. Shay L.K., Wegner G.H. (1986) *J. Dairy Sci.* **69**, 676.
11. Gawel J., Kosikowski. F.V. (1978) *J. Food Sci.* **43**, 1031.
12. Kosikowski F.V., Wzorek W. (1977) *J. Dairy Sci.* **60**,1982.
13. El-Nimr A.A., Badr Eldin S.M., Ghali Y., Youssef Y.B. (1982) *Egypt. J. Dairy Sci.* **10**,1.
14. El-Samragy Y.A., Zall R.R. (1988) *J. Dairy Sci.* **71**,1135.
15. O'Leary V., Sutton C., Bencivengo M., Sullivan B., Halsinger V. (1977) *Biotechnol Bioeng.* **19**, 1689.
16. Marchant R., Nigam P., Brandy D., McHale L., McHale A. (1996) *Biotechnol. Lett.* **18**, 1213.
17. Roukas T., Lazarides H. (1991) *J. Inst. Microbiol.* **7**,15.
18. Bardi E., Koutinas A. (1994) *J. Agric. Food Chem.* **42**, 221.
19. Bardi E., Koutinas A., Soupioni M., Kanellaki M. (1996) *J. Agric. Food Chem.* **44**, 463.
20. Marshall V. (1987) *Journal of Dairy Research* **54**,559.
21. Marshall V., Cole W., Brooker B. (1984) *J. of Appl. Bacteriol.* **57**, 491.
22. Zourari A., Anifantakis E.M. (1988) *Le Lait*, **68 (4),** 373
23. Yoo B.W., Mattick J. F. (1969) *J. Dairy Sci.* **52**, 900.
24. Athanasiadis I., Boskou D., Kanellaki M., Koutinas A.A. (1999) *J. Agric. Food Chem.* **47(10),** 4474.

25. Athanasiadis I., Boskou D., Kanellaki M., Koutinas A.A. (2001) *J. Agric. Food Chem.* **49 (2)** 658-663

26. Koutinas A.A., Kanellaki M., Typas M.A., Drainas C. (1986) *Biotechnol. Letters* **8 (7),** 517.

Chapter 29

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM MATURE COFFEE CHERIES: POTENTIAL APPLICATION IN COFFEE HUSK ENSILING

M.G.B PAGNONCELLI[1], D.BRAND [1,2], S. ROUSSOS[2], I. GAIME-PERRAUD[2], C. AUGUR[2] , C.R. SOCCOL[1]

[1] *Laboratório de Processos Biotecnológicos, Departamento de Engenharia Química, Universidade Federal do Paraná (UFPR), 81531-970 Curitiba-PR, Brasil ;* [2] *Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119; IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925; 163 Avenue de Luminy; F-13288 Marseille Cedex 9, France*

Abstract

Coffee is one of the most profitable crops in Latin America. Brazil is the biggest producer, consumer and exporter in the world. After coffee processing only 5.8% of the total matter is used for coffee production generating a large amount of waste, were no commercial value, that can hardly pollute the environment. The coffee husk conservation can be made by silage. Specific microorganisms can produce a standard ensilage when added in the process. The main goal of this work is to study the natural microflora present in the coffee husk and coffee pulp able to ensilage these by-products. The lactic acid bacteria responsible for this process were isolated and characterised and added in an ensilage process to accelerate the acid lactic production. First, in this work, fresh fruity coffee pulp were put fermented naturally and it was observed that the pH was maintained around 4 during two months. Then many bacteria were isolated from these natural fermented materials. Morphology, fermentative and biochemistry studies were used to identify and characterise the bacteria. These bacteria were able to grown in a coffee extract and change the pH 6 to around 4.4. Then, the second study was added these isolated bacteria in processed coffee husk to ensilage. It shows that is necessary to add the lactic acid bacteria to initiate the ensilage process. In the coffee husk fermentation, 10^7 CFU were added per g of matter and the pH was maintained around 4.8; microorganisms were viable for 2 months.

Introduction

In the last ten years, the average world's coffee production was of 97 millions sacks of 60 Kg. Only two species of the *Coffea* genera are cultivated in great scale in the world, representing 100 % of commercialized coffee, *Coffea arabica* (arabica variety) and *Coffea canephora* (robusta variety). The arabica variety represents 75% of the world's production (**1-3**).

New Horizons in Biotechnology, 321-333, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

The coffee fruit also denominated coffee cherry presents different tissue layers, which covers the grain. The exocarp is the most external part of the fruit. The internal part, rich in sugars and pectin, is the mesocarp (pulp). Finally, covering the grains is the endocarp appearing as a membrane. During processing, the coffee fruit is subjected to different operations with the aim of removing the coffee grains from its envelopes (**4**). There are two technologies to obtain the de-hulled grain: through wet and dry processing. During wet processing, two residues are generated, the pulp and mucilage waters, both rich in nutrients. In dry processing, coffee husk is the sole residue originated. In both processes, pulp and husk elimination occur with the aid of microorganisms, which ferment the fruit and seeds and aid in the elimination of the envelopes (**1,5,6**).

When coffee is ready for consumption as beverage, only 5,8% of the fresh cherry weight is utilized, generating by-products of low commercial value. That may cause important environmental contamination (**7**). The utilization of agro-industrial by-products in biotechnological processes constitutes an alternative with regards to the use of the substrate. It may also help in solving the environmental pollution problem (**8**).

Various studies, utilizing coffee husk and pulp as substrate for fermentation processes, have already been undertaken. Besides the elimination of anti-nutritional factors aiming at its utilization in the nutrition of ruminants, new studies aiming at the utilization of these by-products as fermentation substrates for the production of edible mushrooms and biomolecules of commercial interest such as organic acids, vegetable hormones and aroma compounds, have been done (**9-12**).

The fermentation process, besides conserving and protecting these foodstuffs from pathogenic microorganisms, can improve product digestibility and in certain cases eliminate its toxicity (**13**). Silage is a process by which fermentation of the available sugars with the production of organic acids, mainly lactic acid by lactic bacteria in anaerobic conditions, is achieved (**14,15**). The objective of a silage process is to minimize loss of dry matter and nutritional value and to avoid the development of undesirable microorganisms (**16**).

The lactic bacteria responsible for the fermentation process are normally present on the plant tissue to be ensiled. Nevertheless, the utilization of starters improves the standardization of the fermentation, controlling the plant microbial flora, mainly the aerobic flora and accelerating pH fall (**15,17-19**).

The lactic bacteria can ferment carbohydrates by two means. The homo-fermentative where the final product of the process is exclusively lactic acid and hetero-fermentative, where, besides lactic acid, the presence of other metabolites such as acetic, propionic and butiric acid and ethanol can occur (**20-22**).

The present work had as objectives, the isolation and identification of lactic bacteria present in mature coffee cherries, the evaluation of the potential of these bacteria in the fermentation of sugars available in coffee husk, the determination of the acidification capacity of the media due to the production of organic acids and finally the utilization of strains as starters for the silage of coffee husk.

Coffee husk characterization

The chemical composition of coffee husk may vary depending on the cultivar. In Table 1 the components present in coffee husk of two different cultivars are presented. Sample 1 corresponds to the husk given by Café Damasco and sample 2 was supplied by Cocamar. Analyses were carried out according to the methodologies described by Nelson (**23**), Somogyi (**24**), Goering and Van Soest (**25**) and AOAC (**26**).

For the utilization of a raw material as substrate in a fermentative process, its chemical composition should be considered. The substrate should give the necessary conditions to the development of the microorganisms employed, aiming at a fermented product of high quality. After fermentation, the process should result in a food of high nutritional value.

The values obtained for proteins demonstrate that this substrate possesses an important concentration of organic nitrogen that may be utilized as animal feed. The values obtained for ashes reveal appreciable quantities of minerals in coffee husk, which are favorable to the development of microorganisms. These mineral values are related to the presence of calcium and phosphorus, which meet the 1:3 proportion, which is the ideal condition for the utilization the substrate as animal feed. In order for silage to take place, it is necessary for the material to be used to have approximately 12% of available sugars (**14**). The reducing and total sugars present in sample 2 demonstrated values relatively low, probably to the fact that the cherries, when harvested for processing, were not mature enough. Coffee husk sample 1 was characterized as an ideal substrate for lactic fermentation, because the quantity of reducing sugars showed superior values to the minimal demanded for good ensiling.

Table 1. Chemical composition of coffee husk samples.

Components	Sample 1	Sample 2
Protein (g%)	7.65	9.48
Lipids (g%)	1.57	1.59
Ashes (g%)	6.70	9.62
Calcium (g%)	0.25	0.51
Phosphorus (g%)	0.08	0.12
Reducing sugars (g%)	23.85	3.80
Total sugars (g%)	27.45	8.69
Neutral detergent fibers (g%)	29.14	60.15
Acid detergent fibers (g%)	21.78	49.92
Lignin (g%)	5.83	9.30
Cellulose (g%)	15.27	32.40
Hemicellulose (g%)	7.37	10.23

The physico-chemical analyses of coffee husk samples utilized demonstrate that this residue could be considered as a good substrate for the development of bioprocesses utilizing microorganisms (**8-12**).

Isolation of bacteria from coffee cherries

Mature coffee cherries, manually depulped, were submersed in sterile water and then were placed to ferment naturally for 48 hours at 35°C, with agitation of 150 rpm. Part of the fermented material was transferred to MRS broth and incubated at 35°C for 48 hours (**27**). The isolation occurred in MRS agar after successive dilutions on inoculation plates.

The isolated strains were classified initially by the technique of Gram. The group of strains chosen for further studies were those that showed positive reaction to Gram dye, with rod or coccus-rod morphology. The selected strains were preserved in liquid nitrogen.

The natural fermentation of coffee cherries after 48 hours showed turbidity, indicating the presence of a great number of microorganisms. From the isolated strains, 20 were selected, showing coccus rod morphology and Gram positive reaction, and classified in later fermentative studies (fig 1).

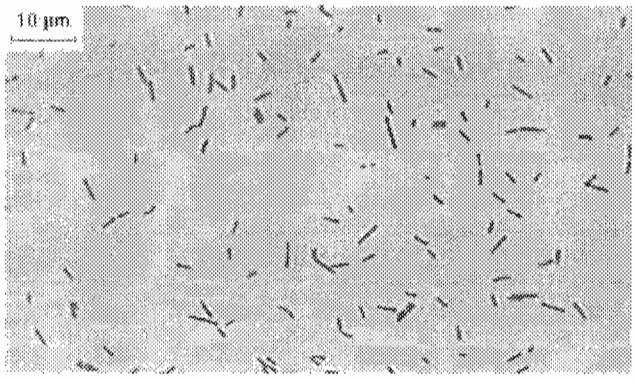


Figure 1. Morphology of Gram-positive bacteria (x1000) isolated from coffee cherries

Selection of bacteria isolated in liquid media containing coffee husk extract

In order to evaluate the potential of each isolated strain in utilizing the sugars present in coffee husk, a natural media was prepared from coffee husk, by cooking 100g of husk (sample 1) in 1L of distilled water at 100°C for 1 hour. The extract obtained was filtered and the pH adjusted to 7.00. The final volume was completed to 1L with distilled water.

Fermentative potential of bacteria in coffee husk extract

The strains were inoculated 10% (v/v) in a liquid medium of coffee husk extract, with initial pH of 6.0 or 7.0 and incubated at 35°C for 48 hours.

This study was carried out with the objective of evaluating the potential of bacteria isolated in relation to its capacity to ferment sugars present in coffee husk. After 48 hours acidification of the culture medium was observed. This is a typical process of acidic bacteria action, where the microorganism is able to transform the reducing sugars of the culture medium into organic acids (**14**). Figure 2 shows the acidification capacity of coffee husk extract by different isolated strains.

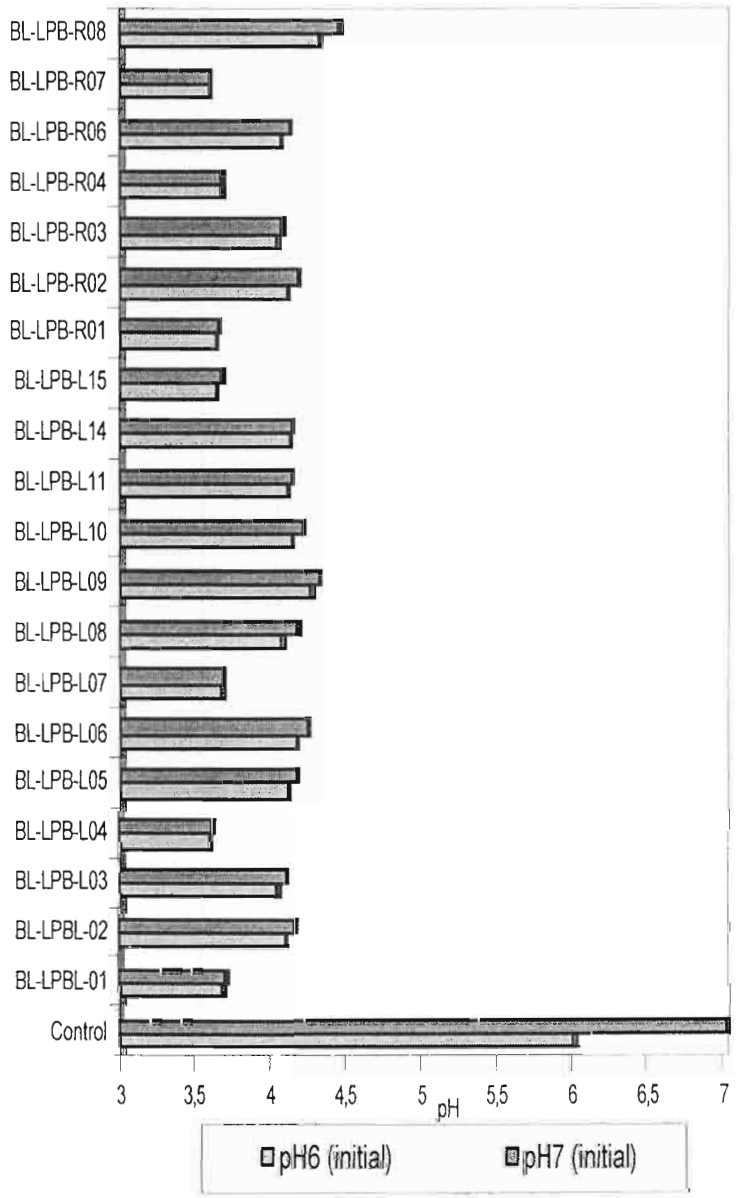


Figure 2. pH in coffee husk extract cultivated with isolated lactic acid bacteria.

It was observed that the majority of the strains studied (BL-LPB-L02, L03, L05, L06, L08, L09, L10, L11, L14, R02, R03, R06 e R08) possess a metabolism that allow the production of acid, reducing the pH of the medium to values close to 4.00. However, a second group represented by the strains BL-LPB-L01, L04, L07, L15, R01, R04 and R07, showed greater acidification power, reducing the pH to values close to 3.50. The strains that represent the second group, by presenting a greater capacity of acidification probably have a bigger potential in being utilized as starters in the silage process of coffee husk.

Identification and quantification of the metabolites produced in coffee husk extract

After the fermentation process, coffee husk extracts, with initial pH 7.00, were centrifuged at 11400 rpm for 15 minutes. The supernatant was diluted in the proportion of 1:10 and filtered through a 0.22 μm membrane and analyzed by high performance liquid chromatography, by utilizing a HPX-87H column (300 x 7.8 nM) – BioRad Aminex, with a temperature of 60°C. The mobile phase utilized was H_2SO_4, 5 nM.

Metabolic products were identified and quantified by comparison to standards such as lactic acid, acetic acid, propionic acid, butyric acid and ethanol. Table 2 shows the products of the metabolism of different isolated bacterial strains through the analysis by HPLC of fermented coffee husk extract.

Some strains produced high concentrations of lactic acid in coffee husk extract, reaching concentrations of 17 g/L, as was the case of the strain BL-LPB- R01. These strains, considered good producers of lactic acid, produced minimal quantities of other metabolites, therefore they can be classified as homo-lactic. On the other hand, another group of strains studied, produced low amounts of lactic acid, and excessive amounts of other metabolites. This was the case of strain BL-LPB-L14 producing 8.14 g/L of lactic acid, 3.08 g/L of acetic acid, 3.77 g/L of propionic acid and 0.97 g/L of ethanol. The strains belonging to this group could be classified as hetero-lactic. Of the 20 isolated strains, 7 presented a homo-fermentative metabolism and the remaining were hetero-fermentative.

Table 2. Metabolites produced by lactic bacteria.

Strains	Lactic acid (g/L)	Acetic acid (g/L)	Propionic acid (g/l)	Butyric acid (g/l)	Ethanol (g/l)
BL-LPB-L01	15,10	0,58	0,26	0	0
BL-LPB-L02	6,88	3,27	0,38	0	0,93
BL-LPB-L03	5,83	4,36	0,32	0	0,75
BL-LPB-L04	13,73	1,20	0,26	0	0

BL-LPB-L05	6,57	3,12	0,34	0	0,87
BL-LPB-L06	7,78	3,12	0,34	0	0,74
BL-LPB-L07	14,15	1,32	0,29	0	0
BL-LPB-L08	7,71	3,10	0,29	0	0,86
BL-LPB-L09	6,99	2,68	0,36	0	0,77
BL-LPB-L10	6,19	3,20	0,34	0	0,77
BL-LPB-L11	6,98	3,19	0,39	0	0,94
BL-LPB-L14	8,28	3,08	3,77	0	0,97
BL-LPB-L15	15,98	1,14	0,30	0	0,68
BL-LPB-R01	17,05	0,50	0,41	0	0
BL-LPB-R02	6,02	2,84	0,49	0	0,46
BL-LPB-R03	6,45	2,62	0,37	0	0,61
BL-LPB-R04	14,26	0,42	0,38	0	0
BL-LPB-R06	6,20	2,69	0,33	0	0,44
BL-LPB-R07	15,16	0,75	0,47	0	0
BL-LPB-R08	4,60	2,15	0,51	0	0

When comparing acidification of the culture medium by the different bacterial strains, it was observed that the strains belonging to the first group had greater capacity to acidify the culture medium, maintaining the final pH around 3.50. As for the hetero-lactic strains, the pH of the medium was close to 4.20. By analyzing these results it was deduced that homo-fermentative bacteria had a greater capacity to acidify the fermentative process. In any silage process, the fast fall of pH is a primordial factor. Lactic bacteria that have greater potentialities for utilization in this process are those that have the best ability for quick acidification.

Biochemical identification of the isolated lactic bacteria

Biochemical identification of lactic bacteria was based on the capacity of these microorganisms to metabolize certain sugars as sole carbon source in their fermentative metabolism to produce organic acids. The biochemical profile of the isolated strains was traced using 49 different carbon sources (API 50CHL – BioMerieux).

Culture media contained the indicator bromocresol purple, which enabled the visualization of the media acidification process. Interpretation of results was based on statistical comparison of the fermented sugars by the bacteria.

Table 3 shows the results of the identification of lactic bacteria which presented a reliability superior to 90% in the use of API 50 CHL.

Table 3. Identification of Lactic Acid Bacteria.

Strains	Species	Probability
BL-LPB-L01	*Lactobacillus plantarum* 1	99,9
BL-LPB-L02	*Lactobacillus brevis* 3	94,3
BL-LPB-L03	*Lactobacillus brevis* 3	94,8
BL-LPB-L04	*Lactobacillus plantarum* 1	99,9
BL-LPB-L05	*Lactobacillus brevis* 3	96,3
BL-LPB-L06	*Lactobacillus brevis* 3	94,8
BL-LPB-L07	*Lactobacillus paracasei ssp paracasei* 3	99,7
BL-LPB-R01	*Lactobacillus plantarum* 1	99,2
BL-LPB-R01	*Lactobacillus brevis* 3	94,8

All the isolated bacteria showing rod morphology and by producing lactic acid as major product of the metabolism belong to the *Lactobacillus* genera. These were then further divided into three different species.

Coffee husk silage

The strains that produced the highest acidification of the fermentation media with coffee husk extract, were selected to be tested as starters of coffee husk silage. The strains utilized in the process were *Lactobacillus plantarum* (BL-LPB-R01) and *Lactobacillus paracasei spp paracasei* (BL-LPB-L07). The inoculation rate utilized was 10^6 CFU/g of coffee husk on a dry weight basis.

Coffee husk utilized in the experiment was sample 1. The husk was re-hydrated in water, to a moisture content between 60 and 70%, inoculated with the selected lactic bacteria and incubated at room temperature.

The material was ensiled over a period of 30 days, with the evaluation of specific fermentative parameters at 0, 3, 10, 20 and 30 days of fermentation. Parameters surveyed included pH, production and quantification of metabolites, consumption of total and reducing sugars and viable cell count.

Inoculation of lactic bacteria facilitated the acceleration of the silage process, in the beginning of the fermentation, as can be observed in figure 3. The silage pH presented a fast fall in the first days of the process, reaching values close to 3.90 after 10 days of fermentation, staying stable until the end of the process (30 days). It can be considered that coffee husk silage can be accelerated with the use of starters. This way, the process could be concluded in only 10 or 15 days. A reduction in the time required to complete the silage process in an important factor for cost reduction resulting in an increase in productivity.

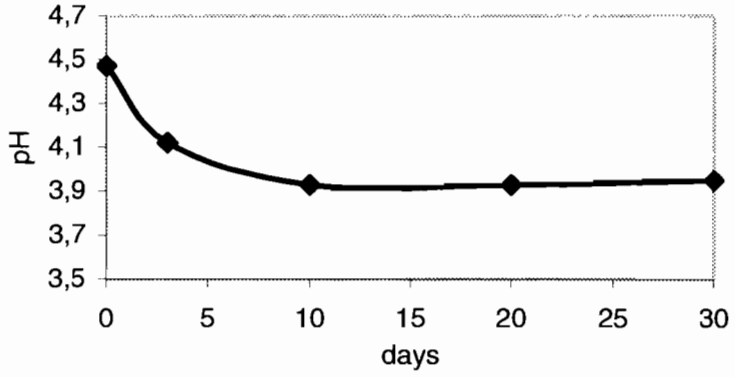


Figure 3. pH evolution of coffee husk silage.

During the silage process, lactic acid was the major metabolite. The production of other organic acids was quite reduced, due to the homo-fermentative metabolism of the starters utilized (fig 4). Ethanol was however produced, probably due to the presence of others microorganisms naturally present in this substrate, such as yeasts.

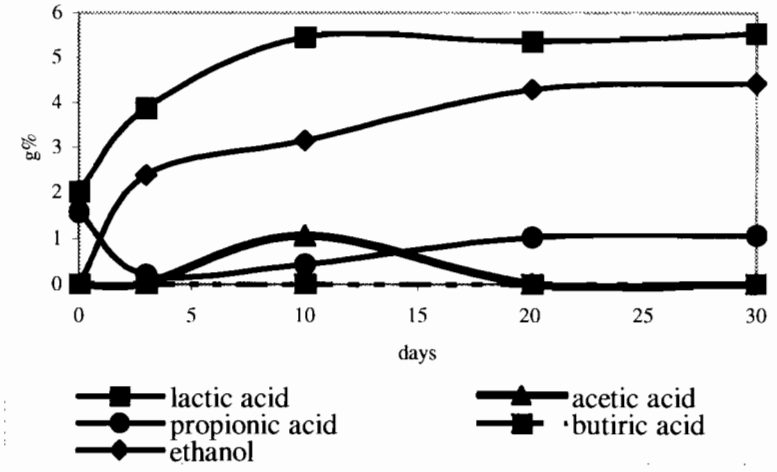


Figure 4. Evolution of the produced metabolites during coffee husk silage.

The microflora was able to metabolize most of the sugars present, within the first ten days. As can be observed in figure 5, a rapid reduction in the reducing sugars was obtained.

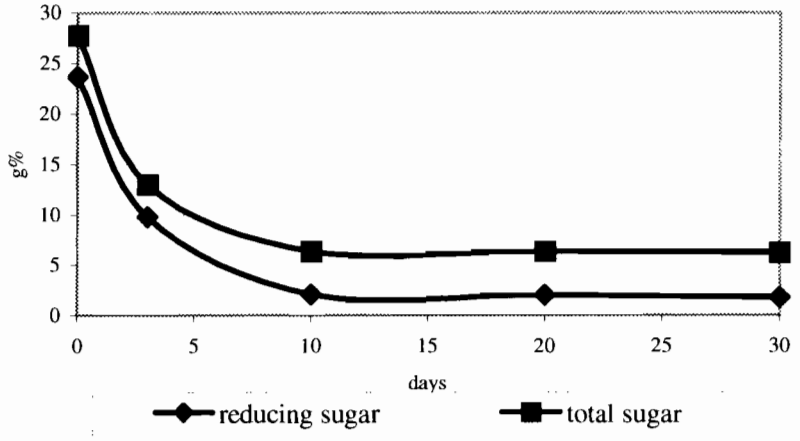


Figure 5. Evolution of total and reducing sugars during coffee husk ensilage.

Figure 6 shows the growth curve of the lactic bacteria during the silage process. In the first days an intense multiplication of cells took place, followed by a decline after 10 days of fermentation. The fall may be explained by the great acidification of the medium in the beginning of the fermentation.

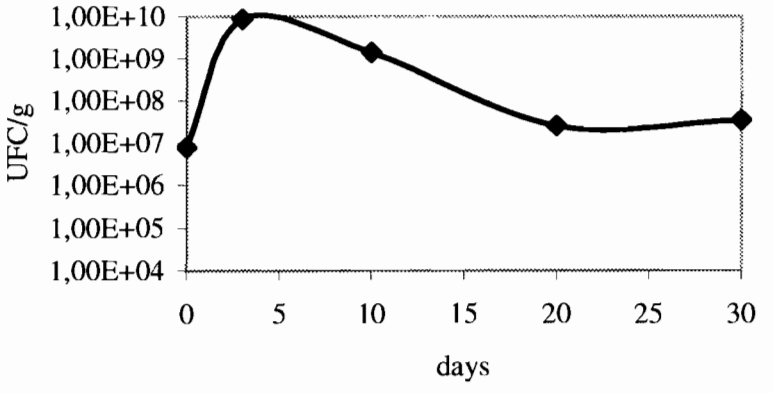


Figure 6. Lactic Acid Bacteria growth curve.

Conclusions

It was possible to isolate from mature coffee cherries a great number of bacteria. Among them, lactic acid bacteria were classified into homo-fermentative and hetero-fermentative groups. The isolated strains were able to produce organic acids from the soluble sugars present in coffee husk. From the 20 selected strains, 8 showed a homo-fermentative metabolism, being classified as homo-lactic, as lactic acid was the major metabolite. The selection of bacteria from coffee husk extract media allowed the selection of bacteria with high acidification power, classified as homo-lactic, able of reduce the final pH of the medium to values around 3.50. The hetero-lactic strains reduced the pH of the medium to values close to 4.20.

The biochemical tests undertaken with the 50 CHL kit allowed the identification of three different species of *Lactobacillus*. The strains were further characterized as *L. plantarum, L. brevis* and *L. paracasei.*

The addition of starters in the silage process of coffee husk accelerated the silage process. After the first ten days of the fermentation, variations were minimal and the pH remained constant until the end of the process.

Strains BL-LPB-R01 and BL-LPB-L07 were tested as starters, demonstrating great potential to be utilized in processes of coffee husk silage.

Acknowledgment

M.G.B Pagnoncelli and C.R. Soccol thank CAPES and CNPq, respectively, for a scholarship, the latter under the Scientific Productivity Scheme.

References

1. Clarke R. J., Macrae R. (1985) Coffee. Londres: Elsevier, v. 1, 305p.
2. Comité Français du Café (1997) A la découverte du café. Paris: Comité Français du Café. 39p.
3. IAPAR (2001) Café no Mundo: produção e consumo. Disponível em http://www.celepar.br/iapar/cafe/m&prodcons.html
4. Bressani R. (1978) *In*: Pulpa de Café: composición, tecnologia y utilización. Braham J.E., Bressani R. (eds), Guatemala: INCAP, p. 9-17.
5. Board R.G. (1988) Introducción a la Microbiología Moderna de los Alimentos. Zaragoza: Acribia, 271p.
6. Silva C.F., Schwan R.F., Dias E.S., Wheals A.E. (2000) *International Journal of Food Microbiology*, **60**, 251-260.
7. Perraud-Gaime I. (1996) Cultures mixtes en milieu solide de bactéries lactiques et de champignons filamenteux pour la conservacion et la décaféination de la pulpe de café. These (Doctorat) – Université de Montpellier II.
8. Pandey A., Soccol C.R., Nigam P., Brand D., Mohan R., Roussos S. (2000) *Biochemical Engineering Journal*, **6**, 153-162.
9. Soares M., Christen P., Pandey A., Soccol C.R. (2000) *Process Biochemistry*. **35** (8), 857- 861.
10. Leifa F., Pandey A., Soccol C. R. (2000) *Journal of Basic Microbiology*, **40** (3), 187-197.
11. Machado C.M.M., Soccol C.R., de Oliviera B.H., Pandey A. (2001) *Applied Biochemistry and*

Biotechnology, **102**, 169-177.

12. Brand D., Pandey A., Roussos S., Soccol C.R. (2000) *Enzyme and Microbial Technology*, **27**, 127-133.
13. Caplice E., Fitzgerald G.F. (1999) *International Journal of Food Microbiology*, **50**, 131-149.
14. Gouet P. (1995) *In*: Microbiología alimentaria: fermentaciones alimentarias. Bourgeois C.M., Larpent J.P. (eds), Zaragoza: Acribia, 2, p.167-177.
15. Shirai K., Guerrero I., Huerta S., Saucedo G., Castillo A., Gonzalez R.O., Hall G.M. (2001) *Enzyme and Microbial Technology*, **28**, 446-452.
16. Souza L.D.N. (1988) Ensilagem e Fenação. Rio de Janeiro: Tecnoprint, 152p.
17. Gouet P. (1994) *In*: Bactéries lactiques. Roissart H., Luquet F. M. (eds) Chemin de Saint Georges: Lorica, 2, p.257-292.
18. Shaver R.D., Batajoo K.K. (1995) *In*: Biotechnology: enzymes, biomass, food and feed. Rehm H. J., Reed G., Pühler A., Stadler P. (eds) Weinheim: VCH, 2. ed, 9, p.767-783.
19. Gardner N.J., Savard T., Obermeier P., Caldwell G., Champagne C.P. (2001) *International Journal of Food Microbiology*, **64**, 261-275.
20. Crueger W., Crueger A. (1993) Biotecnologia: manual de microbiologia industrial. Zaragoza: Acribia, 413p.
21. Larpent J.P. (1995) *In*: Microbiología alimentaria: fermentaciones alimentarias. Bourgeois C.M., Larpent J.P. (eds), Zaragoza: Acribia, v. 2, p. 3-17.
22. Piard J.C., Loir Y., Langella P. (1999) *Biotecnologia Ciência e Desenvolvimento*, **2** (8), 31-39.
23. Nelson N. (1944) *J. Biol. Chem.*, **153**, 375-380.
24. Somogyi M. (1945) *Journal of Biological Chemistry*, **160**, 61-69.
25. Goering H.K., Van Soest P.J. (1970) Forage Fiber Analysis. Handbook nº.379, Agricultural Research Service, USDA, Washington D.C.
26. AOAC (1975) Official Methods of Analysis. Association of Official Agricultural Chemists. 12ª Ed. Washington D.C.
27. De Man J.C., Rogosa M., Sharpe M.E. (1960) *Journal of Applied Bacteriology*, **23**, 130-135.

Chapter 30

LACTIC ACID FERMENTED PRODUCTS AS VEHICLES FOR PROBIOTICS

J.L. PARADA, M.E. SAMBUCETTI, A. ZULETA, M.E. RIO

Universidad de Buenos Aires; Argentina; E-mail: jlparada@qo.fcen.uba.ar

Introduction

For thousands of years microbial cultures have been used to ferment foods and prepare different kind of products. Unstable primary foodstuffs such milk, meat and vegetables can be conserved for relatively long periods of time (Table 1), maintaining their nutritious and caloric value by the use of lactic acid fermentation. Several traditional soured milks such as kefir, koumis, leben and others were used as foods and often therapeutically, before the existence of the actual knowledge on probiotics. The origins of cultured dairy products can be traced in the Bíble and the sacred books of Hinduism. Metchnikoff at the beginning of the XX century proposed the beneficial effects of the lactic acid bacteria (LAB) on health and longevity.

At present yoghurts and other fermented milks using selected strains of LAB are used as benefic foods for the consumers. Some of these bacteria have also been added to domestic animal feed to enhance growth.

In the last three decades, (**1-3**) attempts were made to modulate the indigenous intestinal flora by the exogenous intake of LAB. There is general agreement on the important role of the gastro-intestinal microflora (**4**). The bacterial intestinal flora of humans and animals is the most intimate portion of their biological environment and represents an ecosystem of the highest complexity.

Our understanding of this system and its interactions is still limited. The population may be considered as an open ecosystem, comprising a group of microbial population coexisting in an equilibrium spatio-temporally defined region. The mucosal surface of the small intestine is increased by the folding of the epithelium and by the formation of microvilli in the enterocyte luminal membrane, and that is their normal habitat. The gastrointestinal tract (GIT) of an adult human is estimated to harbour about 10^{12} to 10^{14} viable bacteria. The optimal intestinal flora represents a barrier to the invasion or uptake of pathogenic microorganisms, antigens and harmful compounds from the gut lumen. In addition, the intestinal mucosa is efficient in assimilating antigens and nutrients, providing protection and immunological resistance to harmful agents (**5**).

The balance within the microbial population is maintained by sensitive interactions between living and abiotic compounds (**6**). Several factors can modify the desirable microbial equilibrium and trigger the development of severe and even fatal

New Horizons in Biotechnology, 335-351, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

microbial infections. This situation is termed dysbiosis, and probiotic agents may contribute to restore the functional equilibrium.

Table 1 : Use of lactic acid bacteria in foods

DAIRY FERMENTED PRODUCTS	*Lactobacillus* *Lactococcus* *Streptococcus* *Propionibacterium* *Bifidobacterium*
MEAT FERMENTED PRODUCTS	*Lactobacillus plantarum* *Lactobacillus lactis* *Pediococcus acidalactici*
SARDINE RIPENING	*Pediococcus*
VEGETABLE FERMENTED PRODUCTS	*Leuconostoc* *Lactobacillus brevis* *Lactobacillus plantarum*
SILAGE	*Lactobacillus plantarum*
WINE MALOLACTIC FERMENTATION	*Pediococcus cereviseae* *Leuconostoc oenos* *Lactobacillus plantarum* *Lactobacillus brevis*
WHISKY PRODUCTION	*Lactobacillus delbruekii*
BAKED GOODS	*Lactobacillus plantarum* *Lactobacillus casei* *Lactobacillus brevis* *Leuconostoc* *Pediococcus cereviseae*

Probiotics

From the Greek meaning "for life", certain strains of *Lactobacilli*, *Bifidobacteria* and other microorganisms are termed probiotics. Fuller (**7**) defined the concept as: "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (**8**).

According to Veld and Havenaar (**9**) probiotics may be defined as: "mono or mixed cultures of live microrganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora". This definition does not restrict probiotic activities to the intestinal tract and animals, but also to microbial communities at other sites of the body (urogenital tract), and it is applied to both man and animals.

Probiotics act in two ways:

- by producing lactic acid and other compounds which create a favourable medium for the onset of a desirable flora and hinders the spreading out of harmful microorganisms (**10**).
- by the synthesis of antibiotic-like substances called bacteriocins (**11,12**), which inhibit the growth or the activities of pathogenic or putrefactive microorganisms.

The following criteria and properties must be met before a probiotic can be described as useful:

- The host should gain benefit from harbouring the probiotic (**1**).
- During use, and under storage, the probiotic should remain viable and stable.
- It should be able to survive in the intestinal ecosystem. Examples include strains that have improved resistance to acid and bile secretions.
- The probiotic must be capable of being prepared in a viable manner and on large scale for industrial purpose; it is desirable to have a short generation time.
- Strains isolated from the same species should be selected depending on an enhanced chance of survival and better attachment to the epithelial cells of the gut (**8**). One criterion that has been used to select probiotics is species compatibility.
- They should be generally recognized as safe (GRAS), with minimal possibilities for the transfer of antibiotic resistance.
- When added to feeds, the quality should not be diminished.
- Probiotics may affect the immune system such that improved pathogen resistance occurs (**13**), as well as positive aspects with respect to food allergy.
- Many probiotics are able to inhibit adverse microorganisms by competitive exclusion, production of acid and /or bacteriocins (**14**).

Bacteriocins

Different types of LAB as *Lactobacillus*, *Carnobacterium*, *Pediococcus*, *Leuconostoc*, *Streptococcus thermophilus*, *Enterococcus*, and others are able to produce bacteriocins.

Bacteriocins are biologically active proteins or protein complexes (protein aggregates, lipo-carbohydrate proteins, glycoproteins, etc) displaying a bactericidal mode of action towards Gram-positive bacteria and particularly towards closely related species (**11**). They form a heterogeneous group with respect to production of bacterial species, (Table 2) molecular size, physical and chemical properties, stability, antimicrobial spectrum, mode of action, and so on. Some of the strains may have antagonistic effects when they are introduced in the fermented product in mixture or jointly with other LAB, being necessary to test previously their compatibility in vitro.

Table 2: Different types of Lactic Acid Bacteria able to produce bacteriocins.

MICROORGANISM	BACTERIOCIN	REFERENCE
• *Lactococcus lactis, subsp. lactis*		
ATCC11444	Nisina A	Rogers (1998)
NIZ022186	Nisina Z	Mulders *et al.* (1991)
CNRZ481	Lacticina 481	Piart *et al.* (1990)
• *Lactococcus lactis, subsp. lactis*		

var diacetilactis DPC 938	Lactocina D	Morgan and Hill (1993)
• *Lactococcus lactis, subsp cremoris LMG* 2081	Lactococcina G	Nissen-Meyer et al (1992)
• *Lactobacillus acídophilus M 46*	Bacteriocina M 46	Ten Brink (1990)
• *Lactobacillus casei* B 80	Caseisina 8O	Ramelsberg and Raddler (1990)
• *Lactabacillus plantarum* MI 406	Plantaricina 406	Larsen et al (1993)
• *Carnobactenum psicicola* UI 49	Camocin UI 49	Stoffels et al (1992)
• *Pediococcus acidilactici* SJ-1	Pediocina SJ-1	Schved et al (1993)
• *Pediococcus pentosaceus* L-7230	Pediocina A	Daeschel and Klaenhammer
• *Leuconostoc mesenteroides* UL5	Mesenterocina 5	Daba et al (1991)
• *Streptococcus thermophilus* Sfi 13	Termofilina 13	Maciset & Mollet (1993)
• *Enterococcus fecalis* 226	Enterocina 226 NWC	Villani et al (1993)

The ability of some lactococci to produce inhibitory substances other than organic acids are well known and Nisin was the first bacteriocin reported originally in England. Nisin is a peptide monomer that usually occurs as stable dimers or tetramers and may be inactivated by proteases. It is thermostable depending upon the pH and has good solubility at low pH.

