Advances in Solid State Fermentation

Proceedings of the 2nd International Symposium
on Solid State Fermentation
FMS-95
Montpellier, France

Edited by
S. Roussos, B. K. Lonsane, M. Raimbault and G. Viniegra-Gonzalez

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PRESENTATION

This book is based on the works presented at the meeting of the French Society of Microbiology (SFM) at the 2nd International Symposium on Solid State Fermentation FMS-95, held at Agropolis International in Montpellier, France, February 27-28, 1997 organized by ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), France, UAM-I (Universidad Autónoma Metropolitana-Iztapalapa), México and Agropolis, Montpellier, France.

At the beginning of the book, the International Scientific Committee is presented followed by the preface which gives a general scope of SSF along with comments on the general layout of the nine sections in which the Symposium was structured: General Introduction on FMS-95, Basic Aspects and Parameters Measurements, Bioreactors and Mathematical Models, Upgradation of Agro-industrial Products/Wastes, Enzymes Production and Applications, Secondary Metabolites and Biopesticides, Edible Mushrooms/Fungi, Newer Applications and History of Solid State Fermentation at ORSTOM. Forewords by senior members of ORSTOM and Agropolis as well as four opening lectures stressing the contribution of ORSTOM to the development of the SSF in different countries are then presented as an introduction to the subject and to outline recent achievements of different research groups.

We would like to thank the European Union (DG XII), the Region Languedoc-Roussillon, Montpellier District, ORSTOM-DIST and Finedoc S.A. for their sponsorship. Thanks also go to the members of the French Society of Microbiology for planning this event, the General Organizing Committee at Montpellier, in particular to Nathalie Pujet for her excellent technical assistance and efficiency in producing the book from the manuscripts.

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Foreword

G. HAINNAUX

Departement Milieu et Activités Agricoles, Centre ORSTOM, 911 Avenue d'Agropolis, B.P. 5045, 34032 Montpellier Cedex , France.

Solid state fermentation, popularly abbreviated as SSF, is currently investigated by many groups throughout the world. The study of this technique was largely neglected in the past in European and Western countries and there is now a high demand for SSF, meaning in food, environment, agricultural, pharmaceutical and many other biotechnological applications.

It gives me satisfaction to note that the importance of this technique was realised at my department way back in 1975 since then, our team has put concentrated efforts on developing this technique.

Foreword

M. PUYGRENIER

Agropolis Valorisation, Avenue d'Agropolis, 34394 Montpellier Cedex 5, France.

On the name of the Scientific Community, I would like to express the wish that this International Symposium on SSF should be successful.

Solid State Fermentation is part of biotechnology research. It consists on seeding solid culture medium with bacteria or fungi (filamentous or higher) and on producing, in this medium (solid components and exudates) metabolites and high value products.

In fact, this process is very old. In older industries such the food and agricultural, this technique has been extensively used. An example of this is the production of pork sausages and Roquefort cheese. Pharmaceutical industry could make extensive use of SSF in the production of secondary metabolites of many kinds and development in this direction is soon expected.

Due to the advantages in biotechnology research, we have improved these technologies and opened new fields like waste treatment and upgradation of by-products.

Among these applications, Agropolis is concerned in four fields:

- Agriculture: biological control with biopesticides, the use of mycorrhizas for reforestation, animal feeding with protein enrichment of cattle cake.
- Agribusiness: relating to aroma production by micro-organisms of fungus inoculum production, silage (fermentation) of coffee pulp, and coffee treatment, food microbiology.
- Treatment and upgradation of wastes: biodegradable plastics, worm-composting of wastewater sludge.
- Biotechnology equipment: Solid state fermentation has lead to the design, adaptation and innovation of equipment.

In Montpellier, the research abilities in SSF of Agropolis are very important: ORSTOM, CIRAD, INRA, CNRS, CEMAGREF and Universities have been contributed to enrich this field. Interesting results have been obtained and some of them have been transferred to different regional companies.

Every four years, an International Scientific Symposium on SSF takes place in which the researchers specialised in this field gather to share their experiences. This year, the meeting is held in Montpellier for the second time.

It is very interesting to promote, within Agropolis, non-pollutant processes, and to study cleansing and rinsing, regional and international levels.

When proceedings will be printed, I hope you all will remember this very pleasant and interesting symposium. The proceedings will be an important source of information and inspiration for the future and will also be a very good tool which will promote new scientific and technological co-operation for the development.

Opening lecture I

A. CORNET

Departement Milieux et Activités Agricoles de l'ORSTOM (MAA),
ORSTOM, 213 Rue La Fayette, 75010 Paris, France.

It is my honour to welcome all of you. I am grateful to all of you for participating in this international seminar, FMS-95.

I would be brief in my address for two reasons. First, because I am not a specialist in solid state and food fermentation. My role in MAA is confined to co-ordination of various departments falling under MAA, distribution of funds to different groups and encouraging the work. Second, because I do not like to take away your valuable time, marked for scientific presentations/ discussions, which is of larger interest to all the participants.

I would like to avail this opportunity to brief this august gathering on the evolution of scientific programmes on solid state fermentation at ORSTOM. This evolution commenced 20 years ago. In 1975, up on the initiative of Professor J.C. Senez, the responsibility of developing the first programme in solid state fermentation was entrusted to Maurice Raimbault. Both of these personalities are present in this august gathering and the work involved the protein enrichment of amylaceous tropical plant materials, i.e., cassava, by solid state fermentation.

This was the starting for the building of one team in ORSTOM for research and development work in solid state fermentation. I see that this was an important decision. Thanks to these two initiators, as we have subsequently started investigations on upgrading of agricultural products and sub-products for food aspects, but also now for the environmental aspects. The latter is the interest of our investigation on solid state fermentation, at this hour.

Since this initiation in 1975, a lot of work was realised in this area of solid state fermentation, particularly with the participation of ORSTOM. Our interest is to develop methodologies for upgradation and utilisation of agricultural production for use in developing countries. This is of vital importance, because the development of the developing countries is essentially determined not only by the extent of the production, but also by the utilisation and upgradation of these agricultural products.

In efficient upgradation, the solid state fermentation constitutes a very important element.

ORSTOM has not realised these works alone. It was realised in co-operation. I think, that the best example of this co-operation is your presence here, from near and distant places, and you have relation with our group in this programme. I see that, among the people who are here in this international seminar, we find the representatives/delegations from more or less all the countries of European Community, and also from all the tropical countries, with whom we developed co-operation.

Same is the case with French scientific institutions. I cite here, particularly, CIRAD, INRA, CNRS, CEMAGREF and French Universities, particularly, University of Montpellier II. Additionally, the relation was established with a number of Technical Universities and Institutes like ENSBANA, UTC, INSA Toulouse and INSA Lyon.

Among the international collaborations of ORSTOM for research in solid state fermentation system, I would like to cite the example of Mexico, with which it was developed from long time back and it is even now working smoothly. I think that our efforts with Mexico is the best example of the co-operation. We would like to develop similar type of co-operation with other countries, not only in biotechnology or solid state fermentation, but in more different areas.

The combined investigation work with UAM-Iztapalapa made it possible to establish a centre of excellence in solid state fermentation. Such collaboration permits not only to conduct investigations but also assures the undertaking of teaching work in this area in the collaborating university/country. This aspect of teaching is very important to Mexico and facilitates fellowships in France to investigators from all Caribbean and Latin American countries. We have been able to establish an excellent rapport with this university, from which students come to France to study in different universities. Similarly European students from different countries go to study solid state fermentation in UAM-Iztapalapa.

More recently, we have developed new co-operation with other countries, which were earlier were not having scientific collaboration with ORSTOM. For example, from India, the team of Dr. Lonsane from Central Food Technological Research Institute (CFTRI), Mysore, collaborates with ORSTOM Centre in Montpellier. Another example is Professor Gunasekaran from University of Madurai, India, who is working now in Mexico with our team.

I would like to stress that the area of solid state fermentation is an important activity to our investigation team and it binds us with our international co-operation policies. I am confident that the results and your discussions during this seminar will permit us to visualise more clearly and advance better in this direction.

Before ending my address, I would like to felicitate our pioneers of this programme in our institute, particularly to Professor Jacques Senez, who was originator of this area, Dr. Maurice Raimbault who has been always a very active member of our co-operation in this area and who developed an excellent pole of co-operation with Mexico, with the help of Professor Gustavo Viniegra-Gonzalez.

Opening lecture II

J.-L. GUILLAUMET

Commission Scientifique Sciences du Monde Végétal (CS4),
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Our activities at ORSTOM in the field of solid state fermentation, in collaboration with our partners, have already been critically presented to you by Dr. Cornet. I would just speak a few words, from the view points of the Commission Scientifique Sciences du Monde Végétal.

Before that, I would like to thank Sevastianos Roussos, who is also the elected member of the commission. He has asked me to participate in this international seminar. It is with much pleasure that today I am among this community of solid state fermentation.

This international seminar interests me, because of various reasons. One, to know better this subject, which falls under the jurisdiction of our commission in ORSTOM. I have observed that the specialists in solid state fermentation, are remarkably very well respected. In addition to Jean-Louis Garcia and Sevastianos Roussos, from amongst the members of our Commission No. 43, I see in this gathering a number of our young friends from ORSTOM, whom I know only from their dossiers. It is much more pleasure to meet them today personally.

These young colleagues from ORSTOM answer all the mission quirries in the field of their activities, as per our procedure. These enquiries are mainly at the level of fundamental research on microorganisms, their physiology and biology.

I just recall some of the doctoral theses, which were developed by these youngesters in this field. I can cite some of these from my memory. These were on production of alkaloids by *Claviceps purpurea*, lactic and fumaric acids by *Rhizopus*, citric acid by *Aspergillus*, antibiotics by *Penicillium*, and flavours as well as other biomolecules of pharmaceutical or agricultural interests.

I also recall, that one of our young colleagues, Isabelle Perraud-Gaime, is going to defend her doctoral thesis just day after tomorrow. I would like to stress that this has not detered her in participating in this important seminar. I fondly hope that you all prolong your stay here and go to the meeting on wednesday to watch the defence of

this thesis. It will be a great pleasure to me, to attend this and watch the defence. I would like to wish all the success to Isabelle.

The documents on the studies of microorganisms, which S. Roussos have provided me, are of interest to me, because they approach an aspect of the biodiversity, the topic of my research. The biodiversity is a commonly used word now-a-days and the world around us is also diverse. It is very interesting to know that the filamentous fungi, such as *Aspergillus niger, Rhizopus*, and *Trichoderma*, are of fundamental importance in the fermentation of some food products. I was not fully aware that there is a *Penicillium camembertii* and a *Penicillium roquefortii*. As a botanist, this raises an enquiry, whether there is a special *Penicillium* for each fermented cheese, and, in particular, if there is a *Penicillium pontlevequii*?

The above aspects, are very interesting to me in the field of biodiversity. Starting from a fundamental approach the mechanism working of these microorganisms could be elucidated. Following intensive trying by several groups, I would say that we domesticate them and this domestication appears to be something very fundamental. It is similar to that happening in higher plants, as such domestication leads to future production of these plants, at a larger scale.

I wish to point out that our aims and research actually links us with studies of particular ecosystems concerning the tropical agrofood fermentation, biofilters, etc. Another example is the innovation on degradation of new plastic materials. We feel that this is an important activity of the ORSTOM mission, which is of interest to us and our organization. It opens up a way for utilization of the research in the countries, for whom we make these efforts.

I do not want to take more of your time. I would like to thank, mainly, the founders of this field, which we call as solid state fermentation school, and, in particular, Professor Jacques Senez, whom I have had the pleasure of meeting again today. I have known him, from the time, when he was the president of the technical committee of soil microbiology in ORSTOM. He has contributed to the employment of a younger team of microbiologists at ORSTOM. Jean-Louis Garcia has briefed me on a short history of the evolution of this programme on solid state fermentation in ORSTOM, developed by Maurice Raimbault. I know that your modesty will suffer, but it is necessary to thank you Maurice.

I would also like to thank Professor Gustavo Viniegra-Gonzalez, from UAM-Iztapalapa, Mexico, who also has made efforts together with Maurice Raimbault, since 1980, in forming a young group of researchers in the field of solid state fermentation. The current young researchers, who have been engaged at ORSTOM centre in Mexico, include Christopher Augur, who was employed by our commission, with the accord of the Department MAA.

I wish to stress that fermentation is important and that the international community can take benefits of our efforts.

I congratulate the organizers of Montpellier. I arrived this morning at 8:30 AM and I noticed that the organization was remarkably astonishing. I wish you much success in your work. I assure you my scientific interest and also the support of ORSTOM's Scientific Commission CS4 to this international seminar.

Opening lecture III

R. MOLETTA

Société Française de Microbiologie,
28, rue du Docteur Roux, 75724 Paris cedex 15, France.

I start with apologies, as Charles Divies and Lucien Penasse are not able to attend this international seminar. They asked me to represent the Société Française de Microbiologie. The Société has around 2500 members and it plays an important role in the relationship between research and industry. The Société has a main goal of finding ways to lead the novel research or the development of novel industrial exploitation.

The section of industrial microbiology and biotechnology has been founded by my Professor Gilbert Durand in the seventies. His idea was, I think, to start one section with cross-roads to and strong impressions of industrial microbiology. The solid state fermentation have an important role in this section.

First, I would like to greet Professor Jacques Senez, who is certainly the father of solid state fermentation and also Maurice Raimbault, who has widely contributed to the developments in solid state fermentation. I see that, from the start of this area of solid state fermentation, everybody gives great importance to these two researchers. I, from my very young age, have closely followed the work of Professor Senez. I am very happy to meet him again today.

To cultivate micro-organisms on the solid support, it is necessary to employ a type of the support, which is different from the soluble substrate. The hydrolysis of the solid substrate or the mixture of two or more solid substrates as well as mass transfer mechanisms strongly govern the microbial activities in solid state fermentation. I think that, this is the domain, many things could be done, which could be at the solid substrate level or at the culturing on the solids.

This domain of the application of solid state culture is very large and great diversity of subjects are being approached from almost every part of the world. This will also be evident to all of us during these two days of this international seminar. As in other domains of fermentation, the research in solid state fermentation demands

multidisciplinary approaches from microbiologists, biochemists, mycologists, physiologists, chemical engineers, microbial engineers and automation experts.

In the field of waste treatment, which I know little, the problems of discharge of spent fermentation liquid or solids are always difficult. It is of urgent need to have new processes for upgradation or treatment of wastes. Moreover, demonstrating the potentials of these processes to the industry will be a way for their exploitation.

It is more than obvious to me that we are going to solve the problems encountered in extensive industrialisation of solid state fermentation, by putting up intense efforts on research to obtain knowledge of the functioning of micro-organisms, their physiology as well as kinetics and mastering the technologies and their optimum functioning.

I am very happy about the excellent initiative of Sevastianos Roussos and Gustavo Viniegra. It is absolutely important to hold this type of international seminar. It is also indispensable that such international seminars on solid state fermentation are arranged regularly in the future.

I would like to congratulate the organisers, not only on my behalf, but also on behalf of all of my colleagues from the Société Française de Microbiologie, for having planned, especially for having realised this important seminar on solid state fermentation and for giving it an international dimension.

Opening lecture IV

A. CONESA

Agropolis International, Avenue d'Agropolis, 34394 Montpellier Cedex 5, France.

First, welcome to Agropolis and this building, which is a common house of 18 members of the Agropolis, in which ORSTOM is one of the most eminent.

I would also like to congratulate the organisers of this international seminar and stress that this moment recalls the 20 years of research on the topic of solid state fermentation in ORSTOM. It was started in Senegal, continued in Martinique and was concerned with the upgradation of cassava and banana wastes for their protein content, with a view to provide protein rich fermented substrate for feeding of livestock. I was at that time in the Ministry and we have financed this research, which I found very interesting.

Through the memories, it permits me to state that the above was one efficient approach, which is important for the agro-industrial residue/waste upgradation.

A few words about Agropolis. We are 18 organisations involved in research and developments, which have decided to work together. We can define Agropolis as a synergy, I mean, we do the work together, which we could not have achieved individually. To illustrate this point, I can cite the example of this international seminar. To be more precise, this international seminar is organised by ORSTOM, UAM-I of Mexico and Agropolis. These combined efforts, in a way, will bind our activities with some of you on aspects, which are more focused on industrial needs or technology transfer. Thus, little-by-little, we wish to establish a much larger synergy in this modest manner.

Agropolis, by one point of view, is the agro-food. It represents four big French poles in the domain of transformation of agricultural products/residues. There are many strong points, which joins together the 18 constituents laboratories of Agropolis. For example, the team working on fermentation and biotechnology constitutes one major pole of Agropolis. This pole is faced with severe problems of automation and needs appropriate sensors for process monitoring. These two aspects constitute the activities of another pole of Agropolis, which in turn, is faced with the problems of evaluation of their devices in field conditions. These mutual benefits bind all the members together.

We also have some trumps at Agropolis, Montpellier, and one specificity, which is the Mediterranean domain. We all are here today in a Mediterranean region, but with interest in tropical region.

I come back to the example of Martinique and say that it is very important, what all of us are going to do in this international seminar. It concerns with our co-operation with the South, which we have established step by step, in relation to transformation of agricultural products and upgradation of agro residues/wastes.

In fact, the great majority of these transformation models as well as reflections have been done in the North. We often face a paradoxical situation in the South, which do not upgrade their own products and consequently, they lose considerable natural resources. Some times, these are just thrown away or are upgraded in such a way that the combined value of the material and valorize is at the level of the selling price of the fermented material.

Recently, we have reflected to develop technologies, adjusted to particular situations, which exist in Southern countries. These situations are different from those in the Northern countries. This obstacle in the operation of the technologies needs to be overcome. Thus, from this point of view, the concept of adaptation of the technologies has become very important. It is necessary to examine the models on the technology should be based on, what their limits are and the type of social organisation the user belongs to.

Probably, it might be necessary to replace these technologies. I do not want to divert from the theme of the subject of this seminar. But as a specialist of agrarian systems, please allow me to say that we can not any more ignore the context in which we are going to operate these technologies. This is a significant point, which, no doubt, you also must have examined.

So, here we are ready to deliberate focus in this extremely important area of solid state fermentation. I congratulate the pioneers, they have been cited by all the speakers, but also allow me to cite them. Professor Senez, Dr. Raimbault, Dr. Roussos and Professor Viniegra, all of them have organised this international seminar. I am also very happy to see a some friends again, in particular, the Mexicans. We have a long tradition of friendship and co-operation with Mexico, especially UAM-Iztapalapa. I wish you all good discussions and, of course, all the success in your activities.

Preface

Solid state fermentation (SSF) is an ancient art in the process to become a modern technology. Since a very long time ago, traditional SSF processes were developed in many places of the world. Originally, they were spontaneous invasions of molds growing on wet pieces of foodstuffs. Koji and tempeh which are traditional fermentations of steamed rice or soy beans, are in turn good examples of SSF developed in Asia. Camembert and Roquefort cheeses are European SSF counterparts. Both in Asia and in Europe, food SSF processes have been a little bit updated but have remained essentially the same. But in the last twenty years a renewed interest to understand the principles and increase the applicactions of SSF has been growing around the world and this book is a reflection of such interest.

Several factors seem to support this new aproach to SSF processes. In the first place, most fungal species studied in this field have the property to quickly invade solid substrates and all have evolved the necessary metabolic machinery to transform complex biochemicals in simple nutritional compounds. They do such work using as little water as possible and outgrowing yeast and bacterial infections. From the engineering point of view, such SSF features help to develop non sterile and water saving fermentations. This is on line with the trend to develop large scale clean biotechnology, saving water and energy.

Another special feature of SSF processes is that fungal organisms seem to have an adaptative behaviour to solid substrates when compared to the conventional liquid submerged fermentation (SmF). This key feature is leading some researchers to look for more stable enzymes and unique products developed by SSF techniques.

Finally, fungi are eukaryotic organisms which are easy to grow in large scale fermentation systems but at the same time, they have evolved complex enough reaction systems to modify or "edit" intracellular proteins in a way that resemble protein edition in higher organisms, for example, by glycosilation, phosphorilation or alkylation. Protein edition seems to be a necessary condition for the production of heterologous proteins from animal or vegetal origing whose genes have been cloned

and introduced in fungal cells using the current techniques of genetic engineering. This way, the so called "recombinant proteins" can be excreted and edited in a similar fashion to the original source. Eventhough little is known on the fashion that SSF and SmF techniques modify the edition of proteins there is a mounting evidence showing that such differences exist and their understanding and control may be some of the crucial points to develop cost effective new products of future biotechnology.

Before this new oportunities for SSF arrive, some technical and scientific problems ought to be solved. The good news is that some new commercial SSF processes have been developed within the last five years, for example, in the field of hydrolase or biopesticide production, and many recent developments have taken place in this period of time. This is why this book, that appears just few years after the excelent recent review done by Doelle, Mitchell and Rolz (Solid substrate cultivation, Elsevier Applied Science, London UK, 1992) could be just as timely and atractive as the previous one.

The organisation of this book is like a conceptual voyage to the world of SSF studies. At the entrance of this voyage we encounter the wellcoming station of definitions, biomass estimation and kinetics of fungal growth on solid substrates. After such wellcoming, the visitor enters to the important domain of engineering and SSF reactor design which might help him later to consider the use and applications of the following domains. The trip goes now to the province of agrindustrial waste upgrading which is one traditionally assigned to SSF country. Then it goes to stead fast growing privince of enzyme technology by SSF processes to enter later into the surprising and misterious jungle of secondary metabolites and biopesticides. After this exciting adventure, the road goes to the good all friends of edible mushroom technology, offering new insigths in how to produce the spawn and improve the vegetative stage of fungal development. The last stop is a short visit to the virgin lands of new SSF applications with some unexpected mixtures such as the consideration that worms are solid state living reactors with possible large scale applications.

At the farewell station of our trip an optimistic overview of international cooperation is presented, a brief list of achievements and results of nearly fifteen years of cooperation between ORSTOM researchers and their corresponding colleagues from Africa, Asia and Latin America.

As a coeditor of this book and as an research worker of SSF principles and applications I sincerly hope that a this conceptual trip presented above could be amusing and interesting for all those people of academic and practical walks of life which are in the search for new ways to help the adjustment of this shrinking world. We hope that readers find here enough questions, mysteries and suggestions to consider SSF processes as one of many alternatives to develop biotechnology in a friendly way with our mother Nature.

Gustavo Viniegra-Gonzalez

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Cassava protein enrichment by solid state fermentation

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During this symposium, we have been talking, among other aspects, about my work in the field of solid state fermentation and I can not resist myself in recalling some anecdotes and making remarks on general aspects.

I am an old microbiologist and I started my research work not only in general aspects of microbiology but also on applied aspects of microbiology. My first interest was to develop the process for large scale production of single cell protein (SCP) from hydrocarbons, in collaboration with British Petroleum (BP).

Ultimately, different circumstances, such as political and economical, led to high increase in the cost of petroleums in 1972 and created crisis of petroleum availability thoughout the world. So, the high hopes, which I have formuled about development of the petroleum fermentation arena for producing millions of tonnes of proteins, were smashed at that time. At that moment, I was also working in ORSTOM and I had a number of ideas related to agricultural problems. It struck me that, in general, plants make carbohydrates more easily than they did proteins.

As a trained microbiologist, I wondered, whether we could use the help from micro-organisms to transform plant carbohydrates into proteins, particulary the food proteins. I was fortunate, at this time of my life, to meet Maurice Raimbault, who has helped me to materialize these ideas. Our first interest at the time was in cassava.

Why cassava?

Because, we know that cassava, the starchy plant root, is one of the principal sources of food in tropical areas, basically in developing countries. Particulary, it seemed interesting to use it to produce industrial protein.

Even though these facts are known, please allow me to make a few remarks on the subject.

Even though the initial studies were geared almost exclusively towards protein production for feed purposes, the present applications are numerous, for example, the use of microbial metabolites of economical interest.

About this SSF process, I might allowed to make some more observations. There are two kinds of SSF processes. In the first, the solid state conditions are imparted by the substrate itself, and this type of process has been carried out by ourselves, using cassava flour.

The second one consists of solid state fermentation on inert support. Interestingly, I have noticed during this FMS-95, that there are two kinds of supports. The first one is represented by the chemical polymers whereas the second consists of an inert support of biological origin, particularly sugarcane pith bagasse. I have learnt during this symposium that fermentation results obtained with inert natural organic support are better, as compared to those with the use of synthetic support. At present, we do not have explicit reasons for this fact. It is surprising and I would encourage continued investigations towards an explanation.

I would also like to state that there is a common scope for mixed culture fermentation in these two kinds of processes for production of food proteins, which I imagined to carry out in my life, either by fermentation of petroleum or by SSF of cassava. I expected that protein production could be take place at a scale of one million tonnes/day, either for animal feeding or human consumption, by any one of these processes. It would be an utopia to create a microbial protein manufactured under axenic conditions, without contamination. I remember that we had contamination problems during our studies on petroleum fermentation and carried out intense studies to see why contamination occurred and also investigate the eventual pathogenicity of these contaminating bacteria.

The results of these extensive investigations demonstrated that first, the contaminant bacteria are neither pathogenic nor toxic and, secondly, their population is stable over time.

Why?

Because, in fact, this association between different microrganisms, especially between bacteria and yeasts, is for mutuals benefits. Particularly, the contaminant bacteria, in the case of petroleum fermentation, produce biotin and which helps in the development of yeasts. Contaminant bacteria also eliminate fatty acids, which are accumulated during degradation of petroleum, which otherwise would have inhibited yeast growth.

I would also like to recall an anecdote. I was on the premises of the SCP production plant in Lavéra. The process yielded a stable product, but with bacterial contamination. I was present with Dr. Boris Chain, who received the Nobel Prize in medicine for his work on penicillin, which was carried out with Flemming and

Florey. Boris Chain stated that, if we intend to work in the field of industrial microbiology, it was necessary to work with pure cultures and conduct fermentations in an axenic manner. I remember having urged him to state whether he denied the existence and quality of cheese. Because, traditional fermentations, like those of wines and cheese, are always carried out in a mixed culture fermentation.

To further convince Chain, I told him that in my country, farmers from Normandy make a type of cheese, which is named Camembert. Their process produces Camembert and certainly never leads to Roquefort. This is because the mixed fermentation process, under the same conditions, gives the same product regularly and successfully, as the coexisting microrganisms used are stable.

Maurice Raimbault and myself have observed the same phenomena in SSF of cassava. I wish to stress that we do not sterilize the medium, because mixed cultures were used in the fermentation process.

Using a more serious tone, I would like to bring up a second anecdote. The industrial and economic future of SSF in an unresolved problem, as profitability has not been yet attained. In my opinion, we can not make SSF profitable, without operating it in a continuous culture.

We can undertake continuous cultivation easily in liquid culture, but it represents a difficult challenge in the case of SSF. I think that this point is one aspect on which younger scientists should work very strongly, with concentrated efforts. In fact, it is necessary that we investigate the ways to make a continuous SSF process, if we wish to apply SSF processes at a larger industrial scale.

I have listened all of you here during the last three days and the discussions were very interesting. I have encountered a lot of people, whom I know or those who know me very well.

I had a marvellous time here and thank you all for being here, despite the late hour.

Thank you very much for your patience and listening.

Solid state fermentation: Definition, Characteristics, Limitations and Monitoring

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SUMMARY

SSF has important advantages and drawbacks due to its physico chemical features, namely, relatively low water activity and formation of significant gradients of temperature, nutrients and products. SSF is also qualitatively different from the conventional submerged fermentation (SmF) process in relation to sporulation and production of enzymes as well as secondary metabolites. Biomass estimation by respirometry and physical measurements such as infrared spectroscopy, pressure drop measurements and image analysis are being for different culture systems.

Mass, water and heat balances can be measured accurately and the available data indicate that it is possible to use basic engineering principles to stimulate and characterize the SSF processes. Extrapolation of experimental data can be done on the basis of dimensionless groups related to dispersion and reaction processes. Integration of fermentation and residue disposal is amenable in terns of several commercial products. For example, use of the residue for direct livestock production or composting. The latter could improve the biological properties of the compost. The SSF process has become well understood, according to the rules of bioengineering, biochemistry, microbiology and molecular biology. Microscopic heterogeneity, which was considered as a weakness of SSF, has now become its strength for increasing product yields and appropriately changing the cell physiology. Reduced water level also favours a cleaner industrial operation with low levels of waste water.

Keywords: Solid state fermentation, definition, characteristics, advantages, limitations, biomass, sporulation, enzyme production, secondary metabolites, respirometry, infrared spectroscopy, pressure drop, image analysis, mass balance, heat balance, water balance, integration of fermentation, residue disposal.

RESUME

Fermentation en milieu solide: Définition, Caractéristiques, Limitations et pilotage.

VINIEGRA-GONZALEZ G.

La fermentation en milieu solide (FMS) compte de nombreux avantages mais aussi des inconvénients dus à ses caractéristiques physico-chimiques. La FMS est différente, du point de vue qualitatif, des procédés de fermentations conventionnelles submergées (SmF), en ce qui concerne, la sporulation, la production d'enzyme et de métabolites secondaires. L'estimation de la biomasse par la respirométrie, les mesures physiques telles que la spectroscopie infra-rouge, les mesures de baisse de pression ainsi que les techniques d'analyse d'image sont fiables pour les différents systèmes de culture en milieu solide ou en milieu submergé. Les bilans de matière, eau et chaleur peuvent être évalués de façon précise, et les données disponibles indiquent qu'il est possible d'utiliser les principes basiques d'ingénierie pour simuler et caractériser les procédés de FMS. L'extrapolation des données expérimentales peut être faite en se basant sur des groupes adimensionnels relatifs aux paramètres de dispersion et de réaction. Il est envisageable d'utiliser les fermentations pour valoriser les résidus disponibles afin d'obtenir des produits commerciaux; Par exemple l'utilisation directe pour la production du bétail ou encore comme ensilage; Ce dernier peut améliorer les qualités biologiques du compost. Les mécanisme de FMS ont été maitrisés en ce qui concerne la bioengénierie, la biochimie, la microbiologie et la biologie moléculaire. L'hétérogénéité des phénomènes microscopiques qui était considérée comme le point faible des FMS, est devenu un atout pour augmenter les rendements et changer de façon appropriée la physiologie cellulaire. Au niveau industriel, l'exploitation est d'autant plus favorisée et moins contraignante que la quantité d'eau résiduelle à traiter est moindre.

Mots clés: Fermentation en milieu solide, définition, caractéristiques, avantages, limitations, biomasse, sporulation, production d'enzymes, métabolites secondaires, respirométrie, spectroscopie infra-rouge, baisse de pression, analyse d'images, bilan de masse, bilan de température, bilan d'eau, valorisation des résidus solides.

INTRODUCTION

Some important advantages and drawbacks of solid state fermentation (SSF) are the main focus of this paper.

WHAT IS SOLID STATE FERMENTATION (SSF) ?

It is generally understood that SSF is a microbial process ocurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients.The solid materials could be or not biodegradable. For example, starch and cellulose are solid materials of the first type, whereas, amberlite or polyurethane belong to the second type. It is also required for SSF that microrganisms grow with diffusible nutrients underneath and above a gas.

On the other hand, submerged fermentations (SmF) comprise a large variety of stirred or non stirred microbial processes, where biomass is surrounded completly by the liquid culture medium. The main differences between SSF and SmF are related to the various physico-chemical features, such as a) mixing and diffusion of substrates and products in relation to biomass is much smaller in SSF, b) solubility and diffusion of oxygen and other non-polar gases is greater in SSF, c) heat conduction is much smaller in SSF and d) water content is smaller in SSF. Such differences seem to account for important advantages and drawbacks of SSF, in relation to conventional SmF processes.

SSF IS QUALITATIVELY DIFFERENT TO SMF

During the last ten years, evidence has been accumulated over the support of the view that SSF processes are qualitatively different to SmF processes. This implies that microbial physiology does not work with the same regulation modes in each case, at least for a selected number of cases related to the production of spores, enzymes and secondary metabolites.

Differences in the sporulation of fungal cultures

The best known difference between SSF and SmF is fungal sporulation, since it has been known for a long time that sporulation using SmF is a rather difficult process to control, whereas it is very easy to obtain fungal spores by SSF. In fact, early sporulation is one of the major causes of concern for the safety of SSF processes and can be the cause of health hazards (Gregory and Lacy, 1963). Campbell (1971) has suggested that solid surface cultures are the natural habitats of fungal organism and, because of that, it is easier to conserve and control their morphological cycle by SSF, than by SmF. A practical application of this fact, is the use of SSF as a more

appropriate procedure to produce spores for several types of industrial applications. For example, inoculum production of *Penicillium roquefortii* for blue cheeses and Camembert (Larroche *et al,* 1988), production of *Bauveria bassiana* for use as biopesticide (Desgranges *et al,* 1991a, b) and the production of fresh inocula for starting new SSF or SmF processes.

Differences in the production of enzymes

Industrial enzyme production by SSF process has been carried out from a long time (Takamine, 1914; Underkofler *et al,* 1958). Ayres *et al* (1952) reported that pectinases produced by *Aspergillus foetidus* had noticeable biochemical differences, when produced by SSF, as compared to those by SmF techniques. Alazard and Raimbault (1981) showed that amylases produced by *Aspergillus niger,* using SSF of cassava meal, were more resistant to heat denaturation, than those produced by SmF, using the same strain and substrate. Reports of such type of enzyme differences have been reported (Romero *et al,* 1993; Villegas *et al,* 1993) and reinforced by the observation that the induction and repression patterns of pectinase production by *Aspergillus niger* CH4 are quite different for each kind of fermentation technique (Solìs-Pereira *et al,* 1993). All these reports have supported the claim of Shankaranand *et al* (1992) that there is an idiosyncrasy (*something special and not yet understood*) to the production of enzymes by SSF. They pointed out that microbial strains used for enzyme production by SSF should be different, than those strains used for the same purpose in SmF.

Antier *et al* (1993a, b) suggested a selection protocol of mutants from a wild type *Aspergillus niger* C28B25 (isolated from coffee dumps in Mexico by Boccas *et al,* 1994), in order to produce two classes of mutants, the so called AW99 specialized for the production of pectinases by SmF (AW = 0.99) and the AW96 class specialized for the production of pectinases by SSF with low water activity (AW = 0.96). Recent studies of parasexual recombination (Loera *et al,* 1993; Loera-Corral, 1994) showed that a mutation of the class AW99 seems to induce regulatory changes, which are dominant over regulatory changes of the class AW96. Those and other observations support a model of pleiotropic mutations of *Aspergillus niger* C28B25, that induces changes in the pattern of growth, the resistance to deoxy glucose and regulation of pectinase production (Lorea *et al,* 1993; Lorea-Corral, 1994; Minjares-Carranco and Viniegra-González, 1993; Viniegra-González *et al,* 1993a).

These findings could be relevant to the development of strategies for selection and utilization in SSF industrial processes. For example, it would give support to traditional claims that the artisanal way (SSF, cottage process) of producing inocula yields products with different quality as compared to the industrial way (SmF, large scale process). That could be the case for the large scale production of spores (rye

bread vs. stirred tank techniques) used for Roquefort cheese maturation in France. Also, the strategies for increasing SSF enzyme production processes could be graetly improved, if the regulatory genes responsible for the idiosyncracies required for good adaptation to solid cultures, were known. Finally, perhaps new approaches for enzyme engineering can be developed, if such idiosyncracies were identified in terms of specific amino acid sequences or glycosilation patterns. This could be clearly a new field of research, where the use of advanced techniques of molecular biology could yield significant fruits.

Differences in the production of secondary metabolites

Ziffer and Schelef (1980) reproted in their very early work on penicillin production at the Northern Research Laboratories, that there was a serious consideration for using the rotating drum for SSF, developed earlier by Underkofler *et al* (1958), which, in fact, was an adaptation of the old Japanese koji procedure, imported by Takamine to the USA at the beginning of this century (Takamine, 1914). The reason for considering SSF in penicillin production was related to the observation that penicillin yield is higher by SSF, than by SmF technique (Barrios-González *et al*, 1988). Barrios-González (1994) has also supported the idea that spontaneous mutants of *Penicillium chrysogenum* could be found to adapt in a different way to SSF and SmF processes. Other authors have also studied the production of different antibiotics by SSF (Johns, 1992).

Another interesting secondary metabolite is gibberellic acid. It is widely used as a plant hormone and is produced by *Gibberella fujikuroi.* The preferred industrial production technique, as in the case of penicillin, is SmF. However, Kumar *et al* (1987 a, b) have shown that SSF techniques could yield twice as much the yield of gibberellin, as compared to SmF, and this could mean significant savings in the production cost of such a metabolite (Kumar *et al*, 1987b).

Lakshminarayana *et al* (1975) showed that it was possible to produce large yields of citric acid using *Aspergillus niger* grown on sugar cane chips, with small doses of methanol, thereby indicating that SSF technique does not require the elimination of some metals (Fe, Cu, etc.) to induce the production of citric acid by this organism, as required in the conventional SmF technique. Córdova (1994) has shown that the culture of *Aspergillus niger* No 10, isolated from cassava waste by Raimbault (1980), when grown by SSF technique, had a faster rate of glucose or sucrose utilization, than in SmF technique and also that the SSF process was less inhibited by large amouts of glucose (>200 g/l) (Viniegra-González *et al,* 1993a), as suggested earlier by Oriol *et al* (1988b). The main fermentation product found by Córdova (1994) was citric acid, although other polyols were also accumulated, such as glycerol and erithrol.

These observations suggest the need to reevaluate the use of SSF process for the production of secondary metabolites. Special attention should be paid to such cases, where the quality of an expensive final product is enahanced by SSF. In the field of expensive biochemical products (new antibiotics, gibberellin, antitumoral compounds, etc.), it would be necessary to use clean, solid and recyclable supports, such as Amberlite (Auria *et al,* 1993) or polyurethane (Raimbault *et al,* 1990). But for inexpensive products (citric acid), the agro-industry residual raw materials, i.e., bagasse mixed with concentrated syrups, would allow the development a much cheaper production process.

There is also the need for more basic research on the physiology, biochemistry and molecular biology of secondary metabolism, adapted to the SSF technique and also to new approaches for process control, using respirometry, as suggested by several workers (Cadena *et al,* 1993; Saucedo-Castañeda *et al,* 1994; Villegas *et al,* 1993).

BIOMASS ESTIMATION

Measurements of biomass production by respirometry

The use of heterogeneous solid sopport for SSF makes it difficult for the estimation of biomass content in the fermentation mash, due to interferences of the substrates with current chemical assays and the difficulties in separating entangled biomass from the fibrous or granular structures of solid substrates. One possible way to estimate biomass production is through respirometry, because of the stoichiometric relations between biomass synthesis and oxygen as well as carbon dioxide balances (Cadena *et al,* 1993; Saucedo-Castañeda *et al,* 1994). In this way, it is possible to estimate the rate of biomass production (dX/dt), by measuring the rate of carbon dioxide evolution $R = d(CO_2)/dt$, by considering the respiratory yield coefficient, Y_r

$$dX/dt = R/Y_r \qquad (1)$$

Eq. 1 indicates that the production of carbon dioxide is proportional to the rate of biomass growth, but this is not the general case, because a more rigorous mass balance requires the use of the maintenance coefficient, m, which accounts for the respiratory activity not associated with biomass production, as in Eq. 2

$$R = Y_r(dX/dt) + mX \qquad (2)$$

In many cases, the growth process follows a first order kinetic law (exponential law), with a constant specific growth rate, μ

$$dX/dt = \mu X \quad (3)$$

Therefore, even though the respiratory coefficient, m, is not negligible, the value of μ can be estimated using Eq. 4, from Eqs. 2 and 3, because the solution of Eq. 3 is $X = X_0 e^{\mu t}$ and R can be expressed as proportional to X

$$R = Y_r(dX/dt - mX) = Y_r(\mu - m)X_0 e^{\mu t} \quad (4)$$

Thus, taking logarithms in both sides of Eq. 4, the following semilogarithmic expression is obtained

$$\ln R = \ln A + \mu t \quad (5)$$

The value of A can be related to Y_r, X_0, μ and m as follows

$$A = Y_r(\mu - m)X_0 \quad (6)$$

Being A>0, because μ>m, when microbial growth occurs.

Eq. 6 has been used by Saucedo-Castañeda *et al* (1994) in order to estimate the value of μ of *Schwaniomyces castelli* grown in a tubular reactor, packed with cassava granules, and, also by González-Blanco *et al* (1990) to evaluate the value of μ in SSF cultures of *Aspergillus terreus* on sugarcane residue.

Biomass estimation by physical measurements

Three main physical procedures have been suggested, i.e., *infrared spectroscopy* based on multiple wavelength absorption measurements (Desgranges *et al*, 1991 a, b), *pressure drop changes* in packed bed reactors (Auria *et al*, 1993; Auria and Revah, 1994) and, *image analysis* of fungal cultures (Packer and Thomas, 1990; Viniegra-González *et al*, 1993, 1994; Carlsen *et al*, 1994).

Infrared spectroscopy

This technique is based on the fact that microbial cell walls or membranes have characteristic compounds which are formed during the growth process and have

distinct spectroscopic features. For example, fungal cell walls are rich in glucosamine and fungal membranes are rich in sterols. Both types of compounds can be assayed in the spectrophotometer, especially, if it is equipped with a Fourier transform, in order to make continuous absorption measurements and a large number of spectroscopy correlations. Good correlations have been shown using clay beads as a solid support and *Bauveria bassiana* as a biomass (Desgranges *et al,* 1991 a, b). However, the technique apparently requires fastidious calibration and expensive instrumentation.

Pressure drop measurements

The second technique is based on the fact that in a filter cake, where a fluid is forced to flow, the d'Arcy resistance coefficient is related to the porosity of the cake. If a solid support is granulated or fibrous, with numerous interstitial spaces, and the fluid is air, the porosity would be diminished, if those interstitial spaces are filled by biomass structures.

Auria *et al* (1993) and Auria and Revah, (1994) have shown that the d'Arcy coefficient of tubular reactors, packed with Amberlite beads, is linearly related, in a wide range, to the amount of biomass of *Aspergillus niger* present in the column. The spherical and uniform geometry of Amberlite beads has enabled the interpretation of these results in a straightforward manner, but, this technique has not yet been applied to more irregular substrates, such as bagasse or cassava meal particles, where growth can occur within the solid support and not only on the solid support, as in Amberlite beads. Previous work done by Oriol (private communication) indicated that, with *Aspergillus niger* grown in cassava meal, a simple correlation between air pressure drop and biomass content within the packed bed reactor is absent.

An important result obtained by Auria and Revah (1994) was that the maximal value of biomass density, X_m, compatible with the air flow, and when estimated from the pressure drop measurements, was 22.2 mg/g of support, which thereby and agrees well with the observed value of X_m = 21.5 mg/g, suggesting that, at least for Amberlite columns, biomass saturation of the interstitial space is one of the major constraints for biomass production in a packed bed reactor. Addition of various levels of glucose (90, 130, and 200 g/L), did not change the value of X_m = 20.0 ±1.5 mg/g.

Image analysis techniques

The third type of technique has been used mainly to measure the specific growth rate (μ) of fungal cultures in solid media and study the fermentation kinetics of packed bed reactors (Viniegra-González *et al,* 1993, 1994), although it is being used to study SmF fungal processes (Carlsen *et al,* 1994). It is based on the fact that

mycelial organisms only grow at the tips of their branched structures called hyphae (Barnitcki-Garcìa *et al*, 1989) and also on the observation that μ is proportional to the maximal elongation rate of distal hyphae, U_r (Trinci, 1971). According to these observations, the specific rate of biomass production would be proportional to the amount of tips present in the culture and their number would increase exponentially with branching frequency, ϕ. Using a binary expression, indicated in Eq. 7, the exponential function is derived from a first order differential equation (Eq. 3),

$$X/Xo = e^{\mu t} = 2^{\phi t} \tag{7}$$

Trinci (1971) showed that, for each given distal branch of length L, the rate of elongation followed a saturation law on L, indicated by Eq. 8:

$$dL/dt = U_r L/(K+L) \tag{8}$$

Two extreme cases are to be considered from Eq. 8. A first case would be to assume that, in the population of distal hyphae, small segments with $L<<K$ predominate (first order kinetics of elongation). In such a case, the time τ required for a small tip of initial length D_h (the hyphal diameter) to reach the critical length L_c, at which a new branch will be produced, could be calculated as follows:

$$(U_r/K)\tau = \ln(L_c/D_h) \tag{9}$$

Thus, the value of ϕ can be estimated as the inverse of τ and would yield Eq. 10

$$\phi = (U_r/K)/\ln(L_c/D_h) \tag{10}$$

The second case is to assume that, on the contrary, $L>>K$ (zero order kinetics of elongation) and as a consequence Eq. 11 holds

$$\phi = U_r/L_c \tag{11}$$

Larrade-Corona *et al* (1994) and Viniegra-González *et al* (1994) have shown that, for fungal cultures of *Gibberella fujikuroi* and *Aspergillus niger*, where the actual value of μ has been directly measured from plots of ln X *vs*. t, the best model was Eq. 10,

where K was assumed to be near L_c, which can be measured directly *in situ* by image analysis for SmF, SSF and surface cultures (Petri dishes).

Viniegra-González *et al* (1993, 1994) have shown that the growth curve of *Aspergillus niger* No 10, grown on cassava meal (Raimbault, 1980), can be followed very closely by Eq. 12

$$dX/dt = \mu[X + X_c] [1 - (X/X_m)^p] \quad (12)$$

According to these authors, the values of μ and X_c can be estimated using the following relations:

$$\mu = ln(2)(U_r/L_c)/ln(L_c/D_h) \quad (13)$$

$$X_c = N_o(\pi D_h^2/4)\rho L_c \quad (14)$$

where, N_o is the number of germinated spores and ρ is the biomass density. The parameters U_r, L_c, D_h, N_o and ρ can be estimated by direct optical or physical measurements, whereas the parameters X_m and p ought to be estimated by non-linear variational techniques (minimization of the sum of squared residuals).

In a synchronized culture the parameter X_c corresponds to the amount of germinated spores with $L = L_c$, that is just about to produce the first branch. The parameter X_m is the maximal biomass density to be reached by a certain organism in a given medium and the parameter p is an indication of the degree of self inhibition of the culture. For $0<p<1$, the culture has early inhibition, for $p = 1$ inhibition is equally distributed for different sizes of colonies and for $p>1$, inhibition occurs. When the hyphal density is very high (late inhibition).

Viniegra-Gonzales *et al* (1993, 1994) found that Eqs. 13 and 14, together with a non-linear estimation of X_m and p, gave a very close fit to the actual experimental data published by Raimbault (1980) on the cassava SSF fermentation by *Aspergillus niger* and also, the values of L_c were very close to those observed by electron microscopy. It is notewoothy that the U_r values, used in these similations, were experimental data measured in Petri dishes by Raimbault (1980). This indicates that the maximal elongation velocity, U_r, can be estimated in surface cultures, although it has been found that the values of L_c and D_h should be measured *in situ,* using samples of each given culture medium.

MASS AND HEAT BALANCES OF SSF

Mass balance

According to Raimbault (1980), the mass balance of SSF can be achieved, if the approximate analysis of biomass is known. For example, if the biomass has aproximately 40.5% of C, 6.8% of H, 43.2% of O and 9.45% of N, the stoichiometric equation for aerobic cell growth would be as follows:

$$C_6H_{12}O_6 + nNH_3 + gO_2 \rightarrow nC_5H_{10}O_4N + rCO_2 + wH_2O \quad (15)$$

Now the yield coefficient (Y_X) in terms of substrate consumption (X g biomass per g substrate), can be given by the following equation:

$$Y_X = 148n/180 = 0.822n \quad (16)$$

The mass balance for carbon, derived from Eq. 15, leads to equation 17

$$6 = 5n + r \quad (17)$$

From Eqs. 16 and 17 the following relationship is obtained.

$$r = 6 - 5n = 6(1 - 1.013Y_X) \quad (18)$$

Usually, $0.3<Y_X<0.5$, therefore, $r > 2.96$ (Raimbault, 1980). This indicates that carbon dioxide is produced during the fermentation process :

$$Y_r = 148n/48r = (0.625)(Y_X)/(1 - 1.013Y_X) \quad (19)$$

Water balance

In order to account for water formation during the growth process, it is necessary to make the hydrogen stoichiometric balance, using Eq. 15, as follows:

$$12 + 3n = 10n + 2w \quad (20)$$

From Eqs. 17 and 20, the value of w can be calculated as:

$$w = (12 - 7n)/2 = 6(1 - 0.7093Yx) \quad (21)$$

Again, as $Yx < 1$, it is concluded that $w>0$, which is consistent with the fact that water is produced by a combination of respiration and biomass synthesis (Raimbault, 1980; Oriol *et al,* 1988a), thereby increasing the local amount of water available for biochemical processes.

Raimbault (1980) indicated that amount of water absorbed in biomass should be substracted from the local water available for dissolving the nutrients and, for substrates rich in starch, the amount of water is limiting. But with very hygroscopic materials, (i.e., rich in cellulose), the amount of water is in excess. Using this knowledge, Oriol *et al* (1988a, b) showed that *A. niger,* grown on mixtures of bagasse and cassava meal, had a faster growth rate, than *A. niger* grown on cassava meal alone. This supports the need of using water balance as one of the major macroscopic variables of SSF (Saucedo-Castañeda *et al*, 1994).

Heat balance in SSF

Saucedo-Castañeda *et al* (1990) developed a basic equation for heat balance in SSF, as indicated by Eq. (22).

$$\partial\theta/\partial\tau = [Ld_p/R_a{}^2Pe]\,[\partial^2\theta/\partial p^2 + (1/\rho)(\partial\theta/\partial\rho)] + F_{dm}Da_{III}\,[Rs/Ri] \qquad (22)$$

where, the state variables θ, ρ and τ are the normalized values for the radius of the column, the temperature and the time, defined as follows:

$$\theta = r/R_a; \qquad \rho = T/T_b; \qquad \tau = t/\Theta \qquad (23)$$

R_a is the maximal radius, T_b is the exterior temperature and Θ is the characteristic time = L/u; where, u is the flow velocity of the air within the column, assumed to be strong enough, to destroy the axial temperature gradients along the column length. The dimensionless numbers that characterize the process are, Pe, the Peclet number, related to dispersion phenomena within the column, and Da_{III} the Damköhler number, related to the reaction rate. F_{dm} is the dry mass fraction, d_p is the average particle diam and R_s and R_i are final and initial reaction rates.

Using Eq. 22, Saucedo-Castaneda *et al* (1990) were able to simulate the temperature profiles within a wide column, packed with cassava meal and inoculated with *Aspergillus niger*. These numerical solutions gave a numerical approximation to the respiratory rate of the the column. The Peclet number was found to be consistent with a material having low porosity, in which the heat conduction was limited by a

very low transmission coefficient. The conclusion was that forced air convection ought to be used, as the major way to eliminate heat from the fermentation mash.

These results indicate that it is possible to use basic engineering principles to simulate and characterize the SSF process and in this way, the extrapolation of experimental work can be done on the basis of dimensionless groups related to dispersion and reaction processes.

INTEGRATION BETWEEN FERMENTATION AND RESIDUAL DISPOSAL

Nowdays, a major consideration for new or novel industrial processes is the reduction of environmental hazards and also production costs.

Campos and Viniegra-González (1995) have shown that solid residues coming from SSF processes are a potential source of probiotic material, which enhances the rate of growth of cellulolytic rumen bacteria. These authors compared the effect of yeast and mold extracts from commercial origin, with the residues from SSF of coffee pulp by *Aspergillus niger*, for production of pectinases, which were leached out with the help of a hydraulic press. They found that all samples had similar probiotic activity. Salinas (1994) has isolated various fungal cultures, which grew during the spontaneous composting process and noticed a plant stimulating activity. This suggests that it is possible to compost organic residues in such a way, that the remaining solids are blended with products and organisms having plant regulator properties, during composting under SSF.

Such results indicate the need for developing an integrated approach to SSF processes. In many cases, the SSF industrial process would leave a solid residue, after extracting valuable products (organic acids, enzymes, etc.). These solid residues should be characterized as potential probiotic material, either for direct livestock production or for improved composting materials.

CONCLUDING REMARKS

The ancient process of solid state fermentation (SSF) is becoming a well understood, according to the rules of biochemical engineering, biochemistry, microbiology and molecular biology.

Recent work has found that microscopic heterogeneity, the so called weakness of SSF, is becoming its major strength, as cause for increasing yields and changing cell physiology of appropiate microbial organisms.

Micro-organisms adapted to low water activity levels thrive selectively in many SSF processes, thereby diminishing the cost of upstream processing.

Reduced levels of water content in the fermentation mash of SSF process favour a cleaner industrial operation, with low levels of waste water.

Detailed and automatic water and heat balances of SSF process offer a way to achive the engineering design of large industrial reactors.

There are indications of a possible integration a clean technological operation of many SSF processes, by focusing on the main fermentation product, as well as potential by-products, by means of proper residual recovery and utilization.

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Biomass estimation in solid state fermentation

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SUMMARY

During a solid state fermentation process, the microorganisms are intimately bound to a solid matrix, which poses difficulties for biomass measurements. Unlike submerged fermentation, fungal biomass cannot be quantitatively separated from the solid medium, and hence, direct measurements are impossible.

A brief survey of the different indirect manual biochemical methods, described by several authors, is given, along with the description of four selected biochemical methods (glucosamine, ergosterol, total sugar, carbohydrate consumption). Studies on 22 different fungal strains and different solid media (organic material such as sugar beet pulp, sponge or mineral material as clay micro-granules), showed that the fungal growth kinetics could be followed by glucosamine measurements with a satisfactory accuracy. Infrared estimation of cell components (glucosamine and ergosterol) and medium residues directly allow on-line biomass measurements in solid media the CO2 evolution rate during cultivation. It is not sufficient to measure a total biomass, but it is very important in many processes to be able to measure the viability of cells (for example conidia produced for biological control applications). Hence, methods for estimation of biomass viability are also discussed.

Keywords : Solid state fermentation, biomass estimation, indirect manual biochemical methods, on-line methods, biomass viability estimation.

RESUME

Estimation de la biomasse au cours de la fermentation en milieu solide

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Au cours des procédés de fermentation en milieu solide (FMS), les microorganismes sont intimement liés à la matrice solide, ce qui se traduit par des difficultés au niveau de l'estimation de la biomasse. De ce fait, contrairement à ce qui se passe en fermentation liquide, la biomasse fungique ne peut pas se séparer du milieu solide, et par conséquent la mésure directe de la biomasse est impossible en FMS.

Une brève description de différentes méthodes biochimiques d'estimation indirecte et manuelle est présentée et quatre méthodes biochimiques ont été retenues (glucosamine, ergosterol, sucres totaux, consommation de sucres). Des études réalisées sur 22 souches de champignons filamenteux cultivées sur différents milieux solides (matériaux organiques comme la pulpe de betterave, matériaux minéraux comme les éponges et les granules de vermiculite) ont montré que la cinétique de la croissance mycélienne peut être suivie, d'une manière satisfaisante par l'analyse de la glucosamine. L'estimation, par spectofluorométrie infra rouge, des constituants cellulaires (glucosamine et ergosterol) et des résidus du milieu peuvent être suivis en ligne pour la mésure de la biomasse et l'évolution du CO2 au cours de la FMS. Il n'est pas suffisant de mesurer la biomasse, mais il est important de pouvoir mesurer la viabilité de la biomasse pour certains procédés (viabilité des conidies produites). Dans ce cas, l'éfficacité des méthodes utilisées pour l'estimation de la viabilité de la biomasse est discutée.

Mots clés: Fermentation en milieu solide, estimation de la biomasse, méthodes biochimiques mannuelles indirectes, suivi en ligne, estimation de la biomasse viable.

INTRODUCTION

WHY BIOMASS IS MEASURED ?

In all bioprocesses, biomass constitutes a fundamental parameter in the characterization of microbial growth. The bioprocesses can be divided into two types, the first type aims at biomass production, while the second type involves production of metabolites (primary or secondary).

In the first case, the biomass measurement is very important, because it is the main target of the solid state fermentation (SSF) process.

In the second case, metabolite production is very often correlated and is proportional to the biomass quantity. Therefore, in order to optimize a metabolite production, especially a secondary metabolite, it is necessary to optimize the microbial growth.

However in some cases, for example, when the process concerns the protein enrichment of a by-product, it is important to know the quantity of protein produced, and the biomass estimation in the strict sense, is not of interest.

In the same way, the optimization of the production of an enzyme, which is related to the growth of the micro-organism, can be carried out without the measurement of biomass.

Another consideration concerns the possibility of on-line measurement. In submerged fermentation, on-line biomass measurements are carried out using biophotometer and similar devices, which are based on optical density measurements. In SSF, no method is available for direct on-line biomass measurement. As in the liquid fermentation, the best possibility is to analyze the gases at the reactor output.

Finally, in processes of micro-organism production using different applications, such as biological control, the measurement of the total biomass or the counting of spores produced, is certainly not sufficient. In this case, the viability of the micro-organisms is important.

HOW BIOMASS IS ESTIMATED ?

In SSF, three phases exist, i.e.,a solid matrix (organic, mineral or synthetic), a liquid phase bound with the matrix and a gaseous phase.

The micro-organism is present at all these phases (Durand and Chereau, 1988). Therefore, unlike submerged fermentation, direct determination of biomass in SSF is difficult, due to problems of separation of the micro-organism from the insoluble phase. Nevertheless, in some cases involving spores and unicellular microorganisms (bacteria, yeasts), this separation is possible. However, when the growth of fungi is in the form of mycelium, it is impossible, because the fungal hyphae penetrate inside the solid matrix, and bind the mycelium tightly to the solid phase.

Three categories of methods allowing the biomass estimation can be distinguished (Desgranges *et al*, 1991a), i.e., direct biomass measurements, analysis of biomass components and analysis of biomass metabolic activities.

DIRECT BIOMASS MEASUREMENTS

In this case, the micro-organisms must be unicellular. Depending upon the solid matrix and mainly the micro-organism used, it is possible, as in the liquid fermentation, to measure the biomass directly. Due to the fact that the cells do not bind into the solid phase, it is relatively easy to remove them by mixing a solid sample in water and using the classical methods for biomass estimation (Sato *et al*, 1985).

The cell number is measured using a haemocytometer, plate counts or in some cases filtration for dry weight determination. In our lab, we applied this methodology for the cultivation of yeasts on wheat bran or sponge. The level of recovery was nearly 100 %.

The same procedure can be used for determination of the spore numbers, but it cannot be considered as a total biomass measurement.

A few more specific cases are mentioned in the literature. For example, the recovery of biomass from a specific matrix such as gelatin and carrageenan, was carried out after melting these matrices at a mild temperature (Wei *et al*, 1981). It is obvious that these examples are confined to laboratory studies due to the nature of the solid media involved.

Yet another example concerns the culture of mycelium on a membrane filter (Mitchell *et al*, 1989). As discussed later, this method can only be used for the calibration of indirect methods. Indeed, the biomass dry weight can be obtained simply. The advantage is that the culture can be carried out on a medium having a composition similar to the solid medium used. This method is better than a

calibration from a liquid culture, because the conditions are nearly similar to those in the solid state cultivation.

ANALYSIS OF BIOMASS CONSTITUENTS

Many authors have described different indirect methods, which are based on the analysis of fungal constituents.

These include glucosamin from chitin (Narahara *et al*, 1982), ergosterol (Seitz *et al.* 1979), nucleic acids (Koliander *et al*, 1984), total sugars, and protein using the classical Kjeldahl method.

Among these different constituents, the selection of one or several indicators of biomass concentration must be based essentially on four criteria, such as (1) an indicator well adapted to the micro-organism used, (2) a reproducible and quantitative recovery of this indicator, (3) negligible or little interferences with the culture support and the nutrient solution, and (4) the amount of the selected indicator must be constant throughout the fungal development and must be the same under different cultivation conditions.

Considerable work has been carried out on these aspects. A process for the production of an entomopathogenic fungus (*Beauveria bassiana*) for fighting the corn borer has been developed and patented (Riba *et al*, 1990). This fungus was cultivated on solid media, which involved clay granules (as support) wetted with a nutrient solution.

In order to optimize the growth and the sporulation of this strain, it was interesting to know the biomass formed during the process. For that, the evolution of glucosamine, ergosterol and total sugars in the biomass was studied for the first time (Desgranges *et al*, 1991a).

These different constituents in relation to dry biomass were measured during agar plate cultivation in 3 different nutrient solutions (NS1, NS2, NS3), as shown in Figs. 1a,b and c. Media NS2 and NS3 have exactly the same constituents and only differ in the C/N ratio. NS1 does not contain the same nitrogen and carbon sources.

Fig. 1a shows that the glucosamine content varied according to the nature of the culture medium. Between NS2 and NS3, no significant variation was observed (a mean value of about 4.3 %DM). The value is different (mean value of 7.1 %DM) compared to medium NS1.

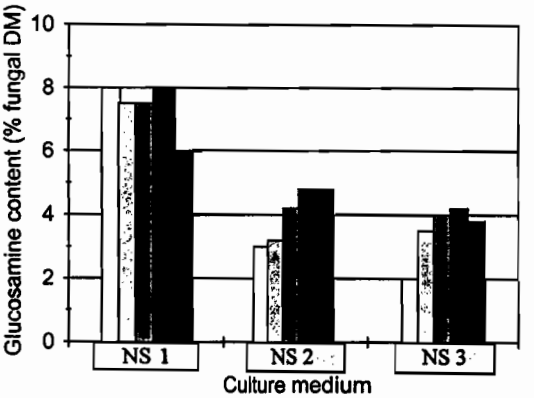


Fig. 1a : Glucosamine content during cultivation of *Beauveria bassiana* on different media.

Thus, it could be concluded that, if the constituents are the same, and the C/N ratio is different, the glucosamine content represents a good indicator for biomass estimation. However, if one substrate in the medium is changed, the results are erroneous. Thus, when screening of substrate is carried out, the glucosamine content is not a good biomass indicator.

Ergosterol (Fig. 1b), increases rapidly to reach a maximum and then remains constant. It also varies according to the medium. The initial values up to 24 h are not presented in the figure, because the amounts were too low for HPLC detection.

Fig. 1c shows that, for NS1 and NS3, the total sugar content is stable from 48 h and for NS2 from 72 h. As soon as the sporulation starts, the total sugar content remains constant. Using NS1 and NS3 media conidiation commenced at 48 h cultivation, whereas with NS2 medium, it started at 72 h.

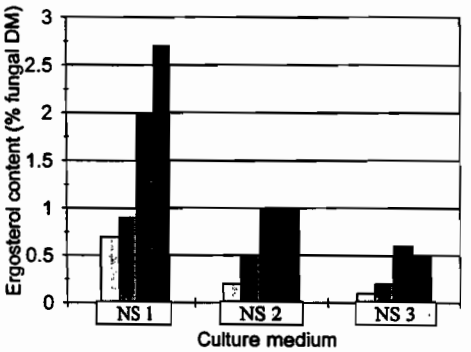


Fig. 1b : Ergosterol content during cultivation of *Beauveria bassiana* on different media.

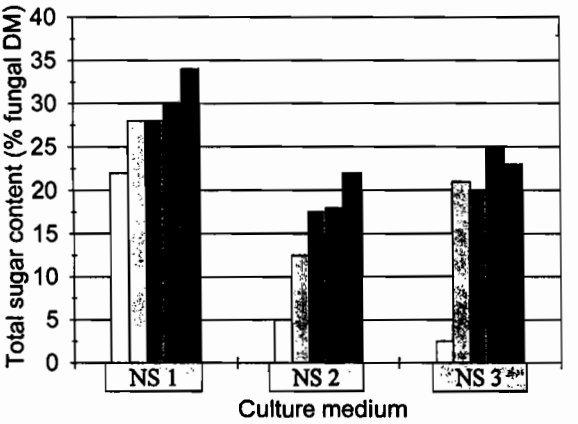


Fig. 1c : Total sugar content during cultivation of *Beauveria bassiana* on different media.

Before using glucosamine for cultures on clay granules, it was necessary to compare results with those obtained on the agar plate. As the biomass dry weight determination was impossible, the ratio of ergosterol to glucosamine (E/G) was calculated to compare the results.

Taking into account the accuracy of the analytical methods, it was observed that this ratio was nearly the same for the 2 culture conditions (agar plate and clay granules). Thus, the results obtained for agar plate could be extrapolated to obtain clay granule cultivations.

Experiments have also been carried out on 22 strains of 12 species (Roche *et al*, 1993) to establish the relationship between the fungal dry matter and the glucosamine amount for each strain. It was significant at a level of 1% (according to Fischer's table).

$$X = \alpha\ GLU + \beta$$

Where X = fungal dry matter (in mg)

GLU = glucosamine amount (in µg)

α = slope (mg/µg)

$1/\alpha$ = glucosamine content (in µg/mg fungal DM)

β = constant

This equation gave satisfactory results for the different strains tested on several solid media.

ANALYSIS OF BIOMASS METABOLIC ACTIVITIES

Some metabolic activities are associated with the growth and can be used as indicators of biomass. These include :

- **Extracellular enzymes.** For example, an α-amylase activity is directly proportional to the mycelial weight for *Aspergillus oryzae* grown on steamed rice (Okazaki *et al*, 1980).
- **ATP content** can also be used as an indicator, but it is not accurate because the biomass may be actively growing in some regions, but may be in stationary phase in other regions (Matcham *et al*, 1984).
- **The consumption of substrate** can also constitute a good indicator. It was used to estimate the growth of *Beauveria bassiana* on clay granules, wetted with a nutritive solution containing sucrose (Desgranges *et al*, 1991a).

Table 1. Relations between mg sucrose consumed and µg glucosamine (GLU) in 4 media having the same nutrients but not at the same concentrations.

Medium	C/N Ratio	Equation	Correlation coefficient
CG 1	27	S = 0.04 GLU - 3.6	0.96
CG 2	1.7	S = 0.02 GLU - 4.0	0.97
CG 3	3.0	S = 0.02 GLU - 2.2	0.93
CG 4	2.0	S = 0.02 GLU - 4.3	0.95

The biomass yield is the inverse of the slope of each equation.

For 3 media (CG2, CG3 and CG4), this yield is the same, i.e., 50 mg glucosamine / 1 mg sucrose consumed.

This yield is different (25 mg glucosamine / 1 mg sucrose consumed) for CG1 medium, which has a very different C/N ratio.

Though many methods of indirect biomass estimation exist for SSF, none is ideally suited to all situations, because practically all the biomass constituents vary according to the growth phase, the nature of the medium and the C/N ratio for a same medium.

However, in many cases, glucosamine constitutes a good indicator for the biomass measurement (Narahara *et al*, 1982). Taking into account the precision of the analytical method (hydrolysis of chitin, coloration measurement), the relationship reported above is satisfactory.

Nevertheless, the major drawbacks of these methods include manual methodology, need for fastidious and time consuming extraction procedure and non-amenability for one-line application.

ON-LINE BIOMASS ESTIMATION METHODS

Essentially, two methods exist for on-line biomass measurement and these include: infrared estimation of some cell constituents and CO2 evolution or O2 consumption.

CO_2 MEASUREMENT

Classically, CO_2 is measured on-line by an IR analyser. A microcomputer can automatically calculate the CPR (carbonoxide production rate) during the culture (Narahara *et al*, 1982). The method is very sensitive and can indicate even low physiological activity. Good correlation has been established, for example, between CO2 and the glucosamine level.

However, like the glucosamine and ergosterol measurements, CO2 estimation does not allow comparison of the growth of fungi cultivated on solid media differing in composition.

It was also observed that the CO2 evolution rate varied with the fermentation conditions. This last aspect could be interesting for industrial applications. Indeed, when the process is very well defined and fixed, the change in the CO2 evolution rate can indicate a problem in the fermentation (Desgranges *et al*, 1991b).

A good reproducibility of the regression can be observed for 3 identical cultures in Table 2. For the 4th experiment, the difference observed is due to a defective air humidity regulation during the first 12 h cultivation.

Table 2. Relationships between CO2 evolution expressed in µmole / g DM and the glucosamine content (GLU), expressed in µg / g DM

Experiment 1	CO_2 = 1.72 GLU - 1236.5
Experiment 2	CO_2 = 1.52 GLU - 1074.0
Experiment 3	CO_2 = 1.68 GLU - 1568.2
Experiment 4	CO_2 = 0.94 GLU - 896.6

INFRARED ESTIMATION

The apparatus used is an InfraAnalyzer 400 (Technicon). Its principle, as shown in Fig. 2, is founded on the measurement of reflected light by the matrix surface at specific wavelengths. It has 19 filters distributed on a wavelength range between 1400 and 2400 nm. The amount of the measured component is calculated by the mathematical relationship :

$$C = F_0 + F_1 \log (1/R_1) + \ldots\ldots\ldots\ldots + F_n \log (1/R_n)$$

where C is the component amount, F_0 to F_n are the calculation constants, R_1 to R_n are the reflection values and 1 to n is the filter number selected by the computer (Durand and Chereau, 1988).

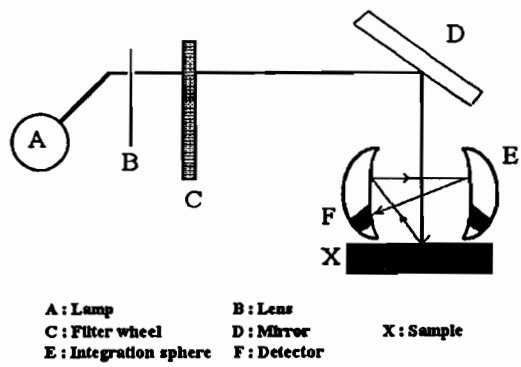


Fig. 2 : Schematic diagram of the infrared measurement principle.

This relationship is established by calibration of the apparatus. The computer calculates a multiple linear regression, by selecting the filters, which are the most characteristic of the component. Of course, the first application of this apparatus is to measure the water content of a solid medium.

When the apparatus is well calibrated by using manual reference methods, (which can be fastidious for some analysis), different constituents of a solid medium can be measured in a few seconds. Therefore, this method can be considered as an on-line method, even if it is necessary to take a sample in the reactor. Table 3 gives an example of the selected filters and the regression coefficients obtained during the calibration (Desgranges *et al*, 1991b).

Table 3. Example of calibration for Infrared measurement of samples from Beauveria bassiana cultivation on clay granules

Constituent	Number of filters selected	Wavelength selected (nm)	Regression coefficient
Glucosamine	2	2100	0.960
		1445	
Ergosterol	4	1982	0.925
		1778	
		1940	
		1734	
Sucrose	3	2336	0.960
		2139	
		1722	
Nitrogen	4	2270	0.840
		2208	
		2100	
		1445	

Unfortunately, some drawbacks also exist. As the principle is based on reflectance, the surface of the sample must be homogeneous, and always with the same granulometry. Therefore, this technique cannot be used for all media. Moreover, as the computer takes into account the nature of the matrix and its composition, the InfraAnalyzer can only be used, when the process is well defined and abolutely fixed.

For an industrial process, this apparatus is very attractive for routine analysis.

Fig. 3 represents an example of results obtained during a culture of *Beauveria bassiana* on clay granules with CO_2 and Infrared measurements, as compared with the classical manual glucosamine determination (Desgranges *et al*, 1991b).

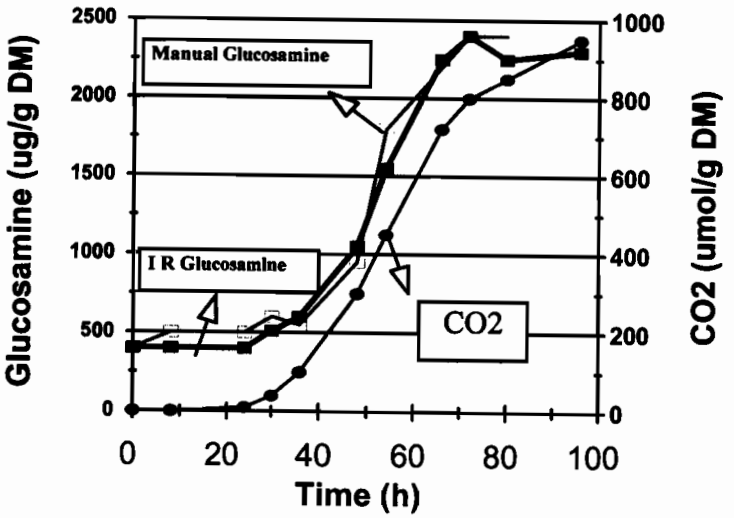


Fig. 3 : Estimation of *Beauveria bassiana* growth by 3 methods (CO_2 measurement, glucosamine determination by manual and infrared measurements).

BIOMASS VIABILITY ESTIMATION

Very often it is necessary to measure the viability of the micro-organisms produced.

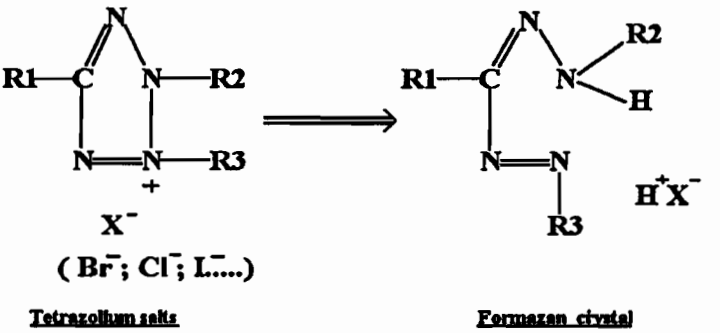
This measurement is of interest for two reasons, i.e., checking an inoculum before inoculation and checking the micro-organisms produced at the end of a process, after downstream processes or storage.

This measurement appraises the quality of an inoculum or a production.

The classical method of measuring the C.F.U. (colony forming unit) is generally time consuming (minimum 2 days incubation), and in some cases, (with mycelium for example), this technique is not accurate. In addition to the respirometry and the ATP content measurement, other methods can be used, such as microscopy after

staining by methylene blue, measurement of labelled compounds incorporated in the cells and measurement of dehydrogenase activity.

This last method is based on the reduction of a substrate by the dehydrogenases present in the micro-organisms (Roehm *et al*, 1991). This reduction, which occurs at the electron transfer level (cytochrome), gives a change of colour:



The substrates used are generally tetrazolium salts (MTT, XTT, INT, etc.) and only the viable cells can reduce these salts to give a formazan crystal, which is coloured. We have developed this methodology to study the viability of conidia of *Beauveria bassiana*.

This measurement can determine small changes in conidia numbers and good correlation between dehydrogenase activity and the conidiation. The influence of thermal treatment on conidia can also be observed (unpublished data).

This method is attractive, because it is simple, rapid (about 4 h) and does not need sophisticated equipments.

CONCLUSION

In solid state fermentation, an universal method for biomass determination does not exist. Anyway, the same problem exists in the classical submerged fermentations. What is more important is to identify the purpose for which the measurement is required. For some research studies or applications, the total biomass measurement is not really necessary.

In some cases, (e.g., yield calculation, optimisation of productivity, etc.), biomass estimation is essential. In such cases, different direct or indirect methods can be used.

The choice depends upon the process and its scale (laboratory or industrial scale), the target (what is produced), the necessity to have a simple and rapid method, the desirable accuracy and the cost.

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Growth characteristics of *Aspergillus chevalieri* and other fungi from under-coating of chocolate truffles

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SUMMARY

Growth characteristics of fungi, which occasionally affected chocolate truffles, have been studied, using moulded and non-moulded samples. A whitish mycelial growth, located at the filling-coating interface is identified as *Eurotium/Aspergillus chevalieri*. The spores of other xerophilic molds (*Aspergillus fumigatus, A. penicilloides* and *Penicillium verrucosum*) were also found at the filling-coating interface. The water activity of chocolate coating was low enough to prevent fungal growth on the external surface of the truffles. However, the water activity of the filling, due to presence of cream, is sufficient enough to allow the growth of xerophilic moulds. The data on growth characteristics of the isolates have been presented in terms of the range of growth temperature and growth at different water activities. The origin of the contaminating fungi is probably from the cocoa beans, but also from the ambient atmosphere during coating of the truffles.

Keywords: Chocolate, truffles, Aw, xerophilic fungi, temperature, *Eurotium, Aspergillus chevalieri, Aspergillus fumigatus, Aspergillus penicilloides, Penicillium verrucosum.*

RESUME

Caractéristiques de la croissance d'*Aspergillus chevalieri* et d'autres moisissures provenant de l'interface fourrage-couverture de truffes de chocolat.

BENSOUSSAN M., ALCARAZ G., ET TOURTEL V.

Les caractéristiques de la croissance de moisissures, qui affectent occasionellement des truffes de chocolat ont été étudiées à partir d'échantillons apparemment moisis et non-moisis. Un mycelium d'aspect blanchâtre visible à l'interface fourrage-enrobage a été isolé et identifié à *Eurotium/Aspergillus chevalieri*. La présence de spores d'autres moisissures xérophiles (*Aspergillus fumigatus, A. penicilloides et Penicillium verrucosum*) à l'interface fourrage-enrobage a été démontrée. L'activité de l'eau de l'enrobage de chocolat est suffisamment faible pour empêcher la croissance fongique à la surface externe des truffes. Cependant, l'activité de l'eau du fourrage est suffisante, en raison de la présence de crème dans la ganache, pour permettre la croissance de moisissures xérophiles. Les caractéristiques des souches fongiques identifiées sont exprimées en termes de domaines de température et d'activité de l'eau favorables à la croissance. L'origine de la contamination fongique est à rechercher au niveau des fèves de cacao, mais aussi dans l'atmosphère qui environne les truffes durant l'enrobage.

Mots clés : Chocolat, truffes, ganache, Activité de l'eau, moisissures xérophiles, température, *Eurotium, Aspergillus chevalieri, Aspergillus fumigatus, Aspergillus penicilloides, Penicillium verrucosum.*

INTRODUCTION

Chocolate, as in the case of many of food products, is a complex mixture of various edible ingredients. In spite of low water activity, it is susceptible to deteriorations during its shelf-life. Such deteriorations have been classified into five groups by Kleinert (1976). These include a) physical deteriorations, such as liquefaction of fats and surface bleaching at high temperature as well as defects associated with alteration of moisture level and crystallization of sugars at the surface under dry atmosphere, b)

aroma defects due to the ability of chocolate to easily absorb odours from the storage environment, c) biochemical modifications due to oxidative or hydrolytic reactions of lipids present in chocolates, d) degradation due to the action of the enzymes present in the product and e) degradation due to microbial action.

Even if the chocolate, as a final product, possesses physico-chemical conditions which prevent the development of numerous micro-organisms, some strains can survive.

The microbial alteration is directly linked to the quality of raw materials and to the hygiene of the personnel and premises during its manufacture (Kleinert, 1976). Up on recept, the average bacterial count is 10^7 CFU/g cocoa beans. There is also a great diversity of yeasts that come from the cocoa fermentation: the most frequently encountered genera are *Saccharomyces, Pichia* and *Candida* (Ravelomanana *et al*, 1985).

The majority of this microbial charge is located on the shell and the grain is infected during the process of shell separation. Torrefaction allows the elimination of a part of this microbial charge, but, in some cases, it is not sufficient for the destruction of *Salmonella* . Post-torrefaction treatments can increase the contamination, if the environment of the premises is not good. The recycling of cocoa doughs, the cooling, the storage and the carriage of the cocoa powder are liable to contamination, in addition to those processes involving the ambient moisture.

Consequently, the chocolate is susceptible to contamination at many points of its manufacture. Thus, the presence of yeasts, fungi or enterobacteria reveals insufficient sanitary conditions, during the manufacture of chocolate (Meursing and Bom, 1984). The growth characteristics of the fungal strains, which have been isolated from chocolate truffles, form the subject matter of this paper.

MATERIAL AND METHODS

SUBSTRATES

Chocolate truffles consist of a parallelepipedic core of soft chocolate mixture, the ganache (chocolate, butter, cream, sugar), as filling. This semi-solid ganache, in turn, is wrapped in a hard dark chocolate, the outer coating, with a low adhesive capacity. The faces of the core show non-sticky areas in several cases.

MICROORGANISMS

Several truffles exhibited the sign of fungal contamination. The mycelium, located between the soft chocolate filling and the internal face of the hard chocolate coating, was isolated.

Two other parts of the filling were also tested for fungal growth, i.e., 1) the surface, which was scrapped and 2) small pieces of the core, which were separated aseptically. The samples (approximately 1 g each) were suspended in 9 ml saline (NaCl solution, 0.9%), using the Stomacher apparatus, further diluted serially, inoculated on a nutritive agar medium and incubated at 27°C.

GROWTH CHARACTERISTICS

Pure isolates were cultivated on three solid media enriched with chloramphenicol (100 $mg.l^{-1}$) to avoid bacterial development: malt extract agar (MEA), potato dextrose agar (PDA) from BioMerieux and Czapeck agar (CZA) from Difco.

Radial elongation of the mycelium on solid media was measured, in triplicate, by estimating the increase in the colony diameter (Pirt, 1967; Trinci, 1971). Biomass production in the liquid medium was measured, in triplicate, after filtration, washing with saline and drying for 72 h in a oven (60°C).

All fungal strains, grown on MEA for one week, were screened for temperature tolerance between 5 and 55°C.

Water activity (Aw) tolerance for growth at 27°C was determined after 10 days in malt extract broth (BioMérieux; 20 g/l^{-1}), using glycerol as a compatible water activity (Aw) depressor. Molarities of glycerol (number of moles of glycerol / 1000 g aqueous nutritive basal medium) were determined, according to Meryman (1966). Water activity measurements (Debeaufort, 1994) on liquid or solid media at 27°C were carried out, using small samples (0.5 g) and an Aqualab CX-2 (Decagon, Washington D.C., USA).

IDENTIFICATION OF STRAINS

Identification of fungal colonies were carried out after one week growth on MEA, PDA and CZA, according to recent methods (Samson *et al*, 1995).

RESULTS AND DISCUSSION

As observed with nutritive substrate, growth of filamentous fungi is also dependent on physicochemical parameters. Then, tolerance ranges of the fungal isolates versus such parameters as temperature and Aw are presented in Tables 1 and 2.

MOISTURE CONDITIONS OF THE CHOCOLATE SUBSTRATES

The external surface of the chocolate coating is free from visible fungal contamination. Aw measurements of this hard dark chocolate showed a value of 0.53 (0.08). This average value is similar to the result obtained by Larumbe *et al* (1991) on the external layer of chocolate bars (Aw = 0.465).

As it is shown in Table 2, these values are adequate to prevent fungal growth on the peripheral chocolate layers of the products. Furthermore, these values are higher than the Aw (0.2 to 0.4) of plain chocolate (Biquet and Labuza, 1988), and have good barrier properties against external moisture.

The ganache filling, because of its composition and presence of cream, has a high Aw, i.e., 0.72 (0.05). This average value corresponds to the minimal Aw values for growth of the storage fungi, particularly *Eurotium* and *Aspergillus* species (Samson *et al*, 1995).

VISIBLE CONTAMINATION AT THE FILLING-COATING INTERFACE

Visible observations of whitish hypha at the interface showed morphological features *Aspergillus* ; however, after cultivation on PDA and MEA, the fungal isolates exhibited more abundant yellow (cleistothecia), characterizing the appearance of the colony to that of the teleomorphic state of *Eurotium*. It was identified as *Eurotium chevalieri* according to Raper and Fennel (1965).

This species was previously isolated from the surface of chocolate bars stored at 25°C for 2 months (Larumbe *et al*, 1991). It is considered as a xerophilic mold: Pitt and Christian (1968) noticed 0.74 as minimal Aw for its growth, Frisvad and Samson (1991) indicated the range of 0.71 - 0.73 Aw. In the present case, after 10 days growth on malt extract broth (Table 2), an Aw of 0.70 allowed the growth to the extent of 5.7% (w/w) of the fungal biomass obtained at 0.99 Aw (1.28 $g.l^{-1}$). No growth was observed at 0.65 Aw.

Toxin production by *Eurotium/Aspergillus chevalieri* has been claimed based on death of animals eating moulded feed (Ali *et al*, 1989; Nazar *et al*, 1984).

Table 1. Comparative growth of fungal isolates incubated on MEA at different temperatures.

	Temperature (°C)						
	5	17	27	30	37	46	55
Eurotium chevalieri	-	++	+	+	-	-	-
Aspergillus fumigatus	-	+	+	++	+	+	-
Aspergillus penicilloides	-	+	+	(+)	-	-	-
Penicillium verrucosum	-	+	+	++	-	-	-

-: No growth ; ++: Colony diam > than at 27°C ; (+): weak growth after 20 days

POTENTIAL FUNGAL MICROFLORA OF THE FILLING

Scrapped samples from non-moulded surface areas of the ganache-filling appeared to contain a potentially rich fungal microflora. *Aspergillus fumigatus*, *A. penicilloides* and *Penicillium verrucosum* were identified according to Samson *et al* (1995). Some of these species are known to include toxigenic strains.

Aspergillus fumigatus is one of the most commonly encountered thermotolerant species of the genus *Aspergillus*. It is present as a contaminant in the products stored at elevated temperature (Frisvad and Samson, 1990) and low oxygen tensions. Because of its pathogenicity, heavy sporulating cultures were stressed to be handled with care (Samson *et al*, 1995). In comparison to other fungal isolates, *Aspergillus fumigatus* showed growth at a large temperature range (Table 1). Similar results were also reported by Lacey and Magan (1991), who indicated a temperature range of 12 - 65°C for this species. The minimal Aw for growth is 0.70 (Table 2), but the growth is about 1% that at 27°C. These results are different compared with the minimal Aw value (0.85 - 0.94) reported by Frisvad and Samson (1991).

Aspergillus penicilloides exhibited a very restricted range of temperature for growth during one week cultivation and, hence the incubation was extended to 20 days. But, only a weak growth was observed at 30°C (Table 1). Pitt and Samson (1990) and Samson and Hoekstra (1994) noticed that this species has a xerophilic growth and is

common on spices, dried fruits and bakery products. They have recommended its isolation and cultivation on low water activity media.

Penicillium verrucosum, which was recently grouped with 7 other species in the *Penicillium aurantiogriseum/verrucosum* complex (Lund and Frisvad, 1994), is an ubiquitous fungus, with an ability to contaminate a large variety of foodstuffs. Its growth was best at 30°C (Table 1) and a little growth was observed at 0.80 Aw (Table 2).

Table 2. Comparison of fungal biomass production as % of growth at 0.99 A_W after 10 days at 27°C in malt extract broth.

	Water activity				
	0.99	0.90	0.80	0.70	0.65
Eurotium chevalieri	100	105	8.3	5.7	0
Aspergillus fumigatus	100	31.8	1.1	1.1	0
Penicillium verrucosum	100	35.7	0.7	0	0
Aspergillus penicilloides	100	23.4	ND	ND	ND

ND: Not Done

Ganache-filling samples from the sub-surface and the core, when tested under the same conditions as that for the surface samples, failed to exhibit fungal growth at 27°C after 20 days and the spore concentrations of these samples were lower than 50 conidia/g. As the ganache-filling was not subjected to a sterilization treatment, the result indicate that the raw materials used in its composition had a very low contamination potential.

Nevertheless, fungal microflora present in the chocolates used for the manufacture of the truffles may have originated directly from beans. Indeed, most common species, isolated from moulded cocoa beans, are xerophiles, i.e., *Aspergillus glaucus*, *A. fumigatus*, *A. flavus*, *A. tamarii* (Roelofsen, 1958). *Penicillium* and *Mucor* species are also commonly found. The fungal species isolated will vary with the moisture and temperature conditions and with the cocoa beans quality (Hansen and Welty, 1970). *A. fumigatus* is known to be present at the beginning of the fermentation of the beans, while *Penicillium* species is linked to drying and storage conditions.

CONCLUSION

The contamination of the filling is mainly due to the ambient atmosphere, which promotes fungal spore deposition before coating. A possible explanation for the fungal growth at the chocolate filling-coating interface, is probably the more rapid solidification of the chocolate coating, which allows enough moisture at the interface, to increase the Aw level of the peripheral layer of the filling. This allows spore germination of several xerophilic molds.

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Kinetics of *Aspergillus niger* growth at high glucose concentrations in different types of the cultures

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ABSTRACT

Solid state fermentation (SSF) systems have been studied for several processes of biomass and metabolites production. Heterogeneity of substrate represents a serious problem in physiological studies to characterize solid state cultures. Thus, comparison of liquid, surface and solid state cultures is difficult to carry out. With the use of an inert support and the same culture medium, it is possible to evaluate the type of culture for growth and glucose consumption in different culture systems. In this work, amberlite IRA 900 and a minimal culture liquid medium were used to study growth of *Aspergillus niger*. This system allowed accurate evaluation of biomass and sugars. Glucose utilization and growth were less affected in solid state and surface cultures than in the submerged culture at high levels of glucose.

Keywords : Solid state fermentation, *Aspergillus niger*, kinetics, Amberlite IRA 900, minimum culture liquid medium, growth, high glucose concentrations, surface fermentation, liquid culture.

RESUME

Cinétiques d'*Aspergillus niger* cultivé selon différents procédés en présence des milieux hautement concentrés en glucose.

FAVELA-TORRES E., GARCIA-RIVERO M., CORDOVA-LOPEZ J., ROUSSOS S., VINIEGRA-GONZALEZ G., GUTIERREZ-ROJAS M., SAUCEDO-CASTAÑEDA G., GUNASEKARAN P. ET HUERTA-OCHOA S.

La fermentation solide a été étudiée pour différents procédés de production de biomasse et de métabolites. L'hétérogénéité du substrat pose un sérieux problème pour l'étude physiologique et la caractérisation des cultures en milieu solide. Aussi, il est fastidieux de réaliser une étude comparative entre les différentes techniques de fermentation : liquide, de surface et solide. On peut cependant comparer la production de biomasse et la consommation du glucose, selon le type de fermentation, en utilisant un support inerte et un même milieu nutritif. Au cours de ces travaux, nous avons utilisé comme support l'Amberlite IRA 900 et un milieu nutritif minimal pour l'étude de la croissance d'*Aspergillus niger*. Ce dispositif permet de suivre l'évolution de la biomasse et des sucres. Les fortes concentrations initiales de glucose, affectent moins la consommation de glucose et la croissance en fermentation solide et de surface que dans le cas de la culture submergée.

Mots clés : Fermentation en milieu solide, *Aspergillus niger*, cinétiques, Amberlite 900, milieu de culture minimum, croissance, hautes concentrations de glucose, surface de fermentation, culture liquide.

INTRODUCTION

The potential of solid state fermentation (SSF) system is increasingly being recognized in recent years (Pandey, 1992, Lonsane *et al,* 1992). Even though, it has many advantages over submerged culture, it also has few limitations (Hesseltine, 1972; Shankaranand *et al,* 1992). One such limitation is the availability of reliable methods for growth characterization studies. The heterogeneity and complex nature of the materials commonly used interferred with the accurate determination of the process parameters. Thus, there are afew reports on growth kinetics of fungi in SSF system, based on the on-line monitoring of CO2 (Saucedo-Castañeda *et al,* 1992) or

by measuring some of the structural components, such as proteins, glucosamine and ergosterol contents (Desgranges *et al*, 1991).

Earlier studies on pectinase production in SSF system revealed that high initial carbon source concentration (up to 50%) was rapidly metabolized by *Aspergillus niger* CH4, without any inhibition in growth and pectinases production (Solís *et al*, 1993). Moreover, it was also observed that the glucose consumption rates were increased when the initial glucose concentration was high. In this work, attempts have been made to characterize growth and substrate utilization by *A. niger* in solid state, surface and submerged cultures. The results of growth kinetic parameters at varying initial glucose concentrations are presented here.

MATERIALS AND METHODS

MICROORGANISM

Aspergillus niger 10 was used in this study. It was inoculated on PDA slants, incubated for 4 days at 35°C and stored at 4°C.

MEDIUM COMPOSITION

The medium contained (g/l): KH_2PO_4, 2.47; $(NH_4)_2SO_4$, 6.6; $CaCl_2$, 0.48; $MgSO_4$ $7H_2O$, 0.38; NaCl, 0.23 and 1 ml of a mineral solution (Clutterbuck, 1994), Glucose concentrations from 50 to 200 g/Lwere used. The initial pH was 4.5 for the solid state cultures and 5.0 for the submerged and surface cultures.

CULTURE CONDITIONS

Submerged cultures were carried out in 250 ml flasks incubated at 35°C on a rotatry shaker at 250 rpm. Surface cultures were carried out at 35°C in 100 mm Petri dishes with 30 ml of agar (1.5% w/v) medium. Solid state cultures were carried out at conditions reported previously (Raimbault and Alazard, 1980). Glass columns (11 x 150 mm) were used. Treated Amberlite IRA 900 (7 g) per column (Cordova, 1994),

impregnated with the inoculated culture medium (1 g amberlite per 1.5 ml of medium), was incubated in a water bath at 35°C with an air flow rate of 3 l/h.

ANALYSIS

Biomass

In the case of submerged culture, biomass was estimated by the dry weight method at 60°C after filtration (Whatman 41) and washing with 250 ml of distilled water. Culture medium and biomass were taken out the Petri dish, in the case of surface cultures. Agar medium was dissolved at 90°C in 250 ml of distilled water. Suspension was filtered (Whatman 41) and solids (biomass) washed with 250 ml of distilled hot water. Total biomass was determined by dry weight at 60°C. Mycelial biomass was estimated by the protein-dye binding method in solid state culture (Bradford, 1976), using biomass grown in surface cultures with the same glucose concentration as the standard. Before analysis, intracellular protein was released with 0.5 M phosphoric acid.

Glucose was measured using an enzymatic analyzer (YSI Model 2000). Moisture content in solid samples was determined, after drying at constant weight at 60°C. Data were analyzed by a logistic type model system (Okasaki *et al*, 1980).

RESULTS AND DISCUSSION

GROWTH AND BIOMASS PRODUCTION

The kinetics of growth of *A. niger* and related parameters, such as biomass production, and substrate consumption, during growth in submerged (SmF), surface (SF) and solid state (SSF) culture conditions at different initial glucose concentrations (50, 100 and 200 g/l) were studied (Fig. 1).

In SSF cultures, the growth began after 20 h incubation and reached a maximum at 40 h. The maximum biomass attained in SSF culture was about 30 g/Land it was independent of initial substrate concentration.

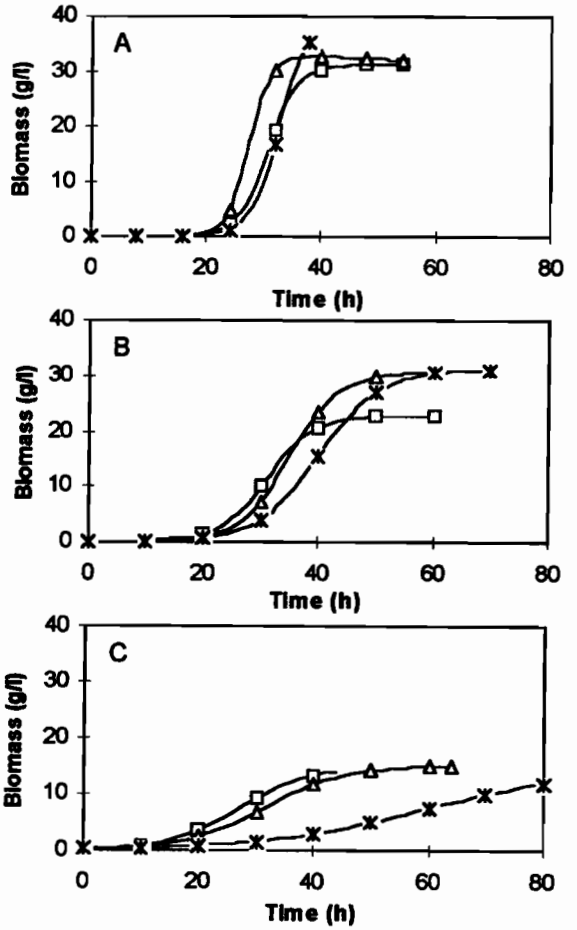

Fig. 1. Profiles of biomass production at different initial glucose concentrations (–, 50; Δ, 100 and X, 200 g/l). **A**: Solid state, **B**: Surface and **C**: Submerged cultures).

The growth of *A. niger* in SF culture also began after 20 h incubation and reached a maximum (20 g/l) at 40 h in the medium containing 50 g/Linitial glucose concentration, but reached a maximum (30 g/l) at 50 to 60 h, in the case of media 100-200 g/Linitial glucose concentration. A slow growth pattern could be observed in the SF culture at 200 g/Linitial glucose concentration. The biomass production in SmF culture was low (below 20 g/l), although growth began after 20 h incubation, as in the case of SF and SSF cultures. The growth pattern of *A. niger* in SmF culture at 50 and 100 g/Linitial glucose concentrations was almost similar, but at 200

g/Lglucose concentration, a considerable lag period (30-40 h) was detected. Furthermore, the strain grew very slowly in SmF culture at 200 g/Lglucose concentration. Thus, the SSF and SF cultures attained equal amount of biomass (about 30 g/L) within 40 h, while it took more than 40 h in the SmF culture. The low biomass obtained in the SmF culture (15 g/L) at more than 60 h was due to the slow growth of the culture.

GLUCOSE CONSUMPTION

The glucose consumption profile of the *A. niger* grown under SSF, SF and SmF culture conditions at three different glucose concentrations is shown in Fig. 2. Maximum amount of glucose was consumed during the vegetative growth period (20 - 60 h) of *A. niger* in SSF, SF and SmF cultures at 50 and 100 g/L of initial glucose concentration.

After 60 h growth, the substrate was completely consumed in SSF cultures at all the three different initial glucose concentrations. Considerable amount of residual glucose was present in SF cultures at 200 g/L initial glucose level and in SmF cultures at 100 and 200 g/Linitial glucose concentrations. In SmF culture, at 200 g/Linitial glucose concentration, the glucose consumption was rather low and only 50 g/Lof glucose was consumed, thereby leaving three - fourths of the substrate unutilized. The poor growth of *A. niger* in SmF culture at 200 g/Lwas due to the effect of the high substrate concentration.

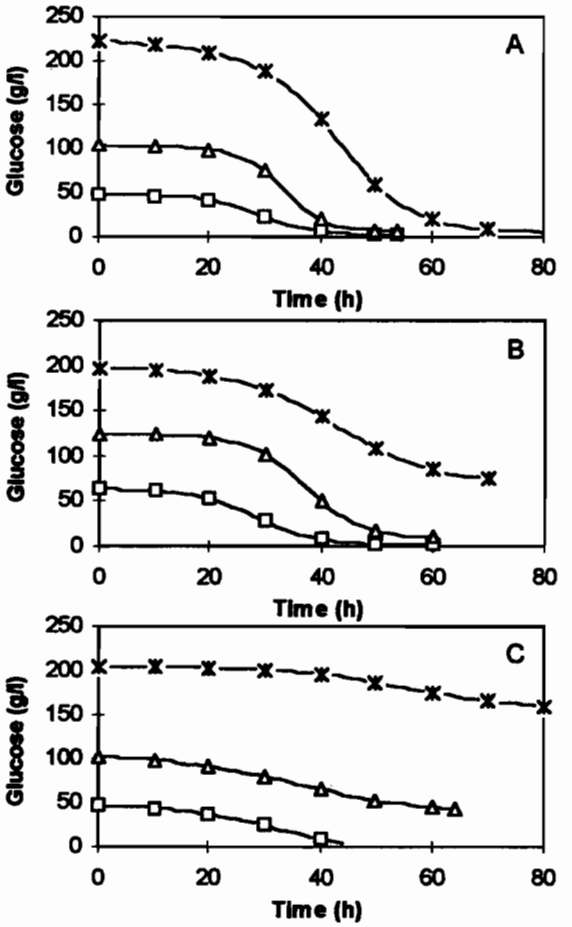

Fig. 2. Profiles of glucose consumption at different initial glucose concentrations (o, 50; Δ 100, and X 200 g/l). **A**: Solid state, **B**: Surface and **C**: Submerged cultures)

KINETIC PARAMETERS AND YIELDS

The calculated specific growth rate, biomass yields and substrate conversions for the three types of cultivation of *A. niger* at different initial glucose concentrations are shown in Table 1. The specific growth rate (μ) of *A. niger* was generally higher in SSF than in SF and SmF cultures. It was interesting to note that the strain showed three-fold higher μ in SSF, than in SmF culture at 50 g/Linitial glucose

concentration. The growth of *A. niger* was limited in the SmF culture rather than the SSF and SF cultures. One of the limiting factors responsible for the growth in SmF culture could be the low availability of oxygen, since the solubility of oxygen is rather low in the liquid culture. But in SF and SSF cultures, the oxygen was readily available and there was no problem of solubility of gases.

Table 1. Kinetic parameters and yields of *Aspergillus niger* in different type of cultures with different initial glucose concentrations (g / l)

	Solid state culture			Surface culture			Submerged culture		
Glucose	50	100	200	50	100	200	50	100	200
μ	0.323	0.313	0.238	0.222	0.232	0.170	0.091	0.083	0.043
Yx/s	0.680	0.357	0.199	0.366	0.272	0.254	0.312	0.25	0.234
%C	91	93	98	96	96	64	95	58	20

The effect of substrate concentration increase on the growth of *A.niger* in three different culture conditions revealed that the growth rate was less affected in SSF culture. It was seen that μ was almost similar (0.32 and 0.31 h^{-1}) at 50 and 100 g/L initial glucose concentrations in SSF culture. At 200 g/Lglucose concentration, it was reduced to 0.24 h^{-1}. A similar trend of the effect of substrate concentrations on the specific growth rate of *A. niger* in SF and SmF cultures could also be observed. The growth rate was not affected significantly with the increase in glucose concentration from 50 to 100 g/Lin SF culture (0.22 and 0.23 h^{-1}) and in SmF cultures (0.091 and 0.083 h^{-1}), although the magnitude of the specific growth rate was rather low in these cultures, as compared to that in the SSF culture. The specific growth rate of *A. niger* at 200 g/Lwas significantly affected in all three cultures, although the culture grew considerably well in SSF culture with a specific growth rate of 0.24 h^{-1}, as compared to the poor specific growth rate in SF (0.17 h^{-1}) and SmF (0.04 h^{-1}) cultures.

Analysis of biomass yield (Yx/s) of *A. niger* in different culture methods revealed that it was generally high in SSF culture. At 50 g/Linitial glucose concentration, it was 0.68 g/g in SSF, 0.37 g/g in SF and 0.31 g/g in SmF cultures. However, an increase in initial glucose concentration severely affected this yield parameter in SSF culture, more than in SF and SmF cultures. For example, increase in concentration of glucose from 50 to 100 g/L resulted in an approximately two-fold reduction of biomass yield (0.68 g/g to 0.35 g/g) in SSF culture. But in other culture methods, although the biomass yield was reduced, the reduction was not as similar

as the SSF culture (0.37 g/g to 0.27 g/g in SF and 0.31 g/g to 0.25 g/g in SmF cultures). Increase in initial glucose concentration to 200 g/L further affected the biomass yield in SSF culture (0.17 g/g). But in SF and SmF cultures, the biomass yield was not further affected significantly at 200 g/Lglucose concentration (0.25 g/g in SF and 0.23 g/g in SmF cultures).

Estimation of the residual substrate in the three cultures of *A. niger* at different glucose concentrations revealed that, in SSF culture all substrate was completely utilized within 70 h, independent of the initial substrate concentrations. At 50 and 100 g/L initial glucose concentrations, the substrate was completely utilized within 40 h growth in SSF culture. In contrast, considerable amount of substrate remained unutilized within 70 h in SF culture grown at 200 g/L initial glucose level, despite the complete utilization of substrate by *A. niger* grown at 100 g/L of initial glucose concentration. However, in the SmF culture, complete utilization of substrate occurred only in the culture with 50 g/L of initial substrate concentration, but about 50% of the substrate remained unutilized, when the culture was grown at 100 g/L of initial glucose concentration. More than 80% of the substrate remained unutilized, if the culture was grown at 200 g/L initial glucose concentration.

These results suggested that inhibition of growth by increasing the substrate concentration in SmF culture, is evident from the reduction in growth rate, biomass yield and residual glucose concentration. This type of effect, despit its existence in SF cultures at 200 g/L initial glucose concentration, was not prominent at 100 g/L initial glucose concentration. On the contrary, the SSF cultures were less affected by the increase in initial glucose concentration. Surprisingly, in the SSF cultures at 50 - 200 g/L initial glucose concentrations, all the substrate was utilized. Significant reduction in growth rate and biomass yield was observed in the SSF culture grown at a initial glucose concentration of 200 g/L, thereby suggesting the possible physiological and metabolic change on the organism for utilizing the substrate to produce fermentation products rather than biomass. Thus, the present study suggests the difference in the pattern of fungal growth and substrate utilization in SSF, SF and SmF cultures as well as the possible differences in their physiology and metabolism.

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Mycelial penetration and enzymic diffusion on soybean tempe

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SUMMARY

Histological observations were made on the penetration of hyphae of *Rhizopus oligosporus* into soybean tempe and defatted soy flour. Hyphae infiltrated to a depth of about 2 mm in 40 hours for soybean tempe, whereas for tempe-flour the depth was about 5-7 mm over the same time. Different staining techniques were followed including the use of Harris's haematoxylin, eosin and Grocoh methenamine silver fungal stain. Different enzymes were produced, including proteases, and their activities are shown. A model relating enzyme diffusion and the movement of hyphae is proposed.

Keywords : Solid state fermentation, soybean tempe, soybean-flour tempe, histological studies, mycelial penetration, enzymic diffussion, *Rhizopus oligosporus*, fungal strains, mathematical model.

RESUME

Pénétration du mycélium et diffusion enzymatique dans les graines de soja au cours de la fermentation tempe.

VARZAKAS T.H., PYLE D.L. ET NIRANJAN K.

Nous avons observé la pénétration des hyphes de *Rhizopus oligosporus* à l'intérieur des graines de soja du tempe ainsi que sur de la farine de soja dégraissée. Le mycélium pénètre à l'intérieur des graines de soja sur environ 2 mm de profondeur en 40 heures, alors que pour la même durée la pénétration est de 5-7 mm dans le cas de la farine de soja. Nous avons utilisé différentes techniques de coloration des champignons, comme la coloration de Harris à l'haematoxiline, la coloration à l'éosine ou à l'argent de methenamine grocoh. Différentes enzymes ont été produites et nous décrivons leurs activités. Nous proposons également un modèle décrivant la diffusion enzymatique et le mouvement des hyphes.

Mots clés : Fermentation en milieu solide, graines de soja, tempe, farine de soja,, études histologiques, pénétrétion mycélienne, diffusion enzymatique, *Rhizopus oligosporus*, champignons filamenteux, modèle mathématique.

INTRODUCTION

Solid state fermentation (SSF) is the utilisation for microbial growth of water-insoluble materials in the absence or near-absence of free water (Mitchell *et al*, 1990). It takes place on solid or semi-solid substrates or occurs in a nutritionally inert solid support, which provides some advantage to the microorganism with respect to access to nutrients. It involves simple equipment. Tempe is Indonesian fermented soybeans produced by the growth of the fungus *Rhizopus oligosporus.* Tempe production involves two distinct fermentations. The first, occurring during soaking, is the bacterial one wich results in acidification and prevents growth of *Bacillus cereus.* The second is fungal fermentation and results in the growth of the mould mycelium.on the bean cotyledons .

Disruption of the cell wall is essential to facilitate the fast growth of *Rhizopus oligosporus*. The diffusion of extracellular enzymes and the degradation of the solid substrate into soluble fragments are very important steps in SSF (Ramana Murthy *et al*, 1993). Enzymes must be excreted deep into the bean mass to facilitate the chemical changes during tempe fermentation. Sudarmadji (1975), using a scanning electron microscope, found that the *Rhizopus* did not penetrate more than two cell layers into the soybeans. He suggested that enzymes should be excreted by the mould and that they facilitate the chemical changes occurring during the tempe fermentation. Jurus and Sundberg (1976) have shown, using staining techniques, that the fungal hyphae occured in the deeper layers of the cotyledons. They also reported that penetration of perpendicular hyphae into soybeans was restricted to the intercellular spaces.

The aim of this work was to elucidate the mechanism of penetration of hyphae, as they are produced by *Rhizopus oligosporus*. It is necessary to study the diffusion of enzymes released by the mycelium at the surface. A hypothetical model has been also formulated and tested.

MATERIALS AND METHODS

TEMPE PROCESSING

Canadian number one soybeans, obtained from a health shop in Reading University (100 g), were soaked overnight at 20°C in 296.5 ml of tap water, acidified with 3.5 ml of 85% (w/v) lactic acid. The beans were then drained, dehulled in a Corona maize mill, washed in hot tap water and husks removed by flotation. Soybean cotyledons were cooked for 60 min under reflux at 100°C in 296.5 ml water containing 3.5 ml of 85% lactic acid. Cotyledons were dried and cooled on absorbent paper under a sterile laminar air flow. The cotyledons were then inoculated with 1 ml of *R. oligosporus* NRRL 2710 spore suspension. The suspension was prepared by growing the mould on Sabouraud dextrose agar in 100 ml Erlenmeyer flasks at 30°C for 7 days and contained approximately 10^7 spores per ml. Mixing followed and cotyledons were packed into each of 10 (9 cm) plastic Petri dishes and incubated at 30°C in the presence of an open beaker of water to maintain humidity. Tempe was finally produced, as described by Nout and Rombouts (1990).

At times corresponding to 0, 12, 20, 24, 30, 36 and 40 hours fermentation, two Petri dishes were withdrawn and checked for pH, the values of which were consistent with the data reported in the published literature. The contents of the Petri dish incubated for 40 hours were freeze dried, ground and defatted using Soxhlet extraction with hexane. This powder (1 g) was extracted by homogenising in Tris (pH:7) buffer in a 10:1 dilution. Centrifuged for 15 minutes, the supernatant was separated and used in an APIzym (Humble *et al*, 1977) to detect the enzymic activities as expressed by the quantity of hydrolysed substrate. The same procedure was followed for tempe produced at the above fermentation times. However, different buffers were used according to the pH values indicated in literature. For pH 3.8 to 5.0, citrate phosphate was used, whereas for pH 5.8 and 6.0, succinate buffer was used.

Tempe was also produced by a similar procedure but from defatted soy flour, made by grinding the soybeans used above.

STAINING EXPERIMENTS

Several beans were randomly selected from tempe. These were fixed using two fixatives: a) 3% glutaraldehyde in 0.05 M phosphate buffer at pH 7.0 for 2 to 3 hours, according to the size of the specimen and, b) formalin, in which the specimen was left overnight. The latter provided the best fungal differentiation. Following that, the beans were cut in half and two different sections were compared. These included a cross section and four successive longitudinal cuts including one surface, two middle and an end section. These were placed in a cassette. All cassettes were put in a dehydration and embedding machine and were ready for blocking out (tissue was placed in a mould). The block was trimmed down so that the tissue surface is free of wax. Samples were then sectioned at 6 microns with a rotary microtome and stained with thionin, orange G and erythrosin as well as on a Shandon linear staining machine. The latter distinguished mould mycelia better from soybean cells. Harris's haematoxylin stained the mould cells, whereas eosin stained the soybean cells. Finally, coverslipping with D.P.X. mounting medium was carried out.

Grocoh methenamine (hexamine) silver fungal stain

Sections as prepared from above were stained using another method. Firstly, they were put into water and oxidised in 4% chromium trioxide for 30 minutes. A silver bath was prepared by adding 2 ml of 5% borax, 25 ml methenamine silver solution, 25 ml deionised water and was placed at 60°C. In addition, silver solution was also made with 6 g methenamine, 5 ml of 10% aqueous silver nitrate and made up to 200 ml with deionised water. Slides were rinsed in distilled water, before incubation in

silver solution. An Aspergillosis control was also used and checked at 20-30°C after 40 minutes and examined microscopically (fungi will be dark brown in colour). Slides were then rinsed in deionised water, toned in 0.2% gold chloride for 5 minutes and then again rinsed in deionised water. Silver was fixed in 5% hypo (sodium thiosulphate) for 5 minutes and slides washed in water. The next step involved counterstaining in light green or haematoxylin and eosin, followed by dehydration, clearing and mounting. Fungal hyphae appear black and the background is according to counterstain (Bancroft and Stevens, 1982).

Estimation of hyphal penetration

An eye piece micrometer was used to determine the maximum hyphal penetration. Four longitudinal continuous 6 μm-thick sections were taken. The depth of one representative hyphae was measured from all four continuous sections and added together. The maximum hyphal diameter was found to be 20 μm. While measuring the depth of penetration, it was made sure that penetrating hyphae counted in one section were not counted in the next one.

Effect of enzymatic activity on tempe produced from beans

An APIzym test was performed to detect the enzymatic activities, which were expressed by the hydrolysed substrate. The numbers on the following table represent activity marks as estimated from an APIzym chart provided by Biomerieux. However, only the enzymes with the highest enzymic activities are shown. five corresponds to 40 nanomoles, 4 to 30 nanomoles, 3 to 20, 2 to 10 and 1 to 5 nanomoles. Leucine and valine arylamidases are aminopeptidases, which specifically hydrolyse the CO-NH bond, releasing leucine and valine, respectively, from arylamides. Chymotrypsin is a proteolytic enzyme and the two phosphatases (alkaline and acid) can hydrolyse S-substituted and O-substituted monoesters of phosphorothioic acid, respectively.

RESULTS AND DISCUSSION

According to the Table 1, leucine arylamidase has a high enzymatic activity at all fermentation times. A similar pattern is followed by alkaline phosphatase after the first 24 hours. Valine arylamidase also has a high activity at 36 and 40 hours. Cellulolytic and hemicellulolytic activities appeared to be very low and were not included in the table. It is well known that during tempe fermentation, *Rhizopus oligosporus* produces polysaccharidases to degrade soybean cell-walls. Sarrette *et al* (1992) grew this mould in a soybean extract model medium, to which glycerol was added to control water activity (a_w). Different enzymes produced were

polygalacturonase, endocellulase and xylanase, the activities of which were influenced by a_w .The optimum a_w for endocellulase was 0.98, at which mycelial growth was significantly reduced as compared to polygalacturonase and xylanase activities, which coincided with that of mycelial growth (0.99-1.00). It appears that the activities of these enzymes, as well as those reported on the above table, are strongly related to the penetration of the mycelium of *Rhizopus oligosporus* into the soybean cell wall.

Table 1. Effect of enzymatic activity in tempe produced from beans

	Activity at time (h)			
Enzyme assayed for :	24	30	36	40s
Phosphatase, alkaline	1	4	5	5
Phosphatase, acid	3	4	3	3
Leucine arylamidase	5	4	5	5
Valine arylamidase	3	3	4	5
Chymotrypsin	1	2	4	3

A simple physical model for the growth of hyphae can be postulated. Branched rhizoids penetrate the soybean after mycelia have been differentiated into them. Rhizoids which provide anchorage for aerial sporangiophores and from which stolons (horizontal hyphae) grow out above the surface of the substrate, produce new rhizoids where they re-enter the substrate. Penetrative hyphae (parallel to surface) are probably stolons which have re-entered the substrate, whereas the perpendicular penetrative hyphae are the rhizoid system (Mitchell *et al*, 1990). Hyphae cause a turgor, pushing soybean cells apart as enzymes degrade them (Figures 1b, 1c, 2c) and thus beans become soft. Figure 1a shows a control of soybean cells depicting the protein and lipid bodies surrounded by the cell wall. There is an increase in hyphal penetration in the case of the defatted flour, over that of soybean tempe probably due to the fact that cells from flour are softer than beans (Figures 2a and 2b).

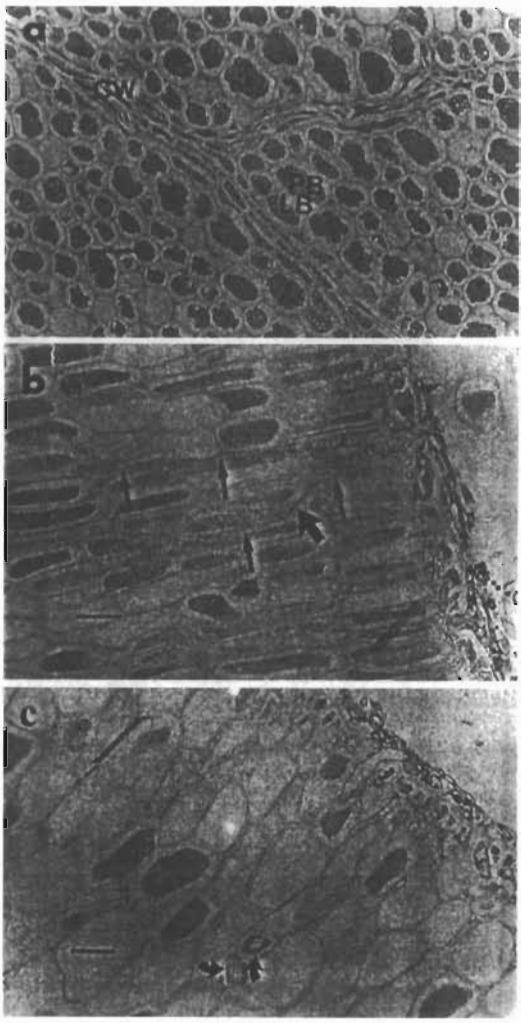

Fig. 1a. Soybean cells (control) showing the protein and lipid bodies surrounded by the cell wall. The magnification on the negative is x70 and 1 cm on the photos represents 15 µm. **Fig. 1b.** Penetration of hyphae (arrow) from the surface into the substrate, disintegrating soybean cells on their way and moving in zig-zag directions (yellow background of microscope). **Fig. 1c.** Disintegration of the cells by hyphae and the formation of haustoria (arrows) inside and outside soybean cells.

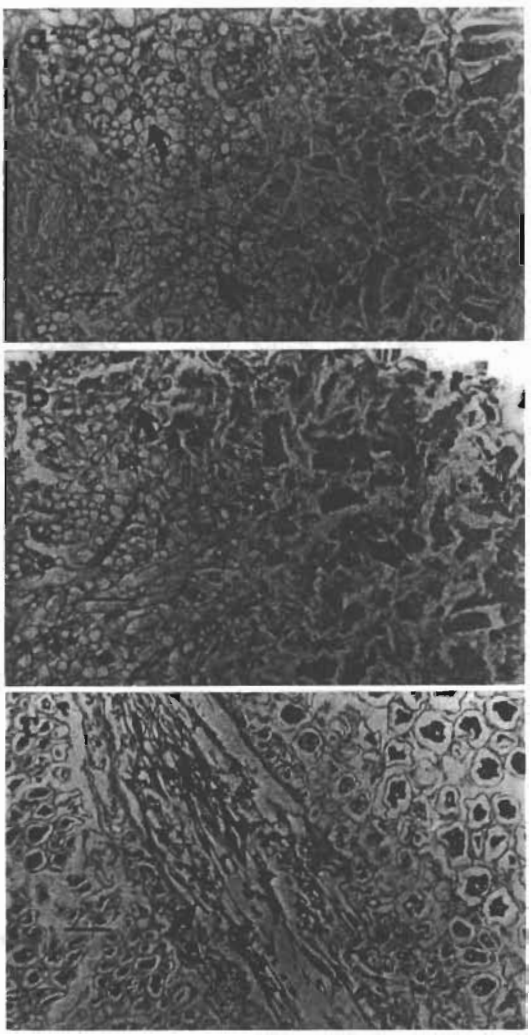

Fig. 2a. Red background section of hyphae (left arrow) penetrating inside defatted soy flour (right arrow). **Fig. 2b.** Deeper penetration of hyphae on defatted soy flour **Fig. 2c.** Blue background section of Grocoh stain showing the penetration of black hyphae into the cell wall of soybean cells moving upwards (arrow). 1 cm on the photos represents 15 μm.

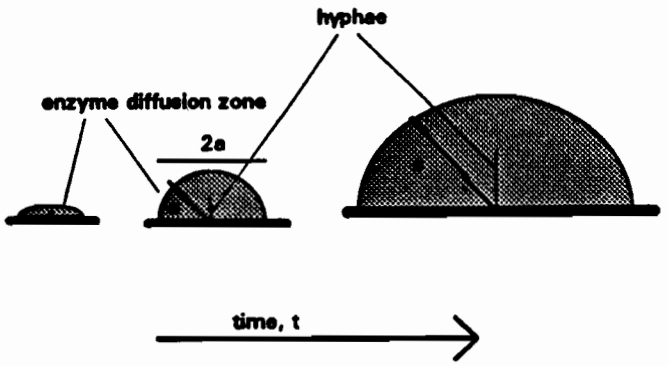

Figure 3a: Representation of proposed model depicting enzyme diffusion from a stationary source and movement of hyphae.

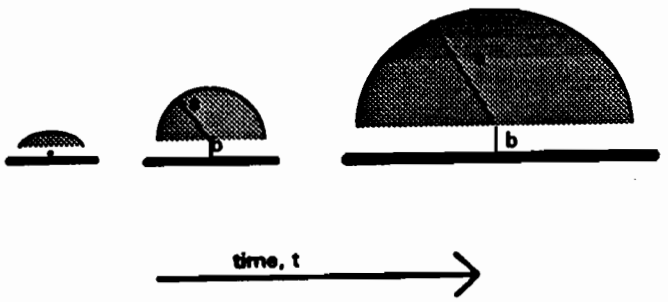

Figure 3b: Model depicting enzyme diffusion from a moving source.

The major stages in growth are summarised below, after having taken into account different models reviewed by Nout and Rombouts (1990). First, extracellular enzymes diffuse to the macromolecular substrates (proteins, lipids, cellulose, xylan and pectin). An enzyme-catalysed breakdown of substrate into smaller, metabolisable units is followed and these small substrates diffuse back towards the hyphae, where they are then taken up by the mould and metabolised to produce hyphal growth (Figure 3a and 3b). The diffusion of small substrates is faster than enzymic diffusion, since diffusion coefficients are inversely related to the solute size, and the rate limiting step is enzyme diffusion.

For tempe beans and flour, the measured hyphal penetration distances (=a+b in Figure 3b) after 40 hours were around 2 mm and 5-7 mm, respectively. The molecular diffusion coefficients of protein in solution are typically of the order of 10^{-11} m^2 s^{-1}, which would be consistent with a diffusion distance a from a stationary source of around 1 mm since $a \sim \sqrt{Dt}$. The results, therefore, suggest that the hyphae act as a moving source of enzymes and play a positive role in pushing through the matrix. The enzymes ahead of, and around the growing hyphae, have a dual role: they help soften the solid structure and liberate small substrate molecules, which provide the necessary source of energy, carbon and other elements. The greater penetration distances observed with tempe flour, probably reflect its relative softness in comparison to the beans. Jurus and Sundberg (1976), revealed hyphal infiltration to a depth of 742 µm or about 25% of the average width of a soybean cotyledon. Their results would suggest a diffusion coefficient of the order of 10^{-12} m^2 s^{-1}.

As for the enzyme transport, it is necessary for oxygen to diffuse through the matrix, in order to help drive oxidative enzyme degradation. Mitchell *et al*, (1990) proposed that oxygen diffusion limits the rate of growth of *Rhizopus oligosporus* into a model solid substrate. This is due to the fact that oxygen must pass through the actively respiring biomass at the substrate particle surface in order to reach the interior and then diffuse through the aqueous phase within the substrate. The diffusion of enzymes and substrate fragments is important and more work needs to be done to characterise these extracellular enzymes and their mode of action in degrading the soybean. If the mass transfer resistance is high, this could be the rate determining step. However, when the porosity of the substrate is low, as in case with the soybean, the major part of degradation will occur at the outer surface. In contrast, if the porosity is high, the degradation can occur inside the structure and the water soluble fragments have to diffuse out. In either mode of enzymatic action, the solid polymeric materials are modified by the enzymes to enable them to enter the cells and serve as carbon or energy sources.

CONCLUSIONS

The observed depth of hyphal penetration was larger than that reported in the literature The results are consistent with a possible diffusion control mechanism. However, tempe/flour has shown deeper penetration. The simple model hypothesis is based on growth experiments and relates the growth of hyphae to the enzymic diffusion. However, the diffusion of these extracellular enzymes needs to be studied in more detail so that the whole mechanism can be understood more clearly.

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The INRA-Dijon reactors : Designs and applications

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SUMMARY

A brief review of reactors for solid state fermentation is presented, along with the description of the INRA-Dijon reactors. These reactors ranging from a prepilot (50 L) to a pilot plant (1.6 m^3), have been developed for research needs, scale-up studies and development of different industrial applications. INRA-Dijon reactors are of the same type, i.e., packed bed reactors with forced aeration through the culture layer and have been used for different non-sterile processes.

Although solid state fermentation processes are very often carried out in non-aseptic conditions, the development of sterile pilot equipment and processes may offer interesting alternatives to submerged fermentation for different applications (pharmaceutical products, human foods, culture of genetically modified micro-organisms, etc). The paper presents detailed information on a small reactor (1 L capacity) for lab-scale studies and a pilot reactor (50 L capacity) for scale-up studies. In addition, the examples of industrial applications developed in the platform for protein enrichment of by-products, enzyme production and production of antagonistic fungi for the biological control are also presented.

Keywords : Solid state fermentation, reactor design, pilot plant, sterile reactors, industrial applications, protein, enzymes, biopesticides.

RESUME

Les bioréacteurs de l'INRA-Dijon: Génie fermentaire et applications

DURAND A., RENAUD R., MARATRAY J. ET ALMANZA S.

Après un bref aperçu et une synthèse des articles concernant l'équipement, une description des réacteurs de l'INRA-Dijon sera présentée. Ces réacteurs, allant du stade prépilote de 50 L au pilote de 1,6 m^3, ont été développés au sein de la Plate-forme pour la recherche, les études d'extension d'échelle et pour le développement de différentes applications industrielles. Tous ces équipements sont basés sur le même principe : réacteurs statiques avec aération forcée à travers la couche de culture. Ces réacteurs sont utilisés pour différents procédés non stériles.

Bien que les procédés de fermentation à l'état solide soient très souvent conduits dans des conditions non aseptiques, le développement d'équipements et de procédés assurant la stérilité peut offrir une alternative à la fermentation submergée pour différentes applications (pharmacie, alimentation humaine, culture de micro-organismes génétiquement modifiés, etc....). Deux réacteurs seront présentés : un réacteur pour des études au niveau laboratoire de 1 L de capacité ; un réacteur pilote de 50 L pour des études d'extension d'échelle.

Dans la dernière partie, seront présentées les applications industrielles développées à la Plate-forme: enrichissement protéique de sous-produits, production d'enzymes, production de champignons antagonistes pour la lutte biologique.

Mots clés : Fermentation en milieu solide - Génie fermentaire - Installation pilote - Réacteurs stériles - Applications industrielles (protéines, enzymes, biopesticides).

BRIEF LITERATURE SURVEY

In submerged liquid fermentations, the equipment used is always almost the same, because the main part of the medium, in terms of physical behaviour, is liquid. In solid state fermentation (SSF), unfortunately, it is absolutely not the case. A wide range of solid matrices can be used (Durand *et al.* 1991).

These can be classified in 3 broad categories :

- organic materials, which are invariably polymeric molecules and insoluble or sparingly soluble in water. The particles of solid matrix have a dual role of providing support and nutrients at the same time. Examples include lignocellulosic and starchy materials, such as sugar beet pulp, sugarcane bagasses, wheat bran, wood chips, straw, copra cake, etc.
- mineral material, such as perlite, clay granules, pouzzolan, etc. In these cases, the materials are only supports and they have to be wetted with nutritive liquid solutions.
- synthetic materials, such as polyurethane foams, sponges, etc. These supports must be wetted by liquid substrates to allow the growth of the microorganisms.

The mineral and synthetic materials are attractive, because of the possibility of using different substrates and thus, can facilitate more studies or improve the applications.

In order to obtain a good definition of a reactors equipment, the nature of the solid matrix used must be considered not only in terms of nutrient sources, but also from a physical point of view, i.e., geometric configuration which provides heat and mass transfer, granulometry, porosity, maximum water holding capacity, resistance to compression and agitation. Therefore, the choice of a reactor design must mainly take into account 4 aspects, such as the structure of the solid matrix used, the type of micro-organism involved, the environmental conditions needed for the process and the type of use: research, industrial applications (Durand *et al.* 1988a).

THE REACTOR EQUIPMENT

Among the reactors described in the literature, three basic types can be distinguished based on the mixing regime and the aeration mode used. These include (i) tray bioreactors and (ii) agitated bioreactors and (iii) packed bed bioreactors (Durand *et al.* 1992).

(i) The first type includes tray fermenters (Ahmed *et al.* 1987, Hesseltine, 1987) and bags of microporous film. These bioreactors are essentially characterized by their simplicity. There is no forced aeration through the substrate layer and generally no mechanical mixing. To avoid overheating and to maintain aerobic conditions, only thin layers of the solids can be used. In the industry, the more representative is the koji type reactor and it has been automated in some cases.

(ii) The second type includes rotating reactors, which are typically the drum-shaped (Rollacell at lab scale), with or without baffles inside (Lindenfelser and Ciegler, 1975), and cement mixers (Han and Anderson, 1975). In another case, the reactor is

immobilised and different agitation devices ensure the mixing of the solid medium (bakery-kneader, screws inside a classical cylinder vessel). The agitation can be occasional or continuous. Usually, temperature control is quite difficult. Hence, continuous agitation is associated with the only problems of shear stresses and damage of some micro-organisms or the solid medium itself (Lonsane *et al.* 1985).

(iii) The last type includes packed bed bioreactors with forced aeration through the substrate layer. Many variations of this design are possible. For laboratory use, these reactors are typically cylindrical columns made up of glass or plastic (Raimbault and Alazard, 1980). The temperature regulation is carried out by locating the columns in a water bath or by using a jacket for thermostated water circulation. These are advantageous for some studies such as quick screening of strains but, can become inadequate for operation with regulations of some parameters in scale-up studies.

CONTROL OF PARAMETERS

If in submerged fermentation, controls of the major parameters (temperature, pH, dissolved oxygen) are trivial, it is not the case in SSF. Moreover, for some parameters, the probes do not exist or are inadequate. For example, the water content of the medium cannot be measured on-line and it is well known that it can affect the water activity and consequently the metabolism of the microorganisms, the structure of the medium, the heat, oxygen and mass transfer. A probe, as shown in Fig. 1, has been developed by Gervais and Bazelin (1986) for submerged fermentation but, it could be adapted to SSF.

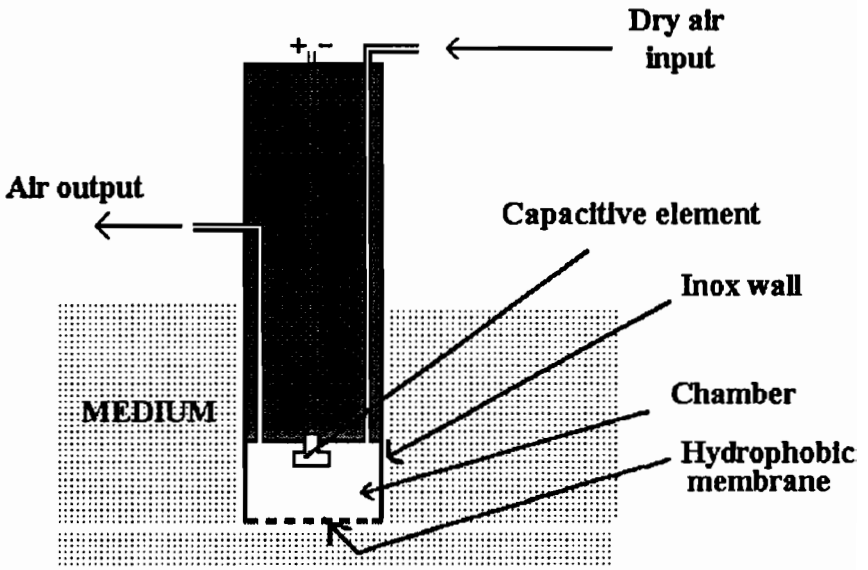

Fig. 1. Principle of the water activity sensor

Temperature measurement is as simple as in submerged cultures, but its regulation, mainly in large reactors, constitutes one of the most difficult problems in SSF. The majority of solid media have weak conductive features. During a culture, the heat accumulation is very rapid. At a laboratory scale, generally, the temperature regulation is carried out by placing the reactors in a temperature controlled room or a water bath. For pilot and industrial reactors, the strategies used are different, but very often unsatisfactory. Thermostated air can be used for the temperature control, but, the heat capacity of the air at maximal water saturation is much less than the heat capacity of water used for cooling in submerged fermentation. Therefore, the SSF needs a large quantity of air that exceeds the amount necessary for microbial respiration, as shown in Fig. 2, which gives an example of K_La measurement (Durand *et al.* 1988c).

Another possibility is to spray water and evaporate it with the air to cool the medium but, continuous agitation is very often necessary.

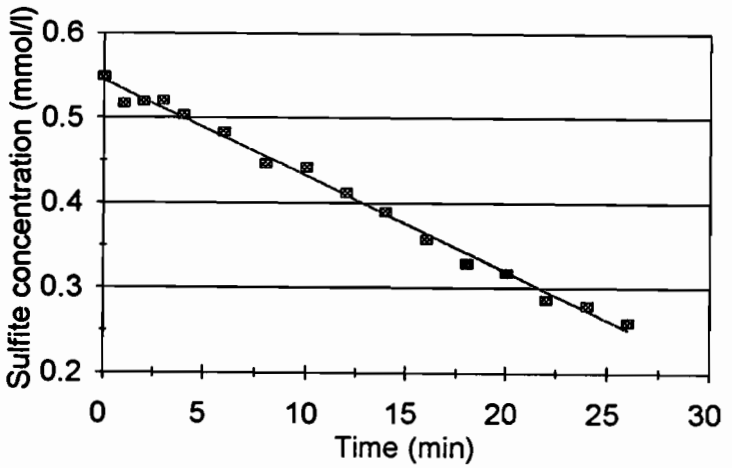

Fig. 2. Example of the KLaC* measurement. Airflow rate 8 l/min.kg DM. Dry matter 32.5%. Regression coefficient 0.996. (KLa = 2250 h-1)

Heat exchangers inside the medium can be also used to remove metabolic heat, but a temperature gradient could can occur when the reactor is larger. Of course, in SSF, temperature and moisture content cannot be dissociated. So, it is essential to combine the control of these two parameters. Some authors have also introduced output gas measurements and mass balance with on-line CO_2 measurements for controlling the moisture content during a culture (Narahara *et al.* 1984).

The pH measurements and its on-line regulation are also not currently possible. Indeed, with some media, pH electrodes cannot be used due to the absence of free water. Nevertheless, some degree of pH control can be achieved through the choice of a combination of nitrogen sources in the solid medium. The use of buffers may also be an interesting alternative but these can increase the salt concentrations and thereby lead to a decreased water activity of the medium. For some specific studies, synthetic non-metabolized buffers can be used but, their prices are prohibitive for industrial applications. When pH measurements are possible, a control can be carried out by spraying pH correcting solutions during the culture (Durand and Chereau, 1988b).

Thus, in SSF process, many different reactors are used, very often built or adapted by a research team for its own needs. However in order to be competitive and represent an interesting alternative to submerged cultures, improvements in the reactor design on a scientific basis are absolutely necessary.

As a general rule, a reactor for SSF must have a number of characteristics, such as 1) its capacity must allow the study of variables inaccessible at a small scale

(aeration rate, effect of layer thickness, settling of substrate, heat build up), 2) monitoring and control devices for different parameters must be well adapted, 3) handling must be simple (empty/refill, cleaning, etc), 4) low cost for construction of several units, and 5) design must allow scale-up.

This last point is particularly important. Indeed, unlike reactors for submerged cultures, where some fundamental or semi-fundamental scale-up criteria exist, the same approach is very difficult in SSF due to lack of criteria.

The only rule-of-thumb used for scaling-up a reactor in SSF is simply to maintain geometric similarity. Different attempts are carried out with errors and modifications until success is achieved. Recently, a criterion, involving the maintenance of heat and water balances during the scale-up has been proposed (Saucedo-Castañeda *et al*,1992) and appears to be very attractive. But, larger efforts are necessary to formulate defined criteria, which can assure similar performances in reactors of different sizes.

REACTORS FOR NON-STERILE PROCESSES

All the reactors presented below have been built in INRA-Dijon either for the development of industrial processes, or for research needs. These packed bed bioreactors with a forced aeration from the bottom of the substrate mass are either provided with agitation devices or agitation is absent, depending on the size and the utilisation.

LABORATORY SCALE REACTORS

These are shown in Fig. 3 (Durand *et al*, 1988a).

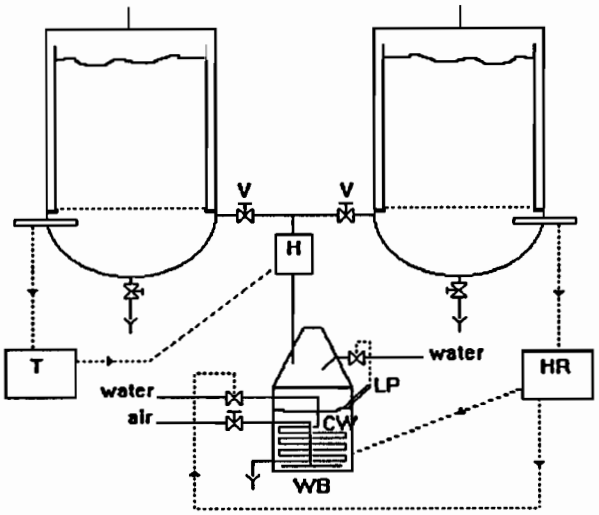

Fig. 3. Schematic diagram of the laboratory scale reactors. (T) Temperature probe and regulator, (HR) Relative humidity probe and regulator, (LP) Level probe, (WB) Humidifier, (CW) Coil for circulation of cold water, (V) Valve for airflow adjustment, (H) Heating box.

In this system, the temperature and the water content of the medium are regulated continuously through the regulation of the temperature and relative humidity of the air inlet. Fig. 4 gives an example of the regulations obtained in these reactors for temperature (TP) and the dry matter percentage (%DM) of the medium during a cultivation of *Trichoderma viride TS* on sugar beet pulp.

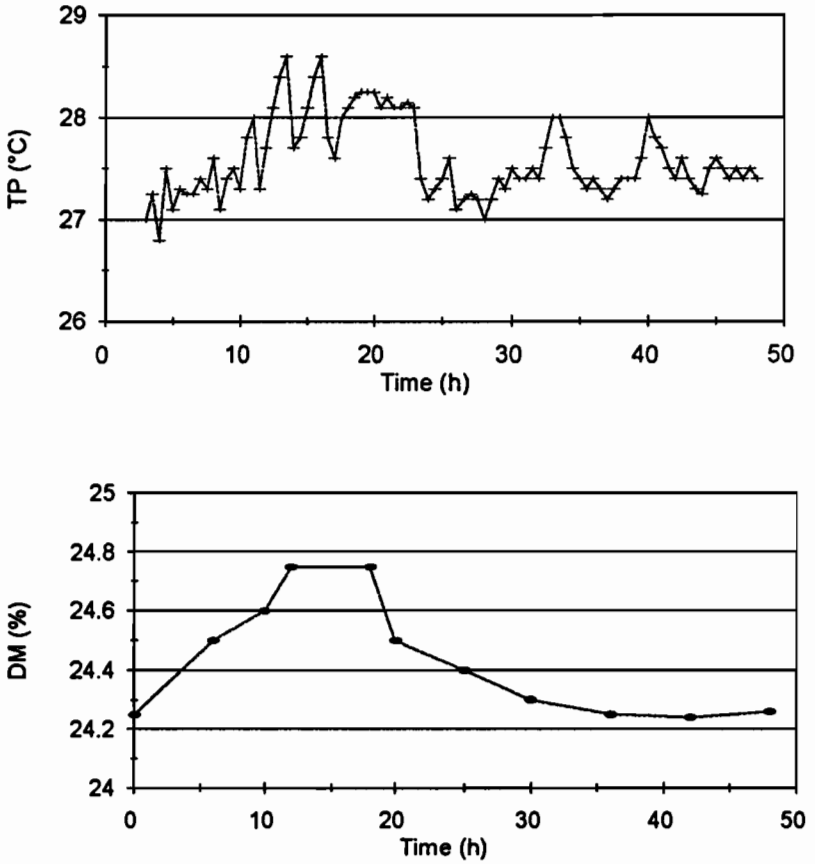

Fig. 4. Example of the regulations of the temperature (TP) and the dry matter percentage (%DM) in a solid medium.

Moreover, outlet air coming from each vat or each compartment of a vat can be pumped and analyzed on a separate gas analysis board, controlled by a computer. With these on-line measurements, the computer calculates and stores the oxygen uptake rate (OUR), the carbon dioxide production rate (CPR) and the respiratory quotient (RQ) of the microorganism during a culture.

Six reactors have been built of this design. These reactors allow a) studies on heat and mass transfers in substrate layers up to 50 cm height, b) studies on temperature, medium moisture level regulations, aeration rate, effect of layer thickness, air channeling, etc. and c) studies on the optimization of culture conditions.

Therefore, many parameters of importance in scale-up to pilot reactor can be studied in these reactors. Only one important parameter cannot be studied and that is the influence of the agitation mode.

NON-STERILE PILOT REACTOR

Pilot plant studies, before possible industrial applications, are of importance in improving the feasibility of a defined process at a larger scale. On the basis of data obtained in the laboratory-scale reactors, a pilot reactor has been built. The main objectives of this prototype were a) to define a technology and particularly an agitation mode, liable to scale-up to production plant level, b) to study heat and mass transfers to adapt culture parameters regulations, c) to optimize processes, d) to supply sufficient quantities of products when necessary and e) to calculate process costs with more accuracy.

Fig. 5 presents the schematic diagram of the measurements and regulations in the pilot plant (1.6 m^3 capacity).

This pilot plant has been used, for example, for protein enrichment of sugar beet pulp with *Trichoderma viride TS*, and the production of spores (*Trichoderma harzianum, Fusarium oxysporum, Beauveria bassiana*, etc.) on different media as sugar beet pulp, wheat bran, perlite and clay granules (Durand and Chereau, 1988b).

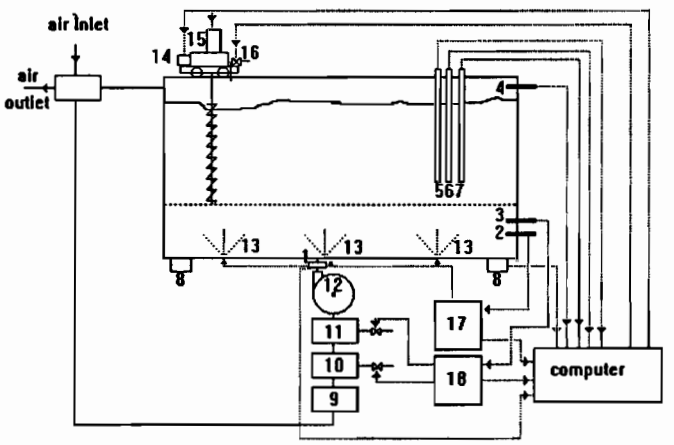

Fig. 5. Schematic diagram of the measurements and regulations: (1) flow meter, (2) temperature probe, (3) relative humidity probe, (4) temperature probe, (5) oxygen probe, (6) pH probe, (7) temperature probe, (8) weight gauges, (9) air filter, (10) cooler, (11) heater, (12) fan, (13) sterile water or steam spray, (14) carriage motor, (15) screw motor,(16) electric valves for spraying solutions, (17) relative humidity regulator, (18) temperature regulator.

Figs. 6 and 7 give the evolutions of the temperature (Tin) and the relative humidity (HRin) of the air inlet, which are of value in maintaining the temperature (TP) and the dry matter percentage (%DM) of the medium during the culture.

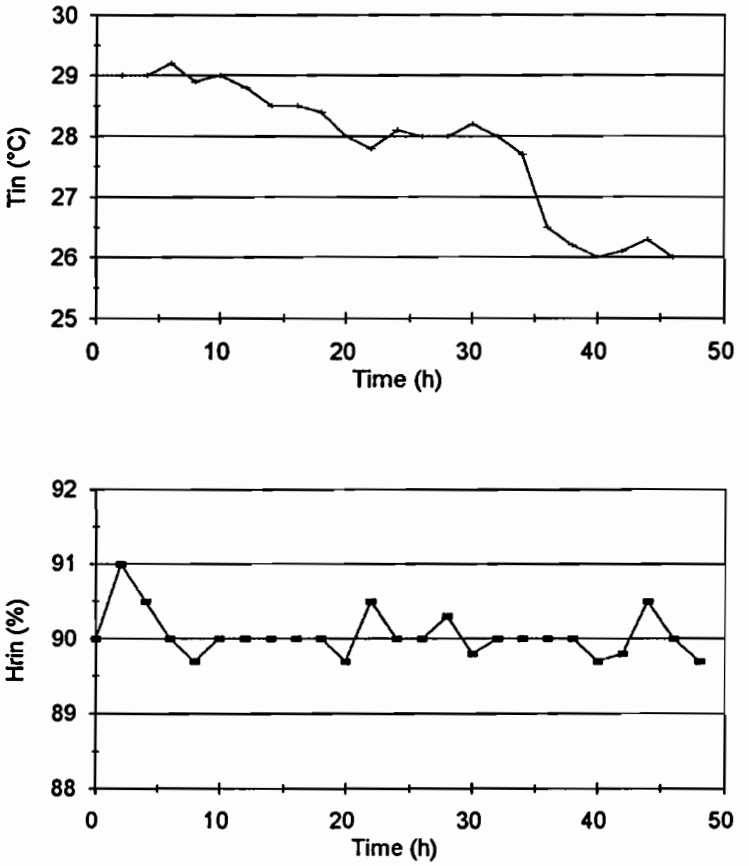

Fig. 6. Example of the regulations of the temperature (Tin) and the relative humidity (RHin) in the air inlet.

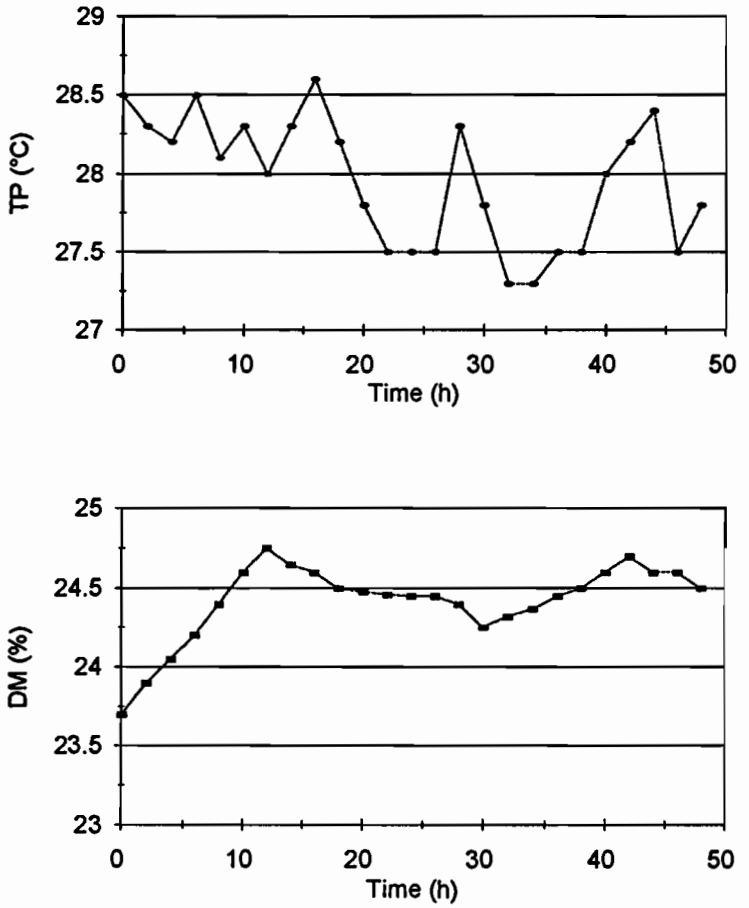

Fig. 7. Example of the regulations of the temperature (TP) and dry matter percentage (%DM) of the medium during a culture.

REACTORS FOR STERILE PROCESSES

Although SSF is usually carried out non-aseptically, in some cases, it may be necessary to develop aseptic processes by sterilizing the substrate, the bioreactor, the air used for forced aeration, and the water used for the humidification.

Sterilization of solid substrates by steam within the reactor is difficult to achieve mainly at large scales. The sterilization time used is much longer in SSF, as compared to submerged fermentation, due to low water content in the solid media used. Moreover, physicochemical alterations of the medium can occur, some nutrients can be damaged and some toxic compounds may also be formed.

Water used for the humidification may be sterilized by filtration or UV light, as also the air stream. For the equipment, chemical sterilization or desinfection is often carried out (alcohol solutions, formaldehyde), because steam sterilization needs closed and pressure-proof bioreactors.

Despite the fact that contamination can be very often prevented by means of dense as well as vigorous inoculum and selective culture conditions, some studies or applications can require total containment and aseptic conditions.

The development of sterile equipment and process may offer interesting alternatives to submerged fermentation for different applications, such as pharmaceutical products, human foods, use of genetically modified microorganisms, strains having a very low growth rate, and production of spores or compounds which trigger allergic or toxic reactions.

LABORATORY SCALE REACTOR

This type of reactor, with a working volume of about 1 L, has been designed for several purposes, such as screening of microorganisms as well as substrates, initial studies on process optimisation, etc.

Entirely automated, it only needs water and air supplies. During a culture, it is possible to take sample near a flame, without problems of contamination. Fig. 8 presents this lab-scale sterile reactor.

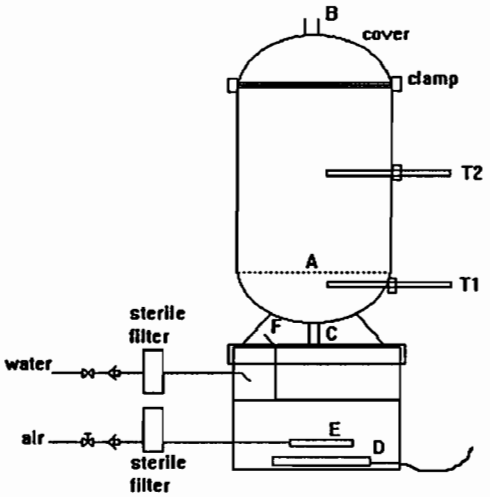

Fig. 8. Schematic of the laboratory scale sterile reactor. (A) wire mesh; (B) air outlet; (C) air inlet; (D) resistive heater; (E) sparger; (F) water level probe; (T1) inlet temperature probe; (T2) medium temperature probe.

Numerous fermentations have been carried out with different organic media (sugar beet pulp, wheat bran, copra cake, etc), as well as mineral and synthetic supports (clay, peat, sponge, etc).

Fig. 9 presents an example of results obtained during *Trichoderma viride* conidia production on sugar beet pulp medium.

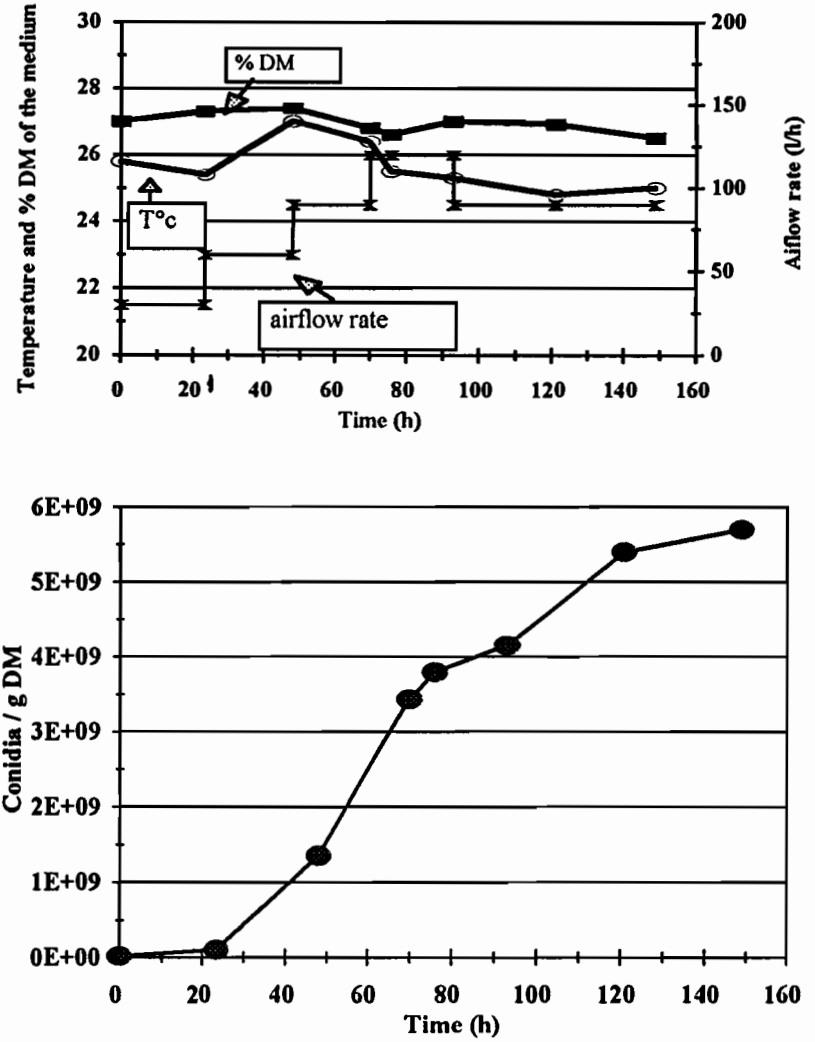

Fig. 9. Example of regulations in the lab scale sterile reactor (Production of Trichoderma viride conidia on sugar beet pulp)

STERILE PILOT PLANT

After experiments in the small sterile reactors, it could be interesting to verify a lab scale sterile process at a larger scale.

At the first attempt, a cylinder shaped horizontal reactor was built, but it proved unsatisfactory, mainly in terms of homogeneity of aeration and temperature. Subsequently, a new one was built and patented. As shown in Fig. 10, it consists mainly of a vertical cylindrical reactor (stainless steel) with a jacket for insulation. With a total capacity of 50 L, it also has a planetary agitation device. All the procedures (sterilisations, regulations, data acquisitions, etc.) are automated via a computer (Chamielec *et al*, 1994).

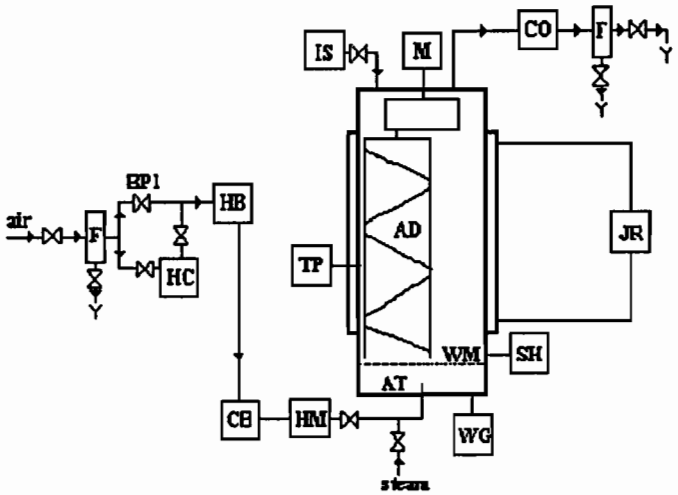

Fig. 10. Schematic diagram of the sterile pilot reactor. (F) air filter; (HC) humidification chamber; (HB) heating battery; (BP1) by-pass; (CB) cooling battery; (HM) probe for air relative humidity measurement; (AT) probe for air temperature measurement; (TP) probe for medium temperature measurement; (WG) weight gauges; (WM) wire mesh; (SH) sterile sample handling; (JR) water temperature regulation in the jacket; (AD) agitation device; (M) motor for agitation; (IS) sterile system for adding inoculum and solutions; (CO) water air condenser.

APPLICATIONS

Only three applications, developed in INRA, Dijon for different industrial applications are presented.

PROTEIN ENRICHMENT OF SUGAR BEET PULP FOR ANIMAL FEED

A process has been developed to increase the protein content of sugar beet pulp by cultivation of a mutant of *Trichoderma viride*. This strain has been screened by Pr STARON, on the basis of different criteria, such as protein content, amino acids composition, low nucleic acids content, no toxicity, no antibiotic production, and ability to consume lignocellulosic substrates. The final product obtained after 48 h cultivation had about 21 % protein. From 1 ton of raw sugar beet pulp, about 0.8 ton of protein enriched product is obtained.

Feeding trials of this product were carried out on lambs and rabbits during fattening period. It was proved that the protein enriched pulp can replace all of soyabean cake in the rations, without any significant difference. For the rabbits, the substitution in the diet gave the same efficiency, i.e.

10% protein enriched pulp = 7 % raw pulp +3% soyabean cake.

In case of lambs, two diets were compared for eleven weeks. The gains in weight were similar during the fattening period. A delay of one week for the diet with protein enriched pulps occured, probably due to the shifting of the animals to this new diet (Fig. 11).

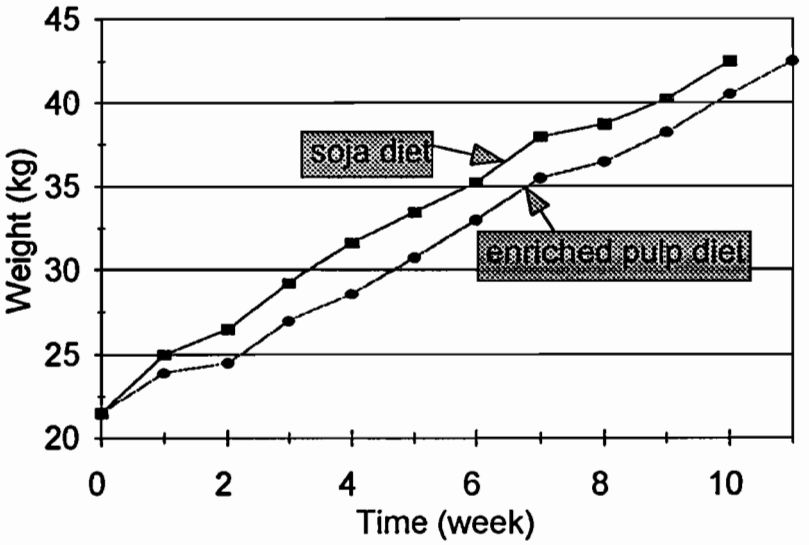

Fig. 11. Evolution of the weight gains of the lambs

Unfortunately, in France, protein enriched pulps for animal feeding cannot be competitive with soyabean cake from an economic point of view.

An economical evaluation of the process for industrial application (50 tons per h) was made. The estimated cost price of this dried animal food was ca. 2 FF/kg dry matter (Table 1).

Table 1. Economical evaluation of the protein enrichment process

Parameter	Percent of total production cost
Depreciations	18.0
Total operating cost	23.0
Raw sugar beet pulp	24.0
Drying	35.0

However, in China , the same process and technology is being used at an industrial level to produce microbial protein by using *Aspergillus tamarii*. Two reactors were built, each of them having a working capacity of 25 metric tons (Xue *et al*, 1992).

This application shows that our technology is easily scaled-up to a large production plant level. Moreover, depending upon the country, this process can be viable on an economic point of view.

ENZYME PRODUCTION

This second application was carried out in collaboration with a French sugar company, GENERALE SUCRIERE.

With 1.6 m^3 reactor and leached sugar beet pulp as solid substrate, a process for pectinolytic enzymes production was optimized.

Using a selected strain of *Aspergillus niger*, the process has been in operation for 4 years at an industrial level. A sub-company of GENERALE SUCRIERE, named LYVEN (CAGNY - FRANCE) has been established. For the first time, polygalacturonase and pectin esterase were produced for applications in oenology, cider-making, etc. Now two industrial plants are able to produce others different enzymes (cellulases, hemicellulases, amylases). One more plant is under construction.

BIOPESTICIDES PRODUCTION

It involves mass production of fungal conidia or mycelium for biological control applications. Up till now, different substrates used were sugar beet pulp, corn stover, wheat bran and straw.

To increase the possibilities of applications and also to eliminate eventual problems of incorporation of such sustrates in soils or on the plants, solid state fermentation processes was developed and patented by using mineral supports wetted with an optimal liquid medium.

Among different supports tested (ceramics, sepiolite, perlite, montmorillonite, etc.), clay microgranules (300-500 µm) was choosen on the basis of some characteristics like granulometry, resistance, density, pH, maximum water holding capacity and porosity.

Different antagonistic fungi (*Trichoderma, Fusarium, Beauveria, Arthrobotrys*) can grow on this support in the non-sterile pilot reactor described earlier.

For example, a process for production of a new bioinsecticide, active against the European corn borer (*Ostrinia nubilalis*) has been developed. An entomopathogenic fungus, *Beauveria bassiana*, is cultivated on the clay microgranules (500 kg dry matter), wetted with an optimal nutritive solution.

During the fermentation, the aeration rate was 300 m^3/h, the temperature and the moisture, respectively, were maintained at 25°C and 40% of the total weight. After incubation for 48 h under these optimal conditions, the culture was dried in the reactor. During drying, the air flow rate was increased two-fold and relative humidity of air decreased to about 15%.

The final product containing mycelium and spores (about 2.10^9 spores/g dry matter), can be directly used after harvesting, without formulation. This process is interesting for several reasons, such as a) the bioinsecticide produced is compatible with the mechanical standard application of pesticides on corn leaves, b) the biomass bound to the microgranules conserves its efficiency after storage for 12 months at 4°C, c) like the chemical insecticides usually used, this product has a field efficiency of 80%. Moreover, this efficiency persists during 3 weeks and d) the application dose (25 kg/ha) and the price are equivalent to the chemical insecticides.

This product has been approved by Commission and a French company NPP (PAU-FRANCE), produces it at an industrial level since the beginning of 1994.

Studies on other applications (for fighting plant diseases) are in progress, as the same company wishes to adapt this process to other antagonistic fungi.

CONCLUSIONS

Solid state fermentations have attracted renewed interest due to their importance in developments concerning solid waste valorization, secondary metabolite production, biopesticide production and also bioremediation. Though SSF is not going to replace submerged fermentation, it may constitute very attractive alternative and may even be strictly necessary for some specific applications.

But, these fermentation processes suffer from the lack of knowledge, mainly in the fields of engineering, reactor design, instrumentations and process controls, research on heat and mass transfers leading to scale-up strategies, and studies on the microorganism physiology in such solid media.

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Laboratory scale bioreactors for study of fungal physiology and metabolism in solid state fermentation system

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SUMMARY

Data on physiology, effect of various parameters on the growth as well as on the metabolism of the culture, monitoring and regulation of parameters are of critical importance in the design of a commercial process. The studies on these aspects at laboratory scale thus dictate the use of efficient laboratory scale bioreactors. A column bioreactor was developed at ORSTOM and the manufactured commercial units are, herewith, described along with various strategies for monitoring and control of different parameters. The design of the modified kneading machine is also reported for upgradation of wastes at a level of 120 L capacity. To overcome the lack of a system for inoculum development, a disk fermentor was developed, which was found to be highly efficient. A static fermenter of the capacity of 50 Kg substrate, named as Zymotis was developed for efficient control of the fermentation temperature and achievement of high productivities. All these bioreactors form one of the most important contribution of ORSTOM to solid state fermentations, based on the use of solid substrates/supports.

Keywords: Solid state fermentation, bioreactors, equipments, Zymotis, column bioreactor, kneading machine, disks fermentor, FMS 16-250.

RESUME

Bioreacteurs de laboratoire pour étudier la physiologie et le métabolisme de champignons cultivés en milieu solide.

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Les données de la physiologie, l'effet de différents paramètres sur la croissance aussi bien que sur le métabolisme de la culture, la supervision et la régulation des paramètres sont d'une importance critique dans la conception de procédés commerciaux. Les études concernant ces aspects à l'échelle du laboratoire impliquent donc l'utilisation des bioréacteurs de laboratoire efficaces. Le bioréacteur en colonne développé à l'ORSTOM et l'unité commerciale construite sont ici décrits, de même que les stratégies de contrôle et supervision de différents paramètres. La conception d'un pétrin de boulangerie modifié est également signalée pour la valorisation de déchets à une capacité de 120 litres. Afin de combler la manque de systèmes de production contrôlée d'inoculum, un fermenteur à disques fut développé, et se révéla d'une grande efficacité. Un fermenteur statique d'une capacité de 50 kg de substrat, nommé Zymotis, fut développé dans le but d'obtenir un contrôle efficace de la température de fermentation et d'atteindre des productivités élevées. Tous ces bioréacteurs forment l'une des plus importantes contributions de l'ORSTOM aux fermentations en milieu solide, basées sur l'utilisation de substrats/supports solides.

Mots clés : Fermentations en milieu solide, bioreacteurs, équipements, zymotis, bioreacteur de type colonne, pétrin de boulangerie, fermenteur à disques, FMS 16-250.

INTRODUCTION

Solid state fermentation offers various advantages in comparison with submerged ones, (Lambert and Meers, 1983). For example, aeration is facilitated through the spaces between the substrate. Substrate agitation, when necessary, is discontinued. The absence of a liquid phase and low substrate humidity levels permit a) reduction of the fermenter volume, b) reduction of the volume of liquid effluents from the process, c) reagents saving during metabolites recovery, d) reduction of bacteria contamination because of low humidity levels, and e) use of non sterile solid

substrate in some cases (Raimbault and Germon, 1976). Culture media are simple, and are mainly composed of agro-industrial residues. Culture growth conditions on solid state media are similar to those in the environment. For some fermentation, the solid support microflora is used as an inoculum (Perraud-Gaime, 1995). Direct use of the fermented substrate is possible in many solid state fermentations.

The main disadvantages are: a) high risks of temperature rise due to excessive metabolic heat generation, b) difficulty in parameter regulation, c) need for pretreatment of solid support/substrate, d) high loss of humidity in fermentations of long duration, e) necessity for high inoculation when natural microflora is not used, and f) the critical role of water and water activity.

Any industrial SSF application requires the understanding of some physiological, physical and chemical parameters, which are characteristic of the strain used and substrate employed. These include data on growth rate, optimum temperature and pH, gas exchange and gas requirement of the strain, water content and activity, nutrient content, etc. (Roussos *et al*, 1991a).

All these parameters have to be considered when designing SSF equipment (Aidoo *et al*, 1982; Moo Young *et al*, 1983; Lonsane *et al*, 1985, 1992; Hesseltine, 1987). Futhermore, the monitoring and controlling of these parameters need to be studied carefully (Saucedo-Castañeda *et al*, 1994).

Some other important parameters, such as inoculum development, in addition to process and equipment scale-up, have to be taken into consideration while studying SSF. (Roussos *et al*, 1991a).

Considering all aspects mentioned above, bioreactors for different purposes have been developed in ORSTOM. These are described in the present paper.

COLUMN BIOREACTOR

The column bioreactor is the first equipment developed by ORSTOM in the years 1975-1980 to study solid state fermentation. Its principle has been patented in 1976 (Raimbault and Germon, 1976). It is composed of a small glass column of 4 cm in diameter and 20 cm in length, having an effective reaction volume of 250 mL. The column is filled up with substrate, as shown in Fig. 1 and placed in a thermoregulated water bath. Several columns can be placed within the same water bath.

Aeration is provided by using a compressor. Air passes through three humidifiers, the second one acting as an air distributor. Air saturation is further assured in the third humidifier which is composed of a small bubbler, positionned at the bottom of a vat, filled with water and placed immediatly below the fermentation column

(Fig. 1). The third humidifier and the fermentation column are submerged in the water bath, thus allowing a good substrate thermoregulation.

The column bioreactor has been widely employed by many researchers (Raimbault and Alazard, 1980; Huerta, 1984; Roussos, 1985; Trejo *et al*, 1992: Oriol, 1987; Dufour, 1990; Saucedo-Castañeda, 1991; Saucedo-Castañeda *et al*, 1992 a,b; Soccol *et al*, 1994). It should shortly be replaced by the recent bioreactor developed by ORSTOM, in collaboration with Gauthier Agro-industries, using the same principle, as described in the last section of the paper.

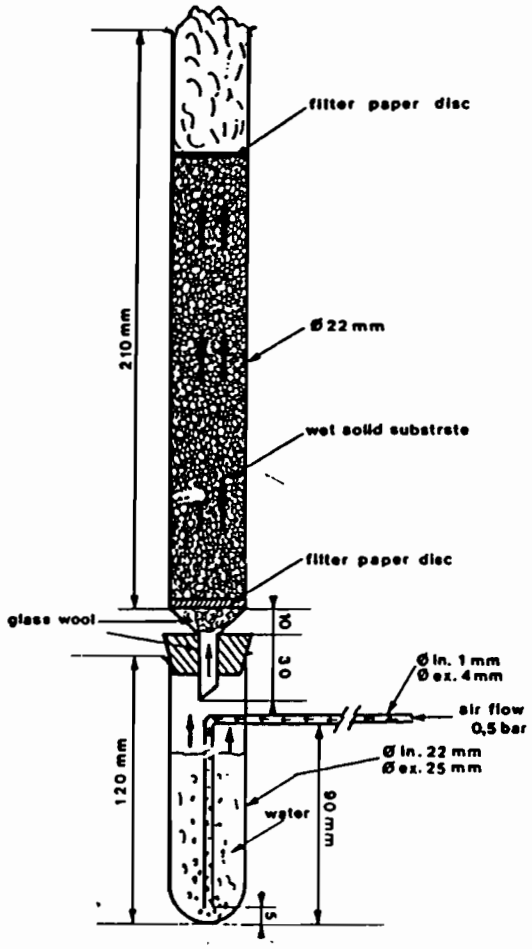

Fig. 1. Column bioreactor

The micro-organisms grown and studied using the column bioreactor, with or without the gas analysis system, have been filamentous fungi (*Trichoderma*,

Rhizopus, Aspergillus, Penicillium), yeasts and mushroom mycelia (*Pleurotus, Agaricus*). The metabolites obtained include enzymes (cellulases, amylases, proteases, lipases), probiotic substances prepared from filamentous fungi, biopesticides, alkaloids, antibiotics, phytohormones, etc. (Table 1).

Table 1. Aplication fields of columns bioreactors in various solid state fermentations.

Application fields	End products	Microorganisms	References
Fermented food	Koji	*Aspergillus oryzae*	Raimbault, 1980
	Pozol	*Lactobacillus* ssp and yeasts	Saucedo-Castañeda, 1987
Protein enriched	Feed	*Aspergillus terreus*	Gonzales-Blanco *et al*, 1990
Enzyme production	Amylases	*Aspergillus*	Oriol, 1987
	Lipases	*Rhyzopus delemar*	Martinez-Cruz *et al*, 1993
	Cellulases	*Trichoderma*	Roussos, 1985
	Pectinases	*Aspergillus*	Dufour, 1990
Secondary metabolites	Aroma	*Ceratocystis fibriata* *Trichoderma*	Christen and Raimbault, 1991 *et al*, 1995
	Penicillin	*Penicillium*	Barrios-Gonzales *et al*, 1988
	Aflatoxin	*Aspergillus flavus*	Barrios-Gonzales *et al*, 1990
Organic acids	Citric acid	*Aspergillus niger*	Gutierrez-Rojas *et al*, 1995
	Gallic acid	*Aspergillus niger*	Raimbault, 1980
	Lactic acid	*Rhyzopus oryzae*	Soccol *et al*, 1994
Alcohol production	Ethanol	*Schwanniomyces castellii*	Saucedo-Castaneda, 1991
Spores production	Insecticide	*Trichoderma harzianum*	Roussos, 1985
Ensilage	Ensiled substrate	*Lactobacillus* spp	Saucedo-Castaneda, 1990
		Mixed cultures	Perraud-Gaime, 1995
Higher mushrooms	Spawn	*Pleurotus spp*	Roussos, *et al*, 1996
		Morchella	Kabbaj *et al*, 1996

KNEADING MACHINE

A kneading machine, used in the bakery for dough preparation, has been adapted in 1979 by ORSTOM and IRCHA, for the study of SSF in large volumes (Meyer and Deschamps, 1979; Deschamps *et al,* 1980). It is composed of a cylindrical open bowl of 1,200 L capacity with a rounded base, freely operating around a vertical axis through a speed regulated motor. It is equiped with a 3 fingers stirrer forming an angle of 30° with the bowl axis (Fig. 2). The bowl has got a double jacket on the bottom side, allowing an air flux through a serie of holes, made in its rounded base. Temperature, pH and humidity are measured and controlled. Temperature and humidity saturation adjustments are obtained by a continuous mixing of the substrate and a simultaneous vaporisation of water mist on substrate surface. This combined operation avoids during of the substrate surface, pH adjustment is implemented by means of addition of a urea solution addition when vaporizing the substrate.

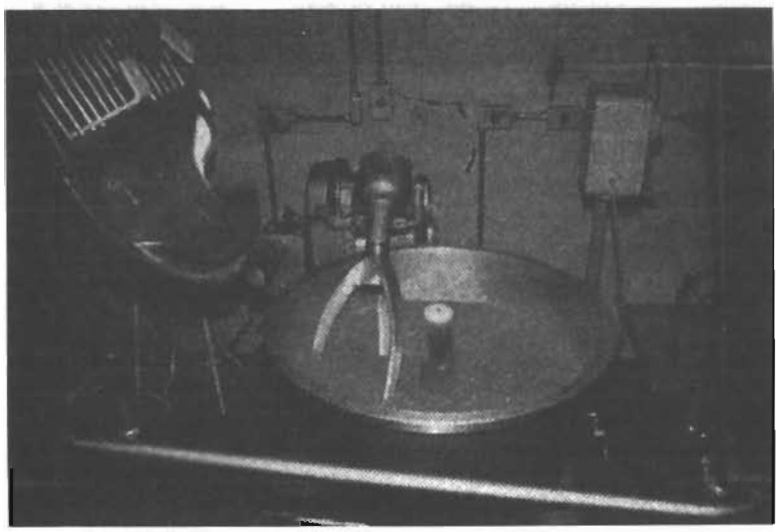

Fig. 2. Kneading machine, implemented by IRCHA for fungal cultivation on solid substrate.

This kneading machine has been used to enrich various amylaceous substrates (cassava, potato, banana) with proteins for animal feed production (Senez *et al,* 1980; Deschamps *et al,* 1980). This type of equipment has also been used for the fermentation of wheat straw (Deschamps *et al,* 1985). An adaptation of this

equipment principle has been made by the researchers of INRA-Dijon (Durand, 1983) for the protein enrichment of beet root pulp.

COMPUTERISED SYSTEM FOR FOLLOW-UP AND MONITORING OF SSF

In order to follow-up and control the fermentation parameters, when using solid state bioreactors, Saucedo-Castañeda *et al*, 1992a, b, have developed a system, which has the ability to continuously monitor the concentration of CO_2 and O_2 in the reactors exhaust air. The system automatically modifies the air flow rate in order to maintain the levels of CO_2 and O_2 at constant values in the gaseous effluents.

For that, air is analysed through classical GC or infrared gas analysers and data are processed using a computer. A program controls air valves for air rate managing.

Such an apparatus can not function in conditions of humidity. Therefore, a sample of the exit gas passes through a chilled water condenser, which separates moisture from gas and then, the gas is directed through a silica gel drying column. Dried air is then analysed. The process operates continuously for each individual column bioreactor through an automatic multiple ways valve, which selects the different outlet of gases to be analysed. Eight to sixteen columns can be analysed, along the fermentation process.

The prototype schematic representation is given in Fig. 3. It consists of a chilled water glass condenser for moisture separation (items No.14 and 15), a silica gel column for gases drying (item No.16), the gases analysers for CO_2 and O_2 (item No.18) with their injection valves (item No.17) and the computer unit with its accessories (item 13).

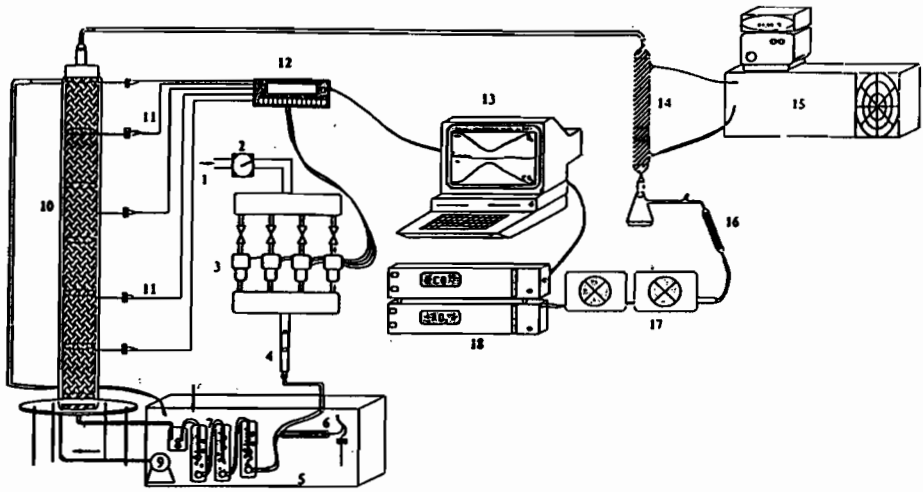

Fig. 3. Diagramatic sketch of the system developed for monitoring and control of exhaust gases in aerobic solid state cultures.

DISKS FERMENTOR

In 1985, ORSTOM researchers adapted a disks fermentor, in order to use it for the study of *Trichoderma harzianum* conidiogenesis. The aim was to develop an efficient technique of spore production, which is not yet available for the industrial applications of solid state fermentation. The micro-organism *Trichoderma harzianum*, was chosen, because of its large potencial in industrial applications: cellulases (Roussos *et al*, 1991b), biopesticides (Elad *et al*, 1993), cassava flour protein enrichment (Muindi and Hansen, 1981) and flavour compounds (Okuda *et al*, 1982). The equipment has been patented in 1985 (Raimbault and Roussos, 1985).

Fig. 4 shows a schematic representation of the disks fermentor. The equipment is composed of a cylindrical glass column of 800 mm in length and 92 mm in internal diameter, with flanged open ends, which can be closed by means of stainless steel plates, "O" rings and winged nuts for asceptic operation. It offers a total capacity of 5 L. Inlet and outlet ports are provided with the column for gas or liquid flux. The column is equipped with 35 disks of 50 mm in diameter, each disk being composed

of 2 steel gratings of 2 mm thickness and having a mesh of 4 mm, thereby giving a working area of 4,960 cm^2. Spacing between the disks is 10 mm. The gratings are fixed on a central shaft, which is connected to a stirring motor. The disks fermentor is positioned horizontally, with the help of appropriate stands for its operation. The cylinder is first filled with an adequate volume of a solution of nutrients as well as agar and sterilized by autoclaving. When cooled to about 45°C, the still liquid medium is inoculated with spores and then allowed to cool down 25-29°C, under slow rotation (30 rpm). This permits the mixed inoculated agar medium to homogeneously solidify on the disks interspace. Before the complete solidification of the agar, rotation is stopped and excess of inoculated medium is drained off from the fermenter (Roussos *et al*, 1991a).

Air is supplied from a compressor and is difated and filtered. It passes through a water bath to get humidified. The disks fermentor, also called sporulator, was used at room temperature, but a temperature control may be obtained by submerging the fermentor in a water bath. The equipment is nevertheless limited to a small production of spores (Roussos *et al*, 1991).

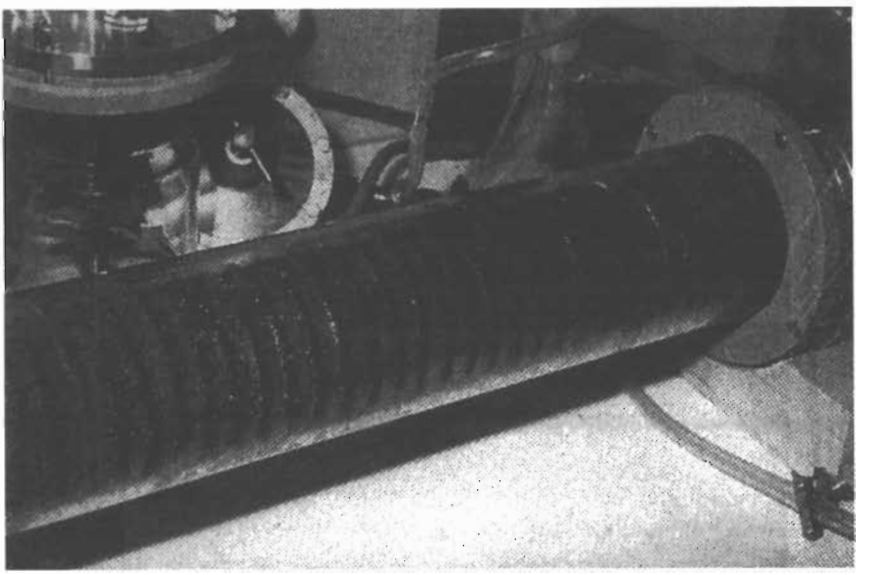

Fig. 4. Schematic representation of disks fermentor.