As additive it has been recognized as generally referred as safety (GRAS) and could be added to heat processed milk and milk drinks, as well in other foods (**15**). This fact is of importance in tropical areas due to its inhibitory effect on sporulated Gram-positive bacteria. The addition of Nisin (10-50 mg/L) to pasteurised milk substantially reduces the bacterial counts and increases shelf life.

Genetically manipulated LAB producing large quantities of nisin may be of interest to be introduced in the GIT to control specific diarrhoeas.

Use of probiotics in Veterinary Medicine

Young animals are particularly exposed to accidents, following a lack of hygiene, a poor and irrational alimentation, weakness, trauma, abrupt changes in food or temperature, or the presence of infectious microorganisms numerous enough to overcome host resistance. These facts may produce as a consequence an upward invasion of the digestive areas normally sterile. Colibacilli or other microbial pathogenic species can suddenly become responsible for very severe enteritis and septicaemia. Attempts are then made to prevent or slow down the diseases with the help of sulphonamides, chemical anti-diarrheics and antibiotics. When these treatments fail, lyophilised lactic ferments can be of great help. An impressively lengthy experimentation has already demonstrated the interest of lactic bacteriotherapy in veterinary medicine. Lyophilisation, a process enabling the production of active lactic ferments, concentrated and long lasting, has greatly contributed to the end results. Doses ranging from some millions to billions of bacterial cells per dose or per day have been administered to animals under experimentation with good results. Same doses are recommended for prophylaxis as for treatment. However, they are preventively administered only once or twice weekly. Concerning poultry, it is recommended to add lactic bacteria to drinking water several days per week. Lactic bacteria may also be added to rations such as milk substitutes, feeds, etc. Such measures are economically profitable and easy to

realize. Lactic bacteria are natural and universal agents of prevention, and they result effective. Scientists have mainly used intestinal *Lactobacilli* and, occasionally, lactic *Streptococci.*

The main species of lactic bacteria commonly found in the gastrointestinal system of animals can be grouped under the *Lactobacillus* group (*L. acidophilus, L. bifidus, L. casei, L. plantarum, L. brevis, L. fermentum*) and *Streptococcus* types, (*S. thermophilus, S. faecium, S. lactis*). *L. acidophilus* and its related lactobacilli and *S. faecium* are the prevailing elements in a normal flora. For experimenting purposes, mostly *L. acidophilus* was used to maintain or restore the protective lactic flora in the animals. Prior to the advent of freeze-drying and the production of effective lyophilized ferments, acidophilus milk was used as the main source of lactic bacteria.

One of the first trials was that of Shaw and Muth, from Oregon State Experimental Station, U.S.A. In 1935, they used acidophilus milk for the prevention and treatment of diarrhoea in calves at the rate of 250 mL per dose, or an equivalent of approximately 100 billion cells. One dose was generally enough. Otherwise, it was repeated. The treatment has proven very effective in the treatment of infectious diarrhoea of calves.

Lactic ferments can be used favourably during convalescence after administration of antibiotics, to supply the intestinal medium with useful microorganisms and avoid the return of pathogenic flora. The substitution of antibiotics by lactic ferments in the feed of veals destined to fattening at a rate of 1 g per day per head up to a live weight of 150 kilos showed the lowest intake of nutritive matter per kilo of increase. Based on these results, it was concluded that the fattening of calves could be successful if antibiotics are replaced by lactic ferments.

Milk fermented products with probiotic agents

Modern processing allows for the production of excellent tasting fermented dairy products with high counts of LAB that may control diarrhoeas caused by pathogens and rotavirus, reduce lactose intolerance, produce immune enhancement (**16**), etc. There are also evidences that reduce cholesterol (**17**) and blood lipids content, as well as harmful intestinal microbial enzyme activity and colon cancer (**18,19**).

In many countries probiotic bacteria are sold in two different formats: food and dietary supplements. The food products containing probiotic bacteria are almost exclusively dairy products, capitalizing on the historical association of lactic acid bacteria with fermented milk.

The general standard of identity for yoghurt requires fermentation with at least *Lactobacillus delbruekii spp bulgaricus* and *Streptococcus thermophilus*, but no levels are officially specified. Probiotic bacteria used in these products include different strains of *Lactobacillus* and *Bifidobacterium* species.

The dairy products associated with probiotic bacteria are primarily fermented fluid milk and yoghurts. Some products utilize approximately 40% of the desired

probiotic bacteria in dairy products. Twenty million people consume products with probiotics in Asia.

Some of the commercially available probiotic strains and the respective producers are:

- *Lactobacillus acidophilus* NCFM (Rhone-Poulenc-Rhodia, USA)
- *Lactobacillus rhamnosus* GG (Valio Dairies, Finland)
- *Lactobacillus reuteri* 1063 (BioGala, USA)
- *Bifidobacterium longum* BB536 (Morinaga Milk Industries, Japan)
- *Lactobacillus plantarum* 299V (ProViva, Finland)
- *Lactobacillus casei* YIT9018-Shirota (Yakult, Japan)
- *Lactobacillus johnsonii* LJ-11 (Nestlé, Switzerland)
- *Lactobacillus casei* CRL431 (Cerela-Sancor, Argentina)
- *Lactobacillus acidophilus* CRL730 (Cerela-Sancor, Argentina)

Fluid milk containing probiotics (unfermented acidophilus milks) became a feasible product concept when bacterial concentration technology improved and led to the commercial production of frozen culture concentrates.

There are some methods of protecting microorganisms, which assures their viability and potency for reasonably long periods. Two treatments are especially efficient: flash freezing at very low temperatures and freeze-drying. Bacteria may be kept alive for many years in liquid nitrogen. This process is practical and is widely used in laboratories. For therapeutic and prophylactic purposes, freeze-drying is preferred. The latter process keeps the bacteria alive long enough to allow their use without inconveniences. However, certain conditions must be fulfilled. Freeze-dried bacteria must be stored under vacuum or they must be mixed with stabilizing (protective) agents. Containers must be water and air proof. Vitality is also prolonged under low temperature.

Another commercial application of probiotics (**20**) is a kefir type drink marketed as a dietary supplement (Gala Geb, Inc) formulated with probiotic lactic cultures (*L. casei* subspecies *casei*, *L. plantarum*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* subspecies *cremoris*) and immunoglobulins from the colostrum of cows. Some studies reported increased resistance to infection. Another example of successful product development of probiotic bacteria is the use of *Lactobacillus rhamnosus* GG for yoghurts in different countries, as food and therapeutical (**21**).

A mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* is sold in the Argentinean market as biotherapeutical milk. Three other types of yoghurts with probiotics are also consumed there.

The required minimum levels of probiotic bacteria in any dairy product is about $2x\ 10^6$ cfu/ml. Culture manufacturers recommend approximately 10^6-10^7 probiotic bacteria per gram of yoghurt or unfermented acidophilus milk at the end of shelf-life, but viable counts may fall below these levels. Bifidum bacteria are particularly sensitive to storage (**22**).

In USA the yoghurt containing live, active cultures for use in any yoghurt or packaging is about 10^8 viable lactic acid bacteria per gram at time of manufacture (**20**). Frozen yoghurts may contain 10^7 /gram.

Even if high numbers of probiotic bacteria are present in dairy products, the selective enumeration of probiotic bacteria in a background of starter bacteria is not a trivial undertaking. This can be influenced by the pH, ability to establish standards for probiotic content and the food company's quality assurance methods for products with multiple LAB strains, species and genera.

The mixture of strains and species in the product and the presence of sub-lethally injured cells can complicate the enumeration. Survival of probiotic bacteria in dairy products is another concern.

The market of supplements contains many different products, formats and contents. A diverse array of bacterial genera and species are represented in these products, including numerous types of *Lactobacilli, Bifidobacteria* and less commonly *Enterococcus, Bacillus*, and *Yeast*. Dietary supplement products are coincident with the overall upward trend in the natural products market (**23**).

Although clinical efficacy is not the subject of this presentation many studies of probiotic bacteria on physiological effects such as diarrhoea, lactose intolerance, activity of faecal enzymes and others, typically show an effect using a daily dose of about 10^9 -10^{10} organism per day. In order to consume $2x10^9$ organisms from an acidophilus milk product that is formulated at $2x10^7$ cfu/ml, 100 ml per day would need to be consumed.

Shanahan (**24**) reported that Steidler and colleagues in 2000 show that dietary administration of the murine enteric bacterium *Lactococcus lactis*-genetically engineered to produce the anti-inflammatory cytokine inerleukin-10 (IL10) within the gut- is therapeutically effective in the mouse model to prevent inflammatory bowel disease. The cytokine IL-10 is secreted in the intestinal lumen of mice by non-pathogenic genetically engineered LAB administered as a food supplement. So, IL-10 traverses the gut epithelium, most probably by a paracellular route as epithelial permeability is increased during inflammation. It suppresses the inflammatory immune response in the gut mucosa by promoting the activity of regulatory T cells that hold effector T_H1 cells in check. In addition, indigenous enteric bacteria that are not genetically modified may also condition the mucosal immune system and influence the cytokine milieu by interacting with the gut epithelium. Probiotic therapy has been effective for treating mice deficient in IL-10 and other animal models of inflammatory bowel disease. This approach is important to eventually control ulcerative colitis and diarrhoea in humans.

New forms of dietary supplement formula should be developed in order to achieve the therapeutical doses. In some places probiotics are sell as capsules or ampules with high concentration of LAB, but their use is still small. This is one of the goals to be achieved in the biotherapeutic use of probiotics. Several factors contribute to the expansion of probiotics and other natural products in the markets.

- People would rather prevent than cure disease. Probiotics may contribute to reduce health-care costs.
- People are more aware of the link between health and nutrition. Consumers already have a negative perception of bacteria that may be hard to change.
- People want to counteract the perceived increase of environmental hazards from pollution, pathogenic microbes, and water and food contamination.
- Controlled scientific studies are providing support for the efficacy of natural approaches to maintain health, with few if any negative side effects. Many probiotics currently being used have been assumed to be safe based on past history of a lack of medical problems.

LAB enzymatic hydrolysis has been shown to enhance the bioavailability of proteins and fat. Bacterial proteases can increase the production of free amino acids, which can benefit the nutritional status of the host, particularly if the host has a deficiency in endogenous protease production. Vitamin B complex should also be increased in fermented foods.

To be accepted as probiotic the strain and products should have at least the following attributes:

- Taxonomy, source origin and purity.
- Animal studies and human clinical studies.
- Safety Pharmacology, Product information
- Target for general population and/or specific risk groups. .
- Food or dietary supplement format and cost evaluation.
- Additional bioactive components registered, storage stability.

There is still a lack of scientific consensus on the benefits of probiotic cultures, particularly when considering some specific effects. Most probiotics have been designated as safe (GRAS) based on their long history of use in food fermentation. However, there have been occasional reports of bacteraemia and endocarditis associated with *Lactobacillus*, generally in immunocompromised individuals. This prompted the suggestion that some type of surveillance must be instituted for probiotics. Serious epidemiological data strongly support the fact that *Lactobacillus* is rarely found in bacteraemial infections and that the current probiotics lack pathogenic potential and are used safely in many countries.

Prebiotics

There is currently a great deal of interest in increasing the number and activities of bacterial groups, which have health-promoting properties. This should be possible by the use of dietary adjuncts, which reach the colon intact and may be specifically utilized. Such material was termed prebiotics by Gibson and Roberfroid (**3**).

Most current attention and success has been derived using non-digestible oligosaccharides, which occur naturally in a variety of plants such as onions, asparagus, chicory, banana and artichoke, which fulfill the prebiotic criteria. Various data have shown that fructo-oligosaccharides (FOS) are specifically fermented by bifidobacteria and in minor extent by other lactic acid bacteria.

Much attention is now therefore being directed towards dietary components that affect the gut in such a manner that the activities of benign or health-promoting bacteria are stimulated, preferably at the expense of pathogens. In this context, one can identify important roles for prebiotics that have a very selective fermentation in the large gut. The human colonic flora has both beneficial and pathogenic potentials with respect to host health.

There is now much interest in manipulation of the microbiota composition in order to improve potentially beneficial aspects. The prebiotic approach dictates that non-viable food components are specifically fermented in the colon by indigenous flora, mainly bifidobacteria and lactobacilli. Any food ingredient that enters the large intestine may be a candidate prebiotic. However, to be effective, selectivity of use by the bacteria and its effective fermentation is essential.

The resident bacterial flora of the large gut comprises about 9% of the total cell in the body representing up to 10^{12} cells /g dry weight faeces, and plays a major role in host nutrition and health.

At least fifty different genera of bacteria reside in the colon, comprising several hundred individual species. The activities of colonic bacteria are affected by a number of physicochemical parameters, with the bacteria present having fluctuating activities in response to substrate availability, redox potential, pH, O_2 tension and distribution in the colon. The numerically predominant bacteria are bacteroides, bifidobacteria, eubacteria, clostridia, lactobacilli, Gram-positive anaerobic cocci, coliforms, methanogens and dissimilatory sulphate-reducing bacteria.

At present most prebiotics are selected on the basis of their ability to promote the growth of lactic acid producing microorganisms.

Most success has been derived from oligosaccharides containing fructose. Oligofructose (degree of polymerization =4) and inulin (degree of polymerization = 10) modified the gut microbiota composition in controlled volunteer trials. They are considered as soluble fibres. Various other oligomers may also be prebiotic. These include some sugar alcohols, lactulose, as well as oligosaccharides that contain xylose, mannose, galactose, maltose and mannose.

Important developments also include the use of prebiotics that have activities with multiple biological functions (**25**). This would clearly involve stimulation of potentially beneficial microorganisms such as the lactic acid microflora. However, further possibilities exist for the use and manufacture of prebiotics that incorporate more than one activity.

Here, the anti-infective nature of fermentable gut substrates can be effective through blocking adhesion sites that may otherwise be occupied by pathogens. For example, oligosaccharides that have mannose side chains may be effective against *E. coli* and related organisms.

Those oligosaccharides, which offer natural attenuative properties, such cellobiose have shown effect against virulence properties of *Listeria monocytogens* and other pathogens.

Bacterial fermentation of chicory fructans and other oligofructoses produces short chain fatty acids in the colon, including a small amount of butyric acid, which has been demonstrated to increase apoptosis in the colon (**3**). Furthermore, there are studies that show that bifidobacteria increase the host immune response.

These observations raise the possibility that selective fermentable non-digestible oligosaccharides that enhance the growth of bifidobacteria in the gut could potentially inhibit colon carcinogenesis. Recent studies on prebiotics have indicated that a FOS dose of about 30 g/day may have prebiotic action.

Dietary administration of prebiotic compounds inhibits the preneoplastic lesions in the colon, suggesting the potential colon tumor inhibitory properties of these compounds. A research avenue of interest is the derivation of oligosaccharides from fibre such as starch-containing polymers.

Synbiotics

Growing numbers of food manufacturers in Western Europe are beginning to explore the commercial opportunities for foodstuffs containing health-promoting microbial food supplements (probiotics) and health-promoting non-digestible food ingredients (prebiotics).

Increasingly, probiotics and prebiotics are used in combination, these being termed synbiotics by Gibson and Roberfroid (**3**).

Throughout history, fermented milk products in particular have been considered beneficial to health, but only in recent years has there been scientific support for these beliefs. Issues considered important to the continuing development of this growing market are proof of safety, proof of efficacy, consumer education, market positioning, price and appropriate health claims strategies. In Europe there is a market of $US 2 billion per annum.

Until recently, much of the innovation in the use of probiotics and prebiotics has been in the dairy sector, which are offered as yoghurt and yoghurt-type fermented milk being made available today to the consumer.

However prebiotics are beginning to find increasing application outside the dairy sector, particularly in baked goods. Behind the broadening application of prebiotics has been the pro-active stance taken by the prebiotic suppliers, Orafti and Cosucra.

To date, market activity in probiotic and prebiotic containing foods has centred around three health propositions, namely improving general gut health (**18**), lowering blood cholesterol and improving the body's natural defences.

Novel products as vehicles for probiotics

Lactic acid bacteria can rarely convert starch into lactic acid. However some strains of *Lactobacillus* and *Streptococcus* can do it (**26**). Giraud *et al.*(**27**) at the IRD of Montpellier have isolated *Lactobacillus plantarum* A6 and characterized the properties of the extracellular amylase activity.

Sour cassava starch is obtained by a natural fermentation and this product is largely appreciated in Africa, South America and other developing countries. *Lactobacillus plantarum* D34 was isolated in Colombia and behaves as an amylolytic strain. Some other strains of LAB as *Lactobacillus spp* SLH6 and *Streptococcus thermophilus* ST4 were also used as inocula to ferment experimental mixtures of milk powder (3g %) plus cassava starch (6g %), or inuline (6g %) as prebiotic. (Fig.1) In the case of some particular strains (D34 and SLH6) a positive response in the growth acidification and carbohydrate utilization using the adjuncts starch or inuline was observed, while the ST4 only consume inuline (Fig 2, Fig 3 and Fig 4). The last parameter was determined by HPLC.

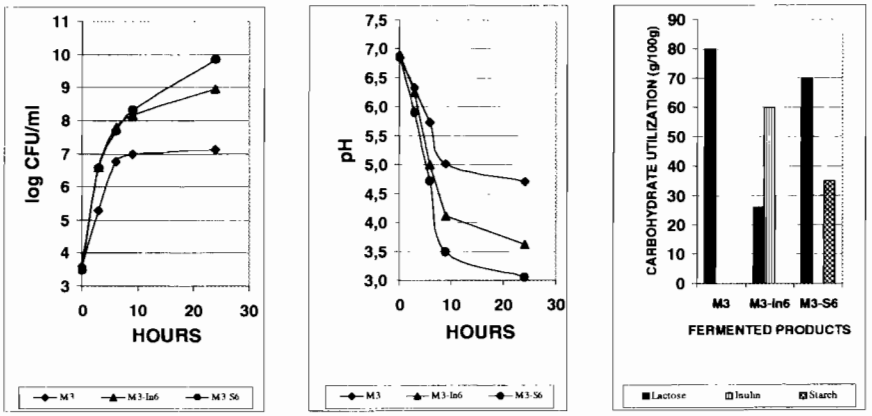


Figure 1: *Lactobacillus plantarum* D34 growth kinetics, acidification and carbohydrate utilization.

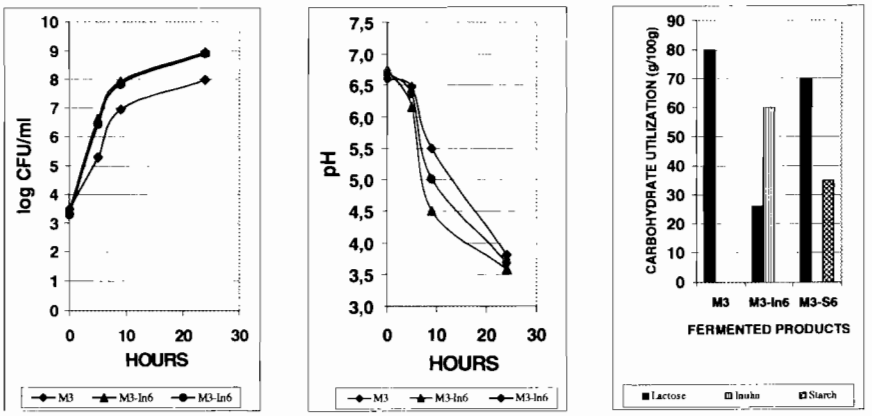


Figure 2. *Lactobacillus spp.* SLH6 growth kinetics, acidification and carbohydrate utilization.

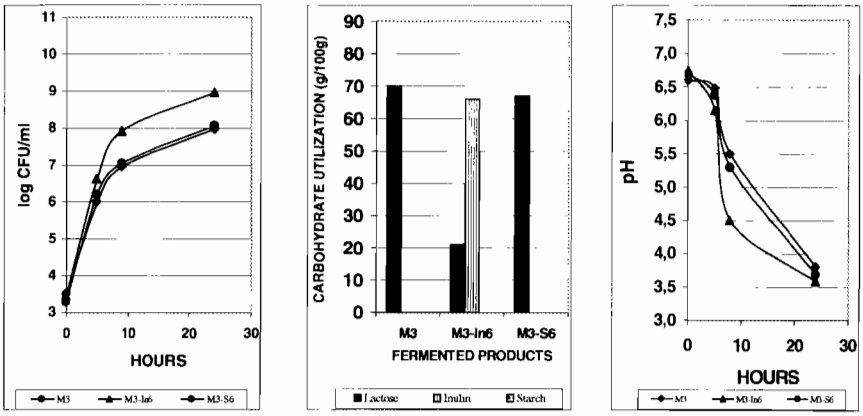


Figure 3. *Streptococus thermophilus* ST4 growth kinetics, acidification and carbohydrate ulization.

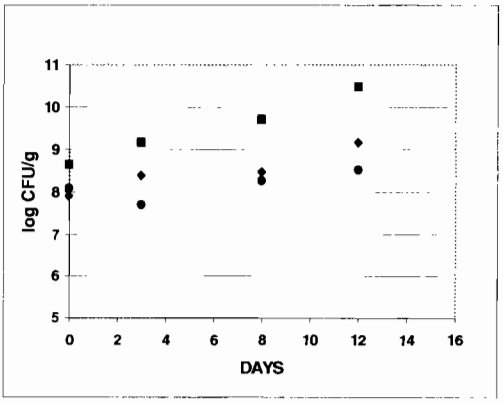


Figure 4. Effect of milk-starch fermented products on LAB counts in rat´s feces. ● Stock diet, ◆ M3-S6 + *Lactobacillus spp.* SLH6, ■ M3-S6 + *Streptococcus thermophilus* ST4.

Survival in the experimental rat model

A model was designed to test in rats the acceptability of the fermented products and the survival and evolution of the microflora. Similar to the method used in the evaluation of impact of nutritional supplements, this model allows following of the evolution of microbial flora in feces. The rats were fed during 4 days with the fermented products (50g/rat/day) and then with only stock diet. In Fig. 5 is shown the result with the SLH6 strain in mixtures milk-inulin M3-In6 g%) and milk-starch (M3-S6g%). Triplicates of rats were used for each sample in this preliminary experiment and the optimal dosis for the young adult rats was established at 50g/rat/day. Results of bacterial enumeration with fermented products using

Lactobacillus SLH6 and mixtures of milk-starch and milk-inuline with the respective control are shown in Fig. 6.

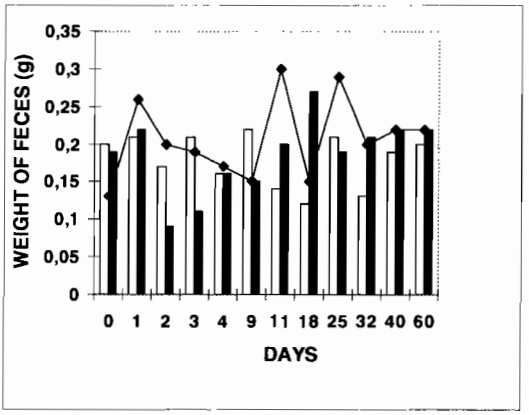


Figure 5. Average weight of rat's feces fed with fermented products using *Lactobacillus spp* SLH6. □ Stock diet, ■ M3-S6.ferm, ♦ M3-In6.ferm.

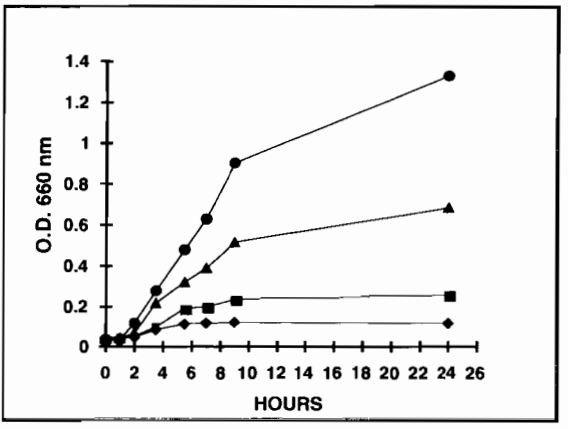


Figure 6. Stimulatory effect of *Spirulina* extracellular products on *Streptococcus salivarius subsp. thermophilus*. In minimal medium with (■) and without (◆) extracts, and in MRS with (●) and without (▲) extracts. From Parada *et al*. (**28**).

The average weight of the feces was determined and was made, after dilutions, in agar MRS medium for *Lactobacillus*. The feces were collected every day and it was noticed that they were more humid and abundant in fermented samples when inuline was used. Further experiments will be carried out in order to confirm the observations and analyse the kinetics of survival for longer periods of time.

Nutritional aspects

The use of this type of milk-oligosaccharides mixtures offers the possibility to reduce the use of milk to 1/3 of that used in regular yoghurts, which can also serve as vehicles for probiotic and prebiotic agents. The product is yoghurt-like and the taste is fairly good and can be offered to those countries that have shortage of milk and are good producers of cassava starch or other starchy products.

The bioavailability of many nutrients in vegetable diets is usually low, and this will significantly contribute to the nutritional inadequacy. In poor countries animal foods and milk although of excellent nutritional value, are not available in enough quantity to these populations, due to their high cost and customs.

Cassava starch provides in many cases, calories, vitamins and minerals to the population in developing countries. Appropriate technologies can be set up to combine starchy materials with a reduced amount of fluid milk or powder milk and prepare lactic acid fermented products in order to improve the nutritional quality of cereals and the nutrient density.

Dos Santos (**29**) in Soccol's Laboratory in Brazil has obtained by fermentation of cassava flour, milk whey and a mixture of probiotic *Lactobacillus* a tasteful lactic drink and a residual cassava flour product with interesting nutritional properties, enriched in LAB and their metabolites.

Svandverg (**30**) describes that staple food is commonly prepared in Africa as a thick porridge from sorghum flour or cassava flour or starch. The thickness may prevent young children from consuming adequate quantities. The thin gruels may be more easily consumed but its energy density is too low to meet the energy requirements of young children. Starchy lactic fermented products using cassava starch or cassava flour and milk may be an interesting vehicle for probiotics and prebiotics improving dietary bulk properties and nutrition.

Viscosity in fermented products is reduced, so it is possible to increase the concentration of starchy substrates. Svanberg in Tanzania using *Lactobacillus plantarum* and sorghum flour already demonstrated this. The low pH of the product increases the bioavailability of calcium and iron in the diet and contributes also to extend the shelf life. The properties of probiotic agents may be a useful tool to control pathogens and recovery from diarrhoeas.

Spiruline as Lactic Acid Bacteria growth promoter

Cyanobacteria (**31**) or blue-green algae are photoautotrophic microorganisms largely distributed in nature. Some of them have been used as human food for many years because of their high protein content (35-65%) an their nutritional value. Spirulina is the best-known genus and it was consumed by the aztecs in Mexico and by the Chaad lake population in Africa. At present some countries, including Argentina are culturing it on a large scale. Some strains also produce bioactive substances that may inhibit or promote microbial growth (**32**). Parada *et al.* (**26**) have found that in both MRS and RM optimal medium for lactic acid bacteria, the extracellular products of Spirulina significantly promote the growth of the LAB

assayed. *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus casei, Lactobacillus acidophilus, and Lactobacillus bulgaricus* were grown in very rich media. MRS, with and without addition of extracellular products obtained from a late log phase culture of *Spirulina platensis* in Zarrouk medium. This stimulatory effect was observed in media with pH adjusted to 5,3, 6,3 and 7,0. No effect was observed in minimal saline medium (MM). It appeared in our experiments that extracellular products of *S. platensis* promoted LAB growth in vitro (Fig. 7).

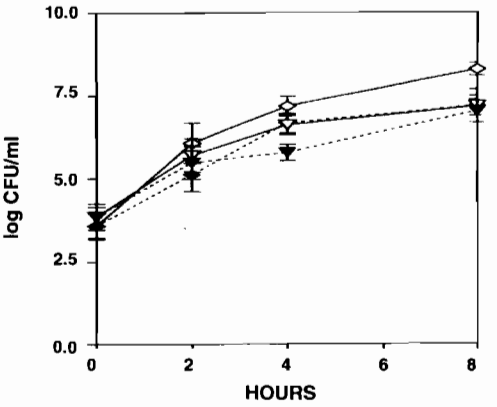


Figure 7. Growth kinetics of *Lactobacillus* L01 (◆ .◊) and *Lactoccoccus lactis* C2 (▼.V) without and with 6 mg/ml *Spirulima platensis* dried biomass (n = 6). From Zulpa de Caire et al. (**33**)

Nowadays, when the dairy industry is supplementing milk with minerals, vitamins and antioxidants, it would be of interest to consider the possibility of adding Spirulina biomass, as a natural product, to fermented milk to induce a faster production of LAB and increase the number of viable cells in the product and in the gut.

In addition we tested the stimulatory effect of aqueous suspensions of *Spirulina platensis* dry biomass extracted at pH 6.8 and 5.5 on four lactic acid bacteria grown in milk (**33**). The addition of dry biomass of Spirulina to milk (6mg/ml) stimulated growth of *Lactococcus lactis* by 27%. The growth of other strains was also promoted.

It will be worthwhile to carry out experiments in rats and humans to determine whether this dietary supplement may be used as a type of prebiotic in order to improve LAB intestinal colonization and human health.

Actual Perspectives

Probiotic bacteria are currently being investigated as live delivery vehicles that can potentially be used to express vaccines, enzymes or antimicrobials (as nisine)

within targeted locations of the intestinal tract or other mucosal surfaces. Definitive assessments regarding health are necessary.

Future research by tracking of microflora changes and survival of probiotic strains using modern molecular methodologies is the new approach. Some of the proposed methodologies are:

- Restriction fragment length polymorphism.
- Direct amplification using polymerase chain reaction.
- Genetic fingerprinting.
- The design of specific gene probes.

To exploit the prebiotic concept more fully, there is a need for assessments of:

- The prebiotic potential of raw and processed food.
- Improved determination of the gut microbiota composition, response and activities.
- The use of molecular methodologies to assess accurately prebiotic identities and develop efficient bacterial probing strategies.
- The health consequences of dietary modulation.

In terms of new prebiotic developments the following aspects may be worthy of research considerations:

- Incorporation into appropriate-food vehicles.
- Stimulation of beneficial flora which also exert anti-adhesive as well as attenuative properties.
- Low dosage forms and non-cariogenic.
- Dietary polysaccharides.
- Good preservation and drying characteristics.
- Viscosity regulation.
- Low caloric value.

Synbiotics have significant potential in improving human health, as well as preventing and treating diarhorrea and other diseases.

Interest in probiotics and prebiotics is likely to increase in our country and all over the world, following trends of other products that have a healthy image. Not all of the products are equal, and research will differentiate them. New forms like energy bars, juices, cereals, cheeses with probiotics and perhaps other health enhancing ingredients will emerge.

"Prevention is better than cure".

References

1. Goldin B.R. (1998) *British J. Nutrition* **80** (Suppl.2), S203-S207.
2. Guililand S.E. (1990) *FEMS Microbiol. Rev.* **87**, 175-188.
3. Gibson G.R., Roberfroid M.B. (1995) *J. Nutrition* **132**, 1401-1412.

4. Holzapfel W.H., Haberer P., Snel J., Schillinger U., Jos H.J., in't Veld H. (1998) *Int. J. Food Microbiol.* **41**, 85-101.
5. Perdigon G, Nader de Ruiz Holgado M.E., Alvarez S., Oliver G., Media M., Pesce de Ruiz Holgado A.A. (1986) *J. Food Prot.* **49**, 986-998.
6. Hove H., Norgaard H., Mortensen P.B. (1999) *European J. Clin. Nutrition* **53**, 339-350.
7. Fuller R. (1989) *J. Appl. Bacteriol.* **66**, 365-378.
8. Conway P.L., Gorbach S.L., Goldin B.R. (1987) *J. Dairy Sci.* **70**, 1-12.
9. Veld J.H.J.H., Havenaar R. (1991) *J. Chem. Technol. Biotechnol.*, **51** (4), 562-567.
10. Reid G. (1999) *Appl. Environ. Microbiol* **65**, 3763-3766.
11. De Vuyst L., Vandamme E.J. (1994) *in* Bacteriocins of Lactic Acid Bacteria, Vuyst De L. and Vandamme E. J. Blackie Academic & Professional, pp 107-131.
12. Piard J.C., Desmazeaud M. (1992) *Lait* **72**, 113-142.
13. De Simone C., Vesely R., Salvadori B.B., Jirillo E. (1993) *Int. J. Immunother.* **9**, 23-29.
14. Guillot J.F., Yvose P., Jule S. (1990) *Microecol. Ther.* **20**, 19-22.
15. Henning S., Metz R., Hammes W.P. (1986) *Int. J. Food Microbiol.* **33**, 135-141.
16. Schiffrin E.J., Rochat F., Link-Amster H., Eeschilimann J.M., Donnet-Hughes A. (1995) *J. Dairy Sci.* **78**, 491-497.
17. Hepner G., Fried R., Jeor S.S., Fusitti L., Morin R. (1979) *Amer. J. Clin. Nutrit.* **32**, 19-24.
18. Reddy B.S., Hamid R., Rao C.V. (1997) *Carcinogenesis* **18**, 1371-1374.
19. Reddy G.S., (1998) *British J. Nutrit.* **80** (Suppl. 2) S219-S223/
20. Sanders M.E. (1998) *British J. Nutrition* **80**, (Suplem.2) S213-S218.
21. Oksamen P.J., Salminen S., Saxelin M., Hanalainen P., Ihantola-Uomisto A., Stiltonen S., Stuckey H., Toppila A., Vapaatalo H. (1990) *Ann. Med.* **22**, 53-56.
22. Medina L.M., Jordano R. (1994) *J. Food Protect.* **56**, 731-733.
23. Young J. (1998) *British J. Nutrit.* **80** (Suppl. 2) S231-233.
24. Shanahan F. (2000) *Science*, **289**, 1311-1312.
25. Delzenne N.M., Kok N., Fiordaliso M.F. Deboyser D.M., Goethels F.M., Roberfroid M.B. (1993) *Amer. J. Clin. Nutrit.* **57**, 8205-8209.
26. Parada J.L., Zapata L.E., de Fabrizio S.V., Martinez A. (1996) *World J. Microbiol. and Biotechnol.* **12**, 53-56.
27. Giraud E., Gosselin L., Marin B., Parada J.L., Raimbault M. (1993) *J. Appl. Bacteriol.* **75**, 276-282.
28. Parada *et al.* (1998) *Int. J. Food Microbiol.*, **45**, 225.
29. Dos Santos M.C.R. (2001) Lactic acid fermented beverage using milk whey, cassava flour and probiotic starters. Master Thesis. Universidad Federal de Parana, Brazil.
30. Svanberg U., Lorri W. (1997) *Food Control* **8**, 319-327.
31. Kulik M.M. (1995) *Eur. J. Plant Pathol.* **101**, 585-599.
32. De Mule M.C., Zulpa de Caire G.Z., de Cano M.S. (1996). *Phyton* **58**, 93-96.
33. Zulpa de Caire G., Parada J.L., Zaccaro M.C., Sorni de Cano M.M. (2000) *World J. Microbiol. and Biotechol.* **16**, 563-565.

Chapter 31

DEVELOPMENT OF A NEW BEVERAGE WITH PROBIOTIC ACTIVITY

M.C.R. SANTOS[1], C.R. SOCCOL[1], N. WASZCZYNSKYJ[2], M. RAIMBAULT[3]

[1] Laboratório de Processos Biotecnológicos, Departamento de Engenharia Química. – UFPR. 81531-990 Paraná Brazil ; [2]Laboratório de Análise Sensorial, Departamento de Engenharia Química. – UFPR. 81531-990 Paraná, Brazil; [3]Probiotec. Cap Alpha. Avenue de l'Europe-Clapiers – 34940 Montpellier Cedex 9 – France

Abstract

The present work was conducted with the following objectives: obtaining an increase in milk whey utilization, effective use of by-product originating from the lactic industry, adding value to cassava flour, a product largely produced in Paraná state, and contributing to the utilization of the amylolytic bacteria *Lactobacillus plantarum* A6, isolated from cassava. A milk beverage and a fermented cassava flour with probiotic activity were developed with mixed cultures of *Lactobacillus plantarum* A6, *Lactobacillus casei* spp Shirota and *Lactobacillus acidophilus*. The optimization of total fermented products (liquid phase + solid) showed that the best parameters were: 16 hours fermentation time, 8% inoculation rate, incubation temperature of 35°C and 20% of cassava flour. After 16 hours of fermentation the beverage had a pH of 4.07; 1050 of titratable acidity in lactic acid, 40 g/L of reducing sugars and 2.8 x10^9 cells / mL of lactic amylolytic bacteria and 2.28 x 10^9 cells/mL of probiotic bacteria. The production of lactic acid measured by high performance liquid chromatography (HPLC) was 1.25% after 16 hours of culture. The lactic beverage maintained its microbiologic and physical-chemical quality after 28 days of storage at 4°C, showing desirable cell count of the specific cultures utilized.

Key-words: Milk whey, cassava flour, *Lactobacillus plantarum* A6, *Lactobacillus casei* spp Shirota, *Lactobacillus acidophilus*, fermented lactic beverage, fermented cassava flour.

Introduction

In the last years, a remarkable increment in the consumption of fermented lactic beverages has been observed. This phenomenon is mainly due to the low viscosity of new drinks and by a significant increase in consumed soft and refreshing beverages (1). Lactic beverages are associated not only with pleasant, slightly acid flavor, but also with a shelf life that is much larger than that of milk.

New Horizons in Biotechnology, 353-364, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

In the dairy product industry, the conversion of whey into beverages, fermented or not, is one of the most attractive options for human consumption, due to process simplicity, use of pre-existing milk processing equipment, without mentioning excellent functional properties of whey proteins themselves (**2**).

Probiotic effects from microorganisms present in fermented milk-based products include the prevention or reversion of clinical states generated by antibiotic therapy, stress, or diets, that have altered natural microflora. Other effects attributed to probiotic products include, the decreasing of cholesterol plasma concentration and anti-tumor activity besides on vitamin synthesis and digestive enzymes (**3**).

Cassava is an important agricultural crop in the state of Parana and constitutes the alimentary basis of a considerable part of the population in extensive areas of Africa, Asia and Latin America.

In 1991, a wild strain of *Lactobacillus plantarum* (denominated A6) was able to hydrolyze starch. It was isolated by Giraud *et al.* (**4**) from fermented cassava starch in the Laboratory of Biotechnology of the IRD (ex-ORSTOM), in Montpellier - France.

This work was undertaken with the following objectives: the production of a fermented beverage and flour production with probiotic activity prepared with sweet milk whey and cassava flour, using mixed cultures of bacteria of the species *Lactobacillus plantarum* A6, *Lactobacillus casei* ssp Shirota and *Lactobacillus acidophilus*, contributing to the study of the utilization of the bacterium *Lactobacillus plantarum* A6, in lactic products. A second objective was to give an added value to cassava flour as well as to milk by-products such as milk whey. These by-products present a high nutritional value and constitute an organic material with great capacity of generating environmental pollution when thrown in fluvial water.

Material and Methods

Microorganisms

Lactobacillus plantarum A6, *Lactobacillus acidophilus* (CCT 0329) and *Lactobacillus casei* ssp Shirota (IAL 0527).