ZYMOTIS FERMENTOR

As the process of spore production at large scale is still not achieved, the ORSTOM group of researchers worked on the design of a new fermenter, taking into account its scale-up potential for industrial purposes. The main problem of solid state fermentation at large scale is the metabolic heat elimination. As the fermenting solid substrate forms a mycelial coat, which restricts air transfer, it seemed almost impossible to envisage large scale SSF production, with high productivity in static conditions. Large scale SSF production was, therefore, mainly carried out, using agitated systems. The equipment developed presents a very efficient cooling system, that solves the metabolic heat problem and allows very high productivity.

Zymotis is the so called new fermentor (the name comes from the Greeek term "zymotiras", which means the fermentor). It is a large scale static solid state fermentor, which offers efficient control of various parameters, such as aeration, temperature and substrate moisture. The equipment has been patented in 1985 (Prébois *et al*, 1985) and its use has been published (Roussos *et al*, 1993).

The fermentation vessel is a rectangular box of 5,000 mm in length, 4,000 mm in width and 6,500 mm in height, made-up of acrylic material and giving a working capacity of 100 L. An acrylic dome-shaped cover fits closed on the top side of the unit, avoiding contamination risks from outside to inside and vice versa. It can, thus, be used even for the production of hazardous micro-organisms or molecules.

Substrate temperature is controlled by water circulation through a total of 10 stainless steel heat exchanger plates, which are provided along the depth of the fermentor vessel and assembled in parallel to each others (Fig. 5), all of them fitted tightly in the fermentor. This makes nine rectangular compartments in the fermenation vessel (Fig. 6). The compartment length can, therefore, be modified by suitably shifting the location of the heat exchanger plates so as to study the effect of substrate thickness, as a function of various growth and production parameters. It is also possible to remove some of the heat exchanger plates, as their water inlet and outlet are individually connected from outside the fermenter.

Aeration is provided, using the same system as that for the disks fermentor. Air is of the same quality (de-oiled, filtered and humidified). It is distributed in each compartment by using nine inlet tubes. For a more precise work, each air inlet can be equipped with its own air control valve, rotameter and humidifier. The system of control and monitoring, developed by ORSTOM (Saucedo-Castañeda *et al*, 1992), can also be used with the Zymotis fermenter.

As the material used for the building of the Zymotis fermentor can not be steamed or autoclaved, it is not possible to work in strictly sterile conditions, but good conditions of sterility can be obtained by cleaning the apparatus with an alcoholic

solution. It is also envisaged to replace acrylic sheets by stainless steel at industrial scale, to obtain a stream resistant equipment, that would be sterilizable.

This unit is of potential promise for industrial exploitation of SSF, as its scale-up is easy. Humidity saturation, temperature and aeration control can be monitored. Futhermore, microorganism growth and metabolite production, with high productivity, can be obtained, using the exhaust gas monitoring system developed by ORSTOM, thereby, making Zymotis an automated high productivity fermenter.

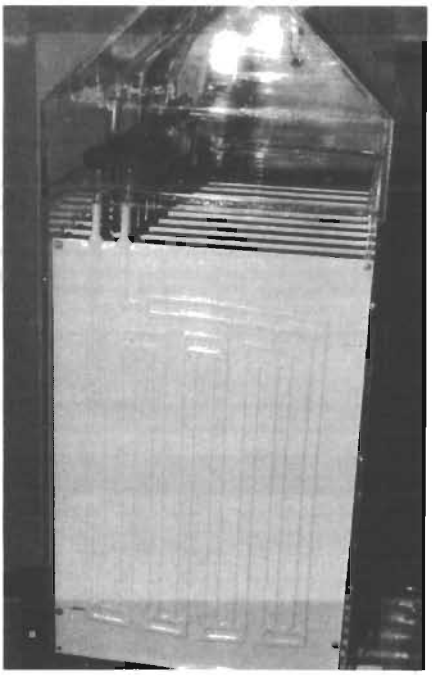

Fig. 5. Zymotis heat exchanger plate showing the tubular pipes for water circulation.

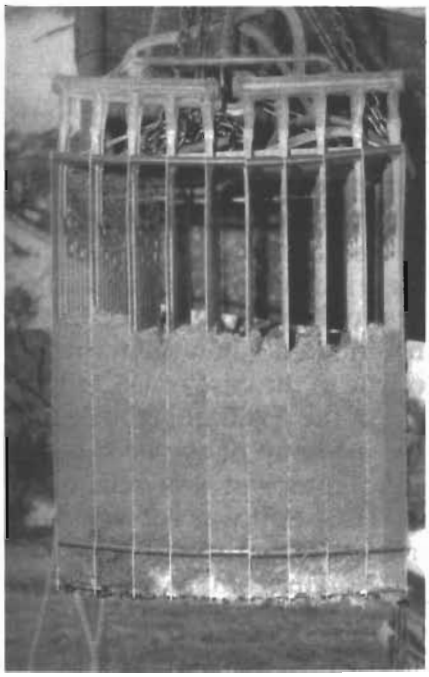

Fig. 6. View showing the rectangular compartments (9 Nos.) of the Zymotis.

MULTIPLE COLUMNS FERMENTOR: FMS 16 - 250

After having worked on SSF process and equipment design, ORSTOM retained 3 systems. The first is the column fermentor, which allows the study of microorganisms, processes or production parameters at a laboratory scale. The second one is the Zymotis fermenter, which allows the production and study of micro-organisms or metabolites at large scale. The third one is the system of control and monitoring of these fermenters.

In order to continue research on SSF processes, which is widely carried out at laboratory scale, ORSTOM desired to obtain a standard SSF equipment, consisting of 16 columns, based on the column principle, and designed for a distribution in the world trade market.

In 1993, Gauthier Agro-industries Ltd obtained licence for the design and manufacturing of the commercial equipment. Some modifications and implementations of the laboratory system took place.

For each laboratory system developed by ORSTOM, Gauthier Agro-industries engineers had selected some principles: fermentation columns made-up of glassware, temperature regulation by subimmersion of the column in a water bath, precise regulation of air flux and possibility of continuous analysis of gaseous effluent. Some others had to be improved, i.e., air humidity saturation, air partition, independent air flow setup on each column, continuous air flow measurement, preservation of substrate humidity along the fermentation, commodity of use in laboratory environment, and commercial design in accordance with ergonomy standards.

The working principle of the apparatus, named FMS 16 - 250, is presented in Fig.7.

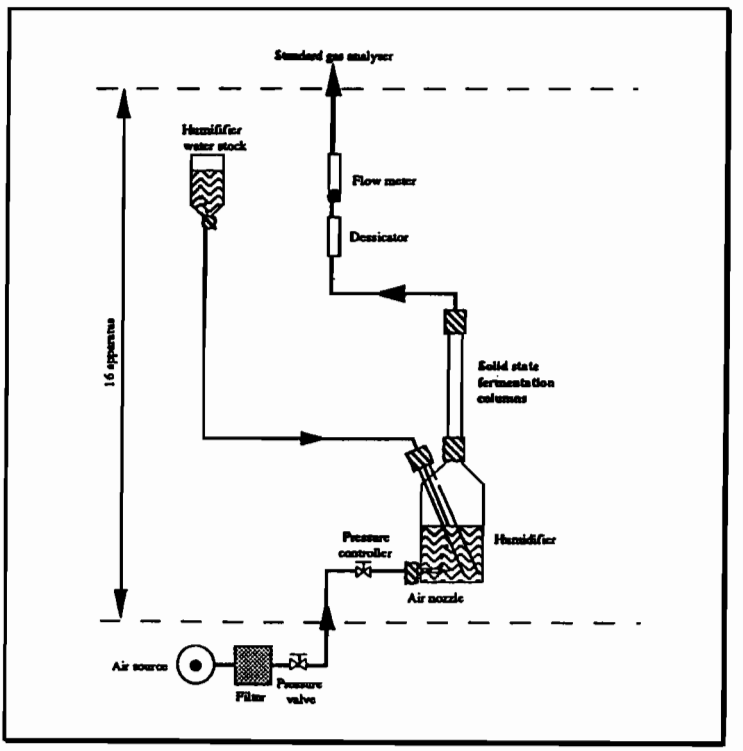

Fig.7. Working principle of FMS 16 - 250.

Air, obtained from a compressor (which can be supplied with the equipment), is difated and filtered through a submicron filter of 0.3 µm pore size. Its pressure is set by passing through a pressure regulator, equipped with a manometer. It is then distributed to feed 16 independant bioreactors of 250 mL each. A pressure setting valve is installed at each bioreactor inlet, allowing a precise setting of air flow below 4 L/h. The loss in pressure on the general circuit, when setting the air flow on a column inlet, is compensated by a high pressure in the upstream circuit, thus allowing the precise regulation of the air flow of a column, without modification of the air flow of another one.

On the bottom section, the bioreactor is composed of a glass humidifier with an air nozzle and water feeding, while the glass fermentation column is on the top part. The air nozzle allows to obtain very thin air bubbles, thus facilitating air humidification. The level of water in the humidifier is maintained at a constant value, through a water reservoir placed on the upper compartment of the FMS 16-250. A glass tube protects the water feeding from being in contact with the air bubbles. It avoids air loss via the feeding system, which otherwise would lead to an over feeding of the humidifier, because of express pressure in the water reservoir. The column (40 mm diameter and 200 mm length), is screwed on the humidifier, using a tight system containing a metallic grid, thereby retaining the solid state support in the column. Tightness is assured by a system of gasket and a positionner.

Each fermentation column outlet is connected to a chilled water condenser, then to a silica gel dessicator and finally to an air flowmeter. The condenser and dessicator remove the excess humidity, allowing a correct use of the flowmeter and the analysis of gaseous effluents by a standard apparatus (GC, IR).

The FMS 16 - 250 apparatus is composed of a metallic painted frame, with 4 stands to support a thermostated water bath (1,650 mm in length, 300 mm in width and 450 mm in height), whose temperature is regulated. The bioreactors (humidifier + column) are submerged in that water bath, so their temperature could be maintained at a constant value. Temperature can be regulated between ambient and 50°C in the standard model, but work below the ambient temperature can be envisaged by installing a chiller on the thermoregulation system. A submerged pump assures the water homogenisation in the bath and can also be used for the bath draining. The bioreactors are arranged in the water bath in 2 rows, each consisting of 8 column bioreactors.

The top of the frame offers 2 compartments, one of which is closed and where the electrical command components are located. The second one, which is illuminated, is equipped with a door and contains the water reservoirs and the systems of gaseous effluents drying. The flowmeters are placed on the door at the height of the eye, thereby facilitating reading and precise adjustment.

The air treatment system is located on the left side of the frame, while the dried gaseous effluent outlets are located on the right side of the frame. The pressure setting valves are located on the front side of the frame, just above the bioreactors at the height of the hand for easy operation. Each system is perfectly referred to the frame and panel. The air and water inlets and outlets are all well equipped with rapid connectors, to facilitate handling. An effort has been made to hide the tubings inside the frame for a better appearance.

Each column can be considered as a single bioreactor. It is independently aerated and humidified. Its temperature is regulated. It is capable of receiving any type of solid state support/substrate. Its air flow is continuously measured after dehumidifying/drying. Gaseous effluent outlets are also equipped with rapid connectors, to facilitate their connection to the standard gas analysis systems. A photograph of the FMS 16 - 250 is given in Fig. 8.

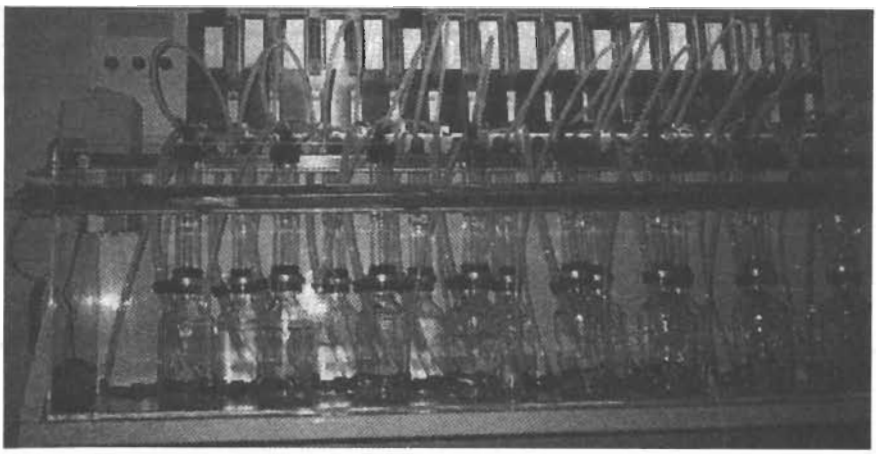

Fig. 8. Photograph of the FMS 16 - 250.

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Efficient and versatile design of a tray type solid state fermentation bioreactor

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SUMMARY

The most popular type of bioreactor, industrially used for solid state fermentation (SSF), is the static tray system. It is essential that oxygen availability is ensured at all points in the substrate bed and heat generated can be removed efficiently, simultaneously with control of water activity at desired level, irrespective of the conditions in the external atmosphere. This requires a careful design of the bioreactor. This paper deals with the development of a versatile design of an industrial tray-type SSF bioreactor, with a number of desirable features, such as efficient mixing and uniform circulation of filtered air, control of relative humidity and temperature under varying external atmospheric conditions.

Keywords: Solid state fermentor, tray-type bioreactor, industrial fermenter, parameter control, control equipments, water activity, O_2 availability, heat removal, forced aeration.

RESUME

Fermenteur industriel à plateaux pour les cultures en milieu solide.

BYNDOOR M.G., KARANTH N.G. ET RAO G.V.

Le bioreacteur le plus communément utilisé en industrie pour les fermentations en milieu solide est le fermenteur statique de type "à plateaux". Au cours des FMS, il est indispensable que l'oxygène soit disponible en tout point du milieu de culture et que la chaleur produite soit évacuée. De même, l'activité de l'eau doit être contrôlée à chaque niveau du bioréacteur, quelques soient les conditions atmosphériques externes. La conception d'un bioréacteur doit donc tenir compte de ces problèmes particuliers. Cet article présente la mise au point d'un fermenteur industriel FMS adaptable de type "à plateaux ". Il dispose d'un dispositif d'homogénéisation du milieu, permet la circulation uniforme de l'air filtré, un contrôle de l'humidité relative et de la température pour différentes conditions atmosphèriques externes, etc .

Mots clés : Fermenteur en milieu solide, bioréacteur à plateaux, bioréacteur industriel, contôle des paramètres, capteurs de contrôle, activité de l'eau, disponibilité d'O_2, transfert de chaleur, aération forcée.

INTRODUCTION

Solid state fermentation (SSF) has been known for a number of years and industrially practised in Asia and Far Eastern countries, but relatively neglected in the Western countries. However, renewed interest is catching up recently in this technique, in view of its potential for enlarged industrial applications. SSF involves mass and heat transfer processes, coupled with biochemical reaction in a heterogenous system of solid substrate particles and gas, under controlled water activity conditions. The nature of the substrate and consequent transport limitation give rise to appreciable temperature and concentration gradients in the reactor, that need to be minimized for optimum performance. Bioreactor design considerations are, therefore, crucial for achieving this. The design and operation of an industrial tray-type koji bioreactor is dealt with in this paper.

CONSTRUCTION OF THE SSF BIOREACTOR SYSTEM

The SSF bioreactor system consists of a specially designed chamber, in which the substrate containing trays are stacked. The chamber has a typical dimension of 12'x12'x12', with brick walls, cement flooring and RCC roof. The walls are coated with a special layer on the inside, providing a vapour barrier as well as superior thermal insulation. The chamber is provided with a snugly closing gasketted doors, through which the trolleys laden with trays are taken in and out. The flooring is provided with appropriate sloping to facilitate draining of condensed water. The atmosphere inside the chamber is accurately regulated for temperature and relative humidity, accounting for seasonal variations in the external atmosphere as well as the SSF reaction conditions. The whole of the regulatory hardware is situated on top of the chamber, which is kept entirely free from the tray stacks. The electronic control system is installed separately outside the chamber.

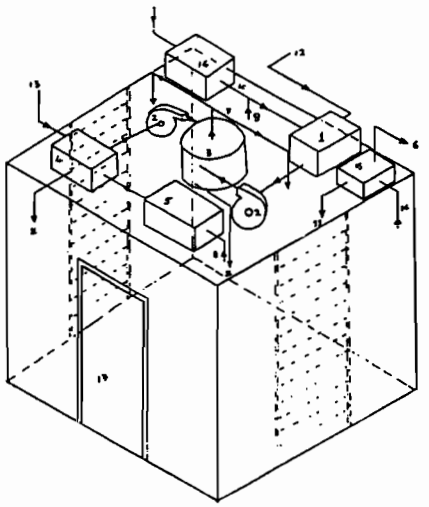

Fig. 1. Koji refrigeration system. 1. Cooling chamber, 2. Blower, 3. Mixer, 4. Humidifier, 56. Heater, 6. Exhaust air, 7. Air supply to chamber, 8. Return to heater and humidifier, 9. Return to humudifier, 10. Filtered fresh air through dehumidifier, 11. Water drain from chamber, 12. Chilled water to cooling coil of cooling or dehumidifier, 13. Filtered water supply, 14. Air filter chamber, 15. Air washer, 16. Return to air washer from koji room, 17. Door.

An isometric view of the koji fermentation system is shown in Fig.1 and a schematic flow diagram indicating the different units is given in Fig.2. Fresh air is drawn by a blower, which passes through a system of prefilter, capable of removing dust of 20 microns to 90% efficiency. Next is a micro-type filter capable of filtering 5 micron particles at 79.5% efficiency or a superfine filter having 70% efficiency to filter 0.3 micron particles. If HEPA filter is used, a booster fan becomes desirable. The type of filter used is based on the dust level in the surrounding and the permissible particle levels inside the fermentor.

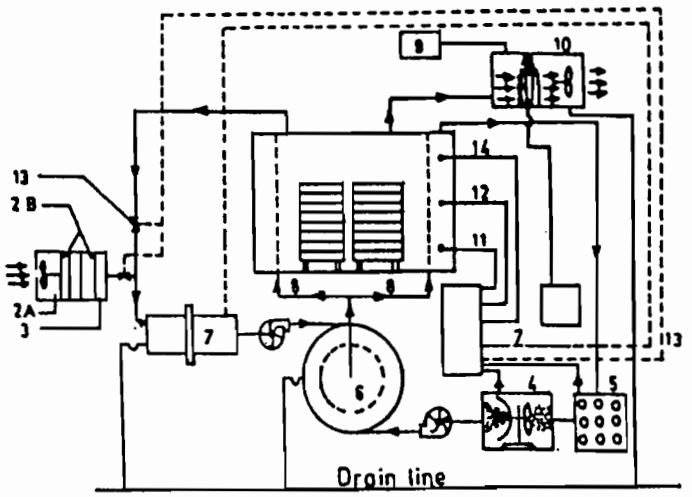

Fig. 2. Process flow diagram of koji room. 1. Air inlet, 2A. Prefilter, 2B. Fine filter, 3. Air inlet, 4. Humidifier chamber, 5. Heat chamber, 6. Mixing chamber, 7. Cooling chamber, 8. Conditioned air distributing ductor, 9. Exhaust gas analyser, 10. Air exhaust filter with blower, 11. Temperature and RH sensors, 12. Temperature and RH indicator controller, 13. Air damper for fresh air and air circulation, 14. Computer interface.

The filtered air mixes with a part of the recycled air stream from the fermentation chamber and passes into a cooling unit. The ratio of the recycle stream to the fresh air stream and the cooling capacity is designed to give the desired temperature inside the fermentor chamber. The cooling capacity can be typically 36,000 BTU/h.

Another recycle air stream from the chamber is drawn through a heating unit with electrical or steam heaters, which passes through a humidifier unit to give an output

of air at 95% RH and 35°C. The inlet water to the humidifier is heated at 35°C for satisfactory operation under winter temperature conditions in the external atmosphere.

The fresh filtered air and the recycled air, after humidification, enter the mixing unit in a tangential way for efficient mixing. Condensation of water occurs in the mixer, which is drained away. This air, at the desired conditions of temperature and RH, passes into the koji chamber, through downward chutes for uniform distribution. A part of the air from the chamber is recycled and the rest is exhausted, after passing through a water spray washing unit. The air exhaust load is much higher at the start up to prepare the chamber for fermentation.

REGULATION ASPECT OF TEMPERATURE AND RH

The variations in the seasons (winter, summer and monsoon) give rise to varying atmospheric conditions (Table 1) and the fermentor control systems must operate in such a way, so as to keep the temperature and RH conditions in the fermentation chamber always uniform. For example, in the typical summer conditions, during the start up, the heating and humidifying chamber will be only functioning whereas the cooling system will not be operative. In addition, the fresh air drawn by the system, through the cooling circuit blower, will be almost zero. This phase will take a few minutes and then the koji room will be opened and the substrate laden trays, loaded on the trolleys, can be brought in quickly, and the door closed. Now fresh filtered air, mixed with recycled air, is cooled in the cooling unit, before entering the mixing chamber. The second recycle stream will also enter the mixing chamber, after passing through the humidified unit.

Table I : Seasonal variations in temperature and relative humidity in the atmosphere at Mysore

Description	Summer	Monsoon	Winter	Desired inside conditions
Dry bulb temperature, °C	41	29.4	12.8	35±2
Wet bulb temperature °C	25.6	27.2	8.9	33.8
Specific humidity grains/Lb air	100	154	38	235
Relative humidity, %	28	82	60	95±5
Dew point temperature, °C	20	26.5	5	33.3
Heat content of air, BTU/Lb	-	-	-	59

The air at the desired temperature (30.8°C, DB temperature) and 95% RH will enter the fermentation chamber and pass over the stacked trays. Initially, there will be no heat generation, but in the later periods, fermentation generates heat, which will be taken up by the air stream for cooling in the cooling chamber. The cooling and the heating units are balanced appropriately to maintain the desired temperature in the koji root at all times.

For the winter conditions, the operation of the system is similar, except that the cooling unit will be having minimum load.

CONTROL FEATURES

The temperature of the fermenting solids is controlled by a chilled water circulation through a heat exchanger and hot air circulation through the humid air. To cool the water, an appropriate capacity refrigeration plant is provided with the necessary solenoid valve to regulate the flow into the heat exchanger. In order to heat the air, a separate heating chamber is provided with adequate capacity of stainless steel finned heaters. The blower from humidity chamber sucks the hot air and blows it into the koji room. The solenoid valve and the heater control operation are controlled by a microprocessor based digital indicating controller. The instrument gets the signal from PT-100 sensors, placed inside the room. In addition to the above, a 20 point data logger is also provided for recording the variation in temperature in different trays, using 20 Nos. of PT-100 sensors, connected to the data logger -cum- printer. The control instrument has a RS-232 interface, through which computer is connected.

The RH is controlled in the room by controlling the humidification. The room humidity is maintained by circulation through a separate humidifying chamber and a blower, controlled by a microprocessor based digital indicating controller. The transmitter is common for temperature as well as RH and is placed inside the room. The signal is processed by the controller and the humidifier is actuated, as per the set point. It also has an RS-232 interface, through which the computer is connected. In addition, there is a recorder to take reading of the temperature and RH continuously.

Moreover, the control panel has the built-in timers for operating the fresh air inlet damper and close loop circulation (diverting) dampers. These dampers aid the manipulation of the required amount of fresh air replacement to the room, as per the process requirement. Once the fresh air is forced into the room, the excess air is automatically pushed out, through the exhaust by the blower.

The exhaust filter chamber has a provision for housing various gas sensors for the analysis of the exhaust gases, such as oxygen, carbon dioxide, etc., which can be continuously monitored and computerised.

CONCLUSIONS

An efficient and versatile tray-type SSF biorector for industry has been designed and fabricated. Major features of the system include efficient mixing and uniform circulation of filtered air, accurate control of temperature and RH in the koji chamber, mixing and humidification outside the koji chamber, minimisation of water condensation in the chamber, and computer control of variables.

ACKNOWLEDGEMENTS

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Practical implementation of a biofilter in a composting/vermicomposting plant: Failures and solutions

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ABSTRACT

Following a primary sorting in the industrially exploited process developed for vermicomposting of household waste the matter is subject to sanitization by a spontaneous moderate heating to about 70 °C aerobic composting, just before the vermicomposting operation. The ventilation system collects all gases and water vapor within the composting facility and exhausts them to the biofilter, for eliminating odours objectionable. The biofilter is simple to install as well as operate, requires little maintenance and has low energy requirements. Unlike others forms of odour control, biofilters adsorb, hydrolyse and oxidize odours in one process by simple biodegradation. Waste gases collected from the composting facility are passed through a perforated piping system located beneath the filter bed and are distributed evenly throughout the filter material. Odorous compounds within the waste gas are adsorbed into water droplets and then oxidized by microbes present within the water droplets. The high concentration of oxygen in the waste gas ensures that the biofilter operates continually under aerobic conditions. The end products of the oxidation process that are released to the atmosphere are carbon dioxide and water vapour, which are safe and odorless compounds. Biofilter has been pilot tested in the laboratory and shown to work under many different conditions in the vermicomposting plant. The data showed the effectiveness and the advantages of this odour control method.

Keywords: Vermicomposting, solid state fermentation, household waste, sanitization, offensive odours, biofilter, microorganisms, flow rate, residence time, biodegradation rate, absorption, adsorption, oxidation, effluent.

RESUME

Réalisation pratique d'un biofiltre dans une usine de compostage/lombricompostage. Difficultés et solutions.

VINCENT, N., BOUCHE M. B.

Après un tri primaire des déchets urbains, traités dans une usine de lombricompostage, subissent une hygiénisation par compostage aérobie vers 70°C puis sont lombricompostés. Au cours du compostage thermophile un système d'aération aspire l'air chargé de gaz et vapeur d'eau et le refoule dans un biofiltre pour éliminer les odeurs nauséabondes. Le biofiltre, d'installation et d'usage simple, ne nécessite qu'une maintenance et énergie réduites. A la différence des autres procédés de désodorisation le biofiltre absorbe, hydrolyse et oxyde les composés malodorants en une opération. Les gaz collectés depuis le compostage sont injectés par des tubulures perforées dessous la couche biofiltrante et traverse l'ensemble de celle-ci. Les composés odorants sont absorbés dans la phase acqueuse du filtre et biodégradés par les microorganismes qui y prospèrent. La richesse en oxygène de l'air traité assure une aérobiose et les produits finaux libérés par le procédé sont du gaz carbonique et de la vapeur d'eau, sans risques ni odeurs. La fiabilité du biofiltre a été éprouvée et maîtrisée en pilote puis pratiquée dans différentes conditions à l'échelle industrielle. Les résultats illustrent l'efficacité, les exigeances et les avantages de ce procédé de désodorisation.

Mots clés : Lombricompostage, fermentation en milieu solide, ordure, hygiénisation, odeurs nauséabondes, biofiltre, microorganismes, débit, temps de résidence, efficience de biodégradation, absorption, oxydation, effluent.

INTRODUCTION

Among air pollutions, odours are the less tolerated by the neighbourhood of the source. Noxious smell elements are also potentially toxic, though the odour is usually perceived at small levels and does not create real harmful effects. The molecules of the odour are diverse and there're only a few reports available underlying each activity, the quality and levels of the odour. The treatment of refuse by vermicomposting, developed by SOVADEC Technologies, requires a phase of aerobic fermentation of the organic waste which generates a noxious smell. Numerous techniques are available to treat odours and these involve the gas transfer

in liquid with or without transformation of the product by oxidation or acid-base reactions, (Arnold, 1974 ; Faujour *et al*, 1979 ; Jarosz, 1979, 1980 ; Langlais, 1983 ; Caillet, 1984).

In addition, gas-solids transfer, based on adsorption phenomena, are widely used. Active coal is the most used material for its capacities of adsorption and regeneration (Tarrada and Boki, 1979 ; Perret, 1983 ; Koe and Seah, 1987). New processes involving biological techniques.are also known (Martin, 1984 ; Dalouche, *et al*, 1987).

The objectives of this study were to evaluate for a first time the levels of the odours, the different concentrations of noxious elements in the bisanitization building and choose the best available solution. The studies in the pilot unit allowed selection and determination of the performances on an industrial scale.

THE PRINCIPLES OF BIOFILTERS

Biofiltering is a process of purification of a noxious gas using biological filters. It is based on two phenomena: physical-chemical transfers (absorption-adsorption), and a biodegradation of noxious components by the micro-organisms present in the biological support.

The suction of the gaseous effluent from the fermentation unit is ensured by a ventilation system. The air is then forced under the mass of the biofilters. The scheme of this installation in shown in Fig. 1. Odorous compounds within the waste gas are adsorbed into water droplets and then oxidized by microbes present within the water droplets. The high concentration of oxygen in the waste gas ensures that the biofilter operates continually under aerobic conditions. The end products of the oxidation process released to the atmosphere are carbon dioxide and water vapour, which are safe and odourless compounds.

MATERIAL AND METHOD

BRIEF DESCRIPTION OF THE UNIT

The Sovadec unit in La Voulte, France, treats on an average of 30 tonnes of refuse per day. The chain of operations starts when the mixed-waste is poured into a pit,

picked up by a grab to sorting chain, transfered where the bags are opened by thermofusion and sorted based on size using the autoselector (R).

The material of less than 160 mm in size is led towards the bisanitazation unit. The continued displacement of the matter, aeration and water sprinkling accelerate the fermentation. The temperature in the matter reaches 70 °C, and ensures destruction of most of the pathogenic germs.

The gaseous effluent coming from the ventilation of the fermentation unit has a flow rate of 10 000 m^3/h and a temperature between 20 and 40 °C. The gas is saturated with water and its composition is given in Table 1.

After 36 days, the matter cools down and undergoes changes to facilitate the action of the worms. The matter is then fed to the worms in a building where aeration and temperature are controlled to allow the best vermicomposting conditions. The lombricubator (R) is a high tower shapeed unit, in which the worms digest the organic content of the refuse. Two months after its introduction, the compost iscollected, dried and stored, before commercialization.

THE PILOT UNIT

The composition of the effluent (Le Cloirec *et al*, 1989),led to selection of the process using biological filters, because most components of noxious gas are biodegradable. The study was carried on in two stages, a) first on a small pilot unit to determine the time required for the purification of the gas and optimization of all the parameters to develop an industrial scale unit, and b) the work under many different conditions in a vermicomposting plant. Data demonstrate the effectiveness and advantages of this odour control method.

DIMENSIONS

The size of the equipment for air filtration depends on three parameters, i. e., the quantity of the airflow to be purified, the nature of the noxious elements to be eliminated, and the concentration of the pollutants in the gas

The components of the odours are numerous and a precise identification of each is difficult (Le Cloirec *et al*, 1988 ; Bouscaren, 1984). It involves aliphatic organic components, sulfurous or nitrogenous inorganic or organic components and aromatic cyclic compounds. The concentration varies according to the temperature, the speed of ventilation, and the quantity of air renewed from the building.

RESULTS

EVALUATION OF THE GASEOUS EFFLUENT : NATURE OF THE ELEMENTS PRODUCING THE SMELL

A first measurement was made on the exhaust gas of the bisanitization unit, by utilizing a suction pump for sampling, with the measuring tubes, indicating a specific colour for the gas to be analyzed. As expected for this type of gas, the gaseous effluent contains low levels of ammonia and sulfurous elements, like H_2S or mercaptans (Table 1). On the other hand, a large amount of volatile acidic, aldehydic or ketonic components, as well as ethanol were encountered.

Table 1 : Main malodorous compounds of the gaseous effluent

Compounds	Concentration (mg/m^3)
Ammonia	0.5
Mercaptans	0.3
Hydrogen sulphide	1
Amines	45
Ketones and Alcohols	25
Organic acids	< 0.1

DETERMINATION OF AIRFLOW TO BE PURIFIED

The amount of polluted air is evaluated by the consumption of oxygen during the degradation of the organic matter and the control of an oxygen level in the building to 18% (in comparison to 21% outside). A security factor to lower the concentration of pollutants and ensure safe atmosphere and slight underpressure in the building was taken into account. Therefore, the effluent coming from the ventilation was calculated to have a flow rate of 10 000 m^3/h.

THE CENTRIFUGAL VENTILATOR

It was designed to have the capacity to extract a fixed airflow (10 000 m^3/h) and ensure a 20 cm of water column pressure (loss of pressure proportional to the height of the filters+loss of pressure due to the length of the pipe).

THE BIOFILTER

It consists of thermally isolated containers with a lateral way-in, without constriction, and a structural body inside to limit the phenomenon of metal dilatation. These are filled with a light organic material, either turf, or humidified (50-80 %) vermicompost.(Martin *et al*, 1989 ; Dalouche *et al*, 1989). The air pipes need be preserved underground between the building and the biofilter.

RESIDENCE TIME

For the gas to be purified, the time of contact between the air and the biofilter should be around 40 seconds, when the velocity of the air is considered to be 90 m/h (0,025 m/s). The biological reactions, being slower than the chemical ones, require relatively longer treatment in the bioelement. Thus, the installations required are fairly larger.

THE SURFACE OF THE FILTER

The computation of the surface was made in relation to the airflow treated (Q) at the surface of the filters (S) :

$Q = SUo$ Uo : travelling speed of the air

$S = Q/Uo = 10\,000/90 = 111\ m^2$

THE HEIGHT OF THE FILTER

The height of the filtering element was estimated to be 80 cm, in order to avoid the compression and the agglomeration of the organic material and to guarantee a constant duration of the treatment of the gas.

THE DISTRIBUTOR

The distributer is an essential element of the filtering process. Its role is to distribute the gas as evenly as possible, throughout the organic material, and support this organic material.

Studies on perforated panels showed a number of limitations such as a) clogging of the panel by the organic material, b) poor distribution of the air and self-selection of preferred airways, c) condensation of the hot and humid air on the panel; the air becomes saturated with water and its temperature is between 20 and 40°C, and d) a risk of freezing in the winter.

For these reasons, a porous environment, such as a layer of pozzuolana of 15 cm, placed on a panel with holes of 5 to 10 mm and below the organic material, was chosen. This system avoids the clogging of the panel, the high humidification of the filter and eliminates the risk of frost.

THE CONDENSER

To avoid the condensation of water in the biofilter, a condenser was installed to dry the air from the bisanitization unit. Some of the noxious molecules soluble in water could also be eliminated and the dimensions of the filter reduced.

THE COST

The cost of an installation (material and maintenance costs) of this biofilter is much less than that of other purification techniques. A study conducted by SAPS Anticorrosion Society (Le Cloarec et al, 1988) gives a comparison between the biofiltering and other techniques of air purification (Table 2).

Table 2. Comparison of biofilters and other odour control methods.

Process	Catalytic oxidation	Thermal oxidation	Carbon sorption	Chemical scrubbing	Biofilters
Material costs (kF)	805	760	175	322	300
Maintenance costs for year(kF)	500	521	730	280	110
Removal rate for ammonia (%)	92	98	>95	>99	>95
Removal rate for amines (%)	98	99,9	>99	>99	>99
Removal rate for sulphur products (%)	96 à 99	99	98	95 à 99	>99

CONCLUSION

This study allowed to clarify the following points on the level of gaseous effluents from a composting unit (the bisanitization), as well as the efficiency of the biofilter to purify the air from its noxious elements. The gas contained alcohol in large proportion, aldehydes and amines. The mercaptans or hydrogen sulphide are present in small quantities. For the actual treatment, a biofilter gives efficient results, but this efficiency decreased quickly because the organic matter was clogged by water condensation. A condenser was introduced on the pipe in the filter, to prevent these phenomena. It allowed the elimination of a large part of the soluble molecules by absorption-condensation. This in turn, allows to increase the life-span of the biofilter, avoids the problems of condensation in the distributor and reduces the size of the installation. The biofiltering is not a new technique but is rarely installed properly. The strong point in the present case is to put together an efficient, low-cost system which is easily adaptable to the requirements.

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A phenomenological model for solid state fermentation of fungal mycelial growth

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SUMMARY

A phenomenological model for the growth of *Aspergillus niger* on inert support in the packed bed is proposed and verified. The model deals with variables such as biomass, sugar, water, oxygen, carbon dioxide and bulk temperature. It is derived from mass and energy balance for each constituent and the kinetic term. To fit experimental results, saturation (Ks, 3.0 kg substrate m^{-3} packed), inhibition constant (Ki, 300 kg substrate m^{-3} packed) and maintenance coefficient of 3.0 E-06 kg substrate (kg wet biomass · s)$^{-1}$, were used.

Keywords: Solid state fermentation, inert support, packed bed, *Aspergillus niger*, phenomenological model growth, model verification.

RESUME

Un modèle phénoménologique de croissance mycéliale de champignons en fermentation en milieu solide

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Un modèle phénoménologique pour la croissance d'*Aspergillus niger* cultivé sur support inerte a été proposé et vérifié. Le modèle intègre 6 variables d'état : la biomasse, les sucres, l'eau, l'oxygène, le dioxyde de carbone et la température. Ce modèle a été construit à partir des bilans de masse et d'énergie de chaque constituant et des paramètres cinétiques. Pour ajuster le modèle aux résultats expérimentaux, nous avons, après une étude de sensibilité paramétrique, défini la constante de saturation du type Monod (Ks, 3,0 kg substrat m^{-3} compacté), une constante d'inhibition (Ki, 300 kg substrat m^{-3} compacté) et du coefficient de maintenance de 3.0 E-06 kg de substrat (kg de biomasse sèche s)-1.

Mots clés: Fermentation en milieu solide, support inerte, *Aspergillus niger*, modèle phénoménologique de croissance, vérification du modèle.

INTRODUCTION

Solid state fermentation (SSF) has received increased attention since a wide variety of compounds (Hang and Woodams, 1987; Barrios-González *et al,* 1993) are efficiently produced. Despite the potential of SSF systems, some physical aspects related to the heterogeneity of the medium became a serious constraint. Problems derived from heterogeneity include: heat (Saucedo-Castañeda *et al,* 1990) and mass (Lonsane *et al,* 1992) transfer limitations, and the accurate measurement of key variables. Physical variables such as bulk and air inlet temperatures (Sargantinis *et al*, 1993), water (Hang and Woodams, 1987) and void space availability (Auria *et al*, 1991) have a strong effect on the physiological and biochemical activities of the microorganisms used and over the global effectiveness. Mathematical modeling for SSF involving these variables is scarce in literature. In SSF, two kind of mathematical modeling efforts can be identified, a) models dealing with microscopic surface growth (Molin *et al*, 1993; Viniegra-González *et al*, 1993), and

b), those dealing with macroscopic variables (Saucedo-Castañeda *et al,* 1990; Auria *et al,* 1991; Sargantinis *et al,* 1993). In the former case, physiological and biochemical research is performed, and the latter models are rather oriented to the physical response. As a consequence, the net physical and biochemical interactions involving these variables are not well understood in practice. Thus, appropriate models dealing with both microscopic and macroscopic observations are required. The objective of this work was to develop a mathematical model to simulate mycelial growth, substrate depletion, water availability, oxygen consumption, carbon dioxide production, and bulk temperature profiles.

THEORETICAL ASPECTS

The SSF system is composed of a porous heterogeneous inert support, in which culture media and inoculum are absorbed. Cell growth takes place under controlled aerated conditions within small packed columns, immersed in a temperature constant water bath. An elementary representative volume (ERV) is chosen as an appropriate size for mathematical modeling. The ERV represents the mean conceptual size in which any local accurate measurements (nutrients, biomass, temperature, etc.) can be made. It is composed of four phases and eight constituents, i. e., i) solid (support), ii). biological (microorganisms), iii). liquid (water, and carbon source), and iv). gas (nitrogen, oxygen, carbon dioxide and water vapor). The interaction among phases and constituents can be summarized as: 1. The inert support is a rigid spherical matrix internally saturated with the liquid phase, the gas phase is considered to be absent. 2. Packed void spaces are filled with gas phase (no liquid phase is found). The gas constituents flow-up at the same phenomenological mean velocity. Biological phase only colonizes interparticular spaces. 3. Microbial growth is only superficial and does not take place inside the support. It depends on: (i) the local temperature, (ii) the concentration of micro-organisms, (iii) the availability of void spaces, and (iv) the growth-limiting nutrient, which at high concentrations causes growth inhibition. Delay due to the lag and product formation is not considered. 4. Oxygen consumption, carbon dioxide production and net water production depend on cell growth rates. 5. Thermal equilibrium among the four phases is considered. The state variables are expressed in kg per cubic meter of packed wet material.

MODEL DEVELOPMENT

General nomenclature is given in previous work (Gutiérrez-Rojas *et al*, 1995) and when not given it is specified in the text. The derived equations from mass and energy balances for each constituent and phase and the associated kinetic terms are the following:

- **Solid phase.** Assuming that support concentration is constant, thus $\rho_a = \rho_a^0 =$ initial dry support.
- **Biological phase.** The mass balance for biomass is given by:

$$\frac{\partial \rho_x}{\partial t} = C_x \tag{1}$$

where C_x is the biomass production rate per packed volume and, according to the assumptions, is given by:

$$C_x = C_{xMax}\,\rho_x \left(1 - \frac{\rho_x}{\rho_{xMax}}\right)\left(\frac{\rho_s}{K_s + \frac{\rho_s^2}{K_i} + \rho_s}\right) \tag{2}$$

the second right member is a logistic type relation (Okasaki *et al*, 1980), and the third right side term is a typical substrate dependence in which an inhibition constant (Ki) is included. C_{xMax} involves the temperature dependence by means of (Saucedo-Castañeda *et al*, 1990):

$$C_{xMax} = \left(\frac{A_1 \exp\left(-\frac{E_{a1}}{RT}\right)}{1 + A_2 \exp\left(-\frac{E_{a2}}{RT}\right)}\right) \tag{3}$$

ρxMax is obtained by taking into consideration the initial void space (ε_0), the available surface for cell growth and the maximum cell growth expected without steric hindrance conditions (R_{cp}), by means of:

$$\rho_{xMax} = \left(\frac{6(1-\varepsilon_0)}{1-\phi}\right)\left(\frac{Rcp}{\Phi}\right) \tag{4}$$

• **Liquid phase**. Considering two key species (sugar and water), the mass balances are:

Sugar: $$\frac{\partial \rho_s}{\partial t} = -C_s \tag{5}$$

where C_S, including maintenance, is given by:

$$C_s = \left(\frac{1-\phi}{Y_s}\right) C_x + m_s \rho_x \tag{6}$$

where: Y_S is the growth yield related to sugar conversion; ø is water content in biomass, and m_S, the maintenance coefficient.

Water: $$\frac{\partial \rho_e}{\partial t} = C_e \tag{7}$$

where C_e includes production and consumption terms, as follow:

$$C_e = \left(\frac{(1-\phi)}{Y_e} - \phi\right) C_x \tag{8}$$

• **Gas phase**: A non-reactive constituent is water vapor (ρ_v) and it changes due to the local temperature variations:

$$\rho_v = (\rho_N + \rho_o + \rho_c)\left(\frac{PMe\, p_e^*}{PMair(P - p_e^*)}\right) \tag{9}$$

where p_e^*, is the water vapor pressure estimated from the following thermodynamic expression:

$$p_e^* = \exp\left(25775 - \frac{5281}{T}\right) \tag{10}$$

Oxygen: $$\frac{\partial \rho_o}{\partial t} = -v_g \frac{\partial \rho_o}{\partial z} - C_o \tag{11}$$

C_O is the reaction term which includes maintenance oxygen consumption:

$$C_o = \left(\frac{1-\phi}{Y_o}\right) C_x + m_o \rho_x \tag{12}$$

Carbon dioxide: $$\frac{\partial \rho_c}{\partial t} = -v_g \frac{\partial \rho_c}{\partial z} + C_c \tag{13}$$

and, as with oxygen:

$$C_c = \left(\frac{1-\phi}{Y_c}\right) C_x + Y_p m_s \rho_x \tag{14}$$

• **Local temperature**. Temperature changes in the bulk (T) are due to convection, conduction and internal metabolic heat:

$$\rho_i c_i \frac{\partial T}{\partial t} = -\rho_g c_g v_g \left(\frac{\partial T}{\partial z}\right) - \lambda\left(\frac{1}{r}\frac{\partial T}{\partial r} + \frac{\partial^2 T}{\partial r^2}\right) - C_i h_i \tag{15}$$

λ is the effective thermal conductivity and h_i is the heat of formation of species i. The whole equation set is transformed into a dimensionless form by introducing the following variables:

$$\rho_x = \frac{\rho_x}{\rho_{xMax}} \qquad \rho_e = \frac{\rho_e}{\rho_e^0} \qquad \rho_o = \frac{\rho_o}{\rho_o^0}$$

$$\rho_s = \frac{\rho_s}{\rho_s^0} \qquad \rho_c = \frac{\rho_c}{\rho_{cMax}} \qquad T = \frac{T - Tp}{Tin - Tp}$$

$$t = \frac{t}{t_f} \qquad z = \frac{z}{L} \qquad \text{and} \qquad r = \frac{r}{Ra}$$

where superscript ^{0} denotes initial mass concentration, L is the reactor height and Ra is the reactor radius.

MATERIALS AND METHODS

Micro-organism, support and culture media, the experimental setup and analytical methods were recently reported (Gutiérrez-Rojas *et al*, 1995). Thermal conductivity was measured in packed columns and a constant value of 0.06 J (m s K)$^{-1}$ was observed. Values for required parameters and initial condition are the same as in previous work (Gutiérrez-Rojas *et al*, 1995). To achieve numerical simulations, a set of twelve procedures in Turbo Pascal 6.0 for a PC-compatible computer were developed. The model was solved by using the finite differences method. The strategy was explicit in time (Δt = 1.0 s), explicit through the axial ordinate (20 nodes were used), and implicit through the radial ordinate (5 nodes). To predict results, a 0.1 m diameter and 0.25 m height reactor (gas phase mean velocity was 0.015 m s^{-1}, initial sucrose concentration, 55 kg m^{-3}; air and water bath temperature, 30°C) was simulated.

RESULTS AND DISCUSSION

Experimental measurements and simulated results for biomass production and sugar consumption are compared in Figs. 1a, b, respectively. In both cases, a good fit with experimental results was obtained. Simulated approach was performed in the centre and at different reactor heights. At a portion close to the reactor wall, no differences, neither in biomass nor in sugar concentration, through the axial coordinate were detected. This may be explained because temperature was constant outside the wall.

Enhanced cell growth and substrate consumption were predicted at the top of the column.

A pattern similar to that of biomass and substrate was observed for void space occupation and water consumption. Major differences, in the center, between top and bottom did not exceed 2%. A considerable amount of remaining water at the end of fermentation is predicted (from 88 to 92% of initial water), thereby suggesting that the water is always available for cell growth.

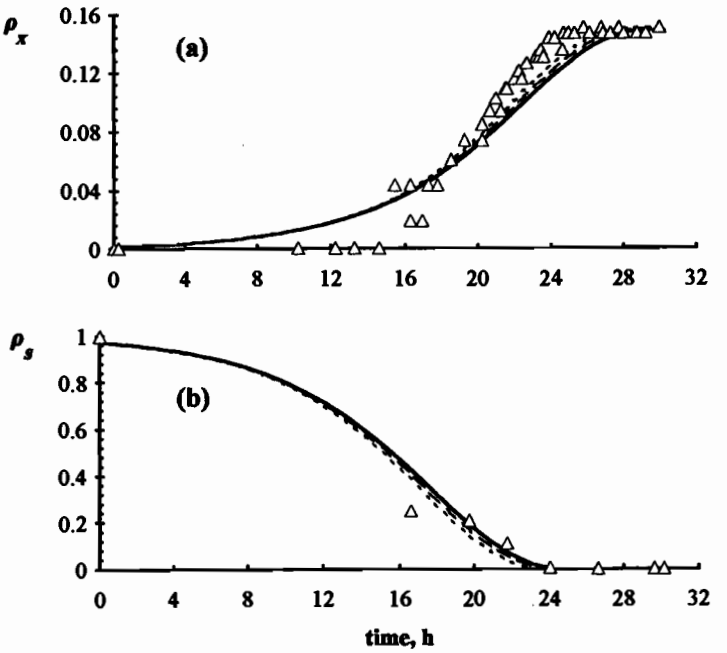

Figure 1. Experimental (Δ) and simulated (a) biomass production as well as (b) sugar consumption in the center and at different reactor heights: (——) 0 m, (— —) 0.125 m and (------) 0.25 m.

Simulated results of oxygen consumption and carbon dioxide production are shown in Figs. 2a,b , respectively. The model exhibits increasing by bristly axial profiles, especially at the top of the reactor.

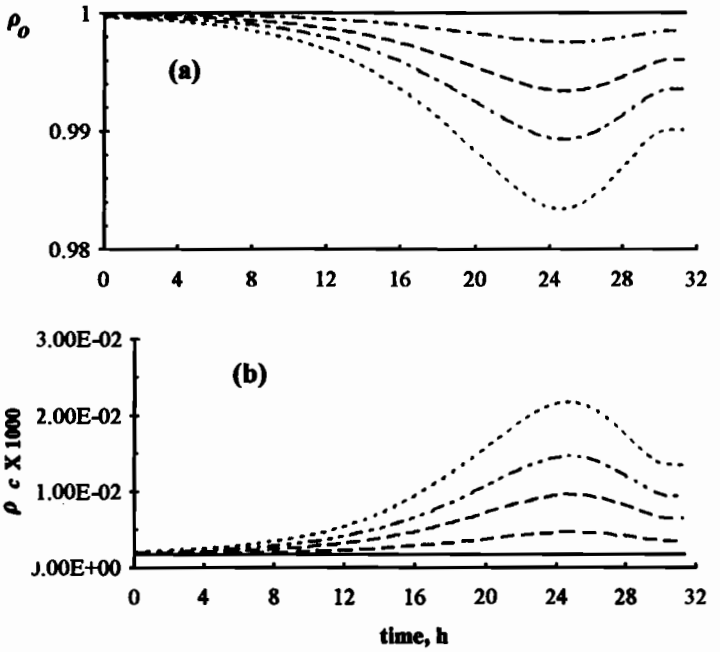

Figure 2. Simulation of (a) oxygen consumption and (b) carbon dioxide production *vs.* time.in the center and at different reactor height. 0 m; (—— - ——), 0.037 m; (— —), 0.125 m; (— - - —), 0.21 m and (-------), 0.25 m.

Experimental data for oxygen consumption rate (C_O) as a function of time are shown in Fig. 3a, along with model predictions. A good agreement is observed between theoretical and experimental values. Equivalent profiles for carbon dioxide production rate (C_C) are shown in Fig. 3b.

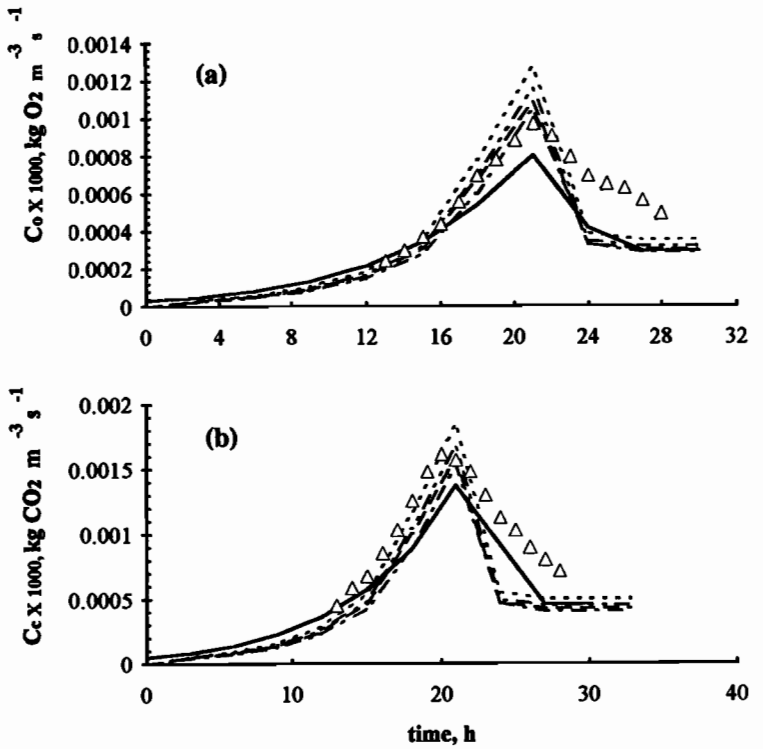

Figure 3. Experimental (Δ) and simulated results (a) of the oxygen consumption rate and (b) carbon dioxide production rate in the center and at different reactor heights. (——), 0 m; (—— - ——), 0.037 m; (—— —), 0.125 m; (—— - - ——), 0.21 m and (------), 0.25 m.

Simulated temperatures profiles are shown in Fig. 4. A sharp-type characteristic profile is obtained and no more than 2.5°C increase was predicted as maximal temperature raised within the packed columns.

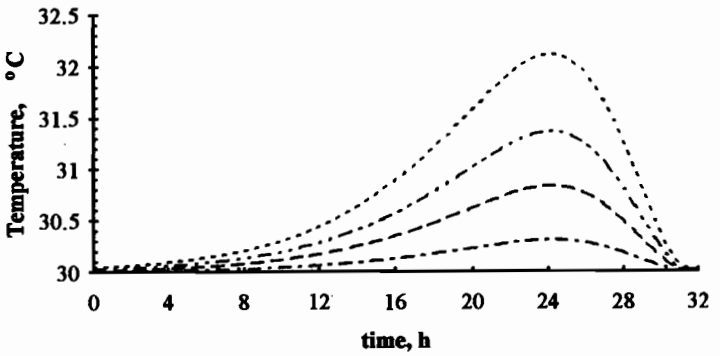

Figure 4. Simulation of temperature *vs.* time in the center and at different reactor heights. (——) 0 m; (— - —) 0.037 m; (— —), 0.125 m; (— - - —), 0.21 m and (------), 0.25 m.

The model presented here provides a good quantitative understanding of the role of common kinetic parameters in solid state fermentation.

ACKNOWLEDGMENTS

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Models for solid-state cultivation of *Rhizopus oligosporus*

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ABSTRACT

Physical and mathematical models for solid state fermentation were developed, using cultivation of *Rhizopus oligosporus* on soy beans as an example. A simple mathematical model was developed which predicts the dynamic behaviour of an adiabatic homogeneous packed-bed reactor accurately during the first 40 hours. Biomass measurements and experimental verification of the substrate composition are required to improve model validation. After 40 hours, the simple kinetic model gives very poor predictions. Axial gradients and bed shrinkage make the packed bed with soy beans an unsatisfactory physical model. Based on the problems encountered, the experimental set-up has been modified: agar beads with an oleic acid emulsion are a suitable model substrate; a scraped-drum reactor offers better perspectives than the packed bed for studies of solid-state fermentation.

Keywords : Solid state fermentation, physical models, mathematical models, *Rhizopus oligosporus*, soy beans, packed-bed reactor, agar beads, scraped-drum reactor.

RESUME

Modèles pour la culture en milieu solide de *Rhizopus oligosporus*

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A partir de l'exemple de la culture de *Rhizopus oligosporus* sur grains de soja nous avons développé des modèles physiques et mathématiques de la fermentation solide. Un modèle mathématique simple a été proposé pour décrire le comportement dynamique d'un réacteur homogène, adiabatique à lit compact pendant les premières 40 heures de culture. Des mesures de production de biomasse et des vérifications expérimentales ont été nécéssaires pour valider et afiner le modèle. Après 40 heures, le modèle cinétique simple donne des informations médiocres. Le lit compacté de graines de soja est un modèle physique peu satisfaisant à cause des gradients axiaux et du rétrécissement du lit. Suite à ces problèmes, nous avons modifié les conditions expérimentales : le substrat modèle sélectionné est une émulsion de billes d'agar et d'acide oléique et le réacteur choisi est équipé d'un tambour à racloir. Ce type de réacteur offre de plus larges pespectives pour l'étude de la fermentation solide que celui à lit compacté.

Mots clés : Fermentation en milieu liquide, modèles physiques, modèles mathematiques, *Rhizopus oligosporus*, graines de soja, réacteur à lit compacté, billes d'agar, réacteur de tambour.

INTRODUCTION

Solid-state fermentations have been used for ages in Asia and Africa for production of fermented foods, starter cultures, enzymes, etc. (Lonsane *et al*, 1992). Despite their long history, the design and scale-up of these systems is still more an art than a technology. Several models combining black-box descriptions of process kinetics with physical transport phenomena have been published recently, but their use is hampered by erroneous assumptions and lack of independently determined parameter values or poor validation (Saucedo-Castañeda *et al*, 1990; Raghava Rao *et al*, 1993; Sargantanis *et al.*, 1993; Rajagopalan and Modak, 1994).

This paper describes the work on modelling of the dynamic behaviour of an adiabatic packed-bed bioreactor. Axial gradients in the bed were minimised by off-gas recirculation, in order to reduce the complexity of the experimental set-up and the model. Growth of *Rhizopus oligosporus* on soy beans was used as model fermentation. Our aim was not to address all scale-up problems involved in packed-bed SSF systems, but to predict the behaviour of a simple physical model with a simple mathematical model based on independently determined kinetics and conservation laws, using only on-line temperature and CO_2 measurements. Based on the problems encountered, the experimental set-up has been modified. The first results obtained with the new set-up are reported.

MATERIALS AND METHODS

MICROORGANISM, SUBSTRATE AND DEFINED MEDIA.

Sporangiospore suspensions of *Rhizopus oligosporus* NRRL 5905 were prepared as described previously (De Reu *et al*, 1993). Yellow seeded soy beans (*Glycine max*) were prepared as described previously (De Reu *et al*, 1993). Agar solutions containing either glucose or oleic acid as sole carbon and energy source (Table 1) were sterilised (121°C, 20 min.) and allowed to solidify in layers (glucose medium) or beads (4-6 mm diameter, oleic acid medium).

INCUBATIONS

Soy beans or agar media were incubated isothermally in closed serum flasks or small aerated packed columns (Raimbault and Alazard, 1980), or in the adiabatic packed-bed or scraped-drum reactor. All flasks and bioreactors were sterilised (121°C, 20 min.) before they were filled with inoculated soy beans or agar.

Table 1. Composition of defined media (g/dm^3)

Compound	Glucose medium	Oleic acid medium
Glucose ($C_6H_{12}O_6.H_2O$)	30	0
Oleic acid	0	40
Tween 80	0	1
Gibco Bacteriological Agar	15	0
Oxoid Technical Agar	0	30
$ZnSO_4.7H_2O$	1	1
$MgSO_4.7H_2O$	1	1
$(NH_4)_2SO_4$	7.5	20
Urea	2.5	7
KH_2PO_4	1	1
K_2HPO_4	1	1
KCl	1.5	0
pH	7.0	7.0

The spore suspension was either mixed through the liquid agar medium (40°C, all experiments with glucose except one, see results) or evenly distributed on the soy beans or the agar surface (one experiment with glucose, all experiments with oleic acid). All manipulations took place in a laminar flow cabinet.

ANALYSES

CO_2 and O_2 in serum flasks were determined by GC. Biomass dry matter was determined gravimetrically or by Kjeldahl nitrogen analysis. Agar samples were melted and filtered over Schleicher and Schuell filters (520B). Filters were rinsed with boiling water and dried at 80 °C for 48 hours or subjected to Kjeldahl analysis. Dry matter was corrected for agar retained on the filter. Kjeldahl nitrogen was determined using Gerhardt equipment and Thompson and Capper, Ltd. special Kjeldahl tabs No. 4. The respiration quotient was determined in closed serum flasks and calculated from the cumulative amounts of CO_2 produced and O_2 consumed from the start of the incubation.

PACKED-BED REACTOR (PBR)

The PBR set-up is shown in Figure 1. A glass cylinder (internal diameter 0.06 m, height 0.26 m) with stainless steel flanges was used. The packed bed (0.1 kg wet beans) was supported by a wire mesh. Two Pt-100Ω sensors were introduced in the bed and one in the off-gas above the bed. The PBR was located in an incubator; the incubator setpoint temperature was maintained at 30°C initially and at 0.1°C below the measured temperature of the PBR as soon as this exceeded 30°C. Fresh air was introduced through a sterile membrane filter; the flow rate (0.42 dm^3/min) was controlled with a mass flow controller. The fresh air was saturated with water vapour at 30°C in a humidifier with independent temperature control. A membrane pump recycled off-gas to the bottom of the bed (2 dm^3/min). CO_2 in the off-gas was measured on-line using an infrared analyser.

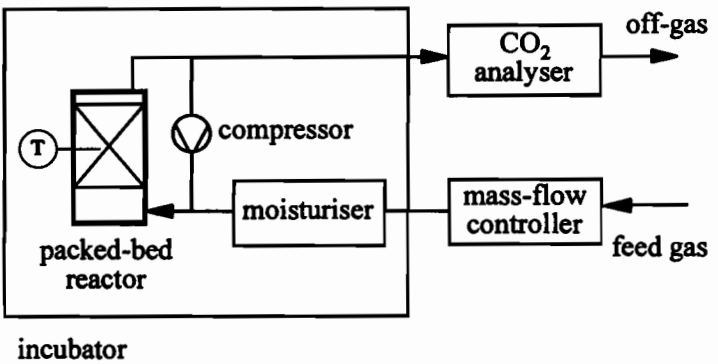

Figure 1. Experimental set-up of the adiabatic packed-bed reactor with off-gas recycle.

SCRAPED-DRUM REACTOR (SDR)

A glass cylinder (internal diameter 0.176 m, length 0.44 m) with stainless steel flanges, equipped with a rotating scraper (not operated during the first 12 hours, thereafter rotation frequency 0.02 Hz) was used in the same set-up, as described for the PBR. The SDR was aerated (1 dm^3/min) through the hollow scraper. Agar beads (335 g wet weight) containing oleic acid medium were used as model substrate. Two Pt-100Ω sensors were introduced in the bed.

RESULTS AND DISCUSSION

SOY BEAN FERMENTATION IN AN ADIABATIC PBR

The main results of our work on cultivation of *R. oligosporus* on soy beans are summarised here; details are described elsewhere (De Reu, 1995; De Reu *et al*, submitted). The effect of temperature on the specific growth rate of *R. oligosporus* was determined by measuring CO_2 accumulation in serum flasks (Figure 2). The very steep decline in specific growth rate above 40°C indicates that severe temperature control problems may occur during SSF.

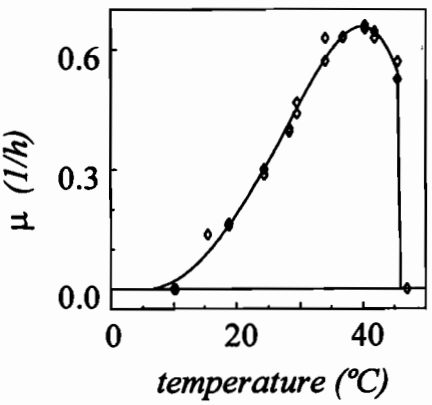

Figure 2. Effect of temperature on specific growth rate of *R. oligosporus* growing on soy beans.

Using these independent measurements of the specific growth rate, mass and enthalpy conservation laws, elemental balances, and empirical relations for the saturated water vapour pressure, a mathematical model for the packed bed reactor was developed. The major assumptions underlying this model are: (1) growth of the fungus is only limited by temperature, (2) the PBR is adiabatic and homogeneous, (3) the off-gas is at equilibrium with the packed bed, (4) lipids are the sole carbon and energy source, NH_3 liberated by proteolytic enzymes is the nitrogen source, biomass, CO_2 and H_2O are the only fermentation products, sulphur and phosphorous are neglected, biomass composition for *R. oligosporus* equals that reported by Sargantanis *et al* (1993), (5) the true yield is $Y_{X/S} = 0.65$ Cmol biomass

per Cmol substrate (Roels, 1983), (6) the maintenance requirements of the fungus and heat capacities and densities of all materials are independent of temperature.

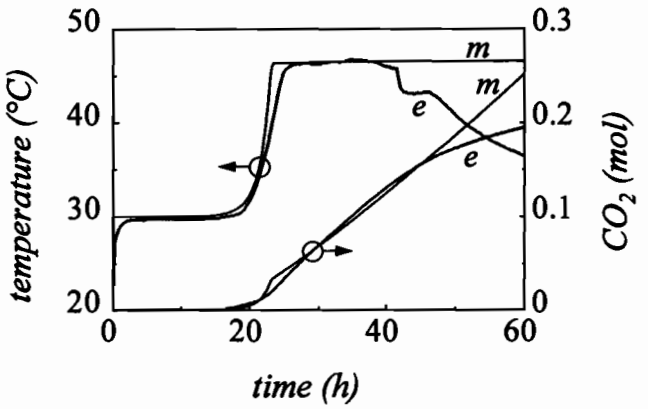

Figure 3. Comparison of measured and predicted temperature and CO_2 development in the PBR (*e* : experimental, *m* : model).

Figure 3 compares the measured and predicted values of the cumulative CO_2 production and the temperature in the centre of the packed bed. After 24 hours, the PBR temperature approached the maximum level that allows growth (Figure 2). Model predictions are accurate during the first 40 hours.

The predictions of CO_2 production are strongly affected by the chosen substrate composition, but the temperature prediction is hardly affected (results not shown). *R. oligosporus* uses lipids during growth on soy beans (Paredes-Lopez et al, 1987; Nout and Rombouts, 1990; De Reu et al, 1994), but we have not yet verified that this is the only substrate. Measurement of O_2 in the off-gas may alleviate the uncertainty about the stoichiometry, but this is extremely difficult because of the high gas flow rate required for cooling.

The point in time, where temperature and CO_2 increase is determined by the initial amount of biomass, was estimated by fitting. In this experiment, the estimated value (2×10^{-6} Cmol) agreed reasonably with four independent measurements of dry weight in a spore suspension ($(4.6 \pm 0.5) \times 10^{-6}$ Cmol), but this was not the case in all experiments. There was also no clear relation between the estimated initial amount of biomass and viable spore counts. Biomass measurements are required to get better validation of the model.

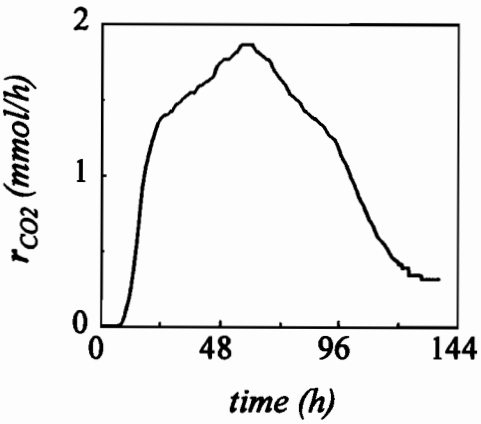

Figure 4. CO_2 production rate during cultivation of *R. oligosporus* on soy beans (29 g wet beans, 30°C).

After about 40 hours, the measured CO_2 production rate and temperature decrease, while the model predicts no such decline. This must be attributed to the very simple kinetic model. During the independent growth rate measurements, only 0.27 mol CO_2 was produced per kg of wet beans, compared to 2 mol/kg in the PBR experiment. A more extensive CO_2 production rate measurement conducted in an isothermal PBR (Raimbault and Alazard, 1980) shows that assumption (1) above is not valid for extended incubations (Figure 4). Probably, substrate, water or space limit fungal growth. The frequently used logistic law also cannot describe the abrupt changes in rate observed after about 24 and 96 hours. More experimental information and a more complex kinetic model will be required to improve model predictions.

Two important complications were observed in the physical model: (1) axial temperature gradients up to 0.5°C/cm occurred, when the bed temperature reached 45°C. Theoretical calculations show that an extremely high recirculation flow rate would be required to decrease these gradients. (2) bed shrinkage occurred after about 24 hours. This causes channelling of the gas, which undermines assumption (3) above.

In order to circumvent experimental problems related to substrate composition, stoichiometry and biomass measurements in the soy bean system, and also to the packed bed, we decided to develop a simpler physical model. Agar beads with defined media using glucose or oleic acid as sole carbon and energy source, and a scraped-drum fermentor with a mixed bed were studied.

DEFINED GLUCOSE MEDIUM

The respiration quotient increased steadily in three experiments with several millimetres thick agar layers, but not in an experiment with an extremely thin (10 μm) layer (Figure 5).

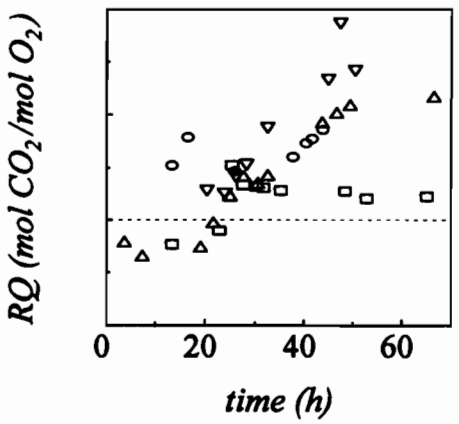

Figure 5. Respiration quotient during cultivation of *R. oligosporus* on agar beads with glucose as sole substrate. Spores distributed in (Δ,∇) and on (m) several millimetres thick agar layers, and in (q) 10 μm thick agar layers.

The ratio of accumulated CO_2 and biomass also increased steadily in the flasks with thick agar layers. This indicates that anaerobic glucose conversion occurred, which was confirmed in an experiment under a 100% nitrogen atmosphere. Consequently, agar beads with a defined glucose medium increase the complexity of the physical model, instead of decreasing it.

DEFINED OLEIC ACID MEDIUM

Figure 6 shows the respiration quotient during incubation of agar beads containing an oleic acid emulsion. The observed values agree reasonably well with the theoretical value (0.515 mol/mol, assuming $Y_{X/S}$ = 0.65 Cmol biomass per Cmol substrate). During these experiments, initially only 0.038 mol O_2 per Cmol oleic acid was present. Fungal metabolism stagnated during 70 hours when the O_2 concentration had dropped to 2-4%. After flushing with air, the O_2 concentration rapidly decreased again to the same level. No anaerobic conversion occurs with oleic acid as a sole carbon and energy source. Therefore, this model system is suitable for further studies.

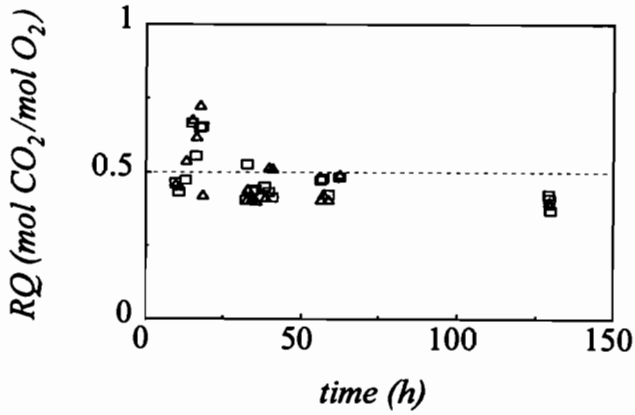

Figure 6. Respiration quotient during cultivation of *R. oligosporus* on agar beads with oleic acid as sole substrate (three experiments).

Results obtained with the SDR are shown in Figure 7. The observed peak temperature agrees with the temperature at which no CO_2 production was detected in independent batch experiments. Biomass production was calculated from Kjeldahl nitrogen, using the biomass composition reported by Sargantanis et al (1993), and from CO_2 production, neglecting maintenance and assuming Yx/s = 0.63 Cmol biomass per Cmol substrate. Both calculations show reasonable agreement, which indicates a closing carbon balance. Consequently, the scraped-drum reactor with oleic-acid/agar-beads offers good perspectives as a physical model to study solid-state fermentation.

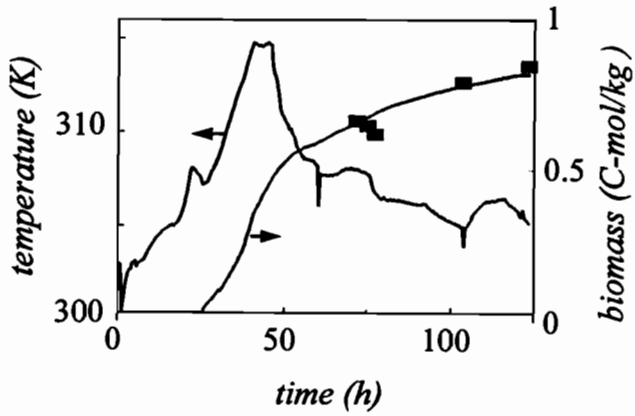

Figure 7. Development of temperature (—) and biomass (calculated from CO_2 production —, from Kjeldahl-nitrogen).

CONCLUSIONS

The simple mathematical model predicts the dynamic behaviour of the adiabatic homogeneous PBR accurately during the first 40 hours. The initial amount of biomass has to be estimated by trial and error for each experiment. Biomass measurements and experimental verification of the substrate composition are required to get better validation of the model. After 40 hours, the simple kinetic model results in very poor predictions. The PBR with soy beans is not fully satisfactory as a physical model, axial temperature gradients cannot be completely suppressed and bed shrinkage causes channelling, which prevents proper equilibration of the off-gas and the bed. Packed bed reactor with soy beans pose experimental problems related to substrate composition, stoichiometry and biomass measurements. These can be overcome by developing a more defined physical model.

R. oligosporus is capable of anaerobic glucose metabolism. Therefore, agar beads with a defined glucose medium increase the complexity of the physical model, instead of decreasing it. No anaerobic conversion occurs with oleic acid as a sole carbon and energy source. Agar with an oleic acid emulsion is a suitable model system. The scraped-drum reactor with oleic-acid/agar-beads offers good perspectives as a physical model to study solid-state fermentation.

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Advances in the development of a control system for a solid substrate pilot bioreactor

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SUMMARY

A preliminary control system for a solid substrate pilot bioreactor is described. The performance of the system in real time experiments is discussed and future improvements are proposed. Reasonable control of temperature and water content of the solid bed were achieved, although the system is not fully automatic and needs human supervision.

keywords : Pilot bioreactor, solid state fermentation, control system, temperature, water content.

RESUME

Mise au point d'un système de contôle pour un bioréacteur pilote de fermantation solide.

FERNANDEZ M, ANANIAS J., SOLAR I., PEREZ R., CHANG L., AGOSIN E.

Nous présentons ici le système préliminaire de contrôle d'un bioréacteur pilote de fermentation solide, d'une capacité de 50 kg. La performance en temps réel du système est discutée et des améliorations futures sont proposées. Bien que nous avons mis au point un système de contrôle de la température et de l'humidité du substrat solide, le système nécéssite encore une surveillance humaine et de ce fait il n'est pas encore entièrement automatisé.

Mots clés: Bioréacteur piulote, fermentation en milieu solide, système de contrôle, température, contenu en eau.

INTRODUCTION

A solid substrate pilot bioreactor of 50 kg nominal capacity has been set up for gibberellins production. The packed bed reactor with periodic agitation (Fig. 1) has been designed to operate aseptically in a semi-batch form for several days. This precludes an attentive manual operation, if both homogeneous quality and reasonable efficiency of the resulting product are desired. Since the cultivation process lasts for several days, it is difficult to keep critical parameters, such as temperature and water content of the bed, constant during night shifts. Thus, it is desirable to facilitate such operation of the equipment and automate it fully.

Control of temperature and water content of the solid bed have been recognized as critical in the operation of solid bed bioreactors (Ramana Murthy *et al,* 1993). The control of both variables is interactive and difficult. Several methods, depending on the type of reactor, have been proposed and experimentally tested. Regarding packed bed reactors, Saucedo-Castañeda *et al.* (1990) used forced air and also increased water content to cool the bed. Since reduction in bed porosity is not dealt with in the

scheme, periodic agitation is necessary. Temperature can also be controlled by discontinuous rotation (agitation) (Reu *et al,* 1993), with the additional advantage of controlling the bed porosity. Sato *et al,* (1982) used wet woody pulp particles, mixed with the solid substrate, to supply moisture for growth of *Aspergillus oryzae.* However, the effective volume of the reactor is reduced. Probably, the most effective way to control bed temperature is by evaporative cooling, experimentally tested by Barstow *et al,* (1988) in a rotary drum bioreactor and later by Ryoo *et al,* (1991) in a novel rocking reactor.

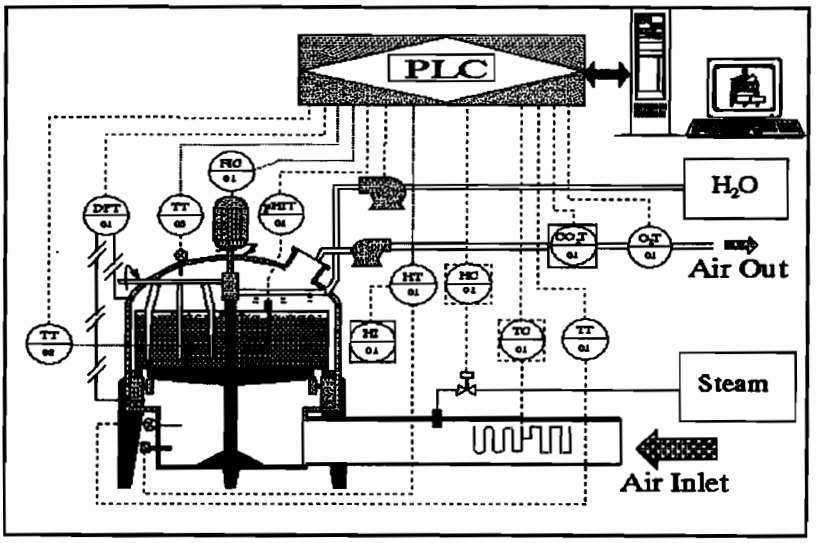

Fig. 1. Process and instrumentation diagram of a solid substrate pilot bioreactor.

In this scheme, relative humidity of inlet air is used to control the amount of evaporation from the bed, thereby, indirectly regulating the bed temperature. Fresh water must be supplied to avoid bed drying. In a packed bed, the rotation or rocking motion must be replaced with discontinuous agitation.

The current state of development of a special purpose control system for a packed bed bioreactor is described in the present paper. The control structure and control logic is described, *i.e.*, the available variables for monitoring and manipulation, the pairings among them and the control algorithms used. The software is briefly described, along with some experimental results, and need for future work is pointed out.

CONTROL STRUCTURE AND LOGIC

Table 1 shows the available measuring instruments. Temperatures can be measured simultaneously at different places inside the bed. This Table also indicates the type of measurement, *i.e.*, on-line or off-line.

Table 1. Available measuring instruments.

Variable	Instrument	Type of measurement
Inlet air temperature	PT100	On-line
Inlet air relative humidity	Vaisala	On-line
Bed temperature	Thermocouple	On-line
Bed pH	Schott pH meter	On-line
Bed water content	Precise balance	Off-line
CO2 in outlet air	IR	On-line
O2 in outlet air	SMC electrochemical	On-line
Air pressure drop	Deflecting diaphragm	On-line

The variables available for manipulation are listed in Table 2.

Table 2. Manipulated variables .

Variable	Control Element	Type of
Heating of inert/outlet air	Electric heater, cooling water	On/off
Addition of water vapour	Solenoid valve	On/off
Bed agitation	Electric motor with variable frequency	On/off, continuous
Addition of fresh water	Peristaltic pump	On/off

The control strategy is based on evaporative cooling (Barstow *et al*, 1988) of the reactor bed, *i.e.*, cascade control through inlet air relative humidity, addition of fresh water to compensate evaporation, and periodic agitation to reduce heterogeneity.

Details of the implemented control loops, which use some of the above variables, are described below:

WATER CONTENT CONTROL

This is a manual control, where periodic off-line measurements (each 90 min) are used to record the amount of water necessary to reach the desired level of water content inside the bed. Fresh water is added through a peristaltic pump, operated through the control software interface. Simultaneously, the agitation is turned on for few min. The added water is intended to compensate evaporation and is calculated by an approximate water balance.

POROSITY AND HOMOGENEITY CONTROL

In order to keep the bed as homogeneous as possible and to avoid excessive interparticular aerial growth, which reduces porosity, a periodic agitation has been employed. The homogeneity is estimated through measurements of water content at different places inside the bed, while the intraparticle aerial growth is estimated by the air pressure loss through the bed, as suggested by Auria *et al*, (1993). This is a semi-automatic loop, but with an heuristic logic.

INLET AIR TEMPERATURE CONTROL

An on-off control algorithm, with dead band and hysteresis, can control the inlet air temperature within certain limits. The controller manipulates an electric heater and a cooling system in the inlet air duct. This control loop assists evaporative cooling during critical periods.

INLET AIR RELATIVE HUMIDITY CONTROL

The inlet air relative humidity is automatically controlled by addition of water vapour, through a solenoid valve, and an on-off control algorithm is used for this purpose.

BED TEMPERATURE CONTROL (EVAPORATIVE COOLING)

This is the main control loop. As shown in Fig. 2, it operates automatically in cascade, continuously measuring the bed temperature and manipulating the set point of the inlet air relative humidity. A PI control algorithm has been tested. The cooling capacity can be improved with the inlet air temperature control and periodic agitation, if necessary.

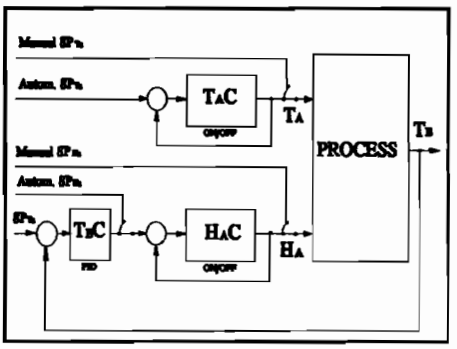

Fig. 2. Main control loop (evaporative cooling).

OUTLET AIR TEMPERATURE CONTROL

Since the bed temperature measurements are too noisy, *i.e.*, the value of the recorded temperature fluctuates as a consequence of inherent noise of the measurement caused by the electronic transducers, it was interesting to see if the bed temperature could be controlled through the outlet air temperature, which is a more stable measurement. A cascade control structure with the inlet air relative humidity, is also used. The control strategy is summarized in Table 3.

Table 3. Control strategy used.

Control variable	Manipulated variables	Type of control	Algorithm
Water content	Addition of fresh water, agitation	Manual	Water balance
Porosity and homogeneity	Agitation	Semi- automatic	Heuristic
Inlet air temperature	Heat input / output	Automatic	On-off, with hysteresis and dead band
Inlet air relative humidity	Vapour addition	Automatic	On-off
Bed temperature	Inlet air relative humidity and temperature	Semi- automatic	Cascade PI, heuristic
Outlet air temperature	Inlet air relative humidity and temperature	Semi- automatic	Cascade PI, heuristic

CONTROL SOFTWARE

The process is controlled by a PC, linked to a PLC, which is used as a data logger. The control software, with a graphic interface, is installed in the PC, where the control calculations are carried out. The process can be operated through the graphic interface. Here, the user can start/stop pumps and motors, select control algorithms, tune parameters, and build tendency graphics of selected variables. A sample screen of the graphic interface is shown in Fig. 3.

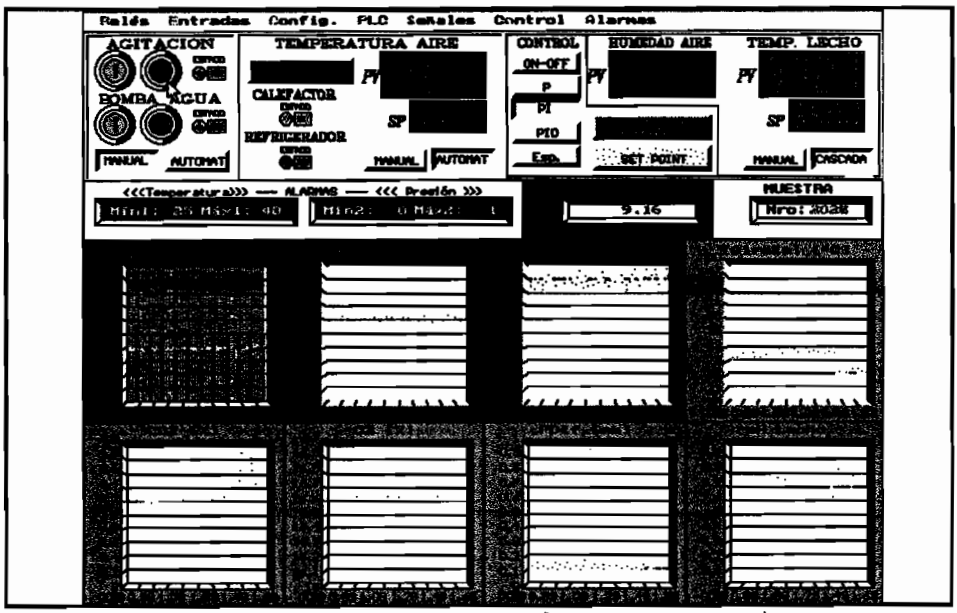

Fig. 3. Sample screen of the graphic interface.

EXPERIMENTAL RESULTS

The above control strategy has been tested experimentally for several days in the packed bed bioreactor. Selected results are given below, in order to illustrate the merits and limitations of the present control strategy.

WATER CONTENT

Fig. 4 shows that, this control works reasonably well. Here, the control system was set up to follow a slowly changing water content reference (SP-Hb). The manual control system can get the final desired value but with large oscillations. These could be attributed to limitations in the measuring system rather than the control algorithm. A sample is taken manually each 90 min and the water content is analyzed in a precise balance. Since the sample is small and the bed is heterogeneous, there are large errors in this measurement.

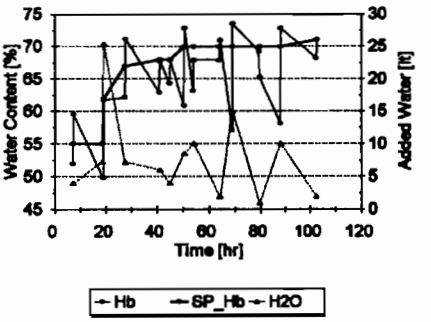

Fig. 4. Water content control. HB: bed water content; SP-Hb : set point; H_2O: added water.

INLET AIR TEMPERATURE

This is controlled precisely. Fig. 5 shows that the temperature does not deviate more than 0.5°C from the reference at steady states. It can also be seen that the heating is much faster than the cooling. The temperature range, that can be controlled, depends on the ambient air temperature. This ambient temperature varies from 5°C in winter to 30°C in summer.

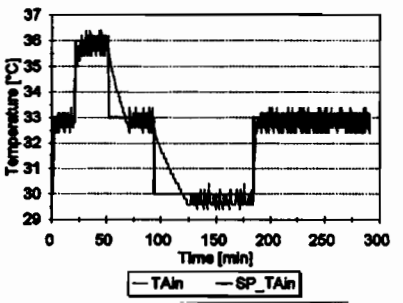

Fig. 5. Inlet air temperature control. TAin : inlet air temperature; SP-Ain: set point.

INLET AIR RELATIVE HUMIDITY

A reasonably good control can be achieved, as shown in Fig. 6. However, during certain periods (at time 0.2, 3 and 3.5 h), the control is poor. This is due to the interaction between relative humidity and air temperature. In order to re-establish

control, at time 1, air had to be cooled. On the other hand, at time 3.5, the air has to be heated, causing a large deviation in the relative humidity.

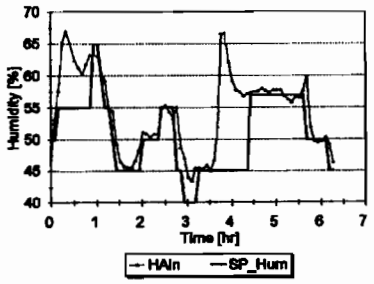

Fig. 6. Inlet air relative humidity control . HAin : measured variable; SP-Hum : set point.

BED TEMPERATURE

Fig. 7 shows open loop step changes to illustrate the effect of the inlet air relative humidity on the bed temperature. It can be seen that the relative humidity can cause variations in the bed temperature between 30 and 50°C. It is also note worthy that the dynamics present a varying delay, which ranges between few minutes (at time 1.3) and 30 minutes (at time 3.3).

The behaviour of the control loop is shown in Fig. 8. At time 5, a disturbance moves away the average bed temperature from the reference, and the relative humidity changes automatically to compensate for this deviation. After an hour, the average temperature returns to the reference value. The bed temperature measurement is extremely noisy, despite being filtered, thereby causing an oscillating control action.

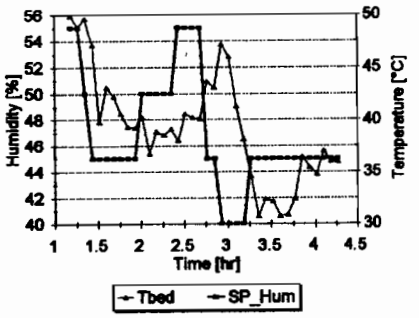

Fig. 7. Inlet air relative humidity step changes. Tbed: bed temperature; SP-Hum: air humidity set point.

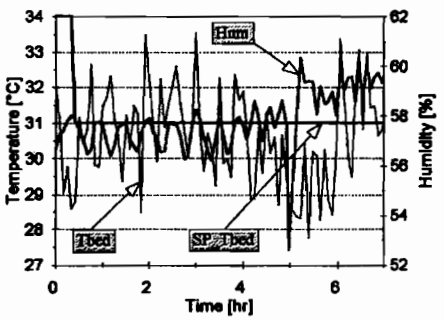

Fig. 8. Bed temperature control. Tbed : measurment, Hum: air relative humidity; SP-Tbed: set point

OUTLET AIR TEMPERATURE

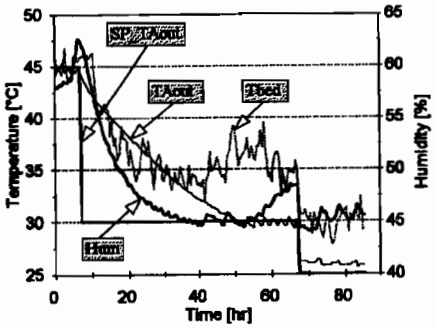

Fig. 9. Outlet air temperature control. Tout: air temperature; Tbed: bed temperature; Hum: manipulated variable; SP-TAout: set point.

Because of the noisy measurement of the bed temperature, the outlet air temperature was controlled, since its measurement was much more stable (Fig. 9). The control is better, than that in the previous case. Different tuning parameters were tried. At time 8, where a slow tuning was used, the controller took 40 h to drive the air temperature from 45°C to the desired value of 30°C. On the other hand, at time 68, the controller, with a different tuning, responded much faster, but it oscillated. A small offset also appeared.

CONCLUSION

The present control strategy is good enough to simplify the bioreactor operation considerably. When it was operated manually, it required permanent attention of at least one operator, and sometimes two operators. On the other hand, the actual control system required only one operator, with a low level of interaction with the process, and also allowed the operation of the bioreactor in certain periods, without direct supervision. Moreover, a precise control of bed temperature and water content was achieved.