Substrates

Milk whey and cassava flour were obtained locally (Curitiba-PR / Brazil). These substrates were subjected to chemical analysis. The time/temperature range of 80°C and 20 minutes was efficient in the destruction of natural microflora present in milk whey (as optimized in a previous study). Cassava flour was sterilized in an autoclave at 121°C for 15 minutes. After treatment, these substrates were blended and cooled to 37°C.

Inoculum preparation

L. casei Shirota and *L. acidophilus* were grown in MRS broth (MERCK) and *L. plantarum* A6 in MRS broth modified with 1% of dissolvable starch. Both strains were incubated at 37°C for 48 h.

Optimization of fermentation parameters

To evaluate the influence of physical conditions, such as the initial inoculation rate and amount of cassava flour, two experimental designs were carried out. The first optimization was undertaken following a complete experimental design 2^{3-0} (two levels and three variables), with 2 repetitions of the central point, resulting in a total of 11 runs. The results were measured by pH variation, variation of the acidity and concentration of cells. With the results obtained in the first fermentation, a second optimization was accomplished. Parameters measured include fermentation temperature, cassava flour concentration and fermentation time. The variables of the first and second optimization are given in Tables 1 and 2.

Table 1 – First optimization variables.

Levels	Inoculation rate (%)	Cassava flour concentration (%)	Fermentation time (h)
-1	1	10	8
0	4,5	25	24
+1	8	40	40

Table 2 – Second optimization variables.

Levels	Fermentation temperature (°C)	Cassava flour concentration(%)	Fermentation time (h)
-1	32	15	8
0	35	20	16
+1	38	25	24

Kinetic study of the fermentation using amylolytic strain and probiotic strain

After 8 hours of fermentation with the amylolytic strain, the probiotic inoculum containing *Lactobacillus acidophilus* (4%) and *Lactobacillus casei* ssp Shirota (4%, was added, under slow agitation. The mixture was incubated at 35°C for 8 hours.

Evaluation of the fermentation

Samplings were carried out in intervals of 2 hours, up to 16 hours of fermentation, to verify the evolution of pH, and acid development in the fermenting substrate. Analyses were carried out by titration with 0,1 N NaOH and by HPLC. Changes in pH were registered with a pH meter (LABSCORE, PL 800). Reducing sugars were analyzed by the Somogyi-Nelson method (**5**). Cell counting of *L. casei* and *L. acidophilus* cells was performed by serial dilutions with 0,1% peptone water, pour plating (in duplicate) using MRS agar plates (**6**). *Lactobacillus plantarum* A6 cells were counted using MRS-modified agar with a substitution of dextrose by

starch 1%. The plates containing modified MRS agar were exposed to iodine vapors in order to check for starch consumption in the medium by the colonies of amylolytic cells. The plates were incubated at 37°C for 48 h. The results were expressed as CFU/mL.

Results and Discussion

Chemical composition of milk whey and cassava starch is shown in the Table 3.

Table 3. Chemical composition of the substrate used (%).

Components	Milk whey (%DM)	Cassava flour (%)
Proteins	12,45	0,75
Lipids	0,6	0,76
Carbohydrates (difference)	76,68	90,85
Starch	-	80,10
Humidity	1,73	4,02
Ashes	8,54	0,70
Fibers	-	2,92

(-): Not detectable

Fermentative parameters optimization

Upon analysis of the three Pareto diagrams (Figs 1, 2 and 3) it can be observed that the concentration of cassava flour was a significant factor as an increase in flour concentration resulted in a decrease of the response (pH variation, titrable acidity and final concentration of cells) until a certain point (40%). At that concentration, the fermentation wasn't occurring because the flour wasn't mixed anymore with milk whey in the Erlenmeyer flasks.

It was also observed that greater fermentation times influenced positively the responses. An increase in the inoculation rate had a significant effect on the pH and as a result on acidity. It was opted to fix the inoculation rate to 8%, due to the fact that in these optimizations, the *Lactobacillus plantarum* A6 strain was used. It is an amylolytic strain being considered as starter of the fermentation. The other strains (*Lactobacillus casei and Lactobacillus acidophilus*) were added after 8 hours of fermentation where there was already a certain glucose concentration in the medium, to be used by these probiotic strains. The inoculation rate of the probiotic strains was also fixed to 8%, resulting in a total fermentation inoculum of 16%.

Second optimization of fermentative parameters

After analysis of the three Pareto diagrams (Figures 4, 5 and 6), during the second optimization phase, it was observed variations in flour concentration, time and incubation temperature did not result in changes that were considered significant.

A constant increase in the fermentation time resulted in a decrease of the final pH. It is known that very low values of pH, highly acidic substrates, are not traits that are sought after as they result in an inhibition of cell growth. The resulting lactic

beverage is therefore acidic and consequently is not well accepted by consumers. Other factors to be taken into consideration include a study of possible variations in pH during the shelf life of the product as cells may ferment the product even at low temperatures.

The central points were considered as optimal for the fermentation. Optimized parameters were as follows: temperature of incubation of 35°C, fermentation time of 16 hours, cassava flour concentration of 20% and inoculation rate of 8% for *Lactobacillus plantarum* A6 in the beginning of the fermentation, 4% for *Lactobacillus casei* and 4% for *Lactobacillus acidophilus* after 8 hours of fermentation.

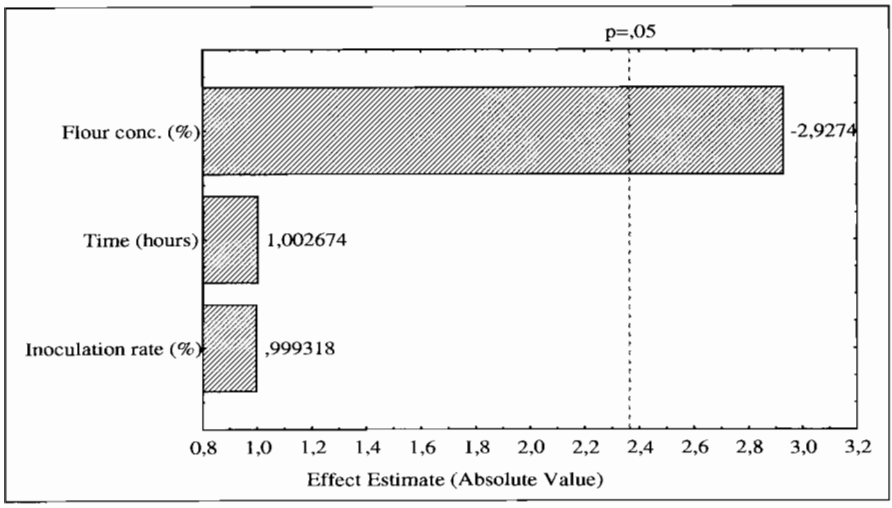


Figure 1. Pareto chart of effects for first optimization for pH variation.

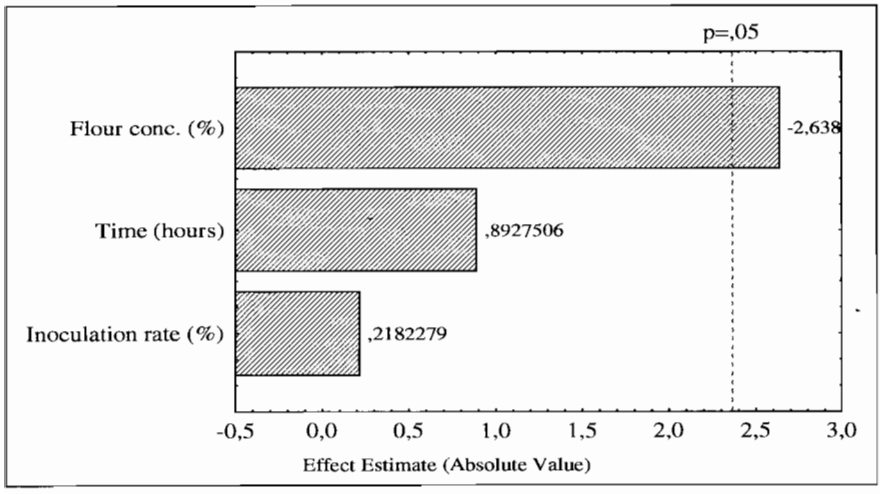


Figure 2: Pareto chart of effects for first optimization for acidity.

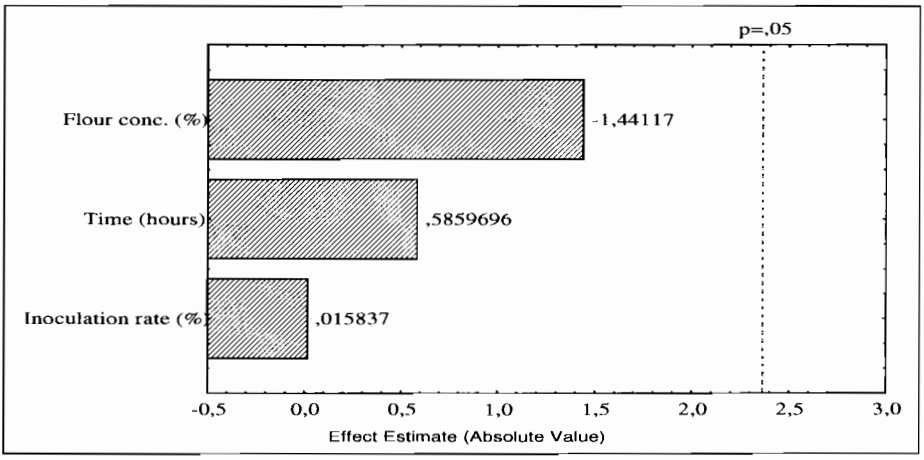


Figure 3. Pareto chart of effects for first optimization for cell concentration.

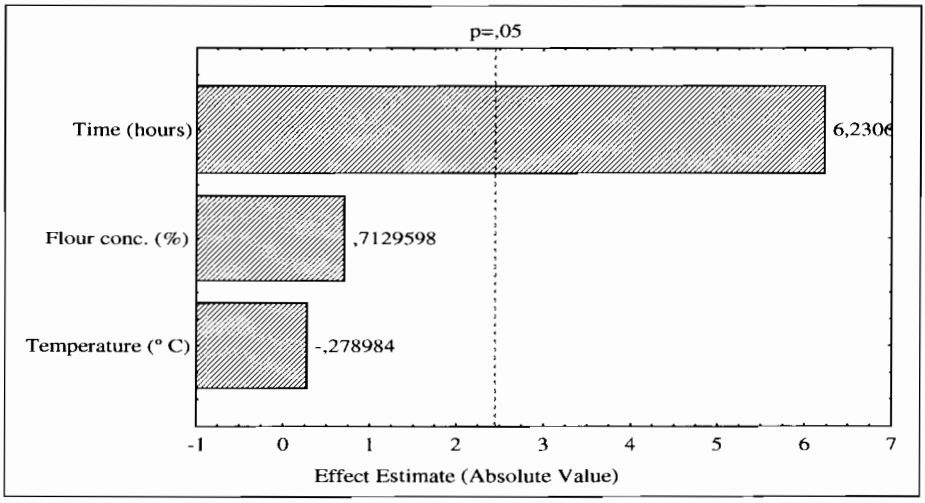


Figure 4. Pareto chart of effects for second optimization for pH variation

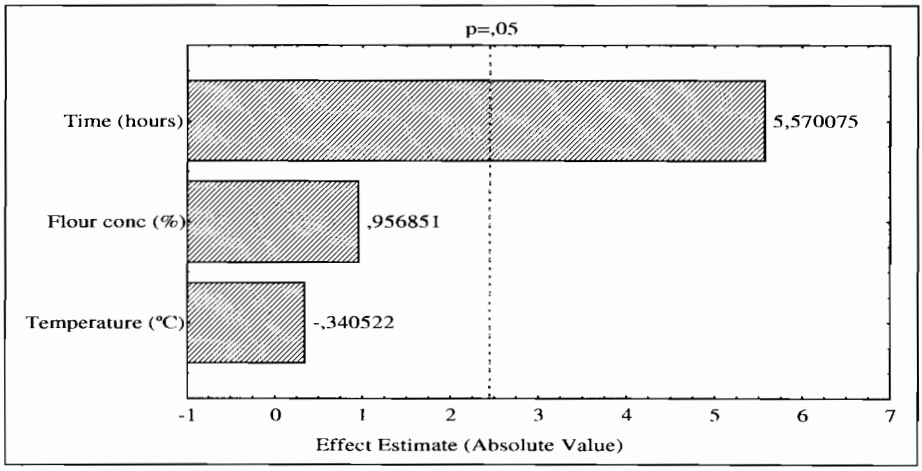


Figure 5. Pareto chart of effects for second optimization for acidity.

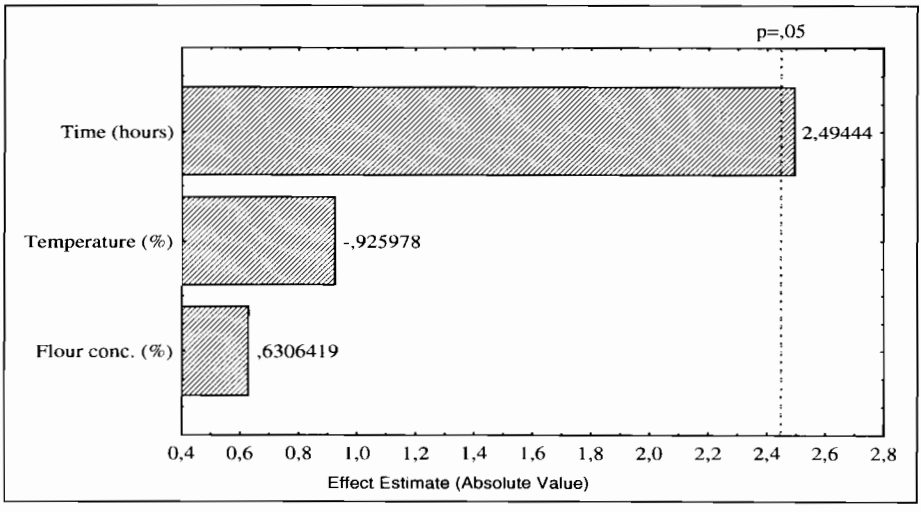


Figure 6. Pareto chart of effects for second optimization for cell concentration.

Fermentation kinetics

A small variation of pH was observed within the first 6 hours of culture, coinciding with moderate growth of the amylolytic cells. This period was an adaptation time of the cells to the substrate (Figures 7, 8 and 9).

After 6 hours of fermentation, an abrupt fall of pH was observed. Cell count as well as titratable acidity increased (Figures. 7, 8 and 9). The fall in pH was then gradual until the end of the 16-hour fermentation (final pH of 3,9 and acidity of 0,776%).

Data regarding lactic acid concentrations were obtained by titration as well as by HPLC analysis. The latter technique gave values that were slightly higher. HPLC analysis of the fermentation products did not result in the appearance of a glucose peak, which would have resulted from starch degradation of the amylolytic strain.

This confirmed earlier data by Giraud *et al.* (**4**) indicating that α-amylase producing bacteria were able to degrade glucose from starch which in turn is rapidly consumed by the bacteria resulting in lactic acid as a final product.

Chromatograms resulting from HPLC analysis of degradation products showed a steady increase in lactic acid over time (Fig 8.).

Reducing sugars, as measured by the Somogyi-Nelson method were abnormally high (50 g/L) (Fig. 9). This may have resulted from the hydrolysis of both starch and lactose. Nevertheless, it can be observed that after 8 hours of fermentation there is an important decrease of total reducing sugars, probable due to cellular growth.

It is also observed that from 8 hours of culture to the end of the fermentation, a significant increase in the amylolytic and probiotic cells counting occurred (arriving at the end with 2,8 x 10^9 CFU/mL for the amylolytic cells and 2,28 x 10^9 CFU/mL for probiotic cells). The amylolytic cell counting was accomplished in MRS agar modified with starch addition, up to 6 hours of fermentation. The probiotic cell counting was carried out in MRS medium (Fig. 10).

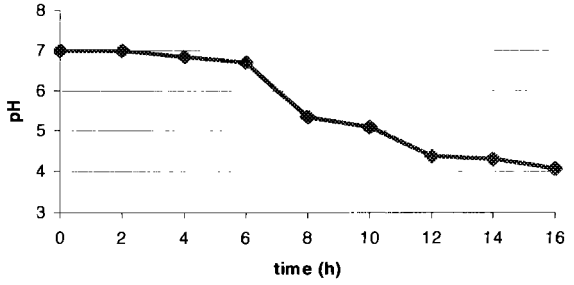


Figure 7. Evolution of pH during 16 h of beverage fermentation.

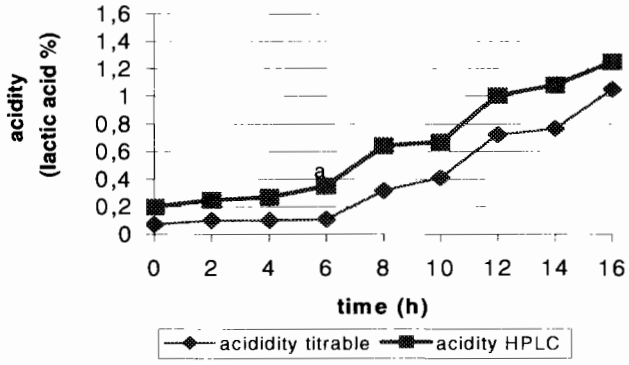


Figure 8. Evolution of the acidity comparing the titratable method and HPLC during the 16 hours of fermentation of the beverage.

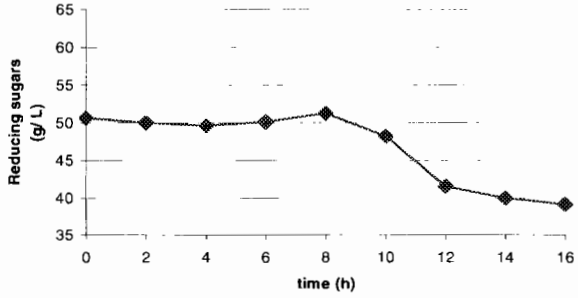


Figure 9. Evolution of the reducing sugars during 8 h of beverage fermentation.

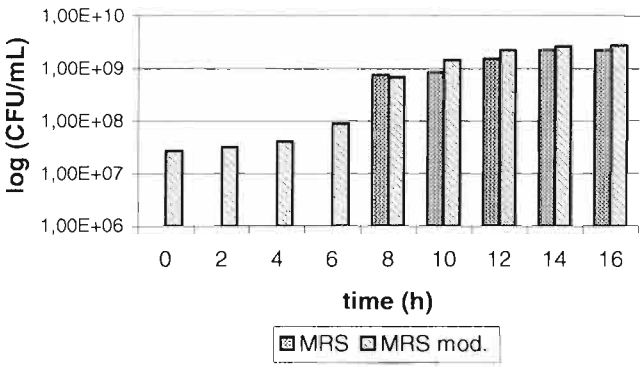


Figure 10. Evolution of viable cells number of *Lactobacillus plantarum* A6 (MRS mod),*Lactobacillus casei* Shirota and *Lactobacillus acidophilus* (MRS) during 16 h of beverage fermentation.

Amylolytic cells were present in higher densities than the probiotic cells. To verify whether the strains that grew in MRS agar weren't amylolytic, certain colonies were isolated and the morphology of the cells were observed by Gram stain (Fig. 10). A defined concentration of cells was added to MRS broth modified with starch. It was observed that the morphology of most of the colonies that grew in MRS agar was that of probiotic strains and that the same ones were not able to grow in modified MRS broth, proving the existence of viable probiotic cells in the fermented product.

Knowing that a good fermented beverage should contain 1x 10^6 to 1x 10^8 culture viable cells, viable cells were counted. For *L. casei* and *L. acidophilus* (probiotic strains) present in fermented beverage after 28 days of storage, cell density was 8,7x 10^8 CFU/mL. Hence, this product is likely to play a beneficial role when consumed.

Conclusions

The optimization demonstrated that the best fermentative parameters were: fermentation time of 16 hours, inoculation rate of 8%, incubation temperature of 35°C and concentration of cassava flour of 20%.

After 16 hours of fermentation the lactic beverage presented a pH of 4.07, titratable acidity in lactic acid of 1,050, 39,2 g/L of reducing sugars, 2,80 x 10^9 CFU/mL of amylolytic lactic bacteria and 2,28 x 10^9 CFU/mL of probiotic bacteria.

The production of lactic acid was accompanied by injection in HPLC, resulting in 1.25% after 16 hours of fermentation. The analysis in HPLC demonstrated that

glucose produced by the microorganism was quickly transformed into lactic acid confirming the data published by Giraud *et al* (**4**).

The lactic beverage maintained its physical, chemical and microbiologic quality after a storage period of 28 days at 4°C, exhibiting desirable cellular count of the used specific cultures.

The elaboration of the lactic beverage using milk whey, cassava flour and probiotic and amylolytic lactic bacteria constitutes an alternative of great economic interest, with low cost of raw materials that are capable of adding nutritional and functional value to the product.

References

1. Otero M., Rodriguez T., Camejo J., Cardoso F. (1995) *Alimentaria.* **260**, 93-95.
2. Gandhi D.N., Patel R.S. (1994) *Cultured Dairy Products Journal*, **29** (1), 25-27.
3. Fuller R. (1989) *Journal of Applied Bacteriology.* **66**, 365-378.
4. Giraud E., Brauman A., Keleke S., Lelong B., Raimbault M. (1991) *Applied Microbiology and Biotechnology.* **36**, 379-383.
5. Somogyi M. (1945) *Journal of Biological Chemistry*, **160**, 61-69.
6. De Man J.C., Rogosa M., Sharpe M.E. (1960) *Journal of Applied Bacteriology.* **23**, 130-135.
7. Macedo R.F., Freitas R.J.S., Pandey A., Soccol C.R. (1999) *Journal of Basic Microbiology.* **39** (4), 243-251.
8. Santos M.C.R. (2001) Desenvolvimento de bebida e farinha láctea fermentada de ação probiótica a base de soro de leite e farinha de mandioca por cultura mista de *Lactobacillus plantarum* A6, *Lactobacillus casei* Shirota e *Lactobacillus acidophilus*. Curitiba,. 105p. Dissertação (Mestrado em tecnologia Química, área de concentração em tecnologia de alimentos) – Setor de tecnologia, universidade Federal do Paraná.

Chapter 32

GENETIC ENGINEERING OF FATTY ACID COMPOSITION OF INDIAN MUSTARD OIL

S.K. GHOSH, A. BHATTACHARJEE, M.K. MAITI, A. BASU, D. GHOSH, S. GHOSH, S.K. SEN

IIT-BREF Biotek, Indian Institute of Technology, Kharagpur- 721 302, India.

Abstract

Seed oils are major components of the human diet. However, these oils often require a modification (hydrogenation process) to increase shelf life and stability. Production of specific fatty acids is enzymatically controlled. Genetic engineering of key elements of the biosynthetic pathway can result in the design of specific fatty acid profiles, with viable commercial applications. One of these is the improvement of Indian mustard oil. To this end, specific thioesterases have been identified, purified and characterized. The corresponding genes have been cloned. Their transgenic expression is presently underway.

Introduction

Vegetable oils or fats contribute significantly to human caloric intake worldwide. Vegetable oil is considered to be one of the plant's most important products with considerable nutritional and industrial utility. However not all of the fats consumed by human beings from this source are safe. Hence, one of the major goals of agricultural biotechnology has been to increase the utility of traditional crops by addition of novel and desirable traits.

Fatty Acid Composition and need for Modification

Vegetable oils or fats which chemically are glycerol esters of fatty acids, viz, triacyl glycerols (TAG) are important agricultural commodities. In fact, fatty acids are the principle components of the dietary fat of human beings. They determine nearly all properties of fat including texture and beneficial and harmful effects. Though a number of different fatty acids are found in plant tri-acyl glycerols, only eight of them are major fatty acids in the economically important vegetable oil, namely: lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), α–linolenic (C18:3) and erucic (C22:1) acids. Indeed these eight account for almost 97% of total fatty acids of seed oil. While fatty acids are undoubtedly important to human beings, we cannot always rely on nature to provide us with the correct intake of this vital nutrient. For example, the essential fatty acid arachidonic acid required for maintenance of normal human physiology

New Horizons in Biotechnology, 365-370, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

and growth, is not synthesized by any higher animal. Similarly, the vegetable oils obtained from seeds of wild plants are not suitable for consumption; they require modification of their fatty acid profile before they are used. In particular, the high level of polyunsaturation in most vegetable oils leads to decreased shelf-life and instability during cooking. Thus nearly 50% of vegetable oils currently produced for commercial food applications are hydrogenated to increase its oxidative stability during storage or frying, or to make the oil solid for edible spreads and margarines. The process of hydrogenation of oil is not only cost intensive but it also creates health hazards due to the formation of trans fatty acid by isomerisation of natural cis fatty acids during catalytic hydrogenation, as the former is energetically favored. Thus, over the last 30 years or so, increasing emphasis has been globally paid on modifying the composition of the major seed oil used for cooking.

The Importance of Acyl-Acyl Carrier Protein Thioesterase

The acyl-acyl carrier protein (ACP) thioesterase is the key determinant of the fatty acid profile of vegetable oils or fats. In plant seeds, the synthesis of fatty acids of up to 18 carbon long chains occurs in undifferentiated plastids. The synthesis is catalysed by a group of enzymatic functions collectively referred to as fatty acid synthase. During synthesis, the growing carbon chain is attached to the acyl carrier protein (ACP) via a thioester linkage. Fatty acid composition of seed oil is primarily determined by the expression of a type of enzyme (acyl-ACP thioesterase) that releases free fatty acids in the plastids depending on enzymes acyl specificity from the fatty acyl ACP thioester. This makes the newly synthesized fatty acyl groups (after conversion to acyl-CoA form) available in cytoplasm of seed cells for the biosynthesis of tri-acyl-glycerols (**1-5**). Thus, the specificities of the thioesterase for different acyl-ACPs influence the ratio of fatty acids produced. Most common oil seeds contain only oleyl (C18:1) preffereing oleyl-ACP thioesterase that makes oleic acid a primary constituent of oil (**6**). Hence, the mechanism by which some plants are able to produce high percentage of a particular fatty acid has been and continues to be an exciting area of investigation. The fatty acyl groups are however, further reduced in the cytoplasm by specific desaturase to linolate (C18:2) and less frequently reduced to linolenate (C18:3) for their deposition in triacyl glycerol, making the oil quite unsaturated. Albeit these oils have been realized to be unsuitable for many types of cooking. Thus, the demand for preferred fatty acid containing edible oil for the consumers remains unfulfilled.

The crucial role of genetic engineering

Plant breeding methods have had some success in addressing the issue of altering the fatty acid profile of vegetable oil. *Brassica napus*, a species of rapeseed the cultivation of which is restricted predominantly to temperate climates, has a very high quantity of erucic acid. The fatty acid profile of the seed oil of this species has been successfully altered (**7,8**) resulting in the high erucic rapeseed HEAR (closest to wild type) and the low erucic rapeseed LEAR (Canola). However, the prospect of

classical breeding in these cases remains limited to the genetic variabilites that are available in the existing genepool. Thus for example it has become impossible to increase the oleic acid content of rapeseed above 80% by breeding methods without obtaining certain undesirable agronomic properties such as reduced cold tolerance. On the other hand with the advent of plant genetic engineering methods there is now a much larger range of possibilities to bring about modifications by introducing genetic traits from any species, including non-plants. Such techniques can also facilitate modification of fatty acids for the production of specific oils in agriculturally and economically attractive crops to a much higher degree than that was previously possible using the traditional techniques of mutagenesis or inter species crossing (**9**). In fact, alteration of the fatty acid composition of seed oil through genetic engineering has now become an experimental reality. With the appropiate genetic elements, it is now possible to redesign the fatty acid profile of either plant membrane or plant storage oils. In the United States (at Calgene) the development of high stearic and high oleic rapeseed (*Brassica napus*) oil with the use of genetical engineering methods have already been implemented. Introduction of a specific ACP thioesterase gene from the California bay tree to low erucic acid containing strain of *Brassica napus* resulted in an up to 50% (by weight) lauric acid content in the seed oil (**10**). This oil type is now in commercial production in North America.

The need for genetic engineering of Mustard oil in India

After years of dependence on imports, India has recently become self sufficient in edible oil and is one of the leading oil seed producing and processing countries in the world. In India the preference for the kind of edible oil consumption has sharp regional preferences. While the area of northern and eastern states have marked preference for the pungent mustard oil, the central, west and southern parts of the country prefer groundnut oil, while some of the coastal parts, coconut oil is preferred. Considering the growing need for the improvement of the productive potential of oil seed crops in India, and in light of the shortcomings of traditional methods of plant breeding mentioned earlier, the importance of genetic engineering techniques needs no emphasis. The analysis of the fatty acid composition of seed oil of Indian mustard (*Brassica juncea*) or Indian rape (*Brassica campestris*) reveal that saturated fatty acids are only 5-6% of total fatty acids, making them unsuitable for edible spreads and margarines. On the other hand, 50% of the fatty acid composition consists of erucic acid. This is unacceptably high, since high erucic acid containing oil tends to accumulate in arterial linings inviting cardiac complications (**11,12**). Not only that but further, the de-oiled cake (DOC) of rapeseed mustard has palatability problems due to the presence of sulphur-based compounds like glucosinolates (**13,14**). Thus, Indian mustard-rapeseed oil contains major nutritional shortcomings. As the result of this, a pressure of demand in the market for groundnut oil and DOC in the absence of the availability of acceptable mustard oil or DOC is always present. Since a consistent productivity of groundnut crop yield every year cannot be ensured, the situation leads to serious problems.

A step taken to improve mustard oil in India

A potential approach to improve the nutritional quality of Indian mustard-rape seed oil would be to genetically alter the fatty acid composition of the seed oil by introducing novel acyl ACP thioesterase gene(s) in order to increase the level of medium (C16:0) to long (C18:0) saturated fatty acid level, while synergistically decreasing the nutritionally harmful (C20:1) and (C22:1) fatty acid levels. To fulfill this experimental requirement, considerable progress has been made in this laboratory to identify and characterise certain targeted acyl-ACP thioesterase genes(s) and their products that are responsible for fatty acid chain elongation processes of seed oil synthesis in certain non-conventional tropical plants. The rationale behind transgenic use of these genetic elements has been to provide new sources of genes which otherwise are absent in mustard/rape.

Bassia Species as a potential source of useful genes for fatty acid modification

Searching in a number of wild tropical non-conventional oil seed plants, we have narrowed down a few plants of which two species of *Bassia* figure prominently. Two highly active fatty acyl-ACP thioesterases (Fats) have been identified from these plants.The enzymes in the immature seed extracts show novel substrate specificity for the fatty acyl group; one (SOFAT) with nearly equal substrate specificity for oleoyl-ACP & stearoyl-ACP; and the other (POFAT) with almost equal specificity for palmitoyl & oleoyl-ACP (**15**).

It is known that plants whose seeds accumulate saturated medium chain rich oil induce at least one medium chain specific thioesterase of FatB class. To find any long chain saturated fatty acyl specific thioesterase in making C16:0 or C18:0 rich seed oil has not been easy (**6,16,17**). Moreover, the demonstration of the presence of a palmitoyl preferring acyl-ACP thioesterase in certain seeds has not concomitantly shown a high yield of high-palmitate seed oil (**18-20**). Hence has not yet been utilized to generate high palimitate seed fat (**18,19**). In fact, an oleoyl-preffering LC-Fat of Fat A class (at least partially) has been utilized for the production of high stearate seed oil in mangosteen, *Garcinia mangostana* (**21**).

In our studies, purification of thioesterase (TE) present in *Bassia* species revealed the existence of high level of TE (SOFAT and POFAT) at all developmental stages. Our observations on substrate specificities of SOFAT and POFAT have not been in conformity with the Fat A-Fat B model proposed by Jones *et. al* (**6**), in spite of the fact that the cDNA sequences of these two thioesterases possess more similarities to the Fat B type than Fat A. We believe that not only has the Fat A type gene evolved from the same primordial Fat B type, but that the SOFAT and the POFAT genes have quite likely also evolved from the same progenitor. We found only one isoform of each of the SOFAT and POFAT genes, and we also found that in the *Bassia* species these two Fat B type genes prematurely release saturated fatty acids into the cytoplasm of embryonic cells endowing these tropical plants with an unusual capacity to synthesize saturated fatty acid (C16:0 and C18:0) rich seed oil.

The seed fats of these two plants thus have unique characteristics and their fatty acid compositions are ideal for preparing cocoa butter and/or margarine. It is known that production of shortenings, margarines and confectionary grade items requires large amounts of stearate and palmitate. Currently, industries manufacturing such products rely on stearate and palmitate produced from partially hydrogenated oils. Most cultivated Indian conventional oil seed crops do not naturally produce abundant amounts of stearate and palmitate. The genes corresponding to SOFAT and POFAT identified through the present study, when expressed trangenically in conventional Indian oil seed plants so as to prematurely release C18:0 or C16:0 from plastid into cytoplasm, would enable the developing seeds to synthesise high stearate or high palmitate fat as desired.

Conclusion

Keeping the above in view, we believe our attempts to genetically improve the Indian mustard-rape oil seed plants with the thioesterase gene(s) from *Bassia* species hold the promise of developing mustard oil for better nutrition.

References

1. Batley J.F., Schimid K.M., Ohlrogge J.B. (1989) *Trends Biotechnol.* **7**: 122
2. Harwood J.L. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**:101
3. Joyard J., Douce R. (1987) *In* Biochemistry of Plants, P.K. Stumph Ed. (Accademic Press,Orlando, F.L.) Vol. **9**, p.215
4. Murphy D.J. (1993) *Prog. Lipid Res.* **32**:247
5. Ohlrogge J.B, Browse J., Sommerville C.R. (1991) *Biochem. Biophys. Acta* **1082**: 1
6. Jones A, Davies H.M, Voelker T.A (1995) *Plant Cell.* **7**: 359-371
7. Downey R.K. (1964) *Can. J. Plant. Sci.* **44**: 499-504
8. Stefansson B.R., Hougen F.W., Downey R.K. (1961) *Can.J.Plant.Sc.***41**: 218
9. Töpfer R., Martini N., Schell J. (1995) *Science.* **268**: 681-686
10. Del Vechino A.J. (1996) *International News on Fats, oils and Related Materials* **7**: 230-243
11. Katan M.B, Zock P.L., Mensink R.P. (1995) *Annu. Rev. Nutr.* **15**: 473-493
12. Nelson G. (1998) *Nutr. Rev.* **56**: 250-252
13. Vaughan J.G., Gordon E.I. (1973) *Ann. Bot.* **37**: 167-184
14. Vaughan J.G., Hemingway J.S., Schofield H.J. (1963) *J. Linn. Soc. Lond. Bot.* **58**: 435-447
15. Bhattacharjee A. (1998) Ph. D Thesis Calcutta University, India
16. Dörmann P., Voelker T.A., Ohlrogge J.B. (1995) *Arch. Biochem. Biophys.* **316**: 612-618
17. Leonard J.M., Slabaugh M.B, Knapp S.J (1997) *Plant Mol. Biol.* **34**: 669-679
18. Martinez-Force E., Cantisan S., Serrano-Vega M.J., Garces R. (2000) *Planta* **211**: 673-678
19. Pritle R.M., Yoder D.W., Iluynh T.T., Nampaisansuk M., Pritle I.L., Chapman K.D. (1999) *Plant cell Physiol.* **40**(2): 155-163
20. Yoder D.W., Nampaisansuk M., Pritle I.L., Chapman K.D., Pritle R.M (1999) *Biochim Biophys Acta.* **1446** (3): 403-413

21. Hawkins D.J., Kridl J.C. (1998) *Plant J.* **13**: 743-754

22. Loof B., Appleqvist. L (1972). *In* Rapeseed, Ed. L. Appleqvistand R. Ohlson, Amsterdam :Elsevier p.101-122.

Chapter 33

PLEUROTUS OSTREATUS VOLATILE AROMA COMPOUNDS IDENTIFIED FROM FRUIT-BODY AND FROM MYCELIUM GROWN IN SUBMERGED AND SOLID-STATE CULTURES

W. KABBAJ[1], S. BREHERET[2], J. GUIMBERTEAU[3], T. TALOU[2], J.M. OLIVIER[3], M. SOBAL[1], M. BENSOUSSAN[4], S. ROUSSOS[1]

[1] *Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119; IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925; 163 Avenue de Luminy; F-13288 Marseille Cedex 9; France; Email : roussos@esil.univ-mrs.fr ;* [2] *Laboratoire de Chimie Agroindustrielle, I.N.P. de Toulouse, 31077 Toulouse, France* [3] *Station de Recherche des Champignons INRA, BP. 81, 33883 Villenave d'Ornon cedex, France ;* [4] *Laboratoire de Microbiologie-UMR-INRA 1082, Université de Bourgogne- ENSBANA, 21000 Dijon, France*

Abstract

Comparative analyses of volatile aroma compounds of the *Pleurotus ostreatus* JMO.95 fruit-body and its corresponding mycelium grown in liquid, on agar surface, or on solid support cultures have been carried out by dynamic headspace concentration using GC/MS and GC/sniffing. The aroma of the fruit-body was due essentially to the presence of octan-3-one and, in a lesser extent, to the presence of octan-3-ol. Other compounds, such as oct-1-en-3-ol, oct-1-en, 2-methylbutanol and α-pinene were also present in low concentrations. The comparison of the aromatic spectra of the fruit-body with the aromatic spectra of mycelia obtained under different culture conditions indicated that the main aromatic compounds present in the *P. ostreatus* fruit-body were also produced by the mycelium and generally in the same proportions, except for culture under submerged conditions.

Key words: *Pleurotus ostreatus*, mushroom fruit-body, mycelium, oct-1-en-3-ol, aroma, headspace concentration, GC/MS, GC/sniffing.

Introduction

Edible mushroom cultivation has greatly increased worldwide in the last decade (**1**). This increase in production has resulted from the interesting flavour characteristics of mushrooms and improvements in their growth processes. Mushrooms such as *Agaricus bisporus, Pleurotus ostreatus* and *Lentinus edodes* are currently cultivated on industrial scale according to well-defined processes. *P. ostreatus* is the second most important mushroom produced in Europe, after *A. bisporus*. The fructification of *P. ostreatus* is simpler than that for *A. bisporus* utilising less elaborated compost over a shorter incubation period (**2**).

New Horizons in Biotechnology, 371-377, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Several papers describing and comparing mushroom flavour compounds produced by the fruit-bodies andby the mycelial biomass exhibiting aromatic properties have been published. Most of them were in relation to the genera *Agaricus*, *Morchella* and *Pleurotus* (**3-9**). Other studies which have focused mainly on widespread species such as *Agaricus bisporus* (**10-14**) revealed the importance of the eight carbon atom compounds (C8) series and notably one molecule: oct-1-en-3-ol. These compounds play a major role in the development of the mushroom's aromatic properties and could represent up to 90% (w:w) of the volatile fraction issued from different fresh fruit-bodies (**11,15,16**). Pyysalo & Suihko (**11**) have shown that the aromatic specificity of seven edible fruit-bodies can be mainly explained by variations in the relative concentrations of these C8 molecules.

Little information is presently available on the aroma composition of both the fruit-body of *P. ostreatus* and its mycelium. The *P. ostreatus* strain JMO 95, which has the ability to fructify in controlled conditions, was chosen for its mycelial vigour and its strong aromatic note.

The aim of the present work was to compare, by means of headspace concentration and GC/SM identification, the volatile compounds exhaled from the *Pleurotus* sporocarpe and from its corresponding mycelium grown in agar surface, in liquid medium and on solid support impregnated with a nutritive solution.

Materials and Methods

Material and maintenance.

P. ostreatus (Jacq.:Fr) Kummer, strain INRA-JMO.95 was isolated in 1995 from dead elm trees in Brittany (France). It is characterised by small caps, a still grey colour, a very short stipe and an abundant fructification with well-isolated fruit-bodies. It was selected among other *Pleurotus* for its fragrant potential. The mycelium was maintained on Potato Dextrose Agar (PDA, Difco) before inoculation. Fruit-bodies and mycelial fractions were treated in duplicate. All mycelial fractions recovered from liquid, surface or solid-state cultures were frozen until chromatographic analysis was performed.

Submerged culture

Mycelial colonies incubated for 5 to 7 days on Raper solid medium (**17**) and having a diameter up to 3 or 4 cm in Petri dishes were carved in aseptic conditions, pounded in a Polytron homogenizer at high speed, in fresh sterile Raper solid medium and cooled on ice. 5 ml of this mycelial suspension were transferred to a 250 ml Erlenmeyer containing 100 ml of liquid Raper medium and incubated at 24-25°C on a shaker with intermittent orbital agitation (100 rpm) with 48 hours periods.

Surface culture.

Cultures of *P. ostreatus* JMO.95 mycelium were grown on agar Raper medium (1.5%).

Solid support culture

The culture system was composed of sugarcane bagasse as an inert support impregnated with the nutritive solution with up to 78% of initial humidity (**18**). The composition of the nutritive solution was (in $g.L^{-1}$): glucose, 30.0; urea, 2.68; yeast extract, 6.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 2.0; $NaH_2PO_4.H_2O$, 1.8. Impregnated sugarcane bagasse fractions (35 g) were introduced in 250 ml flasks and sterilised at 120°C for 20 minutes. Inoculation was carried out with 3 pieces of 1 cm^2 of mycelial colonies of *P. ostreatus* JMO.95 cultivated for 10 days on PDA at 25°C. These inoculum pieces were equally distributed into the flask. Cultures were incubated at 25°C for 14 days without forced aeration.

Culture and fruiting on lignocellulosic substrates

The substrate used for *P. ostreatus* JMO.95 cultivation consisted of pasteurised wheat straw, according to Laborde (1987). Spawn was prepared on rye grains cooked and autoclaved. The spawning rate was 3% (w:w). Each culture unit consisted of a plastic bag filled with 5 Kg of spawned substrate. Spawn running conditions for 16 days were, 25°C, no light, no fresh air and 80% for the relative air humidity. Fruiting was induced by decreasing the temperature to 14-15°C, supplying fresh air at 150 $m^3 h^{-1}$, and 12 hours of cycled illumination a day (by day light lamps) and increasing air humidity up to 92%. First fruit-bodies were picked 16 days after incubation and the fructification continued over a 4-month period with a weak flush effect. Fruit-bodies used for aroma analysis were collected twice during the first flush, early stage (with a rolled margin) and 2 days later (with a flat margin).

Dynamic headspace concentration

Fruit-body or mycelial samples (on an average of 20 g) were placed in a glass cell (0.25 L capacity) directly connected to a dynamic headspace concentrator (CHISA device, SGE). Volatile compounds were concentrated on a TENAX trap with a stripping gas (helium) flow rate of 30 $mL.min^{-1}$ for 20 min at room temperature. Samples were desorbed with a headspace injector (CHISA device, SGE) directly connected to the analytical column. The temperature for desorption was 210°C and volatile compounds were cryofocused at -20°C in the column's head before being injected directly into the column (**19**).

Gas chromatography / Mass spectrometry

Analyses were carried out using a gas chromatograph (5890, Hewlett-Packard) and a mass selective detector (5971, Hewlett-Packard) with a potential of 70 eV for ionisation by electron impact. Headspace analyses were performed by a 50 m x 0.22 mm x 1µm dimethylpolysiloxane BP1 (SGE) fused silica capillary column. The

pressure of the carrier gas (helium) was fixed at 22 psi. The injector and detector temperatures were 210°C and 250°C, respectively. The temperature of the oven was programmed as follows: 50°C to 220°C (3° C min^{-1}).

Gas-chromatography / sniffing

Headspace analysis was carried out by a purge and trap injector (DCI device, DELSI Instruments) connected to a gas chromatograph (DELSI 30) and performed with a 50 m x 0.32 mm x 1μm dimethylpolysiloxane SBP1 fused silica capillary column. Pressure of carrier gas (helium) was 14.5 psi. The detector temperature was 230°C and the temperature of the oven was programmed from 50°C to 220°C (3°C min^{-1}). Temperatures of the trap system for concentration and desorption were -20°C and 250°C respectively (**19**). Odour profile description was obtained using sniffing-port (olfactory detector, SGE) with a ratio FID 30% / Sniffing, 70%. GC/sniffing was performed by three persons using the olfactory referential "Le champ des odeurs" (**20,21**).

All compounds were identified by comparison with the mass spectral library NBS (**22**), literature spectra (**23-25**), Kovats indices data from the literature (**24,25**) and from our own data bank. The Kovats indices were calculated using n-alcanes (C_5-C_{18}); for the headspace technique, 1 μL of the mix was deposited in the glass cell and analysis was carried out as previously described.

Results and Discussion

Direct headspace analysis allowed trapping of the most volatile compounds present in the gaseous state in the closed atmosphere of the mushroom. This analysis reflected an image of the aroma perceived by the human olfactory system (**19**).

The identification of aroma compounds of *P. ostreatus* JMO.95 fruit-bodies collected twice during the first flush (Table 1), demonstrated the presence of octan-3-one as the major volatile molecules, representing 80% of the total GC/MS integrated area. This compound, whose odour was described as sweet (**10**), fruity or mildewy at high concentration (**11**), is responsible for the fruity lemon-like odour of *P. ostreatus*. Octan-3-ol, whose concentration corresponded to an average of 14.3% of the integrated area, participated in the hazelnut and herbaceous sweet note. Nevertheless, the oct-1-en-3-ol, generally described as the key compound of the fresh mushroom aroma, was found to constitute only a very low proportion in the fruit-body profile. The absence of other molecules having eight carbon atoms such as oct-1-en-3-one, oct-2-en-1-ol and octan-1-ol was noted. Furthermore, the composition of the aroma of the *P. ostreatus* fruit-body during the mature stage changed slightly with respect to the proportions of oct-1-en-3-ol and benzilic acid (increase) and of 2-methylbutanol and 2-methylbutanal (decrease).

The *P. ostreatus* mycelium grown in liquid medium, on agar surface, or on solid support cultures, were shown to be able to produce most of the aroma compounds

involved in fruit-body aroma. However, the proportions between these different molecules changed significantly according to the conditions used (Table 1).

Mycelium from solid-state culture showed the highest diversity of aroma compounds, even more than the fruit-body. Nevertheless, the olfactory impact corresponded to the characteristic *Pleurotus* aroma. Thus, oct-1-en-3-one appeared to be responsible for the cooked mushroom note, octanal for a honey-orange like odour and octanol for an orange-rose like odour. Traces of 1,3-octadiene were also present. This compound, having a fruity-green odour, was rarely found among C8 molecules that constituted the typical mushroom aroma. A great similarity in the proportions of octan-3-one and octan-3-ol was observed between aromatic profiles issued from the fruit-body and the mycelium cultivated on sugarcane bagasse impregnated with a nutritive solution.

Table 1. Headspace analysis of aroma compounds from the fruit-body and the mycelium of *P. ostreatus* grown in liquid and solid-state conditions.

Identified a compounds	Kovats indices	Fruit-Body (3 days storage)		Mycelium culture			Odour notes on the sniffing port
		Young	Mature	Solid support (16 days)	Agar surface (21 days)	Liquid medium (21 days)	
2-methylbutanal	639	b 0,2	-	-	-	-	-
1-heptene	683	-	-	0,06	-	-	-
3-methylbutanol	716	-	-	-	-	5,4	-
2-methylbutanol	721	4,1	1,6	9	-	16,2	-
pentan-1-ol	747	-	-	-	0,5	-	-
1-hexanal	777	-	-	2		-	green
oct-1-ene	787	0,9	0,8	3,9	0,1	-	fruity - etherous
*(E)*1,3-octadiene	817	-	-	0,7	-	-	-
*(Z)*1,3-octadiene	819	-	-	0,7	-	-	-
benzaldehyde	939	-	-		1,9	-	mild spicey
a-pinenc	940	-	traces	0,01	-	-	piney
oct-2-en-3-one	957	-	-	3,9	traces	-	fungal
oct-1-en-3-ol	964	0,6	1,6	0,3	1	38,5	fungal
octan-3-one	968	79,9	80,2	72,5	67,4	36,2	fungal - citrus
octan-3-ol	980	14,8	13,8	11,3	26,5	1,7	fungal
octan-1-al	983	-	-	0,4	-	-	orange, honeyed
benzilic acid	1047	traces	0,6	-	-	-	amine like
oct-2-en-1-ol	1050	-	-	1,3	traces	-	orange - rose like
octan-1-ol	1052	-	-	0,04	0,7	-	amine like
anisaldehyde	1234	-	-	-	0,9	-	anised

[a] The identifications of fragmentation spectra from GC-MS analysis were based on Hewlett Packard NBS data.

[b] Relative percentage of the volatile compounds based on the total integrated chromatographic area

On solid agar surface, the aromatic profile of the mycelium resembled that of the fruit-body showing presence of several C8 molecules (Table 1). In this case, the relative proportion of octan-3-ol was the highest comparatively to the solid support and the fruit-body profiles. In liquid culture, the volatile fraction of *P. ostreatus*

mycelium was characterised by the predominance of oct-1-en-3-ol and of octan-3-one (Table 1). Meanwhile, the proportion of octan-3-ol was very low. 2-methylbutanol, found at a relatively high concentration (16.2%), brought a characteristic spicy and repellent odour. These variations in the composition of the aromatic fraction of the mycelium, as compared to these of *P. ostreatus* JMO.95 fruit-body, explain the differences observed in the final aroma. Furthermore, the aromatic intensity perceived for liquid cultures was very low compared to that of fruit-body.

It can be concluded that the composition of the aroma produced by *P. ostreatus* JMO.95 mycelium is directly dependent on the type of culture. Various aromatic profiles were produced under liquid, surface and solid-state culture conditions, but the best aromatic similarities with the fruit-bodies of *P. ostreatus* JMO.95 were obtained with the mycelium grown on sugarcane bagasse impregnated with nutritive solution. Furthermore, the difference noticed between the aromatic spectra of the fruit-bodies and those of the mycelia, developed on agar surface or issued from liquid culture, both obtained with the same culture medium composition (except agar) and environmental conditions, could be explained by the mode of growth.

In liquid culture, mycelial growth involved pellet formation. The microorganism was probably under stress culture conditions, particularly with the low availability of dissolved oxygen. In solid-state conditions, agar surface and solid support, the growth was apical and mycelium development was related with the natural hyphal growth.

The relatively long cycle of fructification, as well as the output of fruit-body production, constituted major disadvantages for *P. ostreatus* industrial aroma production. The production of *Pleurotus* aroma by the mycelium of the selected *P. ostreatus* JMO.95 growing on solid support, which occurred in a shorter period of time and at lesser costs, would be considered as an excellent alternative.

Acknowledgements

Wafâa KABBAJ is grateful to IRD (ex-ORSTOM, France) for partial financial support and scholarship. Authors thank Dr. T.C. Varzakas for helpful corrections in English. This work was funded by the French Ministry of Research N° 94 G 0086 (Aliment 2.002).

References

1. Chang S.T., Miles P.G. (1991) *Mushrooms J.* **504**, 15-15.
2. Laborde J. (1987) P.H.M.- *Revue Horticole* **278**, 13-19.
3. Humfeld H., Sugihara F. (1952) *Mycologia* **44**, 605-620.
4. Litchfield J.H. (1967) *Biothechnol. Bioeng.* **9**, 289-304.
5. Chahal D.S., Khan S.M. (1991) *In* Science and cultivation of edible fungi, (ed. Maher), Balkema, Rotterdam, pp. 709-716.

6. Hadar Y., Cohen-Arazi E. (1986) *Appl. Environ. Microbiol.* **51**, 1352-1354.
7. Hadar Y., Dosoretz C.G. (1991) *Trends Food Sci. and Technol.* **2**, 214-218.
8. Bensoussan M., Tisserand E., Kabbaj W., Roussos S. (1995) *Crypt. Mycol.*, **16**:65-75.
9. Kabbaj W., Bensoussan M., Roussos S. (1997). *In* Advances in Solid State Fermentation (eds. S. Roussos, B.K. Lonsane, M. Raimbault and G. Viniegra-Gonzalez), pp 437-448. Kluwer, Dordrecht.
10. Cronin D.A., Ward M.K. (1971) *J. Sci. Food Agric.* **22**, 477-479.
11. Pyysalo M., Suihko M. (1976) *Lebensm.-Wiss. u.-Technol.* **9**, 371-373.
12. Buchbauer G., Jirovetz L., Wasicky M., Nikiforov A. (1993) *Z. Lebensm. Unters. Forsch.* **197**, 429-433.
13. Grove J.H. (1981) *Phytochem.* **20**, 2021-2022.
14. Fisher K.H., Grosch W. (1987) *Lebensm.-Wiss. u.-Technol.* **20**, 233-236.
15. Latrasse A., Degorce-Dumas J.R., Leveau J.Y (1985) *Sciences des Aliments* **5**, 1-26.
16. Vidal J.P., Toulemonde B., Richard H. (1986) *Lebensm.-Wiss. u.-Technol.* **19**, 353-359.
17. Raper C.A., Raper J.R. (1972) *Mycologia* **64**, 1088-1093.
18. Roussos S., Bresson E., Saucedo-Castañeda G., Martinez P., Olivier J.M., Guimberteau J. (1997) *In* Advances in Solid State Fermentation (eds. S. Roussos, B.K. Lonsane, M. Raimbault and G. Viniegra-Gonzalez), pp 483-500. Kluwer: Dordrecht.
19. Talou T., Roule K., Gaset A. (1996) *Arômes Ingrédients Additifs* **3**, 34-36.
20. Jaubert J.N., Gordon G., Dore J.C. (1987) *Parfums, Cosmet., Aromes*: **78**, 71-82.
21. Jaubert J.N., Tapiero G., Dore J.C. (1995) *Perfum. Flavor.* **20**, 1-16.
22. McLafferty F.W., Stauffer D.B. (1989) The Wiley NBS Registry of Mass Spectral Data. Wiley J. & Sons : New York, USA.
23. Stenhagen E., Abrahamson S., McLafferty F.W. (1976) Registry of Mass Spectral Data. Wiley J. & Sons: New York, USA.
24. Jennings W., Shibamoto T. (1980) Qualitative analysis of flavor and fragance volatiles by glass capillar gas chromatography. Academic Press : New-York, USA.
25. Adams R.P. (1989) Identification of essential oils by ion trap Mass Spectroscopy; Academic Press, Inc.: San Diego, California, USA.

Chapter 34

STANDARDIZATION OF ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY) AND INDIRECT FLUORESCENT ANTIBODY TEST (IFAT) TECHNIQUES FOR CANINE CUTANEOUS LEISHMANIASIS

E.I. CASTRO[1], V. THOMAZ-SOCCOL[1], C. AUGUR[2]

[1]*Universidade Federal do Paraná. Setor de Ciências Biológicas. Departamento de Patologia Básica. Laboratório de Parasitologia Molecular. Curitiba. Paraná, Brasil.*
[2]*Equipe de Mycologie et de Fermentation en Milieu Solide IRD UR-119; IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, Case 925; 163 Avenue de Luminy; F-13288 Marseille Cedex 9; France*

Abstract

In the present work we have standardized two methods to research anti *Leishmania* antibodies in canine cutaneous leishmaniasis. The two techniques, ELISA and IFAT were compared with respect to co-positivity and co-negativity. A strain of *Leishmania (V.) braziliensis* (MHOM/BR/95/CUR6) was used as antigen in this study. For the ELISA technique a soluble antigen was prepared, while in IFAT test, entire promastigotes were used. By ELISA technique, the protein concentration found was 6 mg/ml with antigen concentration at 250ng/well. Serum and conjugate dilution were 1:160 and 1:1000, respectively. Results were considered positive when the absorbance value was more than 0,135 using 2SD (standard deviations) and 0,178 using 3SD. 371 dogs coming from two foci of cutaneous leishmaniasis were examined, 159 dogs from Municipality of Adrianópolis and 212 dogs from Municipalities in North of Paraná State. Leishmanial antibodies were detected by ELISA test in 81 dogs (21,83%) with values of 0,135 or over (2 SD) and in 49 dogs (13,20%) with values of 0,178 or over (3 SD). By IFAT, 22 dogs were found positive (5,9%) with dilution values of 1:40. when data was compared between ELISA and IFAT, 20 dogs were sera positive by ELISA and IFAT, with percentage of concordance between these techniques of 24,6% with 2SD and 40,31% with 3SD. Among eight parasite-positive dogs, seven (87,5%), presented sera-positives for ELISA techniques, while by IFAT technique, six dogs (75%) were positive. One dog (12,5%) was negative in both tests, probably because the lesion was recent. The ELISA technique when compared with IFAT technique presented more sensitivity, but IFAT seemed more specific in detecting leishmanial antibodies.

Index Entries: Standardization, ELISA, IFAT, Canine Cutaneous Leishmaniasis.

New Horizons in Biotechnology, 379-385, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

The genera *Leishmania*, in the New World, occurs from the peninsula of Yucatan, in Mexico, down to the north of Argentina, distributed amongst 17 taxa, and causing diverse clinical effects. Of these, ten taxa are said to parasite humans (**1**). The great majority already have a known epidemiological profile, or, its reservoirs and vectors have already been determined (**1-3**). However, *Leishmania (Viannia) braziliensis* still does not present a definite epidemiological profile. The wild reservoirs of this parasite are, for most, unknown. In most cases, when an animal species is depicted as part of the reservoir of *L. (V.) braziliensis*, the strain of the protozoa was neither isolated nor identified, raising doubts on the true role of this animal species in the maintenance of the parasitic cycle.

In the Northeast and Southeastern regions, endemic of cutaneous leishmaniasis, the dog has been implicated as domestic reservoir of this illness, where infections in human beings are predominantly caused by *Leishmania (V.) braziliensis*. Up to the moment, no wild animal was found parasitized by this species (**4-11**).

In the state of Paraná, despite the epidemic character of cutaneous leishmaniasis, in the north and the Northwest regions since the end of the 80's, no study, to date, has been able to determine the true reservoirs of the parasite. The present study describes a seroepidemiologic study of dogs carried out in two regions of Paraná where cutaneous leishmaniasis is endemic.

To study the role of the dog in the epidemiological chain a methodology is needed, capable of detecting the presence of the parasite or its anti-*Leishmania* antibodies. Antibody detection in canine cutaneous leishmaniasis can be carried out through many serological techniques. Each serological technique has its own advantages and disadvantages. We have therefore standardized two serological techniques to evaluate which presented better results in order to study the role of the dog in *Leishmania* foci of the Paraná State in Brazil.

Materials and Methods

Indirect Fluorescent Antibody Test (IFAT)

Promastigotes of *Leishmania (V.) braziliensis* (MHOM/BR/95/Cur6) were used as antigen after 7 days of culture in NNN (McNeal, Novy, and Nicolle) medium. The promastigotes were washed in phosphate buffered saline (PBS) pH 7,2. The pellet was resuspended in 2% formalin in saline solution at a final concentration of 10^6 parasites/ml. This suspension was diluted with PBS so as to give 40-50 parasites per microscopic field (400x). The suspension of promastigotes were spread on slides and allowed to dry thoroughly and fixed with acetone. Each serum dilution was allotted on the slide. Tests were done at 37°C in a wet chamber for one hour. After incubation, the slides were washed, with two changes of PBS, 10 minutes each, and dried for a few minutes under a ventilator. The conjugate dilution was made in PBS containing Evans blue (10 mg per 1.000mL) and then pipetted on each area (spots)

and incubated and washed again. The slide was then mounted with a cover slip and buffered glycerine. The reactions were judged under a 40X objective in a binocular microscopic provided with HBO 200 light source and BG 12 as exciter filter. The dilutions of antigen, serum samples and conjugate were used after standardization.

Standardization of IFAT technique

Progressive dilutions of a positive and a negative serum (1:20 to 1:1280), were reacted a conjugate in progressive dilutions (1:20 to 1:100) in five slides, as well as a control incubation buffer. Titres were expressed as the reciprocal of the highest serum dilution yielding positive promastigote fluorescence.

Enzyme-linked Immunosorbent Assay (ELISA)

The antigen was prepared with promastigotes of *Leishmania (V.) braziliensis* cultured in NNN medium. The organisms were harvested after 7 days, washed three times with phosphate buffered saline (PBS) pH 7,2 by centrifugation, then were lysed by various cycles of freezing and thawing. The suspension was sonicated and the solid debris removed by centrifugation at 10000 rpm, for 30min, 4°C. The supernatant fluid representing the concentrated soluble antigen was dispensed into small aliquots (1ml) and stored at -20°C until required. ELISA was carried out according to Engvall and Perlamann, (**12**) adapted for polystyrene microtiter plate. The dilutions of antigen, serum samples and conjugate were standardized. At the end of the test, after the addition of substrate, the reaction was stopped and the absorbance was read with a spectrophotometer at 492nm.

Standardization of ELISA technique

The working strengths of the antigen preparation and that of the serum were determined by checkerboard titration. On a test plate, the antigen at different concentrations (500 ng/well to 62,5 ng/well) was reacted upon, using increasing dilutions of a positive and a negative serum (1:40 to 1:640), and a conjugate in increasing dilutions (1:500 to 1:4000) as well as a control incubation buffer. The ideal antigen/serum/conjugate combination, giving the best differentiation between negative and positive without background, was thus chosen and used throughout the test. Although the advantage of this standardization is to be able to test a single dilution for each serum tested, in order to eliminate possible aberrant results, all the tests were repeated at least twice.

Statistical analysis

The techniques of ELISA and IFI were compared with the purpose of evaluation of the indexes of Co positivity (CP), Co negativity (CN), Value of Concordance (C), Value of Positivity Prediction (VPP) and Value of Negativity Prediction (VNP) obtained through the standardization of the technique of indirect ELISA, following the norms proposed by Guimarães *et al.* (**13**). For these evaluations the results of the analysis of 371 serums of dogs were compared using the results obtained by the

IFI method as standard method and comparing with those obtained by the ELISA method.

Positive and negative results were established based on one serological method considered standard for the Leishmaniasis (IFAT) and not due to isolation of the parasite. Co-positivity was calculated by dividing the positive to both tests by the sum of the positive ones to both tests and negative to the test that is measured even if it is positive to the reference test. In the same way, co-negativity was obtained by dividing the negative serums from both tests by the sum of the negatives for both tests and the positive serums for the new test even if it is negative as compared to the reference test (**13**).

It was possible to calculate the true sensitivity using both tests, only for eight animals from which isolation was possible.

Results

Standardization of IFAT technique

Conjugate titres were expressed as the reciprocal of the highest serum dilution yielding positive promastigote fluorescence of 1:40. The dilutions of sera at 1:40 or more, with outline show as green fluorescence, were considered positive. The positive and negative sera at the 1:40 dilution were on all slides, for comparison, when reading the results.

Standardization of ELISA technique

The protein concentration was set at 6 mg/ml and the ideal antigen concentration at 250ng/well. The serum dilution was 1:160 and conjugate dilution was 1:1000.

Cut-off of ELISA technique

The range of ELISA values in 93 dogs originating from indigenous areas (Municipality of Curitiba, Paraná) were 0,005 to 0,189 and only three animals had ELISA values over 0.100. The cut-off value was considered the mean plus two standard deviations (2SD) and three standard deviations (3SD). Results were considered positive when the absorbance value was more than 0,135 using 2SD and 0,178 using 3SD.

Serological diagnosis

Three hundred and seventy one dogs were examined, 159 dogs from Municipality of Adrianópolis (Valley of River Ribeira) and 212 dogs from Municipalities of Apucarana, Arapongas, Cambira, Florestópolis, Jardim Alegre and Sabáudia (North of Paraná State). Leishmanial antibodies were detected by ELISA test in 81 dogs (21,83%) with values of 0,135 or over (2 standard deviations) and in 49 dogs (13,20%) with values of 0,178 or over (3 standard deviations).

The results by region are as follows: Municipality of Adrianópolis – 21 dogs (13,21%) and 10 (6,29%) dogs were found positive by 2 standard deviations (2SD)

and 3 standard deviations (3SD), respectively; North of Paraná State – 60 dogs (28,3%) and 39 dogs (18,39%) were found positive by 2 standard deviations (2SD) and 3 standard deviations (3SD), respectively.

By IFAT (Indirect Fluorescent Antibody Test), 22 dogs were found (5,9%) with dilution values of 1:40 or over. Comparing the data between ELISA and IFAT, 20 dogs were scropositive by ELISA and IFAT, with a percentage of concordance between these techniques of 24,6% with 2SD and 40,31% with 3SD (Table 1).

Table 1: Comparison of ELISA and IFAT techniques in 371 dogs studied of Paraná State, Brazil.

		IFAT		
		POSITIVE ≥ 1:40	NEGATIVE <1:40	TOTAL
ELISA	POSITIVE ≥ 0,138 (2SD)	20	61	81
	NEGATIVE <0,138 (2SD)	2	288	290
	TOTAL	22	349	371

Among eight parasite-positive dogs, seven were seropositive by the ELISA techniques, while by IFAT technique, six dogs were positive. The sensitivity for ELISA test was 87,5% and by IFAT test the sensitivity was 75%. One dog (12,5%) was negative in both tests, probably because the lesion was recent (prepatent period) (Table 2).

Table 2: Comparison of ELISA and IFAT in eight parasite-positive dogs in North Paraná State, Brazil.

	PARASITE-POSITIVE DOGS								
	1	2	3	4	5	6	7	8	SENSIBILITY
ELISA	+	+	+	+	+	-	+	+	87,5%
IFAT	+	+	-	+	+	-	+	+	75%

Discussion

Since 1976, when ELISA was used for the first time in the study of visceral leishmaniasis (**14**), researchers have evaluated its potential as a diagnostic tool, but its use in cutaneous leishmaniasis had not yet led to conclusive results (**15**).

In the present study we have standardized an ELISA test for anti *Leishmania* antibodies and compared with IFAT that is considered a gold standard. By ELISA test Leishmanial antibodies were detected in 81 dogs (21,83%) and 49 dogs

(13,20%) respectively to 2SD and 3SD. By IFAT 22 dogs (5,9%) were detected, with positive dilution values of 1:40 or over. The ELISA test showed best sensitivity (87,5%), while IFAT showed 75% among eight parasite-positive animals. The index of Co – positivity was 90% and co–negativity was 82,5%. Comparative data between ELISA and IFAT showed percentages of concordance between these techniques of 24,6% with two standard deviations (2SD) and 40,31% with three standard deviations (3SD). The ELISA technique when compared with IFAT technique presented more sensitivity, but IFAT seemed more specific in detecting leishmanial antibodies (**16**). It seems that ELISA could be as helpful in detecting low-level parasitic infections with cutaneous leishmaniasis as IFAT (**14**).

Thus, the methodology standardized in the present work permits to study the serology of the dog in endemic zones of cutaneous leishmaniasis. However, difficulties exist, associated with the interpretation of seroprevalence data, comprising the pre-patent period, a fraction of infected dogs may never seroconvert and seroconversion may not be permanent with non-specific cross-reactions (**17**). In this work we used crude extracts of *Leishmania (Viannia) braziliensis* as soluble antigen in ELISA method anti-serum. Non-specific reactions occurred with Chagas' disease (date no shown). The number of false positive results obtained obliges us to look for ways to improve the specificity of this assay maintaining its sensitivity. This could be possible by using protein-purified antigens of the parasite.

To distinguish between homologous and heterologous positive tests, in areas where overlapping of trypanosomiasis and leishmaniasis occur, (**18**) the absorption technique may be used to substitute normal techniques or an antigen purification.

Research should then focus on the development of diagnostic tools with improved sensitivity and specificity to identify infected dogs, using specific antigens for serology (**17**).

Acknowledgements

This study was partially supported by CNPq.

References

1. Thomaz-Soccol V. (1993) PhD thesis, Université Montpellier I. Faculte de Medicine, Montpellier, France.
2. Lainson R., Shaw J.J. (1987) *in*: The Leishmaniases in Biology and Medicine, vol.1, Peters, W. & Killick-Kendrick, R., eds., Academic Press, London, pp.1-120.
3. OMS (1990) *Serie de repports techniques.*, **793**, Geneve, OMS.
4. Dias M., Mayrink W., Deane L., Costa C., Magalhães P., Melo M., Batista S. (1977) *Rev Inst Med Trop São Paulo* **19**, 403-410.
5. Barreto A.C., Cuba-Cuba C., Vexenat J.A., Rosa A.C., Marsden P.D., Magalhães A. (1984) *Rev Soc Bras Med Trop* **17**, 59-65.
6. Coutinho S.G., Nunes M.P., Marzochi M.C.A., Tramontano N. (1985) *Mem Inst Oswaldo Cruz* **80**, 17-22.
7. Falqueto A., Coura J., Barros C., Grimaldi G., Sessa P., Carias V., Alencar J. (1986) *Mem Inst*

Oswaldo Cruz **81,** 155-163.

8. Vexenat J.A., Barreto A.C., Rosa A.De C., Sales C.C., Magalhães A.V. (1986) *Mem Inst Oswaldo Cruz* **81**, 237-238.
9. Pirmez C., Coutinho S.G., Marzochi M.C.A., Nunes M.P., Grimaldi Jr. G. (1988) *Am J Trop Med Hyg* **38**, 52-58.
10. Marzochi M.C.A., Barbosa-Santos E.G.O. (1988) *Mem Inst Oswaldo Cruz* **83**, 391-392.
11. Yoshida E., Correa F., Marques S., Stolf H., Dillon N., Momen H., Grimaldi Jr. G. (1990) *Mem Inst Oswaldo Cruz* **85**, 133-134.
12. Engvall E., Perlamann P. (1972) *J Immunology* **109**, 129-135.
13. Guimarães M.C., Coutinho S.G., Antunes C.M.F. (1987) *Ver Soc Bras Med Trop* **20**, 55-58
14. Hommel M., Peters W., Ranque J., Quilici M., Lanotte G. (1978) *An Trop Med Paras* **72**, 213-218.
15. Garcia-Miss M Del R., Andrade-Narvaez J., Esquivel-Viñas R.E., Simmonds-Diaz E.B., Canto-Lara S.B., Cruz-Ruiz A.L. (1990) *Trans R Soc Trop Med Hyg* **84**, 356-358.
16. Edrissian G.H., Darabian P. (1979) *Trans R Soc Trop Med Hyg* **73**, 289-292.
17. Reithinger R., Davies C.R. (1999) *Am J Trop Med Hyg* **61,** 530-541.
18. Camargo M.E., Rebonato C. (1969) *Am J Trop Med Hyg* **18** , 500-505.

Chapter 35

LEISHMANIA SPECIES IN TWO REGIONS OF PARANÁ, BRAZIL: BIOCHEMICAL CHARACTERIZATION BY ISOENZYME ELECTROPHORESIS

V. THOMAZ-SOCCOL[1], E.A. CASTRO[1], E. LUZ[1], J. DEREURE[2], F. PRATLONG[2], N. MEMBRIVE[3], J.P. DEDET[2]

1- Laboratório de Parasitologia Molecular, Dep. de Patologia Básica, Universidade Federal do Paraná, Centro Politécnico J. das Américas. CEP 81531-990, Phone 041 361 17 01 Fax – 041 266 20 42, E- Mail - vasoccol@bio.ufpr.br ; 2- Laboratoire de Parasitologie et Centre de référence des Leishmanioses – 163, Rue Auguste Broussonet, 34.000 - Montpellier – France. ; 3- Fundação Nacional da Saúde, Regional Arapongas, Paraná

Abstract

Cutaneous leishmaniasis is present in two different regions of the State of Parana, (South of Brazil): in the Northern area and the Ribeira River Valley. In 1994 there was an outbreak with a total of 1395 leishmaniosis cases. Here, we report on the isolation and characterization of 52 *Leishmania* stocks isolated between 1995 and 2000 from humans, dogs, and wild reservoir and phlebotomine hosts. The material, obtained by biopsy (punch no. 4) or by aspiration puncture, was inoculated in two media (NNN and Tobbie & Evans) and maintained at a temperature of 24°C. After isolation, parasites were grown in C.C.S. medium (in Erlenmeyer and bottle of Roux) for protein extract preparation, awaiting identification. Isoenzymatic characterisation was made by electrophoresis in starch thick gel, using 13 enzymatic systems. *Leishmania* stocks were obtained from active lesions of humans coming from 26 counties in the northern region of Parana and 6 from the Adrianópolis borough (Ribeira River Valley). In the northern area of the state stocks were isolated from 8 dogs (*Canis familiaris*); 1 stock from *Nectomys* sp., and 3 stocks were isolated from *Lutzomyia whitmani*. All stocks were identified as *Leishmania (Viannia) braziliensis*. Fifteen zymodemes were characterized. Zymodemes LMP1 and LPM2 showed a large distribution in both regions studied. These zymodemes are present from the south of Mexico to the North of Argentina.

Index Entries: *Leishmania (V) braziliensis*, Isoenzymatic characterisation, Paraná State, genetic diversity.

New Horizons in Biotechnology, 387-395, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

The genus *Leishmania* Ross, 1903 belongs to the Kinetoplastida order, and the Trypanosomatidae family. This parasite causes several infections in humans and is associated with cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniosis (VL). In the infra genus, the species has been classified into two sub-genus: *Leishmania* Saf'Janova (**1**) and *Viannia* Lainson and Shaw (**2**). More than 17 taxa have been described in the New World (**3,4**). These were ordered into phylogenetic complexes based on biochemical techniques and phenetic and cladistic approaches (**4-6**). These taxa have a very large distribution, extending from the South of Mexico to the North of Argentina. It is endemic in most of Central and South American countries (**7**). The existence of a wide diversity of *Leishmania* strains has been reported in those different regions. Several epidemiological surveys were conducted during recent years, which allowed a definition of distribution of strains presently represented (**8-20**).

In the Paraná state, south of Brazil, from 1993 to 1999, an identification of 4873 cases of cutaneous leishmaniosis in 276 municipal districts were done (**21**). The human cases were distributed in two different regions. In the Northern region, during the 80's, cutaneous leishmaniosis was presented as an outbreak. In the region of the Ribeira River Valley, cutaneous leishmaniosis was kept endemic with sporadic annual cases. The question is: are the species of *Leishmania* the same in these two regions?

We report herein the biochemical characterisation of stocks isolated from human, domestic and wild animals and phlebotomine female in two distinct regions of Paraná State, Brazil, between 1996 and 2000. Phenetic analysis was applied to enzymatic proteins, which completed the taxonomy of *Leishmania* from the south of Brazil.

Materials and Methods

Study areas

In the State of Paraná, cutaneous leishmaniosis is present in two different geographical areas. One of these areas, located in the northern region was more recently colonised (within 30-50 years) and the disease has been present, in the last eight years, with an epidemic character. In the second area, Ribeira River Valley, the disease is known from the beginning of the century to have some tendency of peridomiciliar transmission.

North Region of Paraná State

For the 16th regional office of Health for the State of Paraná – National Foundation of Health – which includes about 40 county districts localised between 22°48' to 24°09' latitude south and 51°21' to 51°42' longitude west. The altitude within this egion is 650 m (**22**). The study area is limited by the Tibagi, Paranapanema, Paraná and Iguaçu rivers. This area was colonized in the 40´s and 50´s when the expansion

of coffee plantation destroyed the original forest. The forests were reduced to small pockets, mainly ciliary. Nowadays, the county districts are very populous and the population of the rural area represents, in some county districts, less than 20% of the total population.

Ribeira River Valley Area

Adrianópolis is the Municipality where the work was developed and it is located in the northern part of the first plateau of Paraná, near the border with São Paulo State. This area is represented by a mountainous formation, deep in the Ribeira River (**22**) localised between 24° 39' to 24° 57' latitude south and 48° 31' to 49° 08' longitude west. The altitude varies between 0-100 meters. The area of Adrianópolis is defined geographically to the north by the Ribeira River near the boarder of São Paulo state, to the South by Tunas County, to the west by Cerro Azul and to the East by São Paulo State. It represents an area of 1.333,0 Km^2, with 7.338 inhabitants and a demographic density (hab/Km^2) of 5,50. 10% of these are living in an urban zone. The Rural zone is dispersed forming small agglomerations, where primitive vegetation was substituted by banana and other subsistence cultures. The preserved forest is isolated from residences. Even so, they are generally located in residues of secondary forests. This study was developed in 19 places (valleys).

Parasites Strains

Parasites from skin or mucocutaneous lesions were isolated by skin biopsies or by aspiration of tissue fluid. Tissues from biopsy were triturated aseptically with physiological serum and an antibiotic (penicillin 25,000IU/ml). The materials obtained were transferred to a NNN or Tobbie & Evans medium. Positive cultures were grown on C.C.S. medium at 24°C for 7 days. These steps were taken for the preparation of protein extracts, looking for the identification of the parasites, and enzyme electrophoresis, as reported previously (**5, 23**).

Electrophoresis techniques

All the strains were characterised by electrophoresis of isoenzymes in starch thick gel (**23**). Thirteen enzymes were chosen: malic enzyme dehydrogenase (ME, EC1.1.1.40), isocitrate dehydrogenase (ICD, EC1.1.1.42), 6-phospho-gluconate dehydrogenase (6-PGD, EC1.1.1.44; glucose-6-phosphate dehydrogenase (G-6-PD, EC1.1.1.49), NADH diaphorase (DIA, EC1.6.2.2), purine nucleoside phosphorylase (NP_1, EC 2.4.2.1), purine nucleoside phosphorylase (NP_2, EC 2.4.2.1*), glutamate oxalacetate transaminase 1 and 2 (GOT_1 and GOT_2, EC2.6.1.1), phospho glucomutase (PGM, EC5.4.2.20), fumarate hydratase (FH, EC4.2.1.2), mannose phosphate isomerase (MPI, EC5.3.1.8), and glucose phosphate isomerase (GPI, EC5.3.1.9).

Classification techniques

An automatic classification model was used. Characters are represented by the electromorphs that were numbered according to their mobility. The enzymatic characters, thus identified, allowed individualisation of the zymodeme. The zymodeme was defined by the thirteen enzymatic systems, and named LPM-

(logo for Laboratories of Parasitologie Molecular from Paraná state). Zymodemes thus constituted a taxon whose systematic level was undefined, and was an elementary phenomenon (**24**).

All strains were classified on a numerical basis of taxonomy, namely the phenetic method, using the Jaccard index and ascending hierarchical reconstruction (**24-26**).