Even though the control quality of the process has been improved with the present control system, there is much scope for improvement. In particular, the work is under progress for developing a special purpose bed water content monitor, that would allow direct continuous control of the water addition. This would reduce large water content deviations.

Inlet air temperature control loop performed well. However, the control range is narrow, when lower temperature needs to be achieved, specially during summer. Here, the cooling system must be improved, but the control algorithm can be maintained. This will also improve the dynamic response of the control loop, during cooling.

The control of the relative humidity strongly depends on the inlet air conditions. The present control algorithm behaves well and the observed limitations can be overcome, with an improved cooling system.

The most difficult loop is the bed temperature control. The main limitations include a) strong interaction between the main manipulated variables, *i.e.*, inlet air relative humidity and temperature, b) severe variations on delay, time constant and gain of the process dynamic response and c) extremly noisy measurements. These limitations make it very difficult to tune classic controllers like PIDs.

Need exists to improve the bed temperature measurement system, using different sensors and installing more sensors inside the bed. Then, a more sophisticated control algorithm must be developed, using multivariable, and adaptive or robust techniques. It is possible to control the bed temperature indirectly, with the outlet air temperature. However, as shown in Fig. 9, the air temperature responds slower, than the bed temperature.

ACKNOWLEDGMENTS

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Growth of *Rhizopus* sp. on ungelatinized cassava flour in solid state fermentation for protein enrichment

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SUMMARY

In order to study the potential of bioconversion of crude cassava flour, without gelatiization, it was necessary to apply a preliminary treatment to eliminate the natural microflora (bacteria, yeast and fungal spores), without allowing starch gelatinization, which otherwise would occur in steam sterilization process. Studies on the effect of temperature and moisture content of flour on micro-organisms content and rate of starch gelatinization, allowed the optimization of a dry or semi-dry treatment of cassava flour to obtain a micro-organism-free ungelatinized crude flour. The pretreated flours, as such or after mixing (50/50) with crude soya flour, were inoculated with spores suspension of selected strains of *Rhizopus* sp. *(oryzae, arrhizus, oligosporus)*, and incubated in a column reactor, equipped for on line respirometric analysis in solid state fermentation. The efficiency of each strain, in terms of specific growth rate, protein enrichmen and sugars bioconversion, was determined. Crude cassava flour could be enriched with up to 14% of protein, in contrast to that of 20%, in case of 50/50 mixture of crude cassava and soya flours. The protein enrichment of cassava by *Rhizopus* sp. is of commercial importance due to GRAS clearance of this fungi.

Keywords: Solid state fermentation, *Rhizopus* spp., ungelatinized cassava flour, cassava+soya flours, column fermentors, on-line respirometry, bioconversion, protein enrichment.

RESUME

Culture de *Rhizopus* sp. sur de la farine de manioc non gélatinisée en fermentation en milieu solide pour l'enrihissement en protéines.

RAIMBAULT M. ET RAMIREZ TORO C.

Afin d'étudier les potentialités de bioconversion de farine de manioc non gélatinisée par *Rhizopus* sp., il a été nécessaire de mettre au point un traitement préalable permettant d'éliminer la microflore naturelle (bactéries, levures et spores de champignons) tout en évitant de réaliser une gélatinisation de l'amidon, comme c'est le cas pour le procédé de stérilisation à la vapeur. Après une étude sur l'effet de la température et de l'humidité de la farine sur le contenu en microrganismes, et sur le taux de gélatinisation, il a été possible d'optimiser un traitement de farine sèche ou semi-sèche, de façon à obtenir une farine crue propre, pour les études ultérieures avec *Rhizopus* sp. Les farines de manioc prétraitées ont été utilisées pures ou en mélange (50/50) avec de la farine crue de soja, puis inoculées avec des spores de souches sélectionnées de *Rhizopus* sp. (*R.oryzae, R.arrhizus, R.oligosporus*). Elles ont alors été incubées dans des réacteurs colonnes adaptées à la FMS et à la respirométrie en ligne. Les analyses biochimiques et chimiques initiales et finales ont été réalisées de façon à évaluer l'efficacité de chaque souche à transformer le manioc cru, en terme de croissance spécifique, d'enrichissement en protéines et de bioconversion des sucres. Les résultats indiquent qu'avec de la farine de manioc cru, il est possible d'atteindre 14% de protéines, alors qu'en mélange avec du soja (50/50) on peut obtenir jusqu'à 20% de protéines. Il est rappelé que *Rhizopus* est une moisissure bien connue pour la production d'aliment de bonne qualité pour l'alimentation humaine. De plus *Rhizopus* produit des métabolites d'intérêt alimentaire pour la conservation, la préservation et l'arôme des aliments.

Mots clés: Fermentation en milieu solide, *Rhizopus* sp., farine de manioc non gélatinisée, mélange de farines de soya et de manioc, fermenteurs colonne, respirométrie en ligne, bioconversion, enrichissement protéique.

INTRODUCTION

Studies on solid state fermentation (SSF) of fungi on cassava and amylaceous substrates have been carried out in ORSTOM for more than 15 years. Initially, the

protein enrichment of cassava and other tropical substrate, like cassava, potato and banana, was studied out, using fungi of the *Aspergillus* group (Raimbault, 1981). It was possible to obtain fermented cassava with 18-20% protein on a dry matter (DM) basis (Table 1). Applications were essentially oriented to animal feeding.

Table 1. Protein enrichment by *Aspergillus niger* after 30 h fermentation in solid state fermentation

SUBSTRATE	Initial composition		Final composition	
	Proteins	Sugar	Proteins	Sugar
Cassava	2.5	90	18	30
Banana	6.4	80	20	25
Banana refuse	6.5	72	17	33
Potato	5.1	90	20	35
Potato Waste	5.1	65	18	28

Recently, Soccol *et al* (1993) obtained good results with fungi of the *Rhizopus* group, of special interest in human traditional fermented foods. Work was carried out on the pretreatment, and the effect of cooking on starch availability, protein content and yield of the bioconversion of starch into protein (Table 2). Without any cooking, a selected strain of *Rhizopus oryzae* could transform cassava, containing only 1.68% protein, into fermented cassava, containing 10.89% protein. The results indicated possibility of protein enrichment of raw cassava meal, by using selected strains of *Rhizopus*, with ability to grow on crude ungelatinized starch (Table 2). The application of such fermented cassava was oriented to human consumption.

With respect to amylase biosynthesis, data showed that the amounts of $\alpha-$ amylase and glucoamylase were 10 to 15 times higher in the solid than in the liquid cultures. Moreover, these enzyme titres were higher in crude starch medium than in cooked cassava (Soccol *et al*, 1994b).

Table 2. Growth of *Rhizopus oryzae* in solid substrate fermentation on cassava granules after various treatment (Soccol *et al*, 1994b)

TREATMENT	TOTAL SUGAR		PROTEINS*		Y
	Initial	Final	Initial	Final	Pr/sugar
I	80,01	46,78	1,20	11,69	0,174
II	84,11	60,72	1,61	12,40	0,227
III	82,44	52,57	1,56	13,93	0,208
IV	82,49	56,62	1,47	11,89	0,215
V	82,04	56,62	1,68	10,89	0,190

I : Cassava, autoclaved 30 min, 120°C, freezed, dried, grounded

II : Cassava flour (40% water), autoclaved 30 min at 120°C

III: Cassava flour (30% water), autoclaved 30 min at 120°C

V : Crude cassava flour without treatment

* g / 100 g Dry Matter ; ** g / 100 g Total weight

The work is continued in an EEC program at the bioconversion laboratory of the Universidad del Valle, Cali, Colombia, with the particular view to enhance knowlege about specificity of strains of *Rhizopus*, which are able to degrade the crude granule of starch. This could simplify processing of cassava tuber, having an important ecological aspect, as the plant starch is present in ungelatinized crude form in the natural environment.

It was necessary to get purée flour of crude cassava. In fact, the commercial flour of cassava harbours a large number of natural contaminants to allow microbial studies with fungi, without conventional sterilization and the consequent gelatinization. Therefore, studies were conducted to obtain crude cassava meal, with low content of bacteria as well as fungi, and without significant gelatinization.

MATERIAL AND METHODS

A number of techniques allow to evaluate the gelatinization of starch: enzymatic sensibility to glucoamylase, enthalpy energy, viscoamylogram, crystallography and Lugol reaction. For routine purposes, it was necessary to develop a very simple and rapid method, in view of a large number of determinations. The method involved the use of glucoamylase to measure the efficiency of the hydrolysis of the starch, as an

index of starch gelatinization and proved to be time consuming and costly. Hence, the method of Wootton *et al* (1971) was preferred as it demostrated good correlation coefficient of the calibration curve in well standardized conditions (Fig. 1). This technique involved the measurement of the blue coloration developed by the Lugol reaction. Calibration of the intensity of the reaction, as a function of the extent of gelatinization of cassava starch, was reliable. Starch, with different extent of gelatinization, was obtained by mixing crude (0% gelatinization) cassava flour with well gelatinized (100%) cassava flour. The correlation (0.99) between the optical density at 600 nm and the degree of gelatinization indicated the validity of the technique.

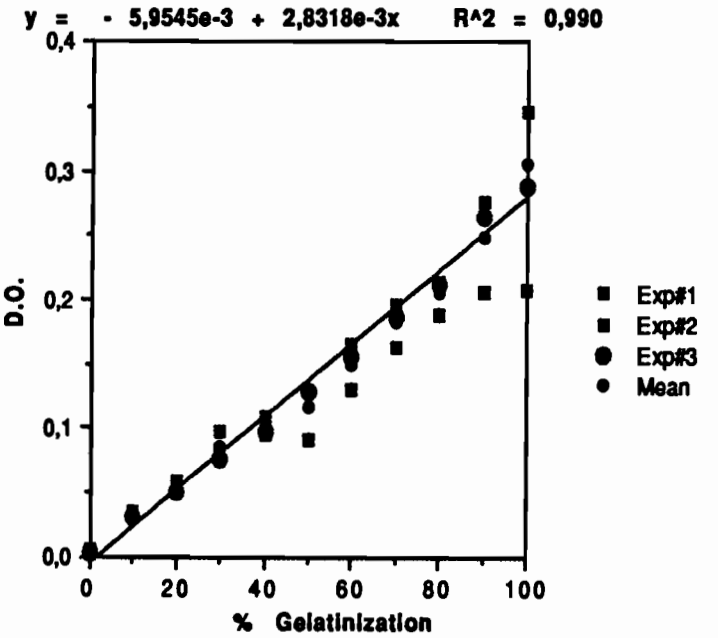

Fig. 1. Calibration curve for the determination of gelatinization ratio.

For the determination of the microflora, the UCP technique was employed using PCA medium for total microflora and PDA medium for spores and yeasts contents, as recomended by Mitchell *et al* (1988).

The technique for *Rhizopus* cultivation in solid state fermentation was described previously (Raimbault and Alazard, 1980). For the measurement of the respiratory metabolism, the automatic gas chromatographic column method was used, as described by Saucedo *et al* (1994).

Strains of *Rhizopus* were the same, as the ones used by Soccol *et al* (1994a): *R. oryzae* 28168; *R. oryzae* 28627; *R. arrhizus* 1526 and *R. oligosporus* 6203.

RESULTS AND DISCUSSION

EFFECT OF DIFFERENT TREATMENT OF DRY CASSAVA MEAL

Crude cassava meal contained about 108-109 bacteria/ g DM, which makes it difficult to develop studies for fermentation of ungelatinized starch in natural conditions. It is, therefore, necessary to reduce the level of bacterial and fungal population, in order to eliminate competition with the spore inoculum (107/ g). Fig. 2 indicates the effect of UV ray, temperature and microwave on the bacterial content of dry cassava meal of less than 10% moisture content.

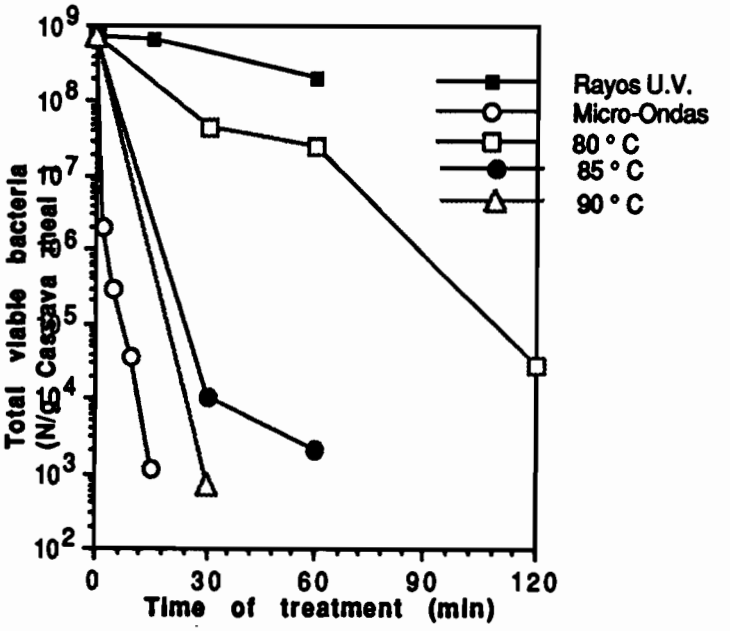

Fig. 2. Total bacterial microflora (PCA) in relation to the treatment and time.

The efffect of UV ray was poor and not sufficient enough to elimine the natural microflora, neither for bacteria, nor for yeast and fungi. On the contrary, microwave reduced fungal content from 108 to 103 / g after 2 min, and bacterial content from 8.108 to 103 bacteria / g after 2.5 min. Dry treatment in oven at different temperatures proved excellent. All the fungal microflora (yeast and fungal spores) were eliminated by the treatment for 30 min at 80°C. For bacteria, the treatment at 95°C for 30 min is required to reduce the number of bacteria under 102 / g. Schiffman (1992) reported similar results on the use of microwave to reduce microbial content. Olsen (1965) also demostrated the high efficiency of microwave and the total elimination of yeast and fungi.

Finally, an autoclave treatment at 120°C for 15 min was applied. The dry meal was kept in a sealed tube for autoclaving, to avoid direct contact with vapor and the resulting gelatinization. The autoclaving treatment produced best results, because no colony was observed on the plate (< 10 colonia / g DM).

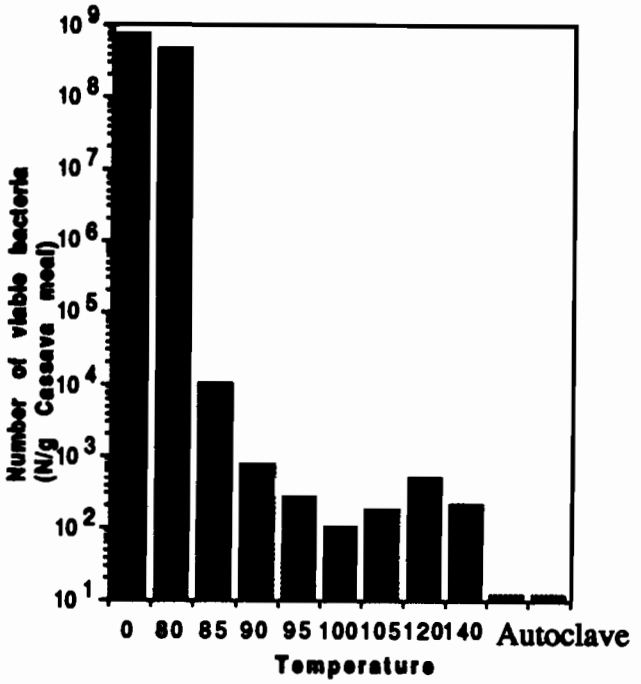

Fig. 3. Effect of temperature on bacterial content of dry cassava meal.

It was concluded that autoclaving treatment of dry cassava meal was sufficient to obtain microbial-free cassava flour for studies on the growth of *Rhizopus* in SSF (Fig. 3).

EFFECT OF HEAT TREATMENTS AND MICROWAVE ON THE GRADE OF GELATINIZATION

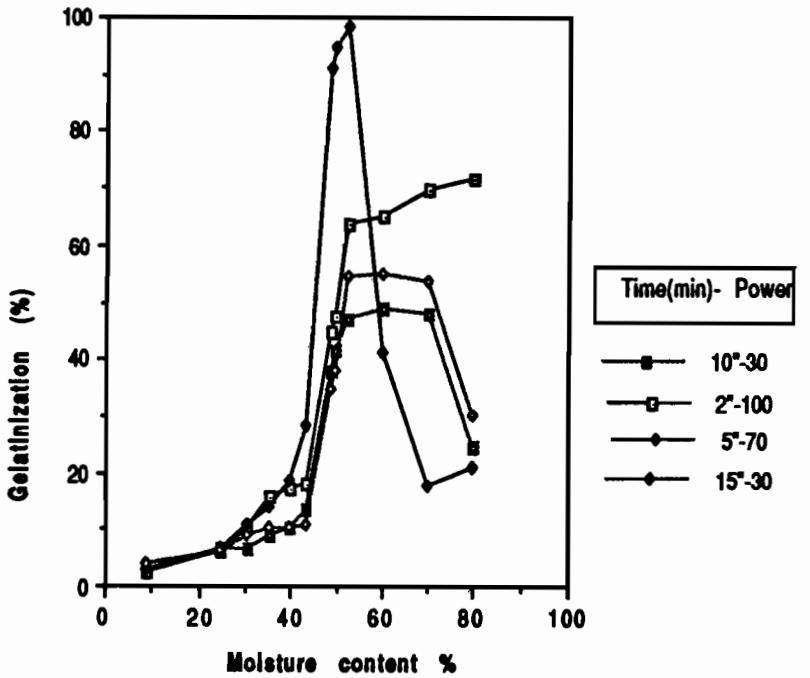

Fig. 4. Effect of micro-waves on gelatinization of cassava meal at various moisture content.

In the case of treatment in a microwave oven, the gelatinization of cassava flour was maximum, when the moisture content was about 50% (Fig. 4). A strange phenomenon of decreased gelatinization was observed for moisture contents higher than 60%, thereby indicating on other kind of starch transformation occurring. However, the aim of the experiment was not to get a high degree of gelatinization of cassava flour. On the contrary, the objective in this study was to find out a procedure of treatment with strong effect on the elimination of contaminant microflora and low

effect on the gelatinization, in view to study the growth of *Rhizopus* in SSF using crude starch. Results obtained by heat treatment were very similar (Fig. 5).

In both cases, it can be observed that, for moisture contents lower than 10% (such as the case of dried meal), both microwave and heat treatment had very low effect on the gelatinization of the starch, even in case of the heating temperature higher than 100°C. This is very important since with such treatment of dry cassava flour, all fungal spore and contaminant microflora could be eliminated, without larger changes in the gelatinization of the starch.

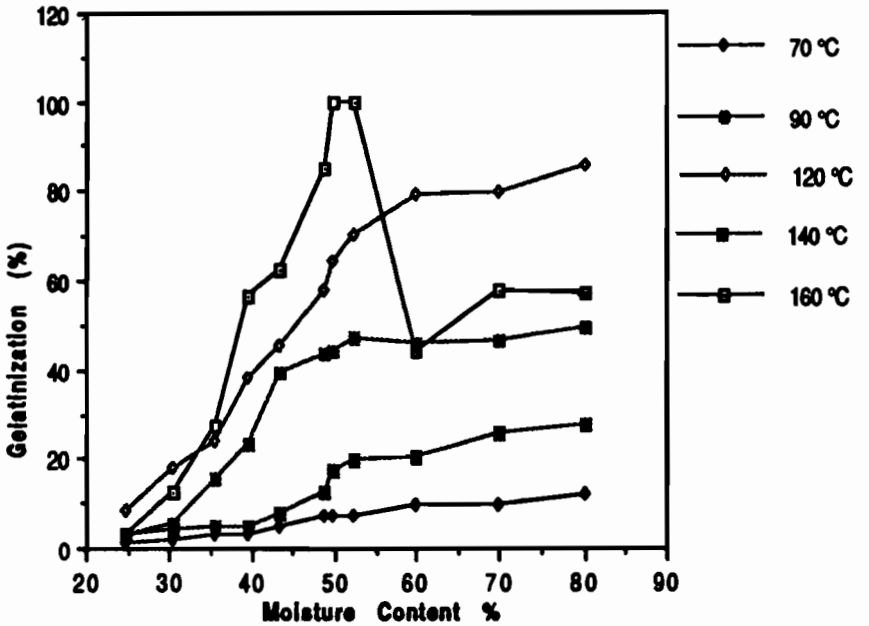

Fig. 5. Gelatinization % at different temperatures for various moisture contents of cassava meals.

Finally, the heat treatment in autoclave in sealed flask was selected for practical reasons at the lab scale. Three kinds of treatment were chosen: a) the dry meal (moisture content< 9%) and gelatinization < 6%, which is considered as crude starch flour, b) the semi-dry treatment (moisture < 30%) and gelatinization = 7%, a very low effect on gelatinization, but allowing prior-humidification of the cassava flour, and c) the wet treatment (moisture content = 40%) with a degree of gelatinization near 30%, similar to the treatment reported by Soccol (1991).

GROWTH OF *RHIZOPUS* ON CASSAVA MEAL OF LOW GELATINIZATION DEGREE

All data on the growth of different strains of *Rhizopus* in the above three categories of cassava flour are not reported here, as was done in various reports on the EEC program. Figs. 6 and 7 show a typical illustration of the respiration kinetics, obtained during the fermentation of *R. oryzae* cultivated in a column containing 150 g of wet product at 50% moisture content, and incubated at 30°C, with an aeration of 100 ml/min. In all these cases, the degree of gelatinization was as low as 7%. A stable RQ (ratio of carbon dioxide production by the oxygen consumption), observed in these studies, indicated a typical vegetative stage and very active growth between 10 and 30 h. On the other hand, Fig. 7 indicates that the exponential phase occurred between 8 and 15 h , was followed by a decrease in growth but *R. oryzae* remained active until 24 h. This simple and direct on-line method allows to determine the specific growth rate of the *Rhizopus* ($\mu = 0.217\ h^{-1}$).

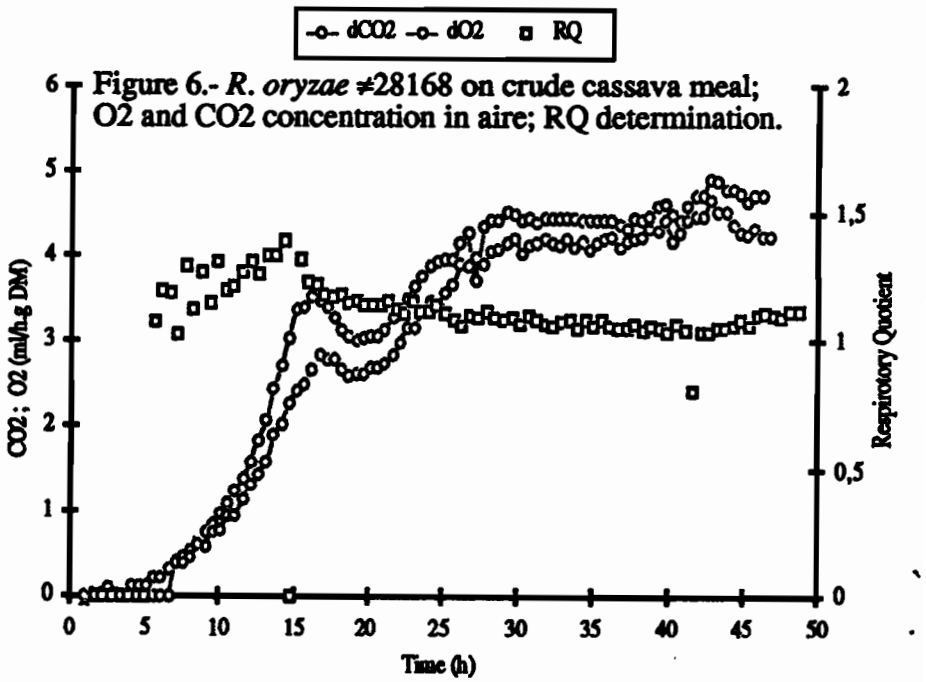

Fig. 6. *R. oryzae* ≠ 28168 on crude cassava meal

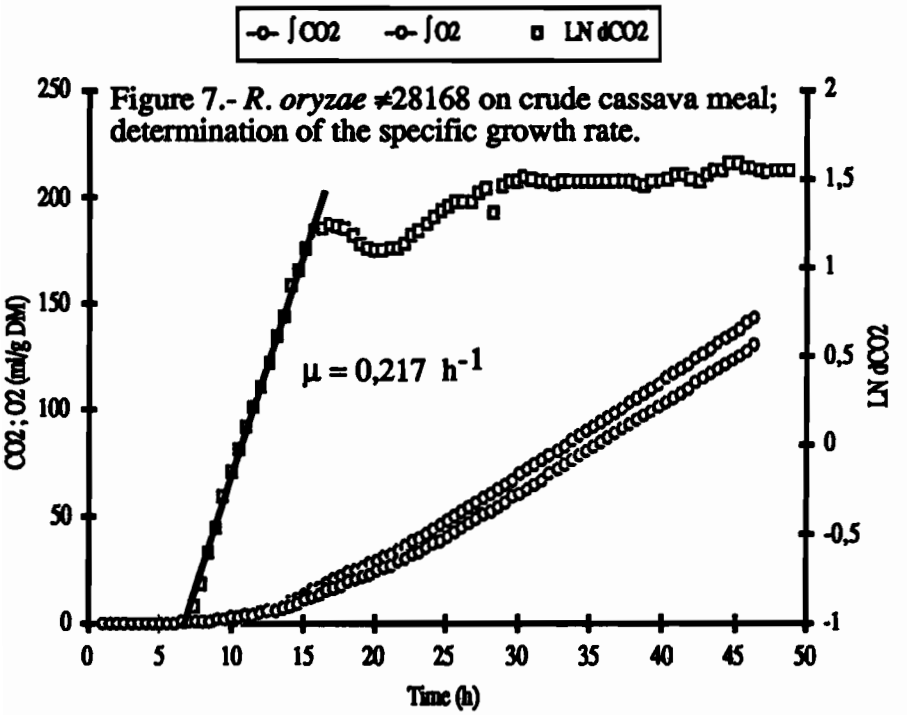

Fig. 7. *R. oryzae* ≠ 28168 on crude cassava meal.

The results on the composition of pure cassava meal and mixed with 50% soybean meal, after autoclave treatment in dry and semi-dry conditions, are presented in Table 3. Very low degree of gelatinization is evident in both cases. With soybean, the mixture is enriched with protein from 11.3 to 20.08%. But the net increase is comparable and in the case of pure cassava meal enrichment, the protein content increased from 4.15 to 14%.

Table 3. Comparison of the growth of *Rhizopus oryzae* on cassava meals treated in autoclave in dry (7% moisture) or semi-dry (30% moisture) for 20 min in sealed containers.

	DRY MEAL	SEMI DRY MEAL
Gelatinization,%	6.31	7.11
μ = Growth rate, 1/h		
Pure cassava	0.056	0.217
Cassava + soybean		
Final protein content		
Pure cassava	4.15	14
Cassava/soybean	11.31	20.08

CONCLUSION

It was concluded that *Rhizopus oryzae* is capable of degrading crude starch, after a light heat treatment that eliminated the contaminant microflora. It was observed that the treatment in semi-dry condition, i.e., at 30% moisture content and 7% gelatinization, improved significantly the specific growth rate and the respiratory activity of *Rhizopus*. However, the mixture with soybean meal did not allow better protein yields. It is necessary to investigate further the screening of *Rhizopus* sp. so that knowledge on the degradation of crude starch by specific strains could be improved. The production and biosynthesis of glucoamylase is under investigation to verify whether the concentration of enzyme is higher, when *Rhizopus* is grown on crude cassava, than on gelatinized starch.

ACKNOWLEDGEMENTS

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Effects of several factors on fungal spore germination in solid state fermentation of coprah cake

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SUMMARY

Optimization of the culture conditions of *Aspergillus carbonarius* (Asca), *Aspergillus* sp (C11B52) and *Penicillium* sp (V26A25), cultured in coprah cake solid state fermentation (SSF), was undertaken, using experimental matrices. The effects of seven culture parameters (humidity = F1, initial $(NH_4)_2SO_4$, $CaCl_2$ and $MgSO_4.7H_2O$ concentrations = F2, F6, and F7, respectively, initial pH = F3, inoculum size = F4 and temperature = F5) on spore germination time and respiratory activities of the strains were studied. In general, the shortest lag phase was observed under the lowest values of the F1, F2, F3 and F7 parameters. Under these conditions *Aspergillus carbonarius* (Asca) and *Aspergillus* sp (C11B52) showed the highest respiratory activities (6.3% and 10.2%, respectively), while the highest respiratory activity (≈ 5%) of *Penicillium* sp was observed at the lower values of F2, F3, F4 and F5 parameters.

Keywords: *Aspergillus carbonarius, Aspergillus sp., Penicillium sp.*, coprah cake, spore germination, solid state fermentation, Lag phase, germination time, respiratory activity, protein enrichment.

RESUME

Effets de différents facteurs sur la germination de spores de champignons filamenteux cultivés sur tourteau de coprah en milieu solide.

MARAKIS S., LAMBRAKI M., PERRAUD-GAIME I., HANNIBAL L. ET ROUSSOS S.

L'optimisation des conditions de culture d'*Aspergillus carbonarius* (Asca), *Aspergillus* sp (C11B52) et *Penicillium* sp (V26A25), cultivés sur tourteau de coprah en milieu solide, a été réalisée en utilisant des plans d'expérience. Les effets de sept paramètres de culture (humidité = F1, concentrations initiales de $(NH_4)_2SO_4$, $CaCl_2$ et de $MgSO_4.7H_2O$ = F2, F6, et F7 respectivement, pH initial = F3, taille d'inoculum = F4 et température = F5) sur le temps de germination de spores et les activités respiratoires des souches ont été étudiés. Les temps les plus courts pour la germination de spores ont été obtenus pour les valeurs les plus faibles des facteurs F1, F2, F3 et F7. Sous ces conditions expérimentales *Aspergillus carbonarius* et *Aspergillus* sp. ont montré les plus grandes activités respiratoires (6,3% et 10,2% respectivement) alors que l'activité respiratoire la plus élevée (≈ 5%) observée pour *Penicillium* sp. a été obtenue avec les plus faibles valeurs des paramètres F2, F3, F4 et F5.

Mots clés: *Aspergillus carbonarius, Aspergillus sp., Penicillium sp.*, Tourteau de coprah, germination de spores, Phase de latence, temps de germination, activité respiratoire, enrichissement en protéines.

INTRODUCTION

The economic and social significances of fungi, as sources of food and biologically active metabolites, stimulated the interest in their growth physiology. One of the important variables of fungal growth is spore germination time, since it determines the duration of the lag phase, and, consequently, the duration of the fungal fermentation. A prolongead lag phase in fungal fermentation leads to increased cost of production of microbial products. It, is, thus, essential to achieve a shorter spore

germination time, either by selecting a fungal strain of short lag phase or by regulating culture conditions, e.g., tannic acid addition (Gaitis and Marakis, 1994).

A strain of *Aspergillus carbonarius*, with high tanninolytic ability, has been isolated from mouldy carob (Marakis, 1980). Although this fungus physiology has been fairly well studied for microbial protein enrichment and metabolite (enzymes) production in submerged shaker cultures (Marakis, 1980, 1985; Marakis and Diamandoglou, 1990), no study on the growth of this strain of *A. carbonarius* in SSF has been carried out, so far. A comparative study of this strain with *Aspergillus* sp and *Penicilium* sp, previously studied for protein enrichment (Roussos *et al*, 1994) and enzyme production (Roussos *et al*, 1995) in SSF system, is attempted.

This paper describes the effects of seven factors on the spore germination time and respiratory activities of *A.carbonarius, Aspergillus* sp and *Penicillium* sp, cultured in coprah cake SSF.

MATERIALS AND METHODS

MICROORGANISMS

Aspergillus carbonarius (Bainier) Thom, was isolated from mouldy carob beans (Marakis, 1980), while *Aspergillus* sp (C11B25) and *Penicillium* sp (V26A25) were obtained from the microbial collection of the Biotechnology laboratory of ORSTOM (Roussos *et al*, 1995).

MEDIA

A mineral solution of 0.36% Na_2HPO_4, 0.3% KH_2PO_4 and 1% urea, on initial substrate dry matter (IDM), was added to coprah residue, while $(NH_4)_2SO_4$, $CaCl_2$ and $MgSO_4.7H_2O$ solution of specific concentration, as shown in Table 1, were then added to obtain the desired initial humidity and mineral contents.

Table 1. Relation between code numbers (limits of factor values) and real values of the factors examined.

Factors	Limits of factor values	
	-1	1
F1 = Initial humidity (% IDM)	60	70
F2 = $(NH_4)_2SO_4$ (%IDM)	5	10
F3 = Initial pH	6	7
F4 = Inoculum size (spores/g IDM)	$2x10^6$	$2x10^7$
F5 = Temperature (°C)	25	30
F6 = $CaCl_2$ (% IDM)	0,3	0,6
F7 = $MgSO_4.7H_2O$ (% IDM)	0,3	0,6

CULTURE CONDITIONS

The values of the seven factors studied are presented in Table 1. The optimization of the culture conditions (Table 2) was approached by using experimental matrices (De Meo *et al*, 1985). Solid state fermentationwas carried out according to the procedure of Raimbault and Alazard (1980).

SPORE GERMINATION DETERMINATION

Spores were considered to be germinated when their germ tube lengths were one and a half times the size of the spore diam (Frossard and Oertli, 1982).

MEASUREMENT OF CO_2

Carbon dioxide in the dry output air flow was continuously measured by using a gas chromatograph, as per the method of Saucedo-Castañeda *et al* (1993).

PROTEIN EVALUATION

Proteins were estimated by total nitrogen titration, as per the total carbonization method (CHN), using a LECO SP 428 apparatus (USA), or by Kjeldhal using Kjeltabs CTQ catalyso, Thomson-Capper Ltd (Prolabo) France. The quantity of ammonium nitrogen was determined colorimetricaly using the Alliance Evolution II automatic apparatus (France) and the value was deducted from the total nitrogen. Results obtained were multiplied by a factor of 5.3 for the unfermented coprah cake and by 6.25 for the fermented products (Rham, 1982).

Table 2. Experimental matrice with code numbers of the factors examined.

Expt.	Limits						
	F1	F2	F3	F4	F5	F6	F7
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1

RESULTS AND DISCUSSION

Spore germination time and respiratory activities were found to depend on fungal strain and culture conditions. All the strain developed well enough on coprah cake in SSF, though with different respirometric profiles. This was not only because of their physiological differences (Roussos *et al*, 1994), but also due to the culture conditions.

A. carbonarius showed the shortest lag phase (2.5 h) and the maximum respiratory activity (6.3% CO_2) after 18 h incubation in experiment No. 1, while the longest

(15 h) lag phase and the lower respiratory activity (3.75% CO_2) was observed in experiment No. 7 (Table 3). Roussos *et al* (1994) also reported a lag phase of 3 h and a respiratory activity of 7%, for the same strain and substrate. On the basis of the above data, and those presented in Fig. 1, the optimization of *A.carbonarius* growth could be achieved if a) substrate initial humidity, inoculum size and incubation temperature values are about 70%, $2x10^7$ and 30°C, respectively, b) nitrogen source and pH are less than 5% and 6, respectively and c) concentrations of $CaCl_2$ and $MgSO_4.7H_20$ are lower than 0.3%. This microorganism has shown similar nutritional or physical requirements, in shaker submerged culture system (Marakis and Diamantoglou, 1990) as well as in solid state fermentation of carob pods (Lambraki *et al*, 1994), thereby indicating that this strain requires same culture conditions, independent of the cultivation system.

Table 3. Growth parameters of *A. carbonarius, Aspergillus* sp. and *Penicillium* sp. uner different culture conditions

Microorganisms	Expt No	Shortest germination Time (h)	Maximum CO_2 production (%)	CO_2 production rate (h^{-1})	Optimum temperature (°C)
A. carbonarius	1	2,5	6,3	0,37	30
Aspergillus sp.	1	15	10,2	0,34	30
Penicillium sp.	1,8	10	?	0,14	25
	2,5	?	4,8	0,14	?

Aspergillus sp. (C11B52) showed growth requirements, similar to *A. carbonarius* (Fig. 1), but the shortest lag phase (15 h) of this strain was 6 fold longer than that of *A. carbonarius*, as observed in experiment No. 1, while the longest lag period was 38 h in experiment No. 7. A temperature of 30°C was found to be optimum for both of the *Aspergillus* strains (Table 3). This result is similar to that reported by Roussos *et al* (1995) in their studies on isolation of fungal populations at different temperatures. They found that *Aspergilli* species were predominant at 30°C (= 75% of the total population), while the population of *Aspergillus* species was 25%, when the isolation temperaturewas 25°C. On the other hand, the maximum CO_2 production (%) in case of *Aspergillus* sp., was 1.7 fold higher than that of *A. carbonarius* (Table 3), though both the strains showed similar CO_2 production rates (0.34 h^{-1} and 0.37 h^{-1} for *Aspergillus* sp. and *A. carbonarius*, respectively).

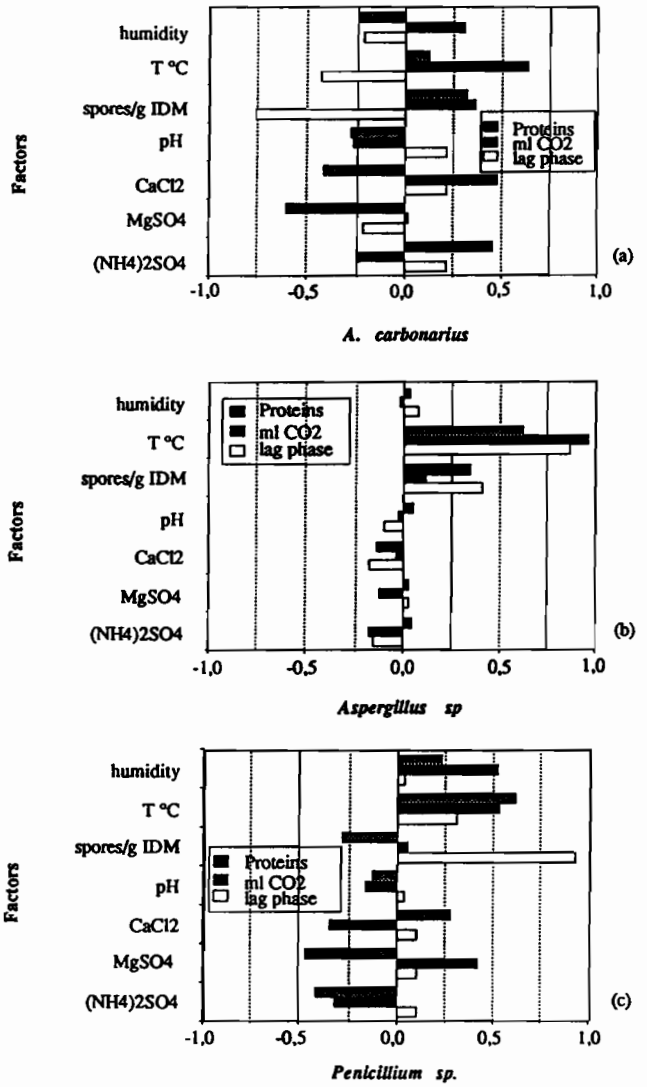

Fig. 1. Protein content, CO_2 production (ml/g IDW), and lag phases of *A. carbonarius, Aspergillus* sp. and *Penicillium* sp. as affected by several culture parameters.

As the extent of CO_2 production indirectly reflects biomass development, the above data indicate that *Aspergillus* sp. C11B52 is most suited for biomass production as well as protein enrichment of agro-industrial byproducts/residues/wastes, while *A. carbonarius* can be of economical benefits in the production of fungal metabolites, such as enzymes, due to its shorter lag phase. Optimization of cultural conditions for each of these two strains for the above exploitation in most efficient manner could be carried out by studing other physico-chemical parameters or by further studing the presently optimized factors at closer range.

Penicillium sp. showed an intermediate (between the other two strains studied) germination time of 10-18 h, but, a comparatively low (4.8%) CO_2 production (Fig. 1). Obviously, this strain is not suitable for fermentation of the above types. Nevertheless, it is interesting to notice that the best and worst growth results for this microorganism were also recorded at experiments Nos. 1 and 7, respectively.

The fact that *Aspergillus* sp. and *Penicillium* sp. were the strains which showed maximum and minimum CO_2 production (%), respectively, is contrary to the report of Roussos *et al* (1994), who cultivated these microorganisms in the same medium. It appears that mineral concentration and aeration flow rate account for these difference in these two kinds of experiments.

Protein values were calculated from the elementary nitrogen composition, from which the amount of ammonium nitrogen was substracted. Titration for the different samples, after 45 h of SSF, revealed that *Aspergillus* sp. cultures had a higher protein content. This was expected as this microorganism presented the highest CO_2 production (Angokai, 1993). *Penicillium* sp. also showed a high protein content, but is characterized by long phase and for this reason it is not economically beneficial.

CONCLUSIONS

Spore germination time and respiratory activity depend on the strain and culture conditions. The culture conditions used in experiment No. 1 proved the most beneficial for the growth parameters of all the fungal strains studied in coprah fermentation. Among these, *Aspergillus* sp. C11B52 is a high-biomass producer, while *A.carbonarius* completes a very quick metabolic cycle.

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Preservation of coffee pulp by ensilage: Influence of biological additives

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SUMMARY

Data on the influence of three biological additives on the ensilage of coffee pulp for its preservation show that the endogenous microflora of the coffee pulp is efficient enough to produce good quality silage, with acceptable levels of organic acid, dry matter loss and final pH. The use of inoculants, as biological additives, showed the efficiency of natural microflora grown on coffee pulp and the monoculture of *Lactobacillus plantarum* A6 in improving the physico-chemical characteristics of the silage, though commercial inoculum was not efficient, due to several reasons. Degradation of caffeine was absent in all the cases. Cellulases as a biological additive showed increased sugar production during ensilage. The results on the kinetics of different microflora development and physico-chemical characteristics during ensilage provide the insight into the microbiology and physiology of the process and point out a number of possibilities for improving the ensilage process as well as the quality of the silage.

Keywords: Coffee pulp, ensilage, endogenous microflora, biological additives, enzyme complex, inoculants, natural microflora inoculant, *Lactobacillus plantarum*, commercial inoculum, lactic acid, acetic acid, butyric acid, ethanol, caffeine, dry matter loss.

RESUME

Conservation de la pulpe de café par ensilage: Influence des additifs biologiques.

PERRAUD-GAIME I. et ROUSSOS S.

La microflore lactique endogène de la pulpe de café séchée au soleil est suffisante pour amorcer un ensilage présentant des caractéristiques physico-chimiques finales acceptables. Cependant, l'apport de differents ferments biologiques (un pied de cuve de pulpe de café ensilée naturellement ; un ferment monosouche (*Lactobacillus plantarum* A6) ; un ferment commercial DpH4 (associant deux souches de bactéries lactiques à un complexe enzymatique) permet d'améliorer la qualité des ensilages : homogénéité de la population microbienne, augmentation du taux d'acide lactique, pas de production d'AGV indésirables ni d'éthanol. La présence de cellulases permet d'augmenter le taux de sucres reducteurs. Les suivis du développement des microflores et les résultats physico-chimiques obtenus au cours des ensilages démontrent une conservation rapide de la pulpe de café par acidification sans perte importante de matière sèche. L'ensilage est un bon procédé de conservation de la pulpe de café humide. La caféine n'a pas été dégradée dans tous les cas.

Mots clés: Pulpe de café, ensilage, microflore endogène, additifs biologiques, ferments, mélange d'enzymes, ferments naturels, *Lactobacillus plantarum*, ferments commerciaux, acide lactique, acide butyrique, ethanol, caféine, perte de matière sèche.

INTRODUCTION

Agro-industrial residues/wastes are generated in large quantities throughout the world. Their non-utilization results in loss of valuable nutrients and environmental pollution (Zuluaga, 1989). Their better utilization by biotechnological means assumes social, economic and industrial importance. Considering these facts, ORSTOM participated into a scientific collaboration with Universidad Autonoma Metropolitana (UAM), Mexico, for the development of biotechnological processes for better utilization of agro-industrial byproducts/wastes, especially the coffee pulp (Viniegra *et al*, 1991).

Coffee pulp, generated to the extent of 40% in the fermentation of coffee berries (Zuluaga, 1989), poses many problems in the coffee producing tropical countries. Its disposal in nature, without any treatment, causes severe environmental pollution,

due to putrefaction of organic matter (Zuluaga, 1989). Hence, the possibility of utilizing coffee pulp in the biotechnological processes for production of different metabolites was investigated thorought by Roussos *et al* (1993).

Coffee pulp, as it is generated, contains 80-85% moisture (Bressani *et al*, 1972), in addition to appreciable quantities of sucrose, proteins, amino acids and other nutrients. All these factors and nutrients allow various microflora to develop quickly on the coffee pulp and the development of the microorganisms cause the putrefaction of coffee pulp (Gaime-Perraud *et al*, 1993 ; Roussos et al, 1995). It is also not practicable to utilize the coffee pulp immediately, after its generation during coffee berry treatment mainly because the season of coffee berry processing lasts for 3-5 months. During this season, the industry cannot divert attention to this waste, as its priority is focused on the quality of coffee seeds during the entire season. Moreover, quick dehydration of the coffee pulp is impracticable, considering the huge quantity of the waste, high energy requirement, larger capacity of machinery needed and heavy investment on space and building, not only for dehydration, but also for stocking of the dehydrated pulp, till its utilization.

Ensilage of coffee pulp, for its preservation and improvement of feed value, is one of the avenues for value-added utilization of coffee pulp. Ensilage, a quick anaerobic process involving lactic acid bacteria, has been extensively used for preservation of forage in the temperate regions. It allows the prevention of putrefaction of the forage with minimum degradation of organic matter. The process is quicker and it also improves the nutritive quality of the forage (Mc Donald *et al*, 1991).

A number of factors are of vital importance in obtaining a good silage. The substrate to be ensiled should have 30-40% dry matter, should be compactable to the desired level, amenable for anaerobiosis and contain utilizable sugars in sufficient quantities (Bertin, 1986). It must also have the colour, which is most nearer to the raw material, the fruity aroma and slightly acidic taste. In terms of chemical characteristics and achievement of the organic matter stability, the ensilage should involve a minimum loss of dry matter and the resulting silage should have a pH value lower than 4.5, higher than 3% lactic acid, but less than 0.5 and 0.3% acetic and butyric acid, respectively (Mc Donald *et al*, 1991).

A number of chemical and biological additives are mixed with the substrate for improving silage or reducing fermentation time. In the case of biological additives, a lactic acid bacterial inoculum is added, as a minimum of 10^5 lactic acid bacteria per g dry matter is required (Gouet, 1994) to convert the carbohydrates into lactic acid, but not into butyric acid. Enzymes are also added, when the rate of assimilation of sucrose by the endogenous lactic acid bacteria is slower (Bertin, 1986).

Ensilage is also practiced in tropical countries, despite the problems in terms of temperature, humidity and rains. Consequently, the rate of ensilage is slower, putrefaction is common and there is need to use a number of additives.

A number of reports have been ptoduced on ensilage of coffee pulp (Bohkenfor and Fonseca, 1974; Murillo, 1978; Carrizalez and Gonzalez, 1984). But, most of these are associated with the development of the ensilage technique or the effect of chemical additives on the process. For example, Murillo (1978) compared the silage of coffee pulp, obtained by natural microflora based fermentation, with that involving the use of molasses or organic acids as additives. After 90 days of ensilage, the loss of dry matter was as high as 26.8%, in the case of the use of organic acids as additive, though it allowed to attain a pH of less than 4.0. Caffeine content of the drained water was reported to increase significantly, in the case of the use of organic acids, probably because it became more soluble in acidic pH.

In the present studies, the ensilage of coffee pulp was investigated, with respect to the microbiology and biochemistry of the process, along with the evaluation of biological additives, for improving the process and also the quality of the silage. Accordingly, the studies involved a) allowing the endogenous lactic microflora to grow on coffee pulp for using the fermented mass as inoculum for the next batch, b) use of monoculture of *Lactobacillus plantarum* A6 as a biological additive and c) the use of commercial inoculum as yet another biological additive. The latter contained two lactic bacteria and an enzyme complex.

MATERIAL AND METHODS

In order to overcome the problem of variation, the same batch of coffee pulp was used throughout the whale experiment. The coffee pulp used was derived from berries of *Coffea arabica* and was obtained from a coffee processing industry from Xalapa, Mexico, where the coffee berries are processed using a wet fermentation method. The coffee pulp was sun-dried by the industry for 10 days to achieve less than 10% moisture and packed in plastic sacs. The dried coffee pulp was coarsely ground and sieved to select the particle size between 0.8 to 2.0 mm. It was hydrated to contain about 70% moisture, before ensilage.

Plastic bottles with closed fitted caps were used as laboratory scale microsilos. Each plastic bottle was charged with 60 or 150 g moist coffee pulp. The moist material in the microsilos was compacted manually for removing the air from inter-particle spaces. The microsilos were incubated at darkness, at 30°C for one month and the samples were removed at desired intervals. Each microsilo constituted a sample.

The moist unsterilized coffee pulp was ensiled under four conditions, i.e., a) dry pulp was only hydrated with water without inoculation, b) dry pulp was hydrated with water and then mixed with natural inoculum provided from (a), c) dry pulp was hydrated and mixed with inoculum containing *Lactobacillus plantarum* A6, d) similar to (c) but inoculated with commercial inoculum. In all the cases, the media were inoculated, before charging into the microsilos, unless otherwise stated.

INOCULUM DETAILS

For development of natural microflora, the unsterilized dry pulp was hydrated and the endogenous microflora was allowed to grow in column fermenters at 30°C for 2 months in anaerobic conditions. This inoculum was added to obtain 10^4 lactic acid bacteria/g dry matter. In the case of monoculture studies, *L. plantarum* A6, isolated from cassava by the Laboratoire de Microbiologie, ORSTOM Centre, Brazzaville, Congo (Giraud *et al*, 1991), was used. The inoculum suspension contained 10^8 bacteria/g dry matter. The commercial inoculum contained two lactic acid bacterial species (10^{10} and 3.10^9 of *L. plantarum* and *Pediococcus acidilactici*, respectively, per g) and the enzyme cellulase (10.000 CMCases units/g). In this last case, the inoculation was done to achieve 10^5 cfu lactic acid bacteria/g dry matter.

TREATMENT OF THE SAMPLES AND ANALYSES

A schematic flow diagram of the sample treatment and analyses is given in Fig. 1. The sample was well mixed, 10% of the moist material was removed and mixed with sterile distilled water, containing 0.001% Tween 8O (dilution: 1/10). The mixture was homogenized at 8000 rpm for 4 min in Ultra-Turrax and used for estimating different microorganisms (Perraud-Gaime, 1995).

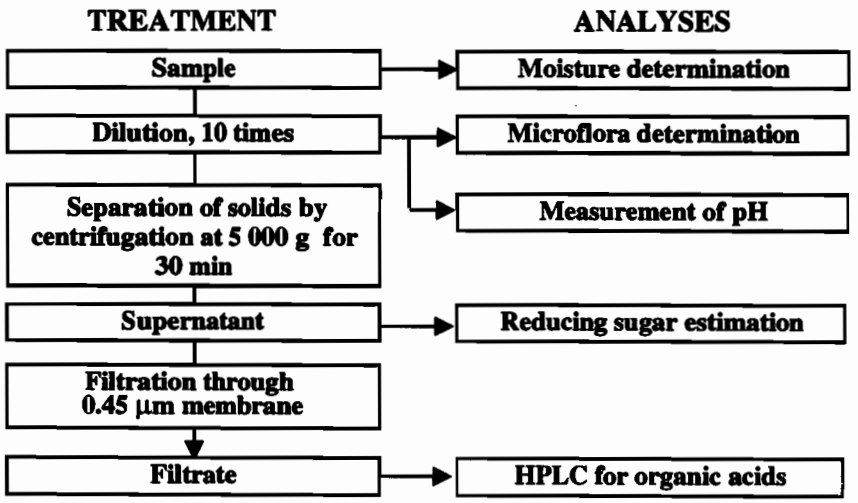

Fig. 1. Schematic diagram for treatment of the sample for microbiological and physico-chemical analyses.

Plate Count Agar was used for determining the total bacterial count, while Potato Dextrose Agar and Sabouraud Dextrose Agar were employed for determining total fungal and total yeast counts, respectively. In both of these media, chloramphenicol (0.25 g/L level) was added to prevent bacterial growth. For the determination of total anaerobes and lactobacilli, MRS agar was used in total anaerobic conditions in the anaerobic incubator (Anaerocult A, Merck). In all the above cases, the incubation temperature was 28°C.

Reducing sugars were estimated by using the dinitrosalicylic acid reagent, as in the method described by Miller (1959). Different organic acids were estimated by HPLC. The conditions employed and the operation of HPLC were described by Giraud *et al* (1991).

RESULTS

The microbial analysis of dry coffee pulp indicated the presence of endogenous microflora, which contained 10^4 lactic acid bacteria/g dry matter. Under favorable atmosphere, such as water and anaerobic conditions endogenous microflora started to grow and 2 months later, the lactic acid bacterial count increased from an initial of 10^4 to 2.10^5 /g dry matter (Fig. 2) in the ensiled coffee pulp based on natural microflora.

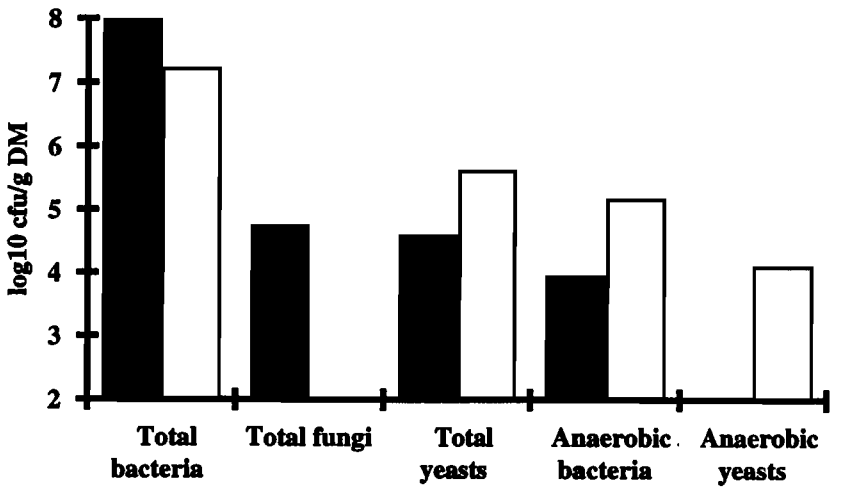

Fig. 2. Evolution of microflora in coffee pulp ensiled without any biological additive. n : Initial coffee pulp, o : Ensiled coffee pulp (2 months)

The microbiological studies indicated highly heterogeneous nature of the microflora, among which the principal genera was *Lactobacillus*. In the ensiled sample, fungi were totally absent, but yeast counts increased significantly, because of the anaerobic conditions prevailing during the ensilage.

The results of physico-chemical analysis of the coffee pulp, ensiled without additives, are presented in Table 1. The ensiled coffee pulp showed a good production of lactic acid (2.39%), a small production of acetic acid (0.29%) and total absence of butyric acid as well as ethanol. The loss of dry matter was very low (0.80%) and the pH was 3.9. However, there was negligible reduction in the caffeine content. These results indicated that ensilage of coffee pulp by natural microflora, without any chemical or biological additives, resulted in a satisfactory silage in many respects. But, the silage contained a lot of yeast, in addition to the presence of highly heterogeneous microflora, and hence it was not of good quality. Therefore, studies were undertaken to evaluate three different biological additives and their effect on the evolution of the microflora in the ensiled material, pH changes, production of organic acids and dry matter loss.

Table 1. Comparative physico-chemical characteristics of the coffee pulp ensiled using natural microflora and biological additives (Perraud-Gaime, 1995).

Parameters	Initial pulp	Ensilage of coffee pulp (28 days)			
		Without additives	Natural microflora	*L. plantarum* A6	Commercial inoculum
Moisture	62,55	61,63	66,78	66,78	66,19
pH	4,44	3,90	3,91	3,92	4,11
DM losses (%)	-	0,80	1,73	1,41	0,38
Lactic acid (%DM)	0,00	2,39	3,35	2,14	0,08
Acetic acid (%DM)	0,00	0,29	0,68	0,48	0,05
Reducing sugars (%DM)	4,72	4,85	4,56	3,67	8,32
Caffeine (%DM)	1,04	0,95	1,02	0,93	0,90

ENSILAGE WITH BIOLOGICAL ADDITIVES

The development of microflora in three ensilage processes is presented in Fig. 3. The microbial population was dominated by aerobic bacteria during the first two days of ensilage. By this time, the lactic acid bacteria grew sufficiently and their number was more or less equal to that of the total aerobic bacteria during the rest of the fermentation period, but only in the case of ensilage with additives of natural microflora or monoculture of *L. plantarum*. In the case of a commercial inoculum, aerobic bacteria continued to be dominant throughout the fermentation period of 30 days.

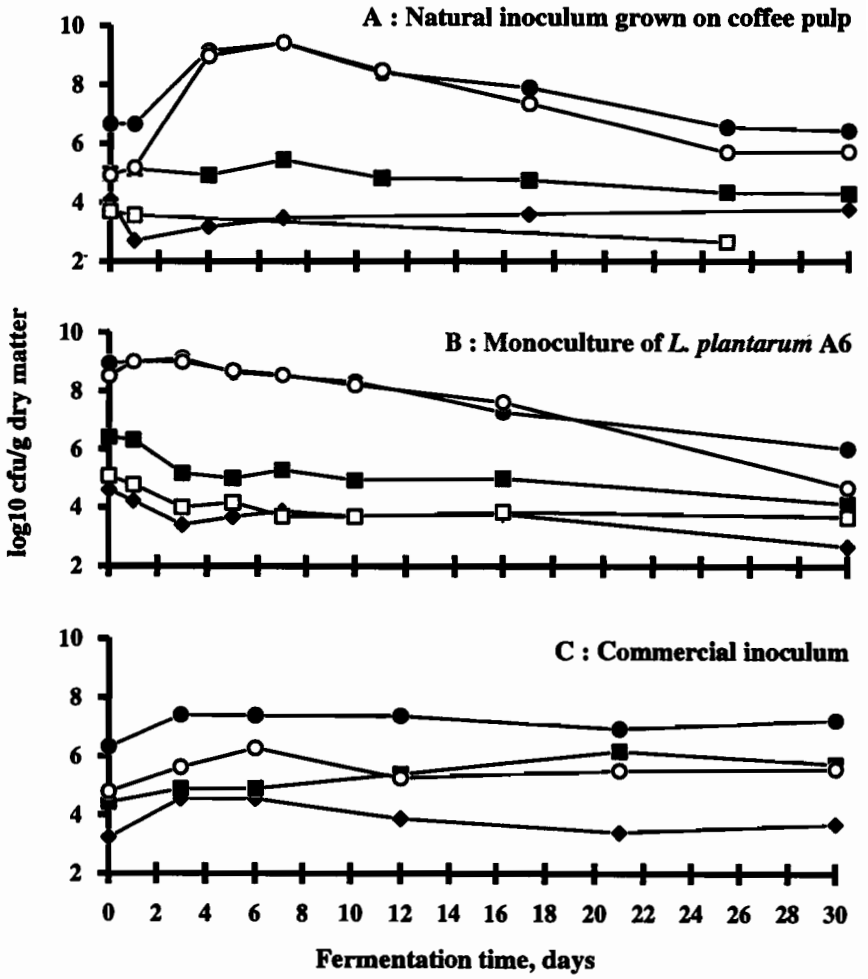

Fig. 3. Development of microflora during the course of ensilage of the coffee pulp with three different biological additives (A, B, C) during 30 days (Perraud-Gaime, 1995). l : Total bacteria, u : Total fungi, n : Total yeasts, m : Total anaerobic bacteria, o : Total anaerobic yeasts.

In ensilage with the addition of natural microflora and monoculture of *L. plantarum*, the initial lactic acid bacterial count of $1.5x10^4$ and $2x10^8$ per g dry matter, respectively, increased to 10^9-10^{10} per g dry matter in about 2-4 days (Figs. 3A, B). In contrast, the lactic acid bacterial population increased from 10^5 to merely about 10^6 even by 6 days, in the case of the commercial inoculum (Fig. 3C). The counts of lactic acid bacteria started decreazsng after 10 days and became stable at 10^5 per g dry matter by the end of ensilage process in all the cases.

Similarly, decrease in the population of the fungi started from the first day of ensilage, but only in the case of ensilage with inoculum of natural microflora and monoculture of *L. plantarum*. The number of total yeasts in the system did not change in the case of use the *L. plantarum* monoculture. Similarly, the anaerobic yeasts, which represent about 10% of the total yeasts, did not change in the medium with addition of natural microflora, in contrast to their decrease in the medium with monoculture of *L. plantarum*. It is of interest to point out that the microscopic examination of the samples revealed that the fungi are present in spore forms, as no vegetative cells were seen. In the case of the commercial inoculum, the picture in terms of the number of yeasts and fungi is totally different than that observed with the other two biological additives. For example, there was no change in fungi during the initial period and these started declining only after 10 days. However, the total yeast count increased slightly. Anaerobic yeasts could not be counted as their number was less than 10^2 per g dry matter.

The evolution of the kinetics of different parameters of ensilage (pH, lactic acid, acetic acid, and sucrose reduction) is presented in Fig. 4, while Table 1 gives the physico-chemical analysis of the coffee pulp ensiled for 28 days.

These results indicate that the quality of the ensiled coffee pulp is satisfactory when natural microflora or monoculture of *L. plantarum* were used as biological additives. The production of lactic acid, in these cases, correlates with the pH changes and these values indicate a good silage. Moreover, the acetic acid formed is also much lower (Fig. 4). In contrast, the silage, made by using commercial inoculum as a biological additive, is of inferior quality, probably because of the poor development of lactic acid bacteria in the system and consequent lower production of lactic acid.

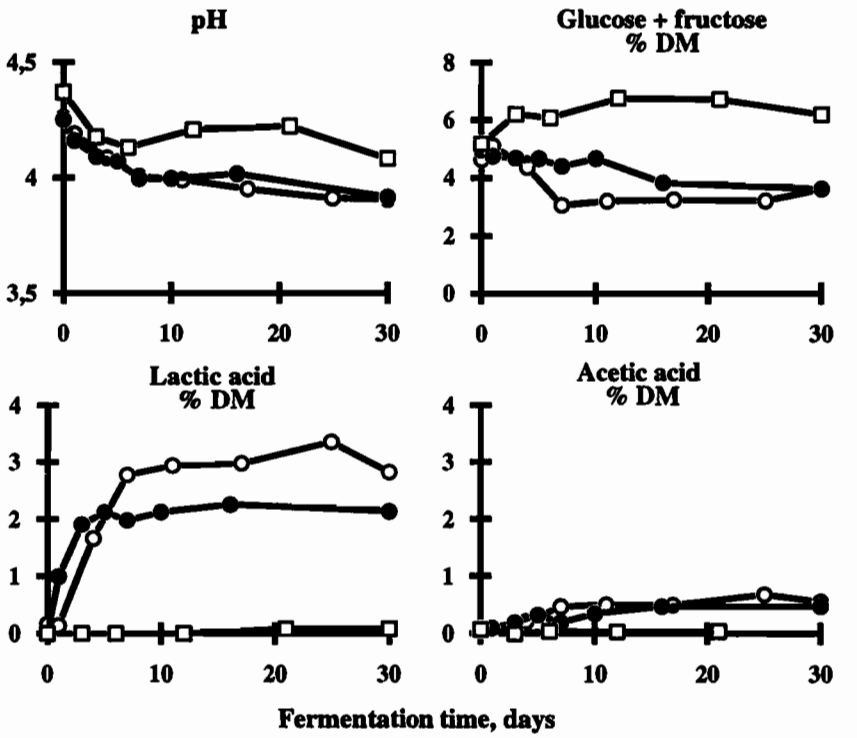

Fig. 4. Kinetics of the changes in physico-chemical characteristics of the coffee pulp ensiled for 30 days with the use of the different biological additives (Perraud-Gaime, 1995). m : Natural microflora grown on coffee pulp, l : Monoculture of *L. plantarum* A6, o : Commercial inoculum.

DISCUSSION

Macroscopic examination of the final product permits to state that the sillage of coffee pulp, obtained during this study, is satisfactory, except in the case of use of commercial inoculum as a biological additive. Of course, the inoculum rate in the case of the commercial inoculum was the lowest among all the experiments, but it was used at that level, as recommended by the society that produces it commercially. The physico-chemical analyses of the coffee pulp ensiled under different conditions (Table 1) support the above statement, as lactic acid is more than 2%, the final pH is less than 4.0, dry matter loss is less than 2%, acetic acid is less than 0.7% and butyric acid as well as ethanol are absent.

The effect of the inoculum as a biological additive has been reported to be positive by Weinberg *et al* (1993), when the endogenous lactic acid bacterial count of the substrate to be ensiled is between 10^2 to 10^4 per g dry matter. The ensilage process becomes slower otherwise and the use of inoculum makes it faster.

In the case of ensilage of dry coffee pulp in laboratory scale microsilos, after hydration and inoculation with the monoculture of *L. plantarum*, the differences in the quality are minor, when compared to the use of inoculum consisting of natural microflora, in spite of the initial lactic acid bacterial count of $2x10^8$ per g dry matter in the former case, opposed to that of $1.5x10^4$ per g dry matter in the latter case. However, it is of interest to note that the lactic acid culture in the former case is homogeneouscompared to the heterogeneous nature of the microflora, in the case of use of natural microflora as an inoculant. Such massive inoculation, in the case of monoculture of *L. plantarum*, allows production of lactic acid at a faster rate, with consequent rapid drop in the pH value.

Total endogenous microflora of the dried pulp was 10^7 per g dry matter and was dominated by total bacteria and, hence, problems will be encountered, when the lactic acid inoculum is low, despite the presence of endogenous lactic acid bacteria in the dry coffee pulp. This may be the reason for unsatisfactory quality of the silage, in the case of use of the commercial inoculum. The level of this inoculum, used in the present studies, was probably lower for the substrate used, i.e., coffee pulp, as this inoculum is made for use in ensilage of forage. Poor development of natural and inoculated lactic acid bacterial population, in the case of use of the commercial inoculum, may also be due to the antagonism between different population of the ensilage coffee pulp. Moreover, the commercial inoculum has been manufactured for use in temperate regions. It is of interest to note that the silage quality was good, when the commercial inoculum was used at a level of 10^8 lactic acid bateria per g dry matter (Perraud-Gaime, 1995).

It has been reported that the endogenous lactic acid microflora of dry coffee pulp is highly heterogeneous (Perraud-Gaime, 1995). In spite of this fact, the use of natural microflora of the coffee pulp gives a good silage. This may be explained by the fact that the endogenous lactic acid flora of coffee pulp have been selected by the substrate itself. Therefore, these are more adapted to grow on the coffee pulp, than the other lactic acid bacterial inoculants grown on liquid media, using sucrose, glucose or lactose as substrate.

The evolution of volatile organic acids is an important indicator of the quality of the silage. It has been reported that the final concentrations of lactic acid should be 3 to 13% (Catchpoole and Henzell, 1981). The silage is also stated to be good, when the production of lactic acid starts the very first day of ensilage, reaches the maximum level by 6 days and then stabilizes (Luis and Ramirez, 1985). In the present studies,

this has been the case, when natural microflora and monoculture of *L. plantarum* were used as biological additives. In both cases, production of lactic acid correlated with pH changes.

Acetic acid is known as an anti-myiotic agent inhibiting the development of fungi and yeasts (Moon, 1983). Therefore, the presence of acetic acid in silage has positive effects. Moreover, the absence of propionic and butyric acids in the silage indicates that endogenous lactic acid flora is mostly homofermentative, while the heterofermentative lactic acid microflora on dry coffee pulp is either absent or represents a minority.

It has been reported that the substrate should have a minimum of 12% reducing sugars, in order to obtain good silage (Demarquilly, 1985). In the case of coffee pulp, the quantity of reducing sugars present is lower, i.e., about 5% (Table 1), but it does not appear to be of any consequences, as the ensiled coffee pulp has the pH value of about 3.9 and, hence, silage is good. As the initial pH of the moist coffee pulp is lower, i.e., 4.3 (Table 1), the quantity of reducing sugars presents in the coffee pulp is probably sufficient to lower the pH to the desirable level. Moreover, the level of reducing sugar does not reduce significantly during the ensilage, as the residual reducing sugar present in the silage is between 3.67 to 4.56% on a dry matter basis (Table 1, Fig. 4). The data, thus, indicate that the production of lactic acid in ensilage of coffee pulp is probably due to degradation of complex sugars, such as hemicelluloses and pentosans (Pettersson and Lindgren, 1990).

Consequently, it is not necessary to supplement the coffee pulp by sugars, such as molasses. It is worth pointing out that Murillo (1974), Ferrer (1984) and Porres *et al* (1993) have used molasses as chemical additive, but the improvement in the quality of the silage was negligible. It seems more reasonable to use biological additives, such as a lactic acid bacterial inoculant and an enzyme complex, to achieve higher degradation of the constituents of the cellulosic components of the vegatative cell-wall (Vanbelle *et al*, 1994). This observation is supported by production of sugars in the silage, in the case of use of the commercial inoculum (Fig. 4). Data, thus, indicate that the conditions (acidic pH, temperature and contact of cellulases with substrate) employed in the present studies are favourable and optimum for the cellulolytic activity present in the commercial inoculum. In fact, the residual sugars in the silage can lead to the production of a higher quantity of lactic acid, using a higher inoculum.

However in all cases, negligible degradation of caffeine was observed in the present studies (Table 1). Moreover, the loss of dry matter was very low without production of water drainage during ensilage (Table 1). These results indicate that the caffeine is not degraded by the lactic acid bacteria. This is in contrast to the report that the caffeine is degraded during the ensilage of coffee pulp, with the use of natural

microflora and added lactic acid as a bacterial inoculum (Porres *et al*, 1993). It, thus, appears that the loss of caffeine observed by these workers is actually not due to the activity of lactic acid bacteria. It was probably due to increased solubility of caffeine in water at low pH values. Hence, the evacuation of water drains during ensilage reduced the caffeine content of the pulp. Studies to support these facts are contemplated.

CONCLUSION

Data allow us to conclude that ensilage is a good technique for preservation of wet coffee pulp. The endogenous lactic acid flora of dry coffee pulp is sufficient enough to produce a good quality of silage. However, addition of biological additives, such as lactic acid bacterial inoculants and enzymes, allows the improvement of the quality of the silage, in terms of augmentation of lactic acid production, without concomitant production of volatile organic acids and ethanol. Caffeine is not degraded during the process and hence it is necessary to decaffeinate the coffee pulp with appropriate fungi by solid state fermentation (Perraud-Gaime and Roussos, 1995), if the ensiled coffee pulp is to be used for animal feeding, as caffeine has antiphysiological effects (Bressani *et al*, 1972).

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Selection of filamentous fungi for decaffeination of coffee pulp in solid state fermentation prior to formation of conidiospores

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SUMMARY

Decaffeination of coffee pulp, to eliminate its antiphysiological effects on animals, was studied by aerobic fungal solid state fermentation, prior to the stage of initiation of conidiospore formation. Comparative data on performance of two strains of *Penicillium* and six strains of *Aspergillus* spp., selected for their high ability to degrade, indicated the potential of *Penicillium* sp V33A25 for caffeine degradation in aerobic solid state fermentation, before the initiation of sporulation by the culture. Kinetic studies pointed out that the evolution of CO_2 is the reliable criterion for the determination of the phase of fermentation, caffeine degradation, increase in medium pH and initiation of sporulation, without taking sample and subjecting it to analyses or disturbing the fermentation. These advantages are not available, if rise in pH the medium is selected as a criterion. Amongst 7 different factors, the fermentation temperature, level of $CaCl_2$ in the medium and autoclaving or non-autoclaving of the medium exhibited strong effects on the initial time of sporulation, extent of CO_2 evolution, pH of the medium and caffeine degradation. The data allow to envisage the use of mixed culture of lactic acid bacteria and filamentous fungi for decaffeination and ensilage of the coffee pulp, or in two stage fermentation, involving any of the simpler order.

Keywords: Solid state fermentation, coffee pulp, aerobic process, filamentous fungi, *Penicillium, Aspergillus*, caffein degradation, spore formation, criterion for fermentation stage, CO_2 evolution, effects of parameters, medium autoclaving, fermentation temperature, calcium chloride.

RESUME

Selection de champignons filamenteux pour la dégradation de la caféine de la pulpe de café en milieu solide et avant la sporulation des souches.

PERRAUD-GAIME I. et ROUSSOS S.

La décaféination de la pulpe de café, pour éliminer son effet antiphysiologique en alimentation animale, a été étudié en fermentation solide en utilisant des champignons filamenteux. L'objectif est d'obtenir la dégradation de la caféine avant la phase de sporulation de manière à pouvoir tester en association avec des bactéries lactiques au cours d'un procédé de conservation du substrat décaféiné. Des études préalables réalisées en coopération avec l'ORSTOM et la UAM d'Iztapalapa de Mexico ont permis de sélectionner 2 *Penicillium* et 6 *Aspergillus* pour leur haute capacité à dégrader la caféine. Après un criblage en Fermentation en Milieu Solide de ces souches, nous avons sélectionné *Penicillium* sp. V33A25. Ce microorganisme dégrade la caféine de la pulpe de café à 94% après 30 heures de fermentation aérobie, avant la phase de sporulation. La croissance du champignon et la dégradation de la caféine sont parfaitement corrélés à la respirométrie, technique qui nous permet de suivre en continu le développement des microorganismes en FMS. D'autre part, un plan d'expérience nous a permis de constater que la stérilisation du substrat n'a pas d'influence sur la dégradation de la caféine.

Mots clés: Fermentation en Milieu solide, pulpe de café, procédé aérobie, champignons filamenteux, *Penicillium*, *Aspergillus*, dégradation de la caféine, formation de conidiospores, critères d'avancement de la fermentation, effets de paramètres, stérilisation des milieux, température de fermentation, évolution du CO_2, chlorure de calcium

INTRODUCTION

It is of economic and industrial importance to note that only 5.8% of the solids of the coffee berry result in the ultimate coffee drink and the remaining 94.2% forms water and various byproducts (Zuluaga, 1989). Among the latter, the coffee pulp is the maximum and represents 40% of the coffee berry in wet form (Tauk, 1986), corresponding to 29% of dry matter (Bressani *et al*, 1972). This large quantity of the

coffee pulp poses problems of disposal to coffee berry producers, due to putrefaction and causes environmental pollution if not disposed after appropriate treatment (Zuluaga, 1989). Due to its high organic matter content, coffee pulp can be utilized for beneficial purposes and intensive research on this topic has been carried out at ORSTOM (Roussos *et al*, 1995) and also in collaboration with Universidad Autonoma Metropolitana (UAM-I), Mexico (Viniegra-Gonzalez *et al*, 1991).

Direct use of coffee pulp in animal feeding poses problems, due to its chemical composition (Viniegra-Gonzalez *et al*, 1991). For example, the coffee pulp of *Coffea arabica* contains approximatelly 1% caffeine and has antiphysiological effects on the animals (Braham *et al*, 1973; Cabezas *et al*, 1974, 1976; Vargas *et al*, 1982). It is, therefore, necessary to decaffeinate the coffee pulp, before its use as animal feed. Moreover, the coffee pulp gets putrified, because of its high content of water and, hence, needs preservation by appropriate economic technique. At ORSTOM, Montpellier, the techniques of ensilage and fungal degradation of caffein by solid state fermentation (SSF) have been selected for preservation and decaffeination of the coffee pulp, respectively, because of their economic character. If these two techniques are applied in succession, it is of vital importance that the decaffeination by fungi is achieved before the formation of conidiospores. In the case of conidiospore formation, it will be essential to sterilize the decaffeinated coffee pulp, before ensiling. However, mycelial cells of fungi can be eliminated during ensiling and hence sterilization step can be avoided to achieve economy (Perraud-Gaime, 1995).

A total of 350 fungi have been isolated from coffee domains (coffee plants, soils of coffee plantation, coffee byproducts, fermenting coffee berries, etc.) during the research at ORSTOM and UAM (Aquiahuatl *et al*, 1988; Viniegra-Gonzalez *et al*, 1991; Roussos *et al*, 1995). From this collection, a total of 8 filamentous fungi, representing two strains of *Penicillium* and 6 strains of *Aspergillus*, were selected for use in the present studies, based on their higher capacity to degrade caffeine to the extent of 90 to 100% in liquid culture (Roussos *et al*, 1989). One of the *Penicillium* strains selected (V33A25) showed negative effect on caffeine degradation, upon the addition of inorganic nitrogen to the medium in SSF process (Roussos *et al*, 1994).

The objective of this study was to select one or more of the filamentous fungi to grow in SSF and to degrade caffeine to the extent of 80%, before the initiation of conidia formation. Work was also carried out to develop a simple criterion, to correlate growth of the fungi, degradation of caffeine and sporulation time, so that it can be used to stop fermentation at the most appropriate stage.

MATERIALS AND METHODS

MICROORGANISMS

The fungal cultures (2 strains of *Penicillium* and 6 strains of *Aspergillus* species) are maintained on sucrose-coffee-medium plates at 4°C by subculturing every 3 months (Perraud-Gaime, 1995). The methodology for inoculum preparation was, as described elsewhere (Roussos *et al*, 1995).

SUBSTRATE PREPARATION AND FERMENTATION

The coffee pulp used in the present study, was obtained from a industry in Xalapa, Mexico, which processes the berries of *Coffee arabica* by a wet fermentation method. The wet coffee pulp was sun-dried by the industry to contain less than 10% moisture and packed in plastic sacs. The dry coffee pulp was coarsely ground and sieved to obtain a particle size of 0.8 to 2 mm (Perraud-Gaime and Roussos, 1995).

The coffee pulp was moistened to contain 68% final moisture, with a nitrogen source free of mineral salt solution, which contained (g/L) KH_2PO_4 1.3, Na_2HPO_4 0.12, $MgSO_4.7H_2O$ 0.3, $CaCl_2.2H_2O$ 0.3 and distilled water 1000. The moist pulp was sterilized at 121°C for 20 min, inoculated with a spore suspension of a culture to obtain $2x10^7$ spore/g dry matter, 80 g moist inoculated medium was charged in to a column and compacted slightly (Raimbault and Alazard, 1980). The column was installed on the humidifier and placed in a constant temperature water bath. The details are presented in Fig. 1.

The fermenter allows continuous monitoring of the parameters, with display on a computer, throughout the fermentation period and every column functions independently. It is possible to pull out a column and the content of the entire column constituted each sample. Exhaust gas was collected from each column and separately analysed for CO_2 and O_2 in gaseous form in the analyzers. These analyzers are equipped for detectors, thermal conductors and a computer to analyze the gases continously, during the course of fermentation (Dufour, 1990; Saucedo-Castañeda, 1991; Soccol, 1992; Trejo-Hernandez, 1992). Thus, the development of the culture in each column can be visually seen on the computor, in terms of O_2 consumption and CO_2 evolution (Fig. 1).

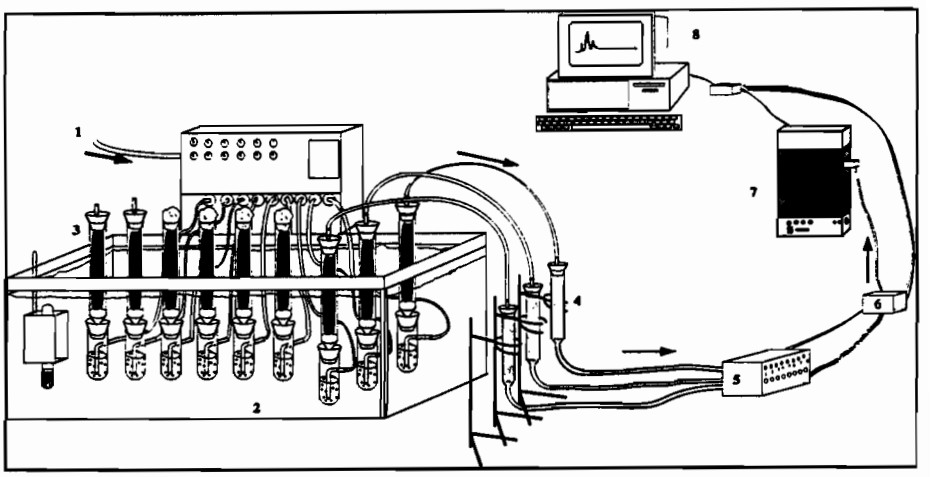

Fig. 1. Protocol for preparation of column for solid state fermentation, with details of set-up and analytical as well as monitoring devices.

1: Air inlet 2: Water bath 3: Column fermenters 4: Silica gel columns 5: Sampler 6: Automatic gas injector 7: Gas chromatograph 8: Computer

SAMPLE PROCESSING AND ANALYSES

Schematic diagram of sample processing and analyses is shown in Fig. 2. Caffeine was estimated by gas chromatography, using the method of Vitzthum *et al* (1974). The details of the methods for estimation of other parameters are similar to those reported elsewere (Perraud-Gaime and Roussos, 1995; Roussos *et al*, 1995).

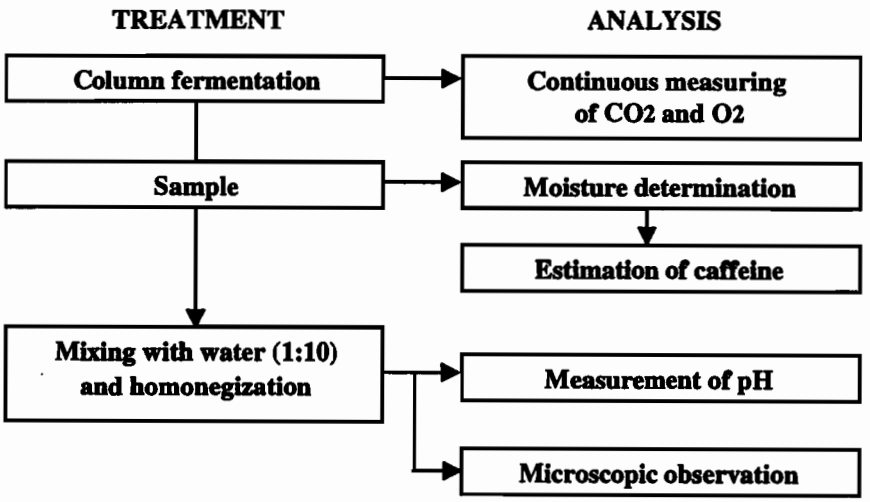

Fig. 2. Schematic diagram of the sample treatment and analyses.

RESULTS AND DISCUSSION

SELECTION OF THE CULTURE

The main objective of the study was to select one or more filamentous fungi, with the ability to degrade caffeine, before initiation of sporulation. This is of importance, as the selected fungal strain is intended to be used for caffeine degradation, before ensiling of the coffee pulp for its preservation. The resulting decaffeinated and ensiled coffee pulp, thus, can find use in animal feeding, without antiphysiological effects of caffeine. The data on growth and metabolism of the 8 fungal cultures studied are presented in Table 1. These fungi have been earlier studied in liquid culture and showed high caffeine degradation capability (Roussos *et al*, 1995).

The comparative analysis of the data allows to eliminate *Aspergillus* sp. strains C16A25 and C23B25, especially because the residual level of caffeine in the medium was higher, even when the strains started to sporulate, as compared to other strains.

The data also allow to eliminate *Aspergillus* sp. C11B25, whose lag phase is very long (20 h) and the level of degradation of caffeine at 30 h is very low (Table 1). *Aspergillus* sp V12A25 sporulates very early (28 h) and is not of interest for the present requirement.

Among the remaining 4 strains, *Penicillium* sp V33A25 and *Aspergillus* sp. C28B25 are the most effective, based on the level of caffein degradation (about 94%) at 30 h (Table 1). These two strains sporulated at 30 and 32 h, respectively, and the lag phase was also shorter (11.0-11.5 h). The data on CO_2 evolution and respiratory coefficient also show a good growth and metabolism of these two fungi on coffee pulp (Table 1).

Table 1. Comparative data on growth and metabolism of the filamentous fungal cultures in column fermenters under solid state fermentation (Perraud-Gaime, 1995).

Strains	Lag phase h	CO_2 production ml/g MSI	Respirometry coefficient h^{-1}	Caffeine degradation at 30 h, %	Time of initiation of sporulation
Penicillium sp. V26A25	12,5	115	0,34	91	32 h
Penicillium sp. V33A25	11,5	95	0,34	94	30 h
Aspergillus sp. C16A25	13,0	100	0,30	80	32 h
Aspergillus sp. V12A25	10,5	130	0,29	82	28 h
Aspergillus sp. C17B25	17,0	65	0,34	87	32 h
Aspergillus sp. C11B25	20,0	65	0,26	12	42 h
Aspergillus sp. C28B25	11,0	100	0,30	94	32 h
Aspergillus sp. C23B25	11,0	85	0,34	79	30 h

Among these two filamentous fungi of good potential, *Penicillium* sp. V33A25, was choosen in the present studies, as this strain is also used in ORSTOM in other studies (Roussos *et al*, 1989; Denis, 1992) and it also fulfills the requirements of this study to the highest extent. For example, the degradation of caffeine in aerobic solid state fermentation is 94% at 30 h, and the sporulation starts only at this stage.

FORMULATION OF CRITERION

The second objective of the study was to formulate a reliable criterion to correlate maximum degradation of caffeine and time of sporulation as an appropriate fermentation parameter, for its use in simpler monitoring of the fermentation of the

culture in columns in solid state fermentation. The results of the experiments conducted for this purpose are presented in Fig. 3, in terms of growth and metabolism of *Penicillium* sp V33A25 on coffee pulp in column fermenters.

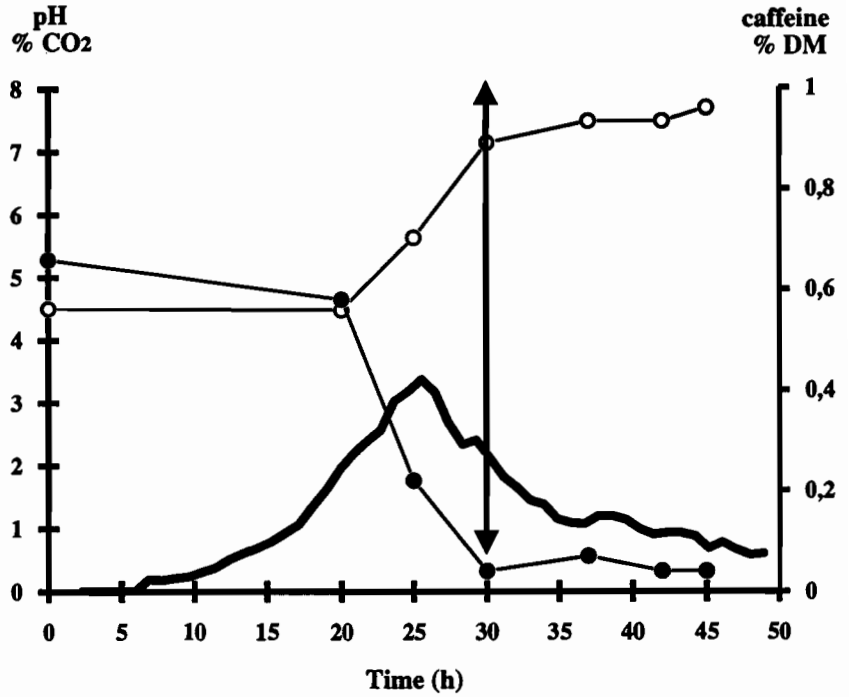

Fig. 3. Profile of pH (m) and caffeine (l) as against CO_2 (—) evolution during the course of the growth and metabolism of *Penicillium* sp. V33A25 on coffee pulp at 25°C. Arrow indicates the phase of initiation of sporulation (Perraud-Gaime, 1995).

Data revealed that the pH of the medium started increasing from 20 h fermentation to reach the level of 6.8 at 30 h, the time at which sporulation was also initiated. The begining of sporulation in the medium at 30 h is represented in Fig. 3 by a verticle arrow. Data on the kinetics of caffeine degradation also perfectly correlate with this stage, at which the pH of the medium starts to increase. Thus, the stage at which pH starts to stabilize could be taken as a criterion to indicate maximum caffeine degradation and initiation of the sporulation stage. However, its utility is

limited, as it is necessary to take the sample and process it to measure the pH before taking the decision to stop the fermentation.

The fermentation system allows to continuously visualize the evolution of CO_2 on the computor, without any necessity to remove a sample, process and analyse. In fact, this does not disturb the fermentation process. Hence, its selection as the reliable criterion is better, as it also allows to distinguish the phases of growth and metabolism during the course of fermentation. For example, the CO_2 evolution curve clearly shows the lag, germination and growth phases, while the last one can also be seen visually by observing the fermenting solids in the column. During the growth phase, the substrate is completely surrounded by the mycelial cells. The spore formation is initiated, after about 5 h from the stage of maximum CO_2 production. Thus, if the fermentation is stopped after 3 h of maximum CO_2 production, it is the fermentation stage at which the level of caffeine in coffee pulp is less than 0.2% on a dry weight basis and there are no spores in the medium.

Hence, data of the respirometry analysis was selected as a criterion to indicate the most important step of the development of fungi and to correlate it with maximum degradation of caffeine, prior to initiation of the conidiospore formation.

EFFECT OF FACTORS ON FERMENTATION EFFICIENCY

As for the last objective of the study, a number of experiments have been carried out to evaluate the effects of seven different parameters on the level of caffeine degradation, CO_2 evolution, final pH of the medium and initiation of sporulation. These seven factors tested were a) with or without the use of mineral solution, b) fermentation under natural light or in darkness, c) aeration at 40 or 70 ml/min, d) fermentation at 25 or 35°C, e) with or without autoclaving of the substrate, f) inclusion of $CaCl_2$ in the mineral solution at 0.1 or 0.3 g/l and h) initial pH of the mineral salt solution at 4.4 or 5.6. All the other factors were constant, i.e., 68% initial moisture content of the medium and inoculum level of $2x10^7$ spores/g dry matter.