Results and Discussion

Between 1995 and 2000, 52 strains of Leishmania from two different regions where cutaneous leishmaniosis is present, were isolated mainly from patients (40), with cutaneous (39) or mucocutaneous lesions (1), or from skin of domestic (8 dogs) or wild mammals (1 *Nectomys* sp. - Cricetidae), or from the digestive tract of phlebotomine sand flies (3 *Lutzomyia whitmani*). The stocks were compared with nine New World reference strains: *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis, L. (L.) amazonensis* and *L. (L.) enriettii* (**5,7**).

The identification of 52 *Leishmania* stocks isolated from different origins (geographic area and host different), indicated that the majority of stocks isolated from human cutaneous lesions showed different aspects, ranging from simple cutaneous lesions, the most frequent (96.05%), to very rare ones such as nodular, verrucose, mucocutaneous, and disseminated lesions. Mucocutaneous leishmaniasis was present in only one patient. The dog-stock isolates were obtained from cutaneous lesions from scrotum, nose, penis, mouth and ear. The isolate from wild mammal (rodentia – Cricetidae) was made from skin and profound organs.

Biochemical characterisation and Taxonomic analysis

Variations in enzymatic sets showed that enzymes with lower polymorphism presented only one each (PGM^{115} GOT^{132} $GOT2^{125}$ GOT^{132} $GOT2^{125}$ FH^{82} and ICD^{55}). These electromorphs were the same as the reference stock of *Leishmania (V.) braziliensis* (MHOM/BR/75/M2903). These enzymes were diagnostic for *Leishmania (V.) braziliensis* and presented only one electromorph independent of the geographic area or host.

The enzymes GPI, NP1, MPI, DIA, G6PD and ME were represented by two electromorphs each ($GPI^{80,87}$ $NP1^{380,385}$ $MPI^{70,71}$ $DIA^{40,45}$ $G6PD^{90,92}$ and $ME^{87,92}$). The enzymes NP2 and PGD had bigger polymorphism, with four electromorphs each. These enzymes resulted in the best separation between stocks by sub-specific degree (Figs. 1a and 1b).

Biochemical characterisation led to identification of 25 different electromorphs. Taking the complete group of the 52 stocks examined, isoenzyme electrophoresis, 15 zymodemes were revealed. The zymodemes have the prefix –LPM and were counted from one to fifteen. Variations within the enzymatic set (PGD, NP2, GPI, ME, MPI, DIA) discriminated these zymodemes: LPM 1 (15 strains), LPM 2 (18 strains), LPM 3 (1 strain), LPM 4 (4 strains), LPM 5 (2 strains), LPM 6 (1 strain), LPM 7 (1 strains), LPM 8 (1 strain), LPM 9 (2 strains), LPM 10 (1 strain), LPM 11 (1 strain), LPM 12 (2 strains), LPM 13 (1 strain), LPM 14

(1 strain) and LPM 15 (1 strain). The zymodeme LPM1 showed the same profile by zymodeme MON*44 and the zymodeme LPM 2 by MON*43 (**1**).

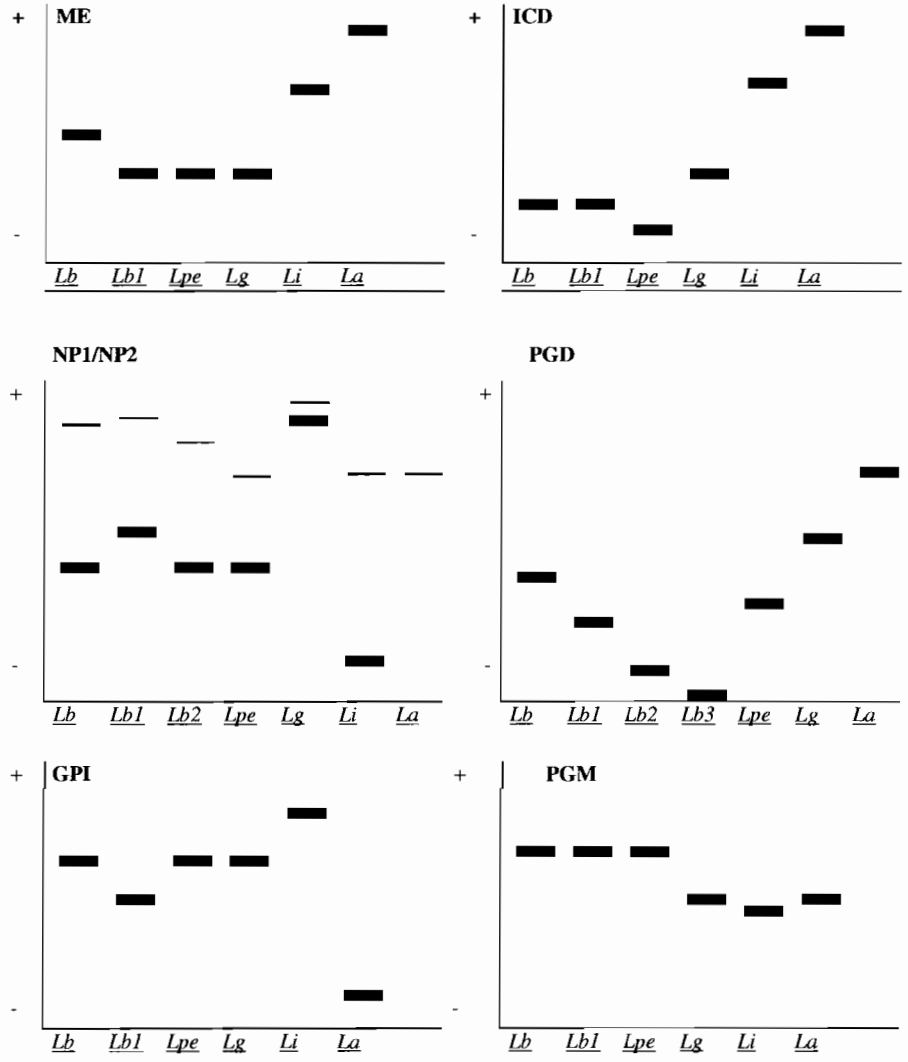


Figure 1a: Schematic representation of 15 loci of 52 *Leishmania* stocks isolated from human, domestic and wild host and sandflies *Lutzomyia whitmani* from two different area where cutaneous leishmaniasis (Paraná south Brazil). Reference strains: Lb = *Leishmania (V.) braziliensis* (MHOM/BR/75/M2903), Lb1= isolate from Paraná state, Lg = *Leishmania (V.) guyanensis*, Lpe = *Leishmania (V.) peruvina*, Lc = *Leishmania (L.) infantum (=L. chagasi)*, *La* = *Leishmania (L.) amazonensis* (MHOM/BR/73/M2269). See text for identification of abbreviated enzymes names.

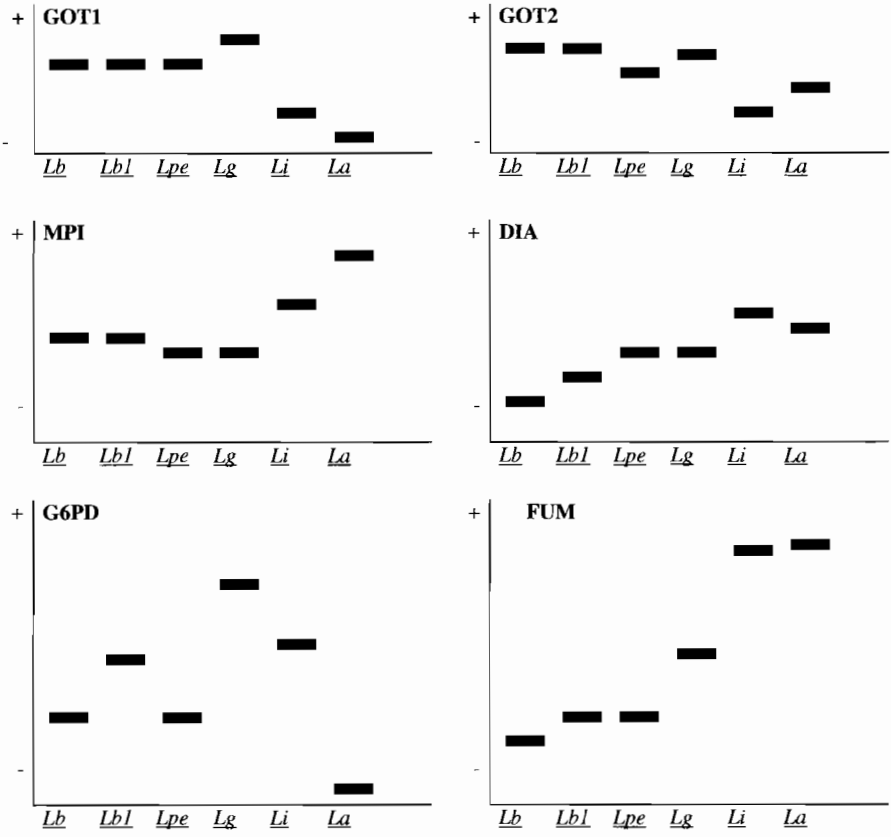


Figure 1b- Schematic representation of 15 loci of 52 *Leishmania* stocks isolated from human, domestic and wild host and sandflies *Lutzomyia whitmani* from two different area where cutaneous leishmaniasis (Paraná south Brazil). Reference strains: Lb = *Leishmania (V.) braziliensis* (MHOM/BR/75/M2903), Lb1= isolate from Paraná state, Lg = *Leishmania (V.) guyanensis*, Lpe = *Leishmania (V.) peruvina*, Lc = *Leishmania (L.) infantum (=L. chagasi), La = Leishmania (L.) amazonensis* (MHOM/BR/73/M2269). See text for identification of abbreviated enzymes names.

The dendogramme constructed by the ascendant agglomerate method individualised into four branches (figure 2) .The first represented the reference strain of *Leishmania (Leishmania) amazonensis,* the second one corresponded to *Leishmania (L.) infantum*, the third to *Leishmania (Viannia) guyanensis* and the fourth grouped 17 zymodemes including reference strains of *Leishmania (V.) peruviana* and *Leishmania (V.) braziliensis.* The dichotomy at degree 0,58 separated the two subgenera *Leishmania* e *Viannia*. Within subgenera *Viannia* a second dichotomy at degree 0,63 separated *L. (V.) guyanensis* to complex *L.(V.) braziliensis*. In the present work the zymodemes were grouped within complex *Leishmania braziliensis*. Within the *L. braziliensis* group complex, four subgroups were formed.

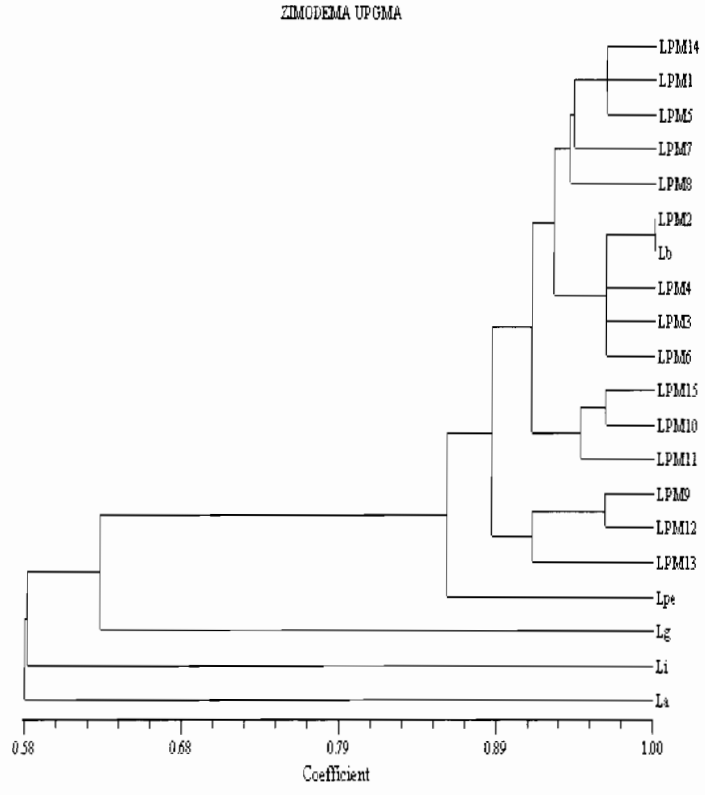


Figure 2 – Dendograme (UPGMA) constructed by distance Jaccard coefficient, obtained by enzymatic data. The strain references and the15 zimodemes studied. Reference strains of *Leishmania: (Leishmania) infantum* (Li) *(Leishmania) amazonensis* (La); *Leishmania (Viannia) guyanensis* (Lg); *Leishmania (V.) peruviana* (Lpe) e *Leishmania (V.) braziliensis* (Lb).

All Paraná stocks were unambiguously attributed to the species *L. (V.) braziliensis*. As a matter of fact, they all showed the specific isoenzyme characters of this species and they all proved to be closely related to the reference stock MHOM/BR/75/M2903. Out of these 13 loci, 8 (*ME, G6PD, DIA, MPI, NP1, GPI, NP2* and *PGD*) were found to be polymorphic in the stocks from Paraná state. Of the 52 stocks examined, the electrophoresis revealed 15 zymodemes (index of 28,84%).

The polymorphism of *Leishmania braziliensis* has been previously studied by different authors and in different regions in the New World (**5,9,11,13,15,20,27-30**).

Leishmania (V.) braziliensis has a very large distribution, extending from the South of Mexico to the North of Argentina (**5,7**). It is equally present in different ecosystems with an altitude varying from sea level to 2.000m altitude and can parasite in different vectors and reservoir hosts (***8,9,11,13-17***).

In studies of the complex *L. (V.) braziliensis* in different Latin American countries, we observe different behaviors in the genetics of mono and polymorphism. In Bolivia and Peru (SA) (**5,9,11,13,27**) and in Belize (Central America) (**5,27,29**), a great number of zimodemes are observed. However, in countries like French Guiana, Venezuela, Ecuador and Colombia, (**5,12,15,20,28**) less variety is observed. Saravia *et al.* (**28**), in Colombia, in reference to *Leishmania (V.) braziliensis*, found 7 zimodemes that were in 91 strains (reason for 8.6%). In another study done by Thomaz-Soccol *et al.* (**20**), the number of zimodemes was lower (4), however, the relationship between this one and the number of strains found (7) was a lot more higher (reason from 57-14%). In Brazil, this behaviour is very diverse as well. In the Northern Region, 7 zimodemes were described, starting with 94 isolates (reason of 7.4%) (10). In the North, a low polymorphism in relation to the *Leishmania braziliensis* was observed (**16,17**).

In the enzymatic study of the strain-set from the New World, Thomaz-Soccol *et al.* (**6**), described 24 zimodemes in 46 stocks (reason 52%). The author highlights the wide geographical distribution that the zimodemes named MON*43 and MON*44 show, having been analysed from Belize to Brazil. In the present work, the northern region of the state as well as the Ribeira River Valley region, showed strains with similar iso-enzymatic profiles.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (BRAZIL).

References

1. Saf'Janova V.M. (1986) *in* Leishmania. Taxonomie et phylogenèse. Aplications éco-épidémiologiques, Rioux J.A.ed., IMEEE, Montpellier, pp.247-255.
2. Lainson, Shaw (1987) *in* The leishmaniases in Biology and Medicine, Peters W., Killick-Kendrick R., eds., Academic Press, London, pp. 1-120.
3. Grimaldi G.Jr., Tesh R.B.T., McMahon-Pratt D. (1989) *Am. J. Trop. Med. Hyg.* **41**, 687-725.
4. Dedet J.P. (1993) *Ann. de l'Inst. Pasteur* **4**, 3-25
5. Thomaz-Soccol V. (1993) *PhD Thesis, Faculté Médecine,* Montpellier, 190p.
6. Thomaz-Soccol V., Lanotte G., Rioux J.A., Pratlong F., Martini-Dumas A., Serres E. (1993) *Annales de Parasitologie Humaine et Comparée,* **68**, 104-106.
7. OMS (1990) *Série de rapports techniques*. OMS, Geneve, **793**, 176 p.
8. Rosa A.C., Cuba-Cuba C., Vexenat A., Barreto A.C., Marsden P.D. (1988) *Trans. R. Soc. Trop.*

Med. Hyg. **82**, 409-410.

9. Arana M., Evans D.A., Zolessi A., Llanos Cuentas A., Arevalo J. (1990) *Trans. R. Soc. Trop. Med. Hyg.* **84**, 526-529.
10. Grimaldi G.Jr., Momen H., Naiff R.D., McMahon-Pratt D., Barrett T.V. (1991) *Am. J. Trop. Med. Hyg.* **44**, 645-661.
11. Revollo S., Dimier-David L., Lyevre P., Camacho C., Dedet J.P. (1992) *Trans. R. Soc. Trop. Med. Hyg.* **86**, 388-391.
12. Bonfante-Garrido R., Meléndez E., Barroeta S., Mejía de Alejos M.A., Momen H., Cupolillo E., McMahon-Pratt D., Grimaldi G.Jr. (1992) *Trans. R. Soc. Trop. Med. Hyg.* **86**, 141-148.
13. Dujardin J.C., Llanos-Cuentas A., Caceres A., Arana M., Dujardin J.P.,Guerrini F., Gomez J., Arroyo De Doncker S., Jacquet D., Hamers R., Guerra H., Le Ray D., Arevalo J. (1993) *An. Trop. Med. Paras.* **87**, 335-347.
14. David C., Dimier-David L., Vargas F., Torrez M., Dedet J.P. (1993) *Trans. R. Soc. Trop. Med. Hyg.* **87**, 7-9.
15. Armijos R.X., Thomaz-Soccol V., Lanotte G., Racines J., Pratlong F., Rioux J.A. (1995) *Parasite* **2**, 301-305.
16. Vasconcelos I.A.B., Vasconcelos A.W., Fe Filho N.M., Queiroz R.G., Santana E.W., Bozza M., Sallenave S.M., Valim C., David J.R., Lopes U.G. (1994) *Am. J. Trop. Med. Hyg.* **50**, 158-164.
17. Barral A., Guerreiro J., Bomfim G., Correia D., Barral-Netto M., Carvalho E.M. (1995) *Am. J. Trop. Med. Hyg.* **53**, 256-259.
18. Raccurt C.P., Pratlong F., Moreau B., Pradinaud R., Dedet J.P. (1995) *Trans. R. Soc. Trop. Med. Hyg.* **89**, 372.
19. Sampaio R.N.R., Ribeiro de Paula C.D. (1999) *Rev. Soc. Bras. Med. Trop.* **32**, 523-528.
20. Thomaz Soccol V., Velez I.D., Pratlong F., Agudelos S., Lanotte G., Rioux J.A. (2000) *Systematic Parasitology* **46**, 59-68.
21. Lima A.P. (2000) Distribuição da leishmaniose tegumentar e análise da sua ocorrência em ambientes antrópicos, no Estado do Paraná, Brasil. Londrina, [Dissertação de Mestrado - UEL, 2000].
22. Maack R. (1981) *Geografia Física do Estado do Paraná*. 2. ed. Livraria José Olympio Editora S.A., Rio de Janeiro, 450 p.
23. Rioux J.A., Lanotte G., Serres E., Pratlong F., Bastien P., Perieres J. (1990) *Ann. Paras. Hum. Comp.* **65**, 11-125.
24. Sneath P.H.A., Sokal,R.R. (1973) Numerical classification. Freeman W.H. and Co. ed., San Francisco, 573p.
25. Serres E., Roux M. (1986) *in* Leishmania. Taxonomie et phylogenèse. Aplications éco-épidémiologiques, Rioux J.A. ed., IMEEE, Montpellier, pp.27-40.
26. Farris J.S. (1979) *Systematic Zoology* **28**, 483-515.
27. Chouicha N., Lanotte G., Pratlong F., Cuba-Cuba C.A., Velez I.D., Dedet J.P. (1997) *Parasitology*, **115**, 343-348.
28. Saravia N.G., Segura I., Holguin A.F., Santrich C., Valderrama L., Ocampo C. (1998) *Am. J. Trop. Med. and Hyg.*, **59**, 86-94.
29. Evans D.A., Lanham S.M., Baldwin C.I., Peters W. (1984) *Trans. R. Soc. Trop. Med. Hyg.*, **78,** 35-42.
30. Cupollillo E., Momen H., Grimaldi G.Jr. (1998) *Mem. Inst. Oswaldo Cruz*, **93**, 663-668.

Chapter 36

INOCULANTS FOR SILAGE IN SUBTROPICAL CLIMATE

Z. G. WEINBERG

Forage Preservation and By-Products Research Unit, Department of Stored Products, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel.: zgw@volcani.agri.gov.il

Abstract

The major crops for silage in Israel are whole-crop wheat and corn. Silage quality must be excellent for the high lactating cows. The climate in Israel is subtropical with warm temperatures most of the year. The high temperature (>40^{0}C) within bunker silos, which results from the ensiling fermentation, persists for many months. Under warm conditions the silages are more susceptible to aerobic deterioration.

Bacterial inoculants can be used in order to enhance the ensiling fermentation. Most previous inoculants included homo- fermentative lactic acid bacteria because they are fast and efficient producers of lactic acid and resulted in fast decrease in pH. However, such strains impaired the aerobic stability of whole-crop wheat, corn and sorghum silages. This was attributed to the lack of enough volatile fatty acids which inhibited fungi, in silages dominated by homo-lactic fermentation. Therefore, new types of inoculants are being tested. *Lactobacillus buchneri* is a hetero-fermentative lactic acid bacteria, which produces high levels of acetic acid during fermentation. Tests with this microorganism gave promising results: silages treated with *L. buchneri* remained stable, while those treated with *L. plantarum* deteriorated upon aerobic exposure.

Key words: silage fermentation, inoculants, aerobic stability, homo- and heterofermentative lactic acid bacteria.

Introduction

Modern agriculture forage crops are harvested at a stage when yields and nutritional values are at their peaks, and are then preserved in order to ensure a continuous and consistent supply throughout the year. Forage crops are preserved either by hay making (field drying) or by ensiling. Hay making is restricted to "thin" crops which can dry quickly, and the process may be interrupted by rain. Ensiling is a preservation method for large masses of moist crops; it is based on solid-state fermentation under anaerobic conditions, whereby lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid. As a result, the pH decreases and the forage is preserved. Ensiling is less weather

New Horizons in Biotechnology, 397-402, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

dependent than hay making, and it is possible to ensile almost any plant material. Therefore, it is widely used, mainly in developed countries, and the annual amount worldwide is estimated at more than 200 million tons of dry matter (**1**). The most important crops for ensiling are whole-plant corn, alfalfa and various grasses. Other crops include whole-crop wheat, sorghum and various legumes.

The ensiling process, which involves many steps, should be timed and controlled carefully in order to ensure successful ensiling with minimal losses. They include harvesting at the optimal stage of maturity, wilting in order to ensure that the dry matter content is adequate to ensure proper fermentation, chopping, loading into a silo, compacting and sealing to exclude air, storing and finally, unloading for animal feeding. Silage making is a sophisticated, costly operation that requires large capital investments in machinery and silos. The cost of a ton of dry matter silage is around 150 US dollars (**1**). The major contributions to the cost of silage production include land and crop production (about half), harvesting and sealing plastics (about one third); the rest arises from silo and additive costs. Silo types include concrete bunkers, towers and plastic sleeves. Recently, big-bale silage has been developed, in which the crop is ensiled in plastic bales of approximately 0.5 ton, which permit more flexible use than traditional silos. Special machinery that wraps the plastics around the baled crop has been developed. For small-holder cattle owners in Africa who cannot afford the high costs of silage making, silages in small plastic bags (10-20 kg) are being tested (**2**).

The biochemical and microbiological events during the ensiling fermentation can be divided into four distinct stages:

- Aerobic, when air is still present between the plant particles and the pH is 6.0-6.5. During this stage plant respiration continues, as well as proteolysis and the activity of aerobic microorganisms such as Enterobacteria, fungi and yeasts.
- Fermentation, which is carried out by a dynamic succession of LABs which change according to the conditions in the silage, starting with Enterococci and Leuconostoc, followed by Lactobacilli and Pediococci (**3**). Lactic acid and other acids accumulate and the pH decreases.
- Stable, when few changes occur during storage, provided that no air penetrates into the silage.
- Unloading for feeding, when the silage is re-exposed to air which re-activates aerobic microorganisms, mainly yeasts and molds, which might spoil the silage.

Good quality silage has a pleasant odor, a typical color and texture, and high nutritional value. If the plant composition is inadequate, or the silage is not prepared properly, undesirable microbiological processes take place which spoil it and result in substantial losses (**4**). If the crop is too moist or lacks enough fermentable sugars, or its buffering capacity is too high, secondary fermentation by Clostridia will take place, resulting in increased pH (through to conversion of lactic into weaker butyric acid), spoilage and losses. Very moist crops result in seepage, which leads to loss of nutrients, and to pollution, since the seepage is high in BOD. Air is detrimental to silage (**5**), and some silages are more susceptible to aerobic

deterioration than others. Aerobic deterioration is undesirable because it is associated with loss of nutrients, decreased intake by the animals, and sometimes, production of mycotoxins in the silage. Its effects depend on silage composition (high dry matter and sugar contents enhance aerobic deterioration), presence of detrimental microbial populations, especially yeasts and molds, the extent of air penetration and exposure to air, and the ambient temperature (**6**). During the aerobic and fermentation phases, the temperature of the silage increases to 40^0C and in warm climate this temperature may persist for many months (**7**). High silage or ambient temperatures accelerate aerobic deterioration because they enhance the activity of aerobic yeasts and molds (**8**).

For successful ensiling it is desirable to shorten the aerobic and fermentation phases, in order to minimize the activity of detrimental microorganisms and to stabilize the silage. It is also important to minimize the exposure of the silage to air during storage and unloading. These aims are achieved by proper management during silage making, storage and unloading, and with the aid of silage additives.

Inoculants for silage

It is possible to use both chemical and biological additives in making silage, in order to reduce fermentation losses, to enhance aerobic stability and to improve the nutritional value. Biological additives are comprised of bacterial inoculants and enzymes. They are safe, easy to use, non-corrosive to machinery, non-polluting and regarded as natural products. In the past, mainly homofermentative lactic acid bacteria (LAB) were included in inoculant formulas, because they rapidly produce lactic acid in the silage and result in fast decrease in pH with minimal losses. In addition, inoculants are also ascribed as having probiotic effects and improve animal performance (live weight gain, feed intake and conversion and milk yields). In many cases improvements in animal performance are the major incentive for inoculant use. Since such inoculants have not always been effective, new types of inoculants were developed (mainly by screening), which are more crop specific and are chosen to counter specific problems such as aerobic instability (**9**). The following section summarizes the experiments performed in our laboratory with inoculants for silage.

Testing inoculants for silage in Israel

The major forage crops for silage in Israel are wheat and corn. Wheat for silage is harvested in April at the milk ripening stage and corn-during summer. Other crops include sorghum and various legumes. The climate in Israel is subtropical with a short cool and rainy winter and a long warm and dry summer. The high temperatures ($>40^0$C) within bunker silos, which result from the ensiling fermentation, persist for many months. Under warm conditions the silages are more susceptible to aerobic deterioration during storage and feed-out.

The dairy cows in Israel are high lactating with annual milk yields of over 10,000 liter and they are very sensitive to feed quality. Therefore, silage quality must be excellent. Our research is conducted to help achieve this goal.

Ensiling experiments in our laboratory are conducted in three modes:

- Under laboratory conditions in small sealed glass jars (mini-silos). In these experiments we can perform time-course studies.
- Under farm conditions in which dacron bags with treated crops are buried in the silo on day of ensiling; when the unloading front reaches the bags they are brought to the laboratory for analysis.
- Full-scale farm experiments in which whole bunker silos are treated and compared to parallel control silos. Such experiments also include studies on animal performance. They are complicated and expensive, and therefore, rare.

Analysis includes chemical measurements, enumeration of LABs, yeasts and molds and aerobic stability tests in the bottle systems developed in our laboratory (**10**). In these tests, visual appraisal, production of CO_2, increase in pH and in yeast and mold populations serves as indicators for aerobic deterioration.

Studies on silage inoculants started in our laboratory about 15 years ago when commercial manufacturers asked us to test their products, comprised mainly homofermentative LAB. Most of these inoculants included *L. plantarum, Ent. faecium* and *Pediococcus* spp. which we usually inoculated at 10^5-10^6 cfu g^{-1}. In most cases they increased lactic acid production in the silage in a short time and so stabilized it with minimal losses. However, when we analyzed for aerobic stability we found that such strains enhanced the aerobic deterioration of whole crop wheat, corn and sorghum silages. We attribute this property to the fact that not enough volatile fatty acids are produced when homofermentative LABs dominates the fermentation; such acids inhibit fungi (**11, 12**). Homofermentative LABs are now used in our experiments to enhance aerobic spoilage and to challenge other microbial strains to overcome this problem.

Since aerobic stability is a concern in our warm climate, following these findings, other bacteria were tested for their ability to stabilize whole crop cereal silages upon aerobic exposure. Among these was a propionic acid bacteria; it was tried with the hope that the propionic acid it would produce in the silage would suppress the yeasts and mold which spoil silages under aerobic conditions. However, this microorganism had only a marginal effect on the aerobic stability of whole crop cereal silages, because it could not sustain under silage conditions (**13,14**).

Recently studies with *Lactobacillus buchneri* in various laboratories gave promising results with regard to aerobic stability (**15,16**). This is a heterofermentative LAB which was isolated from corn silage (**15**) and produces high levels of acetic acid in silage. We tried this microorganism first in mini-silos with wheat and sorghum silages and the results were very good (**17**). The bacterial strains were obtained from Dr. George Szakacs from the Technical University of Budapest, Hungary, with whom we cooperate for a long time. Before going to full-scale farm experiments we conducted recently experiments with wheat and corn in

50-l plastic containers. Treatments included a control (no additives), *L. plantarum* (LP), *L. buchneri* (LB) and a combination LP+LB. The top layer of the control and LP treated wheat silages was moldy whereas those treated with LB were free of mold. The silages treated with LB had higher levels of acetic acid than the control or those treated with LP (30 vs. 10 g kg^{-1}) and they were completely stable upon aerobic exposure (Table 1). In the experiment with corn the results were not as clear cut, but LB improved the aerobic stability of the corn silages as well, as indicated by overall smaller amounts of CO_2, smaller increase in pH and lower yeast and mold counts (Table 2).

Table 1. Results of the aerobic stability test of the wheat silages in 50-l containers.

Treatment	pH	CO_2 (g kg^{-1} DM)	Yeasts	Molds
Control	4.1	4.8^{b}	7.3	7.3
L. plantarum (LP)	4.8	32.2^{a}	8.5	7.6
L. buchneri (LB)	4.1	0^{c}	4.3	3.5
LP+LB	4.0	$1.7^{b,c}$	4.3	<2.0

Table 2. Results of the aerobic stability test of the corn silages in 50-l containers.

Treatment	pH	CO_2 (g kg^{-1} DM)	Yeasts	Molds
Control	5.9-7.3*	36.5a,b	8.1	7.4
L. plantarum (LP)	6.6	50.8^{a}	<2.0	7.2
L. buchneri (LB)	4.2-5.1	9.8^{b}	3.4-7.0	3.3-5.4
LP+LB	4.0-6.5	$20.1^{a,b}$	3.7-4.8	4.5-6.4

Yeast and Mould counts are given as $\log_{10}$ number of cfu g^{-1}.

^{a,b,c} Within a column, means followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test.

*In cases microbiological counts did not agree, values of both replicates are given.

It is concluded that LB has a potential to protect sensitive silages upon aerobic exposure. Now we plan full-scale farm experiments with a commercial inoculant which includes *L. buchneri* which will focus on both aerobic stability and animal performance.

Conclusions

The ensiling process is complex and comprises interactions of different chemical and microbiological interactions. Therefore, the development of inoculants for silage is not as simple as was perceived previously. Now it is understood that new criteria for silage inoculants should be formulated that address specific needs. In Israel, with its warm climate, dry forage crops and sensitive high lactating cows, inoculants should enhance the ensiling process and minimize fermentation losses, protect the silages upon aerobic exposure and further improve animal performance.

After more than a decade of research it seems that inoculants that include *L. buchneri* might provide a solution for the aerobic stability problem.

Aknowledgments

This work was supported by the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No. 401/01-E, 2001 series.

References

1. Wilkins R.J., Syrjala-Qvist L., Bolsen K.K. (1999) *in*: Proceedings of the 12th International Silage Conference. Pauly, T., ed., Uppsala, Sweden, pp. 23-40.
2. Ashbell G., Kipnis T., Titterton M., Hen Y. Azrieli A., Weinberg Z.G. (2001) *Anim. Feed Sci. and Technol.*, **91** (3-4), 213-222.
3. Woolford M.K. (1984) *in*: The Silage Fermentation, Marcel Dekker, Inc., New York, NY. pp. 23-59.
4. Zimmer E. (1980) *in*: Occasional Symposium of the British Grassland Society No. 11, Thomas, C. ed., Brighton, UK. pp. 186-197.
5. Woolford M.K. (1990) *J. Appl. Bacteriol.* **68**, 101-116.
6. McDonald P., Henderson A.R., Heron S.J.E. (1991) *in*: The Biochemistry of Silage, 2nd ed., Chalcombe Publications, Aberystwyth, UK. pp. 81-151.
7. Weinberg Z.G., Ashbell G. (1994) *Can. Agric. Engn.* **36**, 155-158.
8. Ashbell G., Weinberg Z.G., Hen Y., Filya I. (2002) *J. indust. Microbiol. Biotechnol.*, **28** (5), 261-263. (submitted).
9. Weinberg Z.G., Muck R.E. (1996) *FEMS Microbiol. Rev.* **19**, 53-68.
10. Ashbell G., Weinberg Z.G., Azrieli A., Hen Y., Horev B. (1991) *Can. Agric. Engn.* **33**, 391-394.
11. Moon N.J. (1983) *J. Appl. Bacteriol.* **55**, 453-460.
12. Weinberg Z.G., Ashbell G., Hen Y., Azrieli A. (1993) *J. Appl. Bacteriol.* **75**, 512-518.
13. Weinberg Z.G., Ashbell G., Hen Y., Azrieli A. (1995), *J. Industrial Microbiol.* **15**, 493-497.
14. Weinberg Z.G., Ashbell G., Bolsen K.K., Pahlow G., Hen Y., Azrieli A. (1995) *J. Appl. Bacteriol.* **78**, 430-436.
15. Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F. (1999) *J. Appl. Microbiol.* **87**, 585-594.
16. Kung L. jr., Ranjit N.K., Robinson J.M., Charley R.C. (1999) *in*: Proceedings of the 12th International Silage Conference, Pauly, T. ed., Uppsala, Sweden. pp. 272-273.
17. Weinberg Z.G., Szakacs G., Ashbell G., Hen Y. (1999) *J. Indust. Microbiol. Biotechnol.* **23**, 218-222.

Chapter 37

PRODUCTION OF A BIOCOMPOST BY SOLID STATE FERMENTATION AGAINST THE COFFEE NEMATODES

D. BRAND[1,2], S. ROUSSOS[2], F.A.PROCHMANN[1], J. POHL[3], C.R. SOCCOL[1]

[1]Laboratorio de Processos Biotecnologicos, Departamento Engenharia Quimica, Universidade Federal do Parana (UFPR), 81531-970 Curitiba-PR, Brazil; [2]Laboratoire de Microbiologie IRD, IFR-BAIM;Equipe de Mycologie et des FMS; Université de Provence, Case 925; 163 Avenue de Luminy; 13288 Marseille France; [3] Centro de diagnóstico Marcos Enrietti, Secretaria de Estado da Agricultura e Abastecimento (SEAB), 80040-340 Curitiba-PR, Brazil.

Abstract

The main objective of the present work was to produce a biological nematicide by SSF, with an important number of virulent spores of *Paecilomyces lilacinus* and *Verticillium chlamydosporium* by utilising coffee husk as substrate. Studies of the fermentation physical conditions, such as pH, temperature and initial moisture content were done by using an experimental design 2^{3-1} followed by a complete design 3^2 to obtain the best fermentation conditions. The activity of the biocompost obtained with the fungi *Paecilomyces lilacinus* (strains Pl-1, Pl-2 and Pl-3) and *Verticillium chlamydosporium* (strain Vc-1), were evaluated against the nematode *Meloydogyne incognita* race 1. The measured responses were number of females and root weight. The nematophagous fungi *Paecilomyces lilacinus* Pl-1. showed a reduction in the order of 80%, with the exception of the strain of *Paecilomyces lilacinus* Pl-3, the results obtained with the other strains of fungi propitiated a reduction in the number of nematode females in order of 15 – 25 % .

Key words: Biological nematicide, *Meloydogyne incognita*, *Paecilomyces lilacinus, Verticillium chlamydosporium*, Coffee, Solid State Fermentation.

Introduction

Phytosanitary problems caused by nematodes present an important economic incidence all over the world in different agricultural cultures, mainly in coffee plantations. Nematode combat is difficult due to its extreme resistance and its underground life. The most common species that causes largest damages in agriculture belong to the *Meloidogyne* genera, also called root-knot nematodes (**1**).
The more common practices used in nematode combat have been the use of varieties of resistant cultures, rotation of cultures and application of chemicals, mainly phosphates and carbamates. Even so, the latter method, although very efficient and frequently used, is extremely dangerous to men and animals, due to its

New Horizons in Biotechnology, 403-412, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

wide action spectrum. These chemicals disturb the ecological balance of places in which they are used, they add to competition among plagues, they favor action of parasites and they promote organic matter biodegradation. Besides polluting the environment and nutritious products through the accumulation of residues in the soil and underground water, they affect both the health of animals and men. In light of the present facts, a search is underway to find useful alternatives or substitutes to chemical nematicides. Predatory mushrooms, that capture nematodes or destroy its eggs, mycorrhizal mushrooms, toxins produced by microorganisms and plants, could be used shortly with success in the combat against nematodes of different cultures.(**2-5**).

The term Biological control can be defined as the utilization of living organism populations, parasites, predatory pathogens, antagonistic or competitors in maintaining another organism's population density at a lower average than would occur in their absence (**6**). Commonly, more than one microorganism occurs with plant-parasitic or saprozoic nematodes in a particular rhizosphere. Constant association of these organisms in a given ecological niche undoubtedly has a greater impact on the establishment of such nematodes than would be caused by each microorganism alone. Such association results in a biological balance that may manifest itself in the form of direct parasitism by attachment and penetration by one or more pathogenic microorganism in the eggs, juveniles, or adult nematodes, causing death and possibly allowing subsequent invasion by many or selected saprophytic microorganisms. Egg masses, sedentary females, or cysts may be directly invaded by pathogenic or some opportunistic organisms durind various developmental stages of nematodes. (**7-9**).

The use of solid state fermentation (SSF) may provide the elaboration of efficient formulations with fungi that are employed in the biological control of nematodes. The formulations of the active products could be realized with a natural solid substrates or supports for the development of fungi. The use of agricultural residues or by-products such as cassava bagasse, coffee husk and pulp, sugar cane bagasse, could be even more interesting as it may supply good efficiency and stability of the final product (**10,11**)

The main objective of the present work was to produce a biocompost by SSF, with an important number of virulent spores of *Paecilomyces lilacinus* and *Verticillium chlamydosporium* (nematode eggs parasitic fungi) by utilizing coffee husk as substrate. The biocompost produced was then evaluated against the coffee nematode *Meloidogyne incognita* race 1 in vases containing *Coleus.*

Materials and Methods

Micro-organisms and culture media

The strains of nematophagous fungi utilized in the present work were parasites of nematode eggs *Paecilomyces lilacinus* designated as Pl-1, Pl-2 and Pl-3 and one strain of *Verticillium chlamydosporium*, Vc-1 nematode egg and cyst parasites.

They were maintained in Potato dextrose agar (PDA) and cultivated in coffee husk extract media for the production of inoculum. (**12**)
In an agar medium containing 100 g/L of coffee husk extract (**12**) studies of radial growth and biomass production were conducted in order to verify the velocity of growth and the aspects of each microorganism colony. The test was assayed in Petri dishes of 75 mm diameter with 20 mL of culture media over a 12-day period. The inoculation was made with a droplet of a spore suspension at the center of each dish and incubation was held at 28°C. The diameter of each colony was measured every 24 hours and biomass was weighed after 12 days by the dissolution of agar and separation of mycelia on filter paper and dried at 100°C for 24 hours.

Growth Physiology of nematophagous fungi

Strains belonging to the *Paecilomyces* genera showed similar radial growth velocity, 20 mmh^{-1}, 18 mmh^{-1} and 19 mmh^{-1} respectively for Pl-1, Pl-2 and Pl-3. The fungal parasite of nematodes cysts grows slower than the fungal parasites of eggs and presents a radial growth velocity of 13 mmh^{-1}. The strains Pl-1 and Pl-2 produced more biomass in a 12-day period (128 and 132 mg respectively) than Pl-3 and Vc-1 were less efficient in producing biomass but nevertheless were able to assimilate and metabolize the components present in the coffee husk.

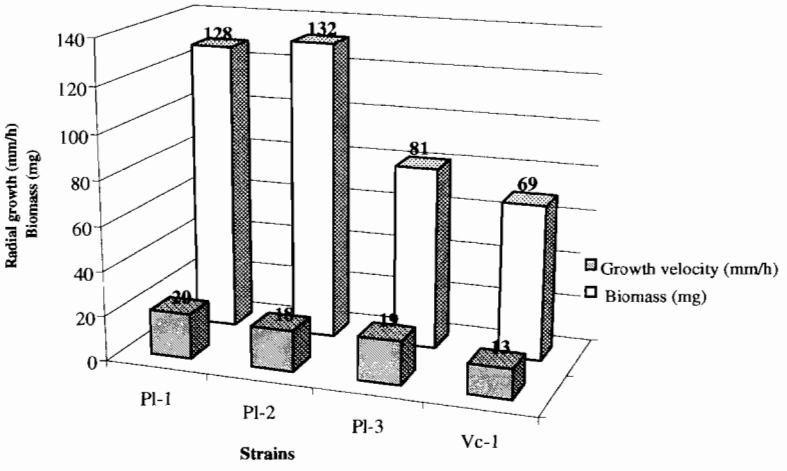


Figure 1. Radial growth of nematophagous fungi.

Solid state fermentation

Experiments were conducted by utilizing coffee husk as substrate. Coffee husk is the major residue of coffee processing in Brazil. The husk was dried, milled and classified. The granulometric fraction comprised between 0.8 and 2.0 mm in diameter was employed. The sterilization of this material must be done without the addition of water otherwise occurs the formation of toxic products to the fungi metabolism mainly to its growth and sporulation.

Fermentation was carried out in glass flasks covered with filter paper for allowing gas exchanges; each flask was filled with 20 g of dry husk. To prepare the inoculum, the strains were grown on coffee husk extract agar and incubated for 10 days at 28° C and spores were counted in a Neubauer cell. Studies of the fermentation's physical conditions, such as pH, temperature and initial moisture content were done initially by using an experimental design $2^{1,3}$. The optimization of this culture conditions was realized in order to obtain better concentration of spores (the response variable). Table 1 shows the real and coded values for all strains tested.

The inoculation rate employed was always 2 E+06 spores/g of substrate in dry weight basis and the incubation period was 7 days at 28°C for each strain studied. After incubation the spores were counted in a Neubauer cell. A total of 5 grams of substrate (wet basis) was vigorously homogenized for 30 minutes with 50 mL of water containing tween 80 and glass beads; the proper dilutions were made.

Table 1. Real and coded values of experimental design 1.

Coded values / Factors	- 1	0	+ 1
Initial moisture (%)	60	65	70
Temperature (° C)	26	28	30
pH	3,5	4,5	5,5

In this first step of optimization, the response variable was evaluated by Pareto chart of effects for each strain employed. The optimized conditions according to the results obtained in this experiment will be done by using a complete experimental plan $3^{2.}$ The results are shown in figures 2 to 5 for each strain employed. By analyzing the figures for the strains of *Paecilomyces lilacinus* Pl-1 and Pl-2 the only significant factor at level of 5% was the pH, and it had a negative effect. This meant that the range employed was above ideal conditions for spore production. Variables such as temperature and initial moisture content were not significant at the 5% level. For the fungus *Verticillium chlamydosporium* the most important factor in spore production was the temperature, the range employed was above ideal.

All strains of *Paecilomyces lilacinus* produced more than 3.5 E+08 spores/g coffee husk (dry weight). The best strain Pl-2 was the one that showed best spore production reaching 6.4 E+08 spores/g. The best conditions of spore production were achieved with the plan 3^2. However, the number of spores didn't increase in a decimal order, due to the fact that conditions employed for each strain were established as initial moisture of 65%, natural pH of the substrate and incubation

temperature of 28°C. With these conditions, fermentations were carried out in order to utilize the biocompost obtained in pot experiments as described below.

Pot experiments

Experiments were based on the activity of the compost obtained by solid state fermentation of coffee husk with the fungus *Paecilomyces lilacinus* (strains Pl-1, Pl-2 and Pl-3) and *Verticillium chlamydosporium* (strain Vc-1), against the nematode *Meloydogyne incognita* race 1.

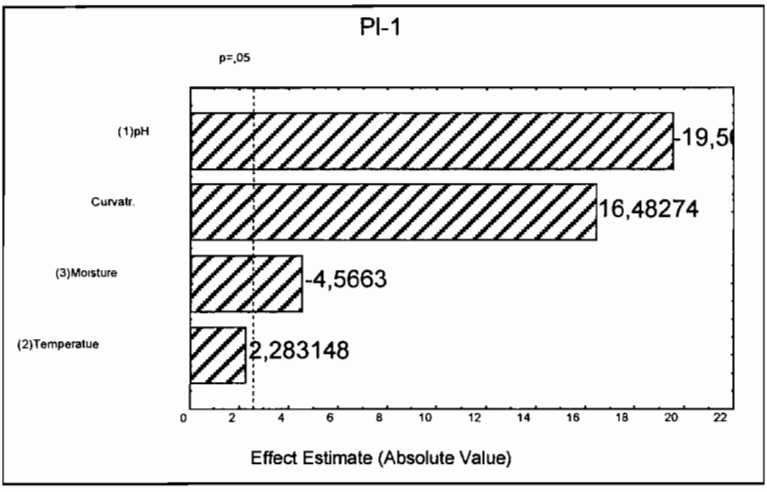


Figure 2: Paredo chart of effect for strain Pl-1.

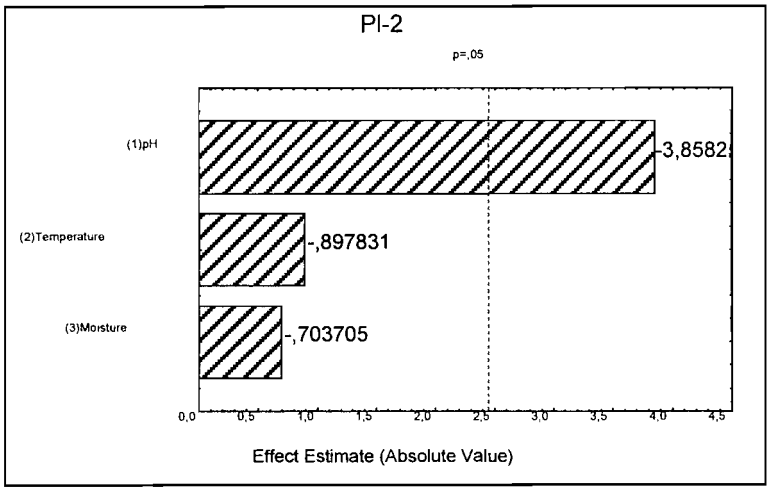


Figure 3: Paredo chart of effcets for strain PI-2.

The nematodes (isolated from a coffee plantation) were reared on tomato as well as in *Coleus*. The experiments were undertaken in a glasshouse without thermal, or illumination controls, therefore subjected to environmental conditions. Two pots containing good quality sterilized soil were prepared for the tests with selected fungi and two more were utilized as control treatments (in which no fungus was inoculated). All experiment were done with two replicates.

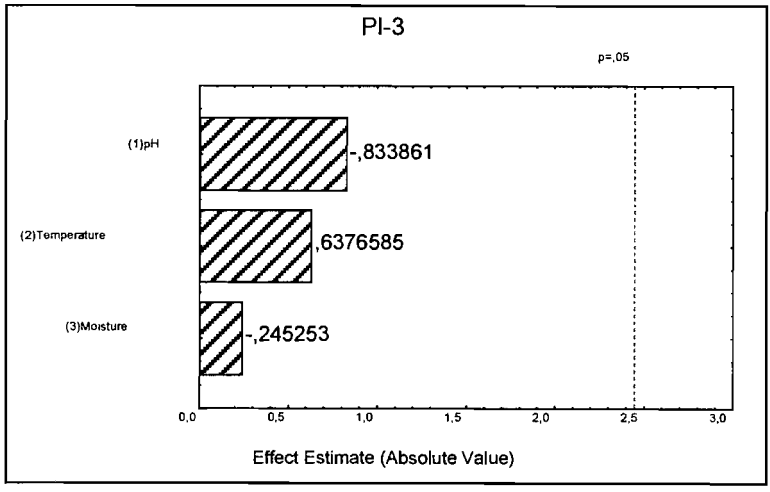


Figure 4. Pareto chart of effects for strain PI-3.

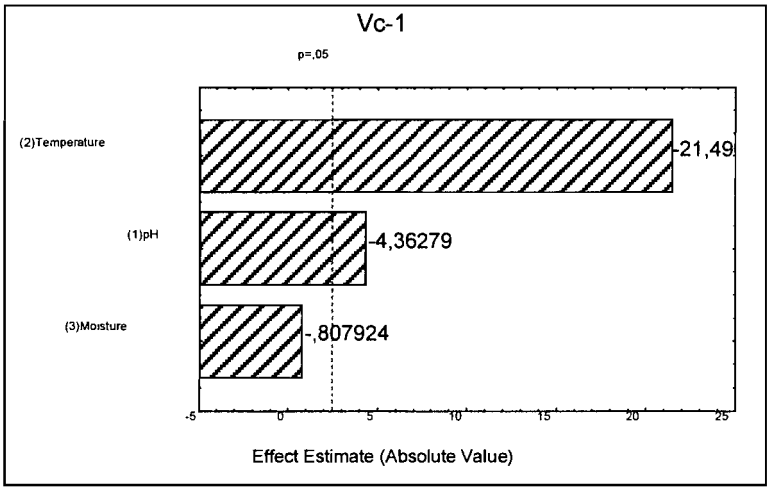


Figure 5. Pareto chart of effects for strain Vc-1.

Each pot received one seedling of the plant *Coleus*, which was chosen for being susceptible to nematode action and for its resistance to other plagues. This experiment was conducted from August 2001 to January 2002. Initially, the analysis of the experiment was stipulated for October 2001, but the low temperatures registered at Curitiba (minimum of 2°C) retarded the development of the nematodes as well as its parasitic action. Temperatures below 18°C reduce the activity of the nematodes as well as the action of nematophagous fungi, amplifying the period of plant infection. Medium temperatures inferior to 5°C may paralyze nematode action. A suspension of nematodes (*Meloydogyne incognita* race 1) was prepared from roots of *Coleus* visually infected, with a great number of galls. Approximately 25 g of the compost obtained by solid state fermentation were homogenized with the soil and inoculated with 100 mL of nematode suspension. Each pot had the same disposition, with layers of soil, biocompost and nematodes alternated around the root of each plant. The concentration of spores was of 10^8 spores/g in wet basis (65 % humidity) and the concentration of the nematodes was of 10000 eggs and juveniles per pot.

The results were analyzed by sampling, due to the impossibility of doing a total nematodes female count present in the galls of plant roots. After removing the plants from pots, roots were isolated, washed and dried at room temperature. The roots were weighed and 5% were evaluated. The results obtained indicated a significant reduction in nematodes count in pots containing the nematophagous fungi Pl-1. The other pots showed great similarity for the gall indexes. The values obtained are demonstrated in Table 2.

Table 2 – Evaluation of nematophagous fungi efficacy.

Fungi	Root weight (g)		Count (female/g root)	
	Pot 1	Pot 2	Pot 1	Pot 2
Vc-1	43,02	33,72	390	345
Pl-3	39,19	13,24	320	462
Pl-1	18,22	19,29	85	89
Pl-2	53,20	34,22	370	360
Control	22,84	29,88	450	433

The values obtained were characterized by a reduction in the number of females in the pots treated with the biocompost containing the strain of nematophagous fungus *Paecilomyces lilacinus* Pl-1. A reduction in the order of 80% was reached when compared to the control pots. With the exception of the strain of *Paecilomyces lilacinus* Pl-3, the results obtained with the others strains of fungi showed a reduction in the number of nematode females in the order of 15 – 25 % inferior to the values observed for the control pots (Fig. 6).

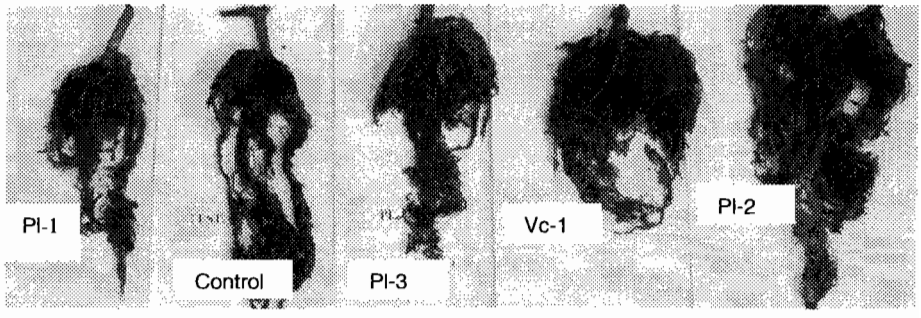


Figure 6. Comparison of roots evaluated in the experiment.

In spite of the lag in the nematode cycle, it was possible to evaluate the experiment. In figure 7 below, reduction of nematodes was approximately 80% in the number of females per g of root. It could be verified that the plant that received the treatment with the biocompost with the strain *Paecilomyces lilacinus* Pl-1 developed excessively well in relation to the control, showing a bigger root system with a much smaller number of galls.

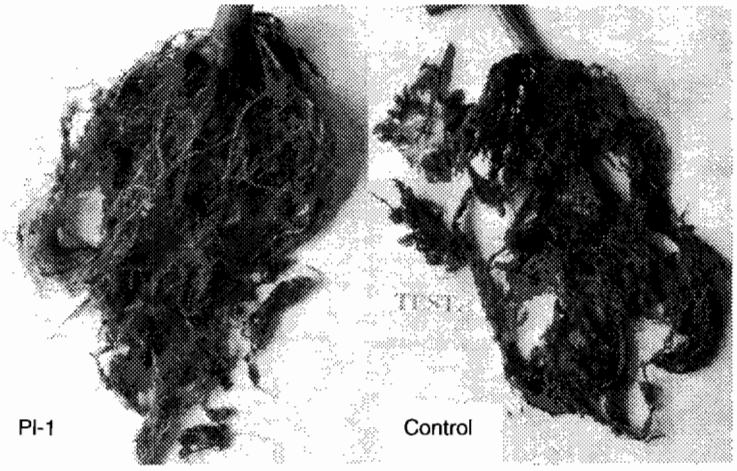


Figure 7. Comparison between control and *Coleus* inoculated with Pl-1.

The results in the reduction of females and root weight didn't have any influence on the sporulation index obtained during fermentation, as it was observed that the strain that produced more spores was Pl-2. The fungus Pl-1 that gave greater reduction is probably better adapted to the soil conditions as well as the nematode species and race employed in the experiment.

Conclusions and future approach

The utilization of a biocompost produced by solid state fermentation utilizing nematophagous fungi and coffee husk as substrate is possible and must be better studied. With the strain *Paecilomyces lilacinus* PL-1 80% of reduction in the coffee nematodes density (*Meloidogyne incognita* race 1) was reached, in a pot experiment. This is a significant result but further studies on the virulence of this strain should be carried out against other species and races of nematodes of the genre *Meloidogyne*. Screening of new strains should also be carried out. Studies on the fermentation conditions will be undertaken in order to enhance spore production and the virulence of the fungus.

Acknowledgements

CRS thanks CNPq for a scholarship under Scientific Productivity Scheme. DB thanks Lactec and IRD (DSF) for a doctorate scholarship.

References

1. Carneiro R.M.D.G., Carneiro R.G., Abrantes I.M.O., Santos M.S.N.A., Almeida M.R.A. (1996) *Journal of Nematology* **28** (2): 177-189.
2. Cayrol J.C. (1989) *P.H.M. - Revue Horticole*, **293**, 53 - 57.
3. Bourne J.M., Kerry B.R. (1999) *Soil Biology and Biochemistry* **31**,75 – 84
4. Ciancio A. (1995) *Appl. Nematol.* **18** (5), 451 - 454.
5. Dijksterhuis J., Veenhuis M., Harder W., Nordbring-Hertz B. (1994) *Advances in Microbial Physiology*, **36**, 111 - 143.
6. Siddiqui Z.A., Mahmood I. (1996) *Bioresource Technology* **58**, 229 - 239.
7. Jatala P. (1986) *Ann. Rev. Phytopathol.*, **24**, 453 - 489.
8. Kerry B.R. (1987) *In* Principles and Practice of nematode control in crops, Edited by Brown R.H., Kerry B.R., Academic Press, 233 - 263.
9. Nordbring-Hertz B. (1988) *Microbiological Sciences*, **5** (4), 108 - 116.
10. Roussos S., Bagnis C., Besnard C., Sigoillot C., Duponnois R., Augur C. (2000) *In* Coffee Biotechnology and Quality Proceedings of the 3rd International Seminar on Biotechnology in the Coffee Agro-Industry, Londrina, Brazil. Sera T., Soccol C.R., Pandey A., Roussos S. (eds). Dordrecht, p.277-286.
11. Soccol C.R, Pandey A. (2002) Coffee waste a new promising substrate to industrial production of microbial bio-products. Concise Encyclopedia of Bioresource Technology. The Haworth Press. Inc, New York, USA (In press).
12. Brand D., Pandey A., Roussos S., Soccol C.R. (2000) *Enzyme and Microbial Technology*, USA, **27**, 127-133.

Chapter 38

CADMIUM AND NICOTINE CORRELATION IN REFERENCE TO TOBACCO PLANT VARIETIES AND TO SLUDGE/SOIL APPLIED RATIOS

D. DOUMA[2], I. PAPAGIANNIS[1], S. DIVANIDIS[3], S. HALIVOPOULOS[3], G. MANOS[2], V. KALFAKAKOU[2]

[1] University of Ioannina, Faculty of Medicine, Physiology Laboratory, Environmental Physiology Unit Ioannina, 45110, Greece ; [2]Technological Educational Institute of Epirus, Plant Production Department, Arta, Greece' [3]Tobacco Institute, Chemistry Department, 66100, Drama, Greece

Abstract

Genetic factors are known to alter the incorporation of heavy metals in tobacco plants (TP). We compared nicotine (Ultra Violet Spectrophotometry) and Cadmium (Cd) levels (Atomic Absorption Spectrophotometry), in 2 Greek TP-varieties, Tsebeli (I) and Mirodata Agriniou (II) grown in increasing proportions (0, 10, 20 and 40%) of sludge added to standard soil. Cd levels decreased in variety I and increased in variety II with increasing leaf height (p=0,000) and a negative correlation between Cd and nicotine levels was confirmed in variety I within the 1st and 3rd harvesting periods (r=-0,643 and r=-0,630 correspondingly). Cd levels, in both varieties, decreased with increasing proportions of sludge. Nevertheless only plants grown in the 40% sludge/soil proportion showed significantly lower values. A negative correlation between nicotine and Cd was observed, only in variety I and in 10%, 20% and 40% sludge/soil proportions. The two tobacco varieties differ in nicotine dependence on sludge/soil proportion. Variety I presents the minimum of nicotine levels at 20% sludge/soil proportion and the maximum at 40% (p=0,000), while variety II presents an inverse pattern.It is concluded that tobacco plant genotype may affect heavy metal and tobacco alkaloid interactions, in relation to sludge/soil proportion and thus biodiversity must be taken in consideration in solid waste management.

Key words: Cadmium, nicotine, tobacco, sewage sludge.

Introduction

Tobacco varieties differ in sugar and nicotine concentrations according to genetically determined patterns of their biosynthesis. Greek tobacco varieties are biotypes of American origin adapted through natural selection to local conditions (**1**). Two classified varieties the Tsebeli variety of low nicotine concentration (1,18%) and sugar (5,77%) percentages per dry weight and Myrodata variety of

New Horizons in Biotechnology, 413-422, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

higher nicotine (1,22%) and sugar (18,90%) levels were our experimental choice (**2**).

Nicotine and sugar concentration determine tobacco organoleptic characteristics as well as its health impact. These characteristics are significantly dependent on soil substrate properties. Heavy metals, such as Cd, Pb, Cr, Zn and Cu, the pH and nitrogen substrate availability affect tobacco properties. Sewage sludge-amended soils cause variation of heavy metal concentration in tobacco crops being strongly dependent on substrate pH (**3,4**).

Total nitrogen and ash are notably higher in sludge-grown tobacco leaves compared to plants grown in either manured or standard soil in relation to heavy metal concentration of the substrate (**5**). Nicotine and protein biosynthesis are both dependent on nitrogen availability whereas metals either as essentials (Zn, Cu) or as toxic ones (Cd, Pb, Cr) may play a regulative role (**6**). Antagonistic or synergistic relations are observed in Cu, Zn and Cd absorption by tobacco plants (**7**).

Nevertheless, the content of total N, total alkaloids and crude ashes increased in proportion to Cd, Zn and Cu concentration applied in tobacco grown substrate while P and reducing sugars were decreased (**8**). In the present work cadmium interference in nicotine biosynthesis is investigated in relation to plant genetic properties, substrate pH and sludge/soil ratios of tobacco plants grown substrates.

Materials and Methods

Municipal sewage sludge (pH=7,1), anaerobically digested was obtained from Ioannina domestic sewage treatment plant. For cultivation, two representative soil samples from Western Greece region with pH = 5,5 → pH1 and pH = 7,4 → pH2 were selected and transported to the area of production (Arta). 32 tobacco seedlings of the Tsebeli variety (variety I) and 32 tobacco seedlings of Mirodata (variety II) were separated and transplanted in 8 different groups of soil/sludge ratios, with 4 replicates in each substrate:

Group 0%	- pH1 :	0% sludge + 100% acid soil
Group 10%	- pH1 :	10% sludge + 90% acid soil
Group 20%	- pH1:	20% sludge + 80% acid soil
Group 40%	- pH1:	40% sludge + 60% acid soil
Group 0%	- pH2 :	0% sludge + 100% acid soil
Group 10%	- pH2 :	10% sludge + 90% acid soil
Group 20%	- pH2 :	20% sludge + 80% acid soil
Group 40%	-pH2 :	40% sludge + 60% acid soil

Tobacco seedlings were transplanted into plastic pots with one plant per pot. The experiment was conducted in the open for 82 days. Starting on the 67th day, leaves from each plant were picked when mature, and sun-dried separately according to their position on the stem. Starting from the bottom of the plant, 4 different leaf harvestings were performed (#1=lower and older leaves, #2=middle lower,

#3=middle upper, #4=upper and higher) when leaves had the same age and maturity level.

The leaves were placed separately in the greenhouse for sun-drying and after recording the dry weights there were stored up for 6 months for fermentation, according to the local practice. A 0,6gr sample of each tobacco plant material was cut and oven dried at 80°C for 24 h. After grinding and weighing, the samples were put in conical flasks of 100 ml with 10 ml of distilled water, 4 ml of concentrated HNO_3 and 4ml of concentrated $HClO_4$ (Merck, Suprapur). The samples were left at room temperature for 18 hours and then they were acid digested in a sand bath (180°C). After filtration, the solutions were transferred to 100-ml plastic volumetric flasks and brought to 10 ml with distilled water. BDH-Spectrosol standrard solutions were used for Cd determination.

Cadmium concentration was measured by means of Atomic Absorption Spectrophotometer (Perkin-Elmer 560 flame-AAS) and calculated by means of the formula:

$$\text{Cd } (\mu g/gr) = \frac{(\mu g/\text{ml solution}) \cdot (\text{solution volume in ml})}{(\text{weight sample in gr})}$$

A sample of each tobacco plant material was oven dried at a temperature not higher than 40°C and then ground to pass a sieve with aperture size 500μm. One gram of ground sample was introduced into the distillation chamber of the distillation apparatus (ISO 3401) under strongly alkaline conditions (20-40gr NaCl, 5ml NaOH and 25ml distilled water).

After adding 15ml 2N H_2SO_4 into the volumetric flask, 220 to 230ml of distillate was collected and removed in a volumetric flask of 250ml and brought to volume with water (V_D). The solution was mixed and used to determine spectrometrically the nicotine concentration. A volume V_V of 25ml of the solution was placed into a second volumetric flask of 100 ml and dilute to the mark with 0,005N H_2SO_4 (V_M=100ml).

Using the spectrometer, absorbance of the filtrate at 236nm, 259nm and 282nm were measured against a reference solution (10ml of 2N H_2SO_4 diluted in 250ml H_2O).

Nicotine expressed as a percentage (%) per dry weight was calculated by means of the reference formula :

$$\text{Nicotine } \% = \frac{100 \cdot A \cdot V_D \cdot V_M}{a \cdot V_V \cdot d \cdot t \cdot ((100-b)/100)} ,$$

A is the corrected absorbance from the observed values of absorbance by means of the formula :

$$A = 1{,}059 \cdot \left(A_{259} - \frac{A_{236} + A_{282}}{2}\right) \quad \text{where :}$$

(A_{236},A_{259} and A_{282} : observed absorbances at 236, 259 and 282nm respectively).
V_D = volume of distillate = 250ml
V_M = volume of diluted solution = 100ml
V_V = volume of distillate used for further dilution = 25ml
a = absorptivity of nicotine in 0,025 mol/l H_2SO_4 in 259nm = 34,3
d = optical path length of the cell (cm) = 1
t = weight sample = 1gr
b = % sample humidity

Statistical Analysis

All values were expressed as mean ± SD, median. Kruskall-Wallis (ANOVA) one way analysis of variance and Mann –Whitney U test were applied to discriminate differences between the groups. Correlations of variables were described by Spearman correlation coefficient. Significance levels were set at $p<0.05$ in all cases.

Results

Variety II presented significantly higher nicotine concentration than variety I (p=0,000). In variety I, nicotine levels in relation to substrates, were significantly higher at sludge/soil ratios of 10% (p=0,000) and 40% (p=0,000) compared to control (0%) (Table1).

Table 1. Nicotine levels of variety I tobacco plants at different substrates and leaf harvesting.

Leaf	Substrates							
	0%		10%		20%		40%	
Height	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median
1	0,66±0,17	0,66	0,79±0,1	0,79	0,54±0,032	0,54	0,89±0,9	0,89
2	0,79±0,032	0,79	0,81±0,01	0,81	0,845±0,25	0,84	0,87±0,17	0,87
3	0,615±0,07	0,61	0,96±0,21	0,96	1,155±0,11	1,15	1,08±0,12	1,08
4	0,4±0,1	0,4	0,89±0,074	0,89	0,64±0,06	0,64	1,045±0,26	1,045
Total	0,647±0,15	0,68	0,862±0,12	0,81	0,79±0,27	0,65	0,97±0,19	0,975

Variety II presents nicotine levels significantly higher at sludge/soil ratio of 20% (p=0,01) in relation to control (Table 2). Nicotine to substrate relation is dependent on the pH of the substrate. Namely variety I presented significantly higher nicotine concentration at pH2 :7,4 → at all sludge/soil ratios.

Table 2. Nicotine levels of variety II tobacco plants at different substrates and leaf harvesting.

Leaf	Substrates							
	0%		10%		20%		40%	
Height	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median
1	0,955±0,15	0,95	0,99±0,15	0,99	0,95±0,42	0,95	1,08±0,106	1,08
2	1,14±0,4	1,145	1,31±0,074	1,31	1,39±0,22	1,39	1,42±0,64	1,42
3	0,98±0,25	0,98	1,02±0,074	1,02	1,275±0,2	1,27	1,025±0,22	1,02
4	1,145±0,53	1,145	1,23±0,32	1,23	1,49±0,64	1,49	1,12±0,96	1,12
Total	1,056±0,36	0,95	1,138±0,22	1,11	1,276±0,32	1,39	1,16±0,2	1,19

The higher the sludge/soil ratio (40%) the higher the nicotine level observed (Fig.1). Variety II as well, at pH2 :7,4 produced more or less higher nicotine concentration in comparison to pH1 :5,5 (Fig.2).

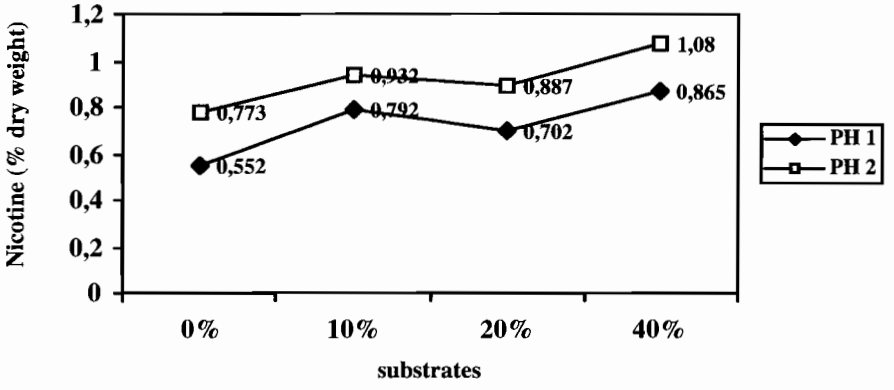
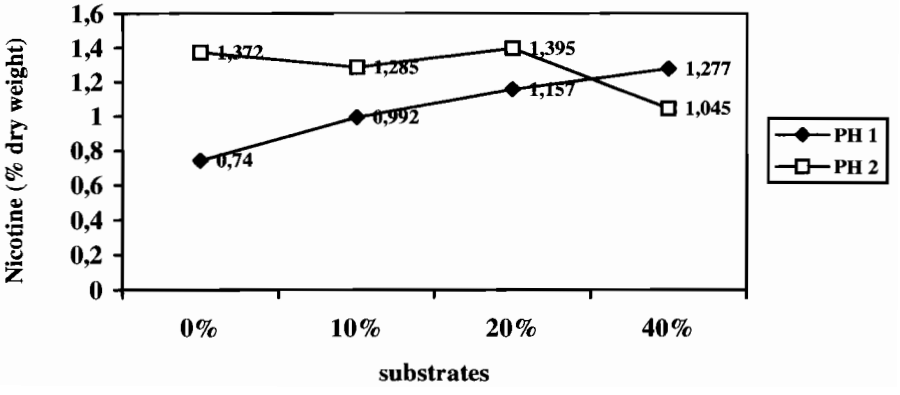


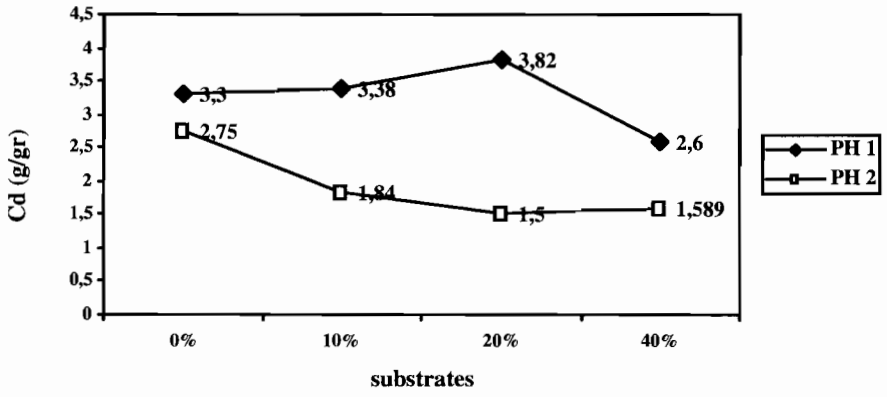
Figure 1. pH effect on nicotine levels of variety I tobacco plants at different substrates.

Figure 2. pH effect on nicotine levels of variety II tobacco plants at different substrates.



Variety I presented significantly higher Cd concentration compared to variety II (Fig.3,4). Both varieties show significantly higher Cd concentrations in pH1: 5,5. Cd decreased significantly with sludge proportion increase, in variety I and pH2: 7,4 (Fig.3). To the contrary, Cd decreased significantly with sludge proportion increase in variety II, pH1: 5,5 (Fig.4).

Figure 3. pH effect on Cd levels of variety I tobacco plants at different substrates.



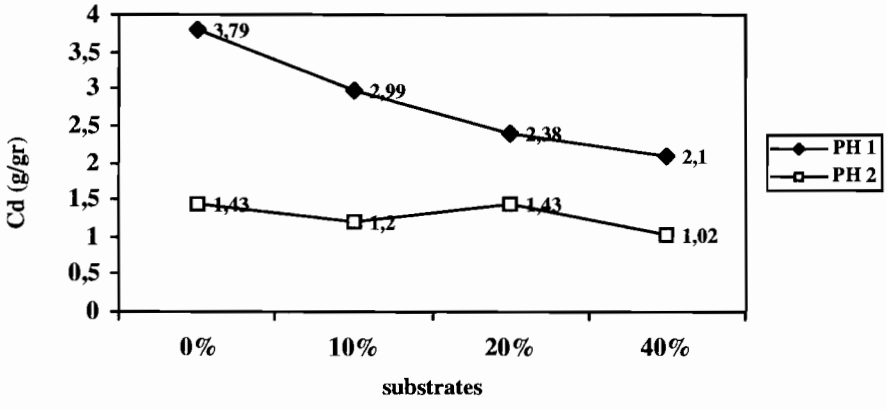


Figure 4. pH effect on Cd levels of variety II tobacco plants at different substrates.

Cd concentration decreases significantly in relation to leaf height, where the higher leaves represent the younger ones, in variety I, in all substrate types (10%, 20%,

40%) and both pH (5,5 and 7,4) (Fig.5). To the contrary, in variety II, Cd increased with leaf height in all substrates and both pH (Fig.6).

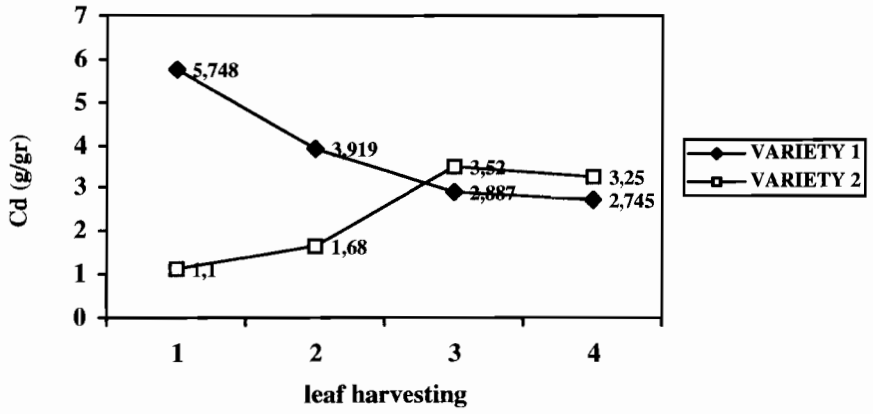


Figure 5. Cadmium (Cd) levels of variety I and variety II at 20% sludge/soil ratio and pH1 at different leaf harvesting.

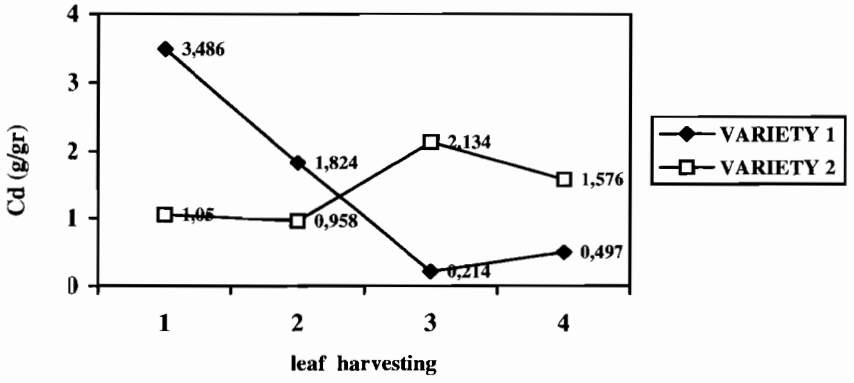


Figure 6. Cadmium (Cd) levels of variety 1 and variety II at 20% sludge/soil ratio and pH 2 at different leaf harvesting.

Cadmium presented significantly negative correlation to nicotine, in all substrate ratios (10%, 20%, 40%) in pH 2: 7,4 in variety I (r=-0,97, r=-0,77 and r=-0,77 respectively).

Discussion

Our results revealed that in both varieties, the higher the sludge/soil ratio the lower the Cd concentration (Tables 3,4 – sludge/soil ratio 40% : VI p=0,004, VII p=0,04). The above findings are in agreement with reports suggesting that sludge application limits Cd bioavailability due to enhanced chelation and absorption mechanisms of the organic substrate (**4,9**).

Table 3. Cadmium levels of variety I tobacco plants at different substrates and leaf harvesting.

Leaf	Substrates							
	0%		10%		20%		40%	
Height	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median
1	4,348±2,08	4,315	4,05±1,132	4,066	4,617±1,21	4,581	3,447±1,25	3,403
2	3,707±0,10	3,7	2,67±0,42	2,644	2,871±1,12	2,829	1,934±0,14	1,962
3	2,06±0,2	2,076	1,4±1,03	1,396	1,55±1,43	1,555	1,389±0,75	1,39
4	1,251±0,06	1,23	2,33±0,718	2,331	1,621±1,2	1,640	1,614±0,06	1,620
Total	3,07±1,59	2,394	2,612±1,27	2,657	2,665±1,73	2,810	2,09±1,07	1,962

Table 4. Cadmium levels of variety II tobacco plants at different substrates and leaf harvesting.