Graphic representation of the relative effects of these seven different factors is given in Figure 4. The results show that the incubation temperature is the most important factor. The fermentation needs to be conducted at 25°C for the best degradation of caffeine and good development of the fungi. These observations are in agreement, with those reported by Roussos *et al* (1989). The rate of aeration, initial pH of the medium, presence or absence of mineral salt solution and fermentation under natural light or in darkness have non-significant influence on all the four parameters studied.

It was felt that it is better to increase the concentration of $CaCl_2$ in the medium to favour the mycelial growth and delay sporulation.

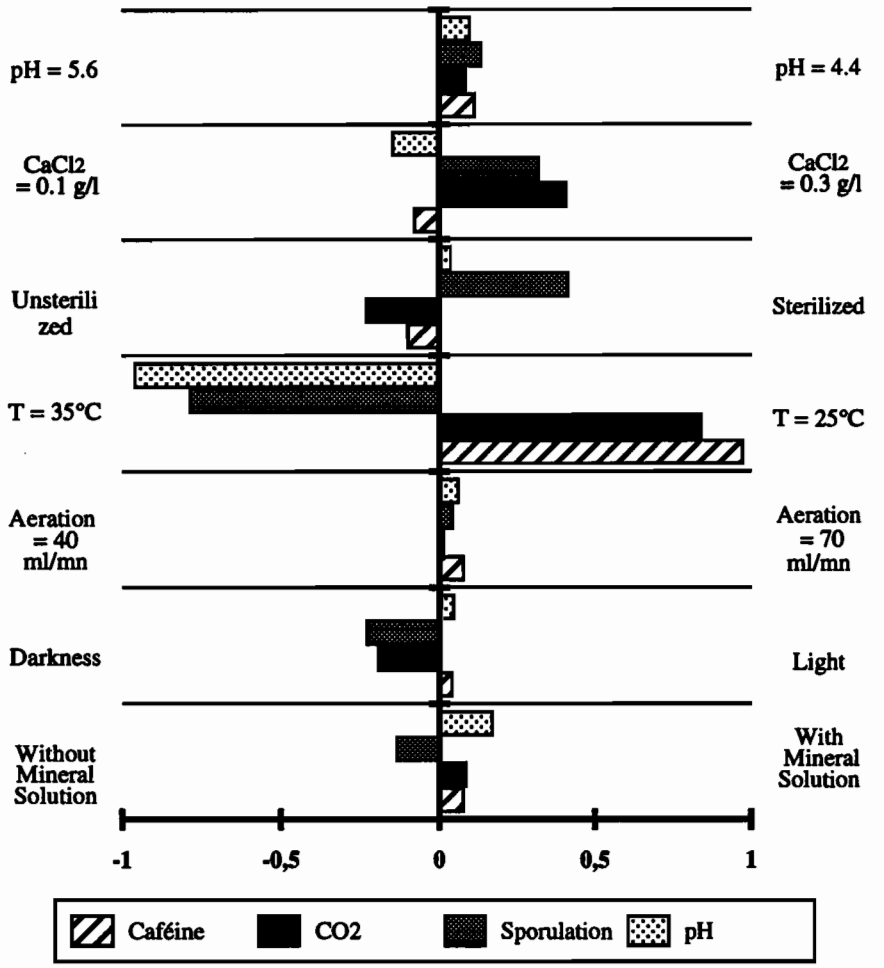

Fig. 4. Graphic analysis of the effects of various factors on the degradation of caffeine, evolution of CO_2, initiation of sporulation and final pH of the moist solid medium (Perraud-Gaime, 1995).

Another economically important result of these experiments is the demostration that the medium sterilization or its use without any sterilization has no influence on the degradation of caffeine (Fig. 4). This observation allows to envisage the use of this process of degradation of caffeine in the coffee pulp by filamentous fungi in SSF,

after its ensilage by lactic acid bacteria, without sterilization. It might be even possible to firstly subject the coffee pulp to degradation of caffeine and then preserve by ensilage.

CONCLUSION

It can be concluded that it is possible to decaffeinate the coffee pulp in 30 h under aerobic conditions by using selected fungal culture, i.e., *Penicillium* sp. V33A25 in solid state fermentation, before initiation of the sporulation by the strain. It is also not necessary to sterilize the substrate. It is, therefore, possible to envisage the inoculation of the coffee pulp with mixed culture of lactic acid bacteria, for the ensilage preservation of coffee pulp, along with the selected filamentous fungi, for degradation of the caffeine. It can lead to decaffeinated and stabilized coffee pulp, which is suitable for animal feeding.

It is also possible that the stages of the fermentation can be observed visually on the computer, through respirometric parameters, without removing the sample and subjecting it to analyses and also without disturbing the culture medium. This factor of CO_2 evolution permits to reliably estimate different phases of the development of *Penicillium* sp. V33A25, in terms of degradation of caffeine and time of the sporulation of the filamentous fungi.

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Grape pulp, grape pips and their mixture: Novel substrates or supports for solid state fermentation

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SUMMARY

Grape marc, comprising of grape pulp and grape pips, is generated in high quantities during ethanol production, but has no significant commercial value. The components of the grape marc, individually or in combination, can serve as substrate or support in solid state fermentation. The potential benefits of sieved fractions (800-3150 µm particle size) was, therefore, evaluated using *Trichoderma viride*, with or whithout enrichment. Based on sorption isotherm profiles, grape pulp showed the best performance, though its ability to promote biomass formation was the lowest. The latter could be improved by enrichment with glucose. It, thus, offers potential as a reusable support. Grape pips promoted high biomass, without any enrichment, but was low in water retention capacity. The performance of the mixture of grape pulp + grape pips (1:1, w/w) was in between that of the individual fractions. Data also indicated that the mycelial fragments frozen at -20°C up to 15 days can be used as an inoculum.

Keywords: Grape marc, grape pulp, grape pips, grape pulp+grape pips, solid state fermentation, sorption isotherms, water activity, novel substrate, novel support, mycelial growth, metabolic activity, thawed mycelia, bioluminescence, ATP, *Trichoderma viride*.

RESUME

Pulpe de raisin, pépins de raisins et leur mélange: Nouveaux substrats ou supports pour la fermentation en milieu solide.

BENSOUSSAN M. ET SERRANO-CARRÉON L.

Le marc de raisin, dont sont issus la pulpe et les pépins, est un sous-produit de la vinification largement disponible en sortie de distillerie, mais sans grande valeur commerciale. Les constituants du marc, individuellement ou en mélange, peuvent être utilisés comme substrats ou supports en fermentation en milieu solide. Ainsi, en présence de *Trichoderma viride*, les aptitudes de fractions solides tamisées, aux dimensions comprises entre 800 et 3150 µm, ont été comparées avec et sans enrichissement. Sur la base des isothermes de sorption, la pulpe de raisin montre la plus grande rétention d'eau, bien que son aptitude à favoriser la formation de biomasse soit la plus faible. Cette dernière peut être améliorée par enrichissement en glucose. Ainsi, la pulpe se présente potentiellement comme un support réutilisable. Les pépins de raisins favorisent les plus fortes productions de biomasse, même sans enrichissement, mais ils ont les plus faibles capacités de rétention d'eau. Les performances du mélange pulpe + pépins (1:1, m/m) correspondent aux valeurs moyennes des fractions individuelles. Les résultats indiquent aussi que le mycélium conservé congelé à -20°C durant moins de 15 jours peut être utilisé comme inoculum.

Mots clés: Marc de raisin, pépins de raisin, mélange marc et pépins de raisin, fermentation en milieu solide, isothermes de sorption, activité de l'eau, nouveau substrat, nouveau support inerte, croissance mycélienne, activité métabolique, mycélium congelé, ATPmétrie, *Trichoderma viride*.

INTRODUCTION

Grape marc is derived from the ethanol manufacturing process. It does not have a significant commercial value, but is available in large quantities from the alcohol distilleries (Mustin, 1987). As alcohol is removed, this waste material is fractionated and two by-products are separated and dehydrated. These included a) the grape pulp,

rich in glycoproteins and pectins, which is generally used as a feedstuff (Vaccarino *et al*, 1992) or as a soil improver (Delas, 1967; Faure and Deschamps, 1990) and b) the grape pips, which have a hard lignocellulosic envelope and provide an edible oil containing a high level (72%) of linoleic acid (Frega *et al*, 1982).

In order to find a potential of the grape marc as a substrate in solid state fermentation (SSF), individual or mixed fractions of pulp and pips were inoculated with a cellulolytic strain of *Trichoderma viride,* previously investigated for lipid accumulation (Serrano-Carréon *et al*, 1992) or aroma production (Serrano-Carréon *et al*, 1993).

MATERIALS AND METHODS

MICROORGANISMS AND INOCULUM PREPARATION

Trichoderma viride was cultivated and stored at 4°C on potato dextrose agar (PDA) slants.

Liquid culture medium for mycelium production contained ($g.L^{-1}$) malt extract 20 and glucose 10. It was inoculated with five days old mycelium (1 cm2-disk) from PDA medium and incubated at 27°C for 72 h on a rotary shaker (100 rpm). The mycelium biomass was recovered after centrifugation (8000 g, 20 min), washed three times and suspended in saline (distilled water with 0.9% NaCl). The mycelium suspension (10 $g.L^{-1}$) was homogenized in a Virtis grinder (The Virtis Co. Inc., Gardiner N.Y.) to obtain viable mycelium fragments of 50 to 200 µm lenght. Part of the mycelial suspension was stored at -20°C in small polycarbonate tubes (1.2 ml) for 7, 15 and 30 days.

THE SOLID SUBSTRATES AND CULTURE MEDIA

Dehydrated grape pulp and grape pips were provided from Fédération Provençale des Distilleries du Gard et de Vauvert (FINEDOC Group). These by products were derived from the processing of two grape varieties: i.e., aramon and grenache. They were slightly crushed. By sieving, several homogenous portions with different size were separated.

The particles used in this study had a size ranging between 800 and 3150 µm. These particles were washed, dried and placed in a 10 g (dry matter, D.M.) quantity for SSF culture in Petri dishes. All samples were enriched with mineral nitrogen (100 $g.L^{-1}$) aqueous ammonium sulfate solution). The initial moisture of the substrates was adjusted to 60%, allowing a water activity of around 0.90. The C/N ratio was 10 in the case of additional carbon sources (either glucose: aqueous solution, 100g. L^{-1} or grape pips oil: aqueous emulsion, 100 ml. L^{-1}). Aqueous solutions and emulsion were separately autoclaved.

All the above moist media, distributed in a uniform layer, were inoculated with a heavy suspension (5 µg mycelium/ g dry matter) of thawed mycelia.

SUBSTRATE ANALYSIS

The concentration of total sugars in the sieved substrates was estimated by the anthrone method (Hassid and Neufeld, 1964). Reducing sugars concentration was measured at 575 nm, following the method of Miller (1959).

The dry matter of the samples (10 g) was measured in triplicate, after drying for 72 h in an oven (60°C). Maximum capacity of the substrate for water retention was estimated after hydration of the samples (10 g). Water activity measurements (Debeaufort, 1994) were carried out in triplicate on samples of the size of 0.5 g, using an Aqualab CX-2 (Decagon, Washington D.C., USA).

Sorption isotherms of pulp, pips and a mixture of pulp and pips (1:1, w/w) were determined by measuring the water activity (Aw) versus the water content (g water/100 g of dry matter) of crude samples.

The pH was estimated, according to Lossin (1970), by suspending 10 g of solid substrate in 50 ml of distilled water. The measurement was made after homogenization with an UltraTurax (20 000 rpm, 2 min).

GROWTH AND METABOLIC ACTIVITY OF THE FUNGAL STRAIN

Radial elongation of the mycelium on PDA was measured in triplicate by estimating the increase in the colony diameter (Pirt, 1967; Trinci, 1971). Metabolic activity of the mycelium on grape marc by-products was estimated by the mycelial ATP level in the culture medium (Cochet *et al*, 1984; Thierry and Chicheportiche, 1988). The assay is based on an ATP-dependent reaction leading to light production (in relative light unit, RLU).

Mycelial ATP concentration was measured using a photon detector (BioCounter 2010A, Lumac-Perstorp Analytical, Paris) and the luciferin-luciferase assay method, as seen in the following reaction:

$$\text{luciferin+mycelial ATP} \xrightarrow[Mg^{++}]{\text{luciferase}} \text{(luciferin-AMP)} + \text{pyrophosphate}$$

$$\text{(luciferin-AMP)} + O_2 \xrightarrow{+light} \text{oxyluciferin} + \text{AMP} + CO_2$$

In this enzymatic reaction, if the other compounds (luciferin and O_2) are maintained in excess, the rate of the reaction only depends on the ATP concentration.

The sample (0.1 g) is suspended in 1 ml aqueous DMSO solution (90%, v/v) to favour permeabilisation of the cell walls (Hysert *et al*, 1976). This suspension (100 µl) was diluted in 900 µl of Tris-HCl (pH 8). The last buffered dilution (0.1 ml) was mixed with an equal volume of the Lumit reactive compound (a complex luciferin-luciferase + Mg^{++}). The measurement of the light produced during 10 sec (RLU 1) was performed using a photon detector. It corresponds to the sum of the intracellular ATP and the free ATP in the culture medium. Each measurement was immediatly followed by a calibration of the integrator by the assay of standard ATP (Lonvaud-Funel and Joyeux, 1982) which gave the value of RLU 2.

The quantity of the mycelial ATP expressed in pg for 100 µl diluted sample is :

$$X = \frac{\text{RLU 1 - RLU 0}}{\text{RLU 2 - RLU 1}} \times A$$

where, RLU 0 is the value obtained in the culture medium before inoculation and A the quantity in pg of standart ATP used for the calibration.

RESULTS AND DISCUSSION

PHYSICOCHEMICAL ANALYSIS OF SUBSTRATE

The mean chemical composition of dehydrated by-products of the grape marc (ITEB, 1988) is presented in Table 1. In the two by-products, crude cellulose constituted a large part of the dry matter.

The crushing treatment, applied before inoculation, will, thus, allow us to brake the hard lignocellulosic sub-fractions, particularly the envelope of the pips and improve the mycelial growth on the substrate.

Table 1. The chemical composition of dehydrated grape marc by-products (ITEB, 1988).

Attribute	PULP	PIPS
Dry matter (%)	85-95	85-95
Mineral matter (% D. M.)	3-13	2-5
Total Nitrogen (% D. M.)	12-17	7-12
Soluble Nitrogen (% N. Total)	9-18	20-40
Crude cellulose (% D. M.)	22-35	42-59
Fats (% D. M.)	5-10	9-18
Tanins (% D. M.)	1.4-3.5	1.1-2.4

Additionnal data have been obtained on the sieved fractions (particle size 800-1350 µm length). Table 2 presents the mean values of 3 samples.

Table 2. Additionnal data from sieved fractions.

Attribute	PULP	PIPS
Dry matter (%)	92.2 (1.2)	92.7 (0.8)
Water retention maxima (%D.M.)	67.0 (3.0)	51.0 (2.0)
Total sugars (% D.M.)	21.2 (0.8)	38.0 (0.5)
Reducing sugars (% D.M.)	6.3 (0.4)	9,0 (0.5)
pH	5.1 (0.2)	5.9 (0.1)

According to data presented in Tables 1 and 2, grape pips exhibited higher levels of nutritive compounds, in terms of the sugars, fats and soluble nitrogen. The acidity of these by-products is favourable for fungal growth.

SORPTION ISOTHERMS

Sorption isotherms have been carried out at 27°C (Fig. 1) on sieved substrate of particles size ranging between 800 and 3150 µm.

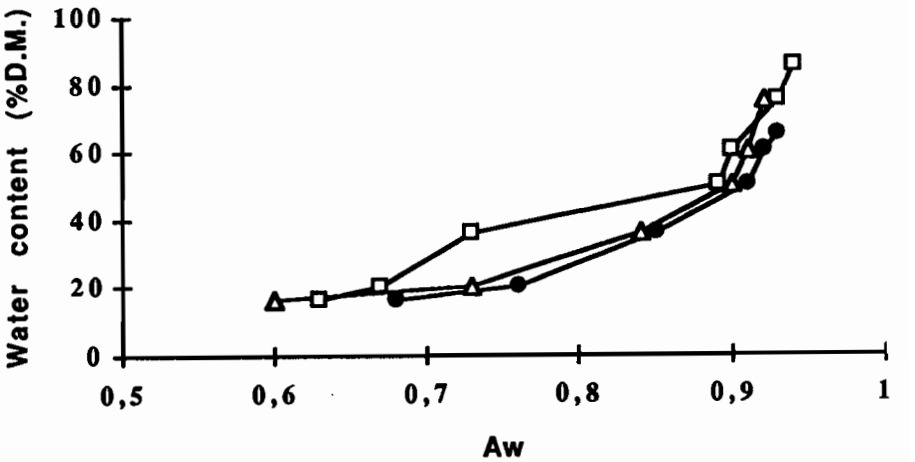

Fig. 1. Sorption isotherms of sieved fractions (800-3150 µm) of grape pulp (❑), grape pips (●) and mixtures (Δ).

Sorption isotherms permitted the estimation of the relationship between water content and water activity (Aw) of moist substrates consisting of the pulp, pips or a mixture of pulp and pips (1:1, w/w).

The sorption isotherm of grape pulp appeared to be clearly separated from those of the pips or of the mixture pulp and pips.

On a large Aw range (0.68 - 0.89) the moisture contents of the pulp samples were higher than those of the pips or the mixture of pulp and pips. The increase in water content (10 to 15%) was rather constant between the Aw limits of 0.73 - 0.85.

GROWTH KINETICS OF THAWED MYCELIUM

Fresh mycelium and mycelium frozen 7, 15 or 30 days were inoculated in triplicate on PDA and incubated at 27°C.

Table 3. Average elongation of fresh and thawed *Trichoderma viride* mycelium incubated on PDA at 27°C.

Freezing time (days)	Radial elongation ($mm.h^{-1}$) at		
	24 h	***48 h***	***72 h***
0	12 (0.01)	0.35 (0.04)	0.67 (0.08)
7	9 (0.02)	0.32 (0.03)	0.63 (0.03)
15	9 (0.01)	0.25 (0.02)	0.51 (0.06)
30	0	0.13 (0.01)	0.33 (0.03)

Radial elongation ($mm.h^{-1}$) of the mycelium is affected by the freezing time (Table 3). Mycelial suspensions frozen for more than 15 days resulted in a lag phase of 24 h, when used as inoculum. Hence, these were not used for inoculation of grape marc by-products.

METABOLIC ACTIVITY OF THAWED MYCELIUM

Solid state fermentation of grape pulps, grape pips and a mixture of them has been carried out with frozen mycelium up to 15 days.

Metabolic activity of *Trichoderma viride* , reflected by the total living biomass, was evident after 96 h, by following the ATP concentration in the culture medium and using the luciferin-luciferase assay. This technique is not time consuming and has been previously positively correlated to ergosterol concentrations, as an indicator of fungal aquatic biomass growing on dried plant material, after being submerged in water (Suberkropp *et al*, 1993).

ATP is a primary metabolite essential for providing energy to the synthesis reactions of the fungal cells. It represents an especially reliable indicator of the metabolic activity, as it cannot accumulate in the culture medium because of its rapid degradation. Fig. 2 reports the average ATP concentration established from three assays of each sample.

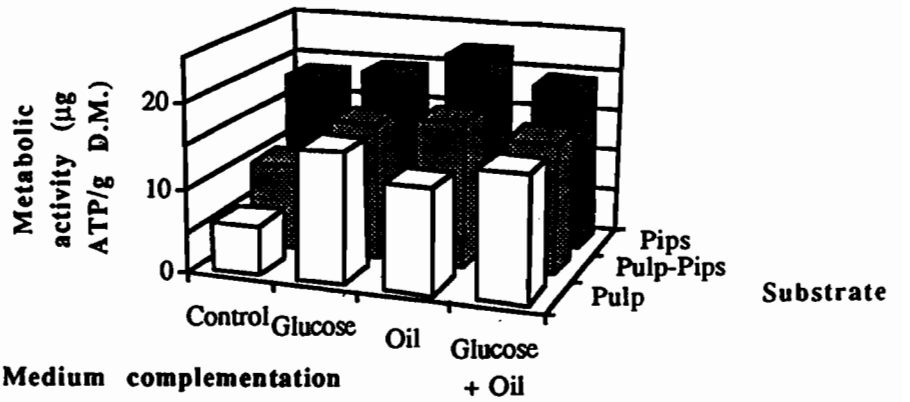

Fig. 2. Influence of the enrichment of solid medium on the metabolic activity of *Trichoderma viride.*

Metabolic activity of the mycelium is low on non-enriched pulp (6 µg ATP/ g dry matter) thereby, indicating a poor nutritive material. Faure and Deschamps (1990) reported a high percentage of lignin in cell-wall components of the grape pulp and the inability of a diversified microflora for its degradation. After enrichment with assimilative carbon, such as glucose or grape pips oil, the fungal metabolic activity on pulp increased (around 15 µg ATP/ g dry matter), but was still lower than the values for the pips (around 18 µg ATP / g dry matter).

In fact, crushing of crude substrates has improved the accessibility of the mycelium to internal compounds. As shown in Tables 1 and 2, the pips appeared not only rich in sugars and fats, but also in compounds capable of entering the fungal metabolism. The enrichment of the pips with glucose or oil did not increase significantly the fungal metabolic activity. The average values remained between 19 and 22 µg ATP/ g dry matter.

CONCLUSION

Grape pips have great potential as a substrate in solid state fermentation, without any enrichment.

Grape pulp appeared to produce less fungal biomass than grape pips. Nevertheless, because of its better ability to retain water, grape pulp might constitute a useful substrate for solid state fermentation involving a re-usable support impregnated with nutritive solution.

Mixture of grape pulp + grape pips also has potential and its efficiency lies in between those of the individual components, but with the advantage of improved water retention capacity than that of the grape pips.

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Enrichment of deseeded carob pod with protein and sucrose or fructose by solid state fermentation

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SUMMARY

The carob bean (fruit of *Ceratonia siliqua,* L.) is an agricultural product of low commercial value. The ripe carob pod (pericarp), although rich in water-soluble sugars, has a very low crude protein content, and contains high levels of tannins, mainly of the condensed type, which minimize its nutritional value. A study of the effect of inoculating deseeded carob pod with mixed culture of *Rhizopus oligosporus* and *Saccharomyces rouxii* or *Saccharomyces cerevisiae* on its protein and sucrose or fructose content, was undertaken. The mixed culture consumed reducing sugars, which resulted in product enriched in sucrose and good quality protein. *S. cerevisiae* hydrolysed the carob sucrose and consumed glucose, thereby producing a product enriched in fructose and yeast protein. Significant amounts of lignocellulose and tannins were also consumed. Both the inoculation reduced the unfavourable effects of the high level of tannins and low protein content on the carob pod nutritional value.

Keywords: Solid state fermentation, deseeded carob pods, mixed culture, *Rhizopus oligosporus, Saccharomyces rouxii,* glucophilic *Saccharomyces cerevisiae*, protein enrichment, sucrose enrichment, fructose enrichment, tannins degradation.

RESUME

Enrichissement de la farine de caroubes en protéines et saccharose ou fructose par fermentation en milieu solide.

MARAKIS S., LAMBRAKI M., MARAKIS G. ET ROUSSOS S.

La caroube (fruit du caroubier, *Ceretonia siliqua*, L) est un produit agricole de faible valeur commerciale. La gousse de caroube (péricarpe) riche en sucres est pauvre en protéines. Sa qualité nutritionnelle est encore diminuée par sa forte concentration en tannins. Nous avons entrepris d'enrichir la farine de caroube en sucrose et lactose, respectivement et en protéines de champignons et de levures ou de levures uniquement. Cela a été réalisé par consommation microsélective du glucose et des autres constituants de la caroube par des cultures mixtes de *Rhizopus oligosporus* et *Saccharomyces rouxii*, souches invertase-négatives, ou par culture simple de *Saccharomyces cerevisiae* par fermentation solide. La culture mixte consomme les sucres réducteurs et la caroube obtenue après fermentation est riche en sucrose et enrichie en protéines de bonne qualité. On observe également la consommation d'une importante quantité de ligno-cellulose et des tannins. Les effets nutritionnels défavorables dus aux fortes concentrations de tannins et aux faibles quantités de protéines ont bien été réduits.

Mots clés: Fermentation en milieu solide, farine de caroubes, cultures mixtes, *Rhizopus oligosporus, Saccharomyces rouxii, Saccharomyces cerevisiae* glucophile, enrichissement en protéines, en sacharose, en fructose, degradation des tannins.

INTRODUCTION

Carob bean (fruit of *Ceratonia siliqua*, L. tree) contains 63-90% husk (deseeded carob pod) and 10-37% seeds, depending on the varieties (Mitrakos, 1968; Marakis *et al*, 1987, 1995). The ripe husk, although rich in water-soluble sugars (30-60% of the husk on dry weight basis), has a very low crude protein content (2.5-6.7%), and contains up to 27% tannins (Marakis *et al*, 1987, 1995; Wursch, 1987), mainly of the condensed type (Tamir *et al*, 1971; Marakis *et al*, 1993), which minimize the nutritional and commercial value of the carob pod (Vohra *et al*, 1966; Tamir and Alumot, 1970).

The nutritional value of carob pod could be improved by eliminating tannins and increasing its protein content. In spite of relevant studies (Drouliscos *et al*, 1976;

Marakis, 1980; Marakis and Diamantoglou, 1990), such an improvement has not yet been achieved.

Oddo (1928) and Kriaris (1957) failed in extracting sucrose from aqueous carob extract, because carob reducing sugars interfered with sucrose crystallization

Marakis (1992) produced sucrose syrup by microselective consumption (the ability of a microorganism to consume selectively one or some of the components of the substrate) of carob reducing sugars using an invertase-negative mixed culture of *Rhizopus oligosporus* and *Saccharomyces rouxii.* Marakis and Marakis (1995) also succeeded in producing carob fructose syrup by microselective fermentation of glucose by a glucophile strain of *S. cerevisiae.* These studies used aqueous carob extract or carob pod slurry. A direct enrichment of the carob pod with protein and sucrose or fructose by solid state fermentation (SSF) is a new promising approach because 1) the water-soluble (sugars, tannins) and water-insoluble (celluloses, lignin) carob components could be fermented and valorized using suitable microorganism(s), 2) the SSF-system is economically better than the conventional submerged fermentation processes, since reactions simulate the fermentation that occur in nature (Lonsane et al, 1985), and 3) protein synthesis by microorganisms in SSF is a useful alternative for replacing protein sources (soybean cake, fish meal) and improving the content in essential amino-acid of various substrates (Roussos et al, 1994).

Therefore we have studied the effect of inoculating carob husk with a mixed culture of invertase negative *R. oligosporus* and *S. rouxii* or a single culture of the glucophile strain of *S. cerevisiae* in SSF-system on the microselective consumption of glucose or other carob components and the resulting increased concentration in fungal-yeast or yeast protein and either sucrose or fructose.

MATERIAL AND METHODS

MICROORGANISMS

An invertase negative mixed culture of *Rhizopus oligosporus* (Rhol-2) and *Saccharomyces rouxii* (Saro-2) and a glucophile strain of *Saccharomyces cerevisiae* (Sace-1) were used for inoculation. *R. oligosporus* and *S. cerevisiae* strains were isolated from grapes of Santorini island according to Marakis (1980). *S. rouxii* strain has been isolated from carob beans (Marakis, 1992).

CAROB POD VARIETIES

The grafted varieties: g-1, g-2 and H-2 were used because they are 40% richer in sucrose than other Greek carob varieties. Variety g-1 is the richest in fructose (28.1%) among Greek carob varieties (Marakis *et al*, 1995). Our previous study (Marakis *et al*, 1993) has also shown that tannins of g-1 and g-2 varieties lack epicatechin, which reduces microbial growth.

CAROB POD FRAGMENTATION-*KIBBLED* CAROB (HUSK) PREPARATION

The dry carob beans were broken into pieces of about 0.5 cm and the seeds were simultaneously separated using a factory mill.

SOLID STATE FERMENTATION PROCEDURE

The *kibbled* carob was wetted with a solution of $(NH_4)_2SO_4$ (0.5% w/w), and NaH_2PO_4 (0.5% w/w) to a final moisture of 60% that was maintained during the incubation after inoculation with 10^7 spores of *R. oligosporus* + 10^7 cells of *S. rouxii* (mixed culture) or $2x10^7$ cells of *S. cerevisiae* (single culture) per g dry matter. pH was adjusted to 5.0-5.5. The *kibbled* carob and $(NH_4)_2SO_4$ and NaH_2PO_4 solutions were sterilized by autoclaving. The *kibbled* carob fermentation was conducted according to Lambraki *et al* (1994).

ANALYTICAL METHODS

Total water-soluble sugars and individual (sugar profile) were determined according to Dubois *et al* (1956) and Marakis (1992). True protein (protein nitrogen x 6.25) was determined and nucleic acids were extracted according to Delaney *et al* (1975). RNA was estimated by the method of Gottlied and Van Etten (1964) whereas DNA according to Dische (1955), using baker's yeast RNA and calf thymus DNA as standards. Amino acids, water-soluble tannins and ash were estimated, as described by Marakis (1985). Lignin was determined by the methods of Van Soest (1963) and cellulose according to Updergraff (1969).

RESULTS AND DISCUSSION

KIBBLED CAROB FERMENTATION BY MIXED CULTURE (FERMENTATION-A)

The mixed culture of *R. oligosporus* and *S. rouxii* consumed reducing sugars after 36 hours of incubation (Table 1) and produced a fermented carob product (FCP) rich in sucrose (about 45% on dry basis) and enriched in protein (up to 17%) with a good amino acid profile (Table 2). The same microorganisms in submerged liquid mixed culture consumed the carob reducing sugars within 34 hours (Marakis, 1992).

Table 1. Gross composition (% on dry weight) of the fermented carob pod produced by mixed culture of *R. oligosporus* and *S. rouxii* in SSF-system.

Incubat. time ,(h)	Carob varieties	Sucrose (%)	Fructose (%)	Glucose (%)	Protein (%)	Tannins (%)	Cellulose (%)	Lignin (%)
	g-1	42.1	7.6	5.0	4.4	4.4	5.1	8.1
0	g-2	43.4	2.2	0.9	4.7	4.7	6.2	9.3
	H-2	45.0	1.9	0.7	4.8	5.0	9.2	7.2
	g-1	42.6	6.2	3.8	5.5	3.6	4.8	7.7
24	g-2	43.9	1.8	0.6	5.4	4.3	5.8	9.0
	H-2	45.8	1.2	0.6	6.8	3.6	7.2	6.3
	g-1	44.1	Trace	ND	11.5	1.8	4.1	7.4
36	g-2	43.7	ND	ND	10.0	2.3	5.2	8.2
	H-2	46.2	ND	ND	12.5	0.5	6.1	5.8
	g-1	44.4	ND	ND	14.5	1.0	3.1	5.4
48	g-2	44.1	ND	ND	12.0	1.6	4.3	7.1
	H-2	46.2	ND	ND	17.0	0.4	3.1	4.4

ND=Not detected

The amino acid profile of the proteins produced by SSF (Table 2) was similar to that obtained in liquid culture (Marakis, 1992). On the other hand, a tannin content of (0.4% on dry basis) indicated that the activities of the microorganisms degrading tannins were 27 times higher in SSF conditions than in shaken liquid culture (Marakis, 1992).

Table 2. Amino acid composition (g/16 g N) of fermented carob pod (H-2 variety) produced by using mixed culture of *R. oligosporus* and *S. rouxii* in SSF-system.

Phe=4.6	Lys=7.4	Arg=5.5	Glu=12.5
Tyr=5.9	Met=2.0	Trp=1.3	Pro=3.1
His=1.8	Cys=1.7	Total EAA=53.2	Cyl=3.4
Ile=4.9	Thr=4.4	Asp=8.3	Ala=4.4
Leu=7.9	Val=5.8	Ser=4.1	Total AA=89.0

The protein contents (17% on dry fermented product), was higher than that (7%) reported by Kokke (1977). From a nutritional aspect, it is important that ash content remains lower than 5% in the compounded feed. Thus, the FCP protein and ash (4.4%) contents were considered acceptable. Marakis and Diamantoglou (1990) reported that a tannin content (0.4%) did not affect the nutritional quality of FCP and dit not appear to cause a toxicological problem or to depress protein digestibility.

Since lignocelluloses are of low nutritional value and affect true nitrogen digestibility, these substances should be significantly reduced (Malefaki-Perela, 1981). Contents of 3.1% in cellulose and 4.4% in lignin of the FCP of H-2 variety (Table 1) are satisfactory from the nutritional point of view.

KIBBLED CAROB FERMENTATION BY SINGLE CULTURE OF *S. CEREVISIAE* (FERMENTATION-B).

S. cerevisiae Sace-2, under SSF conditions, preferentially used glucose with a simultaneous hydrolysis of sucrose to fructose and glucose, thereby leading to higher fructose concentrations (Table 3). This strain lacks a specific kinase responsible for fructose phosphorylation (Cochrane, 1958). The presence of glucose in carob pod did not allow the enzyme synthesis or enzymatic activation for the fructose fermentation, as in the case of other microorganisms, e.g., *Allomyces macrogynus* (Cochrane, 1958). After 72 h of *kibbled* carob fermentation by *S. cerevisiae*, a FCP containing 28.1% fructose on dry weight (Table 3) and enriched in proteins (21.4%) of good quality (Table 4) was obtained. The same strain, in aqueous carob extract of g-1 variety, hydrolysed the sucrose, while fermenting the glucose within 100 h incubation (Marakis and Marakis, 1995).

Table 3 Gross composition (% on dry weight) of the fermented carob pod (g-1 vatiety) produced by a glucophile strain of *S. cerevisiae* in SSF-System.

Incubation time,(h)	Sucrose (%)	Fructose (%)	Glucose (%)	Protein (%)	Tannins (%)	Cellulose (%)	Lignin (%)
0	42.1	7.6	5.0	4.4	4.4	5.1	8.1
24	39.5	8.6	4.5	5.6	4.0	5.0	8.2
48	21.1	17.8	5.5	7.8	3.1	4.3	8.0
68	1.1	27.5	3.1	16.4	2.8	4.0	7.7
72	ND	28.1	Trace	21.4	0.9	3.5	6.7

ND=Not detected

Table 4. Amino acid composition (g/16 g N) of fermented carob pod (g-1 variety) produced by *S. cerevisiae* in SSF-system.

Phe=4.8	Lys=7.3	Arg=5.7	Glu=14.5
Tyr=5.3	Met=1.9	Trp=1.0	Pro=3.4
His=1.3	Cys=1.6	Total EAA=51.6	Cyl=3.6
Ile= 4.9	Thr=4.8	Asp=8.6	Ala=4.0
Leu=7.1	Val=5.3	Ser=4.4	Total AA=90.1

Thus, under SSF conditions the time for sucrose hydrolysis and glucose consumption was reduced, as compared to that in liquid culture.

The balanced amino acid profile (Table 4), low ash (4.1%) and 5.2% RNA contents of the FCP, encourage protein production from deseeded carob pod.

CONCLUSIONS

In both cases (fermentations-A and -B), the protein content of the FCP was acceptable. From a nutritional point of view, the ash content was also acceptable, since it was lower than that is normally observed in compounded feed (5%). Tannin content decreased to less than 0.4%. This level does not show any toxicological effects and does not depress protein digestibility and utilization. Lignocelluloses

Lignocelluloses were decreased significantly, especially in variety H-2, and their levels in FCP were considered satisfactory from the nutritional point of view. The RNA content of the FCP was also low. Since non-toxic microorganisms were used, the FCP could be used as animal feed or food. Having established an exploitable process for the carob pods, their commercial value will increase. Thus, carob producers will be interested in expansion of carob plantations on barren soils, which are often unproductive for any other type of crop. Thereafter, upgrading of the environment is expected, because barren, rocky and dry regions, where carob tree naturally grows, will become reforested.

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Effects of sugar and mineral salts on the growth of *Aspergillus carbonarius* in carob pod solid state fermentation

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SUMMARY

The effects of sugar concentration and mineral addition on the growth of *Aspergillus carbonarius* in carob solid state fermentation were studied. Data on CO2 production, sugar levels and tannins levels, and optical observations, revealed when carob husk with additional mineral salts was used as a substrate, this led to high biomass production. On the other hand, removal of 52% of the water-soluble sugars, prior to fermentation, and elimination of the minerals resulted in a tannin degradation up to 55%, but poor biomass production.

Keywords: Solid state fermentation, carob pods, *Aspergillus carbonarius*, mineral salts enrichments, initial sugar concentration, biomass production, tannin degradation.

RESUME

Effets des sucres et de sels minéraux sur la croissance *d'Aspergillus carbonarius* cultivé sur farine de caroubes en fermentation en milieu solide.

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Les effets de la concentration initiale en sucres et en sels minéraux sur la croissance d'*Aspergillus carbonarius*, cultivé sur farine de caroube épuisée en fermentation en milieu solide ont été étudiés. Les mesures de production de CO2, les analyses des sucres et des tannins ainsi que les observations visuelles de l'évolution de la biomasse ont montré que l'utilisation de sucres contenus dans la farine de caroube additionnée de sels minéraux, est associée à une forte production de biomasse. Par contre, l'élimination préalable de 52% des sucres et de la totalité des sels minéraux contenus dans la gousse de caroube conduit à la dégradation de 55% des tannins et à une faible production de biomasse.

Mots clés: Fermentation en milieu solide, farine de caroube épuisée, *Aspergillus carbonarius*, addition de sels minéraux, concentration initiale en sucres, production de biomasse, degradation de tannins.

INTRODUCTION

Carob bean is the fruit of *Ceratonia siliqua*, L. The ripe deseeded carob pod (husk), although rich in water-soluble sugars (40-60%), has a very low protein level (3-5%) and contains appreciable amounts of total tannins (4-13%) (Marakis and Karagouni, 1985; Marakis *et al*, 1993). The tannins present are of mainly condensed types (Tamir and Alumot, 1970), which minimize the pod's nutritional value (Vohra *et al*, 1966; Tamir and Alumot, 1970).

This worldwide produced product could be variously upgraded (feed, enzymes, probiotics, etc.), after tannin degradation and husk protein enrichment. In spite of the wide use of solid state fermentation (SSF) for the upgradation of several agroindustrial products and the production of secondary metabolites (e.g. enzymes) as well as other microbial products (Lonsane *et al*, 1982, 1985; Roussos *et al*, 1991a, 1994), SSF has not yet been exploited for tannin degradation. Hence, *A. carbonarius*

strain, with high tanninolytic abilities (Lambraki and Marakis, 1993), has been used in SSF of carob pods.

This paper describes the effect of sugar and mineral salts on the growth of *A. carbonarius* in carob SSF.

MATERIALS AND METHODS

MICROORGANISM

A strain of *A. carbonarius* (Bainier) Thom, previously isolated from mouldy carob beans (Marakis, 1980), was used.

MEDIA

Ripe milled carob pods (hereafter referred as 'carob') and ripe milled carob pod from which 52% of their water-soluble sugars had been removed by stirring and filtration (hereafter referred as 'spent carob') were used. These were mixed with sugarcane bagasse (Roussos *et al*, 1991b) in a 5:1 (w/w) ratio. To achieve a humidity level of 65-67%, the above mixture was weted by distilled water or by a mineral solution containing (g/l) $(NH_4)_2SO_4$, 9.7; Urea, 2.4; KH_2PO_4, 5.0 and pH 4.4. Using the above, a total of 4 media were obtained, i.e.: A) carob + distilled water, B) spent carob + distilled water, C) and D) the same as A) and B), respectively, but contained mineral solution, instead of distilled water.

Each medium was inoculated with 10^7 spores/g initial dry weight (IDW) of substrate, incubated at 30°C and aerated at the rate of 1.8 l/column/h (Lambraki *et al*, 1994).

SOLID STATE FERMENTATION SYSTEM

The design and control of the SSF system was, as described by Raimbault and Alazard (1980) and Saucedo-Castañeda *et al*, (1994). During SSF, the gas produced was analysed automatically by GC analyser and results were monitored by an

integration programme in a computer. All fermentations lasted 50 h. Downstream processing was as described by Lambraki *et al* (1994).

SUGAR AND TANNIN DETERMINATIONS

Sugar and tannins extraction is reported elsewhere (Lambraki *et al*, 1994). Total sugars were determined by the method of Dubois *et al* (1956), using glucose as a standard. Total tannin measurement was carried out, according to the Swain and Hillis (1959) method, using gallic acid as the standard.

RESULTS AND DISCUSSION

The most significant growth parameters of *A. carbonarius* are shown in Table 1.

Table 1 Growth parameters of *A. carbonarius* cultured in media containing different sugar and mineral contents.

Media	Sugar conc (%)[a]	Mineral solution addition	Initial humidity (%)	Final humidity (%)	Initial pH	Final pH	Germination time (h)	Maximum CO_2 production (%)
A	100	-	65.3	71.3	5.42	2.67	10	2.4
B	48	-	66.2	75.1	5.59	2.98	5	1.6
C	100	+	65.0	71.7	6.05	3.70	20	7.7
D	48	+	65.4		6.12	6.58	10	9.7

[a] Remaining sugar concentration, % of initial sugar on dry weight

EFFECT OF SUGAR CONCENTRATION OF THE SUBSTRATE

Respiration of A. Carbonarius

The respirometry data of *A. carbonarius*, cultured in carob media of different sugar and mineral contents, are shown in Fig.1. Partial sugar extraction (48% of initial sugar concentration remained) resulted in a significant decrease in spore germination time. This probably indicates the inhibitory effect of high sugar concentration on

A.carbonarius growth. The same effect has been observed in liquid cultures, in which sugar concentration higher than 3% inhibits or delays this strain's growth (unpublished data).

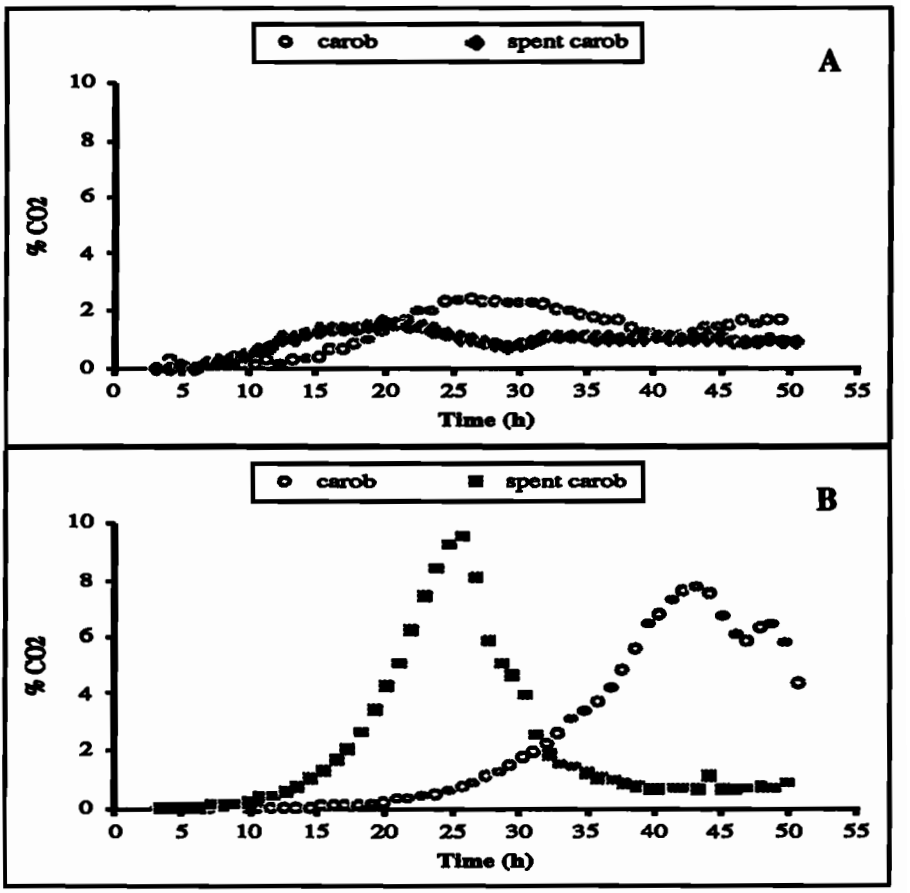

Fig. 1. Effect of sugar concentration of the substrate and mineral addition on the respiration of *A. carbonarius*. A: Without mineral addition, B: With mineral addition.

Sugar and tannin consumption

Sugar and tannin consumptions are shown in Fig. 2. It can be seen that the removal of 52% of the initial sugar weight led to a better utilization of the carbon source of the substrates, in terms of sugars or tannins. The only exception was the medium D), in which the lowest sugar consumption was recorded. In this case sporulation

occured, after 25-30 hours incubation, thereby revealing rather harsh conditions for the microorganism's growth. Under the same conditions, a significant reduction of the medium tannin content was observed, before the first 10 h fermentation, but this is considered as an artifact, caused by absorption and binding of tannins on the spore surface. This hypothesis was confirmed by comparison of spores originating from two different media: a) PDA and b) tannin-containing medium. The microscopic observation of these spores revealed a thick cover around the surface of the spores, obtained from the tannin medium.

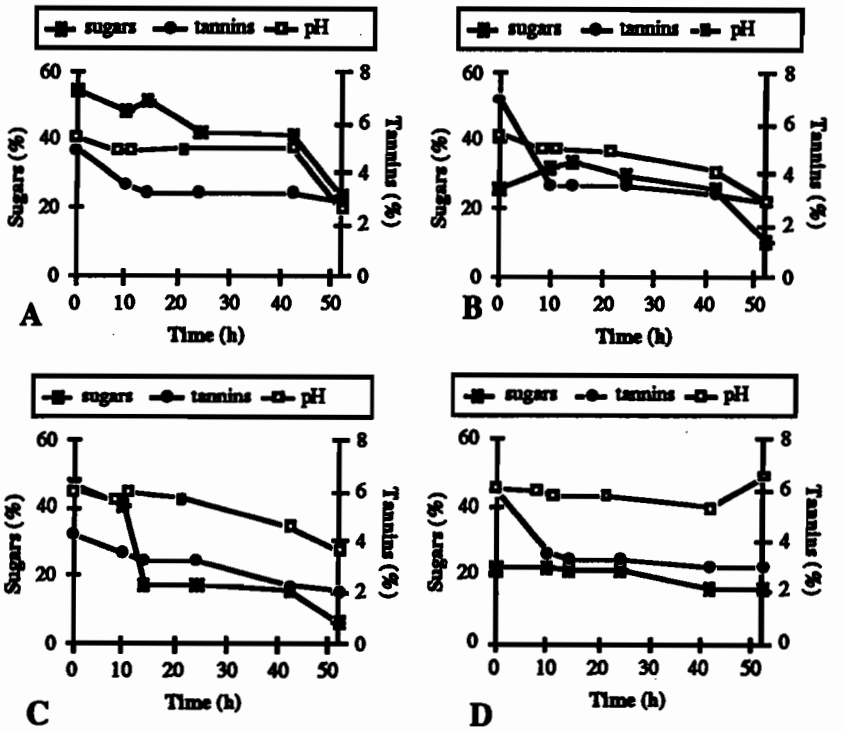

Fig. 2. Kinetics of sugar and tannin consumption (%, initial dry weight of the substrate) and pH evolution in solid state fermentation of 4 carob substrates: A = carob pod, B = spent carob, C and D = carob and spent carob, respectively, enriched with mineral solution.

Highest tannin degradation occurred in medium B, which contained a lower sugar concentration and no mineral addition. It seems that *A. carbonarius* degrades tannins in order to find the necessary nutrients, as no external nitrogen was added to the medium. The fact, that only proteins (and not the tannins) contain nitrogen atoms in

their structure disproves the above hypothesis. Since tannins form complexes with proteins, fungal tannin degradation for liberating and attacking proteins is a likely possibility.

pH evolution

pH values during the course of fermentation in the 4 different media revealed that the removal of 52% of the initial sugars, from the carob pods, did not significantly affect the initial pH of the substrates. The pH values increase of 0.17 and 0.07 were recorded in the low sugar media, without and with minerals, respectively (Fig. 2). The pH remained almost stable or rarely decreased until 42 hours' fermentation and then sharply decreased, probably due to tannin degradation and liberation of acidic products. This result has also been observed in previous work (Lambraki *et al*, 1994). The fact that tannin degradation occurs from the beginning of fermentation, while pH decreases just before the end, can be explained on the basis of high sugar concentration in the media up to 40 hours' incubation. Under these experimental conditions, there was no need for pH regulation, as the substrate itself behaved more or less as buffer and maintained the pH at desirable levels. A similar result was reported by Roussos *et al* (1991a).

EFFECT OF MINERAL ADDITION

Respiration of the fungus

The respirometry data (Fig. 1) of carob and spent carob fermentations, in media with added minerals, showed a significant increase in the quantity of CO2 production, which was about 5 fold higher than that observed with substrates containing no minerals. This can be interpreted as either due to high biomass production seen in medium C) or as a result of stress (medium D, in which the strain could hardly grow and for this reason sporulation occurred). Spore germination time was also affected by mineral addition; in these media its duration doubled compared to those with no minerals. This effect was independent of the sugar concentration of the substrates.

Sugar and tannin consumption

External nitrogen significantly improved sugar consumption in medium C (in which the highest sugar consumption occurred), as compared to medium A, without minerals (Fig. 2). Medium D, did not follow this result, because of sporulation. The highest biomass production was observed in medium C, in which the micro-organism could easily find both the carbon and nitrogen sources needed for its growth. Under such conditions, there is no need for tannin degradation, which would require an extra effort for the strain.

pH evolution

pH increases of 0.53 and 0.63 were observed in carob and spent carob media, respectively, after mineral addition. pH evolution followed the same pattern, as those mentioned above. The only exception was observed in medium D, in which the pH increased slightly after 42 hours' fermentation. This result is directly connected to tannin degradation, as no degradation occurred in medium D, and hence there is no reason for pH decrease.

C/N RATIOS OF THE SUBSTRATES

The solid state fermentation of carob pods, in media containing different concentrations of carbon and nitrogen, revealed a significant effect of C/N ratio on growth parameters and behaviour of *A. carbonarius* . The fungus seems to be well adapted to varying C/N ratios, even for those of very high or very low values. Nevertheless, its behaviour depended strongly on changes in sugar and nitrogen concentrations, and led either to biomass production or tannin degradation. The former occurred, when all the necessary nutrients (carbon and nitrogen sources) were plentiful. The latter occurred, when no external nitrogen sources were added. As tannin degradation started before 10 hours' incubation, it was assumed previously (Lambraki *et al,* 1994), that nitrogen limitation might lead to tannin degradation. The results of the present work confirm this assumption, as tannin degradation occurred in the medium with no available nitrogen and with reduced sugar concentration. A possible explanation could be that the fungus needs a nitrogen source, which is not readily available in the substrate.

Taking into consideration that, the basic carbon sources are sugars and tannins, and, as the tannin content remained almost stable during the pre-treatment of the substrates, the reduction in sugar concentration is considered the factor responsible for the changes in the carbon levels. Furthermore, the nitrogen concentration of the media increased due to the addition of the mineral solution, as the nitrogen content of the carob pod is quite low (0.68% on carob pod dry weight) (Marakis, 1980). Even though it is not easy to calculate the exact C/N value for the media, because their tannin content, is not yet very clear, an estimation of this value can be obtained, considering the carbon of sugars and the carbon of tannins as constant. On this basis, C/N ratio can be estimated by the following equation:

$$C/N = (Cs + Ct) / (Ncp + Nm) = \\ = Cs / (Ncp + Nm) + Ct / (Ncp + Nm) \quad (1)$$

where, Cs : carbon of sugars=6.895, Ct : carbon of tannins, Ncp: nitrogen of carob pod=0.68 and Nm: nitrogen of mineral solution=2.056

After extraction of sugar, the equation becomes:

$$C/N = (Cr + Ct) / (Ncp + Nm) =$$
$$= Cr / (Ncp + Nm) + Ct / (Ncp + Nm) \quad (2)$$

Where, Cr : carbon of reduced sugars=3.577

Using (1) and (2), the C/N ratio of the media can be expressed as:

Medium A: (1) $= 6.895 / (0.68 + 0) + Ct / (0.68 + 0)$

$$= 10.14 + Ct / 0.68 \quad (3)$$

Medium B: (2) $= 3.577 / (0.68 + 0) + Ct / (0.68 + 0)$

$$= 5.26 + Ct / 0.68 \quad (4)$$

Medium C: (1) $= 6.895 / (0.68 + 2.056) + Ct / (0.68 + 2.056)$

$$= 2.52 + Ct / 2.736 \quad (5)$$

Medium D: (2) $= 3.577 / (0.68 + 2.056) + Ct / (0.68 + 2.056)$

$$= 1.31 + Ct / 2.736 \quad (6)$$

Equations (3), (4), (5) and (6) indicate that there is a gradual decrease in the C/N ratio from medium A to medium D, i.e. A > B > C > D

Case (4) proved to be unsatisfactory for the fungal growth and resulted in sporulation. Thus, the C/N ratio in this case is too low. The growth of the strain in medium A was not very satisfactory and resulted in low biomass production. Media B and C, containing intermediate C/N ratios, were most advantageous, each one for a different reason. In medium B, the micro-organism succeeded in the highest tannin degradation, while in medium C, the biomass production was highest (optical observation). It seems that the optimum C/N ratio for both of these procedures lies between the very high and very low values.

CONCLUSION

The solid state fermentation of carob pods containing different amounts of sugars and nitrogen sources resulted either in a high level of biomass production or in tannin degradation, depending on the experimental conditions. Thus, by proper selection of the carbon and nitrogen content of the medium, the desired goal can be achieved.

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Protein enrichment of apple pomace by solid state fermentation

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SUMMARY

The potential of solid state fermentation for enrichment of apple pomace was investigated. A total of 10 strains of *Rhizopus* were screened and *Rhizopus oryzae* 16 was selected. Its growth parameters, such as initial pH of the medium, initial moisture content, inoculum rate, fermentation temperature, KH_2PO_4 concentration and C / N ratios, were optimized to formulate an effective medium. Data on growth kinetics of the strain on apple pomace indicated that the protein content of the fermented substrate was 10.96 g / 100 g dry substrate at 36 h, without any spore formation. The fermented mass was found according to the sanitary legislations with respect to a number of microbial groups / types. It is emphasized that apple pomace was not earlier studied for its protein enrichment by solid state fermentation.

Keywords: Apple pomace, solid state enrichment, protein enrichment, *Rhizopus oryzae*, growth parameters, kinetics, sanitary legislations.

RESUME

Enrichissement en protéines de la pulpe de pomme par fermentation en milieu solide.

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L'enrichissement en protéines de la pulpe de pomme a été réalisé en milieu solide en utilisant 10 souches de *Rhizopus*. Il s'avère que seule la souche de *Rhizopus oryzae* 16 a été retenue. Sa croissance est analysée en suivant l'évolution de plusieurs marqueurs caractéristiques, le pH, l'humidité, la température de la fermentation. D'autres paramètres ont été également étudiés : le taux d'inoculation, la concentration du milieu en phosphate et le rapport C/N. Cela permet d'optimiser les conditions de culture. Dans le meilleur des cas, le contenu en protéines de la pulpe fermentée est de 10,96 g / 100 g de poids sec au bout de 36 h de fermentation, sans production de spores. La pulpe fermentée s'avère conforme aux contraintes de la législation sanitaire en cours au Brésil, notamment en ce qui concerne la présence des différents contaminants microbiens. Ces résultats suggèrent l'emploi de cette pulpe de pomme pour toute production de support enrichi en protéines par fermentation solide.

Mots clés : Pulpe de pomme, enrichissement en protéines, *Rhizopus oryzae*, fermentation en milieu solide, paramètres de culture, cinétiques.

INTRODUCTION

Apple juice processing generates about 15 - 20 % of solid wastes (Hang, 1987). Many studies have been carried out for its utilization and these included processes for the production of ethanol, natural gas, citric acid, and pectin animal food (Hang, 1987). Apple juice processing is dried and, in Russia, is used to enrich flours, breads, candies, cold drinks and confectionery products (Eigor et al, 1984; Kovalskaya *et al*, 1994; Mironenko, 1985; Patt, 1994).

Apple pomace constitutes a rich source of organic matter (Hang, 1987). It could be efficiently utilized for various purposes, such as upgradation of its protein value by solid state fermentation. Therefore, the present work was undertaken to study the utilization of apple pomace as a bioresource material for protein enrichment through

solid state fermentation by *Rhizopus*. Use of apple pomace for the production of fungal proteins has not been conducted earlier.

MATERIALS AND METHODS

MICRO-ORGANISM

Ten different strains of *Rhizopus oryzae* LPB 68 from the collection of the University were screened. Stock culture of fungus was maintained in potato dextrose agar (PDA) tubes. Inocula were prepared from 8-day culture slants at 30°C. After inoculation and incubation, each tube was maintained at 4°C over several weeks.

SOLID SUBSTRATE

Apple pomace was used as a substrate. Its chemical constituents are shown in Table 1. Apple pomace with a moisture content of 75 % was dehydrated at 55°C for 30 h in a tray oven with air circulation. After cooling, it was packed in polyethylene bags.

Table 1. Physical and chemical characteristics of solid substrate.

Components	Quantity (%)
Protein	3.33
Total sugars	27.50
Fibers	13.40
Moisture	8.00

INOCULUM AND MEDIUM PREPARATION

Inoculum was produced in Petri dishes containing 30 ml of PDA medium and incubated at 28° C for 8 - 10 days. The spores were harvested using a platinum loop in 10 ml sterile distilled water and a drop of Tween 80. After dilution, they were counted in a Malassez plate. Spore suspension was routinely stored at 4°C. These

provedures were carried out in a laminar flow chamber. Apple pomace was humidified to 70% with a mineral solution containing 4.75 % KH_2PO_4; 4.65% $(NH_4)_2SO_4$ and 1.15% urea. Its pH was adjusted to 6.5 with 3 N Na_2CO_3. This mixture was inoculated with spore suspension to obtain a final concentration of 2 x 10^7 spores /g dry pomace. Incubation lasted 48 hours at 30°C in a water saturated environment. Finally, the material was dehydrated in an oven at 60°C until 6 % moisture was reached.

Sample Analyses

The moisture content was estimated by drying the sample (5 g) at 105°C for 24 h. pH was determined following homogenization the sample (5 g) with distilled water (50 mL). Protein content was estimated according to Vervack (1973). Protein nitrogen was determined by Kjeldahl method (Bradstreet, 1965). Estimation of sugars was done by the DNS method (Miller, 1959), after acid hydrolysis.

Microbiological Analyses

Total microbial population, associated with apple juice extraction pomace, during the course of SSF, were counted using a nutrient agar medium and employing the plate technique. Inoculated plates were incubated at 30°C for 3-5 days. Specific analyses were carried out for mesophile bacteria, total coliforms, faecal coliforms, fungus and yeast, *Staphilococcus aureus* , *Bacillus cereus* and *Salmonella* sp. All these analyses were carried out using a 25 g material.

RESULTS AND DISCUSSION

Strain selection

Ten different strains of *Rhizopus* were tested for their ability to grow and produce protein in an apple pomace medium (Table 2). Among these, the strain *R. oryzae* 68 showed an excellent development and it was selected for further studies.

Table 2. Screening of *Rhizopus* strains for growth and protein production in apple pomace medium.

Strains	Growth	Proteins (g/100 dry powder g)
R. circicans 67	+++	6.6
R. oryzae 95	-	5.3
R. formosa 22	-	5.0
R. microsporus 36	-	4.9
R. arrhizus 79	++	6.2
R. sp 75	-	4.7
R. oryzae 27	+++	7.0
R. oryzae 68	++++	8.0
R. delemar 12	++	6.2
R. arrhizus 25	+	5.1

Apple pomace contained 4.2 g protein/100 g dry powder. - : no growth, + : weak growth, ++ : regular growth, ++ : regular growth, +++ : good growth, ++++ : excellent growth.

OPTIMIZATION OF CULTURE CONDITIONS

Different factors (pH, moisture, temperature, inoculation rate, KH_2PO_4 concentration and C/N ratios were studied independently.

Moisture effect

Growth of *R. oryzae* 68 in apple pomace was evaluated at five different initial moisture content levels (50, 60, 65, 70, 75 %). Fig. 1 shows that on initial moisture of 70 % is the best in terms of protein biosynthesis. Other moisture levels lead to lower protein synthesis.

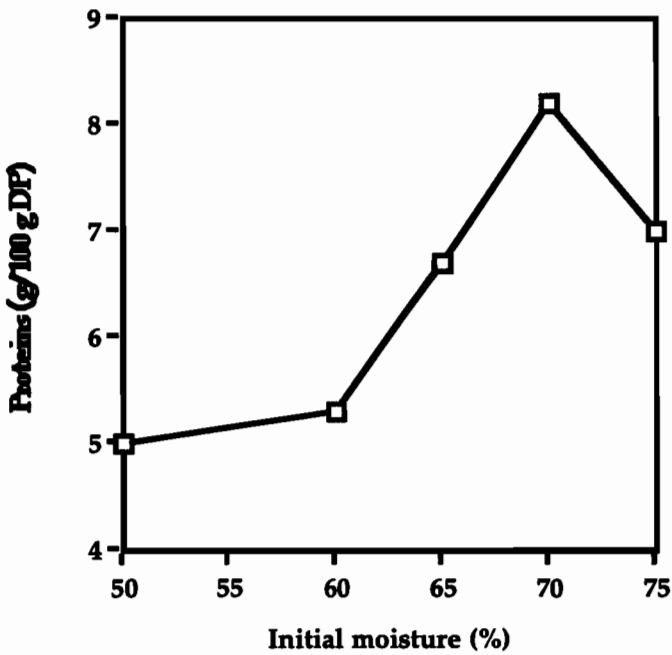

Fig. 1. Effect of initial moisture content of the medium on protein production by *R. oryzae* LPB 68.

pH effect

The values of initial pH of the medium were 3.2 (pH of the substrat itself) : 3.9, 5.0, 5.9, 6.8, 8.0 and 9.0. The pH 6.8 was the best and the strain produced 8.02 g protein / 100 g dry powder. Similar results were obtained by Soccol (1994) and Soccol *et al* (1995), working with different species of *Rhizopus* in solid state fermentation of cassava bagasse.

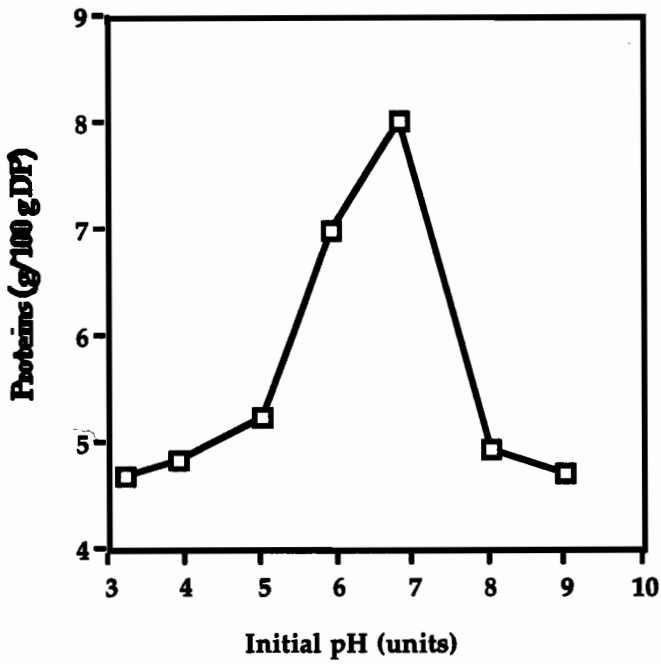

Fig. 2 - Effect of initial pH of the medium on protein production by *R. oryzae* LPB 68.

Temperature effect

Study of the effect of temperature on the growth of *R. oryzae* 68 on apple pomace showed that the optimum temperature for the growth of the fungi was 32°C, where protein levels reached 8.2 g / 100 g dry powder (Fig. 3). The protein formation was lower at other fermentations.

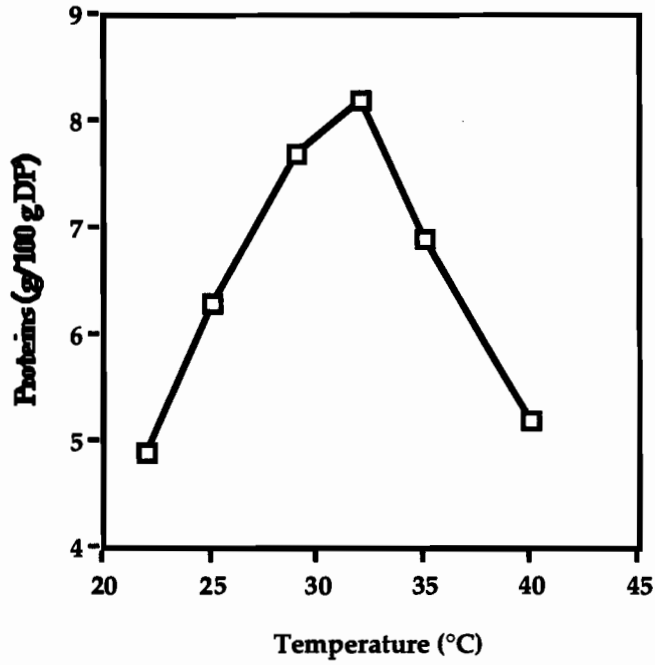

Fig. 3 - Effet of temperature on protein formation by *R. oryzae* LPB 68.

Effect of inoculum rate

Results of the inoculum rate on the growth of *R. oryzae* 68 are shown on Fig. 4. Data showed that inoculum rate at 10^8 spores / g of dry powder is the ideal. Raimbault (1980) and Soccol (1992) pointed out that spores inoculation rate must be sufficient to ensure an homogeneous inoculation of the substrate as well as a quick start of growth to prevent the development of contaminants. On the other hand, inoculation rate should not be very high because a very high density of spores can induce inhibition phenomena and reduce germination percentage (Laukevicks *et al*, 1985 ; Barrios-Gonzalez *et al*, 1989). According to these authors one dose of 2 x 10^7 spores / g of dried support is sufficient enough to obtain a good growth.

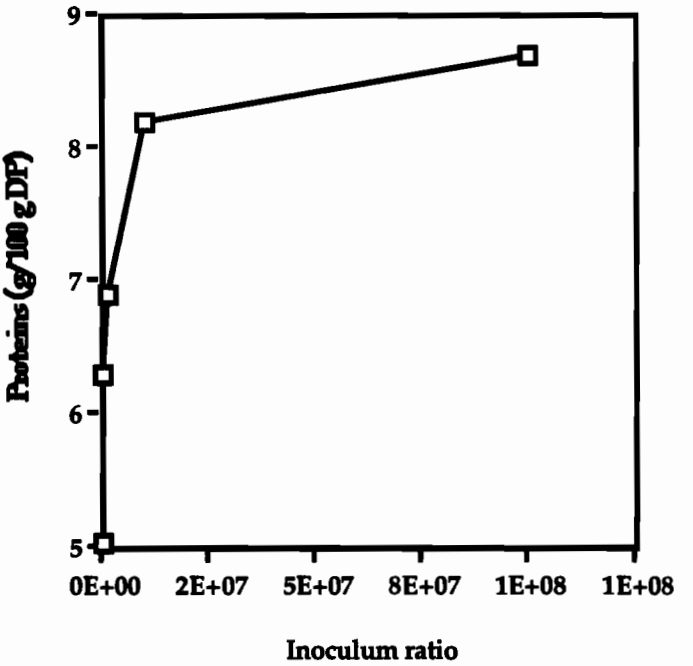

Fig. 4 - Effect of inoculation rate on protein formation by *R. oryzae* LPB 68.

Effect of KH_2PO_4 concentration

Fig. 5 shows that the addition of KH_2PO_4 to the apple pomace has a positive effect on the increase protein formation. Protein synthesis reaches its maximum level at a salt concentration of 1.33 g / 100 g dry powder.

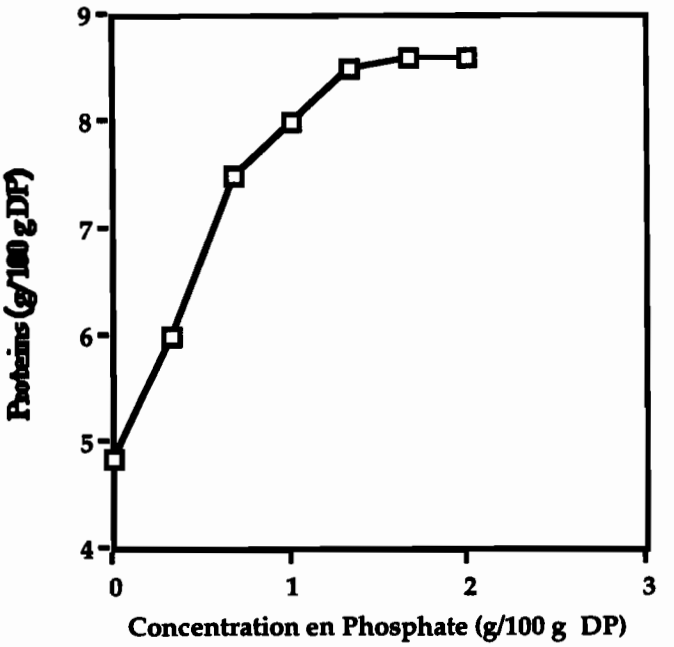

Fig. 5 - Effect of KH_2PO_4 concentration on protein formation by *R. oryzae* LPB 68.

Effect of C / N ratio

The effect of C/N ratio was studied using different concentrations of a mixture of $(NH_4)_2SO_4$ and urea. The highest rates of growth were at lower ratios, i.e C/N 3.6 to 9 (Fig. 6).

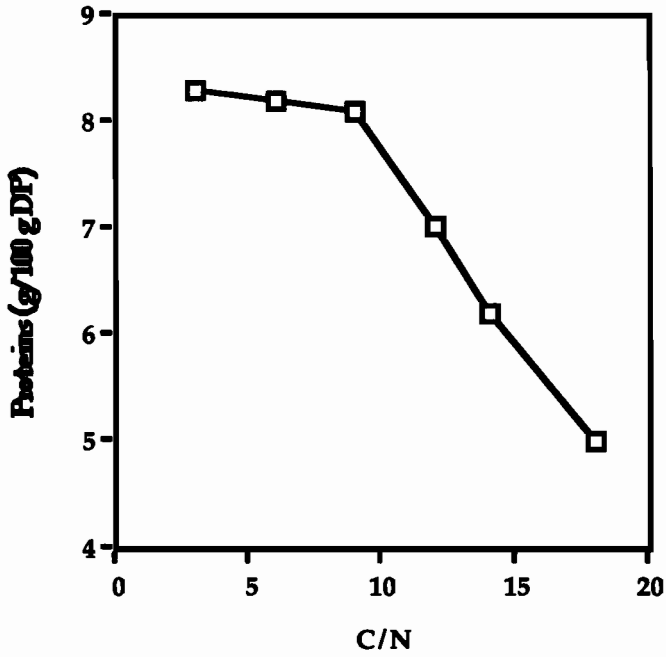

Fig. 6 - Effect of C/N ratio on protein formation by *R. oryzae* LPB 68.

Growth kinetics on apple pomace

These were carried out using optimum levels of all the standardized parameters. It contained : dried apple pomace, 100 g ; KH_2PO_4 , 1.7 g ; $(NH_4)_2SO_4$, 5.4 g ; urea, 1.33 g ; water, 233 ml and pH 6.5. Other parameters used were : temperature, 32°C ; inoculation ratio, 10^7 spores / g dry powder ; fermentation time, 48 h.

Figs. 7 A and 7 B show the time-course of some factors during the growth of *Rhizopus* on apple pomace in solid state fermentation. Protein content was used as a criterion to evaluate fungal biomass produced during growth. No lag phase was observed and during the first 12 h of fermentation significant changes in total sugar and protein content occurred. The protein content of the fermented pomace increased progressively during the course of fermentation from 3.51 g / 100 g dried pomace (0 h) to 11.37 g / 100 g dried pomace (48 h). No sporulation was observed at 36 h and protein content was 10.96 g / 100 g dried pomace.

At the beginning of fermentation the amount of total sugars was 36 g / 100 g dried pomace pomace whereas, at the end of fermentation, it decreased to 17.9 g / 100 g dried pomace (Fig. 7 A)

The change in initial pH, from 6.8 (0 h) to 5.3, was compared to the change of pH during the rest of fermentation, 5.3 (24 h) to 7.5 (48 h) (Fig. 7 B).

Moisture content of the fermented pomace increased from 69.9 % (0 h) to 71.8 % (48 h). The increase of the moisture content of the substrate during fermentation might be due to the production of metabolic water (Yang, 1988).

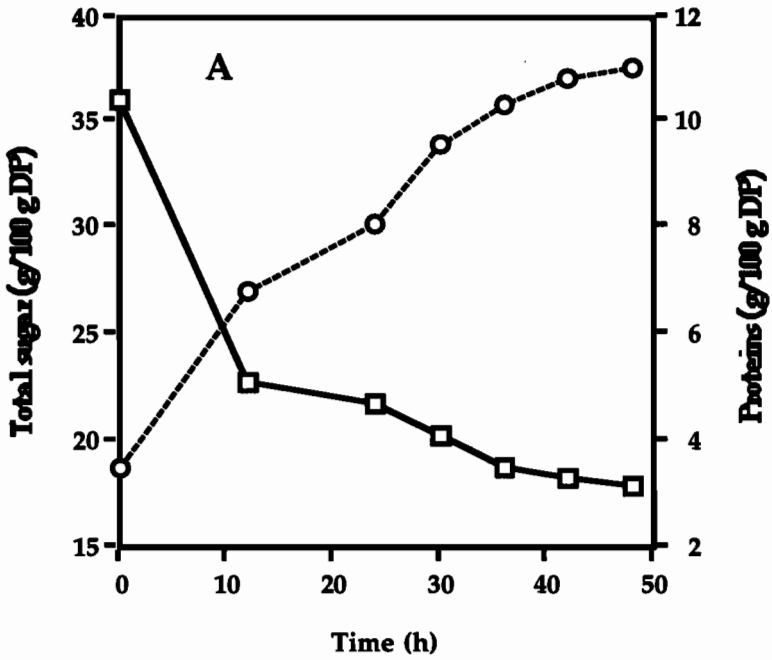

Fig. 7A - Kinetics of the growth of *Rhizopus oryzae* LPB 68 on apple pomace.

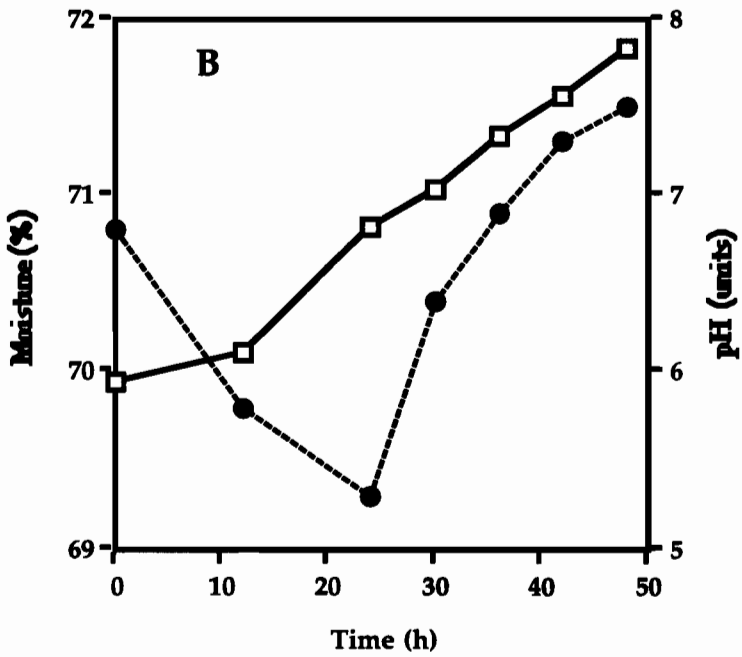

Fig. 7B - Kinetics of the growth of *Rhizopus oryzae* LPB 68 on apple pomace.

Microbiology evaluation

Results of microbiologic evaluation of the fermented apple pomace are reported in Table 2. The product meets all the sanitary legislation. That is probably due to a biocide action of *R. oryzae* LPB 68. These results confirm those obtained by Wang *et al* (1969).

Table 2. Microbiology evaluation of fermented apple pomace.

Microbiology evaluation	Results	Legislation
Mesophile bacteria (UFC/g)	Negative < 10	5×10^5/g
Total coliforms (NMP/g)	Negative < 3.0	-
Faecal coliforms (NMP/g)	Negative < 3.0	10 / g
Fungus and yeast (UFC/g)	Negative < 10	10^3 / g
Staphylococcus aureus (UFC/g)	Negative < 10	-
Bacillus cereus (UFC/g)	Negative < 10	10^3 / g
Salmonella sp / 25 g	Absence	Absence

CONCLUSION

The present study suggests that apple pomace could be utilized as asolid substrate for protein enrichment. Studies on the optimization of the parameters and the growth kinetics of *Rhizopus oryzae* LPB 68 showed its high capacity to grow and produce protein biomass in solid state fermentation. The best results were obtained after 36 h fermentation.

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Solid state fermentation of lignocellulosics into animal feed with white rot fungi

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SUMMARY

The high content of polysaccharides in lignocellulosics, particularly cereal straws, is a potential source of dietary energy for ruminants. However, their nutritive value is limited because of the poor digestion of complex polysaccharide in the rumen. In order to increase the digestibility of lignocellulose, biological methods of delignification can be used. The principle of these methods is the splitting of the cellulose-lignin complex by extraction or decomposition of lignin. The main problem of biological upgrading of lignocelluloses into feed is to find suitable micro-organisms, with metabolic patterns different from those of the rumen flora and fauna and a cheap large scale process. Ideal micro-organisms for upgrading of lignocellulosics into animal feed should have a strong lignin metabolism, with a low degradation of cellulose and hemicellulose. In this review, principles of solid state fermentation and fungal activities on lignocelluloses are briefly summarized.

Keywords : White rot fungi, lignocellulosics, upgradation, selective delignification, parameters, bioreactor, solid state fermentation.

RESUME

Fermentation en milieu solide de matériaux lignocellulosiques avec des champignons pour l'alimentation du bétail.

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La concentration élevée en polysaccharides des matériaux lignocellulosiques particulièrement des pailles de céréales, représente une source d'énérgie potentielle pour l'alimentation des ruminants. Cependant leur intérêt nutritif est limité par la mauvaise digestibilité des complexes polysaccharides qu'ils contiennent. La digestibilité de la lignocellulose peut être augmentée par traitement biologique. Le principe de ces traitements biologiques consiste à casser le complexe lignocellulosique par extraction ou décomposition de la lignine. La principale difficulté est de trouver un microorganisme adapté, ayant un métabolisme différent de ceux de la microflore du rumen, et dont l'adaptation à grande échelle soit compatible avec une production bon marché . Il faut également pour transformer des déchets ligno-cellulosiques en aliments du bétail, un microorganisme capable de dégrader la lignine sans hydrolyser la cellulose et l'hemicellulose. Dans cet article, nous rappelons les principes de la fermentation solide et de l'activité ligno-cellulolytique des champignons.

Mots clés : Champignons de la pourriture blanche, lignocellulose, biodégradation, délignification sélective, facteurs, réacteurs, FMS.

INTRODUCTION

The presence of lignin in plant materials, when used as feed, causes a rapid decrease in digestibility by rumen micro-organisms. In order to increase the digestibility of lignocellulose, physical, chemical and biological methods of delignification can be used (Baker *et al*, 1975; Jackson, 1978). These methods involve the splitting of the cellulose-lignin complex by extraction or decomposition of lignin.

Rumen of domestic and wild animals, modified during evolution, is an ideal fermentor, digesting lignin and cellulose containing plant residues, i.e.,

lignocelluloses (Stewart, 1981). Digestion proceeds discontinuously by anaerobic bacteria, fungi and protozoa. Cellulose and hemicellulose are partially metabolized during a relatively short time of 2 - 3 days.

The process of upgrading must be characterized by strong lignin decomposition and consequent accumulation of digestible substances. Ideal microorganisms, for upgrading of lignocellulosics into animal feed, should have a strong lignin metabolism, but with a low degradation of cellulose and hemicellulose.

The process must be simple and shouldbe carried out using low cost technology, as in the case of processes to convert plant residues into human food by cultivation of edible fungi (Chang and Quimio, 1982). The ability of fungi to grow and form fruiting bodies depends on the type, composition and treatment of lignocelluloses. For some fungi, certain additives into the substrate are sufficient to obtain good yields. Spent fungal substrate can be used as animal feed (Zadrazil, unpublished results).

The main problem in the biological upgrading of lignocelluloses into feed is to find suitable microorganisms, with metabolic patterns different from those of the rumen flora as well as fauna, and good cultivation potentials in a cheap large scale process.

In this review, fungal activities on lignocellulosics and biological possibilites of upgrading these agricultural wastes in to animal feed are discussed.

ECOLOGICAL BACKGROUND OF CONVERSION OF LIGNOCELLULOSICS INTO ANIMAL FEED BY WHITE ROT FUNGI

INFLUENCE OF FUNGAL SPECIES

Lignolytic microorganisms are mainly wood inhabitating fungi. These are able to colonize different plant residues (Zadrazil, 1976a, 1979) and increase the digestibility of the substrate (Kirk and Moore, 1972). The influence of fungal species on the decomposition of wheat straw and the *in vitro* digestibility as well as decomposition of lignin is comprehensively discussed by Zadrazil (1985). The physiological behaviours of these fungi, with respect to lignocellulose degradation, can be used to divide them into four groups:

- Fungi in group (a) decompose the substrate, without lignin degradation (brown rot fungi). *In vitro* digestibility is negative, in comparison with untreated straw. Examples are *Agrocybe aegerita* and *Flammulina velutipes*. Similar results are obtained by cultivation of lower fungi, bacteria or yeasts on cereal straw.
- The second group (b) includes fungi, which decompose lignin well, but other substrate components are only partially decomposed. *In vitro* digestibility increases. Examples are *Dichomitus squalens, Abortiporus biennis, Stropharia rugosoannulata, Pleurotus eryngii* and *Pleurotus sajor caju.*
- The third group (c) includes fungi, which decompose lignin and other substrate components slowly, but *in vitro* digestibility decreases. This may be due to the toxicity of the compounds, formed during fermentation, and the rumen micro-organisms used for the determination of digestibility.
- The fourth group (d) consists of fungi, which decompose lignin and other components rapidly, but only partially change the *in vitro* digestibility. An example is *Sporotrichum pulverulentum,* which decomposes about 3% of the organic matter daily, but causes non-significant changes in the digestibility of cereal straw (Zadrazil and Brunnert, 1982; Zadrazil, 1985).

INFLUENCE OF SUBSTRATE COMPOSITION AND QUALITY

Different substrates, including beech sawdust, rape straw, reed straw, and rice husks, were tested with different fungi for speed of decomposition of organic matter, lignin decomposition and *in vitro* digestibility, (Zadrazil, 1980). Each substrate was subjected to 60 days of solid state fermentation by each fungus. All these factors are strongly dependent on fungal species and the plant waste substrate. *Pleurotus* spp., *Dichomitus squalens, Abortiporus biennis, Stropharia rugosoannulat,a* etc., showed good lignin decomposition and increased the *in vitro* digestibility of all substrates, except for the rice husks. *Agrocybe aegerita, Flammulina velutipes* and other brown rot fungi decomposed lignin only to a small extent and decreased *the in vitro* digestibility of the rice husks. This effect is probably caused by the high incrustation of rice husks with SiO2.

INFLUENCE OF FERMENTATION TEMPERATURE

The fermentation temperature not only influenced the decomposition speed of the organic matter, but also the sequence of decomposition of the substrate components. Using all tested fungi, the increase of temperature between 22-30°C, led to an

increase. The decomposition speed of the organic matter, (Zadrazil, 1977, 1985). Large differences in the rates of straw decomposition, caused by temperature, were observed *for Pleurotus cornucopiae* and *Stropharia rugosoannulata.* A positive correlation between increases in temperature and lignin decomposition or *in vitro* digestibility was only observed for *Stropharia rugosoannulata.*

INFLUENCE OF DURATION OF FERMENTATION

In the fungal life cycles, the periods distinguished included colonization of the substrate, maturation of fungus, induction of fruiting bodies and autolyses.

During the first stage of fungal growth (colonization of the substrate), the content of digestible substances for ruminants decreases (Zadrazil, 1977). During maturation of fungus, the *in vitro* digestibility of fungal substrate increases, but it decreases, thereafter, in older substrates, which have a relatively high content of accumulated minerals. Under favourable conditions, some fungi can totally mineralise cereal straw during a 80-100 day fermentation (Zadrazil, 1985).

INFLUENCE OF NITROGEN SUPPLEMENTATION

Variation in NH_4NO_3 changed the decomposition rate and also the sequence of decomposition of substrate components. *Stropharia rugosoannulata, Agrocybe aegerita* and *Pleurotus sp. Florida* were stimulated during decomposition of the substrate, with the use of lower NH_4NO_3 concentrations, while *Pleurotus eryngii* was inhibited. The lignin decompositon rate was relatively unchanged by NH_4NO_3 variations (Zadrazil and Brunnert, 1982). *In vitro* digestibility of substrate mixture decreased in all fungi, when substrate received higher concentrations of NH_4NO_3.

INFLUENCE OF THE RATIO OF LIQUID TO GASEOUS PHASE OF SUBSTRATE

With increased water content and a constant substrate volume, the air content of the substrate decrease. This resulted in increased water tension and swelling of the substrate. All investigated fungi showed good growth on substrates with varying water contents (from 25 to 150 ml water/25 g straw). Both at the lowest and the highest water contents, the decomposition rate of the total organic matter decreased, along with that of lignin and accumulation of digestible substances. The tested fungi

showed specific growth optima for various air and water contents of the substrate (Zadrazil and Brunnert, 1981; 1982).

INFLUENCE OF COMPOSITION OF GASEOUS PHASE

The losses in organic matter and lignin, after fermentation of wheat straw with *Pleurotus sajor caju,* were found to be highest in a 100% oxygen atmosphere (Kamra and Zadrazil, 1986), followed by those in air atmosphere, in the case of *P. eryngii.* Carbon dioxide, at 1-20% level in the atmosphere, influenced neither organic matter loss nor lignin degradation. However, at 30% concentration, the organic matter loss increased slightly, but lignin degradation decreased considerably. Lignin was degraded at a much lower rate, at less than 20% oxygen in the atmosphere. The increase in the *in vitro* digestibility was highest in pure oxygen, followed by that in the air atmosphere. Carbon dioxide, at 1-10% level, positively influenced the increase in digestibility, but at a higher concentration, the digestibility was reduced (Kamra and Zadrazil, 1986).

Gaseous metabolites of fungal degradation of straw have strong influence on mineralization of organic matter, loss of lignin and *in vitro* digestibility (Zadrazil *et al,* 1991). For large scale processes, the composition of gaseous phase is identified as the key-factor. Influence on composition of the substrate, following different treatments with gaseous metabolites, is sumarized by Buta *et al* (1989) and Chiavari *et al* (1989).

LARGE SCALE CONVERSION OF LIGNOCELLULOSICS: SCALE UP OF SOLID STATE FERMENTATION

The analysis of *Palo podrido* samples (Phillippi, 1893; Knoche *et al,* 1929; Zadrazil *et al,* 1982), showed that the use of white rot fungi for upgrading lignocelluloses into feed is possible, at least on a laboratory scale and in natural processes. On the other hand, a little is known about the large scale process. Solid state fermentation of lignocelluloses in deep layers might be proposed (Schuchardt and Zadrazil, 1982, 1988).

DEFINITION OF SOLID STATE FERMENTATION

Solid state fermentation can be defined as a process, in which solid substrates are decomposed by known mono- or mixed-cultures of micro-organisms (mainly fungi, which can grow on and in these substrates), under controlled environmental conditions and with the aim of producing a high quality standardized product or products. The substrate of different particle sizes is characterized by a relatively low water content. Since much of the water is chemically or physically bound to the substrate, physical properties, e.g., porosity and density are uniform. The substrate is not mixed or moved during the process (Zadrazil *et al,* 1990a,b). Temperature is controlled by air circulation in the reactor.

Recently, many different reactors for solid state fermentation have been designed, developed and constructed. Some of these are used in koji process for production of soya bean sauce, or in the production of substrate for the cultivation of edible fungi, i.e., *Agaricus bisporus* (white mushroom) (Gerrits, 1988) and *Pleurotus* spp. (Schuchardt and Zadrazil, 1982, 1988). Another proposed use for this fungal substrate is the conversion of lignocellulosics into animal feed (Laukevics *et al,* 1984; Zadrazil, 1977, 1980, 1985), chemical feedstocks (Hatakka and Pirhonen, 1985) and for biological pulping (Kirk *et al,* 1980; Eriksson *et al,* 1990). Prepared substrates can also be used as a biofilter in environmental control (Hüttermann *et al,* 1988), or for decontamination of xenobiotics in soil and waste materials (Hüttermann *et al,* 1988, 1989; Martens and Zadrazil, 1992).

Many factors influence the course of solid state fermentation, but only some factors, such as temperature, humidity and composition of gas phase, can be controlled and changed during the process.

THE PRINCIPLES OF SSF

The pretreated substrate is filled into the reactor in 1.5-2.0 m deep layers and incubated by passing the gas phase through the substrate. The temperature in the substrate is indirectly controlled, by passing conditioned gas. In the cultivation of *Agaricus spp.*, fresh air is passed in order to cool the substrate. In contrast, in the production of wood decaying fungi, high carbon dioxide and low oxygen concentrations are required, during the period of colonization (Zadrazil, 1975).

DESCRIPTION OF SSF-REACTOR

The principal design of a small scale reactor and its control have been described by Schuchardt and Zadrazil (1982, 1988). The reactor used in the Institute for Soil Biology, FAL, Braunschweig, FRG, is based on previous experience. The reactor is constructed of polyurethane foam sandwich panels which are covered on both sides with polyester board. The filling height is approximately 2.0 m, the internal width 2.3 m and the length 2.0 m. This results in a net filling volume of 9.2 m^3, equivalent to 1.5 tonne straw or 3.0 tonne wood chip substrate.

Two similarly insulated swing doors are situated at the front of the reactor. The width of the doors is equal to the width of the reactor itself. Inside the reactor, there is a raised slatted floor, covered firstly with a gliding net and then with a drag net. Two reactors of the same shape and construction are used. One reactor is used for substrate pretreatment and the second for substrate colonisation (Zadrazil *et al,* 1990a,b). Following the movement of substrate from one reactor to another inoculation proceeds.