Leaf	Substrates							
	0%		10%		20%		40%	
Height	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median	Mean±sd	Median
1	1,018±0,26	1,09	1,476±0,87	1,5	1,077±0,04	1,06	1,009±0,54	1,03
2	2,24±1,18	2,024	1,537±0,94	1,54	1,316±0,38	1,31	1,041±0,28	1,04
3	3,778±2,1	3,77	2,782±0,89	2,8	2,829±0,74	2,84	2,03±0,78	2,04
4	3420±1,53	3,41	2,606±1,12	2,59	2,410±0,89	2,42	2,157±0,7	2,15
Total	2,614±1,75	1,91	2,1±1,09	2,16	1,908±0,94	1,63	1,561±0,79	1,38

In relation also to substrate pH, a parameter known to affect soil cation exchanging capacity- CEC, our data showed that Cd was higher at pH1: 5,5 in both tobacco varieties (Fig.3,4).

Related references agree in that Cd-concentration in tobacco decreased as soil pH increased to pH: 6,0 but no decrease was noted at higher pH. Sludge rate effect was significant at low pH but diminished as pH increased (**10**).

Miner *et al*, (**4**), also conclude that best-fit regression models incorporating soil parameters such as pH, clay and organic carbon- OC, along with extractable metals described plant metal concentration with high R^2.

Cadmium effect on nicotine concentration on the other hand, is a very important issue since both the metals and alkaloids comprise major health impacts.

Nicotine represents the 85-95% of total tobacco alkaloids (**11,12**). The alkaloid exerts acetylcholine- Ach actions binding to nicotinergic receptors and thus producing dependence to tobacco smokers (**13,14**). Nicotine is also related to cancer pathogenesis by 1) mitogen-activated protein-MAP activation resulting to bcl-2 enhanced expression and cancer cell apoptosis inhibition, and 2) protein-kinase C-PKC and extracellular signal regulated kinase -ERK 2 activation by

counteracting the anticarcinogenic actions of therapeutically administered opioiods (**15**).

According to our data, both varieties II and I present significantly lower nicotine levels at pH1: 5,5 with the exception of variety II in the 40% sludge/soil ratio. Also our results show that tobacco variety I was in general characterized by lower nicotine levels and higher Cd concentration as compared to variety II (Tables 1, 2, 3, 4. Fig. 1, 2). Consequently, low nicotine concentration may be attributed to inhibiting effects of Cd on biosynthesis of the alkaloid. Nevertheless, reports on Cd effects on nicotine synthesis are conflicting. Li *et al*, (**6**), are in agreement with our results suggesting that nicotine decreases with the increase of Cd. Wen's findings (**8**) suggest that total nitrogen and total alkaloids increased in tobacco leaves in proportion to Cd, Zn and Cu applied to tobacco plant. In our opinion Cd and nicotine interaction is a multiparametric relation, proven actually by multiple regression analyses of our data (not shown here). These multiple regression analyses revealed that among the investigated parameters (Cd, Total N, NO_3, Cu, Zn, Pb, Cr, Cl) in variety I , Cd concentration was the important predictor of nicotine levels considering pH 5,5 to 7,4 (Beta : -1,13 with p=0,000 and Beta= –1,17 with p=0,0002, correspondingly). In variety II on the other hand, Cu was the important predictor of nicotine levels at pH 5,5 (Beta : -0,78 with p=0,000).

A determinative parameter of Cd kinetics in the tobacco plant was the age of the leaves directly related to leaf height or otherwise leaf harvesting. King and Hajjard, (**10**), report general decline in Cd, Ni, Zn concentrations with increasing leaf height, suggesting a limited mobility of the metals in the younger leaves. They also report that over pH:5,8, Cd levels remain more or less steady even at higher sludge/soil ratios and regardless leaf age. In our data, leaf harvesting (#1) represented the older and lower leaves while leaf harvesting (#4) represented correspondingly the younger and higher ones. All substrate proportions according to our data, produced in general Cd decreases with increasing sludge/soil ratio in both varieties. In Figs. 5 and 6, it is shown that Cd significantly decreased in younger leaves in variety I, while in variety II increased, at sludge/soil 20% ratio and pH :5,5 or 7,1. Therefore, genetic features may explain that Cd and Cu are two discrete important predictors of nicotine levels in variety I and variety II, correspondingly.

In conclusion, sewage sludge/soil ratios in reference to its metal concentration and the tobacco varieties may be determinant tools in the tobacco technology regarding nicotine levels and hence tobacco organoleptic properties.

References

1. Akehurst B.C. (1981) Tobacco, Tropical Agriculture Series, 2nd edition, Longman Inc., New York.
2. Divanidis S., Halivopoulos S. (2000) Data of Tobacco Institute.
3. Gigliotti G., Giusquiani P.L., Businelli D. (1993) *Annali della facolta di Agraria Universita degli studi di Perugia,* **44**(1):563-575.
4. Miner G.S., Gutierrez R., King L.D. (1997) *Journal of Environmental Quality*, **26**:989-994.
5. Gutenmann W.H., Bache C.A., Lisk D.J. (1982) *J. of Toxicology and Environmental Health*, **10**: 423-431.

6. Li Y.,Wang H.X.,Wu Y.S. (1992) *Acta Ecologica Sinica*, **12** (2):147-154.
7. Fergusson J.E. (1990) *Chemistry, Environmental impact and Health Effects*, **10**:377-405.
8. Wen T.C. (1983) *Bull. Taiwan Tob. Res. Inst.*, **19,** 21-33.
9. Bruwaene R.V., Kirchmann R., Impens R. (1984) *Experienta*, **40**: 52.
10. King L.D., Hajjar L.M. (1990) *J. Environ. Qual.*, **19**:738-748.
11. Hoffman D., Brunnemann K.D., Prokopczyk B., Djordjevic M.V. (1998) *J. of Toxicology and Environmental Health*, **41**: 1-52.
12. Scmeltz I., Hoffman D. (1977) *Chem. Rev.*, **77**:295-311.
13. Guyton A., Hall J. (1997) Human Physiology and Mechanisms of Disease, 6th edition, W.B. Saunders company, London,.
14. Krasnegor N.A. (1979) *Natl. Inst. Drug Abuse Res. Monogr. Ser.*, **23**:194.
15. Hesch W.L., Mneckjee R. (1998) *Carcinogenesis*, **19** (4): 551-6.

Chapter 39

APPLICABILITY OF FLY ASH IS SOUND FOR RHIZOSPHERE, BIOMASS YIELD, ENHANCED BANANA PRODUCTIVITY AND SUSTAINABLE ECO-SYSTEM

N.V. PHIRKE, S.B. CHINCHOLKAR, R.M. KOTHARI

School of Life Sciences, North Maharashtra University, Jalgaon-425 001 (India); Email: rmkothari@rediffmail.com

Abstract

Applicability of fly ash (FA) was explored for qualitative and quantitative improvement(s) in banana orchards, in light of its physico-chemical properties, imparting porous texture to soil, water holding capacity, increase in aeration, supplement calcium and phosphate and micronutrients cost-effectively. For this purpose, FA was applied at 1.2 kg/plant (i.e. 5 MT/hectare) without and with soil conditioner (SC), consortium of four bio-fertilisers (BF) and plant growth regulator (PGR), individually and in combinations. Upon statistical analysis at p = 5 %, it was found that addition of FA (i) did not affect per cent survival of plants upon transplantation, (ii) improved an uptake of NPK, (iii) in conjunction with SC and BF stimulated the growth of biomass as judged from measurement of height and girth of pseudostem, (iv) enhanced degree of ramification of root system, (v) did not adversely affect microbial profiles in rhizosphere, (vi) partially increased VAM spore count as well as its infection, (vii) maintained minimal 40-50 % siderophore units in the mycorrhizosphere, (viii) afforded incrementally more chlorophyll content in the leaves, (ix) possibly rendered plant protection and finally (x) provided 15-20 % increase in the yield of banana. Ion chromatographic system revealed almost total absence of As, Cd, Cr, Hg and Pb (the toxic elements present in fly ash in mg quantities) in pseudostem and banana. The trend of these observations persisted in both trials conducted at the 1-acre and 4-acre scale at separate places during the year 1998-99 and 1999-2000. The keeping quality of banana improved ten fold, while qualitatively bananas were sweeter and softer compared to those grown by traditional methods, under identical conditions. Thus, in the light of the above data, it appeared that application of fly ash is ecologically safe, economically desirable, logistically practicable and in favour of sustainability for farmers/consumers.

Key words: Banana cultivation, Traditional approach, IPNM approach, Soil Conditioner, Consortium of Biofertilisers, Plant Growth Regulator, Fly ash, Drip irrigation, Sustainable productivity.

New Horizons in Biotechnology, 423-436, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Introduction

Banana cultivation is presently undertaken through traditional farm practices marginally refined on the basis of (i) personal experience of the farmers and (ii) commercial advertisements on the efficacy of certain inputs. These practices involved indiscriminate application of (i) chemical fertilisers for nutrition, (ii) pesticides for plant protection and (iii) frequent irrigation by flooding, while forgetting the application of organic carbon. Continuation of these practices over 25-30 years has led to (a) increase in the cost of inputs, (b) reduction in the population of beneficial microflora in soil, (c) increase in its salinity and (d) stagnation/reduction in the productivity of banana. To remedy this scenario, integrated plant nutrition management (IPNM) appeared to be a sustainable proposition (**1**). The IPNM concept involved an application of soil conditioner (SC), plant growth regulators (PGR), consortium of biofertilisers (BF) and drip irrigation (DI). Besides, in view of banana composition, requirement of calcium, phosphate and micro-nutrients for its cultivation appeared vital and remediable by the application of fly ash in a logistically viable manner. Due to its (i) inherent ability to amend soil characteristics for porous texture (for coarse as well as fine grained soils), (ii) cost-free availability for enhanced water holding capacity (for coarse grained soils), rendering overall cost: benefit ratio in favour of farmers, (iii) ability to reduce soil bulk density and increase aeration by crack formation in clayey soils (**2,3**) and (iv) use, meriting reduction in the cost of phosphatic fertilisers by 25-40 %, FA incorporation in banana orchards appeared worth exploring. However, whether fly ash would provide these attributes for rapid/healthy growth and productivity of banana, while keeping soil texture and its microflora undisturbed has long been debated (**2,4-7**). To address these issues, efforts made in safe application of fly ash are discussed in the present article.

Materials and Methods

Banana cultivar

Elite banana suckers (dwarf Cavendish AAA, *Basrai* variety, designated as *Shrimanti*), cultivated extensively, were chosen.

Soil conditioner

It was prepared from banana pseudostem after banana harvesting by solid state fermentation under warm, humid and aerobic conditions in eight weeks (**8**).

Plant growth regulator

It was prepared by acid hydrolysis of upgraded corn gluten (75% protein) as discussed earlier (**9**).

Consortium of biofertilisers

An exponentially growing inoculum of efficient *A. chroococcum* for nitrogen fixation, *A. awamorii* for phosphate solubilisation, *T. thiooxidans* for sulphur oxidation, *T. imbricatum* and local VAM population for moisture arresting was prepared as described earlier (**10,11**).

Fly ash

It was procured cost-free from Deepnagar Thermal Power Station, Bhusawal and analysed (**12**).

Drip irrigation

It was commissioned by M/s. Jain Irrigation Systems, Jalgaon.

Chemical fertilisers

Urea, single super phosphate (SSP) and muriate of potash (MOP) were locally procured.

Experimental set up

Experimental trials to explore the utility of fly ash in conjunction with IPNM were conducted in triplicate during July, 1998-October, 1999 and repeated during July, 1999-October, 2000 by giving 12 treatments in random block design (RBD) as summarised in Table 1.

Table 1: Profiles of fly ash application in conjunction with IPNM

Tr.	Description	Tr.	Description
T-1	50 % Chem. Fert.	T-7	SC 10 $Mtha^{-1}$
T-2	100 % Chem. Fert.	T-8	SC + BF
T-3	150 % Chem. Fert.	T-9	SC + FA
T-4	BF	T-10	SC + FA + BF
T-5	FA 5 $MTha^{-1}$	T-11	PGR
T-6	FA + BF	T-12	SC + FA + BF + PGR

Tr. = Treatments; Chem. Fert. = Chemical fertilisers; BF = Biofertilisers; FA = Fly ash

Farm practices

Plantation was carried out at a distance of 150 cm between two plants as well as between two rows, with the application of SC (10 MT/ha), PGR (by sucker drenching and three foliar sprays), BFs (each 20 L/ha), FA (5 MT/ha) and chemical fertilisers (50 % of the recommended dose (**13**), N at 450 kg/ha; P_2O_5 175 kg/ha and K_2O at 450 kg/ha). While SSP and MOP were applied in two splits (first as a basal dose and second dose two months after plantation), urea was applied through DI on daily basis up to six months, for which DI was commissioned (**14**). Similarly, post-plantation care was taken as suggested in Souvenir (**15**).

Parameters monitored

During growth phase (four months from plantation) per cent survival of banana plants, their height, girth, number of leaves, number of suckers and banana bunch weight were monitored by standard methods (**16**). N, P, K were analysed after eight months from a composite foliar sample derived from the randomly selected plants from each replication (**12**). Average chlorophyll content of leaf lamina, third from emergence, was estimated (**17**). Number of VAM spores (from composite rhizospheric sample of each replication) and % VAM infection (from hairy

absorbing root samples collected at the depth of 25 cm) were respectively monitored (**18,19**). Per cent siderophore units were estimated (**20**). Presence/ absence of As, Cd, Cr, Hg and Pb was monitored by ion chromatography system (ICS, Shimadzu, Japan). Results of all these parameters were subsequently analysed statistically using the ANOVA software developed by M/s. Indostat Services Pvt. Ltd., Hyderabad.

Results and Discussion

Jalgaon district is situated on central peninsular India, covered by vertisols and associated soils, commonly called as black cotton soils (**21**). Though agricultural research in the last three decades contributed to higher yield of banana, the approach lacked sustainability due to lack of consideration on soil texture, porosity, salinity, water logging, microbial population and accumulation of pollutants. It was experienced that black cotton soils get compact when dry and disperse upon tillage. When soil gets compacted, its bulk density increases, thereby reducing porosity, aeration, infiltration rate and impeding drainage (***22***). A narrow range of moisture in them allows growth of root system and its lack creates adverse growth conditions. To overcome these problems and its adverse effects on banana plantation, incorporation of SC was recommended (**23**), also substantiated by vast data from Soil Testing Laboratory, Jalgaon. In the light of analysis of experimental farms, these considerations led to application of SC and FA in the present work.

Physico-chemical and microbial analysis of soil

The analysis of soil of experimental farms at NMU and BAT is in Table 2.

Table 2: Analytical profiles of research farm soils at NMU and BAT.

Sr. No.	Soil characteristics	NMU soil	Status	BAT soil	Status
1.	pH	7.9	Normal	7.4	Normal
2.	Total soil salinity (mmhocm^{-1})	0.47	Normal	0.56	Normal
3.	Water holding capacity (%)	52.4	Moderate	58.7	Moderate
4.	Soil density (gcm^{-3})	1.28	Normal	1.12	Low
5.	Organic carbon (%)	0.13	Very low	0.43	Medium
6.	Available N (kgha^{-1})	147.3	Low	194.4	Low
7.	Available P_2O_5 (kgha^{-1})	17.0	Low	20.0	Low
8.	Available K_2O (kgha^{-1})	678	Very high	1084	Very high
9.	Exchangeable Ca (meq/100 g soil)	61.6	--	26.4	--
10.	Exchangeable Mg (meq/100 g soil)	10.5	--	4.20	--
11.	Exchangeable Na (meq/100 g soil)	50.0	--	21.5	--
12.	Mn (μgg^{-1})	36.6	Enough	8.9	Enough
13.	Cu (μgg^{-1})	11.0	Enough	2.6	Enough
14.	Fe (μgg^{-1})	3.0	Enough	21.0	Enough
15.	Zn (μgg^{-1})	2.1	Enough	0.9	Enough
16.	Total microbia (x 10^5 cfug^{-1})	31	--	430	--
17.	VAM spore density (g^{-1})	14.0	--	18.0	--
18.	Nematode population (g^{-1})	0.8	--	0.6	--

meq = milliequivalent; cfug^{-1} = colony forming units per gm of soil

From Table 2, it was apparent that NMU and BAT farm soils were comparable in (i) nitrogen and phosphorus deficiency, (ii) slight alkalinity, (iii) moderate water holding capacity and (iv) very high K content and differed in soil density, organic carbon and total microbial count. These differences reflected in their texture and in

turn some effect on growth and productivity of banana per hectare. Whether biotech inputs envisaged in IPNM levelled up such differences and gave a similar trend in productivity was worth examination.

Fly ash

It is a major waste of coal based thermal power stations. It was procured cost-free with US $ 33 per trolley containing 5 MT for transportation charges. Since this amount was applied per hectare, the cost of FA application was US $ 33. Its composition from 11 thermal power stations has confirmed that its overall properties are comparable, with a marginal variability depending upon grade/composition of coal, combustion conditions, efficiency of combustion, type of emission control devices and method of disposal (**24**). Physically, FA is a mixture of small, glassy, hollow particles with size from 0.01-100 μm and specific gravity from 2.1-2.6, giving rise to amorphous and crystalline phases and thereby imparting porous texture and water holding capacity.

Chemically, fly ash (i) is devoid of N and organic carbon due to volatilisation during combustion of coal, (ii) contained mg quantities of toxic elements like arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg) and lead (Pb), (iii) is poor in manganese (Mn) and zinc (Zn), (iv) has comparable phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and copper (Cu) and (v) has high sulphur (S), boron (B), iron (Fe) and molybdenum (Mo) (**4**). As a result of combustion of coal, FA ingredients are in the form of oxides. While (i) SiO_2, Al_2O_3 and Fe_2O_3 accounted for 92-94 %, (ii) CaO and MgO accounted for 3-5 % and (iii) minor (0.5-1.5 %) constituents comprised of 20 micro-nutrients and some heavy/toxic metal oxides. Their quantum in fly ash appeared to be comparable with that in sandy loam or loamy sand soil as summarized in Table 3 (**25**).

Factors pertinent in fixing FA dose

The following factors were kept in mind while fixing the dose of 1.2 kg FA per plant in banana farm application: (i) high dose of FA develops compacted layers which reduce aeration, water infiltration and root penetration (**4**), (ii) immediate response of application of its heavy dose is an increased alkalinity/salinity of soil which gradually weathers out, presumably by leaching through rainfall and irrigation, (iii) excess addition of FA leads to formation of carbonates of CaO and MgO by reaction with atmospheric CO_2, leading to pH 8.2 of aqueous suspension of fly ash (**26**). On the basis of (a) uptake of micro-nutrients studied for vegetables, cereals, pulses and forestry crops (**3**), (b) comparability established (**25**) and (c) the above facts, application of 1.2 kg FA per plant was considered neither low nor high; it appeared adequate to meet Ca, PO_4 and micronutrient requirements.

Table 3: Comparison of fly ash characteristics with soil types.

Elements	NTPC-Fly ash	Sandy loam soil	Loamy sand soil
Al	14.8	1.4	2.4
As	1.8	0.8	0.5
B	18.8	0.4	0.3
Ca	353.3	333.0	310.0
Cd	0.2	0.1	0.1
Co	0.2	0.2	0.1
Cr	0.3	0.4	0.2
Cu	2.4	5.5	0.7
Fe	38.3	23.1	10.5
Hg	2.3	0.4	1.3
K	99.5	127.0	76.0
Mg	95.4	134.0	64.0
Mn	2.4	22.3	11.0
Mo	3.4	0.3	0.3
Ni	0.8	0.7	0.2
P	32.4	18.6	15.0
Pb	1.1	0.8	0.7
S	99.8	16.5	10.0
Zn	1.4	7.0	1.6

Content of each element is expressed as $\mu g g^{-1}$ of soil.
Values expressed in bold are harmless and normal nutrients of plants system.

Experimental design

In both trials, twelve treatments were given. Amongst them, with the application of chemical fertilisers alone at different doses, T-1 to T-3 were considered as traditional (control), while with the application of biotech inputs and FA, T-4 to T-12 were considered as IPNM (experimental). Cultivation in RBD and statistical analysis of the data permitted to establish a cause and effect relationship as depicted in Fig. 1-10.

Effect of FA on per cent survival of banana plants

It is clear from Fig. 1 (a and b) that FA did not affect per cent survival of banana suckers in both trials. This gave the first proof that although FA contains toxic elements, they do not affect vital life processes pertinent to transformation of suckers into tender plantlets. Had it exhibited slight toxicity, rate of germination would have been delayed vis-à-vis control. This also did not happen. About 20 % higher survival upon transplantation in experimental plants (T-4 to T-12) compared to control plants (T-1 to T-3) confirmed it to be harmless.

Effect on vegetative growth

Application of FA in conjunction with SC and BF increased the height of pseudostems significantly in both trials vis-à-vis FA alone (Fig. 2). The same pattern was repeated in the case of diameter of girth of pseudostems, albeit not as distinctly as in the case of height (Fig. 3). In the case of leaves, since no comparable pattern emerged from both trials (Fig. 4), correlation between cause and effect

relationship was difficult. However, the number of suckers were significantly high due to SC (T-7) and comparable to those in T-9, T-10 and T-12, indicating a synergistic role of FA in conjunction with SC, BF and PGR (Fig. 5). Thus, from the profiles of height, girth, and number of leaves/suckers, a broad pattern emerged that FA has exhibited no toxicity; instead it played a synergistic role in the presence of biotech ingredients of IPNM, quite probably a contribution from its soluble micronutrients.

Theoretically, with the incorporation of SC and FA in soil, its water holding capacity had enhanced and hence, there was no need for wider ramification of root system for absorption of nutrients/moisture. Yet wider ramification in the presence of FA alone or in combination with individual ingredients of IPNM suggested that FA played a positive role in ramification of root system (biomass growth). These findings are in concurrence with an earlier report (**3**), which indicated that upto 25 % (w/w) fly ash fortification showed increased plant growth and yield by about 40 % and uptake of heavy metals, if any, was within permissible limits.

Effect on vigour

By and large, banana plants with FA input appeared turgid, healthy and greener compared to those of control. While FA alone (T-5) contributed in a divergent manner to chlorophyll content in both trials (Fig. 6), for reasons difficult to establish, in conjunction with SC, BF and PGR, it exhibited maximum synergistic effect, which was most pronounced when all IPNM ingredients were present (T-12). Increase in chlorophyll content with individual ingredients of IPNM indicated that its soluble micronutrients, notably Mg (which is constituent of chlorophyll) must have contributed to increasing the vigour of banana plants.

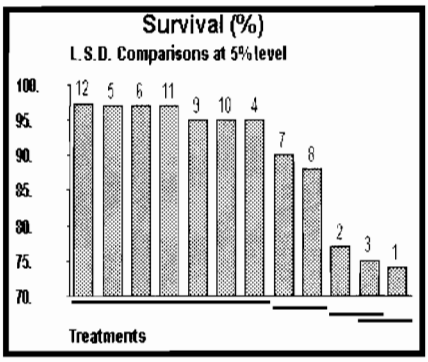
Effect on VAM spores and per cent infection

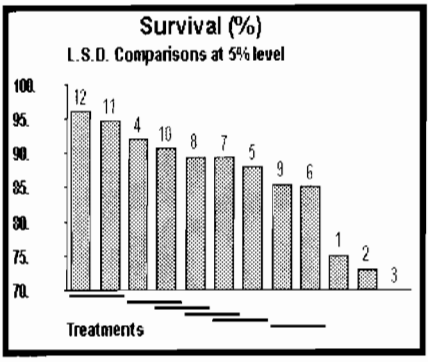
In comparison to control (T-1 to T-3), a number of VAM spores were significantly more in T-9, T-10 and T-12 and quite comparable with T-5 (FA alone) treatment, reflecting the contribution by FA (Fig. 7). This was subsequently confirmed from per cent VAM infection (Fig. 8). Both these observations have exposed the myth that FA exhibits an adverse effect on life processes. Had this been a reality, number of VAM spore, percent VAM infection and above all NPK uptake would not have improved in FA fortified treatments. Presence of less VAM spores and less % VAM infection in control vis-à-vis in presence of FA alone in comparison with increase in T-12 treatment indicated that basically moderate amount of FA incorporation is not toxic to plant life. Increased number of spores and per cent infection is also due to contribution by BF (T-6) and SC (T-9) in the increasing order. Had it been toxic, more spore formation should have been inducted, reflecting adverse physiological conditions to combat alleged toxicity due to its toxic elements.

Effect on microbial population and per cent siderophore units

Generally, a question is raised about the entry of PPM level toxic elements of fly ash in the food chain. To avoid this contingency, addition of SC was made, rendering conditions more favourable for soil microbe interaction due to provision of organic carbon as a source of energy and nitrogen for microbial growth. This is

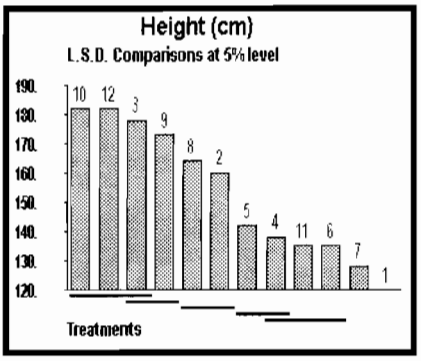
supported by others too (**27, 28**). Actively thriving microflora throughout the trial period and presence of siderophores suggested pseudomonas type rhizobacteria, responsible for the synthesis of siderophores. A non-coherent profile of percent siderophore units (Fig. 9) indirectly supports a view that with the presence of adequate amount of Fe and actively propagating microflora contributed by BF, possibly Fe was immobilised as phosphate, while presence of FA alone contributed to 50% siderophore units in both trials.

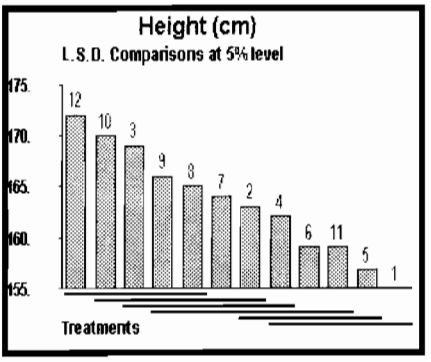




a. At NMU farm b. At BAT farm

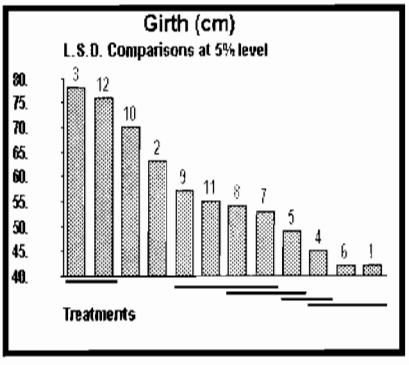
Fig. 1: LSD comparative profiles of percent survival as a function of traditional and biotech inputs.

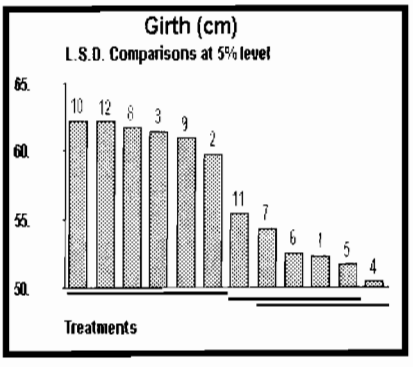




a. At NMU farm b. At BAT farm

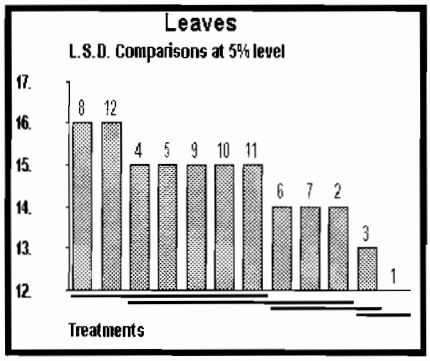
Fig. 2: LSD comparative profiles of height as a function of treatment of traditional and biotech inputs.

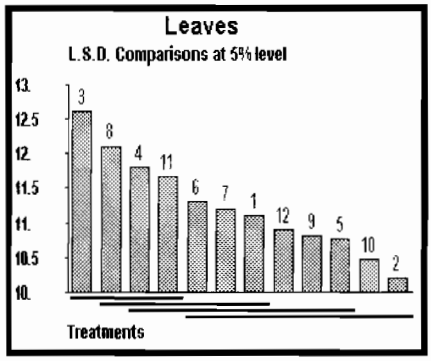




a. At NMU farm b. At BAT farm

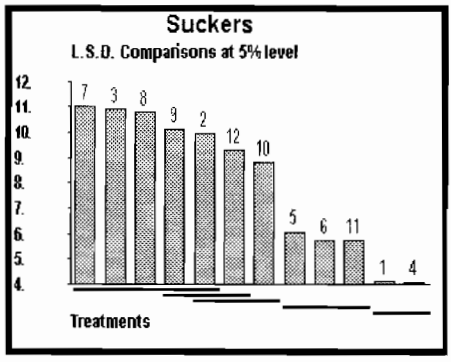
Fig. 3: LSD comparative profiles of girth as a function of traditional and biotech inputs.

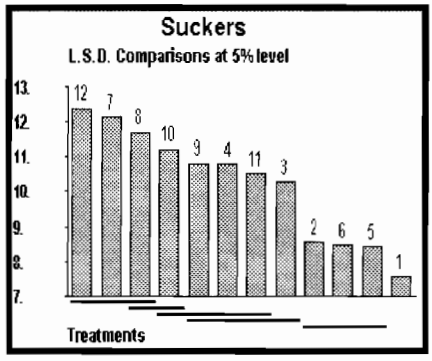




a. At NMU farm b. At BAT farm

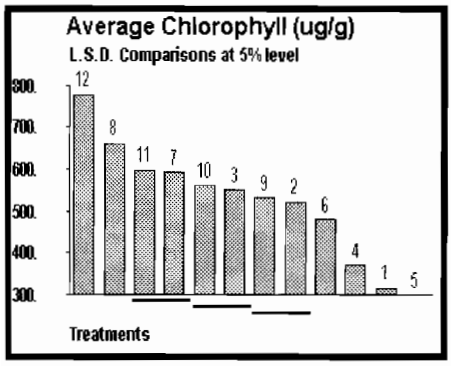
Fig. 4: LSD comparative profiles of leaves as a function of traditional and biotech inputs.

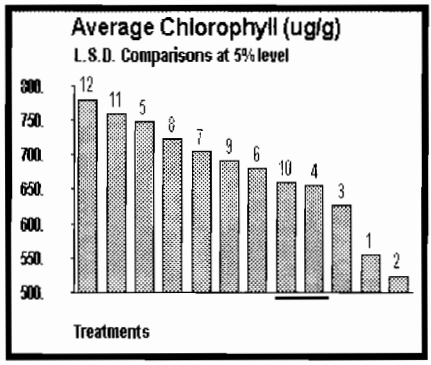




a. At NMU farm b. At BAT farm

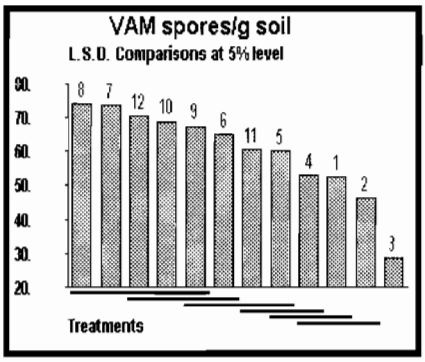
Fig. 5: LSD comparative profile of suckers as a function of traditional and biotech inputs.

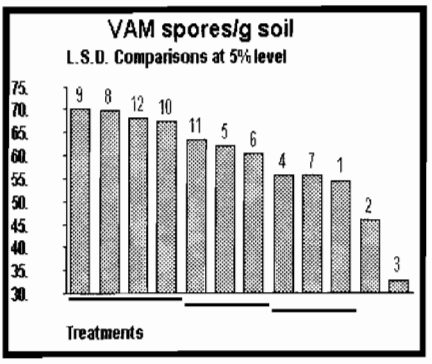




a. At NMU farm b. At BAT farm

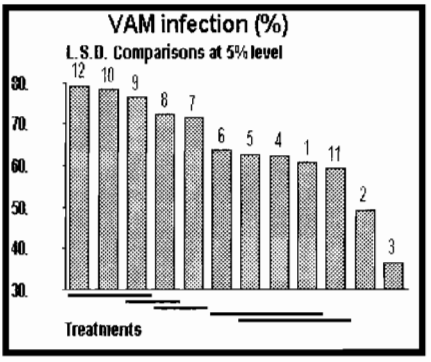
Fig. 6: LSD comparative profile of average chlorophyll as a function of traditional and biotech inputs.

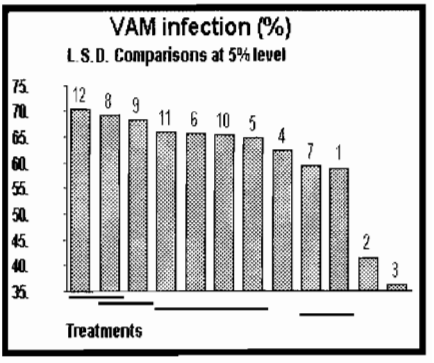




a. At NMU farm b. At BAT farm

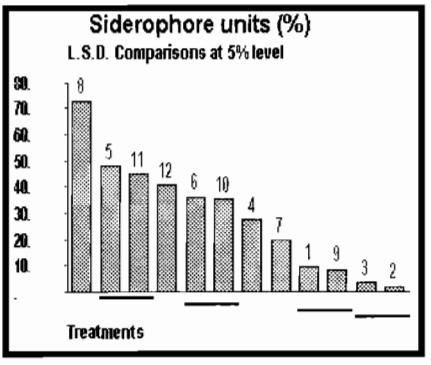
Fig. 7: LSD comparative profile of VAM spores g^{-1} soil as a function of traditional and biotech inputs.

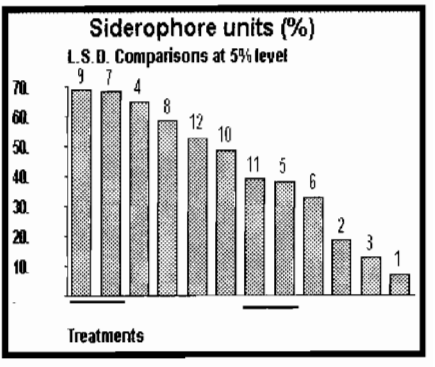




a. At NMU farm b. At BAT farm

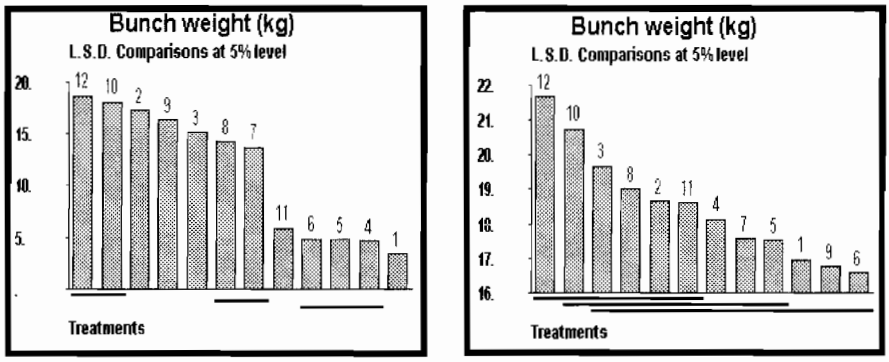
Fig. 8: LSD comparative profiles of percent VAM infection as a function of traditional and biotech inputs.





a. At NMU farm b. At BAT farm

Fig. 9: LSD comparative profiles of percent siderophore units as a function of traditional and biotech inputs.



a. At NMU farm b. At BAT farm

Fig. 10: LSD comparative profile of average bunch weight as a function of traditional and biotech inputs.

Effect on banana productivity

FA was applied in the form of a ring, 30 cm around the banana sucker @ 5.0 MT/ha as a one time input at the time of plantation for improvement in (i) soil porosity and aeration, (ii) water holding capacity and (iii) availability of macro- and micro-nutrients for enhancement of plant growth (**6,7**). It has afforded an increase in the yield by minimum 15 % compared to control (Fig. 10).

In isolation, 15 % increase in banana yield may not appear significant. However, considering (a) 50 % less application of chemical fertilisers and (b) consumption of 40-50 % less water (and hence, less electricity), 15 % increase in banana output in T-10 and T-12 is significant. This is yet noteworthy from the trend in two trials in different geo-climatic conditions by virtue of trials being carried out (a) in different quality of soil as indicated in Table 2, (b) at different places and (c) in different years. Further noteworthy is the keeping quality of experimental banana for fifteen days vis-à-vis traditional banana for three days.

Ingredient-wise effect of FA on soil amendment for banana

From the above results, it is clear that basically there is no scientific rationale not to use fly ash as a source of trace nutrients and supplementary fertilisers. Its major (50-55 %) component is SiO_2, an inert matter and a major component of all types of soils. Its second largest (20-25 %) ingredient is Al_2O_3. Although it is toxic, its physical presence in soil does not find entry in pseudostem of banana, leave alone banana fruit due to (a) dilution factor by soil as a result of amendment and (b) its sparing solubility in water. In fact, vast stretches of land in Kutch (Gujarat) and Orissa, rich in Al_2O_3 (Bauxite), cultivating respectively bajara and rice from ages have not reported any toxic effect as a result of consumption being exhibited on plants, cattle and human beings. Mn and Zn being poor in FA in comparison to an average soil composition (Table 3), they are not inhibitory to banana plant metabolism. Ca, K, Mg and Cu being comparable to soil composition (Table 3),

they too do not pose any problem in FA application. Fe, Mo, B and S are in higher amount compared to their amount in an average soil (Table 3). However, being in the form of oxides, they are not anticipated to be injurious to banana by virtue of their dilution by soil and insolubility (**29**).

Thus, by and large FA application for micro-nutrient supplements in banana cultivation does not pose any threat either to soil texture, microflora and banana itself. This has been experimentally found to be true by ICS wherein As, Cd, Cr, Hg and Pb were barely detectable as a blip in comparison to a sharp peak of their respective standards at 1 ppm level. Finally, the presence of ectomycorrhiza *T. imbricatum* and native 10 species of endomycorrhizae found in the soil are anticipated to micro-encapsulate these elements, thereby prohibiting their entry in the food chain (**30**).

Apprehension of FA toxicity is ruled out

The dose of FA applied in the present studies is 1.2 kg and it gets mixed with minimum 120 kg of soil around every plant. Thus, 100-fold dilution automatically dilutes mg quantities of toxic elements of FA into µg quantities. In the microenvironment of soil, they are simultaneously subjected to (a) permeation to lower strata of soil, (b) immobilisation by local acidity created by phosphate solubilising microbes, (c) complexation by a variety of organic acids generated by local microflora, (d) micro-encapsulation by endomycorrhizae, and (e) diluted by a variety of forces not known presently. As a cumulative effect of these activities solubilisation of µg quantities and dilution to nanogram quantities has a remote chance of entering the food chain. Data contrary to their entry is already on record (**28**). Accordingly, it was suggested that SC fortification in soil has buffering effect on pH fluctuations and FA application in conjunction with mycorrhizae has further moderating effect on the entry of insoluble elements in the food chain. With an inherently insoluble nature of these oxides fortified in soil, it is indeed difficult to envisage as to how selectively they would enter the food chain to pose a threat to human health and eco-system.