The substrate is filled into the reactor, using a specially designed machine, which deposits it on the drag net. A removable front panel keeps the substrate from falling out of the container, during filling. The substrate is removed from the container, by attaching the drag net to a winch. The substrate is loosened, as it is pulled through a set of toothed bars, before falling on the elevator. Then, it is filled into another reactor, either for incubation or treatment of the fully colonized substrate. This translocation from one reactor into the another, decreases the bulk density of the substrate and reduces the stream resistance of the gas phase by the substrate. Stream resistance could be a parameter for fungal growth activity.

AIR-CONDITIONING

Fungal growth and heat exchange from the substrate are controlled by recirculating the air within the reactor. For the cultivation of *Pleurotus spp.* and other wood decaying fungi, the gaseous phase is recycled and gas composition is controlled by monitoring CO_2 and O_2 concentrations (Schuchardt and Zadrazil, 1982, 1988). To control and reduce the growth of competitive microorganisms, CO_2 could be passed at the begining of the fermentation process.

The reactor has an air-conditioning system, installed on the roof or in the gas-tubes. A system of aluminium air ducts (supply duct below, and return ducts on the roof), ventilates the substrate from below. The total quantity of circulated air can be varied by changing the speed of electronically controlled fans. A centrifugal type fan was chosen, to ensure that the various processes would be controlled adequately. It has a

capacity of 800 m^3/h at a static pressure of 1200 Pa, or approximately 200-500 m^3 air per tonne substrate per hour (depending on its specific volume weight). The most suitable fans, for this purpose, are of the centrifugal type, with backward curved blades, as their capacity varies only slightly with decreasing resistance.

In order to humidify the air and raise the substrate temperature for pretreatment of substrate (e.g., pasteurisation), a steam injection pipe is installed under the slatted floor. Steam injection is controlled by a 2-way valve, operated by a servomotor. Heat exchange takes place by refrigeration cooling in the air-circulation system, controlled by a servomotor driven 4-way valve. The lowest temperature of cooling liquid can be -15°C.

Fresh air, oxygen, or carbon dioxide can be introduced, after gas analysis, by computer-controlled valves. Exhaust gases leave the fermenter, through an over-pressure valve, located above the substrate.

CONTROL OF GAS HUMIDITY

The humidity of gaseous phase was controlled by hygrometers (Hygrotest 720), placed in different parts of the reactor. The air humidity fluctuates between 95 and 100%. Commercially available hygrometers are not sensitive enough in this region, to give more precise control.

CONTROL OF WATER EVAPORATION

Saturation deficit of water in the gaseous phase increases during the penetration of gas through the substrate. The gaseous phase has a lower temperature than the substrate and, hence, water evaporates. From the circulating gases, water condenses on the cooling equipment as well as within cold areas of substrate and reaches 100% relative humidity again. Evaporation of water in circulating gases was measured by the water loss from a 20 cm^2 ceramic disc (Czeratzki, 1968), placed in the space above the substrate.

WATER TRANSLOCATION

Evaporation of water from the substrate and condensation on the cold parts of reactors as well as the cooling unit are undesirable, but cannot be eliminated. The translocation of water could be a measure of the technical standard of the reactor and

the efficacy of the control system. All condensed water is led into the container, at the base of the reactor, and the quantity can be measured periodically.

METABOLIC HEAT

For scientific studies, the ducts must be insulated to eliminate the uncontrolled heat loss from the reactor. The rate of heat liberation during the fermentation process can be measured by a flowmeter, installed in the cooling system of the reactor.

TEMPERATURE CONTROL

The temperature of the gas phase was monitored, by placing 4 PT100 resistance thermometer sensors at the inlet and outlet ducts. Temperature of substrate is measured at 4 different layers with 8 thermometer sensors and recordet using 2 independent computers.

Minimum and maximum limits for temperature can be adjusted. If the temperature of the air rises above or falls below the temperature limit, the heating or cooling system is activated and an alarm could be calibrated for critical temperature areas. Different possibilities, mathematical and physical models for temperature control, are discussed by Teifke and Bohnet (1990).

AIR SUPPLY AND CONTROL OF CIRCULATED AIR

The amount of air, circulated in the reactor through the substrate, is determined on the supply side of the fan, by measuring the difference in pressure across a gauge ring. The volume of circulated gas is indicated in m^3. The accuracy of this apparatus is good and air resistance is very low. The required fresh air is added with an air pump and measured with a flowmeter.

DIGESTIBILITY AND HOMOGENITY OF PRODUCT

The digestibility of the fermented substrate (Tilly and Terry, 1963) increased by 13.8 digestibility units on average. The highest increases (18.7 and 18.3 units) were found in the two layers near the substrate surface and the lowest (7.0 units) in the bottom layer. The observed increases in the digestibility of cereal straw, following

fungal treatment on a large-scale, were comparable with results obtained by sodium hydroxide or ammonia treatment (Sundstol and Owen, 1984).

After incubation, the substrate was also used for production of edible fungi. The colonized substrate was placed into a container for fructification. The yield of fruit bodies was comparable to that obtained using other cultivation systems.

The final product (fungal substrate) differs in water content and digestibility. One may assume that differences between the water contents of different layers have an influence on the digestibility of substrate. It may be possible to eliminate this phenomenon by better control of the gas phase.

CONCLUSIONS

1. *In vitro* digestibility of fungal substrates decreases at the beginning of colonization by white rot fungi and increases afterwards (Zadrazil, 1977; Zadrazil and Brunnert, 1982). During incubation, the contents of soluble substances (partly sugars) increases (Zadrazil, 1976b; Lindenfelser *et al*, 1979).
2. The increase in digestibility depends on the fungal species (Zadrazil, 1979), cultivation time, temperature, water/air ratio in the substrate as well as the product, CO_2/O_2 ratio in the gaseous phase and composition of the substrate (Zadrazil and Brunnert, 1981, 1982).
3. The *in vitro* digestibility of lignocelluloses by white rot fungi decreases with the addition of inorganic nitrogen (Zadrazil and Brunnert, 1980).
4. The SSF-pilot reactor described and utilized at the Institute of Soil Biology, FAL Braunschweig, since 1985, can be used, after some technical modifications as a model for large-scale technology.
5. Insufficient knowledge exists about solid state fermentation and suitable reactors for this process. More basic and applied research must be done, before this mushroom technology could be transferred into another area of exploitation of biotechnology.
6. For each organism, special strategies for the design of the reactor and the control of the process, must be developed.
7. Cheap and easily-controlled reactors must, therefore, be developed for the application in developing countries.

Based on the studies reported here and elsewhere, one may conclude that there is a need for further research on several aspects of the SSF process. These include :

1. Development of new designs and constructions of SSF reactors for meeting the requirements such as homogenous conditions during processing and minimal differences between process parameters (growth conditions) at different layers of substrates.
2. Development of equipment and sensors for the control of the SSF process, with respect to the control of the air speed in different parts of reactors, control of air humidity (95-100% relative humidity), control of water evaporation from substrate and control of water translocation.
3. Development of efficient strategy for process control.
4. Development of mathematical models for the SSF process.
5. Verification of results in laboratory and pilot-scale reactors.
6. Comparative economic studies with other lignocellulose-upgrading processes.
7. Research and development of solid state fermenters or reactors that are cheap, easy to use and suitable for developing countries.
8. Training and development programs for scientists in developing countries, essential for the success of technological transfer in this modern field of research.

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Protein enrichment of sugar beet pulp by solid state fermentation and its efficacy in animal feeding

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SUMMARY

Sugar beet pulp (SBP) with a moisture level of 80.0-85.0% was fermented with *Trichoderma viride, T. reesei, Phanerochaete chrysosporium, Dichomitus squalens* and *Phlebia radiata. T. reesei* was chosen, due to its high bioconversion rate and high degradation of hemicellulose and cellulose. The fermentation increased its protein content by 70.0%, (from 13.3 to 22.5% D.M), mainly at the expense of hemicellulose, which decreased by 54.0% (from 36.7 to 16.7% DM), while cellulose was slightly reduced. The lignin and ash content of the substrate remained largely unchanged. Feeding trials on mice showed a weight gain during the 30-day feeding period. However, palatability was lower in fermented sugar beet pulp (FSBP) diet, as compared to the control.

The protein digestibility of the fermented pulp was higher, as compared to the unfermented material. Fermented pulp can be a potentially good feed supplement suitable for both monogastric and ruminants, to replace their normal fodder at a certain extent.

Keywords: Sugar beet pulp, microbial protein, solid state fermentation, animal feeding, *Trichoderma reesei,* weight gain, palatibility, protein digestibility;

RESUME

Enrichissement en protéines de la pulpe de betterave par fermentation en milieu solide et son efficacité pour l'alimentation animale.

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La pulpe de betterave (P.B) fraîche, à 80.0 - 85.0% d'humidité a été fermentée avec *Trichoderma viride, Trichoderma reesei, Phanerochaete chrysosporium, Dichomitus squalens* et *Phledia radiata*. Parmi ces souches, *T. reesei a été sélectionnée* pour son haut rendement de bioconversion et de la dégradation élevée de l'hemicellulose et de la cellulose. La pulpe de betteraves fermentée (PBF) a été enrichie de 70,0% en protéïnes (de 13,3 à 22.5% en matière sèche) au dépend de l'hemicellulose qui diminue de 54.0% (de 36.7% à 16.7%) alors que la concentration en cellulose varie plus lentement. La lignine et les cendres contenus dans le substrat ne varient pas d'une manière significative. Des essais nutritionnels ont été réalisés sur des souris à qui on a administré un régime complémentaire de PBF à la ration de base pendant une période de 30 jours de régime. Cependant l'acceptabilité (palatabilité) de la PBF a été inférieure à celle du régime de contrôle. La dégradabilité des protéines de PBF, estimée dans le rumen de moutons fistulés, a été plus élevée que celle de la PB non fermentée. La pulpe de beterrave fermentée peut être un bon complément pour l'alimentation des animaux monogastriques et ruminants, en remplacement de leur ration normale en protéine jusqu'à un niveau.

Mots clés: Pulpe de café, protéines microbiennes, fermentation en milieu solide, aliments pour le bétail, *Trichoderma reesei*, gain de poids, appetissant, digestibilité de protéines.

INTRODUCTION

Agricultural by-products have a high nutritive potential and ,when properly utilized, can contribute to a better and cheaper feeding of livestock (Israilides *et al*, 1979; Pemir and Anil, 1988). Sugar beet pulp is a lignocellulosic by-product of the sugar industry. In Greece, the annual production of sugar is about 300,000 tonnes from five refineries. This results in the production of about 250,000 tonnes fresh, molasses- free SBP, plus another 100,000 tonnes SBP with molasses (Iconomou, 1993).

Most of this SBP is not effectively utilized, except for some uses up to 30 and 15% of the diet of ruminants and pigs respectively, after supplementation with various protein sources. The protein content of SBP (9.50 ± 0.20% on the basis of dry matter, (D.M.) is considered low, compared to the requirements (in general 10.0-15.0%) of most ruminants and those of the monogastric animals, which are even higher (ARC, 1965). Presence of other compounds, like the lignocellulose complex, has also been reported, as a reason for the lower availability of the nutrients in lignocellulosic by-products (Iconomou, 1982).

The nutritive value of SBP for feedstuffs may be improved after a solid state fermentation with various lignocellulolytic microorganisms. This is achieved mainly by increasing its protein content and lowering its fiber (cellulose and hemicellulose) content. Many lignocellulolytic fungi have been reported for the bioconversion of various cellulosic substrates (Peitersen, 1975; Eriksson, and Larsson, 1975; Zadrazil, and Brunnert, 1982; Durand *et al*, 1983; Bakshi *et al*, 1986; Iconomou, 1982, 1993).

Solid state fermentation of SBP with the lignocellulolytic fungus *Trichoderma reesei* was the approach of this study for the production of a animal feed. Nutritional evaluation of the fermented sugar beet pulp (FSBP) has also been studied.

MATERIALS AND METHODS

MICROORGANISMS

The micro-organisms used to ferment fresh SBP included a) *Trichoderma reesei* (N.R.R.L. 6156, Syn. Q.M 9414), b) *Trichoderma viride* from the collection of microorganisms of the Institute of Technology of Agricultural Products, Greece, c) *Phanerochaete chrysosporium* (B.K.M. Provenance Madison No 37), d) *Dichomitus squalens* (No 30), and e) *Phlebia radiata* (No 1.4.17.A, No 25). The last three strains, were from the Institut National Agronomique (I.N.A), Paris - Grignon (France).

The micro-organisms were grown and maintained in a medium containing (g/L): Glucose, 20.0; NH_4NO_3, 2.0; Peptone, 1.0; yeast extract, 0.5; KH_2PO_4 1.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; Agar, 1.5; and pH, 5.0. The incubation temperature was 28 ± 1°C.

SUBSTRATE

Raw pulp was heated at 110°C for 30 min. After cooling, the substrate (moisture 80.0%) was mixed with 5.0% (v/w) of a nitrogen-mineral solution, which served both as a buffer as well as nitrogen sources and contained (g per 100 ml), NH_4NO_3, 7.0; NH_2CONH_2, 3.0 (Israilides *et al*, 1994).

FERMENTATION AND EQUIPMENT

The microorganisms grown on the above liquid medium for 48 h, were filtered and inoculated into the substrate (50 ml culture broth/100 g dry pulp), and mixed aseptically. The mixture was placed in 2 liter Erlenmayer flasks as well as in specially designed trays, which were loaded in an incubator chamber under controlled conditions for a solid state fermentation, without agitation (Iconomou, 1993). The temperature was maintained at 28 ± 1°C and the relative humidity was kept constant at 85.0 ± 1% in the chamber. The initial pH was 5.0 and fermentation was continued for 3 and 5 days. The FSBP from the incubation chamber provided enough material for the nutritional evaluation.

ANIMAL FEEDING

Feeding trials were carried out on sheeps and mice.

Three "Karaguniko wethers" sheeps, weighing 70 kg live weight on average, were fitted with a permanent rumen cannula of 40 mm in diam. Six nylon bags, each containing 5 g fresh materials, were introduced into the rumen for 2 successive days. These were removed from the rumen at 1.5,3,6,9,12 and 24 h after placement, washed under tap water and dried at 60°C for 48 h. The disappearance of dry matter and nitrogen was calculated from the initial amount and the amount left in the bags after incubation, as decribed by Kandylis and Fegeros (1986). The rate of passage of protein particles from the rumen was determined by treatment with sodium bichromate, as described by Ganev *et al* (1979). The nylon bag technique and the parameters measured for the estimation of protein digestibility were as described by Orskov, et al. (1981) and Orskov and McDonald (1979).

Feeding trials on mice of the "Wistar" strain were conducted, as described previously by Israilides *et al* (1994). Feed consumption, weight gain and acceptability trials were run on mice fed with a basic diet (control, C) mixed with FSBP at levels 15.0% (A1 diet) and 30.0% (A2 diet).

ANALYTICAL PROCEDURES

Dry matter(D.M.) in the residues was determined by drying in an oven at 60°C for 48 h. Residual nitrogen was estimated by the Kjeldahl method. Crude protein was estimated by multiplying the value by 6.25, according to the method of AOAC (1984).

For determining microbial protein production, samples were washed thoroughly to remove soluble nitrogen and sugars. These were then dried, milled to 1 mm length and the residual (N) and protein were determined as previously. In the filtrate, polygalacturonic acid was determined, according to the method of Bitter and Muir (1962). Protopectin was determined according to McGready and McComb (1952). Cellulose, hemicellulose, lignin and ash were determined by the method of Goering and Van Soest (1970). FSBP was tested for aflatoxins B1, B2, G1 and G2 by ELISA and T.L.C. methods (Cuartero *et al*, 1990; OJEC, 1976).

Unless otherwise specified, all other chemical analyses were carried out, according to the AOAC (1984) methods.

RESULTS AND DISCUSSION

In all samples tested, no aflatoxins were detected. Fermentation parameters and protein yield (g protein produced / g cellulose and hemicellulose consumed) are given in Table 1. The variation in protein at time 0, is due to different amounts of biomass in the same volume of inoculum broth. Data in Table 1 showed that *T. reesei and Phan. chrysosporium* were most efficient in converting the SBP into fungal protein (yields 39.5 and 41.3%, respectively). For the feeding trials, the FSBP obtained with the use *of T. reesei.* was selected and its protein enrichment was 70.0%. The hemicellulose portion of the FSBP decreased by 54.0%, while the cellulose decreased by 16.5%.

Table 1. Fermentation parameters with fermented sugar beet pulp(FSBP) using different strains of fungi in semi-solid fermentations.

Strains	Time : Start End	Produced Protein (%D.M) Start End *Dif:*D1*	Cellulose (%D.M) Start End *Dif:*D2*	Hemicellulose (%D.M) Start End *Dif:*D3*	Yield (%) *D1 /D2+D3 X100*
Trichoderma	0	13.25	20.60	36.65	39.50
reesei	96	22.50 D1=9.25	17.10 D2=3.40	16.65 D3=20.00	
Trichoderma	0	14.20	25.10	28.70	36.40
viride	144	23.00 D1=8.80	14.05 D2=11.05	15.60 D3=13.10	
Phaneroch..	0	14.05	23.50	37.30	41.30
chrysosporium	120	24.75 D1=10.70	16.80 D2=6.70	18.10 D3=19.20	
Phebia	0	14.0	26.10	39.40	31.20
radiata	12	24.90 D1=10.90	14.60 D2=11.60	16.05 D3=23.35	
Dichomitus	0	15.05	25.50	39.30	34.40
squalens	120	24.45 D1=9.40	14.35 D2=11.10	22.10 D3=17.20	

Fermentation periods were 120 hours, except for 96 and 144 hours for *T. viride.*

I= Initial, F= Final

Yield: (Microbial protein producer, 1 / cellulose 2 + hemicellulose) x 100

The chemical composition of unfermented sugar beet pulp (USBP) and FSBP is shown in Table 2. The microbial fermentation increased the total crude protein content to 22.6 + 0.20 (3 days) or 24.8 ± 0.20% D.M. (5 days). No free added nitrogen was left at the end of fermentation. In the USBP filtrate sample, the trace amounts of polygalacturonic acid (P.A.) were detected. After 5 h fermentation, the content of P.A. was raised to 9.40 g/100 g DM, thereby releasing most of the protopectin determined in dry beet pulp (12.4 g per 100 g DM). This was probably due to the microbial enzymatic action. At the end of the 5 days fermentation period, the P.A. was absent. Thus, the P.A. also contributed to the biomass production

(Iconomou, 1993). The lignin and ash content of the fermented pulp remained practically unchanged, while there was apromaxitely a 2.5 fold increase in fat.

Table 2: Chemical composition (% of dry matter) of unfermented (USBP) and fermented sugar beet pulp (FSBP) with T. reesei for 3 and 5 days.

Substance	USBP	FSBP	
		3 days	5 days
Moisture	7.0 ± 0.2	7.0 ± 0.2	7.3 ± 0.2
Crude Protein	9.5 ± 0.2	22.6 ± 0.2	24.8 ± 0.2
Cellulose	21.5 ± 0.5	20.8 ± 0.1	20.6 ± 0.2
Hemicellulose	30.0 ± 0.5	14.0 ± 0.5	12.0 ± 0.5
Fat	0.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.2
Lignin	3.9 ± 0.1	4.2 ± 0.1	4.2 ± 0.2
Ash	2.2 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
Undetermined fraction	33.0 ± 0.2	34.8 ± 0.2	34.6 ± 0.2

Table 3. Per cent dry matter (DM) and Nitrogen (N) disappearance (potential degradability) of the USBP and FSBP with T.reesei for 3 and 5 days from the in sacco incubation in the rumen of sheep.

Sugar beet pulp	% D.M.	% Nitrogen disappearance (Effective degradability) *
USBP	61.0 ± 1.4	40.0
FSBP 3 days	56.0 ± 4.5	70.0
FSBP 5 days	60.0 ± 4.7	70.2

* p=a+bc/c+k, where a= the rapidly soluble protein fraction,b= the fraction which is subject to degradation, c= the rate of degradation, e= the natural logarith, and k= the fractional outflow rate per hour. a,b,c: constants fitted by a least square procedure

The mean values for dry matter and N disappearance from the supplements following various incubation times in the sacs, placed in the rumen of sheep, are presented in Table 3. The % nitrogen loss increased from 40.0% to 70.0% during the incubation, due to the disappearance of the most soluble fraction in the

fermented samples. The effective protein digestibility (% N disappearance) was similar (70%) for both 3 and 5 day FSBP, while the respective degradation of the original material was much smaller (40%). Dry matter remained practically unchaged in the 3 and 5 day FSBP.

The mice gained weight in the case of both the test diets and control. With diet containing 15.0 and 30.0% FSBP, the average weight gain (g/mouse) was 5.5 and 3.5 g respectively in a 30 day feeding trial (Israilides *et al*, 1994). Feed consumption and weight gain of mice was the same for the control diet and the A1 diet. Feed consumption and weight gains were 1.5 times lower for the A2 diet, than for the control and the A1 diets. In free choice feeding trials, the control diet was preferred over the test diets. However, the acceptability did not differ significantly between the two test diets ($p>0.01$).

CONCLUSION

Trichoderma reesei was chosen for the solid state fermentation of SBP, due to its high microbial protein production and degradation of hemicellulose as well as cellulose. The FSBP with *T. reesei* increased the protein content by 70.0% (from 13.3% to 22.5%), mainly at the expence of hemicellulose, which decreased by about 54.0% (from 36.7% to 16.7%), while cellulose was slightly consumed (about 16.5%). The relatively moderate protein degradation rates found in the two fermented sugar beet supplements were much less, in comparison to the original material. Feed efficiencies in mice (g weight gain / g feed) were not significantly different between all diets.

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Solid state fermentation of wheat straw: Methods for detecting straw quality and improving biodegradability of poor quality straw

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SUMMARY

The quality of lignocellulosic materials is known to vary, even in a single type, such as wheat straw. During solid state fermentation (SSF) experiments at a laboratory-scale with different straw samples, it was observed that measurements of soluble compounds and cellulose availability were efficient tools to estimate the quality of straw for use in SSF, in terms of the straw biodegradability. When the quality is not optimal, its degradability could be improved by addition of low quantity polysaccharidases adsorbed on amylodextrins. This additive was proved to act by inducing the production of cellulases and by improving bacterial development.

Key words : Solid state fermentation, wheat straw, quality of wheat straw, quality estimation method, improving poor quality straw, biodegradation, cellulose, polysaccharidases, *Bacillus* spp., composting.

RESUME

Fermentation en milieu solide de la paille de blé: Méthodes pour détecter la qualité de la paille et pour améliorer la biodégradabilité des pailles de pauvre qualité.

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La qualité des materiaux lignocellulosiques peut être très variable, même lorsque l'on s'intéresse à un seul type comme la paille de blé. Cette variabilité n'est généralement pas prise en compte lors de l'utilisation des pailles en fermentation en milieu solide (FMS). Au cours d'expériences à l'échelle du laboratoire avec différents lots de pailles, nous avons observé que la mesure des éléments solubles et l'accessibilité à la cellulose étaient des outils efficaces pour estimer la qualité des pailles pour leur utilisation en FMS. Cette qualité est définie par la biodégradabilité. Quand elle n'est pas optimale, la dégradabilité peut être améliorée par l'addition de faibles quantités de polysaccharidases adsorbées sur des amylodextrines. Il est montré ici que cet additif agit par induction de la production de cellulases et améliore le développement bactérien.

Mots clés : Fermentation solide, paille de blé, qualité des pailles, méthode d'estimation de la qualité, amélioration de la qualité, biodégradation, cellulose, polysaccharidases, *Bacillus* spp., compostage.

INTRODUCTION

The world-wide abundance of cereal straw and its high polysaccharide content favour efforts for using it as a ruminant feed and substrate in SSF. Although, information is available on the effect of genetic variation, location, fertilizer application, stage of harvesting and storage on the quality and feeding value of straw (Theander and Aman, 1984; Herber and Thomson, 1992; Kernan *et al*, 1993), the research on variability in straw quality for SSF and its consequences has received lesser attention. However, selection of microorganism and success of SSF will be dependent on the quality of solid substrates used. To control and improve the biotransformation of a lignocellulosic material to biomass or metabolites, it is necessary to determine its ability to be degraded by microorganisms. *In vitro* and *in vivo* digestibility tests are used to obtain feeding values. Chemical analysis, measurements of *in vitro*

degradability and *in situ* biodegradation during SSF were compared in the present investigation to define methods and estimate straw quality for SSF. Microorganisms in SSF are often fungi and the consequences of increased use of fungicides during cereal cultivation could be a problem. Variability in straw quality due to fungicide application was evaluated in this and previous studies for the purpose of culturing *Agaricus bisporus* (Savoie *et al,* 1992; Chalaux, 1993).

Chemical treatments are currently used to increase straw digestibility and research on biological treatments is in progress (Rai *et al,* 1989). The purpose of using different treatments is to render the straw polysaccharides more available to microorganisms. An alternative way of treatment is to stimulate the degradative abilities of microorganisms. We succeeded in increasing the velocity of straw degradation and production of polysaccharidases by a microbial community during wheat straw composting (Libmond and Savoie, 1993; Savoie and Libmond, 1994). Data supporting an explanation of this stimulation are presented here to permit the use of the activators in other SSF processes.

MATERIALS AND METHODS

The effects of fungicide application were studied on 5 different wheat cultivars. The treatments included, not treated (F-), treated (F+) or treated twice (F++) and the fungicides treated cultivars were grown in randomized block designs. Straw was chopped into 5 cm lengths and stored under a plastic shed or in the laboratory. For the fermentation, moisture content was adjusted to 65% and $(NH4)_2SO_4$ (5% dry matter) as well as $CaCO_3$ (10%) were added. The substrate was introduced into thermostated stainless steel reactors of 5 liters (Bono *et al,* 1992). Sterile air was humidified and heated to the temperature of that of the reactor and blown continuously throughout the substrate, from the bottom to the top, at a rate of 100 ml/min. The exhaust gas was continuously trapped in 1 N H_2SO_4 and then in 5 N NaOH. The CO_2 trapped in NaOH was measured by a colourimetric method. A Technicon autoanalyser method was used for the analysis of NH_3, which was trapped in H_2SO_4. Every 2 or 3 days, the reactors were opened, the fermenting mass was mixed and samples were collected for analyses. Cellulase and xylanase activities were assayed after extraction from the straw (Libmond and Savoie, 1993). One U of FPase activity was equivalent to 1 µmol/hour of glucose released from 50 mg Whatman No 1 filter paper (Mandels *et al,* 1976). One U of xylanase activity was equivalent to 1 µmol/min of xylose released from xylan. Samples were dried in an oven at 80°C and ground to pass through a 0.5 mm sieve. The neutral detergent fiber (NDF)

content and sometimes the cellulose, hemicellulose and lignin contents, were measured (Van Soest and Wine, 1967). Water soluble, hot ethanol soluble and ash contents were also measured (Savoie *et al,* 1992). The *in vitro* degradability of polysaccharides was estimated by measuring the solubilisation of sugars by polysaccharidases. The number of colony forming units (CFU), after incubation at 48°C was determined on selective media for bacteria and fungi (Libmond and Savoie, 1993).

For the study on induction of CMCase production by *Bacillus* spp., the culture medium plus the biomass were centrifuged at 19 000 g. The residue was suspended in 1 N NaOH and boiled for 10 min. Proteins from the bacterial biomass were assayed colorimetrically (Bradford, 1976). CMCase activity was measured by incubating 0.5 ml of supernatant with 0.5 ml of 3% CMC in 0.1 M acetate (pH 5.0) for one hour at 45°C. One unit of activity was equivalent to 1 µmol glucose / min.

RESULTS AND DISCUSSION

DETERMINATION OF WHEAT STRAW QUALITY AND ITS VARIABILITY

Hot ethanol solubility was shown to provide an estimate of plant residue degradability (Spalding, 1979) and its relation to an easily available C-pool was reported (Reinertsen *et al,* 1984). Rapid colonization by microorganisms could result from growth on these components. Cell-wall components are, however, the main C resource in plant wastes. Lignin is considered to be the major biodegradation obstacle, however, either none or significant positive correlations between the lignin content and biological or enzymatic degradation of straw cell wall polysaccharides were observed (Savoie *et al,* 1992, 1994). In fact, the chemical nature of lignins and how they are linked with the other cell-wall polymers are as important as the total amount of lignin present (Cornu *et al,* 1994). Enzymatic methods could enable a good evaluation of plant residue quality. With 6 wheat straw samples, a positive correlations in cell wall degradation by a mixture of polysaccharidases and by 3 fungi in pure culture was observed (Savoie *et al,* 1994).

The efficiency of biochemical analyses of wheat straw to estimate its quality for SSF and determine variability in straw quality were tested in an experiment on the importance of fungicide treatments during wheat cultivation. Five cultivars from 2

sites of cultivation, treated once (F+), treated twice (F++) or not (F-) with fungicides, were used (Savoie *et al,* 1992). *In situ* degradation of the straw samples by the naturally occurring microbial populations was studied in laboratory-scale reactors at 45°C for 19 days (Chalaux, 1993). For each cultivar, fungicide schedules showed consequences on straw biodegradation and microbial respiratory activity at the beginning of the process (Table 1). No effect on NDF degradation due to fungicides was observed. For each cultivar extractable cellulase activities were higher during the initial days of the process in F+ than in F- and, generally, a higher respiration was observed.

The present data are partly related to the quality of F+ samples compared to F- samples in terms of (i) higher concentrations of hot ethanol-soluble (HES) compounds, (ii) higher cellulose/ lignin ratio, (iii) lower or equal *in vitro* degradability of cellulose (Savoie *et al,* 1992). Otherwise, fungicide residues detected in straw were sufficient to limit the growth of some fungi (Chalaux et al, 1993) and could then affect SSF.

Table 1 : Changes in microbial activities and straw decomposition during composting of straw samples from 5 wheat cultivars cultivated with (F+, F++) or without (F-) fungicides.

	'Capdur'			'Goelent'			'Baroudeur		'Soisson'		'Thésée'	
	F-	F+	F++	F-	F+	F++	F-	F+	F-	F+	F-	F+
mg CO_2/g												
3 d	80	109	131	75	94	99	112	60	138	129	141	191
19 d	311	366	382	394	360	447	357	238	441	403	406	578
Cellulases U / g												
2 d	19	33	28	20	28	31	28	34	23	45	22	43
5 d	38	42	42	15	20	27		44	40	43	27	44
12 d	45	50	32	50	51	49	27	19	47	44	10	25
Degradation of NDF, (%°)	38	62	49	49	29	47	48	42	45	40	38	39

The overall straw decomposition was a balance between opposite effects of fungicide treatments and the result was a higher or a lower degradation of straw, depending on the cultivar and the site of cultivation (Chalaux, 1993). It was, therefore, difficult to estimate the overall effect of fungicide schedules on the behaviour of straw during

SSF, but biochemical measurements of the straw biodegradability could provide information on the beginning of the process. Correlations between measurements of the microbial activity development during SSF and parameters of straw quality estimation (Savoie *et al,* 1992) were determined (Table 2).

Table 2 : Correlation coefficients between microbial activities during SSF and parameters of straw quality measured before SSF on 12 different straw samples.

	mg CO_2 /g		Cellulase U			NDF
	3 d	19 d	2 d	5 d	12 d	degraded, %
mg CO_2 /g, 19 d	0.76*					
Cellulases, 2 d	0.42	0.45				
Cellulases, 5 d	0.35	0.11	0.54*			
Cellulases, 12 d	-0.31	-0.08	-0.11	-0.26		
NDF degraded,%	-0.06	-0.20	-0.01	0.07	0.01	
Straw components						
Hemicellulose	0.01	-0.08	-0.50*	-0.05	0.49*	-0.18
Cellulose	0.43	0.30	0.26	0.77*	-0.39	-0.29
Lignin	-0.25	-0.26	-0.45	-0.81*	0.06	0.21
HES NH_2-N	0.12	0.49*	0.25	-0.07	0.59*	0.40
HES sugars	-0.09	0.31	0.57*	-0.18	0.33	-0.30
In vitro cellulose degradability	0.41	0.32	0.29	0.16	-0.54*	0.43

* Significant correlations at $P<10\%$.

The overall NDF degradation was negatively correlated with microbial activities, but was positively correlated with *in vitro* cellulose degradability. The rapid development of biomass was positively correlated with the rate of cellulose content and its degradability, whereas the total respiration was correlated with ethanol soluble nitrogen. These corresponded to a rapid production of cellulases, which was also negatively correlated with the concentration of lignin and hemicellulose. When cellulose was less degradable, higher cellulase activities were observed latter in the SSF, whereas a higher production of cellulases after 2 days was correlated with higher contents in ethanol soluble sugars. Thus, straw samples with sufficient quantities of soluble sugars and potentially available cellulose were more favourable

to a rapid development of microbial activities during SSF, whereas both lignin and hemicellulose had negative effects.

IMPROVEMENT OF WHEAT STRAW BIODEGRADATION BY STIMULATING THE PRODUCTION OF POLYSACCHARIDASES

In Table 2, higher production of cellulases during the first days of composting as linked to higher production of CO_2 and higher concentrations of soluble sugars in the straw. When the quality of raw material (wheat straw) is not optimum, improvement of the biological mechanisms of lignocellulose degradation at the beginning of the process could be obtained by activation of some microbial activities.

The addition of low quantities of polysaccharidases adsorbed on amylodextrins (Czym,) to decomposing wheat straw or to the raw materials of environmentally controlled composting of straw plus poultry manure had three consequences: (i) supplied and released low quantities of readily available sugars; (ii) increased the cellulase activities in the substrates and (iii) increased the number of aerobic bacteria (Libmond and Savoie, 1993; Savoie and Libmond, 1994).

The effects of the enzymatic fraction (E) of the amylodextrins (A) and the commercial product (C) were compared to an untreated control (U) during SSF of 'Baroudeur' F- straw samples at 48°C for 12 days simultaneously in 4 laboratory-scale reactors. The NDF degradation, the respiratory activity and the production of polysaccharidases were stimulated by the three treatments but higher effects were obtained with C (Fig. 1). The data supported previous observations that the enzymes, and not only the amylodextrins, were responsible for the stimulating effect of Czym, (Libmond and Savoie, 1993) and showed a synergistic effect between the components. When the enzymes present in Czym, were applied *in vitro* on water extracted wheat straw, no release of sugar was measured up to 48 h at a concentration X equivalent to that used in SSF. By using 100 to 830 X, a linear regression was determined :

$$(1)\ Y = 0.74\ 10^{-4}\ X + 25.9\ 10^{-4}\ (r = 0.97;\ P = 0.003).$$

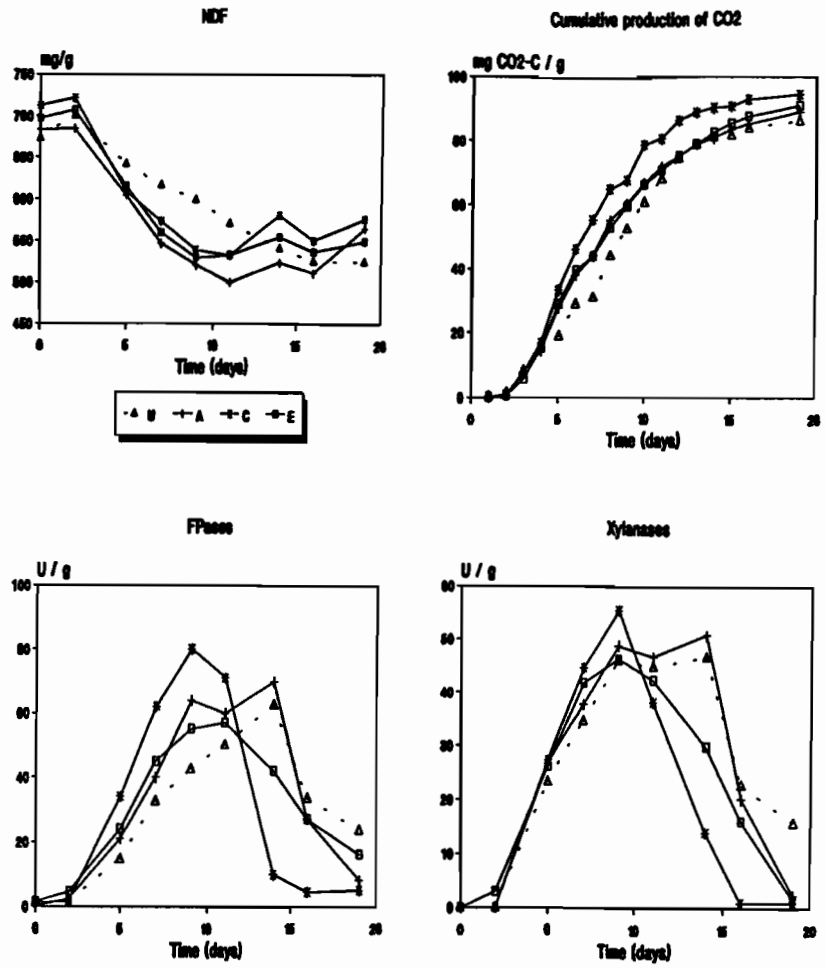

Fig. 1. Cell wall degradation and microbial activities during SSF of wheat straw with its natural microbial community at 45°C for 19 days. Δ Δ = Untreated; - = Enzymes , + = Amylodextrines, * = Czym .

The estimation of the parameters of equation (1) showed changes with different straw samples. From equation (1), it is, however, possible to observe that, at the concentration used for SSF, Czym could release approximately 3 mg of glucose equivalent / g straw / 10 h at 45°C, pH 5, whereas the soluble sugar content in straw varied from 30 to 120 mg/g (Chalaux, 1993). Therefore, a direct effect of carbon source did not seem to be probable.

The production of cellulolytic enzymes is regulated by induction and catabolic repression. It is generally assumed that induction occurs through assimilation of some soluble degradation products by bacteria (Linden and Shiang, 1991). *Bacillus* spp. is the major genera of cellulolytic bacteria isolated from composting straw. One isolate was cultivated in a liquid minimal medium without C source, except for xylose, glucose, cellobiose or cellodextrines at 5 mg/l. CMCase activity in the culture medium as well as cellular proteins were measured after 5 h and 6.5 h incubation. In previous studies on the induction of cellulase from *Bacillus*, the lowest concentrations of sugar used were 20 mg/l (Au and Chan, 1986). Induction was determined by calculating the CMCase/cellular proteins ratio in order to eliminate the effect of bacterial growth on the increase of CMCase activity.

Significant inductions of CMCase activity were observed with cellobiose and cellodextrins (Table 3). The concentration of sugars is approximately equivalent to the one obtained by the action of Czym on wheat straw.

Table 3: Induction of CMCase (U/mg of cellular proteins) production by *Bacillus* spp. with sugars at concentration of 5 mg/l.

Incubation	Treatments				
period, h	Control	Xylose	Glucose	Cellobiose	Cellodextrines
5.0 h	0.19	0.18	0.18	0.28	0.28
6.5 h	0.22	0.23	0.26	0.33	0.29

The data are, thus, in agreement with the hypothesis that Czym, acts through a regulation of production of cellulases by bacteria. The efficiency of Czym could be linked to a location effect. Inducers of polysaccharidases are released at the site of polysaccharides degradation by bacteria at concentrations compatible with induction and not with repression. The correlations between the availability of cellulose measured *in vitro* and the higher production of cellulases during the first days of composting (Table 2) were probably partly the result of identical mechanisms of induction.

CONCLUSIONS

The results presented here are in agreement with previous observations showing that the variability in wheat straw is important. Moreover, this variability affects straw biodegradation and simple methods are proposed to estimate straw quality for SSF. These methods allow choice of good raw materials. In the case that the choice is not possible, the activation of degradative activities through enhancement of the induction of the production of polysaccharidases is a way to improve the efficiency of the biodegradability of poor quality whaet straw

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Mannanase production by filamentous fungi in solid state fermentation

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SUMMARY

Mannan degrading enzymes are useful in the processing of instant coffee, biopulping of softwoods, and clarification of fruit juices. Production of mannanases in solid state fermentation by filamentous fungi has not been widely studied. Therefore, production of mannan degrading enzymes was studied in SSF using several strains of filamentous fungi, such as *Aspergillus ochraceus*, *Penicillium fellutanum*, *Mucor rouxii*, and *Rhizopus oligosporus*. Mannanase production on domestic coffee waste, copra meal, and a liquid medium absorbed on sugarcane bagasse was compared. Fermentations were carried out in small static column bioreactors. The highest enzyme activity was obtained, when *A. ochraceus* was incubated in copra meal.

Keywords: Mannanase production, solid state fermentation, *Aspergillus ochraceus, Penicillium fellutatum, Mucor rouxii, Rhizopus oligosporus,* copra meal, domestic coffee waste, nutrients absorbed sugarcane bagasse, column fermenter.

RÉSUMÉ

Production de mannanases par les champignons filamenteux cultivés en milieu solide.

ALCARAZ-SANDOVALV., SAUCEDO-CASTAÑEDA G. ET HUERTA-OCHOA S.

Les mannanases sont des enzymes couramment utilisées dans les procédés de fabrication de café instantané, pour le biopulpage des bois mous et pour la clarification des jus de fruits. La production de mannanase par fermentation en milieu solide (FMS) n'a pas été déjà réalisée. Cependant la production des enzymes degradant les mannanes a été étudiée en FMS en utilisant plusieurs souches de champignons filamenteux comme: *Aspergillus ochraceus, Penicillium fellutanum Mucor rouxii* et *Rhizopus oligosporus.* La production de mannanases a été obtenue directement sur des sous produits solides comme: les déchets de café domestiques, le tourteau de coprah et avec un milieu synthétique absorbé sur bagasse de canne à sucre. Les fermentations ont été réalisées au laboratoire en utilisant un réacteur statique de type colonne. La meilleure activité enzymatique a été obtenue , lorsque *A.ochraceus* a été cultivé sur le tourteau de coprah.

Mots clés : Production de mannases, fermentation en milieu solide, *Aspergillus ochraceus, Penicillium fellutatum, Mucor rouxii, Rhizopus oligosporus,* tourteau de coprah, pulpe de café, nutrients absorbés sur bagasse de canne à sucre, fermenteur de type colonne.

INTRODUCTION

Mannan degrading enzymes are useful in the processing of instant coffee, biopulping of softwoods, and clarification of fruit juices (Wong and Saddler, 1993; Viikari e*t al,* 1993). Mannanases have been identified from a variety of sources including plant seeds (McCleary, 1979), bacteria and fungi (Torrie *et al,* 1990; Stalbrand *et al,* 1993). Production of mannanases by filamentous fungi has been studied previously using submerged culture. However, very few efforts have been devoted to study

mannanase production by solid state fermentation (SSF), an alternative process to use fibrous waste from the agro-industry. These fibrous materials have an adverse effect on the environment, when they are discarded into the rivers. Besides, products obtained in SSF can be used to improve processes, where fibrous waste are produced. The aim of this work was to study the production of mannan degrading enzymes in solid substrate fermentation by several strains of filamentous fungi.

MATERIALS AND METHODS

MICROORGANISMS AND INOCULA PREPARATION

The strains used were: *Aspergillus ochraceus* 2093, *Penicillium fellutanum* 2277, *Mucor rouxii* 2655 from the Spanish Type Culture Collection; and *Rhizopus oligosporus* sp. isolated in our Lab. Strains were reactivated with an isotonic solution and inoculated in Petri dishes on PDA medium. Strains were inoculated in PDA slopes and incubated at 30°C for 7 days. Spore suspensions were obtained by adding 50 ml of distilled water containing Tween 80 (0.01%) to cultures grown for 7 days at 30°C.

SUBSTRATES

Three different substrates were used and included domestic coffee waste, copra meal, and a synthetic medium absorbed on sugarcane bagasse. Substrates were selected according to the following characteristics: domestic coffee waste, because it is a subproduct of coffee extraction; copra meal due to its high galactomannan content of about 40-50% (Park and Chang, 1993); and the liquid medium absorbed on sugar cane bagasse (Huerta *et al*, 1994) as a control. The liquid medium contained (g/L) :KH_2PO_4, 2.0; $CaCl_2$, 0,302; $MgSO_4$ $7H_2O$, 0.3; $FeSO_4$ $7H_2O$, $5x10^{-3}$; $MnSO_4$ H_2O, $1.6x10^{-3}$; $ZnSO_4$ $7H_2O$, $6.4x10^{-3}$; $CoCl_2$ $6H_2O$, $7.95x10^{-3}$; $CuSO_4$ $5H_2O$, $2.5x10^{-4}$; H_3BO_3, $5x10^{-4}$; $(NH4)_6Mo_7O_{24}$ $4H_2O$, $5x10^{-5}$; peptone, 1.0; locust bean gum, 10.

SOLID SUBSTRATE CULTURE

Solid substrates were inoculated, packed in small tubular static bioreactors (Raimbault and Alazard, 1980) and incubated in a water bath at 30°C for 7 days. Air flow through the packed bed was 0.4 l/h.

EXTRACTION OF CRUDE ENZYME

Fermented samples were harvested, humidified with one volume of distilled water and mixed. The leaching of the product from the humidified samples was obtained by hydraulic pressing according to the methodology described by Roussos *et al,* (1992). The leachate was centrifuged at 3000 rpm for 5 min and the supernatant was used for protein and mannanase activity assays.

ANALYTICAL METHODS

For the enzyme assay, the enzyme extract (0.5 ml) was added to 0.5 ml of 50 mM acetate buffer solution (pH 4.8), containing 0.5% locust bean gum. Samples were homogenised and incubated for 30 min at 50°C. Reducing sugar concentration was determined by the dinitrosalicylic acid method (Miller, 1959). Protein concentration was determined using the Lowry's method after TCA precipitation (Lowry *et al,* 1951). Water activity was measured at 30°C using a Decagon CX-1 Aw equipment.

RESULTS AND DISCUSSION

SURFACE CULTURE

Growth rates of the fungal strains were studied on Petri dishes containing PDA medium and the liquid medium described above. Petri dishes were inoculated and incubated at 30°C. Radius of the colony formed was measured every 24 hours. Growth rates of the colonies were obtained by linear regression of data and the results are plotted in Fig. 1. *A.ochraceus* had the lowest growth rate in both media, and the highest growth rate was obtained with *R. oligosporus* (Fig. 1).

SOLID STATE CULTURE

The protein concentration of the crude enzyme extracts is plotted in Fig. 2. The highest protein concentrations were obtained with copra meal and the lowest with bagasse. This fact might result from protein solubilized from the solid matrix, because of the use of hydraulic press for extraction of the enzyme.

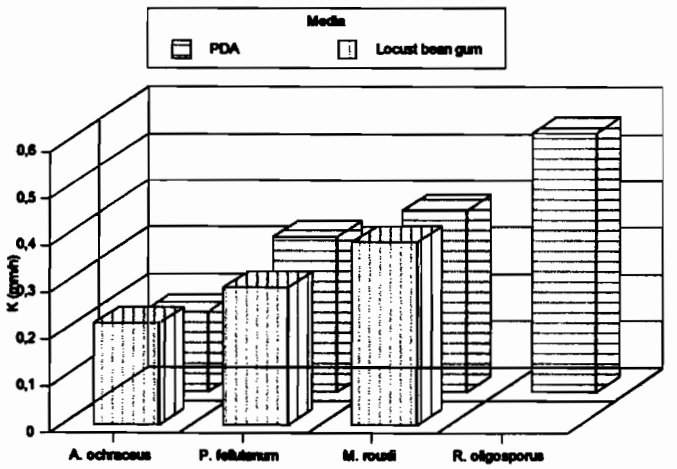

Fig. 1. Growth rates of the colonies on surface culture

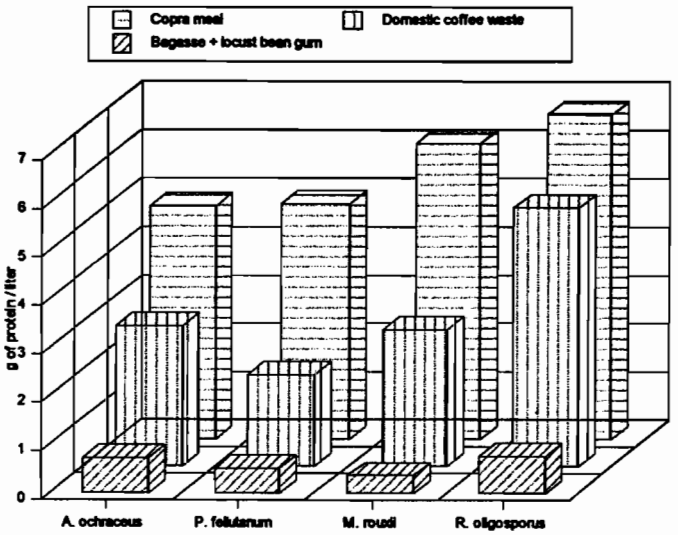

Fig. 2. Protein concentration of the crude enzyme extracts

The protein content in copra meal is around 20% and in bagasse it is almost negligible. After fermentation, protein concentration in the extract increased, mainly because of extracellular enzyme production.

The mannanase activity production was detected only in two strains, i.e., *A. ochraceus* and *P. fellutanum* (Fig. 3). It is also observed that copra meal is a better substrate for enzyme production. Initial moisture content and water activity values of the substrates used are presented in Table 1. It is observed that the water activity value are different even when initial moisture content was similar for the solid substrates . Water activity is one of the main factors affecting cell growth and enzyme production in solid state fermentation processes. Several authors have found that relatively low water activity might enhance the production of some extracellular enzymes (Kim *et al*, 1985; Grajek and Gervais, 1987; Pandey *et al*, 1994).

Table 1. Initial moisture contents and water activities of the solid substrates

Solid substrates	Water activity, (a_W)	Moisture, (%)
Copra meal	0.965	61.0
Domestic coffee waste	0.976	59.5
Bagasse + locust bean gum	0.979	59.0

Despite higher protein concentrations in enzyme extracts obtained from copra meal, when inoculated with *M. rouxii* and *R. oligosporus* (Fig. 2), mannanase activity was not observed (Fig. 3). Therefore, other extracellular enzymes might be produced by these strains.

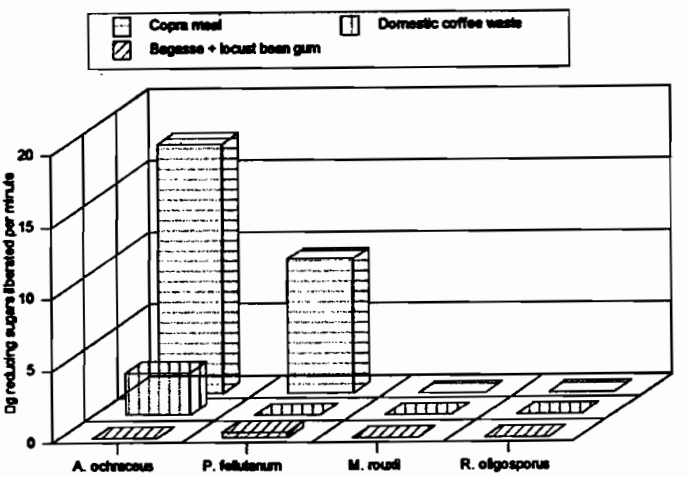

Fig. 3. Mannanase activity of the enzymatic crude extracts

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Lipase production by *Rhizopus delemar* grown on a synthetic support in solid state fermentation

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SUMMARY

Lipase production by *Rhizopus delemar* has been studied by growing on a polymeric resin (Amberlite IRA 900), after absorbing a medium previously optimized in solid state fermentation (SSF) and also in submerged fermentation (SmF). The activity of *R. delemar* lipase, produced in liquid culture (LC), could be adsorbed to an extent of 24% on 1 g Amberlite. Desorption, carried out using 5 g NaCl / g Amberlite at pH 5, allowed to recover 35% of the adsorbed lipase. Data indicated that the resin displayed a thermo-protective effect, since only little loss in activity was observed, when the adsorbed enzyme was heated at 80°C for 24 hours. In solid state fermentation (SSF), the fungus produced high amounts of enzyme (93 U/g dry Amberlite at 24 hours, against 14.1 U/ml at 48 hours in submerged culture), when dextrin was used as a carbon source. Significant activity was also detected with maltose and surprisingly with glucose (68 and 57 U/g dry Amberlite, respectively). The strong inhibitory effect of glucose, observed in liquid culture, was reduced in SSF.

Keywords: Solid state fermentation, submerged fermentation, lipase production, *Rhizopus delemar*, synthetic support, Amberlite IRA 900, comparative titres, thermo-protective effect, glucose inhibitory effect.

RESUME

Production de lipases par *Rhizopus delemar* cultivé sur un support synthétique en fermentation solide.

CHRISTEN P., ANGELES N., FARRES A., ET REVAH S.

La production de lipases, par *Rhizopus delemar* cultivé sur un support synthétique (Amberlite IRA 900) imprégné d'une solution nutritive optimisée, a été étudiée d'abord en fermentation en milieu solide (FMS) et ensuite en fermentation submergée (SmF). L'activité lipase de *R.delemar*, produite en SmF, a pu être absorbée à 24% pour 1 g d'Amberlite. La desorption, réalisée en utilisant 5 g de NaCl/ g d'Amberlite à un pH 5, a permis de récupérer 35% de la lipase absorbée. Les résultats indiquent que la résine montre un effet thermo-protecteur vis-à-vis des lipases, étant donné qu'une faible perte d'activité enzymatique a été observée lorque l'enzyme a été absorbée et portée à 80°C pendant 24 h. En milieu solide (FMS) en présence de la dextrine comme source de carbone, le champignon filamenteux produit des quantités élevées d'enzymes (93 U/g poids sec d'Amberlite à 24 h, au lieu de 14,1 U/ml à 48h en fermentation submergée (SmF). Des activités importantes ont été détectées avec du maltose ou avec du glucose (68 et 57 U/g poids sec d'Amberlite, respectivement). L'hinibition forte provoquée par le glucose a été uniquement observée pour les cultures liquides alors que cette inhibition été nettement réduite en FMS.

Mots Clés : Fermentation en milieu solide, Fermentation submergée, Production de lipases, *Rhizopus delemar*, Support synthétique, Amberlite IRA 900, Production comparée liquide-solide, Effet thermo protecteur, Inhibition par le glucose.

INTRODUCTION

Lipases are the widely used enzymes, which can be obtained from animals, plants and microorganisms. Microbial lipases are used in the food industry, mainly in dairy products, and are also important to detergents, pharmaceutical, cosmetics and leather processing industries (Seitz, 1973). The enzyme modified cheeses (EMC) are also an interesting application involving lipases (Revah and Lebeault, 1989). New trends are directed towards the use of immobilized lipases in organic solvent for ester

synthesis, triglycerides hydrolysis and flavouring compounds synthesis (Christen and López-Munguía, 1994).

Solid state fermentation can be a suitable method for producing enzymes, such as pectinases, amylases, or cellulases (Lonsane and Ghildyal, 1992), but few papers have dealt with lipases. Nevertheless, Yamada (1977) reported that, in Japan, most of the microbial lipases originate from *Aspergillus* strain cultivated in SmF and SSF. More recently, Rivera Muñoz *et al,* (1991), using *Penicillium candidum* grown on wheat bran, found that SSF has many advantages over the SmF for lipase production.

To better understand the fungal growth, inert supports, impregnated with a nutritive solution, have been employed (Auria *et al,* 1990; Christen *et al,* 1994). The aim of this work was to study the growth of and the lipase production by *Rhizopus delemar* on Amberlite, an anionic resin. Data on characterization of the lipolytic activity in SmF and SSF experiments and interaction between support/enzyme are also presented.

MATERIALS AND METHODS

MICROORGANISMS AND CULTURE MEDIA

Two *Rhizopus delemar* strains were tested: CDBB H313 (CINVESTAV-MEXICO) and NRRL 1472. These were periodically transferred on potato dextrose agar (PDA) slants and stored at 4°C. Spores were produced in Erlenmeyer flasks on PDA at 29°C during 6 days. The nutritive medium, previously optimised by Martinez Cruz *et al* (1993), was used both in SmF and SSF. SmF was studied in 250 ml Erlenmeyer flasks placed on a rotary shaker. Initial conditions were: temperature, 29°C; pH, 6; inoculum size, $1x10^7$ spores/ml; and agitation speed, 180 rpm. In solid state cultures, an anionic resin (Amberlite IRA-900, Rohm and Haas) was used and prepared according to Christen *et al* (1993). Nutritive medium was added to the dried support to achieve 58% final water content, the maximum absortion capacity of the resin. The cultures were carried out in small columns placed in a temperature controlled bath. Initial conditions were: temperature, 29°C; pH, 6; inoculum size, $1x10^7$ spores/g initial dry matter (IDM) and aeration rate, 0.5 l/h.g IDM.

ANALYTICAL PROCEDURES

In SmF, growth was followed by dry weight determination. In SSF, respirometry was used to calculate CO_2 production rate, as previously described (Christen *et al*, 1993). Water activity, moisture content and pH were also determined at the end of the fermentation. Lipolytic activity was assayed by the method used by Nahas (1988) with some modifications. The substrate was a 5% tributyrin emulsion, prepared in a 1% Tween solution in 2.5 M tris-maleate buffer (pH = 6) by homogenizing in an Ultraturax apparatus (8000 rpm during 2 min). The reaction mixture contained 18 ml substrate and 12 ml enzyme extract. In the case of adsorbed lipase, 1 g Amberlite was added to the reaction medium. The reaction was carried out using a Mettler DL 21 pH stat, at 37°C and pH adjusted to 6. The butyric acid released was titrated with 5 mM NaOH solution during 5 min reaction. One unit (U) was defined as the amount of enzyme releasing one mmol of free fatty acid per min.

ADSORPTION/DESORPTION STUDY

R. delemar lipase adsortion study was carried out using entire, (average diam : 0.53 mm), or ground (average diam : 0.10 mm) Amberlite. Amounts varying from 0.5 to 6 g Amberlite were contacted with 50 ml enzymatic extract in 250 ml Erlenmeyer flasks placed on a rotary shaker (150 rpm) for 24 h. Temperature was 29°C and pH was adjusted to 6. Desorption study was carried out using 2 g Amberlite in 50 ml of sodium chloride solution concentrations ranging from 10 to 120 g/l and at different pH. Conditions were similar to those of the adsorption studies. Results are reported as : units absorbed, units expressed by the resin, total units desorbed and units desorbed as the % of the total expressed activity.

RESULTS AND DISCUSSION

EVALUATION OF THE *R. DELEMAR* STRAINS

Lipolytic activity of the 2 strains was evaluated, according to Corzo (1993), by growing the mould in Petri dishes on PDA containing emulsified tributyrin (1%).

The diameter of the clearing zone around the colony, corresponding to the hydrolysis of the substrate, was measured after 3 days. The CDBB H313 strain gave an average diam of 2.75 mm calculated from one hundred colonies, against 2.28 mm for the NRRL 1472 strain. The former strain was used in all further experiments.

GROWTH AND LIPASE PRODUCTION KINETICS IN SMF

SmF was used to produce the enzymatic extract needed for the adsorption/desorption experiments (Fig. 1). It can be seen that maximum activity (14.1 U/ml) corresponds to maximum growth (12.3 mg/ml). These values were reached within 2 days and are similar to those obtained by Martinez Cruz *et al,* (1993). Centrifugation at 5000 rpm for 5 min resulted in a loss of more than 50% lipolytic activity, probably due to proteolytic activity and/or denaturation of the protein. This was not observed when Amberlite (2 g/50 ml) was present in the medium (Angeles, 1995). The extract was more stable at pH 5, while the optimum activity was obtained at 6.5 (Fig. 2).

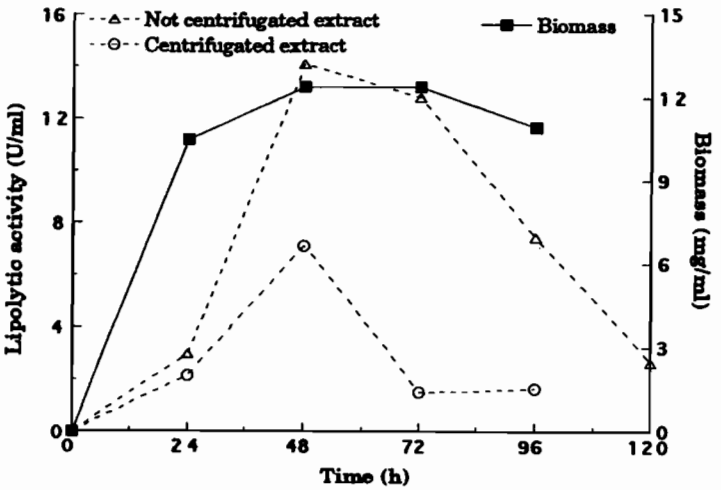

Fig. 1 : *R. delemar* lipase production kinetic in LC effect of centrifugation on lipolytic activity.

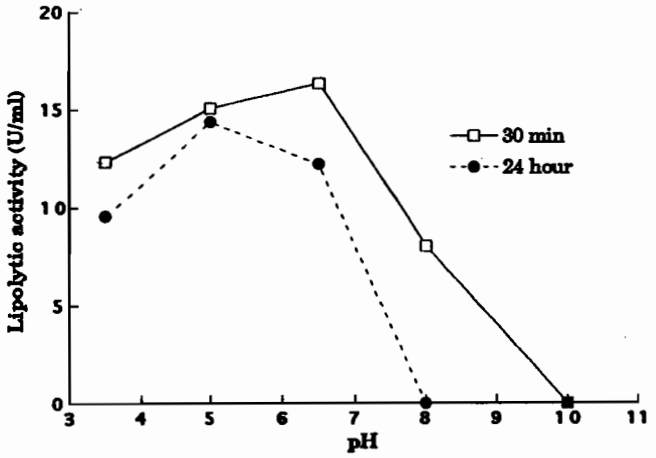

Fig 2. : Extract stability at different pH.

GROWTH AND LIPASE PRODUCTION IN SSF

Glucose is known to repress lipase production in SmF (Haas and Bailey, 1993). One of the particular aims of the experiments in SSF was to see if this catabolite repression could be partially or totally overcome. Three carbon sources (glucose, maltose and dextrin, each at (20 g/l) were used in the SSF experiments.

Results are presented in Figs. 3 and 4. The growth, followed by CO_2 production rate, was maximum between 15-20 h, a very short time in SSF, and did not display significant differences between the 3 substrates (Figs. 3 and 4). Maximum lipase production was found at 24 h, corresponding to the lower pH in the medium. Best production was found with dextrin (95.6 U/g IDM), against 68.2 U/g IDM with maltose and 57.7 U/g IDM with glucose (Fig. 4). The equivalent in U/ml reactor (Table 1) shows that SSF gave a higher productivity with the same substrate (dextrin) than in SmF. This activity decreased after in all 3 cases.

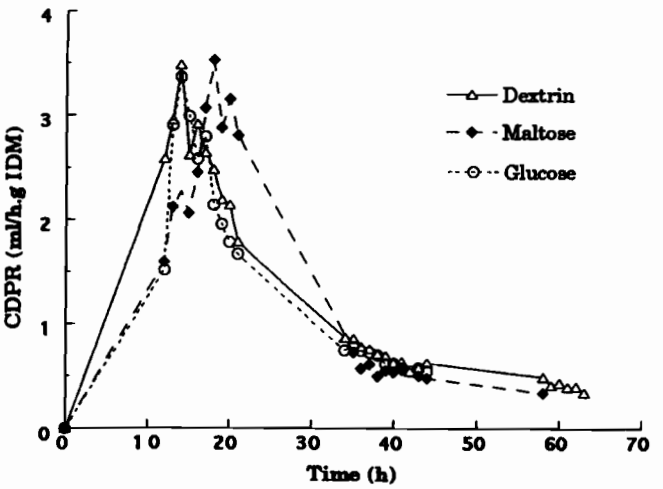

Fig. 3 : Respirometric activity of *R. delemar* grown in SSF with 3 different carbon sources.

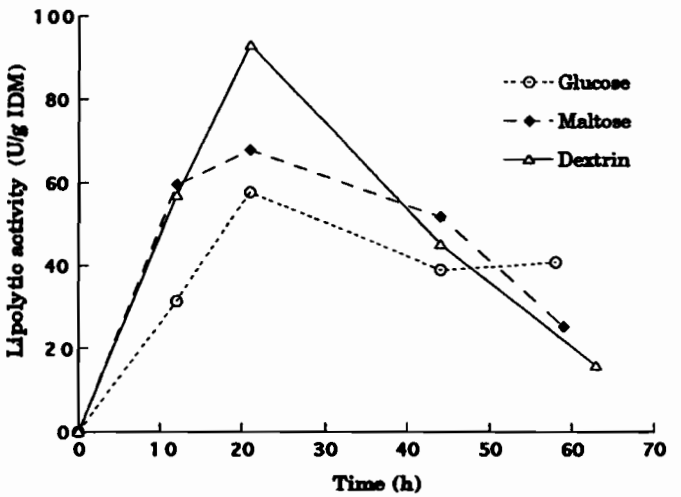

Fig. 4 : Lipolytic activity for *R. delemar* grown in SSF with 3 different carbon sources

Table 1. Comparative data of SmF and SSF for lipase production.

Carbon source	SmF Dextrin	SSF Glucose	SSF Maltose	SSF Dextrin
T max (h) *	48	18	18	15
CO_2 production rate, (ml/h.g IDM)	-	2.8	3.5	3.5
R.Q. (range)	-	1-1.3	1-1.3	1-1.4
pH max *	-	5.7	4.9	5.6
Final Aw	-	0.998	0.999	0.994
Lip. Act. (U/g IDM)	-	57.7	68.2	95.6
Vol. Lip. Act. (U/ml)	14.1	10.2	11.3	15.7

* Refers to time and pH of maximum lipase production.

Data summarized in Table 1 show that the enzyme is produced faster and with a better productivity in SSF. Moreover, Amberlite is an adequate support for this purpose, as it provided a good stability for pH, moisture content and Aw, all of these being key parameters in SSF. The best carbon source was dextrin as in SmF (Martinez Cruz *et al*, 1993), but in SSF, the catabolite repression due to glucose was not as strong as in SmF (only 40% decrease against dextrin). Respiratory quotients (RQ) observed are typical of the oxidative use of the carbon sources.

R. DELEMAR LIPASE ADSORPTION/DESORPTION STUDY

Adsorption study

Data (Fig. 3) showed that the amount of lipase adsorbed per g Amberlite decreased for higher amounts of resin, while an opposite trend is observed for the residual activity. Only 3% of the adsorbed enzyme was active when 0.5 g resin was used, but this increased to 26% for 6 g. The losses observed in terms of the expressed activity may be due to the partial denaturation of the protein, inactivation of the active sites due to the anionic properties of the support or partial diffusion of the protein inside the resin.

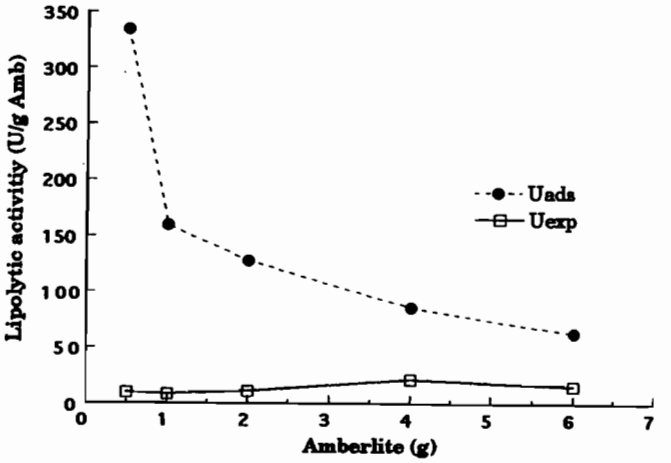

Fig. 5 : *R. delemar* lipase adsorption on Amberlite.

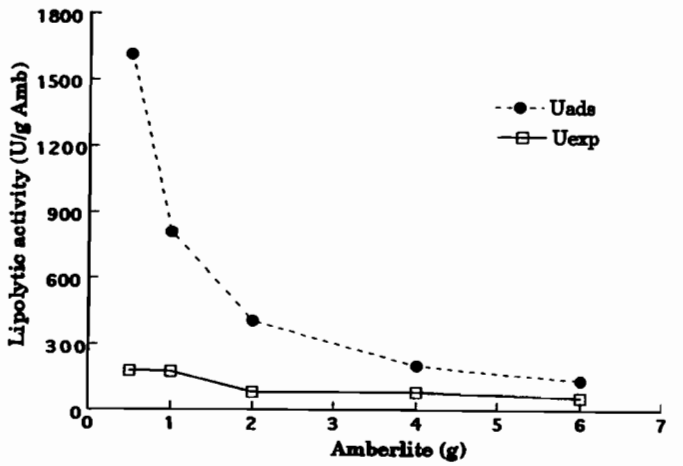

Fig. 6 : *R. delemar* lipase adsorption on ground Amberlite.

In the case of ground Amberlite (Fig. 6), as low as 0.5 g support was sufficient to adsorb all the lipase present in the reaction medium. The relation between expressed and adsorbed lipase increased from 12.4% to 34% with the increase of amount of resin in the medium. Nevertheless, the values for adsorbed and expressed activities are higher than those for entire Amberlite, probably due to the increase in the contact area of the resin and decrease in the limitation of diffusion.

All the lipase present in the medium is adsorbed on ground Amberlite within about 2 hours, while, entire Amberlite was saturated after 8 hours, with about 20% of the lipase present in the reactive medium (Fig. 7). These experiments showed that all the lipase was adsorbed on Amberlite in a maximum of 8 hours. The amount of adsorbed lipase and the adsorption dynamics depend strongly on the size of the resin. Moreover, the adsorption on Amberlite displayed a thermo-protective effect, since no loss in activity was observed, after a sample of adsorbed lipase was kept for 24 hours at 80°C (Angeles, 1995). The adsorption of the enzyme on Amberlite during growth on SSF may also serve as a method to concentrate it simultaneously.

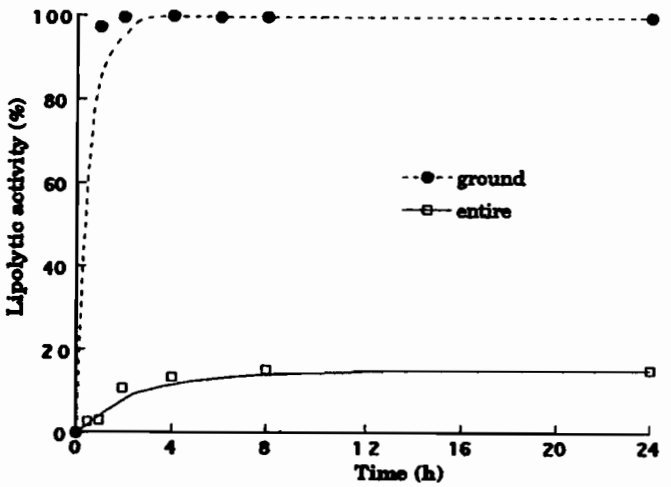

Fig. 7 : *R. delemar* lipase adsorption kinetic on ground and entire Amberlite.

Desorption study

To study the recovery of the adsorbed *R. delemar* lipase on Amberlite, the entire particles were used at 29°C for 24 hours (150 rpm). The effect of NaCl concentration and pH was explored (Figs. 8 and 9). Addition of NaCl, previously used by Corzo (1993) for lipase desorption, allowed a 38% of desorption at 100 g NaCl/L level and an optimum pH of 5.

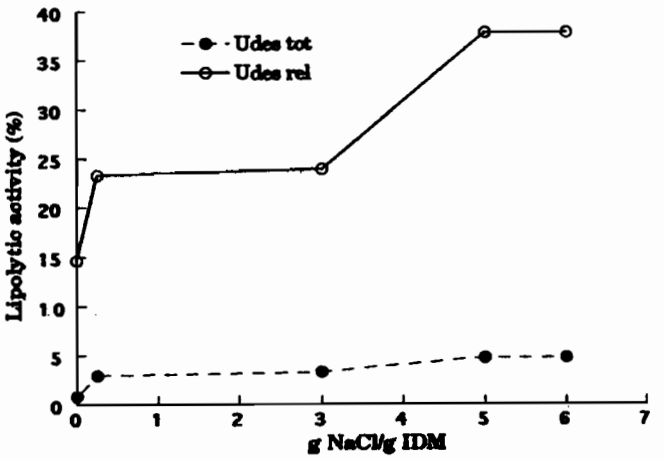

Fig. 8 : Influence of (NaCl) on lipase desorption (pH=6).

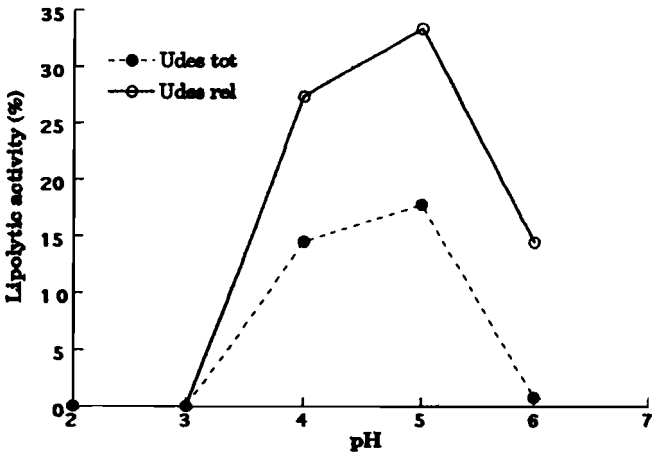

Fig. 9 : Influence of pH on lipase desorption (NaCl) = 2g/g Amberlite).

CONCLUSION

R delemar CDBB H313 strain was a better lipase producer than NRRL 1472. In SmF, the negative effect of centrifugation on lipase recovery was observed. It was established that the enzyme was more time stable at pH 5.0, while its optimum activity was at pH 6.0. In SSF, the mould showed a good capacity to grow on Amberlite with various carbon sources (dextrin, maltose and glucose). Best lipase production was found with dextrin, while lower glucose repression was observed than in SmF. Entire Amberlite was saturated with 24% of the lipase, while ground Amberlite was able to adsorb all the lipase present in the medium (about 700 U). There is an important difference between the enzyme adsorbed Amberlite (defined as initial adsorbed and the residual activity after desorption) and actual active lipase on Amberlite (only 26% and 34% for entire and ground support). The recovery of the adsorbed enzyme was lower (only 38% with 2 g NaCl/g IDM at pH 5.0). It will be preferable to use the enzyme

adsorbed on Amberlite than to desorb it. Furthermore, such lipase displayed a good thermostability. The use of Amberlite as a support opens interesting possibilities to study simultaneous enzyme production and immobilization in SSF.

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Lignin-degrading enzymes produced by *Pleurotus* species during solid state fermentation of wheat straw

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SUMMARY

Three enzymes, i.e. manganese peroxidase (MnP), aryl-alcohol oxidase (AAO) and laccase, presumptively involved in lignin degradation, were detected and quantified during the solid state fermentation (SSF) of wheat straw with five *Pleurotus* species. The highest levels were produced by *P. pulmonarius*. In the course of the study, it was realized that several aspects of the physiology of ligninolysis during SSF differed from those reported in liquid culture. In particular, MnP activity appeared during a more extended period of time, and it was not dependent on the presence of peptone in the medium or inhibited by Mn^{2+}, as in liquid cultures of *Pleurotus* species. It is suggested that these differences are the consequence of the artificial growth conditions prevailing in liquid cultures of basidiomycetes. In particular, the separation between growth phase and secondary metabolism can be observed only in liquid culture. Under SSF conditions, a third type of metabolism probably occurs, characterized by close relationships between hyphal growth and secondary metabolism events, including production and release of ligninolytic enzymes for enabling substrate colonization. Finally, the use of ^{14}C-lignin straw provide indirect evidence for MnP involvement in lignin degradation under SSF conditions (probably via Mn^{3+} formation), since the addition of Mn^{2+}, which did not increase MnP levels, strongly stimulated lignin mineralization by *P. pulmonarius*.

Keywords : Solid state fermentation, lignin-degrading enzymes, manganese peroxide, aryl-alcohol oxidase, laccase, wheat straw, *Pleurotus spp.*, *P. pulmonarius*.

RESUME

Les enzymes de la dégradation de lignine produites par des espèces de *Pleurotus* cultivées sur paille de blé en milieu solide.

CAMARERO S., MARTÍNEZ M.J., ET MARTÍNEZ A.T.

Trois enzymes supposées impliquées dans la dégradation de la lignine : une manganèse peroxydase (MnP), une aryl-oxydase (AAO) et une laccase ont été détectées et quantifiées au cours de fermentations en milieu solide (FMS) sur paille de blé de cinq espèces de *Pleurotus*. Les taux les plus élévés de ces trois enzymes ont été détectés pour *P. pulmonarius*. Nous avons également constaté que la physiologie de la dégradation de la lignine en FMS diffère sur plusieurs aspects de celle rapportée pour la fermentation liquide. Contrairement à la fermentation liquide, l'activité de la Mnp est observée pendant une plus longue durée en FMS. Elle ne dépend pas de la présence dans le milieu de peptone et n'est pas inhibée par Mn^{2+} . On suppose que cette différence est due aux conditions de croissance "artefact" de la culture liquide des basidiomycètes. Ainsi le passage de la phase de croissance au métabolisme secondaire ne peut être décrit que pour les cultures liquides. En FMS, les conditions de culture sont plus proches de l'environnement naturel des champignons lignolytiques, et un troisième type de métabolisme doit être envisagé. Il se caractérise par une relation étroite entre la croissance de l'hyphe et le métabolisme secondaire et permet la colonisation du substrat par la production d'enzymes lignolytiques extracellulaires. Enfin, en utilisant de la paille marquée à la lignine ^{14}C, il a été prouvé l'implication de la MnP dans la dégradation de la lignine en FMS (probablement via la formation de Mn^{3+}) puisque l'addition d'ions Mn^{2+}, qui n'augmente pas l'activité de la MnP, stimule fortement la minéralisation de la lignine par *P. pulmonarius*.

Mots clés: Fermentation en milieu solide, enzymes dégradant la lignine, manganèse peroxyde, aryl-alcool oxydase, laccase, paille de blé, *Pleurotus sp. P. pulmonarius.*

INTRODUCTION

A general scheme for the enzymatic mechanism of lignin degradation cannot be depicted at the present. During the last 12 years, abundant information on physiology, enzymology and genetics of lignin degradation by the white-rot fungus, *Phanerochaete chrysosporium*, has accumulated (Kirk and Farrell, 1987; Schoemaker

et al, 1994). However, several pieces of evidence suggest that other ligninolytic fungi exhibit different regulation mechanisms and enzymatic activities. Moreover, the physiology and enzymology of ligninolysis has been near exclusively investigated in liquid cultures and very little is known about the mechanism of lignin degradation under solid state fermentation (SSF) conditions.

Pleurotus species are being investigated for biopulping of agricultural wastes (Martínez *et al,* 1994a). These fungi are well adapted to grow on wheat straw and, some of them, remove lignin preferentially during straw SSF with a very limited degradation of cellulose (Kamra and Zadrazil, 1986; Valmaseda *et al,* 1990). Under these conditions, the attack on straw constituents by *P. chrysosporium* is remarkably unspecific, being comparable with the degradation pattern caused by cellulolytic ascomycetes (Valmaseda *et al,* 1991).

Until recently, laccase was the only ligninolytic enzyme known in *Pleurotus* species. Lignin-peroxidase (LiP), described in *P.chrysosporium* 12 years ago, has not been detected in *Pleurotus.* The first efforts to detect LiP in *P. eryngii* led to the description of aryl-alcohol oxidase, an H_2O_2-producing oxidase of anisyl and related alcohols (Bourbonnais and Paice 1988 ; Guillén *et al,* 1988, 1992a). Recently, the use of peptone as N-source has led to the detection and characterization of two isoenzymes with MnP activity in *P. eryngii* (Martínez and Martínez, 1994), which can utilize the H_2O_2 produced by AAO and could play an important role in lignin degradation by this fungus.

MATERIAL AND METHODS

To study fungal delignification of wheat straw, as a pretreatment for paper pulp production (Martínez *et al,* 1994a), a rotary fermenter, including six bottles of 2-l capacity, each of them containing 150 g straw, was used (Valmaseda *et al,* 1991). However, the experiments using ^{14}C-lignin straw and the parallel estimation of enzymatic activities were performed using 1 g straw. The wheat straw, received from SAICA (Zaragoza, Spain), was chopped and sieved to 2-10 mm size for these small-scale laboratory experiments. For the assay of the enzymatic activities, straw was extracted with hot water (110°C for 1 h) to remove water-soluble materials, which cause interferences in spectrophotometric determinations. The Mn content in initial and extracted straw was estimated by atomic absorption, after combustion at 600°C.

The fungal strains used for wheat straw SSF include *Pleurotus sajor-caju* CCBAS 666, *P. eryngii* CBS 613.91 (= IJFM A169), *Pleurotus floridanus* MUCL 285.18, *Pleurotus ostreatus* CBS 411.71, *Pleurotus pulmonarius* CBS 507.85, *P.*

chrysosporium ATCC 24725 and *Trametes versicolor* IJFM A136. For inoculum preparation, stationary cultures in 500 ml-flasks containing 50 ml of medium described elsewhere (Guillén *et al*, 1992a), were used. After development of a fungal mat, it was recovered, homogenized and incubated at 170 rpm for 4 days in the same medium. The pellets obtained were washed, suspended in water, and used to inoculate straw (using a 3: 1, water: straw ratio, v/w). When the effect of the addition of Mn, Fe or peptone to the straw was studied, the pellets were suspended in a solution of $MnSO_4$ H_2O, $FeSO_4$ $7H_2O$, or peptone (final concentration 300 µM in the two former cases and 7.5 g/l in the case of peptone), and used as inoculum. Triplicate flasks were prepared and incubated at 28°C.

For the analysis of the enzymatic activities, whole flasks were sampled during a period of 40 days. Twenty ml of water was added to each flask, and the flask was agitated at 200 rpm for 30 min, vacuum filtered and then filtrated through the filter of pore size of 0.8 µm. Several enzymes involved in lignin degradation were measured in the extracts: aryl-alcohol oxidase (AAO), lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. LiP (EC 1.11.1.14) activity was assayed as the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 (Tien and Kirk, 1988)

AAO (EC 1.1.3.7) activity was measured as ligninase activity without the presence of H_2O_2. MnP (EC 1.11.1.13) was assayed by formation of tartrate-Mn^{3+} complex after oxidation of Mn^{2+} (Paszczynski *et al*, 1988). Laccase (EC 1.10.3.2) activity was estimated by formation of the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) cation radical (Martínez *et al*, 1994b). One unit of activity was defined as the amount of enzyme transforming 1 µmol of substrate per min.

Lignin content in dried samples, after fungal SSF, was estimated by the Klason method, and straw polysaccharide composition was analyzed by gas chromatography as alditol acetates, following the standard Tappi methods T 222 om-88 and T 242 cm-85 (TAPPI 1993).

The ^{14}C-lignin straw, used to quantify fungal mineralization and solubilization of lignin during SSF, was labelled *in vivo*. ^{14}C-phenylalanine was provided to wheat plants, and extractives, water-soluble material, and protein were removed after radioactivity incorporation (Camarero *et al*, 1994). A radioactivity of 4.2 kBq/flask, containing 1 g straw, was used in subsequent experiments to study mineralization (by measuring $^{14}CO_2$ displaced with O_2 and trapped in phenethylamine:ethanol:water, 2:1:1) and solubilization (material extracted at 110°C for 15 min). The eventual role of MnP and laccase mediators in lignin degradation was investigated by adding Mn^{2+} and ABTS (final concentration 300 µM) to 11 day SSF cultures, and estimating $^{14}CO_2$ released during subsequent fungal growth.

RESULTS AND DISCUSSION

In the course of a previous SSF study using ^{14}C-lignin wheat straw, the kinetics of lignin mineralization by the same *Pleurotus* species was analyzed and compared with *P. chrysosporium* and *T. versicolor* (Camarero and Martinez 1993). After 40 day incubation, *T. versicolor* mineralized 40% of the initial radioactivity, whereas *P. chrysosporium*, *P.pulmonarius*, *P. sajor-caju* and *P. ostreatus* showed mineralization values of about 35% (lower values were obtained with the two other species). Moreover, the *P. pulmonarius* exceeded the mineralization value reached by *P. chrysosporium* and *T. versicolor* in later periods of SSF. This fact indicates that, in spite of no production of LiP activity by *Pleurotus* species (Guillén *et al*, 1992b), they are able to mineralize lignin during wheat-straw SSF. Consequently, the first objective of the present study was to identify the ligninolytic enzymes produced by *Pleurotus* species during wheat-straw SSF, in order to explain the degradation of straw lignin produced by these fungi.

However, results were negative for LiP activity during straw treatment in any of the *Pleurotus* species studied, as occurred in liquid cultures. However, LiP activity was also absent in SSF samples from *P. chrysosporium* and *T. versicolor*, in contrast with the fact that LiP was produced in liquid cultures of both fungi. Kerem *et al* (1992) could not detect LiP activity during degradation of cotton stalks by *P. chrysosporium*. Although this could be explained by the presence of compounds interfering with LiP estimation in the extracts, it seems that the genes encoding lignin-degrading enzymes are expressed differently during SSF, than in the liquid culture (Datta *et al*, 1991).

In all the *Pleurotus* species tested, MnP, AAO and laccase were present (Table 1), although the levels varied.

Table 1. Enzyme levels and composition of the final substrate during fungal SSF of wheat straw

	Enzymes[a]			Composition[b]	
	MnP	**AAO**	**Laccase**	**Lignin**	**Cellulose**
Pleurotus ervngii	3.0	0.04	0.66	11.9	71.1
Pleurotus floridanus	2.3	0.08	0.60	9.4	68.2
Pleurotus ostreatus	3.7	0.03	0.68	13.2	84.9
Pleurotus pulmonarius	4.6	0.18	0.59	10.4	67.0
Pleurotus sajor-caju	4.2	0.03	0.43	9.3	57.0
Phanerochaete chrysosporium	0.7	0	0	11.2	30.0
Trametes versicolor	0.2	0.04	0.12	14.3	27.6
control straw	0	0	0	16.9	73.1

[a]Maximal activities (U/g initial straw); [b]g/100 g straw after 140 days

Low activities of the three enzymes were also produced by *T. versicolor*, but only MnP was found in the straw treated with *P. chrysosporium*. The comparatively high MnP levels produced by all the *Pleurotus* species studied need to be observed. It seems that the higher degradation of lignin corresponds to the higher MnP levels in *P. pulmonarius* and *P. sajor-caju*, although AAO and laccase could also play an important role in the degradation of this polymer. In this way, AAO can provide H_2O_2 to MnP and laccase can degrade phenolic lignin units by about 20% in wheat-straw (Camarero *et al*, 1994).

In the course of the present study, *P. pulmonarius* produced the highest MnP and AAO levels, and the evolution of enzyme activities during straw SSF with this species is presented in Fig. 1.

As observed in liquid culture, laccase appeared earlier than MnP during straw SSF. However, both enzymes were detected during the extended time period. On the other hand, AAO appeared in a comparatively earlier growth period, than that in liquid culture.

Mn^{2+} regulates the production of LiP and MnP in liquid cultures of *P. chrysosporium* (Bonnarme and Jeffries, 1990) due to an inductive effect on MnP activity and a repressing effect on LiP.

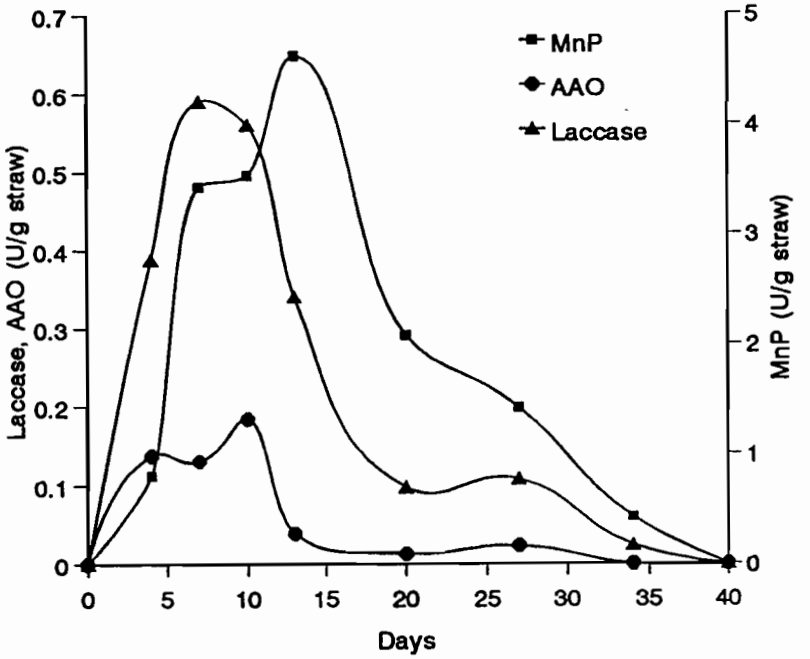

Fig.1. Enzymatic activities during straw SSF with *Pleurotus pulmonarius*

On the contrary, this does not occur in liquid cultures of *Pleurotus* species, where LiP is absent and Mn^{2+} has an inhibitory effect on the production of MnP activity (Martínez and Martínez, 1994).The latter case is difficult to explain, since Mn^{2+} should be necessary for the activity of MnP, being responsible for closing its catalytic cycle. The Mn^{2+} content of the whole wheat-straw was 11.4 ppm, while the amount of Mn^{2+} in the water-extracted straw did not exceed 3.5 ppm. This seems too low to affect LiP or MnP production. Therefore, the effect of Mn^{2+} addition to the straw on both activities was analyzed. Moreover, as LiP and MnP are hemoproteins, the possible effect of Fe^{2+} addition to the substrate was studied too. The results showed that the straw supplied with these metals was neither a better substrate nor a worse one for the production of ligninolytic enzymes by the fungi.