Conclusion :

Its use is a pollution containment strategy by recycling for increasing the productivity through the availability of Ca, PO_4 and micro-nutrients. Recycling of SC, fly ash, PGR, all of which are waste derived products of society being a corner of good bio-resource management, fly ash incorporation at a negligible cost in banana cultivation rightfully deserves consideration in IPNM.

Acknowledgements :

Thanks are due to (i) Prof. S. F. Patil, Vice-Chancellor for providing research farm facilities, (ii) Development Board for the Rest of Maharashtra, Mumbai for financial assistance and (iii) Mr. R. P. Patil, Garden Superintendent for providing critical inputs for conducting these studies meaningfully.

References

1. Phirke N.V., Patil R.P., Sharma R.K., Kothari R.M., Patil S.F (In press) *in* The environment 2001-Global changes and challenges, Prakash, R. ed., Sci. Publ., Meerut, India.
2. Chang A.C., Lund L.J., Page A.L., Warneke J.E. (1977) *J. Environ. Qual.* **6**, 267-270.
3. Mandal S., Saxena M. (1998) *J. Environ. Studies & Policy*, **2**, 29-38.
4. Bradshaw A.D., Chadwick M.J. (1980) The restoration of land, University of California Press, Berkley, CA, USA.
5. Adriano D.C., Page A.L., Elseewi A.A., Chang A.C. (1980) *J. Environ. Qual.* **9**, 333-334.
6. Aitken R.L., Campbell D.J., Bell L.C. (1984) *Aust. J. Soil Res.* **22**, 443-453.
7. Jacobs L.W., Erickson A.E., Berti W.R., MacKellar B.M. (1991) *in*: Proc. 9th Int. Ash use symp. **3**, 59-61.
8. Ramamurthy V., Sharma R.K., Kothari R.M. (1998) *in* Adv. Biotechnol., Pandey A., ed., Edu. Publ., New Delhi, India, pp. 433-438.
9. Sharma R.K., Yadav K.R., Kothari R.M. (1994) *Technovation*, **14**, 31-36.
10. Kothari R.M. (1995) *in* Popular lecture series, Punjabi University, Patiala by Punjab State Council for Science and Technology, Chandigarh and Dept. of Biotechnology, New Delhi, India, pp. 1-11.
11. Talegaonkar S.K. (2000) Ph.D. dissertation, North Maharashtra University, Jalgaon, India.
12. Somwanshi R.B., Tamboli B.D., Kadu P.P. (1997) A laboratory manual, MPKV Res. Publ. No. 18, MPKV, Rahuri, India.
13. Pawar V.P., Kathmale D.K., Deshmukh Z.V., More T.A. (1997) *J. Maharashtra Agric. Univ.* **22**, 166-168.
14. Phirke N.V. (2001) Ph.D. dissertation, North Maharashtra University, Jalgaon, India.
15. Souvenir (1999) Seminar on Technological advancement in banana production, handling and processing management (March 27-28), Jalgaon, India.
16. Deo D.D., Sadawarte K.T., Shelke B.D., Paithankar B.H., Mahorkar V.K. (1998) *PKV. Res. J.* **22**, 47-49.
17. Jayraman J. (1996) Laboratory manual in biochemistry, New Age Internat. Publ., New Delhi, India, pp. 171-172.
18. Gerdemann J.W., Nicolson J.H. (1963) *Trans. Brit. Mycol. Soc.* **46**, 235-244.
19. Phillips J.M., Hayman D.S. (1970) *Trans. Brit. Mycol. Soc.* **55**, 158-161.
20. Schwyn B., Neilands J.B. (1987) *Analyt. Biochem.* **160**, 47-56.
21. Murthy R.S., Bhattacharjee R.J., Landey R.J., Pofali R.M. (1982) Trans. 12th International Congress of Soil Science, New Delhi, India, pp.3-22,
22. Kadam J., Jagtap B. (1994) Soil fertility and Crop Production, Utkarsha publication, Pune, India.
23. Varade S.B. (1994) *in* Natural Resources management for sustainable agriculture and environment, Deb. D.L. ed., Angkor Publ. (P) Ltd., New Delhi, India, pp 159-166.
24. Soni D.K., Bedi R.B.L., Bansal R.K., Jain A. (1995) *in* Natl. Conf. on fly ash waste or wealth, TIET, Patiala, India, pp. 257-261.
25. Singh D., Kansal B.D. (1995) *in* Natl. Conf. on fly ash-waste or wealth, TIET, Patiala, India, p.1-7.
26. Adriano D.C., Page A.L., Elseewi A.A., Chang A.C. (1982) *J. Environ. Qual.* **11**, 197-203.
27. Wong M.H., Wong J.W.C. (1986) *Environ. Pollut. Ser. A*, **40**, 127-144.
28. Klubek B., Carlson C.L., Oliver J., Adriano D.C. (1992) *Soil Biol. Biochem.* **24**, 1119-1125.
29. Budavari S. (1989) The Merck Index (XIth Edn.), Merck & Co. Inc. New Jersey, USA.
30. Varma A., Hock B. (1995) Mycorrhizae: Structure, function, molecular biology and biotechnology, Springer-Verlag, Berlin, Germany.

Chapter 40

IMPACTS ON VESICULAR-ARBUSCULAR MYCORRHIZAL (VAM) FUNGI DIVERSITY ASSOCIATED WITH DEGRADATION OF SOILS IN HUMID TROPICAL FORESTS

S.C. TIWARI, S.S. SINGH

Department of Forestry, North Eastern Regional Institute of Science and Technology (NERIST), P.O. Nirjuli-791 109 (Itanagar), Arunachal Pradesh, India

Abstract

VAM spore population was studied under degraded, least degraded and undegraded forest soils with an objective of assessing the influence of soil degradation on VAM spore distribution at two soil depths, surface layer (0-20 cm) and sub-surface layer (20-40 cm). Physico-chemical properties of soils in all the three sites were also studied. The increase in degree of soil degradation caused a reduction in VAM spore population at both the soil layers of the three sites. Highest VAM spore population was recorded from the undegraded site followed by least degraded and degraded sites respectively. There was a reduction in VAM spore population of up to 70 per cent in the degraded site and 30 per cent in the least degraded site in comparison to the undegraded site. The VAM populations in soils were positively correlated with soil moisture content (P=0.01), water holding capacity (P=0.01) and pH (P=0.05) in the case of surface soil layer. Bulk density was negatively correlated with the VAM spore population at the surface (P=0.05) and sub-surface soil layers (P=0.01). Altogether five genera (*Acaulospora, Entrophospora, Glomus, Gigaspora, and Scutellospora*) having forty-four species were recorded.

Key words: Forest soil, *Glomales*, humid tropics, soil degradation, VAM fungi

Introduction

Soil disturbance due to deforestation and associated agricultural practices causes undesirable changes in physico-chemical and biological characteristics of the soil. This leads to the ultimate decline in soil fertility and lowers crop productivity (**1-5**). The degraded lands with poor soil quality are unable to support good vegetation due to slow regeneration potential and finally turns into unproductive wasteland until restoration measures are taken up.

Soil microflora represents an important index of soil health, as its abundance and diversity are sensitive to changes occurring in the soil environment (**6,7**). Such changes in microfloral population following soil disturbance contribute to poor plant growth (**8**). One of the severely disturbed groups of microbes in the soil is mycorrhiza which are associated to the roots of most of the higher plants.

New Horizons in Biotechnology, 437-444, S. Roussos et al. (eds)
2003 Kluwer Academic Publishers, Printed in the Netherlands.

Vesicular-arbuscular mycorrhizal fungi (VAMF) are an important group of soil microflora as they increase the absorptive surface area of plant roots and supply of micro-nutrients particularly phosphorous during plant growth (**9,10**). One of the fundamental roles of mycorrhizal fungal hyphae may be to bridge the annular space, producing a physical connection between the root surface and surrounding soil particles (**11**). Mycorrhizal hyphae are also involved as an important cementing agent in forming soil aggregates and in maintaining its stability (**12**), thus conserving moisture and plant nutrient.

Any undesirable change in the association between these fungi and host plant roots due to soil disturbance leads to reduction in propagule population of mycorrhizal fungi (**13,14**) as well as in slow forest regeneration as evidenced by most of the degraded tropical forest soils. It was reported that a decrease in the number of mycorrhizal propagules occurs when soil is disturbed or is partially removed (**15**). Bellgard (**8**) also suggested that density of VAM fungal propagules present in a given soil system will determine to what extent soil disturbance also reduces root colonization. There have been reports on distribution of VAM species under the soil of important tree species in other parts of the northeastern hilly region of India (**16,17**).

VAM species diversity and population status of the fungi in degraded soils of Arunachal Pradesh has not been studied so far where shifting cultivation, locally called "jhum" agriculture, is practiced at a large scale on the hill slopes. Under this practice the area is cultivated for 1 or sometimes 2 years, and then left abandoned as "jhum fallow" for restoration of nutrients and forest regeneration (**3,5**).

The shifting cultivation practice is responsible for fast decline in fertility, crop productivity as well as decrease in biological diversity of the hilly soils associated with enhanced soil erosion. Therefore, it is required to examine to what extent the population status of vesicular-arbuscular mycorrhizal fungi (VAMF) is related to the degree of soil degradation for a successful restoration strategy of these abandoned lands. The present study investigates the distribution pattern of VAM spore population under different degrees of soil degradation in the humid tropical soils of Arunachal Pradesh, North Eastern India.

Material and Methods

Study site

The Banderdewa forest range was selected as an experimental site for the present study. Three study sites, namely a degraded site, a least degraded site and an undegraded natural forest site, which are located within 2 sq. km. range were selected for the study. The study sites are located at 27° 6′ N latitude and 93° 49′ E longitude at an elevation of 500 meters. The mean annual precipitation ranges from 1850 to 2100 mm of which 80 per cent is received during March to August. The mean ambient temperature is 22°C. The textural class of the soils in all the three different study sites falls under sandy loam.

The natural vegetation consists of semi-evergreen and mixed deciduous forests. *Ailanthus grandis* Prain, *Altinga excelsa* Noron, *Arthocarpus chaplasha* Roxb, *Bombax ceiba* Linn, *Canarium strictum* Roxb, *Chukrasia tabularis* Andr. Juss, *Duabanga grandiflora* (Roxb. ex. D.C.) Walp, *Gmelina arborea* Roxb, *Kydia calycina* Rob, *Magnolia caveana*, *Phoebe goalparensis* Hutch, *Pterispermum acerifolium* Willd, *Terminalia myriocarpum* Huerck & Muell Arg, and *Toona ciliata* Roem. are the important tree species growing naturally at the study sites. The presence of a protected forest area in the range allowed us to select an undegraded natural forest as control site and two others, degraded forest and least degraded forest sites for the study. The selection of study sites was based on land use history, vegetal cover distribution and occurrence of tree density.

The undegraded site has not been used for any type of cultivation in the past. The tree density is comparatively higher in this site (87 trees 100 m^{-2}). The forest floor is covered with thick layer of litter throughout the year. The site consists of natural trees and there has been no trees introduced so far. The least degraded site has not been used for any type of cultivation as per the official record of the forest department but forest people have been clearing and sometimes burning the forest floor every 2 to 3 years for selective logging and introduction of new forest plantations. The forest floor is covered with litter and grasses throughout the year. The species composition of the trees in the site is similar to that of the undegraded site but the tree density is lower (62 trees 100 m^{-2}).

Degraded forest site is a regenerating "jhum fallow" which had been used for 13 years for cultivation with 1 to 2 years of fallow period from 1980 to 1993 without input of proper nutrients based on inorganic and organic fertilizers. The important crops grown on the site were rice, maize, finger millet, and tuber crops. Since then, the site has not been used for cultivation of crops and is currently lying as an abandoned land. The soil surface is covered with luxuriant growth of *Osmunda calytoniana* Linn, *Phagopteris auriculata* Linn. (ferns), *Imperata cylindrica* Linn. and *Cyperus* grasses during summer and rainy season while dried and decomposed biomass of the ferns and grasses cover the soil surface in winter and dry seasons. The shrub *Lantana camara* Linn. is also found growing in all the seasons. The tree density in the degraded site is very low (11trees 100 m^{-2}) and comprised of only four tree species *Manglieta caveana* HK. f. & Th, *Dillenia indica* Linn, *Duabanga grandiflora* (Roxb. ex. D.C.) Walp, and *Gmelina arborea* Roxb.

Soil sampling

Soil sampling was done from all the three sites in the middle of October 1998 after the rainfall had stopped in the month of September. Soil samples were collected from each of the study sites at two depths i.e. for surface layer (0-20 cm) and for sub-surface layer (20-40 cm) after discarding 0.5 cm of soil in between them. Twenty-five soil samples were collected randomly (2 meters away from a tree trunk and 3 meters diagonal distance between two samples) from a quadrate of 50 m^2 from each site for both the depths to analyze the VAM spore population.

Physicochemical parameters

Soil moisture content was determined by drying 10 g of fresh soil at 105°C for 24 h in a hot air oven. Water holding capacity (WHC) was measured by using Keens' box apparatus. Soil texture, bulk density, and pH were determined using the procedures given by Okalebo et al. (**18**).

VAM population

VAM population was assessed after adapting the method given by Smith and Skipper (**19**) and Gaur and Adholeya (**20**). VAM spores were identified by using the manual of Schenck and Perez (**21**) under stereozoom microscope (Leica MZ6) using PVLG (polyvinyl alcohol, lactic acid and glycerin) as mounting media. The data were statistically analysed by calculating correlation coefficient (r) values between the selected soil properties and VAM spore population.

Results

The value of soil bulk density was highest in the degraded site followed by the least degraded and undegraded sites both for surface (1.27 g cm^{-3}) as well as sub-surface (1.39g cm^{-3}) layers (Table1).

Table 1: Physicochemical properties of degraded, least degraded and undegraded forest soils at two soil depths.

Study sites	Soil depth (cm)	BD (gcm-3)	SMC (%)	VHC (%)	pH
Degraded	0-20	1.27	12	46	5.0
	20-40	1.39	14	47	5.0
Least degraded	0-20	1.14	18	50	5.7
	20-40	1.16	14	51	5.5
Undegraded	0-20	1.11	22	52	5.9
	20-40	1.15	20	52	5.6

Values are means of 25 replicate samples BD, bulk density; SMC, soil moisture content; WHC, water-holding capacity.

The soil moisture content increased from 12 per cent in degraded site to 22 per cent in undegraded site at surface soil layer. The same trend was also noted in the sub-surface soil layer. The highest value (52%) of water holding capacity (WHC) was recorded from the undegraded site followed by least degraded site (50%) and degraded site (46%) respectively. The soil in degraded site was slightly more acidic than the other two sites (Table 1).

The VAM population was comparatively high in the undegraded site (805 spores 100 g^{-1}soil) which was followed by the least degraded site (495 spores 100 g^{-1} soil). Lowest population (215 spores 100 g^{-1} soil) was recorded in the degraded site at the surface soil layer (Fig. 1A).

Similar patterns of VAM spore population were observed in the sub-surface layer of the study sites (Fig.1B). The sub-surface soil layers of undegraded, least degraded

and degraded sites contained 50 per cent, 30 per cent, and 20 per cent less VAM spore populations than respective surface soil layers.

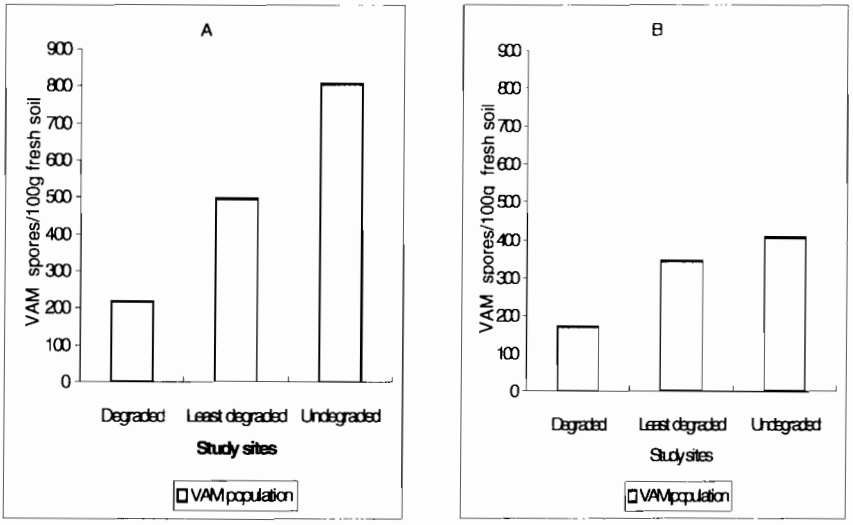


Figure 1: Distribution of VAM spore population in degraded, least degraded and undegraded forest soils at surface (A) and sub-surface (B) soil layers.

The higher values of soil moisture, water holding capacity and soil reaction were positively correlated to the higher population of VAM spores in undegraded site except for bulk density which showed negative correlation to the distribution of VAM population in this site (Table 2).

Table 2: Correlation coefficient (r) between VAM spore population and selected soil properties.

Soil Properties	VAM spore population	
Soil depth	Surface layer (0-20 cm)	Sub-surface layer (20-40 cm)
Bulk density	-0.914174*	-0.977491**
SMC	0.997479**	0.849782*
WHC	0.966693**	0.998243**
pH	0.92814*	0.993169**

SMC=Soil moisture content; WHC=Water holding capacity

* values are significant at $p<0.05\%$ ** values are significant at $p<0.01\%$

Qualitative study of soil samples revealed that undegraded soil had the maximum number of VAM fungi species (35) followed by least degraded (25) and degraded (14) sites respectively. Altogether four genera (*Acaulospora, Entrosphospora, Glomus, Gigaspora, and Scutellospora*) having forty-four species were recorded from all the three sites (Table 3).

Table 3. Distribution of VAM fungi species in degraded (A), least degraded (B) and undegraded (C) forest soils.

Species name	A	B	C
Acaulospora delicata Walker et al[f].	+	+	+
A. denticulata Sieverding & Toro[f]	+	+	+
A. elegans Trappe & Gerd[b].	-	+	-
A. foveata Trappe & Janos[d]	+	-	+
A. lacunosa Morton[c]	-	-	+
A. spinosa Walker & Trappe[d]	+	-	+
Entrophospora infrequens Hall[a]	-	-	+
Glomus albidum Walker & Rhodes[d]	+	-	+
G. clarum Nicol. & Schenck[f]	+	+	+
G. boreale (Thaxter) Trappe & Gerd[f].	+	+	+
G. canadense (Thaxter) Trappe & Gerd[a]	-	-	+
G claroideum Schenk & Smith[a]	-	-	+
G. constrictum Trappe[f]	-	+	+
G. diaphanum Morton & Walker[d]	-	+	-
G. fasciculatum (Thaxter) Gerd,. & Trappe[e] emend. Walker & Koske	-	+	+
G. fulvum (Berk & Broome) Trappe & Gerd[e].	-	+	+
G. globiferum Koske & Walker[c]	+	-	-
G. glumerulatum Sieverding[b]	-	+	-
G. heterosporum Smith & Schenck[f]	-	+	+
G. intraradices Schenk & Smith[a]	-	-	+
G. *lacteum* Rose & Trappe[a]	-	-	+
G. maculosum Miller & Walker[e]	-	+	+
G. microaggregatum Koske et al[e].	-	+	+
G. microcarpum Tul. & Tul[e].	-	+	+
G. monosporum Gerd. & Trappe[b]	-	+	-
G. mosseae (Nicol. & Gerd.) Gerd. & Trappe[f]	+	+	+
G. multicaule Gerd. & Bakshi[e]	-	+	+
G. pulvinatum (Henn.) Trappe & Gerd[a].	-	-	+
G. reticulatum Bhattacharjee & Mukerjee[c]	+	-	-
G. rubiformis Gerd. & Trappe[a]	-	-	+
G. sinuosa Gerd. & Bakshi[b]	-	+	-
G. tenebrosum (Thaxter) Berch[b]	-	+	-
G. tenerum Tandy emend. Mc Gee[f]	+	+	+
G. tortuosum Schenck & Smith[f]	+	+	+
Gigaspora albida Schenck & Smith[a]	-	-	+
G. candida Bhattacharjee et al[b].	-	+	-
G. decipiens Hall & Abbot[a]	-	-	+
Scutellospora aurigloba (Hall) Walker & Sanders[a]	-	-	+
S. coralloidea (Trappe et al.) Walker & Sanders[a]	-	-	+
S. gregaria (Schenck & Nicol.) Walker & Sanders[f]	+	+	+
S. heterogama (Schenck & Nicol.) Walker & Sanders[f]	-	+	+
S. pellucida (Schenck & Nicol.) Walker & Sanders[f]	+	+	+
S. persica (Koske & Walker) Walker & Sanders[a]	-	-	+
S. reticulata (Koske & Walker) Walker & Sanders[a]	-	-	+
	14	25	35

Note: (+) species present; (-) species absent; *a*, VAM species found only in the undegraded site; *b*, VAM species found only in the least degraded site; *c*, VAM species found only in the degraded site; *d*, VAM species found common to undegraded and degraded sites ; *e*, VAM species found common to undegraded least degraded sites ; *f*, VAM species found common to undegraded, least degraded and degaraded sites

Twenty-one species were found abundant in all the three sites whereas distribution of fourteen species was restricted to undegraded site, seven species to least degraded site and only two species to degraded site. The diversity of VAM species was highest in undegraded forest soil and minimum in degraded forest soil. The distribution of VAM spore population in all the sites demonstrates a clear impact of soil disturbance.

Discussion

The results of the studied physicochemical parameters and VAM population structure in all the sites reveal that the degree of soil degradation had a significant influence on the distribution of VAM spore population. The reason for lower population structure in both depths of the degraded site may be due to compaction and hardening of soil as supported by higher value of bulk density. This might have restricted and delayed the penetration of plant roots in the soil system thereby reducing movement and colonization of VAM spores in the regenerating site. Ahmad (**13**) reported 30-50 per cent reduction in VAM propagules when forest soils were severely disturbed through heavy soil mechanical compaction, exposure and erosion. This is in correspondence to our finding of 30 per cent reduction in degraded site and 70 per cent reduction in degraded site when the soils at these sites were disturbed for selective logging and agricultural use in comparison to the undegraded natural forest site.

The lower moisture and organic carbon contents and more slightly acidic condition of the degraded site might have also resulted in reduced VAM spore germination and subsequent growth of the hyphae in the hardened and compacted soil. Since the degraded site was clear cut and burned every time prior to cropping, the continuity in the life cycle of VAM fungi in the soil through host plant roots must have been disturbed repeatedly. Thus reduction in the rate of infection, formation and colonization of VAM fungi propagules occurred as reported by Jasper *et al.* (**22,23**); McGonigle *et al.* (**24**) and Bellgard (**25**) in the disturbed soils. It was found from the present study that there was significant reduction in VAM spore density in the degraded site following slash and burn agriculture in comparison to the undegraded natural forest site. Even after five years of fallow period from 1994 to 1998 for restoration of physico-chemical, biological properties and soil fertility, VAM fungi were not able to re-establish to their original population level and many species were unable to recover at all. The absence of a number of VAM fungi species in the degraded site might be due to the impact of uncontrolled burning done for every 1, 2 or 3 years which might have killed VAM propagules at the surface layer. Another reason may be that the spores in the soil could not complete their life cycle due to the absence of a suitable host root even if VAM propagules are present in the site. Roldan *et al.* (**14**) also reported a decrease in AM fungi population following agriculture use of soils and their recovery appearing to be influenced more by the presence of host plants in the semi-arid areas. The study revealed that higher degree of soil degradation due to shifting agriculture practices have a significant adverse

influence on the distribution of VAM fungi in the fragile hilly soils in the humid tropical region in the long term.

Acknowledgements:

The authors are thankful to CSIR, New Delhi, Director, NERIST and Forest Department Officials, Govt. of Arunachal Pradesh, respectively, for financial assistance, facilities and co-operation.

References

1. Lal R., Cummings D.J. (1979) *Field Crop Science*. **2**, 91-107.
2. Ramakrishnan P.S. (1995) *in* The Biological Management of the Tropical Soil Fertility, Woomer P.L. and Smith M.J. eds., John Willey & Sons, West Susex, U.K, pp 189-207.
3. Ramakrishnan P.S., Toky O.P. (1991) *Plant and Soil*. **60**, 41-64
4. Stromgaard P. (1984) *Plant and Soil* **80**, 307-320.
5. Tiessen H, Salcedo I.H., Sampaio E.V.S.B. (1992) *Agriculture, Ecosystems and Environment*. **38**, 139-151.
6. Kennedy A.C., Papendick R.I. (1995) *Journal of Soil and Water Conservation*. **50**, 243-248
7. Turco R.F., Kennedy A.C., Jawson M.D. (1994) *in* Defining Soil Quality for a Sustainable Environment, Doran J.W., Coleman D.C, Bezdicek D.F. and Steward B.A. eds., Soil Science Society of America Special Publication No. 35, pp. 73-90.
8. Bellgard S. E. (1994) *Mycorrhiza News*. **6**, 1-5.
9. Jacobson I. (1999) *in* Mycorrhiza: Structure, function, molecular biology, and biotechnology. 2nd ed., Varma A. and Hock B. eds, Springer-Verlag, Berlin, Heidelberg, pp. 305-335.
10. Sanders F.E., Tinker (1971) *Nature*.**233**, 278-279.
11. Miller R.M. (1987) *in* Ecophysiology of VA Mycorrhizal Plants. Safir G.R. ed. CRC Press, Boca Raton, Finland, pp. 135-170.
12. Miller R.M., Jastrow J.B. (1992) *in* Mycorrhizal Functioning: An integrative plant fungal process. Allen M.F. ed., Chapman and Hall, London, pp. 439-437.
13. Ahmad N. (1996) *Journal of Tropical Forest Science*, **9**, 137-146.
14. Roldan A., Garcia C., Albadalejo J. (1997) *Arid Soil Research and Rehabilitation*. **11**, 211-220.
15. Miller R.M. (1979) *Canadian Journal of Botany*, **57**, 61-623.
16. Sharma S.K, Sharma G.D., Mishra R. R. (1986) *Journal of Indian Botanical Society*. **66**, 266-268.
17. Pradhan M., Sharma G.D., Mishra R.R. (1996) *Journal of Hill Research*, **8**, 325-330.
18. Okalebo J.R., Gathua K.W., Woomer P.L. (1993) *in* Soil Science Society of East Africa Technical Publication, **1**,TSBF Nairobi, pp. 13-21.
19. Smith G.W., Skipper H.D. (1979) *Journal Soil Science Society*. **43**, 722-725.
20. Gaur A., Adholeya A. (1994) *Mycorrhiza News*. **6**, 10-11.
21. Shcenck N.C., Perez Y. (1990) Manual for identification of VA Mycorrhizal Fungi. 3rd ed., Synergistic Publications, Gainsville, Florida.
22. Jasper D.A., Robson A.D., Abbott L.K. (1987) *Australian Journal of Botany*. **35**, 641-652.
23. Jasper D.A., Abbott L.K., Robson A.D. (1989) *New Phytologist*. **112**, 93-99.
24. McGonigle T.P., Evans D.G., Miller M.H. (1990) *New Phytologist*. **116**, 629-639.
25. Bellgard S.E. (1993) *Mycorrhiza*. **3**, 25-29.

Authors index NHBT

Agate A.D., 215
Athanasiadis I., 311
Augur C., 321, 379,
Banerjee A.C., 193
Baratti J., 43
Basu A., 365
Bekatorou A., 311
Bensoussan M., 371
Bhamidimarri R., 221
Bhattacharjee A., 365
Bickers P.O., 221
Bisaria V.S., 123
Boom R.M., 17
Brand D., 321, 403
Breheret S., 371
Brizuela M.A., 163
Bueno G., 163
Cabeza Pulles D., 57
Campos Sofia M., 57
Castro E.A., 379, 387
Castro G.R., 113
Chincholkar S.B., 423
Dedet J.P., 387
Delgado G., 163
Dereure J., 387
Deshpande C.V., 241
Divanidis S., 413
Douma D., 413
Economou E.D, 285
Ficara E., 133
Filer K., 1
Gaime-Perraud I., 321
Gambhir A., 153
Garg S.K., 251
Ghosh D., 365
Ghosh S.K., 193, 365
Ghosh S., 365
Gonzalez Morla A., 57
Guimberteau J., 371
Halivopoulos S., 413
Hoogschagen M.J., 17
Houde A., 35, 43
Hu Z.B., 221
Iconomopoulou M., 311
Ismaili-Alaoui M., 35, 43
Jain J.K., 123
Jisaka M., 199
Jitbunjerdkul S., 63
Kabbaj W., 371
Kademi A., 35, 43
Kalfakakou V., 413
Kamal M., 35
Kannan V., 27
Kaul S.N., 241, 271
Kishimoto A., 199
Kothari R.M., 423
Kotowska U., 267
Koutinas A.A., 297
Kumar A., 153
Kundu A., 193
Latha P., 27
Lindner C., 311
Lu S., 199
Luz E., 387
Madamwar D., 97
Maeta K., 199
Maiti M.K., 365
Malhotra B.D., 153
Manos G., 413
Martínez-Carrera D., 89
Mas Diego S.M., 57
Mathur R.M., 123
Membrive N., 387
Miksa B., 153
Mishra S., 123
Morin A., 35, 43
Nagaya T., 199
Nagel F.J.I., 17
Nampoothiri K.M., 27
Nandy T., 241
Nishimura K., 199
Olivier J.M., 371
Oostra J., 17
Pagnoncelli M.G.B., 321
Pandey A., 27
Papagiannis I., 413
Papamichael E.M., 285
Parada J.L., 335
Paschalis T., 311
Pathe P.P., 241
Phirke N.V., 423
Pokhrel D., 231
Prachumratana T., 63
Prasertsan P., 63
Pratlong F., 387
Prochmann F.A., 403
Pohl J., 403
Psarianos C., 311
Rahardjo Y.S.P., 17
Raimbault M., 353
Ramteke P.W., 251
Remigi E., 133
Rinzema A., 17
Rio B., 89
Rio M.E., 335
Rodriguez D.E., 163
Rodríguez-León J.A., 163
Roussos S., 35, 43, 89,
321, 371, 403
Rozzi A., 133
Sabu A., 27
Sahai V., 123
Sambucetti M.E., 335
Santos M.C.R., 353
Schutyser M.A.I., 17
Sen S.K., 365
Serrano P., 163
Singh S.S., 437
Slomkowski S., 153
Sobal M., 89
Soccol C.R., 73, 321, 353,
403
Soni K., 97
Srinath T., 251
Srivastava A.K., 173
Stepniewska Z., 267
Szakacs G., 27
Szpyrkowicz L., 241, 271
Takata I., 199

Talou T., 371
Thayalakumaran N., 221
Thomaz-Soccol V., 379, 387
Tiwari S.C., 437
Torres S., 113
Trairatananukoon, 63
Tramper J., 17
Vaimakis T.C., 285
Visvanathan C., 231
Voliotis S., 311
Waszczynskyj N., 353
Weber F.J., 17
Weinberg Z.G., 397
Yokota K., 199
Zhu Y., 17
Zuleta A., 335

Subject Index NBHT

Acinetobacter, 221
Activated sludge, 133
Aerobic stability, 397
Airlift bioreactors, 173
Alcalase, 63
Alcoholic fermentation, 297, 311
Ammonia, 241
Anaerobic MBR, 231
Arachidonic acid, 199
Aroma, 73, 371
Aspergillus ficuum, 27
Aspergillus niger, 27, 35, 73, 193
Aspergillus oryzae, 1
Bacillus coagulans, 251
Bacillus licheniformis, 113
Bacillus subtilis, 113
Bacteriocins, 335
Banana cultivation, 423
Biobeneficiation, 215
Biocatalysis, 97
Biochemical engineering, 17
Biocontrol fungus, 17
Biological detoxification, 73
Biological nematicide, 403
Biomass production, 73
Biomass regeneration, 251
Bioprocessing techniques, 215
Bioremediation, 215
Biosorption, 251
BOD, 231
Brazil, 73
Cadmium, 215, 413
Canine cutaneous Leishmaniasis, 379
Carbonic anhydrase II, 285
Cassava flour, 353
Cell growth, 311
Ceratocystis fimbriata, 73
Chromium (VI), 251
COD, 231
Coffea arabica, 321
Coffea canephora, 321
Coffee cherries, 321
Coffee husk, 73, 89, 321, 403
Coffee pulp, 89
Column solid state fermentation, 57
Commercial, 1
Condensed tannins, 89
Conductive cooling, 17
Coniothyrium minitans, 17
Consortium of biofertilisers, 423
Cyclooxygenase, 199
Denitrification, 133, 241, 271
Dissolution of carbonates, 285
DNA (cDNA), 193
Dostat, 133
Drip irrigation, 423
Dynamic solid state fermentation, 57
EBPR, 221
Edible mushrooms, 73, 89
Eicosanoid, 199
Electromagnetic fields, 57
ELISA, 379
Elution, 251
Enzymatic prebleaching, 123
Enzymes, 1, 17, 73, 97
Esterase, 43
Evaporative cooling, 17
Expression-secretion system, 193
F/M ratio, 231
Fatty acids, 365
Feed industry, 1, 35
Fermentation, 163
Fermented cassava flour, 353
Fermented lactic beverage, 353
Filamentous fungi, 1, 17, 27, 35, 43, 57, 193, 403
Flamulina velutipes, 73
Fly ash, 423
Forest soil, 437
Full-scale plant performance, 271
Fungal strains, 35
β-galactosidase, 297
GC/MS, 371
GC/sniffing, 371
Genetic diversity, 387
Genetic engineering, 365
Genetic manipulation, 193
gibberellic acid GA_3, 73
Giberrela fujikuroi, 73
Glomales, 437
Granular biomass, 297, 311
Headspace concentration, 371
Heavy metals, 215, 251
Heterofermentative lactic acid bacteria, 397
Homofermentative lactic acid bacteria, 397
Host factors, 193
Humid tropics, 437
hydrolysate from tuna viscera, 63
Hydroperoxyeicosatetraenoic acid, 199
Hydroxyeicosatetraenoic acid, 199
IFAT, 379
Immobilized *kefir* yeast, 297
Industrial effluents, 231
Industrial enzymes, 1, 17
Inhibition, 271
Inoculants, 397
Intra-particle gradients, 17
IPNM approach, 423

Irrigation fields, 267
Isoenzymatic characterisation, 387
Kefir, 297, 311
Kluyveromyces fragilis, 297
Kluyveromyces marxianus, 297
Kraft pulp, 123
Lactic acid bacteria, 321
Lactobacillus acidophilus, 353
Lactobacillus amylovorans, 27
Lactobacillus casei spp Shirota, 353
Lactobacillus plantarum ,321,335, 353
Lactobacillus rhamnosus, 163
Leishmania (V) braziliensis, 387
Lentinus edodes, 73
Linoleic acid, 199
Lipase activity, 43, 113
Lipolytic enzyme, 113
Lipoxygenase , 199
Maintenance coefficient, 163
Mechanism of action, 285
Melanocarpus albomyces, 123
Meloydogyne incognita, 403
Membrane bioreactor, 231
membrane filtration, 231
Methanogenesis, 133
Microbiological treatment, 241
Microemulsion, 97
Microspheres, 153
Milk whey, 353
Mixed-bed fermenters, 17
Mucor miehei, 215
Municipal wastewater, 267
Mushroom fruit-body, 371
Mycelium, 371
Myo-inositol, 27
Nicotine, 413
Nitrification, 133, 241, 271
Nitrogen, 267
Nitrogenous fertilizer industry, 241
Nuclear factor, 199
Oct-1-en-3-ol, 371
Olive cake, 35
Organic solvent, 43
Organogel, 97
Packed bed fermenters, 17
Paecilomyces lilacinus, 403
PAOs, 221
Paper manufacture, 123
Paraná state, 387
P*ediococcus,* 335
Penicillium chrysogenum, 215
Peroxisome proliferator-activated receptor, 199
PHA, 221
Phosphorus, 267
PHstat, 133
Phytases, 1, 27
Phytic acid, 27
Plant cell reactor, 173
Plant growth regulator, 423
Pleurotus ostreatus, 73, 371
p-nitrophenyl, 113
Podophyllotoxin, 173
Podophyllum hexandrum, 173
Polyphenols, 89
Polypyrrole, 153
Prebiotics, 335
Probiotics, 335
Promotion, 311
Prostaglandin, 199
protease activity, 63
Protein enrichment, 35
Raisin extract, 311
Respirometry, 133
Reverse micelles, 97
Rhizomucor pusillus, 43
Rhizopus arrhizus, 215
Rhizopus homothallicus, 43
Saccharomyces cerevisiae, 297
Sewage sludge, 413
Silage fermentation, 321, 397
Soil conditioner, 423
Soil degradation, 437
Solid state fermentation, 1, 17, 35, 43, 73, 403
Standardization, 379
Streptococcus thermophilus, 335
Submerged liquid fermentation, 1
Sugar consumption balance, 163
Sugar fermentation, 297
Sustainable productivity, 423
Synbiotics, 335
Tannases, 89
Tannery wastewater, 271
Thermoascus aurantiacus, 27,
Thermophilic fungi, 43
Thermophilic strains, 27
Thermoresistant microbial, 113
Thioesterases, 365
Thunnus albacares, 63
Titration biosensor, 133
Tobacco, 413
Traditional approach, 423
Transgenic expression, 365
Treatment wetlands, 267
Trichoderma reesei, 57
Tuna viscera, 63
Urea bio-hydrolysis, 241
Urea biosensor, 153
Urea, 241
Urease, 153
VAM fungi, 437
Verticillium chlamydosporium, 119
Wastewater treatment, 231, 241

Water restricted microenvironment , 97
Whey, 297, 311
Xylanase, 123
Yield, 163
β-galactosidase, 297

New Horizons in Biotechnology

S. Roussos, C. R. Soccol, A. Pandey and C. Augur (Eds.)

The practice of biotechnology, though different in style, scale and substance in globalizing science for development involves all countries. Investment in biotechnology in the industrialised, the developing, and the least developed countries, is now amongst the widely accepted avenues being used for economic development. Long-term use of biotechnology in the agricultural, food, energy and health sectors is expected to yield a windfall of economic, environmental and social benefits. Already the prototypes of new medicines and of prescription fruit vaccines are available. Gene-based agriculture and medicine is increasingly being adopted and accepted. Emerging trends and practices are reflected in the designing of more efficient bioprocesses, and in new research in enzyme and fermentation technology, in the bioconversion of agro-industrial residues into bio-utility products, in animal healthcare, and in the bioremediation and medical biotechnologies. Indeed, with each new day, new horizons in biotechnology beckon.

Developed from presentations made at the international conference on New Horizons in Biotechnology, this volume offers biotechnologists, industrialists and policy makers alike an insight into many of the most recent advances and opportunities emerging from this exciting field.

IRD Éditions

KLUWER ACADEMIC PUBLISCHERS

ISBN 1-4020-1718-9

9 781402 017186