Moreover, the presence of peptone in liquid media has been reported to strongly enhance MnP production by *Pleurotus* species. It is known that this enzyme is difficult to detect in media containing ammonium tartrate as N-source (as generally used to study MnP in *P. chrysosporium*). To evaluate the effect of peptone during SSF, an experiment was carried out, adding peptone to wheat straw, and the different

ligninolytic enzymes were analyzed during the SSF. In this case also, LiP could not be detected but, in contrast to results reported in liquid cultures, the MnP activity during SSF was not significantly affected by the presence of peptone. Some positive effect of peptone on laccase activity could be explained by an increased fungal growth in the presence of additional nutrients. Different low molecular-weight mediators seem to play an important role in the enzymatic degradation of lignin (Evans *et al*, 1994). The involvement of Mn^{3+}, produced by the oxidation of Mn^{2+} by MnP, is generally accepted. However, the role of some laccase substrates, enabling the oxidation of non-phenolic compounds (Bourbonnais and Paice, 1990), is still to be shown under natural conditions. Anyway, this has been demonstrated under *in vitro* conditions, using ABTS (a synthetic laccase substrate) and laccase purified from *P. eryngii* (Martínez *et al*, 1994b). In order to investigate the involvement of MnP and laccase mediators in the degradation of wheat-straw lignin, Mn^{2+} and ABTS were added during *Pleurotus* SSF of wheat straw. The results obtained with *P. pulmonarius* are presented in Fig. 2. A very slight increase in mineralization by both fungi was observed, after ABTS addition.

However, the addition of Mn^{2+} strongly increased mineralization by *P. pulmonarius*, thereby suggesting the involvement of MnP in the lignin degradation process and the mediator role of Mn^{3+} produced by the enzyme. The slight increase of mineralization by Mn^{2+} in the case of *P. eryngii* (similar to that caused by ABTS), showed that a different factor was limiting ligninolysis. Although additional experiments are necessary, it is suggested that, under the present conditions, the supply of H_2O_2 could be the limitation factor, due to the low AAO activity detected in this fungus, compared to that in *P. pulmonarius* (Table 1).

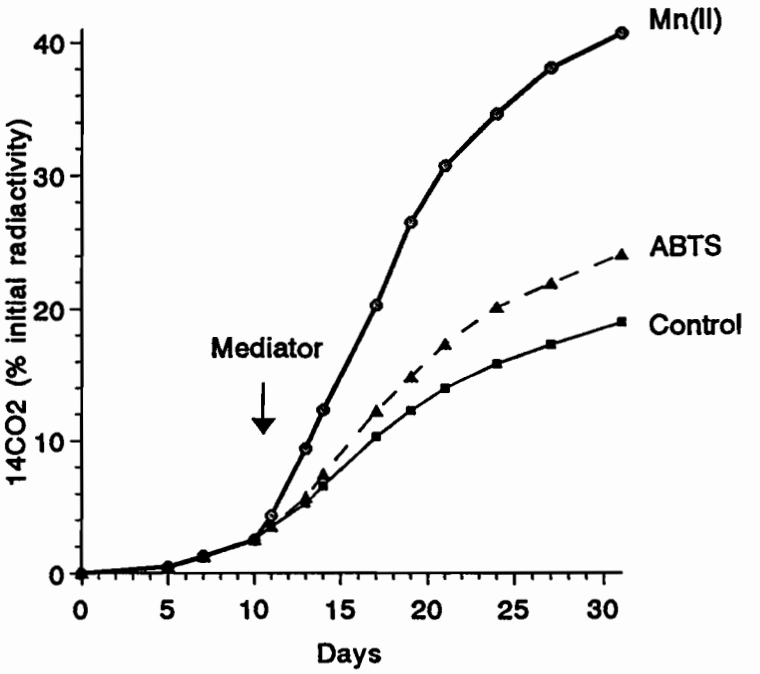

Fig. 2. Influence of the MnP and laccase mediators Mn^{2+} and ABTS, on lignin mineralization by *Pleurotus pulmonarius* during straw SSF

In conclusion, the results suggest that LiP presence is not indispensable in the ligninolytic machinery of all white-rot fungi. Moreover, MnP seems to play a definitive role in lignin degradation by some fungi. This is the case in the *Pleurotus* species studied here, but also in *Rigidoporus lignosus* (Galliano *et al*, 1990) *or Dichomitus squalens* (Périé *and Gold*, 1991). The different ligninolytic machinery of the white-rot fungi would be, in this way, derived from combinations of a wide assortment of enzymes.

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Comparative studies of pectinase production by *Aspergillus niger* in solid state and submerged fermentations

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SUMMARY

Pectinases, the enzymes of wide application in food and food processing, are produced by fungi in solid state or submerged fermentations. A screening of 248 strains resulted in selection of a hyper-producer for solid state fermentation (SSF) of coffee pulp. Digital imaging technique allowed the characterization of the mutant strains, based on morphological traits. Further UV irradiation of selected strains led to mutants adapted to either SSF or submerged fermentation (SmF). Mutants adapted to SSF produced five times higher enzyme, as compared to the same mutant placed in SmF and *vice versa*. The differences are in the requirement of water activity and may also be in their genetic make-up as well as physiological properties. The data also revealed differences in protein bands, proteins biosynthesized by the same mutant, when placed either in SSF or SmF.

Keywords : Solid state fermentation, submerged fermentation, pectinases, *Aspergillus niger*, mutants, water activity, comparative production, hyper-production, digital imaging, protein banding.

RESUME

Etudes comparées sur la production de pectinases par *Aspergillus niger* cultivé en milieu solide et en milieu liquide.

MINJARES-CARRANCO A., VINIEGRA-GONZALEZ G., et AUGUR, C.

Les pectinases, enzymes d'un interêt commercial certain pour l'agroindustrie, sont produites essentiellement par les moisissures en fermentation en milieu solide (FMS) et liquide (FML). La sélection d'une souche hyperproductrice en FMS a été obtenue par un criblage de 248 souches sur pulpe de café. Une technique d'analyse d'images a permis une caractérisation précise des souches suivant des traits morphologiques. Suite à une irradiation aux ultra violets des souches adaptées, soit à la FMS soit à la FML, ont été sélectionnées. Les mutants adaptés à la FMS produisent cinq fois plus de pectinases que les mêmes souches en FML et *vice versa*. Les données expérimentales montrent des différences au niveau des protéines biosynthétisées par une même souche placée soit en FMS soit en FML.

Mots clés : Fermentation en milieu solide, fermentation en milieu liquide, pectinases, *Aspergillus niger*, mutant, activité de l'eau, production comparative, hyperproduction, image digitale, protéines.

INTRODUCTION

Filamentous fungi have been largely exploited for over fifty years for the production of industrial enzymes. Among them, one of the most widely used fungi, in view of its potential for enlarged industrial applications, is *Aspergillus niger*. It has been commonly utilized to produce commercially important enzymes, such as pectinases. The industrial applications of these enzymes are rather diverse, from the clarification of fruit juices (Ward, 1985) and the extraction of nectars from tropical fruit (Kilara and Benchura, 1990) to the upgrading of agroindustrial waste (Peñaloza *et al*, 1985). Pectinases also play an essential role in the yield and quality of the coffee bean. Industrially, pectinases are produced using both solid state fermentation (SSF) and submerged fermentation (SmF) cultivation techniques, with strains of *A. niger*. However, SSF is generally considered more suitable for rendering higher yields of specific pectinases, in particular, pectin esterases and polygalacturonases (Rombouts

and Pilnik, 1980). According to Bailey and Pessa (1990), the decrease in pectinase production in submerged cultures may be due to the disruption of mycelia during culture conditions. In order to better comprehend the differences in pectinase production by *A. niger* in either SSF or SmF, an extensive screening programme for the selection of fungal strains, adapted to either SSF or SmF, was undertaken. A screening of 248 strains, isolated in Mexico's coffee-growing areas, enabled the isolation of pectinase-hyperproducing strains from coffee pulp in SSF (Boccas *et al,* 1994). These strains were then UV-irradiated and, through a selection procedure, pectinase-hyperproducing *A. niger* mutants, adapted either to SSF or SmF, were identified (Antier *et al,* 1993).

MATERIALS AND METHODS

ORGANISM AND INOCULUM DEVELOPMENT

A. niger strains were isolated and maintained as described by Boccas *et al,* (1994).

ENZYME PRODUCTION IN SSF AND SMF

The methodologies used have been described in detail by Antier *et al* (1993).

ANALYTICAL METHODS

For the analysis of SSF samples, 10 g fermented coffee pulp was mixed with 10 ml water and pressed at 1000 psi in a hydraulic press. The extract obtained was then centrifuged at 6000 g for 5 min. Supernatants were used for further studies. The pectinase activity was assayed through the measurement of the reduction of viscosity of a pectin solution (20 g/L, citrate-phosphate buffer 0.1 M, pH 5.5), using a Brookfield viscometer. Assay involved a thorough mixing of 18 ml of pectin solution and 1 of ml enzyme solution, and incubation at 45°C for 10 min. One unit (U) of pectinase activity was defined as the amount of enzyme which reduced the initial viscosity by 50% in 10 min (Minjares, 1992).

For the analysis of SmF samples, cultures were filtered through Whatman paper No. 4; the filtrate was collected and used for the enzyme assays, as described above.

Water activity was measured at equilibrium by means of a water activity system (Decagon device).

RESULTS AND DISCUSSION

Mutants were further characterized, according to their rate of colonial expansion as well as their ability to sporulate early. As shown in Fig. 1, the various groups of mutant strains, along with the parent strain (C28B25), could be easily distinguished according to the two parameters described below, using a digital imaging technique.

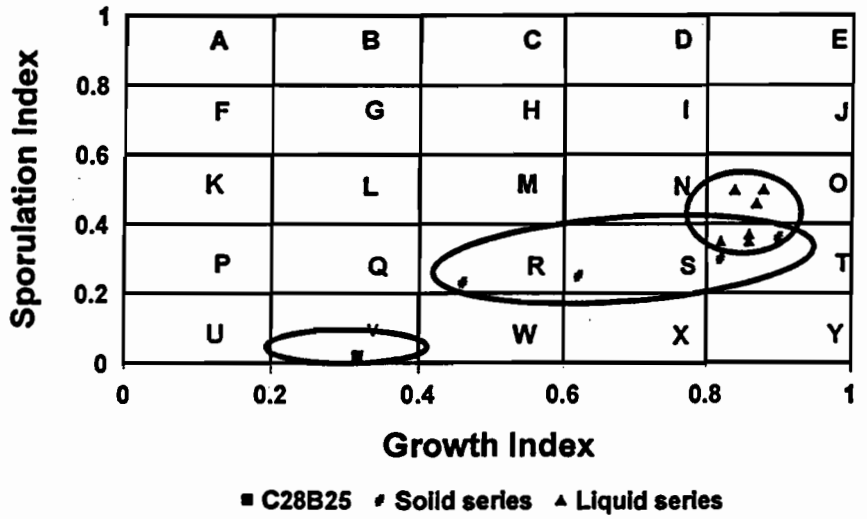

Fig. 1. Strain characterization by digital imaging.

All three groups of strains were grown on the same defined growth medium, composed of dextrose (10 g/L) and deoxyglucose (0.5 g/L). It should be observed that the mutant series, which hyperproduce pectinases in SmF (liquid series), have, under these conditions, the highest growth index as well as sporulation index. It is also interesting to note that all the pectinase-hyperproducing mutants of a particular

series (liquid or solid) have the same growth patterns. Therefore, fungal strains with specific characteristics can be distinguished, by observing morphological traits only. At present, this technique is being further developed towards an easy typing of fungal strains.

Pectinases from *A. niger* have been used in our laboratory, in the past few years, as a model system to study differences between SSF and SmF. The lack of the ability of fungal strains to efficiently produce pectinases in SSF, and the lack of an understanding of the regulatory mechanisms, which control enzyme production in different fermentation processes, prompted the present study. Initially, it was observed (Solis *et al*, 1991) that the production of pectinases by the parent strain (C28B25) decreased with a decrease in water activity (0.99 in SmF and 0.95 in SSF). Pectinase-hyperproducing mutants adapted either to SSF (Sol-3) or to SmF (Liq-3), were selected and grown in both SSF and SmF, with coffee pulp as a source of pectin. Growth conditions were described as elsewhere (Minjares, 1992).

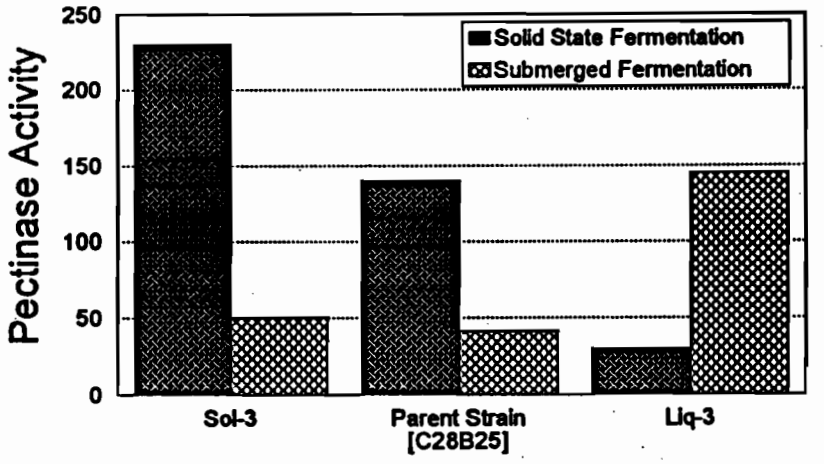

Fig. 2. Comparison of pectinase activities of *A. niger* mutants and parent strain, grown in SSF and SmF

As shown in Fig. 2, pectinase activities vary among the strains, when grown in either SSF or SmF. More importantly, when Sol-3 mutant is grown in SSF, it produces five times more pectinase activity, than when it is grown in SmF. The opposite is observed with the Liq-3 strain. The difference in both cases lies in the water activity, which seems to be a key factor. The two classes of mutants of *A. niger* C28B25 may have quite different genetic and physiological properties, which could be the basis for their adaptation to SSF or SmF. A molecular approach is, therefore, being taken to determine whether the observed differences in pectinase

activities were due to transcriptional or translational differences of the corresponding genes involved, or a combination of both.

The pectinases that are being studied are a group of fungal secreted enzymes with the ability to degrade a polygalacturonic homopolymer, when used as a substrate. Consequently, the pectinase activity determined is due to a number of different and specific enzymic activities. The enzymes responsible for these activities can be classified as endo- and exo- polygalacturonases, as well as lyases and methyl esterases. Each enzyme contributes towards the degradation of the corresponding polymer. At present, it is not known, whether one or more of the above mentioned enzymes are responsible for the changes in pectinase production, when both classes of mutants of *A. niger* C28B25 are grown either in SSF or SmF.

The initial approach taken was to study the pattern of total extracellular proteins produced by each mutant strain in SSF or SmF. For this, Sol-3 and Liq-3 mutants were grown in SSF as well as SmF, as described previously (Minjares, 1992). Mycelium was spun down and the supernatant from each sample, containing pectinase activity, was collected. The protein content could not be measured by the classical methods, due to presence of the compounds in the supernatant, which interferred with known colourimetric assays. Protein content was, therefore, evaluated by running an aliquot of each sample in a 15% polyacrylamide gel in denaturing conditions, followed by silver staining of the proteins present (data not shown).

It was observed that the extracts from SmF contained around five fold less total protein, than the extracts from SSF. Moreover, the separated proteins showed different banding patterns. The same mutant strain (Sol-3 or Liq-3) produced different proteins, when each was grown in either SSF or SmF. Some proteins from Sol-3 are present in SSF, but absent in SmF extracts, while others are present in SmF and absent in SSF. The same observations were made for the Liq-3 mutant. It is interesting to note that there are differences in the banding patterns in the 35,000 dalton range, which is the approximate molecular weight of endo-polygalacturonases.

Studies are now underway, using the Westrern Blot technique, with both monoclonal and polyclonal antibodies against an endo-polygalacturonase from *A. niger*, to determine whether the observed changes in pectinase activity and in protein production are due to changes in gene expression of endopolygalacturonases of the mutant strains in different fermentation techniques.

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Production of gibberellins by solid substrate cultivation of *Gibberella fujikuroi*

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SUMMARY

The kinetic parameters for growth and production of gibberellins by *Gibberella fujikuroi*, grown on liquid (LC), and solid substrate (SSC) cultivation, were compared. For the latter, organic (OSC) and inert (ISC) support cultures were investigated. Significantly higher biomass content and product yields were found for SSC, as compared to the LC system. However, similar specific productivities were found for LC and ISC, thereby suggesting that physical conditions are not necessarily a determining factor for the elevated product yields in SSC.

A significantly higher specific productivity and product yield for OSC, as compared with ISC, were observed. This could result from limited carbon catabolite repression, that might prevail in the former system. A further possibility could be the presence of an unknown compound in wheat bran, that strongly induced GA_3 biosynthesis.

Major secreted gibberellins were GA_3, GA_1, GA_{13}, GA_4, GA_7, GA_9 and GA_{14}, for all studied culture systems. A constant GA_3/GA ratio was found throughout the cultivation for both SSC systems, thereby suggesting that the production of GA_3 is a good indicator of the biosynthetic pathway expression for all gibberellins.

Keywords : Gibberellins, *Giberella fujikuroi*, kinetic parameters, solid substrate cultivation, wheat bran, inner support culture, liquid cultivation, productivities.

RESUME

Production de gibberellines par *Gibberella fujikuroi* en fermentation en milieu solide.

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Nous avons comparé les cinétiques de croissance de *Gibberella fujikuroi* et de production de la gibberelline, pour deux techniques de culture : en milieu liquide (FL) et en milieu solide (FMS). Pour la FMS, les cultures ont été réalisées à la fois sur support organique (OSC) et sur support inerte (ISC). On observe en FMS une production de biomasse et des rendements de production nettement supérieurs à ceux obtenus en FL. Cependant la productivité spécifique mesurée est identique pour les deux techniques de fermentation: les caractéristiques physiques du support n'apparaissent pas comme le facteur déterminant les hauts rendements de production obtenus en FMS.

Nous avons également observé une productivité spécifique et un rendement de production plus élevé pour le milieu OSC que pour ISC. Cela peut s'expliquer par une répression catabolique limitée dans le premier milieu, par la présence dans le son de blé d'un composé inconnu fortement inducteur de la biosynthèse de la GA_3.

Quelque soit la technique de fermentation utilisée, les gibberellines majoritairement produites sont les GA_3, GA_1, GA_{13}, GA_4, GA_7, GA_9 et GA_{14}. On mesure un rapport GA_3/GA_3 constant au cours de la fermentation , pour les trois types de culture. On peut supposer que la production de GA_3 est un bon indicateur de la voie anabolique des gibberellines.

Mots clés: Gibberellines, *Giberella fujikuroi*, paramètres cinétiques, cultures sur substrats solides, son de blé, culture sur support inerte, culture liquide, productivités.

INTRODUCTION

The gibberellins (GAs) are a large group of biosynthetically related tetracyclic diterpenes, that are naturally present in plants. Most C19-GAs are biologically active compounds, which act as hormones, and regulate many different plant growth and development processes (Bruckner and Blechschmidt, 1991).

All commercially available gibberellins are produced by liquid cultivation (LC) of the ascomycetous fungus, *Gibberella fujikuroi* (Borrow *et al*, 1964). Gibberellins are

typical secondary metabolites. Upon exhaustion of assimilative nitrogen sources, exponential fungal growth ceases. At this point, secondary metabolism is triggered, with the concomittant biosynthesis of gibberellins. The most important resultant product is gibberellic acid (GA_3).

Solid substrate cultivation (SSC) is a culture system, where microbial growth and product formation occurs at or near the surfaces of porous solid materials, in the absence of free water. SSC has traditionally used substrates, such as agricultural products and by-products, including wheat, rice, corn, wheat bran, etc. These play a dual role both as a nutrient and support for the micro-organism. For the purpose of this study, we will label this type of SSC system, as an organic support culture (OSC). OSC generally leads to much higher yields and lower capital and operational costs than LC. The Lonsane group, for example, recently reported a maximum yield for GA_3 production of 1.5 g/kg of wheat bran after 7-8 days cultivation (Kumar and Lonsane, 1987a,b, 1990).

A recent development in SSC, the inert support culture (ISC) system, is based on the sorption of a defined nutrient solution in an inert surface matrix. This technique has been evaluated for the production of several metabolites, such as fungal spores (Durand, personal communication), penicillin (Barrios-Gonzalez *et al*, 1988) and citric acid (Lakshminarayana *et al*, 1975). ISC shows several advantages over traditional SSC methods (Auria *et al*, 1990). It enables higher product purity, constant substrate geometry throughout the cultivation, higher control of microbial metabolism through culture medium manipulation, and higher sorption capacity of the culture medium. However, ISC has several drawbacks, such as higher substrate costs, the requirement of concentrated media, which could lead to catabolite repression problems, low water activity and a laborious system operation.

This paper compares the kinetic parameters for growth and the production of GA_3 by *G. fujikuroi* grown in the three culture systems referred above. The evolution of other major gibberellins is also examined.

MATERIALS AND METHODS

MICROORGANISM AND CONDITIONS FOR INOCULUM PREPARATION

Gibberella fujikuroi UC 5, a gibberellic acid hyperproducer, was used throughout this study. Inocula were prepared in a liquid culture medium, containing (g/L): glucose, 80; ammonium tartrate, 1.84; potassium phosphate, 5; magnesium sulphate, 0.45 and 10 mL nutrient salts solution. Culture flasks were incubated for 72 h on a rotary shaker at 200 rpm for 60 h.

CULTIVATION SYSTEMS

Solid substrate cultures were run in the columns (Raimbault and Alazard, 1980), containing either 6 g organic support or 4 g inert support. Columns were incubated in a water bath at 28°C, and a constant saturated air was passed through the fermenting substrate at a rate of 4 L/g/h. The packing density for organic and inert supports was 0.3 g/cm^3. Liquid cultures (LC) were carried out in 250 mL Erlenmeyer flasks with 100 mL culture medium. Flasks were incubated at 28°C in an orbital shaker at 200 rpm. All the experiments were carried out in triplicate.

CULTURE MEDIA

The organic support culture (OSC) medium essentially consists of a wheat bran-starch based medium, as described by Kumar and Lonsane (1987b). Expanded vermiculite, sieved to obtain 20-40 mesh size, was employed in inert support cultures (ISC). Ten mL of a nutrient solution, containing (g/L) 240 maltodextrins, 4.0 ammonium tartrate, 12 g potassium phosphate, 1.2 magnesium sulphate and 0.2 yeast extract, was filter sterilized, inoculated with vegetative mycelia cells (0.5%, v/v) and added to an autoclaved vermiculite-containing column. Cultivation conditions were as those for OSC. The initial moisture content was 55% for wheat bran and 67% for vermiculite-based media.

Optimized liquid culture (LC) medium contained (g/L) maltodextrin, 100; sunflower oil, 5; ammonium tartrate, 1.8; yeast extract, 0.2; and, potassium, phosphate as well as magnesium sulphate, as described above.

ANALYTICAL PROCEDURES

CO_2 evolution

The gas effluent of the columns was continuously flushed into 10 mL of 3 N NaOH to trap the CO_2 produced. The latter was periodically quantified by back-titration of the remaining 3 N NaOH, as described by Agosin and Odier (1985). The specific growth rate was then calculated, according to Carrizales and Rodriguez (1981).

Gibberellin extraction

Acidified water (3x2 mL) was added to 2 g of wet fermented material, pressed in a manual laboratory press, and then centrifuged. For GC analysis, 50 ml of 2 N NaOH was added to 0.5 mL supernatant, and the latter was partitioned against 3x500 mL of ETOAc. The remaining water phase was acidified to pH 2.5 with HCl and extracted three times with 500 mL each of ETOAc. The organic phases were combined and concentrated to dryness in a stream of N_2.

Gibberellin determination

GA_3 concentration was directly estimated from the centrifuged supernatants using fluorometry (Kavanagh and Kuzel, 1958). All of the GAs were separated and quantified by GC, after MSTFA trimethylsilylation of the dried samples. Analyses were then carried out in a Shimadzu GC8A gas chromatograph with an FID detector, equipped with a fused silica BP-1 WCOT capillary column (50 m x 0.33 mm i.d. x 1,0 µm film thickness). Helium was used as the carrier gas at 15 psi pressure.

Selected samples were also analyzed by GC/MS, using a Hewlett Packard 5980 gas chromatograph, coupled to a 5872 mass spectrometer. Samples in MSTFA (1 µL) were injected into the same capillary column as above, at an oven temperature of 60°C, maintained for 1 min. Temperature was raised at 20°C/min to 240°C, and then at 4°C/min to 300°C. The temperatures of injector, interface and MS source were 220, 280 and 200°C, respectively. The ionisation energy was 70eV. GAs were identified by comparison of their mass spectra with those of authentic samples and/or with published mass spectral data (Takahashi, 1986)

Sugar and nitrogen determination

Reducing sugars were measured, following the Somogy-Nelson procedure (Nelson, 1944). Ammonia was measured, using the method of Muftic (1964). Total nitrogen in dried samples was determined by microKjeldhal digestion. Nitrogen biomass content was 5.5%.

Biomass estimation

For the ISC medium, the total biomass was quantified, using the respective yield from nitrogen, while for the LC medium, mycelial dry weight was measured. The CO_2 produced during cultivation was evaluated to determine the biomass for OSC.

For this purpose, the relationship between biomass and CO_2 , as determined in ISC, was employed.

RESULTS AND DISCUSSION

GROWTH

The kinetic parameters, related with fungal growth of *G. fujikuroi* in the three culture systems under study, are shown in Table 1.

Table 1. Kinetic growth parameters of *Gibberella fujikuroi* grown on different culture systems.

Parameter	Symbol	Units	LC	OSC	ISC
Specific growth rate	μ	hr^{-1}	0.08	0.09	0.08
Cell content	X_m	g /kg IDM	17.00	80.00	94.00
Cell concentration	X_v	g/L	17.00	24.00	28.00

IDM: Initial Dry Matter

The total biomass in ISC medium was 94 g/kg dry matter, equivalent to 28 g/L. A similar level was reported by Laukevics *et al* (1985) in their studies on SSC biomass production, where the highest cell content was 120 g/kg IDM, corresponding to 30 g/L. For the OSC medium, biomass was estimated from the CO_2 production rate, considering that nitrogen sources in wheat bran are complex and not fully available to the fungus. A maximum level of 80 g/kg IDM was reached, equivalent to 24 g/L. These results were considerably higher, than those reported earlier (16 g/kg IDM) by Kumar and Lonsane (1987a, 1990) for a similar medium.

PRODUCTION

SSC technology shows advantages in both production and productivity of gibberellins, over liquid fermentation techniques (Table 2). Product yields (Yp/idm) for organic (OSC) and inert (ISC) support media were 6.7 and 5 times higher,

respectively, as compared with LC medium. Product concentration was 6 times higher than that reported by Kumar and Lonsane (1987b). This could be explained, at least in part, by the employment of a different, hyperproducing strain in the present case.

Table 2. Kinetic parameters for gibberellic acid (GA_3) production by *Gibberella fujikuroi grown* on different culture systems.

Parameter	Symbol	Units	LC	OSC	ISC
Cultivation time	t	h	230	190	190
Product concentration	P	mg GA_3/L	750	7600	3000
Mass product yield	$Y_{p/idm}$	mg GA_3/kg	750	6800	3800
Specific production	$Y_{p/x}$	mg GA_3/g	44	85	40
Mass productivity	Q_{idm}	mg GA_3/kg/day	78	860	480
Specific productivity	q_p	mg GA_3/g/day	4.5	12	5

One of the main advantages of SSC is the smaller equipment size (Hesseltine, 1977), resulting in lower costs for both the reactor and auxiliary plant. Assuming a packing density of 0.3 g/cm^3 in SSC, the effective reactor volumes for GA production by OSC and ISC will be 35 and 60% of that required for LC.

FINAL PRODUCT CONCENTRATION

A key parameter, which determines the dimension of downstream operations, is the final product concentration in the fermented solids. In SSC, final product yields were 10.8 and 4.0 g/kg of final dry matter for OSC and ISC, respectively. Preliminary results show that 90% of the GAs in the fermented solids could be extracted with water at a solid:liquid ratio of 1:3 for OSC and 1:1 for ISC. Hence, resulting downstream processing volumes in OSC and ISC, would be 25 and 20% of those required for GA processing in LC medium. These values indicate significant reduction in the costs of extraction, purification and concentration operations could be achieved, if SSC technology is employed for GA3 production.

SPECIFIC PRODUCTION OF GA_3

Similar specific GA_3 production values were found for ISC and LC. However, these were significantly lower, than those observed for OSC (Table 2). In the former systems, low GA_3 biosynthesis could be the result of carbon catabolite repression (Muñoz and Agosin, 1993) or the use of the carbon in divergent storage compounds (Borrow *et al,* 1964). This would be minimized in OSC, where carbon is usually found as complex polymers. Indeed, the very low concentrations of soluble sugars, which have been found in OSC cultures after 72 h cultivation, would support this assumption (Corona and Agosin, unpublished).

A further possibility could be the presence of one or more unknown compound(s) in wheat bran, which induce strong GA3 biosynthesis.

GA_3 PRODUCTIVITY

Maximum production yields were reached in lower cultivation times in SSC, than in LC. The difference must have occured during the production phase, as the duration of the growth phase was similar for all cultivation systems (results not shown). Final productivities were 11 and 6 times higher for OSC and ISC, respectively, compared with those for LC (Table 2).

Similar specific productivity levels were found for both LC and ISC, which would suggest that physical conditions are not necessarily a determining factor in the elevated metabolite production in SSC systems. Therefore, the composition of the medium and/or the high biomass content obtained through ISC could be considered as possible explanations of the resulting product yields. The maximum instantaneous productivity reached in ISC medium, however, was 40 times higher than that obtained in an optimized LC medium (results not shown). Furthermore, the carbon source is completely exhausted after 170 h cultivation in ISC. This results in mycelial cell lysis and the arrest of GA_3 biosynthesis. Monitored feeding with a carbon source at this point should allow for obtaining the much higher product yields from the ISC system.

GIBBERELINS PRODUCED

The same gibberellins, *i.e.* GA_3, GA_1, GA_{13}, GA_4, GA_7, GA_9 and GA_{14}, were produced under all cultivation conditions (Fig. 1). Moreover, the relative proportion of the different GAs was similar in all media. As expected, the major gibberellin was GA_3, followed by GA_{13} and GA_1.

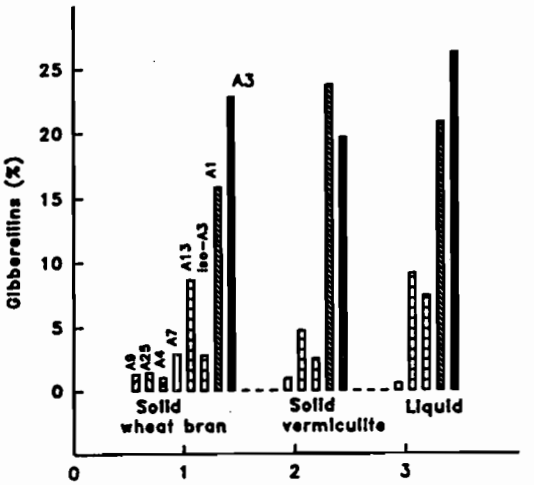

Fig. 1. Relative proportion of gibberellins produced by *Gibberella fujikuroi*, grown in the different cultivation systems.

A constant GA_3/GA ratio, equivalent to 40%, was found throughout the cultivation, surprisingly for both SSC systems (Fig. 2A). In this regard, a considerable difference between OSC and ISC media was anticipated. LC was not studied. The consistent GA_3/GA ratio in SSC systems strongly suggests that the expression of GA_3 is a good indicator of the biosynthetic pathway expression for all gibberellins.

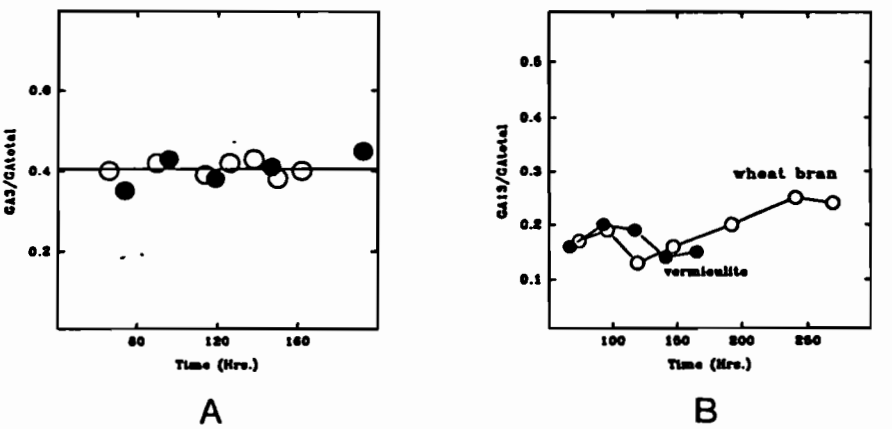

Fig. 2. Evolution of the GA_3/GA (A) and GA_{13}/GA (B) ratios during cultivation of *Gibberella fujikuroi* in an ISC (∘) or OSC (O) medium.

Nevertheless, GA_{13}/GA ratio increased during cultivation in both SSC systems (Fig. 2B), at the expense of others. This indicates that the oxidation of GA_{36}, the precursor of GA_{13}, is probably not enzymatic. GA_{13} is one of the final products of the GA pathway in *G. fujikuroi*. This GA is a dicarboxylic acid, without any biological activity.

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NOMENCLATURE

EtOAc	ethyl acetate
GAs	gibberellins
GA_3	gibberellic acid
IDM	initial dry matter, kg
ISC	inert support culture
LC	liquid culture
MSTFA	methylsilyltrifluoroacetic acid
OSC	organic support culture
μ	specific growth rate, h^{-1}
SSC	solid substrate culture
P	product concentration, mg GA_3/L pressed broth
Q_{idm}	mass productivity, mg GA_3/kg IDM/day
q_p	specific productivity, mg GA_3/g biomass/day
$Y_{p/idm}$	mass product yield,mg GA_3/kg IDM
$Y_{p/x}$	specific production , mg GA_3/g biomass

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Fruity aroma production by *Ceratocystis fimbriata* in solid state fermentation

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SUMMARY

Production of fruity aroma in solid state fermentation (SSF) by the fungus *Ceratocystis fimbriata* has been studied, with respect to the nature of the support/substrate, the importance of added precursors in the medium and the aeration. The aroma were characterized by the sniffing technique and GC headspace analysis, while the growth was followed by respirometry. Wheat bran, cassava and sugarcane bagasse were adequate for growth and aroma production. The nutritive media with a higher glucose concentration (200 g/l) gave a strong apple aroma, while media containing amino acids precursors, such as leucine and valine, gave strong banana aroma. Aroma production was growth dependent and the maximum aroma intensity was detected a few hours, before or after the maximum respirometric activity (0.8 to 1.6 ml CO_2 / h. g dry matter, after 24 h). Experiments carried out under various aeration rates (5 and 50 ml/h. g dry matter) showed that this parameter was not limiting for growth, even if the exit gas was very poor in oxygen at the lower aeration rate, giving in this case the most intense aroma. For experiments carried out without forced aeration, the same aroma were also found with higher intensity. Fourteen compounds have been separated by GC headspace and 11 of these were identified. Major compounds included acetaldehyde, ethanol, ethyl acetate, isoamyl acetate and isoamyl alcohol.

Keywords: Solid state fermentation, *Ceratocystis fimbriata*, fruity aroma, substrates, support, precursors, aeration, respirometry, apple aroma, banan aroma, growth dependance.

RESUME

Production d'arômes fruités *par Ceratocystis fimbriata* cultivé en fermentation solide.

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La production d'arômes fruités en fermentation solide (FMS) par un champignon filamenteux *Ceratocystis fimbriata* a été étudiée en vue d'estimer l'influence de la nature du substrat et du support, de la présence dans le milieu de culture de précurseurs d'arômes et enfin du taux d'aération des cultures sur la qualité des arômes obtenus. Les arômes produits ont été caractérisés par la technique du «sniffing » et la croissance microbienne a été suivie par respirométrie. Le son de blé, le manioc et la bagasse de canne à sucre ont été utilisés pour la culture et la production d'arômes. Les milieux de cultures à forte concentration de glucose (200 g/l) donnent un puissant arôme de poire ou de pomme, alors que les milieux riches en acides aminés tels que la leucine et la valine favorisent la production d'arôme de banane. Il a été démontré que la production d'arôme est fonction de la croissance et l'intensité maximale a été detectée quelques heures après l'activité respirométrique maximale (elle varie entre 0,8 et 1,6 ml CO_2/h.g matière sèche après 24 heures). Des essais réalisés avec différents taux d'aération (5 et 50 ml/h.g matière sèche) ont montré que ce paramètre n'est pas limitant pour la croissance, même si pour des taux d'aération faibles, les effluents gazeux à la sortie du fermenteur sont pauvres en O_2. Dans ce dernier cas, comme pour des fermentations réalisées sans aération forcée, les mêmes arômes ont été détectés, mais à des intensités plus fortes. Quatorze composés ont été séparés par CPG parmis lesquels onze ont pu être identifiés. Les composés majoritaires ont été les suivants: acétaldéhyde, éthanol, ethyl acétate, isoamyl acétate et isoamyl alcool.

Mots clés: Fermentation en milieu solide, *Ceratocystis fimbriata*, arômes fruités, substrats, support, précurseurs, aération, respirométrie, arôme de pomme, arôme de banane, croissance limitée.

INTRODUCTION

Microorganisms play an important role in the generation of natural flavouring compounds, particularly food aroma. A number of the extensive reviews dealing

with flavour generation by microorganisms are available (Latrasse *et al*, 1985; Welsh *et al*, 1989; Janssens *et al*, 1992). As pointed out recently by Bigelis (1992) and Christen (1995), filamentous fungi are especially interesting, because they are able to produce flavouring compounds or flavours-related enzymes. Recently, the production of aroma in SSF has been reported. Yamauchi *et al* (1989) obtained a fruity odour by growing a *Neurospora* strain on pregelatinized rice. Gervais and Sarrette (1990) studied the production of coconut aroma by *Trichoderma viride* on agar, while Humphrey *et al* (1990) patented a process, where an *Aspergillus* strain grown on cellulose fibers produced methyl ketones from coconut oil. Moreover, the capacity of some moulds from the genus *Ceratocystis* to produce fruit-like aroma has already been demonstrated (Hanssen and Sprecher, 1981; Senemaud 1988; Christen *et al*, 1994). In this work, the ability of *Ceratocystis fimbriata* to produce aroma in SSF was explored. It involved the study on the influence of the substrates/supports, the aeration flow rates and the precursors, on both growth and aroma production.

MATERIAL AND METHODS

ORGANISM AND CULTURE MEDIA

Ceratocystis fimbriata CBS 374-83 was used. It was periodically transferred onto potato dextrose agar (PDA) slants and stored at 4°C. Four substrates / supports were used: wheat bran, sugarcane bagasse, cassava and an anionic resin (Amberlite IRA-900, Rohm and Haas, USA). These were prepared according to Christen *et al* (1993). When forced aeration (packed bed) was used, the cultures were carried out in small columns placed in temperature controlled bath. Experiments without forced aeration (surface culture) were carried out in 500 ml Erlenmeyer flasks, covered with gauze or tight-sealed. The reactors were filled with 7.5 g initial dry matter (IDM) for wheat bran and Amberlite, and 5.25 g IDM for bagasse and cassava. For all experiments, initial conditions were: temperature, 30°C; pH, 6; and inoculum size $1x10^7$ spores/g IDM. Aeration rates were 50 or 5 ml/h.g IDM. Initial water content was calculated, according to the maximum adsorption capacity of each support (wheat bran, 50%; Amberlite, 58%; sugarcane bagasse, 63% and cassava, 65%). Culture conditions are given in tables 1 and 2; Nutritive medium SM is the synthetic medium optimized by Christen and Raimbault (1991), and contained 200 g/l of glucose. In all cases, an oligoelement solution, previously used by these authors, was added. Urea, leucine and valine (167 mmol/l) were used as a nitrogen source and/or precursor of the aroma.

ANALYTICAL PROCEDURES.

The odours of the cultures were determined by sensory evaluation by a non-trained panel, consisting of six members, with no restriction in descriptive terms. Growth was characterized by respirometry and measured by gas chromatography. For packed column experiments, this allowed the calculation of the carbon dioxide production rate, the oxygen uptake rate and the respiratory quotient (RQ) (Christen *et al*, 1993). For surface cultures, O_2 consumption and CO_2 evolution were followed. Water activity and pH were also determined at the end of the fermentation. Volatiles produced during the fermentation were characterized by gas chromatography (Hewlett-Packard 5890, equipped with a Megabore HP-1 column (length, 5 m) and with a flame ionization detector) of headspace vapor from the cultures (only for experiments without forced aeration). Temperatures were: injector and detector, 210°C, oven held at 40°C during 2 min and then programmed at 10°C/min to 150°C. The nitrogen gas flow rate was 1.5 ml/min and split ratio 1:32.

Table 1. Culture conditions for experiments witrh forced aeration

Run	Substrate/Support	Nutritive media	Aeration rate (ml/h.g)
1	Amberlite	Potato broth	50
2	Amberlite	Potato broth	5
3	Amberlite	SM	5
4	Wheat bran	Urea	50
5	Wheat bran	-	5
6	Wheat bran	Urea	5
7	Wheat bran	Leucine	5
8	Wheat bran	Valine	5
9	Sugarcane bagasse	SM	5
10	Sugarcane bagasse	Potato broth+glucose	5

Table 2. Culture conditions for experiments without forced aeration Each experiment was made in both gauze and tight-sealed flaskes.

Run	Substrate/Support	Nutritive media
11	Wheat bran	-
12	Wheat bran	SM
13	Wheat bran	Leucine
14	Wheat bran	Urea
15	Cassava	-
16	Cassava	SM
17	Cassava	Leucine
18	Cassava	Urea
19	Sugarcane bagasse	SM + leucine

RESULTS AND DISCUSSION

PACKED CULTURES EXPERIMENTS WITH FORCED AERATION

At the higher aeration rate (50 ml/h.g IDM in runs 1 and 4), negligible or poor aroma was detected (Table 3). It can be assumed that the high aeration rate swept away the volatiles produced and/or oxygenated conditions reduced the synthesis of such molecules. This is why a very low aeration rate was then used (5 ml/h.g IDM). In such cases, the overall aroma detected (pear/apple and banana) were stronger. Leucine and valine, when added to the medium, served as a precursor for the development of the banana aroma (runs 7 and 8). This fruity aroma appeared very rapidly (before the first 24 h) in these cases. When no precursor was used, pear/apple aroma was also detected (runs 4, 5, 6, 9 and 10). In runs 9 and 10, two successive kinds of aroma were detected, first the pear/apple one and then at 5 days a strong peach aroma. Using this aeration rate, lower maximum CO_2 production rates were observed (less than 1.3 ml/h.g IDM), which indicated that, growth was limited and volatile metabolites production was favoured in poorly aerated media. In terms of support evaluation, wheat bran (supplemented or not) and supplemented bagasse, gave a better result compared with Amberlite. In all cases, water activity was maintained at a satisfactory level, but the pH at the end of fermentation was alkaline

(for wheat bran) or acidic (for bagasse). In all cases, no compounds were detected in headspace analysis of the cultures.

Table 3. Aroma production in packed culture under forced aeration

Run	Aroma and intensity	Tmax (h) #	CDPR max (ml/h.g)	Aw final	pH final
1	-	-	2.58	0.996	6.76
2	Banana +, then pear/apple +	67 / 91	0.95	0.973	3.05
3	Banana +	91	0.89	0.990	2.65
4	Pear/apple +	42	2.45	0.983	8.76
5	Pear/apple ++	17	1.30	0.988	9.06
6	Pear/apple ++	39	1.04	0.985	8.95
7	Banana +++	17	0.72	0.989	9.03
8	Banana +++	17	0.79	0.990	9.06
9	Pear/apple +, then peach +++	20 / 91	0.84	0.993	2.71
10	Pear/apple ++, then peach ++	68 / 116	0.70	0.998	6.24

-: none, +: weak, ++: medium, +++: strong. #: T max, time of maximum aroma perception. CDPR: CO_2 production rate, ml/h.g IDM.

The maximum aroma was detected, just before or after the maximum in CO_2 production rate was attained. Interestingly aroma production was found to be related to growth.

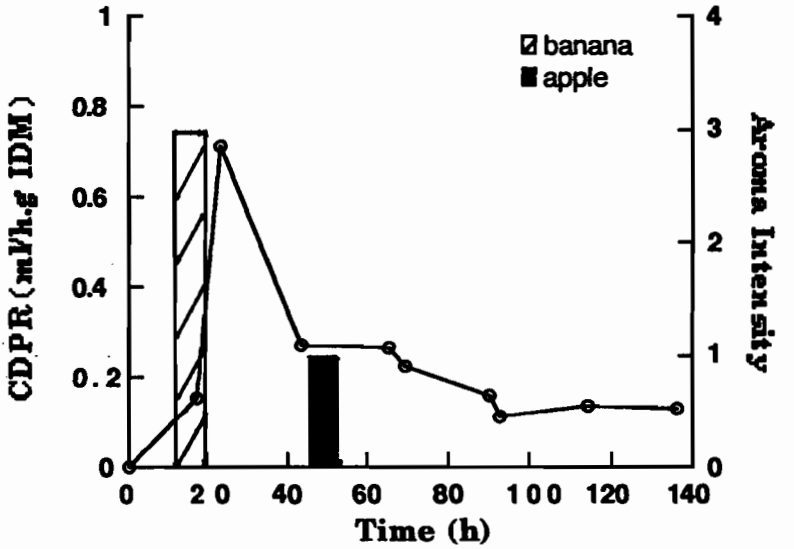

Fig. 1. CO_2 production rate and aroma production by *C. fimbriata* grown on wheat bran supplemented with leucine (aeration rate = 5 ml/h.g IDM).

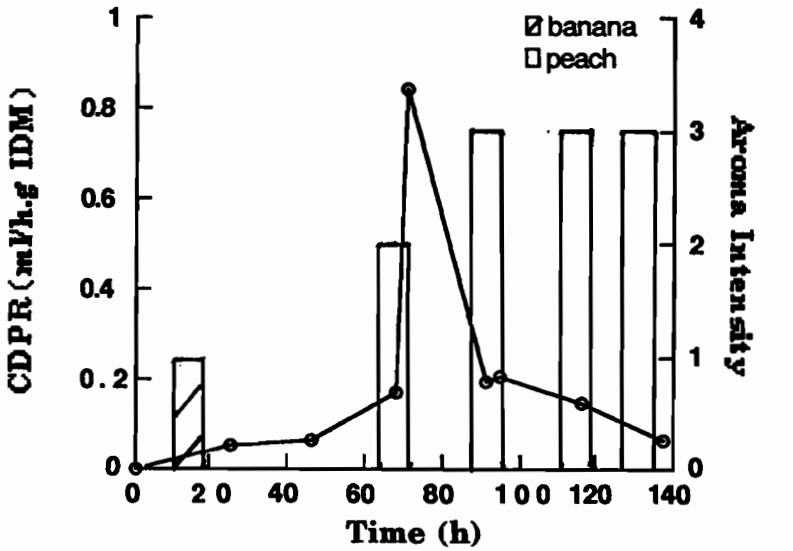

Fig. 2. CO_2 production rate and aroma production by *C. fimbriata* grown on sugarcane bagasse supplemented with synthetic medium (aeration rate = 5 ml/h.g IDM).

SURFACE CULTURES EXPERIMENTS WITHOUT FORCED AERATION

In this case, 9 combinations were tested, and in each case, experiments were carried out using a gauze cover (static culture) tightly-sealed (without aeration). Sensory evaluation was only possible in the former case. In the second case, respiration was characterized by O_2 (%) consumption and CO_2 (%) accumulation in the flask. Results are presented in Table 4.

Table 4. Results of aroma production in surface cultures without forced aeration.

Run	Aroma and intensity (1)	T max (h) #	CDPR max (ml/h.g) (1)	CO_2 max (%) (2)
11	-	-	1.15	11.3
12	apple / pear ++	44	0.06	81.3
13	banana +++	36	0.16	31.5
14	apple / pear ++	41	1.23	22.3
15	banana ++	40	0.2	36.7
16	-	-	0.9	62.5
17	banana +++	40	0.2	29.6
18	-	-	0.45	7.3
19	banana +++	39	0.08	46.9

-: none, +: weak, ++: medium, +++: strong. #: T max, time of maximum perception of the aroma. CDPR: CO2 production rate in ml/hour/g IDM. 1 refers to gauze covered flask cultures and 2 to tight-sealed flask cultures.

All three substrates/support were found to allow growth and aroma production in aerated conditions. Aroma production was higher in forced aeration. The strongest aroma detected (banana) corresponded to that case where media were enriched with leucine (runs 13, 17, 19), while pear/apple aroma, of a lower intensity, was obtained in wheat bran culture with synthetic medium and urea (runs 12 and 14). These aroma had major intensity between the first and the second days. In tight-sealed flask cultures, growth was observed. As growth and substrate fermentation evolved, CO_2 was produced and the internal pressure increased. This pressure was released during sampling, which provoked the increase in CO_2 concentration up to 81%. These values of CO_2 were coupled with low O_2 (less than 2 % of residual oxygen), which channelled the metabolism towards the fermentative route. No Sensory evaluation

was made for tightly-sealed flask cultures, but large amounts of volatiles were produced (Fig. 3).

SEPARATION AND IDENTIFICATION OF GC DETECTED COMPOUNDS

Fig. 3 gives headspace chromatograms of gauze covered aerated and tight-sealed cultures of run 19. Fourteen compounds were detected. Eleven compounds were identified through retention time comparison with a standard and could be classified according to their relative quantity (peak area). Ethanol, ethyl acetate, ethyl propionate and isoamyl acetate in major; acetaldehyde, isoamyl alcohol and isobutyl acetate in intermediate and 1-propanol, 2-propanol, 1-butanol and amyl acetate in small amounts are formed. Among them, isoamyl acetate and isoamyl alcohol are known to be the major compounds in the banana aroma, while acetaldehyde, ethanol and ethyl acetate are always present in fruit aroma. Other minor compounds, like ethyl propionate and isobutyl acetate are also reported to be present in fruit aroma. Some differences can be observed between gauze covered and tightly-sealed cultures. Acetaldehyde peak is bigger, ethyl propionate peak is smaller and unknown peak #13 is absent in the latter. Unfortunately, it is not possible to evaluate directly the impact of these differences on the aroma.

CONCLUSION

Wheat bran, cassava and sugarcane bagasse were found to be adequate substrates/support for aroma production by *C. fimbriata*. Amino acids, such as valine or leucine, direct banana-like aroma. Other aroma (peach, apple) were also detected in the absence of any precursor. The corresponding compounds of banana aroma (isoamyl alcohol and isoamyl acetate) were detected in the headspace of the culture at relatively high amounts. A total of 14 compounds were separated by GC, and, among them, 11 were identified (1 aldehyde, 5 alcohols and 5 esters). Work is currently continued to identify the unknown peaks and to quantify the identified compounds. Very low aeration (5 ml/h.g IDM) or passive diffusion favoured the production of strong aroma. Results were highly improved in these conditions, in comparison with those obtained by Christen *et al* (1994) at higher aeration rates. In the tightly-sealed flasks, the fungus was able to ferment the carbohydrates present in the medium (glucose in the case of bagasse, starch in the case of wheat bran and cassava). The fact that very low or no aeration is required opens interesting technological perspectives for the production of fruity aroma by *C. fimbriata*.

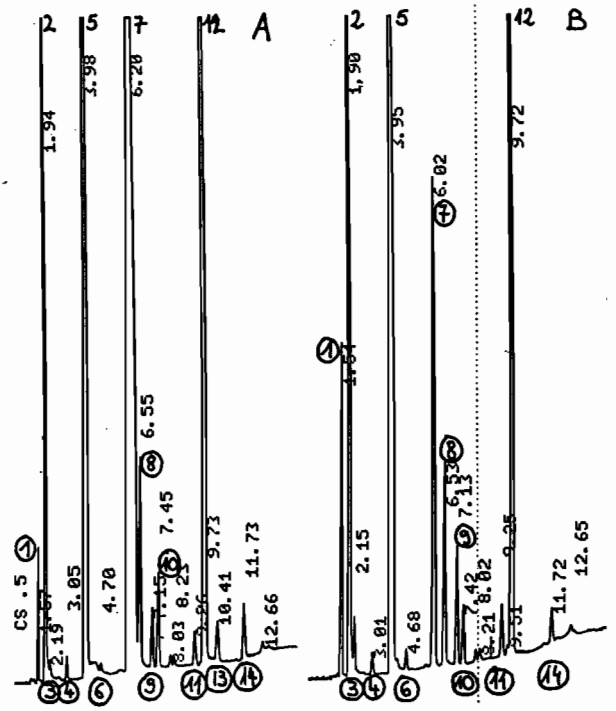

Fig. 3. Headspace chromatograms of 48 h cultures of gauze covered (A) and tight-sealed (B) cultures of *C. fimbriata* grown on bagasse + synthetic medium (glucose = 200 g/l). 1: acetaldehyde, 2: ethanol, 3: 2-propanol, 4: 1-propanol, 5: ethyl acetate, 6: 1-butanol, 7: ethyl propionate, 8: isoamyl alcohol, 9: unknown, 10: isobutyl acetate, 11: unknown, 12: isoamyl acetate, 13: unknown, 14: amyl acetate.

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Coconut-Like aroma production by *Trichoderma harzianum* in solid state fermentation

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SUMMARY

From the present work, it is now established that, by solid state fermentation (SSF), it is possible to produce 6-pentyl-α-pyrone (6-PP), an unsaturated δ-lactone with a strong odour of coconut, by using *Trichoderma harzianum*. 6-PP has been produced on a solid substrate, impregnated with a culture medium of a very high carbon/nitrogen (C/N) ratio, after an initial biomass production stage in a liquid culture medium at a low C/N ratio. 6-PP is the main component. Other volatile compounds accompanied this biosynthesis, and became more diversified with the progress of fermentation. The amount of 6-PP produced during SSF, following a 5 day culture, was 2.8±0.5 mg/g dry matter. Therefore, the 6-PP concentration produced during SSF is greater than that reported in literature during liquid culture. 6-PP does not inhibit growth during SSF. Moreover, with a nitrogen deficiency, the strain sporulated on a solid substrate. Semi-continuous 6-PP production by medium inoculation may, therefore, be possible.

Keywords: Solid state fermentation, sugarcane bagasse, dessiccated coconut, *Trichoderma harzianum*, coconut-like aroma, 6-pentyl-a-pyrone, physiology, spore production, C/N ratio, criterion, fermentation monitoring, microscopic examination, macroscopic examination.

RESUME

Production d'arôme noix de coco par fermentation solide de Trichoderma harzianum

SARHY-BAGNON V., LOZANO P., PIOCH D., AND ROUSSOS S.

En fermentation en milieu solide (FMS), il a été possible de produire la 6-pentyl-α-pyrone (6-PP), un composé à odeur dominante noix de coco, par *Trichoderma harzianum* . La 6-PP a été produite sur un support solide imprégné d'un milieu de culture dont le rapport carbone/azote C/N est très élevé après une étape préalable de production de biomasse dans un milieu de culture liquide dont le rapport (C/N) est faible. La 6-PP est majoritairement présente. D'autres composés volatils ont accompagné cette biosynthèse. Ces derniers se sont diversifiés au cours du temps de culture. La quantité de 6-PP produite en FMS au bout de 5 jours de culture a été de 2,8±0,5 mg/g Matière Sèche (MS). La concentration de 6-PP produite en FMS paraît être plus importante qu'en culture liquide (données bibliographiques). Il semblerait qu'il n'y ait pas d'inhibition de la croissance par la 6-PP en FMS. De plus dans des conditions de carence d'azote, la souche a sporulé sur support solide. Une production semi-continue de 6-PP par ensemencement par pied de cuve est donc envisageable.

Mots clés: Fermentation en milieu solide, bagasse de canne à sucre, coco râpé, *Trichoderma harzianum* , arôme noix de coco, 6-pentyl-α-pyrone, physiologie, production de spores, rapport C/N, critère, suivi de fermentation, examen microscopique, examen macroscopique.

INTRODUCTION

Demand for flavour compounds has increased substantially during the recent years. Extraction from natural raw materials and chemical synthesis are the conventional ways of producing flavour compounds, but they have their drawbacks. For example, agricultural production of aromatic plants is seasonal and of limited quantities. The quality of the essential oils is governed by uncontrollable factors, such as climatic and geographical conditions. Chemical synthesis leads to the so-called artificial compounds, which are not often appreciated by consumers (Gross and Asther, 1989). Volatile compounds production by micro-organisms - yeasts, bacteria and filamentous fungi (Janssens *et al*, 1992) - seems to be an interesting alternative. In

fact, the process is easily manageable, from both a qualitative and quantitative point of view. Moreover, European legislation considers biotechnological aroma as natural substances, on the sole condition that the precursors involved in their synthesis being natural (CEE, 1988).

Micro-organisms are capable of producing aroma, notably fruity aroma, such as aldehydes, terpenes, and lactones. For example, γ-decanolactone, with its peach smell, is produced by different bacteria, yeasts and filamentous fungi (Farbood and Willis, 1983).

Amongst lactones, 6-pentyl-α-pyrone (6-PP), an unsaturated δ-lactone, has an interesting aromatic potential. It has a predominantly coconut-type fruity smell, but 6-PP is not naturally found in the coconut meat. It is one of the aroma compounds found in peaches and nectarines (Sevenants and Jennings, 1966). It is produced chemically, after seven reaction steps, under a high working temperature (Pittet and Klaiber, 1975). Hence, its biotechnological production is a good alternative.

It was Rifai (1969), who first described and classified the different *Trichoderma* species, including *T. harzianum*, which produce a characteristic coconut aroma. 6-PP was first detected in *Trichoderma viride,* and its presence was correlated to the coconut smell (Collins and Halim, 1972). Considerable work has been carried out on the optimization of production of this lactone in a liquid medium (Zeppa *et al*, 1990; Sastry *et al*, 1985). However, Serrano-Carreon (1992) was the first to study the metabolic pathways involved in the formation of this unsaturated lactone. Similar to the saturated lactones, 6-PP seems to stem from lipid metabolism by successive β-oxidation of fatty acids. Serrano-Carreon *et al* (1992) showed that limited *T. viride* growth promotes 6-PP production by enabling lipid accumulation in the cell. Serrano-Carreon *et al* (1993) proposed a hypothetical diagram for 6-PP formation: under the effect of a lipoxygenase, linoleic acid seems to be converted into 6-PP through successive steps involving β-oxidation.

Lozano *et al* (1995) described a new aroma preparation procedure, notably for coconut aroma, by solid state fermentation (SSF) of various agricultural substrates. These results were used as guidelines in the present study of 6-PP production in SSF.

The aim of the work was to monitor 6-PP production by *T. harzianum* cultured in SSF system, using 80% sugarcane bagasse as support, 20% dessicated coconut followed by impregnation of the solids with the liquid medium at a C/N ratio of 60. In this first stage of the work, the interest was focused on utilizing dessicated coconut as a substrate to develop the process (Lozano *et al,* 1995). The second stage involved the quantification of the volatile compounds formed and comparison of the results with those reported for liquid culture. In the next stage, the interest

concentrated on the physiology of the fungus growth, particularly the evolution of pH changes, the dry matter and spore counts.

MATERIALS AND METHODS

MICROORGANISMS

Trichoderma harzianum (IMI 206040) was used. The strain was cultured and maintained on potato dextrose agar (PDA), in Petri dishes. The cultures were incubated at 22°C. The 5-day-old mycelium obtained under these conditions was used to inoculate the biomass production medium. The strain was maintained on PDA at 4°C.

BIOMASS PRODUCTION IN LIQUID MEDIUM (STAGE 1)

As *T. harzianum* strain used does not sporulate on solid media, it was impossible to inoculate the solid medium using a spore suspension. A liquid medium was, therefore, used for mycelium production. The liquid medium (50 ml), containing malt extract (20 g/l) and glucose (10 g/l) was poured into 250-ml Erlenmeyer flasks. The medium was autoclaved at 115°C for 20 min, before inoculation with 1 cm^2 of mycelium-impregnated gel took place. It was incubated at 22°C for 48 h on a rotary shaker (100 rpm). The mycelium formed was separated from the medium by decantation and rinsed twice with physiological saline (0.9% NaCl). It was then suspended in 14 ml of the same saline solution, and the suspension (2 ml) was used to inoculate two solid media.

AROMA COMPOUNDS PRODUCTION IN SSF (STAGE 2)

The solid substrate, comprising of 3.6 g bagasse and 0.9 g dessicated coconut, was placed into 250-ml flasks. It was impregnated with 13.5 ml of a medium, which promoted lipid accumulation (high C/N ratio, glucose 30 g/l and $(NH_4)_2SO_4$ 0.94 g/l). The solid culture medium was autoclaved at 115°C for 20 min. Following cooling, the flasks were inoculated with 2 ml of mycelial cell suspension. The flasks were incubated at 22°C.

EXTRACTION OF AROMA COMPOUNDS

Samples (5 g) were removed from solid state *T. harzianum* cultures and placed into 250-ml flasks, with 50 ml distilled water. The aroma compounds were extracted from the samples with 10 ml pentane for one hour by simultaneous distillation-extraction using Likens-Nickerson apparatus, modified by Godefroot *et al* (1981). After extraction, the mixture was evaporated and then dissolved in 1 ml solvent.

ANALYTICAL METHODS

pH measurement

The pH was determined directly on a suspension of 2.5 g fermented substrate in 100 ml distilled water, using a Knic digital pH meter.

Dry matter (DM) measurement

The dry weight was determined by the weight difference. Around 2-3 g fermented substrate was weighed on a Sartorius R160 D precision balance and then dried to constant weight at 105°C.

Macroscopic and microscopic description of Trichoderma

The macroscopic description was carried out using a binocular microscope, and the state of the culture was recorded in terms of substrate colonization (white filaments, bagasse set in a mass) and culture colour. The latter provided an idea of the state of strain sporulation (*Trichoderma* spores are green). The microscopic examination of the mycelium was carried out under a microscope (magnification x 400). The physiological state of the strain (spores, germination, mycelium, conidiophores) was recorded.

Spores counting

The spores were counted in the suspension of 2.5 g fermented substrate in 100 ml distilled water, after blending for 2 min in an Ultraturax at 20,000 rpm. They were either counted in the direct fermented substrate suspension or after dilution, using a Malassez cell. The results are expressed as the number of *T. harzianum* spores produced/dry matter.

Qualitative analysis of aroma compounds

The aroma compounds were identified, using a quadripolar mass spectrometer (Fisons Trio 1000), with ionization by electronic impact (70 eV). The apparatus was connected to a Carlo Erba GC 8000 gas chromatograph, fitted with a DB Wax column (internal diam: 0.32 mm, length: 60 m, film thickness: 0.25 µm). The splitless injector and detector temperatures were 200 and 300°C, respectively. Oven temperature was increased from 100 to 200°C, at a rate of 10°C/min.

Quantitative analysis of aroma compounds

A Hewlett Packard gas chromatograph, fitted with a flame ionization detector, was used. The constituents were separated on a Carbowax capillary column (internal diam: 0.32 mm, length: 30 m, film thickness: 0.25 μm) using a split injection mode. The temperature was increased from 40 to 180°C, at an initial rate of 10°C/min for 14 min, followed by a rate of 3°C/min to 200°C. Quantitative analysis of 6-PP was carried out using the internal calibration method, with γ-undecanolactone (99%, Aldrich) as the internal standard.

RESULTS AND DISCUSSION

EVOLUTION OF AROMA COMPONENTS

The compounds, produced by culturing *T. harzianum* for seven days on a solid medium, were extracted by simultaneous distillation-extraction using the Likens-Nickerson apparatus, modified by Godefroot *et al* (1981). The compounds found in the samples were identified by GC/MS coupling. Fig. 1A is the chromatogram for the aroma extract of non-inoculated dessicated coconut. Figs. 1B, C and D are the chromatograms of *T. harzianum* cultures, fermented for 2, 5 and 7 days, respectively.

Fig. 1A shows that 6-PP is not naturally found in the non-inoculated substrate. It did not appear clearly until the substrate had been fermented by the filamentous fungi for 2 days (Fig. 1B). The next chromatograms (Figs. 1B, C and D) obtained after 2, 5 and 7 days, show the presence of 6-PP, in addition to the molecules, such as alcohols, ketones, fatty acids, etc. These molecules led to additional aroma categories arised during culture.

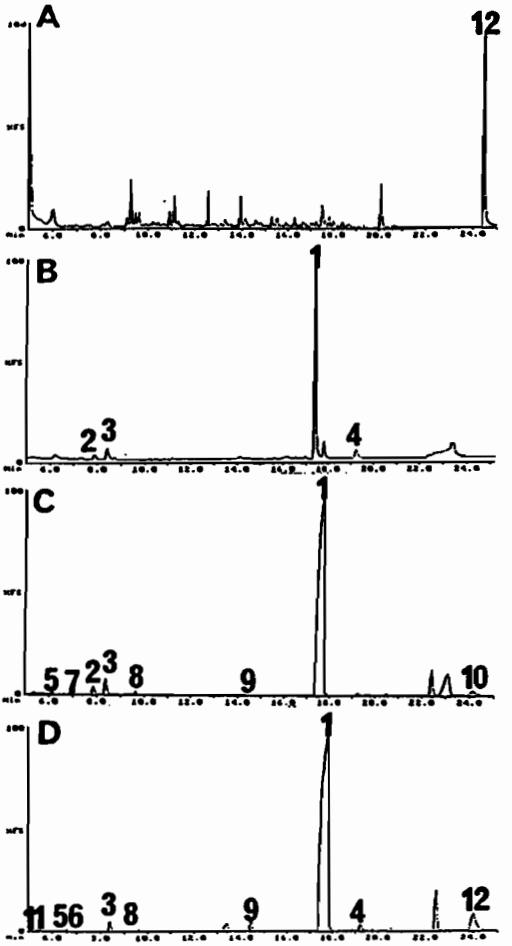
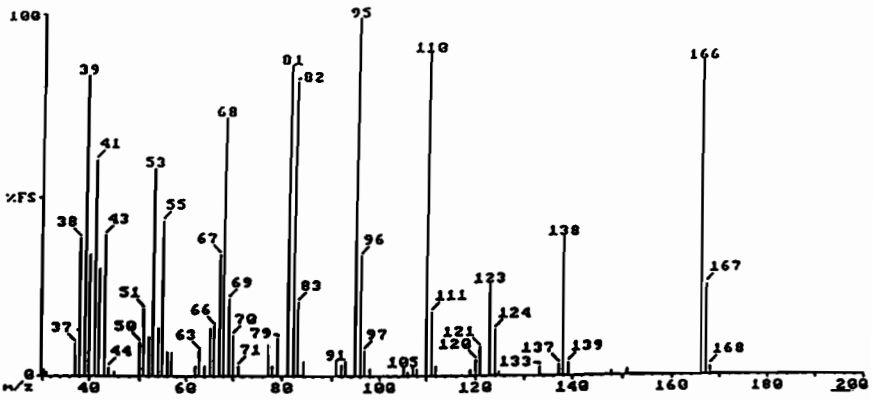
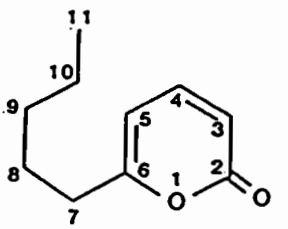


Fig. 1. Chromatografic profile (GC/MS coupling) of aroma extracts of *T. harzianum* cultures grown in SSF on a mixture of bagasse and desiccated coconut for two days (B), five days (C) and seven days (D). The chromatogram for the aroma extract of non-inoculated desiccated coconut is shown in A. (The sensitivity of the photomultiplier is the same for the 4 samples: 500V). List of some compounds identified by CPG/SM: **1.** 6-pentyl-α-pyrone, **2.** 2-decanone, **3.** 2-undecanone, **4.** decanoic acid, **5.** 2-nonanone, **6.** 1-octen-3-ol, **7.** 2-nonanol, **8.** 2-undecanol, **9.** octanoic acid, **10.** tetradecanoic acid, **11.** 2-heptanol, and **12.** δ-lactone.





6-PENTYL-α-PYRONE

Fig. 2. Mass spectra of 6-PP from fungal solid state culture and structure of the molecule

6-PP was identified by its mass spectra obtained from liquid and solid fungal cultures (Fig. 2). Data were compared to the previous studies on the production of 6-PP in liquid culture (Collins and Halim, 1972; Kikushi *et al*, 1974; Sevenants and Jennings, 1974). Electron impact mass spectral data indicated a molecular ion peak (M+) at m/z 166. The formula is $C_{10}H_{14}O_2$. The biosynthesis was accompanied by the production of other compounds, and the range of compounds widened in number with culture time. However, 6-PP remained by far the main component. More than twenty components were identified, using the mass spectra in the files of the GC/MS apparatus. There might be some discrepancies for certain peaks, which could result from unresolved groups of compounds. Anyhow, this part of the work will be continued during a further stage of the project.

The compounds produced by culturing *T. harzianum* for 5 days were extracted and quantified by GC, using the internal calibration method. The response factor of C11/C10 (γ-undecanolactone/ γ-decanolactone) was used, which is 0.8. The amount of 6-PP recovered was 2.8±0.5 mg/g dry matter (DM). Assuming that the fermented product contained 75% moisture, it could be estimated that about 930 mg of 6-PP was produced per litre of liquid solution adsorbed on the substrate. In liquid culture, the maximum concentration recorded, using Amberlite XAD-2 as an adsorbent, was 248 ppm, which allows to overcome growth inhibition by 6-PP (Prapulla *et al*, 1992). A 6-PP concentration of 90 to 110 mg/l would be enough to inhibit growth and prevent further 6-PP production. The 6-PP concentration obtained in SSF was, therefore, greater than that in liquid culture. Moreover, there was no evidence of growth inhibition by 6-PP in SSF.

STUDY OF THE GROWTH PHYSIOLOGY OF *T. HARZIANUM* CULTURED ON A SOLID MEDIUM WITH A VIEW TO PRODUCE 6-PP

T. harzianum growth on a solid medium was monitored under a microscope and by determining the pH, moisture content and sporulation. The morphological appearance of the mycelium and data on the different parameters are presented in Table 1.

Table 1. Morphological appearance and parameters of *T. harzianum* development on a solid substrate under nitrogen deficiency conditions (C:N = 60)

Time (days)	pH	DM (%)	No. of spores/g DM	Macroscopic description	Microscopic description
0	4.8	24.3	0		Uniform inoculum
2	4.6	22.5	0	Substrate set in one mass	Long, tangled filaments
5	4.6	23.7	$4.0x10^8$	Substrate set in one mass. White and green clumps	Long filaments with phialides and spores
7	4.6	22.6	$2.1x10^9$	Green clumps	Spores
14	4.5	22.3	$4.0x10^9$	Green surface	Spores

DM : Dry Matter

Macroscopic description of the cultures provided a picture of the physiological state of *T. harzianum* growth. Following a 2 day culture in SSF, the mycelium was well developed, which meant that the substrate got set in one mass. Between 4 and 5 day cultures, phialides developed and conidiospores were released into the culture medium. The surface turned green, reflecting the release of conidiospores. This microscopic observation enabled the monitoring of culture development during SSF and correlation of these results with coconut aroma production. The pH was hardly varied during SSF, remaining constant at around 4.6. However, DM decreased slightly, from 24.3 to 22.3%.

The *T. harzianum* strain, used for aroma production, did not sporulate on solid media, and its choice for aroma production in a liquid medium was based on this criterion. On a solid medium containing bagasse, the strain sporulated after 5 days. As the medium used had a C:N ratio of 60, a nitrogen deficiency must have led to sporulation of the strain. Once enough spores were available, semi-continuous culture by inoculating the medium was possible. The number of spores produced after 5 day culture was around $4x10^8$/g DM. The number of spores stabilized at around $4x10^9$ between 7 and 14 days. As a result, inoculation of the culture medium

could be considered, so as to obtain 10^7 spores/g DM, which is the usual inoculation rate for SSF (Roussos, 1985).

CONCLUSIONS

6-PP production has only been demonstrated and optimized by liquid culture recently. The present study demonstrates that 6-PP production is possible by SSF in two stages, i.e., biomass production in a liquid culture medium at a C/N ratio of 14, followed by biosynthesis on a solid medium impregnated with a culture medium at a C/N ratio of 60.

A study of the aroma profile showed that 6-PP was produced once the medium was inoculated. The amount of 6-PP produced, after 5 day culturing under nitrogen deficiency conditions, was four times greater than that in the liquid culture. The absence of the growth inhibition by 6-PP, seen in liquid culture, may be the cause for this more substantial production. SSF, therefore, seemed to be appropriate for 6-PP production.

As the strain used did not sporulate on solid media, the mycelial inoculum had to be produced first in a liquid medium. The solid medium used for 6-PP production was then inoculated with a mycelium suspension, and not a spore suspension, as it is the case in SSF. The study of the physiological development of the strain during culture showed that the strain sporulated on bagasse, whereas it dit not sporulate on other solid media. Environmental stress (nitrogen deficiency) enabled strain sporulation. Semi-continuous culture by inoculating the medium may therefore be possible. The recovery of the product in a lesser quantity of extract in SSF made this technique even more interesting for an industrial scale production.

ACKNOWLEDGEMENTS

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Pigments and citrinin production during cultures of *Monascus* in liquid and solid media

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SUMMARY

As a part of the investigations on red pigment production by *Monascus* species, an antibacterial compound was isolated from the supernatant fungal cultures and identified as the nephrotoxic agent, citrinin. It was produced both by *Monascus purpureus* as well as *M. ruber* and was synthesized the pigments, through the polyketides pathway. As *Monascus* red pigments are commonly used in Asia as food additives, where these are produced on rice, the commercial preparations obtained by SSF were analyzed. These contained no citrinin at all, while our preparations obtained by submerged cultures contained a non-negligible fraction of citrinin. In the liquid medium, *M. purpureus* and *M. ruber* produced citrinin at concentrations of 240 and 370 mg/l, respectively, while in SSF on rice, the production was 100 and 300 mg/kg of dried fermented rice powder, respectively.

Keywords : *Monascus purpureus*, *M. ruber*, citrinin, pigments, solid state fermentation, submerged fermentation, ang-kak, red mould rice.

RESUME

Production de pigments rouges et de citrinine lors de cultures de *Monascus* en milieu liquide et solide.

BLANC P.J., LORET M.O. ET GOMA G.

Lors d'études sur la production de pigments rouges par des souches de *Monascus*, un composé antibactérien a été isolé de surnageants de cultures et identifié comme étant un agent néphrotoxine connu sous le nom de citrinine. Ce composé est produit aussi bien par *Monascus purpureus* que *par M. ruber* et comme les pigments, il est synthétisé par la voie des polycétides. Les pigments rouges étant utilisés en Asie comme additifs alimentaires où ils sont produits sur riz, des échantillons commerciaux obtenus par FMS ont été analysés et ils ne contenaient pas du tout de citrinine, alors que nos échantillons obtenus en cultures immergées en contiennent une quantité non-négligeable. En milieu liquide, *M. purpureus* et *M. ruber* produisent de la citrinine à des concentrations respectives de 240 et 370 mg/l alors qu,en FMS sur riz, leurs productions sont respectivement égales à 100 et 300 mg/kg de poudre sèche de riz fermenté.

Mots clés : *Monascus purpureus*, *M. ruber*, citrinine, pigments rouges, fermentation en milieu solide, fermentation immergée, ang-kak, riz fermenté rouge.

INTRODUCTION

In a Chinese herbal medicine book, published in the first century, the term ang-kak or red mould rice was first mentioned. Red mould rice has been used as a food colourant or as a spice in cooking. Van Tieghem (1884) isolated a purple mould on potato as well as linseed cakes and named it *Monascus ruber*. This ascomycete was so named, as it has only got one polyspored ascus. Following that, Went (1895) isolated *Monascus purpureus* from the red mould rice, obtained from the market in Java, Indonesia. Subsequently, several others species were isolated around the world. *Monascus* is encountered often in oriental foods, especially in Southern China, Japan

and Southeastern Asia. Besides red-mould rice, it has been used traditionally in red bean curd or red Chinese cheese, red vinegar and red rice wines. The powdered red-mould rice was used as a meat disinfectant as well as in Chinese herbal medicine to cure several diseases, including indigestion, dysentery, bruises of muscles, pain, and anthrax. Most of the studies conducted during the earlier decades concentrated on the industrial production of *Monascus* pigments, which can be used as a colourant in food industry.

The present study is concerned with isolation of an antibacterial compound, its chemical identity and production by two strains under various cultural conditions.

BIOLOGY OF *MONASCUS*

Monascus is an ascomycete, classified in the family Monascaceae (Table 1).

Table 1. Classsification of *Monascus*

Phyllum	Ascomycotina
Class	Plectomycetes
Order	Eurotiales
Family	Monascaceae
Genus	Monascus

Hawksworth and Pitt (1983) have divided the genus *Monascus* into three species : *M. pilosus*, *M. purpureus* and *M. ruber*, which comprise the majority of the strains, mainly isolated in Oriental food (Table 2).

Table 2. Classsification and origin of *Monascus* according to Hawksworth and Pitt (1983)

Earlier classification	Origin	Classification as per Hawksworth and Pitt (1983)
M. albidus	Tofu	*M. purpureus*
M. albidus var. *glaber*	Tofu	*M. purpureus*
M. anka	Anka	*M. purpureus*
M. anka var. *rubellus*	Anka	*M. purpureus*
M. araneosus	Moldy bran	*M. purpureus*
M. barkeri	Red koji	*M. ruber*
M. fuliginosus	Moldy bran	*M. ruber*
M. kaoliang	Anka	*M. purpureus*
M. major	Anka	*M. purpureus*
M. paxii	Plant	*M. ruber*
M. pilosus	Moldy bran	*M. pilosus*
M. pubigerus	Moldy bran	*M. pilosus*
M. purpureus	Anka	*M. purpureus*
M. ruber	Plant	*M. ruber*
M. rubiginosus	Anka	*M. purpureus*
M. rubropunctatus	Moldy bran	*M. pilosus*
M. serorubescens	Tofu	*M. pilosus*
M. vitreus	Tofu	*M. ruber*

FUNGAL METABOLITES

Fungi produce a wide variety of secondary metabolites, which are formed from intermediates of the primary metabolism, and are classified according to their precursor. Some of these secondary metabolites are not obtained from acetate, but these are rare among fungi. Those derived directly from glucose, such as kojic acid, are produced by various species of *Aspergillus*. Those derived from benzene are formed through the shikimic acid pathway. Acetyl CoA is certainly the most important intermediate in fungal secondary metabolism and can lead to various molecules, such as terpenes (carotenoids, ergosterol, gibberellic acid), fatty acid

derivatives (polyacetylene), derivatives from amino acids (alkaloids, penicillins, cephalosporins) and polyketides.

Polyketides

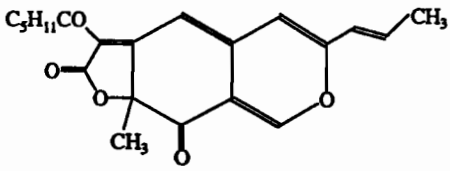
Polyketides are formed, through the condensation of one acetyl CoA with one or more malonyl CoA, with a simultaneous decarboxylation, as in the case of lipidic synthesis. Malonyl CoA is obtained from the enzymatic action of acetyl CoA carboxylase on acetyl CoA. The polyketides are classified, according to the number of units of 2 carbons contributing to the synthesis of the polyketomethylene. The secondary metabolite is obtained by successive reductions, dehydrations, methylations and cyclisations. Polyketide synthesis is characteristic of fungi, especially for ascomycetes. Nevertheless, their biosynthesis is still unclear, even though Turner (1971) has recorded hundreds of polyketides. As with many fungi, *Monascus* can produce many molecules of industrial interest, according to the substrates used.

Free and complexed pigments

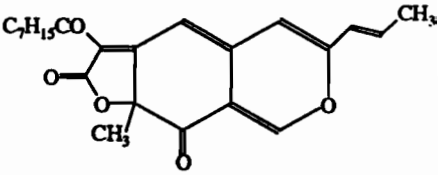
Monascus pigments are a group of fungal metabolites, called azaphilones, which have similar molecular structures as well as chemical properties. Azaphilones also consist of sclerotiorin and rotiorin from *Penicillium*. Molecular structures of the *Monascus* pigments are shown in Fig. 1. Ankaflavine and monascine are yellow, rubropunctatine and monascorubrine are orange and rubropunctamine and monascorubramine are purple in colour. The same colour exists in two molecular structures, differing in the length of the aliphatic chain.

The free *Monascus* pigments are not soluble in water, but are soluble in organic solvents. A number of methods have been patented for manufacturing water-soluble pigments. The principle behind these methods lies in the substitution of the replaceable oxygen in monascorubrin or rubropunctatin by the nitrogen of the amino group of various compounds, such as amino acids, peptides as well as proteins, and changing the color from orange to purple. *Monascus* pigments can be reduced, oxidized and reacted with other products, especially amino acids, to form various derivative products, sometimes called the complex pigments. From the broth of a submerged fermentation, the complex pigments shown on Fig. 2 have been isolated (Blanc *et al*, 1994).

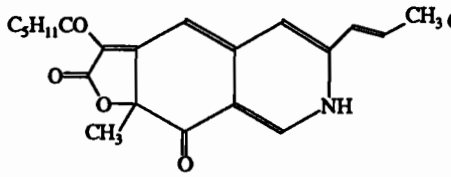
Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity and time. It was shown that these pigments, when added in the sausages or the pies, remained stable during 3 months of storage at 4°C, the stability being 92 to 98% (Fabre *et al*, 1993).



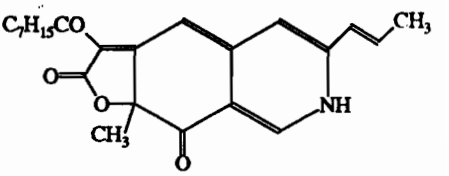
n° 1 - Rubropunctatine M=354



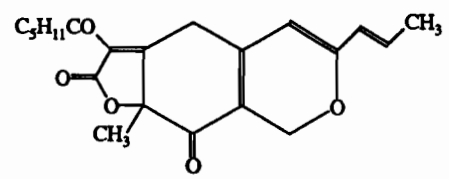
n° 2 - Monascorubrine M=382



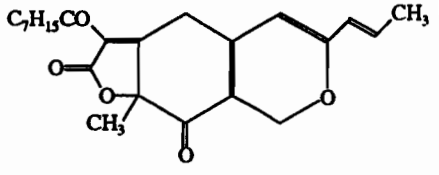
n° 3 -Rubropunctamine M=353



n° 4 - Monascorubramine M=381

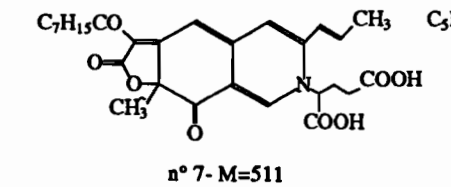


n° 5 - Monascine M=358

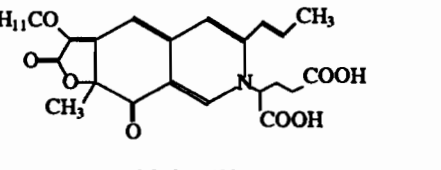


n° 6 - Ankaflavine M=386

Fig. 1. Structure of the free pigments of *Monascus*



n° 7- M=511



n° 8- M=483

Fig. 2 Structure of the coloured components obtained by *Monascus ruber* and *Monascus purpureus* on chemically defined medium (organic substrate : glucose or ethanol)

Monascidin A/citrinin

Antibacterial properties of *Monascus* were first mentioned by Wong and Bau (1977). The so-called monascidin A was efficient against *Bacillus, Streptococcus* and *Pseudomonas*. It was shown that this molecule is citrinin and its production by various *Monascus* species was determined, using different culture media and conditions (Blanc *et al*, 1995).

Monacolins

A series of hypocholesteremic agents have been isolated from *Monascus* and these are named monacolin J, K, and L. These polyketides were first isolated from cultures of *Penicillium citrinum*, can inhibit specifically the enzyme, and control the rate of cholesterol biosynthesis.

Flavours (methylketones)

Monascus is an interesting organism for flavour production, as it is able to produce methylketones from fatty acids, via the classical β–oxidation pathway. For example, 2-nonanone is produced from decanoic acid. These ketones constitute the flavours of the blue cheese of Roquefort (Kranz *et al*, 1992)

PRODUCTION BY SUBMERGED FERMENTATION

Considerable contradiction exists in the published work, as to the best carbon source for red pigment production. Traditionally cultured on breads and rice, *Monascus* grows on every amylaceous substrate. Lin and Demain (1991) have shown that *Monascus* grows quite well on starch, dextrines, glucose, maltose and fructose, Chen and Johns (1994) achieved high-production of pigments, using glucose and maltose. The nitrogen source seems to have more importance, than the carbon source. Chen and Johns (1993) found that ammonium and peptones, as nitrogen sources, gave superior growth and pigment concentrations, as compared to nitrate. Production of the red pigment, with the use of various carbon and nitrogen sources and different cultural conditions, are summarized in Table 3. The best results were obtained, using ethanol and monosodium glutamate as substrates in a controlled fedbatch (Santerre *et al*, 1995). The preference for this amino acid was also shown by Lin and Demain (1991).

Table 3. Production and productivity of red pigments by *Monascus* using various substrates.

Carbon source	Nitrogen source	Cultural conditions	Production (g/l)	Productivity (mg/l h)
GLUCOSE	MSG	batch	1.40	11
	Soyapeptone	batch	1.05	12
	MSG	batch[1]	1.20	7
ETHANOL	MSG	batch	1.15	15
	Soyapeptone	batch	0.90	11
	MSG	batch[1]	2.00	11
	MSG	manual	5.25	9.5
	MSG	fedbatch[1]	*(35 ODU)*	
	MSG	controlled	9.00	20
	MSG	fedbatch[1]	*(60 ODU)*	

[1] The inoculum was prepared on a minimal medium

Juzlova *et al* (1994) found that ethanol, when used as sole carbon source at a concentration of 20 g/l, was better than maltose for pigment production by *Monascus*. Amino acids supported the biosynthesis of both yellow and red pigments. They also found that a two-stage cultivation, using maltose and ethanol in the first and second stages, respectively, may be useful in increasing the efficiency of ethanol utilization for pigment production.

The C/N ratio was shown by Wong *et al* (1981) to be important. About 50 g/g growth while about of 7-9 g/g pigmentation, could be achieved at a proper C/N ratio.

PRODUCTION BY SOLID STATE FERMENTATION

The classical Chinese method involves the inoculation of steamed rice grains, spread in big trays, with a strain of *Monascus anka* and incubation in an aerated and temperature-controlled room for 20 days.

Effect of moisture content

The moisture content is a very important parameter. Lotong and Suwanarit (1990) produced red pigments in plastic bags, containing rice grains. They observed that

pigmentation occurred only at a relatively low initial moisture level (26-32%). Initial substrate moisture content regulated pigmentation, as it was found that glucoamylase activity increased with an increase in the initial substrate moisture content. Therefore, at high moisture content and high enzyme activity, glucose was rapidly liberated, in amounts (120 g/l) that inhibited pigmentation. The sugar was then transformed into ethanol.

In the case of rice culture, Johns and Stuart (1991) found an optimal pigmentation at an initial moisture content of 56%, while lower moisture contents led to a large decrease in pigment formation. They confirmed that solid culture was superior to liquid culture for red pigment production by *Monascus purpureus*. This result has been attributed to the derepression of pigment synthesis in solid systems, due to the diffusion of intracellular pigments into the surrounding solid matrix. In the submerged culture, pigments normally remained in the mycelium, due to the low solubility of the pigment in the usually acidic medium.

Effect of gas environment

Han and Mudgett (1992) found that levels of oxygen and carbon dioxide in the gas environment influenced pigment production significantly and also the growth to a lesser extent in SSF. With *Monascus purpureus* cultivation on rice, maximum pigment yields were observed at 0.5 atm of partial oxygen pressure in closed pressure vessels. However, high partial carbon dioxide pressures progressively inhibited pigment production, inhibition being complete at 1.0 atm. In a closed aeration system with a packed-bed fermentor, partial oxygen pressures ranging from 0.05 to 0.5 atm, at constant partial carbon dioxide pressures of 0.02 atm, gave high pigment yields with a maximum at 0.5 atm of oxygen, whereas lower partial carbon dioxide pressures, at constant partial oxygen pressures of 0.21 atm, gave higher pigment yields. Maximum oxygen uptake and carbon dioxide evolution rates were observed at 70-90 and 60-80 h, respectively, depending on the gas environment. Respiratory quotients were close to 1.0, except at 0.05 atm of oxygen and 0.02 atm of carbon dioxide partial pressures.

Effect of the extraction

The addition of a sterilized nonionic polymeric adsorbent resin, directly to the growing submerged culture, did not enhance the pigment production, thus indicating that pigment extraction is probably not a factor (Evans and Wang, 1984). *Monascus* cells immobilized in a hydrogel were studied and exhibited decreased pigment production, probably due to the diffusional resistance of the pigment, through the hydrogel beads. Addition of the adsorbent resin, to the immobilized *Monascus* culture, increased both the maximum pigment yield and the production rate, above

those of the free-cell fermentations. The provision of a support for the mycelium may explain enhanced pigment production by the SSF. These results indicate that product diffusion from immobilized cell systems can be the limiting factor and that *in situ* extraction is one possible way to circumvent this problem.

Effect of various cereal media

Lin and Iizuka (1982) have studied various cereal media (Table 4) and obtained the best results with the use of mantou meal (yeast-fermented wheat meal).

Table 4. Effect of various cereal media (Lin and Iizuka, 1982) on the pigment production

Cereal media	Pigment production (Units of absorbancy / g dry matter)	
	400 nm	500 nm
Polished rice meal	1500	1700
Dehulled rice meal	1570	1850
Bread meal	3500	4000
Wheat bran	2300	2900
Corn meal	2300	2700
Kaoliang meal	2600	3000
Mantou meal	5100	5430
Wheat meal	3100	3600

COMPARISON OF PIGMENTS PRODUCTION IN SSF AND SUBMERGED CULTURES

Table 5 compares the pigment production, according to various parameters (strain, mode of culture and substrate). The best performances were again obtained with mantou meal (90-times more than that reported by Blanc *et al* (1994) in liquid cultures.

Table 5. Comparison of the production of red pigments.

Strains	Cultural conditions	Substrate	Production 400 nm	500 nm	Reference
M. anka	Submerged	Rice		5.25	cited by
M. kaoliang	Submerged	Rice	145	92	Lin and
M. sp.	Submerged	Glucose	-	17	Iizuka
M. anka	Submerged	Rice	119	156	(1982)
M. ruber	Submerged	Prickly pear juice	-	13	Hamdi *et al* (1995)
M. ruber	Submergedf ed batch	Ethanol + MSG	-	60	Santerre *et al* (1995)
M. anka	SSF	Polished rice	-	174	cited by Lin
M. anka	SSF	Bread meal	-	1690	and Iizuka (1982)
M. kaoliang	SSF	Mantou meal	5100	5430	Lin and Iizuka (1982)

CITRININ PRODUCTION

According to our work, the antibiotic already mentioned by Wong and Bau (1977), was identified as citrinin and it was produced both by *Monascus ruber* and *M. purpureus* (Table 6). With *Monascus purpureus*, the maximum production was obtained in submerged cultures (YES medium), with a citrinin concentration of 240 mg/l, or in the solid state culture, with a citrinin concentration of 100 mg/kg dried matter. The maximal production in the case of *M. ruber*, in pO_2-controlled fermenter with ethanol as a substrate, was 380 mg/l in the submerged fermentation or 300 mg/kg dried matter in the solid state culture.

Table 6. Production of citrinin.

Cultures	Citrinin production (mg/l)				
	Synthetic medium (glucose 13 g/l)	Synthetic medium (ethanol 9 g/l)	Synthetic medium (acetate 10 g/l)	YES medium (static)	Rice powder (static)
M. purpureus CBS 109.07	5	9	3	240	100
M. purpureus CBS 109.07 (mutant)	0	3	0	-	-
M. purpureus DSM 1379	-	-	-	20	-
M. ruber	18	59	19	370	300
M. ruber	0	0	0	-	-
M. pilosus	0	0	0	-	-

For submerged cultures, the production of red pigments is expressed as units of absorbance/ml. For SSF, the productions are expressed as units of absorbance/g of dried medium.

CONCLUSION

The present work contributes to the characterization of the chemical structure of monascidin A and the identification as well as quantification of one compound, among the pigments of *Monascus*. As *Monascus* is being used as a food additive, it might be necessary to focus the investigations on the detoxification of the pigments (in order to eliminate citrinin from the commercial colored preparations), the use of non-citrinin producing strains, or fermentative conditions of non-production, either in submerged cultures or SSF (effects of temperature, carbon/nitrogen ratio or mixing). These are the subjects of our attention now.

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Factors controlling the levels of penicillin in solid state fermentation

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SUMMARY

Antibiotic production in solid state fermentation (SSF) has revealed a greater potential, since higher yields are obtained in shorter times, as compared to submerged fermentation (SmF). The effect of several factors, that can be divided into environmental and genetic factors, has been studied on penicillin production. Results indicated the importance of using high initial moisture contents and concentrated media, to obtain a high penicillin production. Results show that these parameters do not control the idiophase directly. Production is controlled by the proportion of the support and the other two components in the solid medium (nutrients and water). Highest titres were obtained, when the solid medium had low bagasse content (10.3 to 12.5%). Results of the genetic control indicate that penicillin biosynthesis is also regulated in SSF by catabolic repression, ammonium concentration and feedback regulation. Similar to SmF, the strain turned out to be a key factor. It was found that special strains are needed for an efficient production in SSF. Methodology has been designed to generate high producing mutant strains, particularly efficient in solid culture, from high-yielding strains of SmF.

Keywords : Solid state fermentation, submerged cultivation, penicillin, environmental factors, genetic factors, yields, fermentation time, biosynthesis regulation.

RESUME

Facteurs contrôlant les niveaux de production de penicilline en fermentation en milieu solide.

BARRIOS-GONZALEZ J., DOMINGUEZ M., FLORES V., MEJIA A.

La production d'antibiotiques par fermentation solide (FMS) a suscité beaucoup d'intérêt depuis que des rendements élévés ont été obtenus plus rapidement que dans le cas de la fermentation submergée (SmF). Les effets de différents paramètres, environnementaux ou génétiques, sur la production de pénicilline par FMS ont été étudiés. Les résultats ont montré l'importance d'utiliser une humidité initiale élevée et des milieux nutritifs concentrés pour obtenir une production élevée en penicilline. Ces deux paramètres ne contrôlent pas directement l'idiophase. La production de penicilline en FMS est contrôlée par la composition des principaux constituants de la solution nutritive (nutriments et eau). Les taux de production de penicilline les plus élévés ont été obtenus quand le rapport support (bagasse) / milieu de culture était faible (10,3 à 12,5%) . Les résultats obtenus indiquent que la biosynthèse de la pénicilline en FMS est régulée par un système de répréssion catabolique, la concentration d'ammonium et par une régulation feedback. Comme dans le cas de culture submergée, les souches dépendent d'un facteur clé pour leur fonctionnement. On a trouvé que certaines souches étaient mieux adaptées et produisent plus de penicilline en FMS. Une méthodologie a été mise au point pour donner naissance, à partir de souches performantes en milieu liquide, à des souches mutantes particulièrement efficaces en culture solide.

Mots clés: Fermentation en milieu solide, fermentation submergée, penicilline, facteurs environnementaux, facteurs génétiques, rendements, temps de fermentation, régulation de biosynthèse.

INTRODUCTION

There has been great interest in solid state fermentations (SSF) in the last 15 years (Mudget, 1986), and, as a consequence, this culture method has been modernized and applied in many new fields. SSF could become an alternative production method for antibiotics and other secondary metabolites, since it has shown great potential (Barrios-González *et al*, 1988b, 1993a, 1993b; Kumar and Lonsane, 1987; Lin and Iizuka, 1982).

Our group developed a novel SSF system, involving an inert support (sugar cane bagasse) with absorbed liquid medium (Barrios-González *et al*, 1988a; Raimbault *et al*, 1989), which not only produces very high yields of enzymes and metabolites, but is also very convenient for basic studies. This SSF system was successfully applied to penicillin production by Barrios-González *et al* (1988b). Yields were several times higher and obtained in shorter times, as compared to those in submerged fermentation (SmF), under similar conditions.

Penicillin was used, as a convenient model of microbial secondary metabolite production and the effect of several variables on penicillin production in SSF was studied. These can be divided into environmental and genetic factors.

Among the environmental factors, initial moisture, as well as nutrients concentration (in the liquid phase) stand out because of their strong impact on penicillin yields. Oriol *et al,* (1988), working with a similar SSF system, studied the growth phase of *Aspergillus niger*, and showed that initial moisture content did not affect growth rate or germination, while these parameters were controlled by the medium concentration (water activity, A_W). On the other hand, Barrios-González *et al,* (1988b) showed that an initial moisture content greatly impacts idiophase (penicillin production), showing an optimum of 70% (w/v). Results, in the same work, indicated that the concentration of the liquid medium (absorbed on the support) had to be high (twice the concentration recommended for SmF), in order to obtain satisfactory yields in SSF.

However, solid medium is formed by the support (sugarcane bagasse), nutrients and water. The first two represent the solids, and have to be considered to calculate the medium's initial moisture, in such a way that an increase in one parameter (like moisture) would be compensated by decreasing one or both solid components

This means that a certain initial moisture or medium concentration value can be adjusted, varying the other two components in different ways, probably giving very different production results. These different ways are, in fact, different combinations of the three components.

The aim of this part of the work was to determine the effect of varying support/nutrients/water combinations on penicillin production in SSF.

The first section of this paper presents the recent results on the effect of environmental factors, while the second section reviews our research on genetic factors.

MATERIALS AND METHODS

Details of the strain used, fermenter, general methodology, control of parameters, extraction of the antibiotics and its assay method are, as reported elsewhere (Barrios-Gonzales *et al*, 1988b, 1993a; Garcia *et al*, 1992).

A triangle of all theoretical combinations of support/nutrients/water was created, and the zone where SSF could be carried out was determined experimentally (study zone = 10-24% bagasse; 58-80% moisture; 4-26% nutrients). The study zone was covered by points aligned in three axes, which were, in fact, three experimental series. In series one, a constant water/bagasse ratio (5 ml/g) was maintained in the solid media used. Media in series two had a constant nutrient/bagasse ratio of (1.1 g/g), while media in series three had a constant nutrient/water ratio of (0.228 g/ml), that is a constant concentration of the liquid phase.

The differences between these series of experiments can also be viewed, as different strategies to vary one parameter. In series one, increases in moisture content were accompanied by increases in support and decreases in the nutrients content. In series two, these increases in moisture were compensated by decreasing support and nutrient content, while, in series three, these variations in moisture were compensated by decreasing the support content.

RESULTS AND DISCUSSION

ENVIRONMENTAL FACTORS

Effects of support/nutrients/water ratios on penicillin production in SSF

Very different penicillin titres and production kinetics were obtained within each experimental series. Moreover, trends in the penicillin yields differed, when initial moisture content was increased, using different strategies (Fig. 1a). The same phenomenon was observed, when nutrients concentration was varied (Fig. 1b). However, penicillin production increased, when bagasse content decreased, independently of how the other two components were modified (Fig. 1c).

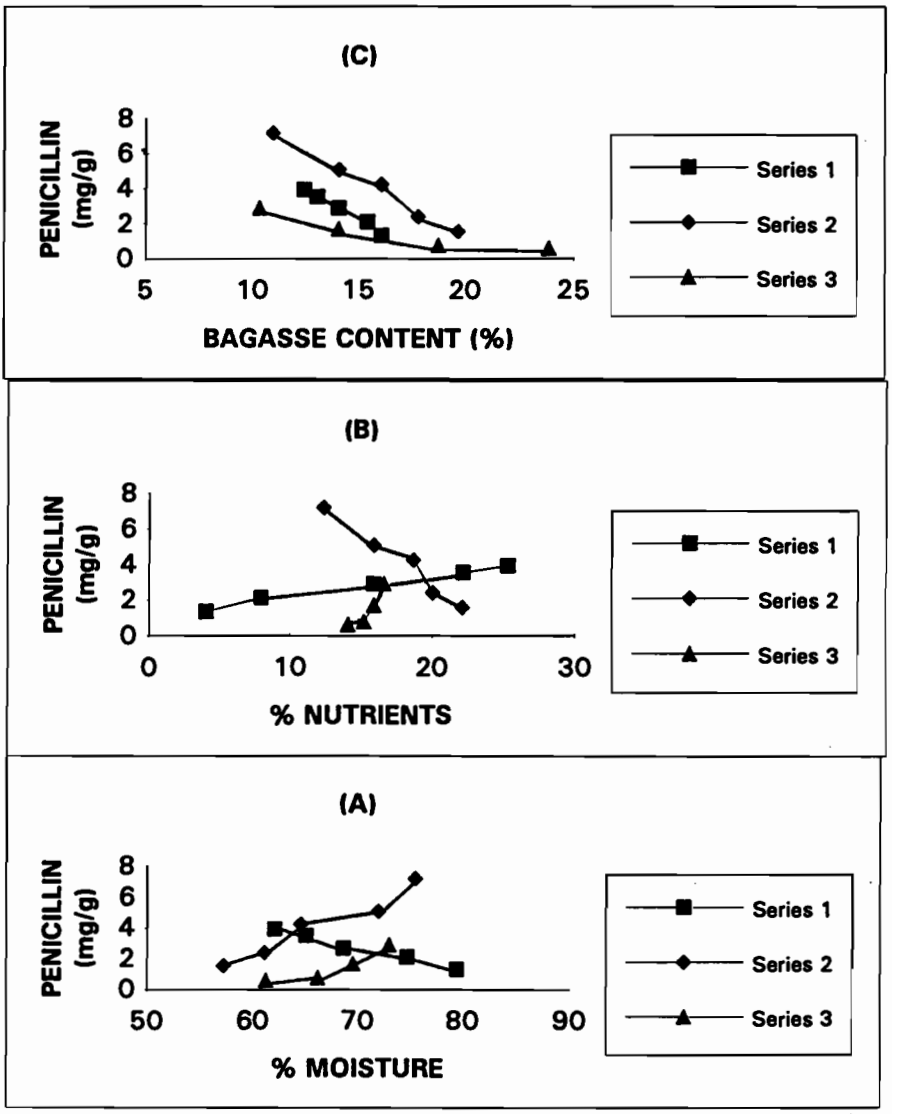

Fig. 1. Peak penicillin production by *P.chrysogenum* P2-4, obtained in solid media with different bagasse/nutrients/water combinations. Increases in moisture (A), nutrients (B) and bagasse content (C) were compensated, using different strategies (Series). Penicillin concentration is expressed as mg penicillin G/g dry solid medium.

If the conditions of maximal production are localized in the triangle of combinations (data not shown), the points fall in a narrow fringe of low support content (10 to 12.5%). This strip includes zones of low moisture and high nutrients content (62% water; 26% nutrients), as well as zones of high moisture (75.5%) and a middle level of nutrients concentration (12.4 to 16.6%). A similar view is seen in a 3-D graph shown in Fig. 2.

Respiratory kinetics suggested that a low baggase content in the solid medium slows down metabolic activity. In this study, it was not possible to determine a typical QCO2 (gravimetric production rate) value for high penicillin production. Nevertheless, the QCO2 (during idiophase) of cultures, under high penicillin production conditions, was always much lower, than in the control or in the low production conditions (between 15 and 96%).

It is possible that higher bagasse content facilitates nutrients and water transport in the solid medium (capillarity). This means that, under conditions of low support content, nutrient provision is slower, thereby, generating better metabolic conditions (higher values of μ during idiophase) for antibiotic production (Pirt and Righelato, 1967), and probably making it last longer.

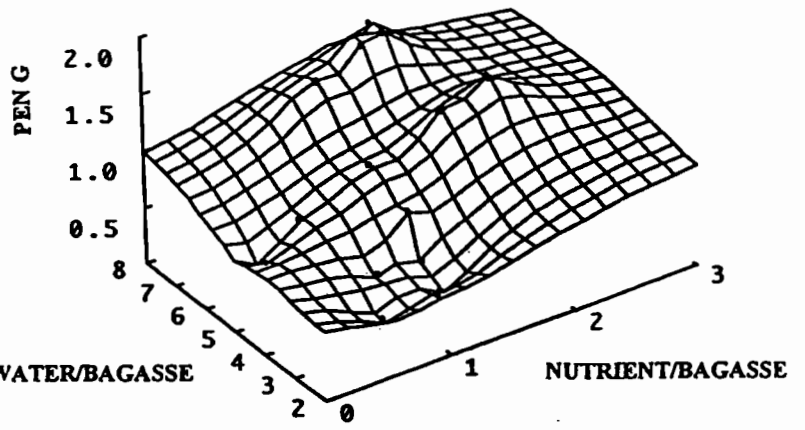

Fig. 2. Response surface of penicillin yields in solid cultures, with different water/support (W/S) and nutrient/support (N/S) ratios. Penicillin production was normalized by dividing it by production value, in the control experiment (under standard conditions).

GENETIC FACTORS

Regulatory Mechanisms of the Pathway

Classical experiments in the liquid medium have shown that penicillin biosynthesis is regulated by 1) carbon catabolite repression, 2) feed-back regulation and 3) ammonium regulation (Martín and Demain,1980). Initially, it seemed likely that penicillin synthesis in solid medium could be sub-regulated, due to the limitations in mass transfer in this system. However, research on this subject has shown that catabolite repression also regulates penicillin production in the solid medium. Moreover, the glucose thresholds estimated were similar for SmF and SSF i.e., 20 to 28 vs. 15 to 36 g/l, respectively (García *et al.* 1992).

A study on feed-back regulation of penicillin G biosynthesis by penicillin V in SSF indicated that this mechanism was also active in the solid medium (Flores *et al*, 1994). Results suggested that, as was found in the liquid medium (Martín and Demain, 1980), the regulatory threshold of a strain is directly related to its productivity. However, the thresholds estimated in SSF appeared to be higher, than that in the liquid medium.

Strains for SSF

Although penicillin titres, obtained in SSF, were several times higher, than those obtained in the liquid culture, the strains used in these comparative studies more resemble the wild type strains more than the overproducers used in the modern industry. It was interesting to know, if an industrial strain would have such an impressive performance in SSF, or if different strains have to be developed for solid medium.

Barrios-González *et al,* (1993a) studied penicillin production using five strains of *P. chrysogenum* of different production capacities, in solid and liquid media. To quantify the efficiency of a strain to produce penicillin in SSF, the relative production was defined as the peak production by the strain in SSF, divided by its peak production in SmF, e.g., PS/PL. Roughly, this parameter indicated how many times production in solid state fermentation was greater, than that in the liquid medium (if PS/PL > 1). Results showed that higher producing strains (in SmF) also tend to be higher producers in SSF. This fact agrees with the above mentioned idea, that the antibiotic synthesis is regulated in a similar way in solid and liquid media. In this way, mutations that were useful to overproduce penicillin in liquid medium, will also be useful to overproduce in SSF.

However, these strains tend to be less efficient to produce penicillin in SSF, than in SmF, meaning that high yield strains of SmF showed a relative production of 0.1 to 4, whereas low yield strains showed a relative production of up to 17. In this work,

the same experiments were carried out using at 40 clones (spontaneous mutants) from each strain. It was found that the clones showed the same tendency, meaning that clones generated from high producing strains usually gave higher yields in SSF. On the other hand, 80%, of the clones that displayed high PS/PL values, were derived from the low producing strain (Wisconsin 54-1255).

Table 1. Genetic improvement of the best clones obtained.

P. chrysogenum	Clone	Production (μg/g)	%Increase
P2	Parental	2,084	0
P2	# 4	10,555	506
P2	# 9	9,695	465
ASP-78	Parental	1,366	
ASP-78	# 20	8,750	640

It is noteworthy that some clones, generated from high producing strains, presented a relatively high PS/PL ratio. Clearly, these mutants combined a high production capacity with an efficient performance in SSF. In other words, the latter were high producing strains, particularly suited for SSF. These findings were the basis to isolate special strains for SSF. A very high increase in rapid penicillin production was obtained with the resulting system (Table 1).

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Application of on -line measurement of oxygen uptake and carbon dioxide evolution to penicillin production in solid state fermentation

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SUMMARY

On-line measurement of oxygen uptake and carbon dioxide evolution in penicillin production by *Penicillium chrysogenum* mutant strain P2-4 in solid state fermentation were studied, with respect to the proportions of the nutrients, support and water. The data indicated their importance in achieving higher production of penicillin. The on-line measurement system for the gases allowed the detection of subtle changes in the metabolism of the culture and the correlation of respiratory changes with the start and end of penicillin production. The results established that the environmental conditions, which permit high penicillin production, are those, which result in lower respiration rates.

Keywords: Solid state fermentation, *Penicillium chrysogenum*, penicillin G, respirometry, nutrients, support, water, respiration rates, biosynthesis.

RESUME

Application de la respirométrie pour suivre la production de penicilline en fermentation en milieu solide.

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Le suivi en ligne de l'évolution de l'oxygène et du dioxyde de carbone, au cours de la production de penicilline par le mutant P2-4 de *Penicillium chrysogenum* cultivé en fermentation en milieu solide a été étudié pour analyser l'effet des nutrients, du support inerte et de l'eau. Les résultats obtenus confirment l'importance de ces trois paramères pour la production élevée de penicilline. Grâce au suivi analytique des effluents gazeux de la FMS on a pu détecter de légers changements dans le métabolisme de *Penicillium chrysogenum* et correler des variations respirometriques au début et à la fin de la production de la pénicilline. Ainsi, il a été démontré que les conditions environnementales permettant de fortes productions de penicilline étaient celles qui induisaient des faibles taux respiratoires.

Mots clés : Fermentation en milieu solide, *Penicillium chrysogenum,* penicillin G, respirometrie, taux respiratoire, nutrients, support, eau, biosynthèse.

INTRODUCTION

Many studies have been carried out on antibiotics production by solid state fermentation (SSF) (Barrios-González *et al,* 1988; Ohno *et al,* 1992; Jermini and Demain, 1989). The use of inert support impregnated with liquid medium has been reported as an attractive alternative for penicillin production (Barrios-González *et al,* 1988, 1993a, 1993b). In this system, the micro-organism grows closery bound to the solid matrix, and fungal biomass cannot be separated from the solid medium, thereby making direct measurement of biomass practically impossible. These difficulties hamper basic studies on fungal physiology.

To overcome this problem, several methods of indirect biomass estimation for use in SSF have been reported. Narahara *et al,* (1982) measured chitin, the fungal wall constituent, Desgranges *et al,* (1991a) evaluated other cellular constituents, such as glucosamine and ergosterol, whereas Degranges *et al,* (1991b) measured the CO2

production using an infrared analyzer. Oriol *et al,* (1988) estimated the growth from oxygen uptake rate, using a paramagnetic analyzer.

In this work, the use of an automated system of on-line measurement of both oxygen uptake and carbon dioxide evolution, for the estimation of biological activity during primary and secondary metabolism of *Penicillium chrysogenum* in SSF, was reported.

MATERIALS AND METHODS

MICROORGANISM AND MEDIA

A mutant strain, P2-4, derived from *Penicillium chrysogenum* ATCC 48271, was used. *Bacillus subtilis* ATCC 6633 was used for bioassay of penicillin. Both the strains were kept lyophilized or as a spore suspension in 20% glycerol at -20^{o}C. Spore inoculum was obtained in Power medium which contained a 1:1 mixture of Czapeck (Luengo *et al,* 1980) and PM1 (López-Nieto *et al,* 1985). The production medium, which is used to impregnate the solid support media, was a modified Somerson *et al,* (1961) medium, which contained (g/liter) lactose, 110; glucose, 14; $CaCO_3$, 20; corn steep solids, 70; $MgSO_4.7H_2O$, 6; KH_2PO_4, 14; phenylacetic acid, 12.5; pH 6.5 (after sterilization in the solid medium).

SOLID STATE FERMENTATION

The cultures were left under non-aseptic conditions, as described previously (Barrios-González *et al,* 1988). Column fermentors with 12 g solid medium contained: sugar-cane bagasse pith impregnated with inoculated liquid medium ($2x10^6$ spores/ml). Bagasse was ground and sieved (10-30 mesh) before pretreatment. The fermenters were incubated at 25^{o}C, with an aeration rate of 0.2 l h^{-1} g^{-1} of solid medium.

ANALYTICAL PROCEDURES

Penicillin was extracted from SSF samples with 0.01 M phosphate buffer (pH 5.5). The extract or a convenient dilution (60 ml) was used to determine antimicrobial

activity against *B. subtilis.* Production was expressed in mg penicillin G/g dry fermented material.

RESPIROMETRY

Measurement of oxygen uptake and carbon dioxide production

The outlet air from column reactors was dried by passing through a silica gel column. The dry air was injected into the gas chromatograph (Gow-Mac Instrument Co.) The GC conditions included a concentric column (Alltech CTR1) at room temperature, injector and detector at 70°C. Hydrogen was used as carrier gas at 65 ml.min-1 flow rate. Detector's current was set to 120 v.

Sampling control system

It consists of one computer, which controls the sampling frequency. At sampling time, the column reactor is selected by means of a multiple valve (rheodyne). This valve is controlled by the computer through a pneumatic system, which is electrically activated (Fig. 1). The software also processes the chromatographic data, optimizes integration criteria (like sensitivity, base line, minimal area, etc.), determines the concentration of each component, generates reports and transfers data to other software, like *Excel.*

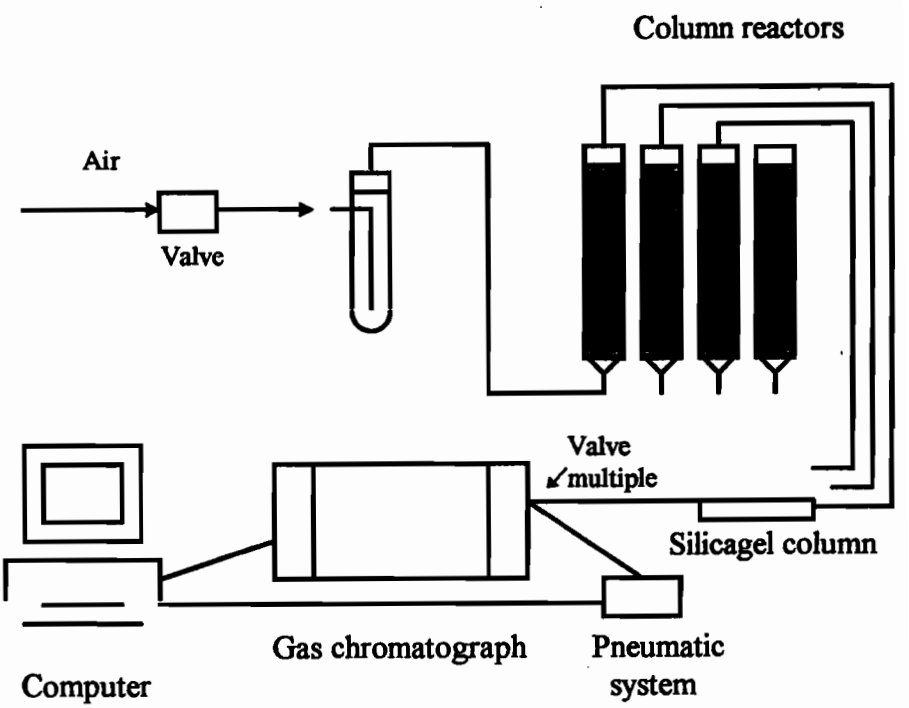

Fig. 1. Automatic on-line measurement system for oxygen uptake and carbon dioxide production.

RESULTS AND DISCUSSION

Studies on the effect of different proportions of nutrients, support and water on penicillin production and respiration of the culture showed their importance in the SSF.

Carbon dioxide production kinetics were obtained by integrating different production rates (derivatives) in the course of the fermentation.

Initially, the effect of bagasse content was studied, keeping the nutrient/water ratio constant (0.228 g/ml).

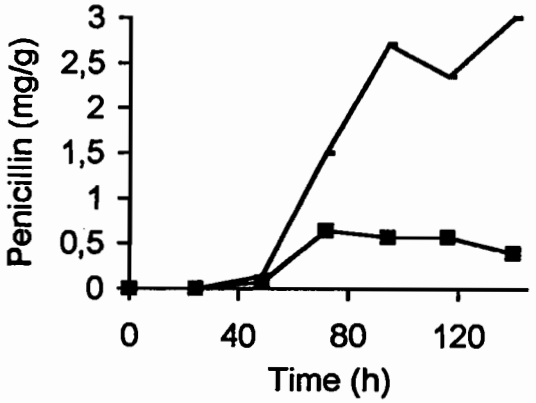

Fig. 2. Time course of penicillin production in SSF by *P. chrysogenum* with different support (pith bagasse) content: 23.9%(n) and 10.3%(-).

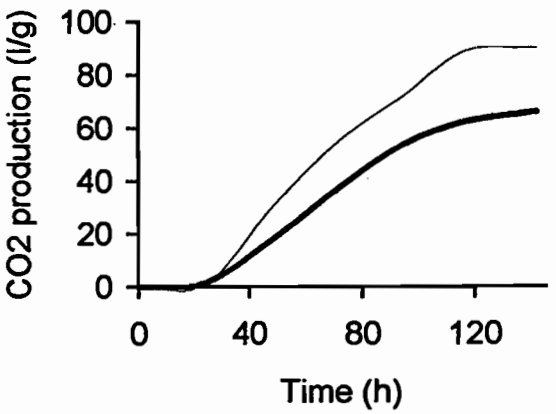

Fig. 3 : Time course of CO_2 production in SSF by *P. chrysogenum* with different support (pith bagasse) contents: 23.9% (-) and 10.3% (-).

Figs. 2 and 3 show that, when CO2 production rate is slow, penicillin production is high. The same effect was observed when initial moisture content was varied, keeping the bagasse/ nutrient ratio constant. (1.1 g/g) (Figs. 4 and 5).

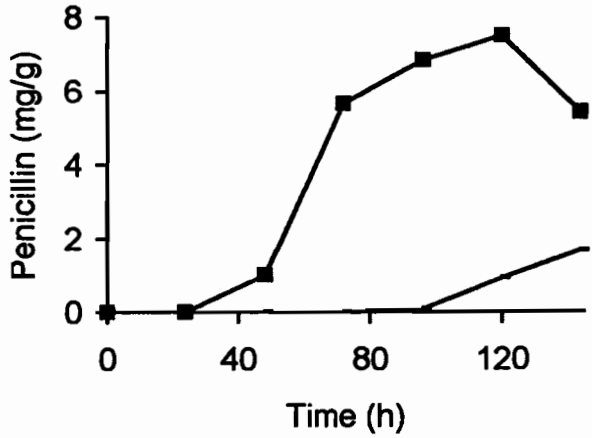

Fig. 4. Time course of penicillin production in SSF by *P. chrysogenum* with different moisture contents : 57.2% (-) and 75.5% (n).

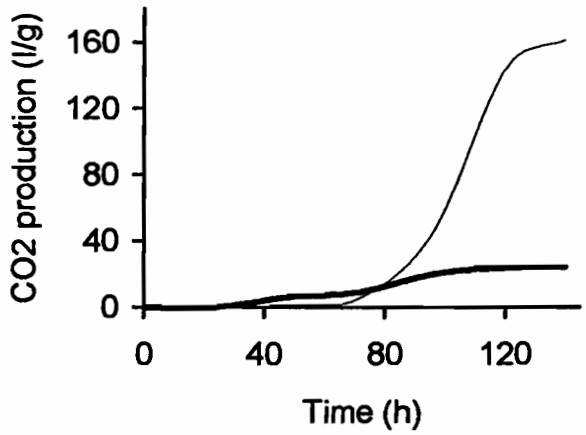

Fig. 5. Time course of CO2 production in SSF by *P. chrysogenum* with different moisture contents : 57.2% (-) and 75.5% (-).

Finally, nutrients concentration was varied, keeping the bagasse/water ratio constant (Figs. 6 and 7). It was observed that, at low nutrients concentrations, CO2 production and penicillin synthesis started earlier (at a moderate rate). On the other hand, with high nutrients concentration, CO2 production rate was relatively high between 60 and 90 h, correlating with a low penicillin production rate. After this period, CO2 production rate decreased drastically correlated with a high increase in penicillin production rate.

Oxygen uptake kinetics proved to be the inverse of the CO2 production kinetics.

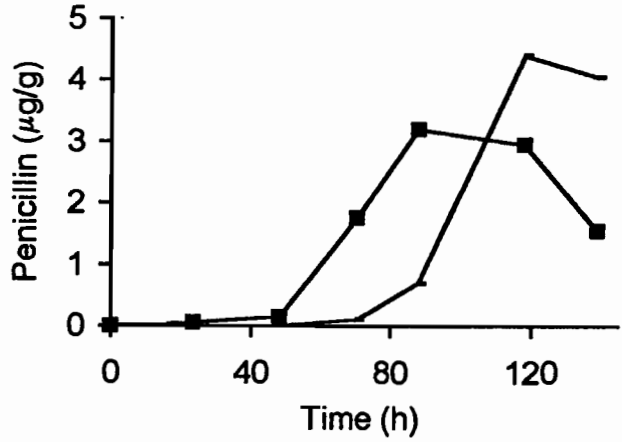

Fig. 6. Time course of Penicillin production in SSF by *P. chrysogenum* with different nutrient concentrations : 3.2 (-) and 2.0 (n).

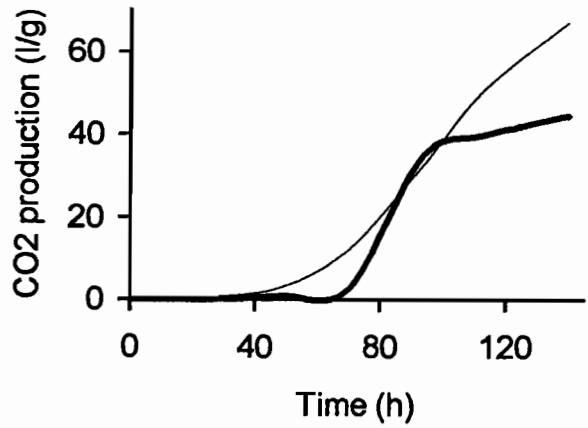

Fig. 7. Time course of CO2 production in SSF by *P. chrysogenum* with different nutrient concentrations : 3.2 (-) and 2.0 (-).

It can be concluded that respiration is a good indicator of the metabolic state of the micro-organism, and that satisfactory penicillin yields in SSF require conditions that support low respiration rates.

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Potential of solid state fermentation products for probiotic capacity as indicated by a newly developed reliable bioassay

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SUMMARY

A fermentation kinetic study was made to evaluate the effect of a commercial probiotic and a mould based on copra cake SSF products on the growth of *Selenomonas ruminantium*, lactate consumption and volatile fatty acid production. Lactate consumption by *S. ruminantium* was higher in *A. niger* SSF samples (4.12 g/L), than in the *P. italicum* (3.06 g/L) and *Penicillium* sp (2.60 g/L) SSF samples as well as commercial probiotic (1.02 g/L). Lactate consumption of *S. ruminantium* was enhanced (2.8 fold) by the fungal SSF production in comparison to that produced by commercial probiotics. Between the probiotics of moulds, the maximum differences observed in lactate consumption rate were less than 11%. Data indicate the potential of using extracts of fungal solid state cultivation as probiotics. A newly developed reliable bioassay involving the use of lactic acid consuming rumen *S. ruminantium* HD4 also proved reliable.

Keywords: Solid state fermentation, probiotic activities, *Penicillium* spp, *Aspergillus* spp, copra cake fermentation, bioassay for probiotic activity, lactose consuming rumen bacteria, *Selenomonas ruminantium*.

RESUME

Potentilités d'utilisation comme probiotique des produits fermentés en milieu solide confirmées par un nouveau bio-essai mis au point.

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Une étude a été réalisée afin de comparer l'effet d'un probiotique commercial à celui de différents lots de tourteau de coprah fermentés en milieu solide, sur la croissance de *Selenomonas ruminantium*, l'utilisation du lactate et la production d'acides gras volatils par cette bactérie du rumen. La consommation du lactate par *S.ruminantium* a été supérieure pour les échantillons de tourteau de coprah fermentés par *A.niger* (4.12 g/L); elle a été moindre pour les échantillons fermentés en FMS par *Penicillium italicum*, *Penicillium* sp (2.60 g/L) et le probiotique commercial (1.02 g/L). La consommation du lactate par *S. ruminantium* a été 2.8 fois supérieure pour les produits fongiques par rapport au probiotique commercial. Entre les divers produits fermentés utilisés, la différence observée pour la consommation du lactate a été de 11%. Les résultats obtenus confirment les potentialités d'utilisation comme probiotique des extraits fongiques obtenus par FMS. Un Bio-essai , basé sur l'augmentation de la consommation du lactate par *S. ruminantium* cultivée en présence d'extraits de produits fermentés a été mis au point .

Mots clés: Fermentation en Milieu Solide, Activités probiotiques, *Penicillium*, *Aspergillus*, Tourteau de coprah, Biomasse avec activité probiotique, Utilisation du lactate par les bactéries du rumen, *Selenomonas ruminantium.*

INTRODUCTION

RUMEN FUNDAMENTALS

Rumen could be considered as a complex fermenter, where synergism and antagonism among bacteria, fungi and protozoa take place (Hungate, 1966). Carbohydrate metabolism leads to the formation of organic acids (lactic, acetic, propionic, butyric). Around 70% of energy required for meat and milk production is obtained from these organic acids. The proportion of different micro-organisms in the rumen depends on type of feeding. When cattles are fed with a high carbohydrate diet, lactic acid bacteria are predominant. Consequently, a high lactic acid is produced, which decreases the pH of rumen and this phenomenon is recognized as

acidosis (Slyter, 1976). A pH below 6.0 affects the activity of cellulolytic micro-organisms and, therefore, the overall efficiency of the rumen.

Nevertheless, to overcome this problem, there are lactate consuming bacteria such as *Selenomonas* and *Megasphaera* in the rumen, which allow maintenance of a dynamic equilibrium of rumen activity. Nutritional needs of *Selenomonas* species are complex. They require n-valerate and PABA, when growing on glucose, and biotin has a stimulatory effect. On the other hand, when growing on lactate, biotin is essential for growth, while n-valerate has a stimulatory effect on growth (Bryant, 1956; Kanegasaki and Takahashi, 1967).

PROBIOTICS

It is felt by different authors that rumen activity can be enhanced by some products or compounds or a mixture of them, which are called as probiotic (Williams, 1991; Males and Johnson, 1990). The role of probiotics in human nutrition is related to the built-up of intestinal microflora. In the case of animal nutrition, they are used as additives in order to increase animal yields. In this work, the term probiotic will be used exclusively for ruminant nutrition purposes.

The probiotics should be non-toxic, non-pathogenic, efficient in small doses (1-3%), with the ability to stimulate overall activity of rumen bacteria, and the micro-organisms producing probiotics should be easily cultured and conserved (Males and Johnson, 1990; Hose and Sozzi, 1991). The mechanism of action of probiotics is not well elucidated and more research work is needed in this direction.

IMPORTANCE OF BIO-ASSAY FOR PROBIOTICS

Evaluation of probiotics *in vivo* is an expensive and laborious test. Consequently, several authors have proposed the use of some rumen bacteria to test the stimulatory capacity of probiotics (*in vitro* assays), especially regarding bacterial growth and lactate consumption (Nisbet and Martin, 1990, 1991; Russell and Baldwin, 1979; Tapia *et al*, 1988).

The aim of this work was to test the potential of different solid state fermention products for their stimulatory effect on growth and lactate utilization by *Selenomonas ruminantium* HD4. In this direction, solid state fermentation (SSF) products present an interesting alternative to conventionally produced probiotics (Pandey, 1992). Newly developed simple and reliable bioassay method for probiotics is also presented.

MATERIALS AND METHODS

MICROORGANISM

A strain of *Selenomonas ruminantium* HD4, provided by Dr. Martin, was used throughout this study. The strain was kept in glucose medium (as described below) at -76°C, before activation for different trials.

CULTURE MEDIUM AND CONDITIONS

A basal medium, modified from literature data (Prins, 1971; Tinari *et al,* 1968; Hobson *et al,* 1963) was used. It contains in mg/L: Yeast extract 500, Peptone of casein 500, K_2HPO_4 292, KH_2PO_4 292, $(NH_4)_2SO_4$ 480, resarzurin 1, PABA 1, NaCl 480, $MgSO_47H_2O$ 100, $CaCl_2$ 50, cystein 600, Na_2CO_3 4000, biotin 0.1. Volatile fatty acids (VFA) were added to the culture medium as follows (mM):acetic acid 30, propionic acid 8.1, butyric acid 3.4, isovaleric acid 1, valeric acid (as required).

Glucose and lactate were used as carbon sources. Sugar was used at 2 g/L level, while maintaining valeric acid at 1 ppm. Lactate was used at 13 g/L level, keeping valeric acid concentration constant at 0.2 ppm, and adding sodium acetate at 30 mM in this case. Cultures were grown at 39°C at pH 6.5-7.0, under a reduced CO_2 atmosphere, after autoclaving at 120°C for 15 min. Inoculation was carried out at 10% (v/v). All trials were carried out in duplicate.

ANALYTICAL ASPECTS

Bacterial growth was determined by optical density at 600 nm. Glucose, VFA's, and lactic acids were measured by HPLC, as described elsewhere (Giraud *et al,* 1991).

PROBIOTIC

A commercial yeast probiotic (submerged culture, SmF) and 4 solid state fermentation (SSF) products were tested. All SSF products were obtained from copra cake fermentations. Strains used were *Aspergillus carbonarius* No.57, *Aspergillus niger* No. 2.10, *Penicillium sp* No. 1.4 and *Penicillium italicum* No. 2.25. The last three samples were tested after o and 31 hour fermentation, while the commercial product was based on *Saccharomyces cerevisiae.*

SSF fermentation products were lyophilized after fermentation. Samples (4 g) quantity were mixed thoroughly with 50 ml distilled water, filtered through Whatman No.1 filter paper, then sterilized by passing through a 0.45 µm Millipore membrane. Probiotic extracts were added to the culture media at 2% (v/v).

RESULTS AND DISCUSSION

KINETIC STUDY

Kinetics were carried out in order to test the effect of addition of an *Aspergillus carbonarius* SSF product and a commercial probiotic upon lactate utilization and growth evolution, during cultures of *S. ruminantium* HD4. Figs. 1 and 2 show the results obtained for biomass formation and lactate utilization, respectively. Higher values of biomass were obtained in the case of addition of a SSF extract, compared with the commercial product or the control (without additive) culture (Fig. 1). Maximum values were attained after 12 hour cultivation for SSF extract. In the case of the commercial product, maximum value was reached at 50 hour . Addition of an *Aspergillus carbonarius* SSF extract presented a stimulatory effect on biomass level and growth rate.

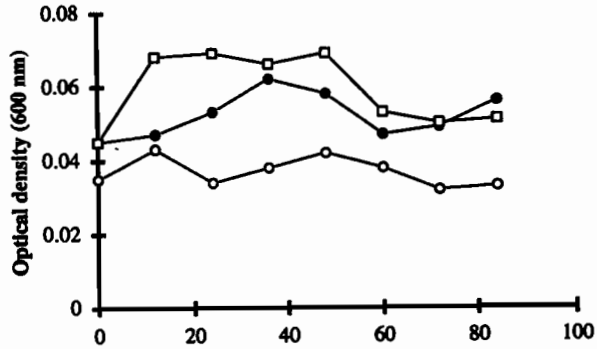

Fig. 1.- Effect of addition of a commercial probiotic (Yea-Sacc), a SSF extract on evolution of *S. ruminantium* HD4 biomass.
❑:*A.. carbonarious* SSF extract, ●: Yea-Sacc and, ○: Control (without additive).

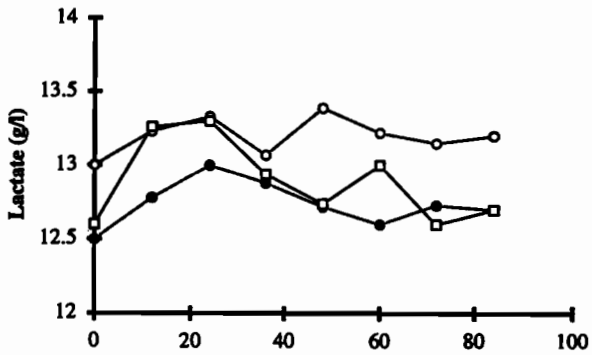

Fig. 2.- Effect of addition of a commercial probiotic (Yea-Sacc), a SSF extract on lactate evolution during cultivation of *S. ruminantium* HD4 biomass.
❑:*A. carbonarious* SSF extract, ●: Yea-Sacc and, ○: Control (without additive).

For all three treatments, maximum values of lactate concentrations were reached after 24 hour cultivation, followed by a progressively slow decrease (Fig. 2). The same pattern was observed when glucose (2 g/L) was used as a carbon source (data not shown). Higher lactate consumption was obtained using a SSF extract (89 µM/h) compared with the commercial product extract (44 µM/h) and the control (17 µM/h). These results may be considered of low magnitude. Nevertheless, the reported affinity of *Selenomonas* against lactate is extremely low. In the case of *M. eldenii,* the value of Ks was of o.37 mM (Russell and Baldwin, 1979). The rise in lactate concentration during first 24 hour cultivation (Fig. 2) can be attributed to the presence of readily utilizable nutrients, such as peptone and yeast extract.

BIOASSAY

The last trial showed that the increase in lactate concentration was followed by a culture phase, where lactate was consumed by *S. ruminantium* HD4. According to this observation, a bioassay was carried out to test the stimulatory effect of 3 SSF products (before and after 31 hour solid state cultivation) upon lactate and biomass concentrations during cultivation of *S. ruminantium* HD4. Analyses were carried out after 0, 24 and 80 hour cultivation of rumen bacteria. Fig. 3 shows the results of biomass concentration. Higher levels of biomass were found in the case of SSF extracts. Nevertheless, two unfermented inoculated SSF samples (*Penicillium* sp and *P. italicum*) presented a higher stimulatory effect, than in the case of using the fermented SSF product (Fig. 3). It can be explained based on the presence of readily utilizable nutrients in copra cake.

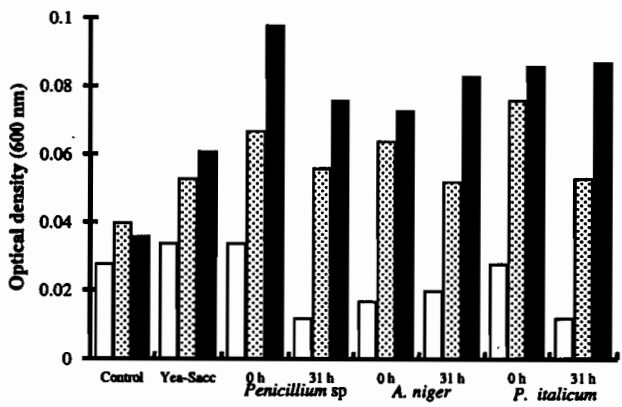

Fig. 3.- Effect of addition of a commercial probiotic (Yea-Sacc) and different SSF extracts on biomass of *S. ruminantium* HD4. Analysis were realized after 0 (□), 24 (▨) and 80 h (■)cultivation of rumen bacteria. Numbers for each fungus strain correspond to the fermentation time in SSF.

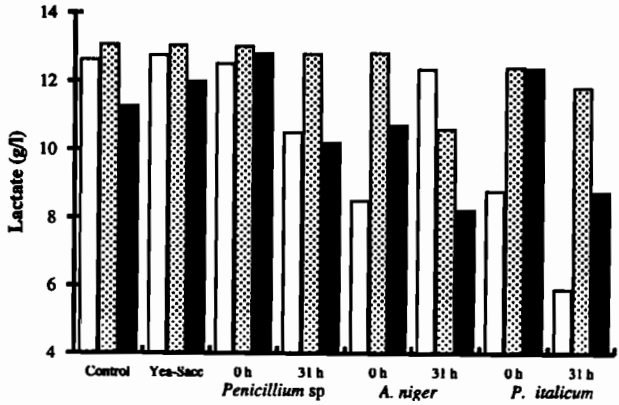

Fig. 4.- Effect of addition of a commercial probiotic (Yea-Sacc), and different SSF extracts on lactate concentration during cultivation of *S. ruminantium* HD4. Analysis were realized after 0 (❑), 24 (❑) and 80 h (■) cultivation of rumen bacteria. Numbers for each fungus strain correspond to the fermentation time in SSF.

Lactate increased from 0 to 24 hour (Fig. 4), then its consumption was observed. These results are in agreement with those reported earlier in this work. In the case of unfermented samples of *Penicillium* sp *and P. italicum*, no consumption of lactate was observed from 24 to 80 hour cultivation of the rumen bacteria. In the case of unfermented samples *of A. niger*, a consumption of 2.11 g/L of lactate was observed during the same period of time. It is probably due to the enzymatic activity of *Aspergillus* spores. After 24 hour cultivation of *S. ruminantium* HD4, a consumption of lactate was observed in fermented samples (31 hour, SSF) of *Penicillium* sp and *P. italicum*. *A. niger* extracts presented a continuous utilization of lactate from 0 to 80 hour cultivation. Lactate consumption was higher (Table 1) for *A. niger* SSF samples (4.12 g/L), followed by *P. italicum* (3.06 g/L), *Penicillium* sp (2.60 g/L) and commercial probiotic (1.02 g/L). Lactate consumption rates were higher (2.8 fold) in samples derived from fungal SSF compared with those from commercial probiotics. In the case of moulds, maximum differences of lactate consumption rate were less than 11% (Table 1).

Table 1. Effect of the addition of mould SSF extracts on consumption of lactate during cultivation of *S. ruminantium* HD4.

	Lactate consumption	
Strains	Total, (g/L)	Rate (mg/L h)
S. cerevisiae	1.02	18.2
Aspergillus niger	4.12	51.5
Penicillium sp	2.60	46.4
Penicillium italicum	3.06	54.6

In this work, evidence has been presented for the potential of using mold extracts from solid state cultivation, as probiotics in ruminant nutrition, especially taking into account the lactic acid consuming activity. It must be emphazised that the function of probiotics is to give essential elements other than carbon and nitrogen.

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Factors affecting physiology of mycelial growth and mushrooms aroma production in solid state fermentation

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SUMMARY

Effects of different nitrogen sources and C/N ratios on mycelial growth and intensity of volatile aroma production, by three species of *Morchella* and one *Pleurotus* species, were studied in agar and solid support media. The results formed a base for selection of sodium nitrate as a nitrogen source, C/N ratio of 10 and *M.esculenta* as potential fungi for production of morel mushroom aroma by mycelial cells in solid state fermentation. Gas chromatographic analysis revealed wide differences in aroma compounds, produced by *M. esculenta* and *P. cornucopiae*. As the aroma compounds produced by mycelial cells of *M. esculenta* are identical to those formed by the fruiting bodies, the present results open up a never, simpler and economic strategy for production of highly priced morel mushroom aroma.

Keywords : Aroma, solid state fermentation, support solid medium, *Morchella esculenta, Pleurotus cornucopiae*, mycelial cells, nitrogen sources, C/N ratio, apical growth, aroma profile.

RESUME

Effets de la source d'azote sur la physiologie de croissance mycélienne et la production d'arôme de champignons cultivés sur support solide.

KABBAJ W., BENSOUSSAN M. ET ROUSSOS S.

L'effet de différentes sources d'azote et du rapport C/N sur la croissance mycélienne et sur l'intensité aromatique dégagée par trois espèces du genre *Morchella* et d'une espèce du genre *Pleurotus* a été étudié sur agar puis sur support solide. Les résultats ont été à la base du choix du nitrate de sodium comme source d'azote, du rapport C/N de 10 ainsi que du mycélium de *M. esculenta* comme source potentielle pour la production de l'arôme de morille. L'identification des molécules contribuant à l'arôme réalisée par CPG-SM a révélé de larges différences entre *M. esculenta* et *P. cornucopiae*. Dans la mesure où l'arôme produit par le mycélium rappelle celui du carpophore, ce résultat constitue une méthode simple, rapide et économique pour la production de l'arôme morille .

Mots clés : Arôme, fermentation en milieu solide, *Morchella esculenta, Pleurotus cornucopiae*, mycélium, sources d'azote, croissance apicale, rapport C/N, profil aromatique.

INTRODUCTION

Morchella species, a group of highly priced edible mushrooms, are well known for their ability to produce volatile metabolites of pleasant aroma (Litchfield, 1967). The produce is consumed as a delicacy. Moreover, *in vitro* cultivation of *Morchella* is difficult and, hence, recovery of aroma compound from morel mushrooms on industrial scale is not yet carried out.

Most edible fungi form aroma compounds during fruiting body development (Gross and Asther, 1989). However, morel mushrooms have a rare ability of forming similar aroma compounds both at mycelial and fruiting body stages (Gilbert, 1960). The biotechnological advances in easier cultivation of the mycelium biomass of edible fungi by solid state fermentation can, therefore, be exploited to produce these aroma compounds, without the necessity of waiting upto the fruiting body development stage. Such aroma will be of commercial value, especially due to paucity of good natural aroma for use in foods and food-products.

A number of reports on the mycelial cell production of *Morchella* in liquid culture are available (Fron, 1905; Brock, 1951; Willam et al., 1956; Gilbert, 1960; Litchefield *et al*, 1963, Le Duy *et al*.., 1974; Buscot, 1993; Bensoussan *et al*, 1995). However, only one report is available on mycelium production of *Morchella* by solid state fermentation (Launay, 1989).

Solid state fermentation (SSF) is well known to offer many advantages over the conventional submerged fermentation, especially in the case of fungi (Hesseltine, 1972; Mudgett, 1986). The conditions of solid state fermentation are very similar to those involved in the fungal growth that occurs in nature (Roussos, 1985). Moreover, the porosity of the substrate facilitates easier aeration and oxygen transfer (Raimbault and Alazard, 1980). The technique is simpler and the liquid effluents formed are minimal (Lambert and Meers, 1983). In particular, the oxidation of lipids and fats is stimulated in solid state fermentation, due to the limited use of water in the media (Loncin, 1976). Consequently, it is possible to obtain 1-octen,3-ol, which represent 80% of the aroma fraction in fungi, through the enzymic oxidation of linoleic acid in solid state fermentation (Wurzenberger and Grosch, 1982, 1984 a,b).

The present study focuses on the aroma production and apical growth of three species of *Morchella* (*M. esculenta, M. crassipes and M. hortensis*) and one species of *Pleurotus* (*P. cornucopiae*) in solid state fermentation in response to different nitrogen sources and C/N ratios. The volatile aroma compounds formed by *M. esculenta* have also been characterized and compared to those formed by *P. cornucopiae.*

It is emphasized that *M. esculenta* was never grown earlier on sugarcane pith bagasse in solid support fermentation, nor its aroma compounds were characterized.

MATERIALS AND METHODS

FUNGAL CULTURE

Three species of the genus *Morchella* were used, along with one species of *Pleurotus* for comparative purposes. *M. esculenta* 91.9 (isolated from Danish forests), *M. hortensis* MH 88.7 (isolated from Provence region), *M. crassipes* MCR 92.24 (No. 28963, CBS, Baarns) and *P. cornucopiae* AVPL Corrèze (isolated from Provence region) were obtained from the collection of station d'Agronomie et de Mycologie, INRA, Clermont Ferrand, France. These cultures were maintained on Potato Dextrose Agar (DIFCO, Detroit USA) slants at 4°C and subcultured every 3 months.

CULTURING IN PETRI DISHES

Media with five different nitrogen sources (asparagine, glycine, tryptophane, sodium nitrate and ammonium sulphate) were used to select the best nitrogen source, based on the elongation of apical growth of mycelial cells. All these media had a constant C/N ratio of 10 and contained nitrogen at a level of 2 g/L. Consequently, the concentration of glucose used in the media ranged from 6.03 to 10.50 g/L. The other common components of the media included (g/L) KH_2PO_4 1, $MgSO_4$ 0.5, agar 15 and distilled water 1000. The pH of the media was adjusted to 6.9 before autoclaving at 121°C for 20 min. Inoculum used was 1 cm^2 piece of mycelial growth from PDA medium grown at 20°C for 7 days. The plates were incubated (static) at 20°C for 80 h and the elongation of the apical growth of the cells was measured.

CULTURING IN BOTTLES

Round bottles of 250 ml capacity, provided with autoclavable caps, were used and each bottle was charged with 40 g sugarcane pith bagasse (as inert support), after impregnating with nutrients. It occupied about one third of the total volume of the bottle. The nutrient medium (C/N ratio of 10 and 20), absorbed on a pith bagasse, contained (g/L) glucose 30, sodium nitrate 4 or 8 (as per desired C/N ratio), KH_2PO_4 4, Na_2HPO_4 1.6, $MgSO_4$ 4, $ZnSO_4$ 0.04, $MnSO_4$ 0.04, and distilled water 1000.

Inoculation was done, using 3 pieces of 1 cm^2 size of the mycelial growth from PDA medium, by placing these at an equal distance in the medium, along the walls of the bottle and at 1.5 cm height from the bottom of the flask. All these operations were carried out under aseptic conditions. Other details are as described above for culturing in Petri dishes. Samples were taken for determining apical growth of the mycelium at different time intervals. The sniffing for aroma notes was done at 216 h.

PARAMETERS MEASUREMENTS

The apical growth of the fungi was estimated in terms of the elongation of mycelial cells was expressed in mm. The changes in the pH of medium were determined, as with the method used by Raimbault and Alazard, 1980. Qualitative measurements of the aroma produced by the culture were taken by sniffing and were classified into four categories, i.e., absent -, just perceptible +, strong ++, and very strong +++.

EXTRACTION OF AROMA

The fermented support solid medium (10 g) was mixed with 40 ml distilled water and subjected to ultrasonic treatment at ambient temperature (20° C) for 20 min, with a view to break the cell wall. The resulting mass was subjected to extraction-distillation of the aroma compounds in a Likens-Nickerson apparatus, modified by Godefroot et al (1981). Samples were simultaneously distilled with hexane over a period of 60 min.

IDENTIFICATION OF AROMA COMPOUNDS

The volatile compounds present in the distillates from solid state culture of *M. esculenta* and *P. cornucopiae* were individually subjected to gas chromatography (Fison-Trio 1000), equipped with a flame ionization detector. The column used was DB5 of 30 m length. The oven temperature was raised from 50 to 300°C at a rate of 2°C/min. The injector temperature was 250°C in a splitless mode. The retention time was compared with the reference compounds for their identification.

RESULTS AND DISCUSSION

NITROGEN SOURCES VS GROWTH

Data on the effect of three organic and two inorganic nitrogen sources, with respect to the elongation of mycelial cells of three species of *Morchella* and one *Pleurotus* species, in the medium with C/N ratio of 10, showed different responses to the sources studied, at 80 hours (Table 1). However, the maximum increase in the apical growth of all the cultures was observed in sodium nitrate medium, though the efficiency of asparagine was equal to that of sodium nitrate in the case of *M. hortensis*. Ammonium sulphate promoted negligible or very slight increase in the elongation of the mycelial cells of all the cultures, except for a definitive increase in the case of *P. cornucopiae*. The negative response to ammonium sulphate by *Morchella* species might be due to formation of H_2SO_4 and the consequent reduction in the pH of the medium. This reason is supported by the fact that the optimum pH for the growth of *Morchella* is between 6.93 to 8.30 (Brock, 1951).

Table 1. Effect of different nitrogen sources on the apical elongation of the mycelial cells (mm) at 80 h in Petri dishes culturing on agar medium.

Nitrogen sources	Apical elongation of mycelial cells (mm)			
	M. esculenta	*M. crassipes*	*M. hortensis*	*P. cornucopiae*
Asparagine	22	20	40	5
Glycine	17	21	22	11
Tryptophane	12	14	23	0
Sodium-nitrate	25	22	40	13
Ammonium-sulphate	+/-	+/-	+/-	11

The results indicate that the utilization of different amino acids, as nitrogen sources, by all *Morchella* species, was slower than that of sodium nitrate, except for a more or less similar results in the case of *M.crassipes* growing on asparagine and glycine. Among the amino acids, the efficiency of tryptophane was lowest in the case of all the cultures, except for its equal efficiency with glycine in the case of *M. hortensis*. However, the growth of *P.cornucopiae* was absent in medium containing tryptophane.

Based on the above results, sodium nitrate was selected as the best nitrogen sources for all the cultures. Its use was also advantageous, as it was much cheaper than the amino acids and did not drastically lower the pH of the medium, as happed with ammonium sulfate.

EFFECT OF C/N RATIO ON GROWTH AND AROMA PRODUCTION

The grading of the aroma intensity, as determined by sniffing, is presented in Table 2, with respect to two different C/N ratios and the cultivation of the fungi on support media in the culture bottles. *M.hortensis* was not capable of producing aroma, even at a C/N ratio of 20, while the aroma production was equal at both ratios in the case of *M. crassipes* and *P. cornucopiae*. In the case of *M. esculenta* , the aroma production was lower at a C/N ratio of 20, as compared to that of 10.

Table 2. Effect of different C/N ratios on the intensity of aroma production at 216 h in bottle culture under solid state fermentation involving use of sugarcane pith bagasse as inert solid for absorbing nutrient.

C/N ratio	Intensity of aroma production			
	M. esculenta	*M. crassipes*	*M. hortensis*	*P. cornucopiae*
10	+++	+++	-	++
20	++	+++	-	++

- : Absent, + : perceptible, ++ : strong, +++ : Very strong.

In terms of growth of the mycelial cells, the results gave excellent insight on the effect of C/N ratios (Fig. 1). In the case of all cultures, the use of C/N ratio of 20 resulted in a longer lag phase and lower growth. In the case of *M.esculenta* the difference was dramatic at a C/N ratio of 20, since the lag phase was very long and the growth was less than 50%, even at 125 hours as compared with that at 75 hour, having a C/N ratio of 10. The colonization of the support by *M. esculenta* was rapid and complete at C/N ratio of 10.

Such rapid growth is of particular importance, when the cultivation is carried out at a larger scale, due to the possibility of development of contaminant microorganisms. No contamination was observed in lab scale column fermentors, due to the care taken during the entire fermentation process. No such contamination problem will be encountered in bottle cultures, because of easier maintenance of aseptic conditions, but this will be difficult in the case of larger column fermenters.

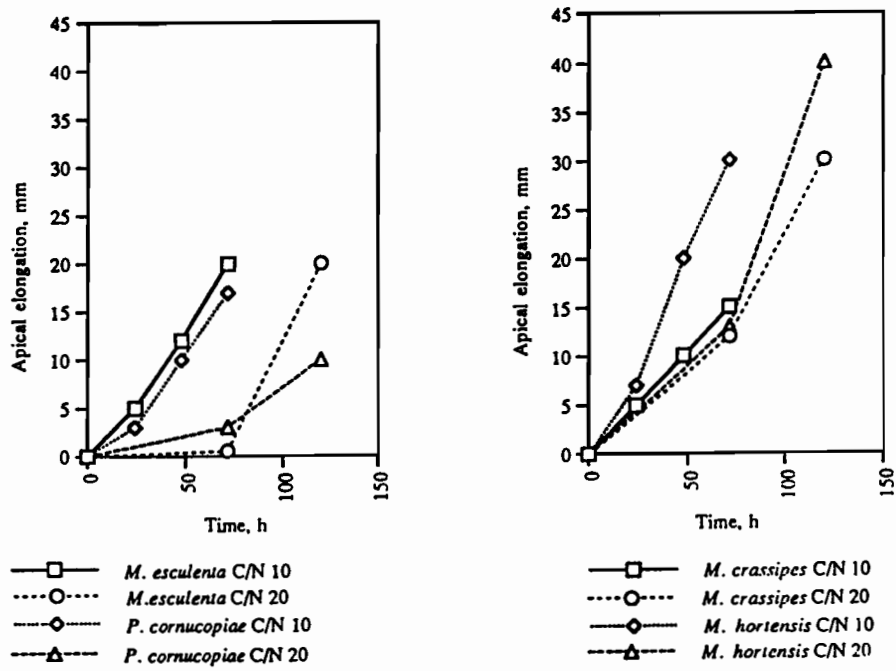

Fig. 1 : Elongation (mm) of apical mycelial cells of the cultures in media with two different C/N ratios under solid state culture on support in the cultures bottles.

It is of interest to point out that the mushroom aroma is the secondary metabolite (Grosch, 1987). Its production, however is not affected by the C/N ratio, except , for a slight reduction at a higher ratio in the case of *M. esculenta.*

Based on the above results, *M. esculenta* was selected for further studies, using a C/N ratio of 10 and sodium nitrate as the nitrogen source.

IDENTIFICATION OF AROMA COMPOUNDS

The aroma was produced under optimized conditions using *M. esculenta*. *P. cornucopiae* was also studied for comparative purposes. The chromatograms obtained by CG-MS are presented in Fig. 2.

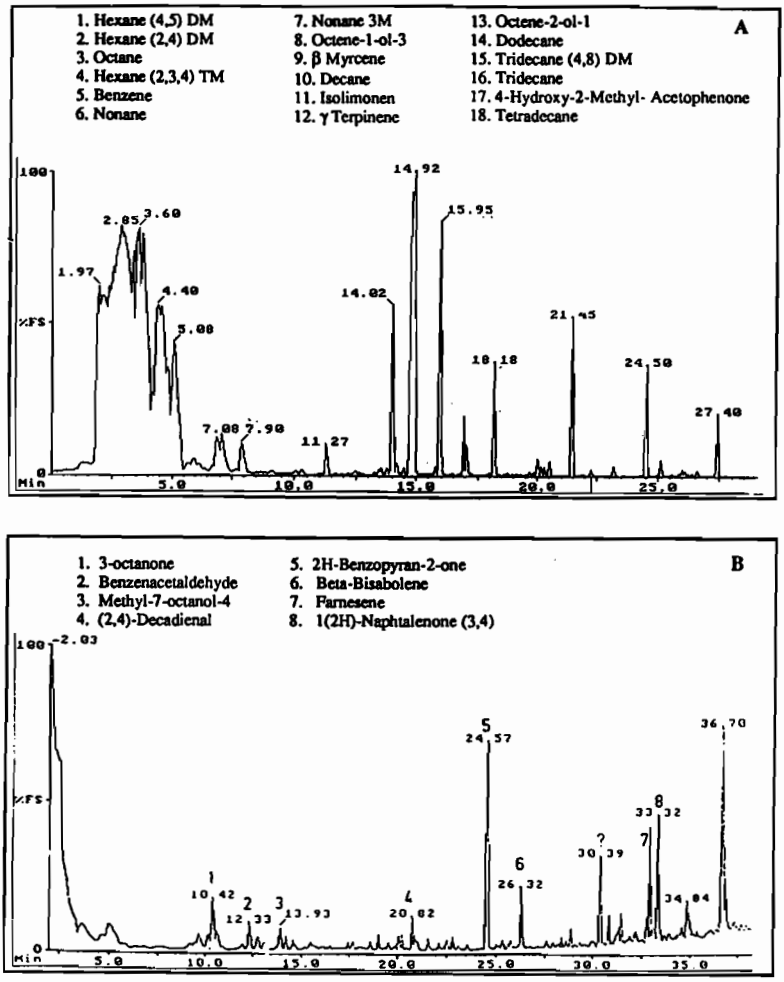

Fig. 2 : Chromatograms of aroma compounds produced by *M. esculenta* and *P. cornucopiae* at mycelial cell stage of the growth. A : *M. esculenta*, B: *P. cornucopiae*. The peaks are numbered and their identification indicated.

In the case of *M. esculenta*, a total of 18 fractions have beeen identified, with only 6 of them being aromatic in nature and with possible contribution to the aroma of *M. esculenta*. For example, 1-octen,3-ol confers the typical mushroom like aroma and this compound has been identified in the extract of several species of edible fungi, such as *Cantharellus cibarius, Boletus edulis, Lactarius trivialis, Lactarius torminosus, Lactarius rufus, Gyromitra esculenta , Agaricus bisporus* (Pyysalo and Suihko, 1976; Grove, 1980).

ß-myrcene and isolimonen, the compounds which impart pleasant and lemon-like odours, respectively, have also been identified in the extract of *M. conica* at the fruiting stage (Audoin et al, 1989). In fact, Isolimonen is the main aroma compound produced by *M. esculenta*. 2-octen,1-ol another compound produced by *M. esculenta,* is common for many different mushrooms and has a medicinal oily odour.

4-H -2- methyl acetophenone, the compound produced by *M. esculenta*, has a odour like that of orange-blossom. It is also produced by *M. esculenta* at the mycelial stage in liquid culture (Bensoussan *et al.*, 1995).

A total of eight compounds have been identified. Four of them are aromatic in nature (Gallois *et al*, 1990). None of these compounds are identical to those identified in the extract of *M. esculenta*.

The 2-H- benzopyrane-2-one, the major aroma compound in the extract of *P. cornucopiae*, smells like flower at a lower concentration, but smells like rubber at a higher concentration. The other three aroma are present in minor quantities and include 3-octanone (flower or lavender like odor), benzenacetaldehyde (almond like odor) and 2,4 Decadienal (fresh fruity like odor).

CONCLUSION

Data show the potential for growing *Morchella esculenta* mycelial cells on a support in solid state fermentation for aroma production. The source of nitrogen has a significant effect on the production of aroma by mycelial cells. Nitrate seems to be the most appropriate for the mycelial growth of *M. esculenta* and *P. cornucopiae*. In the case of the use of ammonium salts as a nitrogen source, the drop in the pH of the medium affects the growth of the culture.

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Citric acid production on three cellulosic supports in solid state fermentation

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SUMMARY

Six strains of *Aspergillus niger* were screened in liquid medium and strain LPB 21 was selected for further studies on three different agro-industrial wastes/residues for production of citric acid by solid state fermentation. Cassava bagasse was found to be a better substrate than vegetable sponge and sugar cane bagasse. Citric acid production and yields were 13.64 g/100 g dried substrate and 41.78 %, respectively with the use of cassava bagasse. Studies on optimization of parameters for production of citric acid by *A. niger* LPB 21 in cassava bagasse medium indicated that 50% of initial moisture content, initial pH of 2.0, aeration rate of 60 ml/min/column and 26°C fermentation temperature are the optimum values. Under these optimized conditions, the production of citric acid was 280 g/kg dry substrate at 120 hours, which corresponds to the yield of 70% based on sugars consumed. Data on kinetics of pH changes, moisture level, loss of weight of the substrate, starch utilization and alpha-amylase production give insights in the process.

Keywords : citric acid, solid state fermentation, *Aspergillus niger,* cassava, bagasse, vegetable sponge, moisture, aeration, temperature.

RESUME

Production d'acide citrique par fermentation en milieu solide sur trois supports cellulosiques.

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Parmi les six souches *d'Aspergillus niger* qui ont été criblées en milieu liquide, l'une d'entre elles, la souche LPB 21 a été retenue pour les études de valorisation de trois sous-produits agro-industriels en vue de produire de l'acide citrique par fermentation en milieu solide. La bagasse de manioc s'avère le meilleur substrat par rapport à la bagasse de canne à sucre et à l'éponge végétale. La production d'acide citrique a été de 13,64 g / 100 g de substrat sec. Le rendement s'élève à 41,78%. Les différents facteurs intervenant dans cette production ont fait l'objet d'une étude d'optimisation. Les meilleures conditions retenues ont été les suivantes : une humidité initiale de 50%, un pH initial de 2.0, une vitesse d'aération de 60 ml/min/colonne et une température d'incubation de 26°C. Dans ces conditions, la production d'acide citrique a été de 280 g/kg de substrat sec après 120 h de fermentation, ce qui correspond à un rendement de 70% (sur la base des sucres consommés). Les résultats rapportés dans cette communication précisent l'évolution du pH; de l'humidité, de la perte de poids en substrat, de l'utilisation de l'amidon et de la production de l'alpha-amylase au cours de la fermentation en milieu solide.

Mots clés : Acide citrique, fermentation en milieu solide, *Aspergillus niger*, bagasse de manioc, éponge végétale, humidité, pH initial, aération, température de la fermentation, cinétique fermentaire.

INTRODUCTION

Citric acid was first isolated from lemon juice and crystallized as calcium citrate as early as in 1784 (Milson and Meers, 1985). Wehmer (1893) was the first to observe the presence of citric acid as a product of *Penicillium glaucum* growing on sugar. A great number of problems had to be overcome, before an effective fermentation process could be used commercialy (Lockwood and Schweiger, 1967).

Citric acid is of industrial interest, mainly used in the food industry (Rohr *et al*, 1983). Another important area of application is the pharmaceutical and the cosmetics industries. Increasing amounts of citric acid are being used in industry, e. g., as raw material for the manufacture of derivatives in the plastics industry. Actually, more than 300,000 tons of citric acid were produced per year, with a growing up demand of about 20 % per year. Most of the world's supply of citric acid is produced by carbohydrate fermentation with selected strains of *Aspergillus niger*. Citric acid is produced either by submerged or liquid surface fermentation processes, employing sugar beet molasses as substrate (Milson and Meers, 1985). About 80 - 85 % of the weight of the sugar used in the medium can be recovered as citric acid, the fermentation being usually conducted over a period of 5- to 14-day at 25 to 33°C (Peller and Perlman, 1979). However, solid state fermentation constitutes an other alternative with good yield, with the use of specific strains and the nature of the substrates employed (Flores, 1994; Shankaranand and Lonsane, 1993, 1994; Hang *et al.*, 1987; Hang and Woodames, 1984, 1986).

The objectives of the present work were to characterize the conditions of citric acid production by *Aspergillus niger* in solid state fermentation on different supports, such as vegetable sponge (Luffa cylindrica Roem), sugar cane and cassava (*Manihot esculenta* Crantz) processed residue. Therefore, several high citric acid producing strains of *Aspergillus niger* were studied in SSF under different parameters.

MATERIAL AND METHODS

MICROORGANISMS

Six citric acid producing strains of *Aspergillus niger* (LPB 21, LPB 28, LPB 32, LPB 34, LPB 70 and LPB 99) were used. All fungal strains were cultivated on agar plates of Czapek agar (50.5 g / L) and malt extract (40.0 g / L). After growth at 28°C for 7 days, the spores were harvested with platine loop under aseptic conditions and transferred into 20 mL test tubes, containing 10 mL of sterile 0.1% Tween 80. The spore suspension was mixed thoroughly and the number of spores per mL was determined using a hemacytometer. The spore suspension was stored at 4°C.

STRAINS SELECTION

Strain selection was carried out as described by Prescott and Dunn (1959). Inoculum was prepared in a 500 mL Erlenmeyer shake flask containing 150 mL medium, containing (g/L) sucrose, 140.0 ; NH_4NO_3, 2.231 ; KH_2PO_4, 1.0 ; $MgSO_4.7H_2O$, 0.23. The pH of the medium was adjusted to 1.6 - 2.2 with 1 N HCl solution. After sterilization at 121°C for 15 min and cooling to about 30°C, it was inoculated to contain 10^7 spores / mL. The flasks were shaken reciprocally at 100 rpm at 28°C for 10 days.

PREPARATION OF SUPPORTS

Solid materials were washed, dried in an oven at 60°C and sieved to obtain 8 - 20 mm fraction. Unless otherwise specified, they are moistened to 70% with a mineral solution containing (g / L) NH_4NO_3 , 4.46 ; KH_2PO_4 , 0.20 ; Mg $SO_4.7H_2O$, 0.46 ; $ZnSO_4.7H_2O$, 0.375 ; $CuSO_4.7H_2O$, 0.002. The pH of the medium was adjusted to 3.0 with 1.0 N HCl. When vegetable sponge and sugar cane bagasse were used, the medium was supplemented with sucrose (70 g/L). For cassava bagasse, residual solids from starch extraction process were used as carbon source.

SOLID-STATE FERMENTATION

Solid state fermentation was carried out in column type bioreactors (Raimbault and Alazard, 1980). The humidified substrate was inoculated with a suspension of 10^7 spores / mL placed in columns of 0.60 g/mL capacity and compacted (Soccol, 1994). The columns were placed in a temperature regulated water bath at 22°C and aerated at a rate of 100 mL/min. Studies were conducted using the same methodology as described above on the effect of initial moisture content, initial pH of the medium, aeration rate, temperature of fermentation and kinetics of citric acid production.

ANALYSES

Citric acid in the extract was determined by the colorimetric method of Marier and Boulet (1958) as modified by Hartford (1962). The analysis for starch was done by iodine-iodure method (Thivend *et al*, 1965) while the reducing sugar formed were estimated by the method of Bernfeld (1955). The total sugars were estimated by the method of Dubois *et al* (1956) after hydrolysis with HCl. Residual sugars were estimated by DNS method (Miller, 1959). Water content was measured, as described elsewhere (Soccol, 1992).

RESULTS AND DISCUSSION

SELECTION OF *ASPERGILLUS NIGER* STRAINS

After 10 days of submerged fermentation, all six strains of *Aspergillus niger* showed the ability for citric acid production. Strain LPB 21 produced the highest quantity of citric acid (Fig. 1) and hence was selected for futher studies.

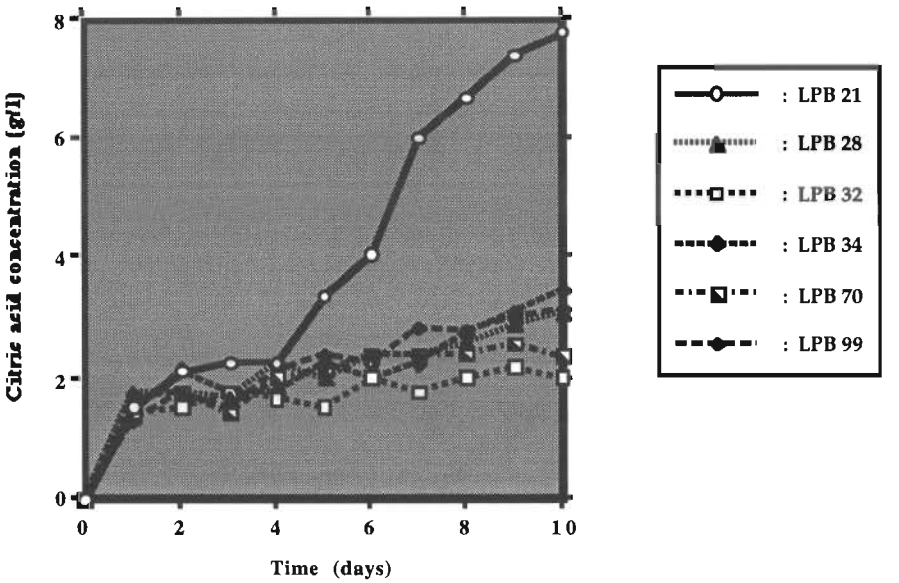


Fig. 1 - Comparison of citric acid production by several strains of *Aspergillus niger* in submerged fermentation.

SUPPORT CHARACTERISTICS

The three cellulosic materials were found to have good characteristics for use as support in solid state fermentation (Table 1). Cassava bagasse was characterized by a high content of total sugars. Consequently, when used, this support was not supplemented in sucrose.

Table 1. Physical and chemical characteristics of cellulosic materials used as support during solid state fermentation.

Support	Moisture (%)	Ashes (%)	Water absorption (g H_2O /g support)	Total sugars (g/100 g sup.)	Starch (g/100 g sup.)
Vegetable sponge	11.54	0.33	3.25	0.75	-
Sugarcane bagasse	7.72	1.24	5.75	0.25	-
Cassava bagasse	14.04	1.72	4.07	46.50	41.55

SOLID-STATE FERMENTATION IN DIFFERENT CELLULOSIC SUPPORTS

Table 2 shows that cassava bagasse, in comparison to sugar cane bagasse and vegetable sponge, is the best support to produce citric acid by solid state fermentation. The citric acid production and yield were 13.64 g/100 g dry cassava bagasse and 41.78 %, respectively.

Table 2 - Solid-state fermentation in different cellulosic support.

Support	Produced citric acid (g / 100 g dried support)	Consumed sugar (g / 100 g dried support)	Yield (%)
Vegetal sponge	4.85	59.09	8.21
Sugar cane bagasse	5.64	60.16	9.34
Cassava bagasse	13.64	32.66	41.78

Initial Moisture : 70 %, Yield : Citric acid produced / Sugar consumed ; Initial sugar : cassava bagasse, 46,5 g / 100 g dried support ; sugarcane bagasse, 70.25 g / 100 g dried support ; vegetal sponge, 70.75 g / 100 g dried support.

EFFECT OF INITIAL MOISTURE CONTENT OF CASSAVA BAGASSE

The water content of solid support has been reported as an important limiting factor for solid state fermentation (Lonsane *et al*, 1992 ; Pandey, 1992). Consequently, the effect of moisture present in cassava bagasse was found to be in the range of 45-65 % initial moisture. Data showed that the production of citric acid at 8 days increased with an increase in initial moisture content of the medium up to 50% (Table 3).

Table 3. Effect of initial moisture content of cassava bagasse on citric acid production by *Aspergillus niger* LPB 21.

Moisture (%)	Citric acid produced (g / 100 g dried support)	Sugar consumed (g / 100 g dried support)	Yield (%)
45	16.35	36.92	44.28
50	27.25	40.10	67.96
55	25.32	36.83	68.75
60	22.15	39.63	55.89
65	19.39	37.19	52.14

Initial sugar : 46.5 g / 100 g dried support, Yield : g citric acid produced / g sugar consumed.

A further increase in the initial moisture content of cassava bagasse by 10% had an adverse effect on the synthesis of citric acid. Thus, the initial moisture content of 50% could be considered as ideal to produce a high quantity of citric acid (272 g/kg cassava bagasse). In this case, fermentation yield was approximately 70%.

EFFECT OF INITIAL PH

The medium pH for production of citric acid must be between 1.6 to 3.5 (Kolicheski, 1995). Table 4 shows the results for the production of citric acid by SSF using different pH values. Very low values of pH (1.0) reduces the production of citric acid. Higher values favour the production of citric acid and the maximum production was at pH 2.0, where the concentration of citric acid was 246 g/kg dried cassava bagasse with a yield of 71.45 %. These results confirm those obtained by different authors (Meers *et al*, 1991; Muller, 1981).

Table 4. Effect of initial pH of the medium on citric acid production by *A. niger* LPB 21 on cassava bagasse.

pH	Citric acid production (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
1	18.12	28.32	63.98
2	24.64	34.54	71.45
3	21.93	34.19	64.14

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced / sugar consumed

AERATION EFFECT

Aeration is an important factor in the production of citric acid in column type bioreactors because the air helps to dissipate metabolic heat and also provide the essential oxygen for the growth of the microorganism. The results of the aeration effect are shown in Table 5. The optimal aeration flow is between 50 and 60 ml/min and the aeration rate above or below these values leads to a fall in citric acid production.

Table 5. Effect of aeration rate on citric acid production by *A. niger* LPB 21 in cassava bagasse.

(ml/min/column)	Citric acid produced (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
40	13.60	38.36	35.45
50	25.42	36.13	70.36
60	27.12	37.54	72.24
70	19.45	37.51	51.85
80	17.32	32.61	53.11

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced/Sugar consumed

TEMPERATURE EFFECT

Optimum temperature for fermentation varies according the microorganism used, and is usually between 25 and 35°C (Prescott and Dunn, 1959). Highest production of citric acid occurred at 26°C (Table 6).

Table 6. Effect of temperature on citric acid production by *A. niger* LPB 21 in cassava bagasse.

Temperatures	Citric acid production (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
24	15.64	37.81	41.36
26	28.45	37.66	75.54
28	23.91	36.88	64.83
30	20.80	35.97	57.83
32	19.73	37.67	51.96

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced/Consumed sugar

KINETICS OF CITRIC ACID PRODUCTION

Fig. 2A shows the evolution of consumption of starch and citric acid production during fermentation. The production of citric acid starts in the initial period of fermentation although its concentration reaches 74 g/kg dried cassava bagasse at 48 h. Between 48 and 72 h fermentation, citric acid productivity reaches its highest point (201.34 g/kg dried cassava bagasse). Between 72 and 120 h, the final concentration in citric acid reaches a value of 280 g/kg dried cassava bagasse, which correponds to a yield of 70 % in relation with the starch consumed. These results are higher as compared to those of other authors working with different substrates (Hang *et al*, 1987; Omar *et al*, 1992; Shakaranand and Lonsane, 1993).

Alpha-amylase production is higher during the first 72 h fermentation (192 IU/g dried cassava bagasse) (Fig. 2B). Significant alpha-amylase production in the first 72 h is associated with higher consumption of starch in the same period (Fig. 2A).

pH is gradually reduced as citric acid is accumulated in the medium and reaches its lowest value (pH 0.5) after 96 h fermentation (Fig. 2B).

Fig. 2C shows the increase in moisture content of the medium and loss of weight of support during fermentation. An increase of moisture from 60.32 to 64.18% is due to fungal metabolism (Soccol, 1992 and 1994). The loss of weight is higher in the first 24 hours and it reaches about 7.93 %. After 120 hours of fermentation, the total loss of weight is 10.57%. This loss of weight from the support is due to starch tilization.

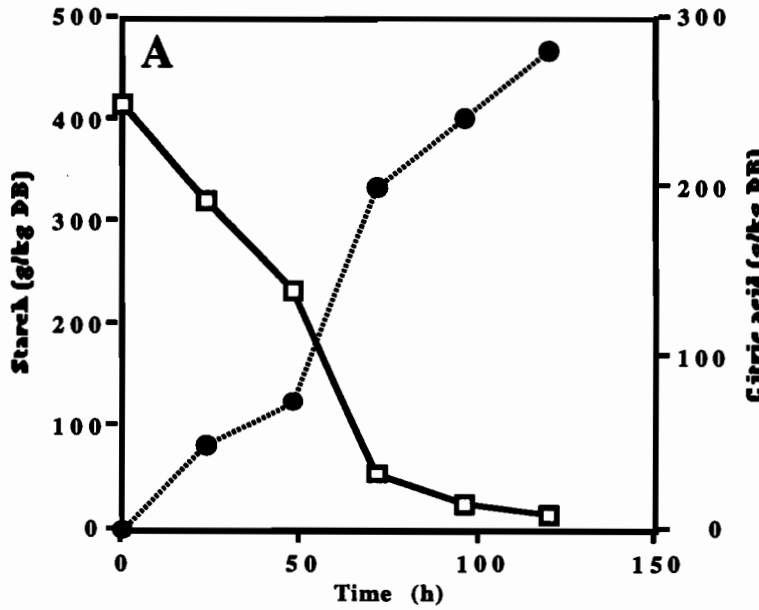
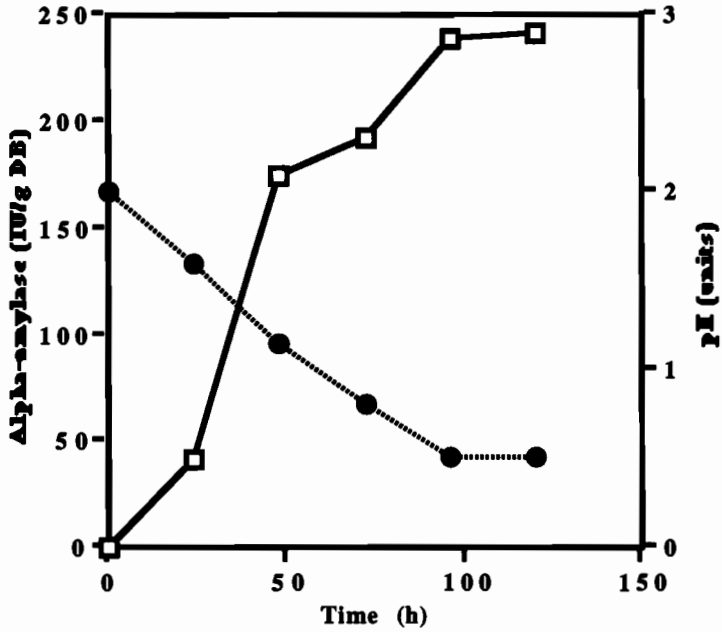
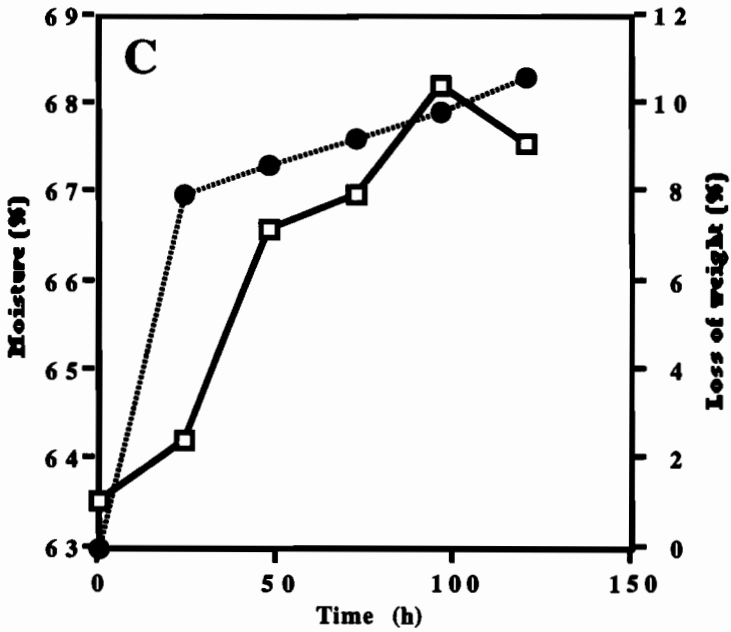


Fig. 2A. Kinetics of citric acid production by *A. niger* LPB 21 in SSF.





Figs. 2B-C. Kinetics of citric acid production by *A. niger* LPB 21 in SSF.

A : Starch utilization. B : Changes in pH and alpha-amylase levels. C : Changes in moisture and loss of weight during fermentation.

CONCLUSIONS

Aspergillus niger LPB 21 showed good potential for citric acid production in solid state fermentation based on the use of cassava bagasse as a substrate. The optimization allowed to improve the production and also the yield based on sugars consumed.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Brazilian Agencies CNPq and CAPES.

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Comparative properties of *Trichoderma harzianum* spore produced under solid state and submerged culture conditions

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SUMMARY

Aerial spores of *Trichoderma harzianum* P1, a potential biocontrol agent, were produced in solid substrate cultivation (SSC)and their suitability for use in biological control programmes was compared, against those produced in liquid media. The former provided higher production yields, greater UV-resistance and had a longer shelf-life. These spores were produced in clusters, had a thick outer wall, and few organelles in solid substrate cultivation.

Spores produced in submerged liquid culture, however, were mostly collapsed, exhibited many cytoplasmatic organelles and had a much thinner outer wall. In addition, they were hydrophilic, against a highly hydrophobic nature of aerial spores formed in solid substrate cultivation. During analysis, hydrophobicity was related to the presence of one major low molecular weight protein, but this hydrophobic-like protein was nearly absent in extracts from the walls of spores, produced by submerged fermentation. An involvement of the outer cell wall layer in the resting state of *T. harzianum* spores is proposed.

Keywords : *Trichoderma harzianum*, aerial spores, submerged spores, biocontrol agent, solid substrate cultivation, submerged cultivation, spore characteristics.

RESUME

Propriétés comparées des spores de *Trichoderma harzianum* produite par cultures en milieu solide et en milieu submergé pour les programmes de lutte biologique

AGOSIN E., COTORAS M., MUÑOZ G., SAN MARTIN R. ET VOLPE D.

Nous avons comparé les efficacités pour le contrôle biologique, des spores de *Trichoderma harzianum* P1, produites soit par culture sur milieu solide (FMS) (par la partie aérienne du mycélium), soit par fermentation liquide (spores "submergées"). La FMS donne des taux de production supérieurs; les spores obtenues sont plus résistantes aux UV et montrent une viabilité supérieure. Elles sont produites en grappes, elles possèdent une paroie épaisse et un nombre réduit d'organites.

Nous avons observé que la paroie des spores "submergées" est plus fine et fortement afaissée et qu'elles contiennent beaucoup d'organites. Contrairement à celles produites par FMS, qui sont hydrophobes, ces spores sont hydrophiles. Les analyses ont montré que la propriété hydrophile est associée à la présence d'une proteïne de faible poids moléculaire, quasiment abscente de la paroie cellulaire des spores de fermentation submergée. Nous supposons que la paroie joue un rôle déterminant sur la dormance des spores.

Mots clés: ***Trichoderma harzianum***, spores aériennes, spores submergées, contrôle biologique, culture en milieu solide, culture submergée, caractéristiques des spores.

INTRODUCTION

Trichoderma harzianum has an ability for efficient control of several phytopathogenic fungi. Three types of propagules are formed by *Trichoderma*, i. e., hyphae, chlamydospores and conidiospores (Papavizas, 1985). Among these, the last has been the most widely employed in biocontrol programmes (*Elad et al*, 1993). These propagules can be obtained either by submerged (Elad and Krisshner, 1993) or solid substrate (Lewis and Papavizas, 1983) cultivation techniques.

Mass production of *Trichoderma* spp spores for biocontrol purposes is mainly confined to submerged cultivation technology (Jin *et al*, 1991). Reasonably high

spore production yields have been obtained, but, the resulting spores exhibited low germination capabilities, particularly after spore drying.

Trichoderma spores, with enhanced desiccation tolerance, were produced in liquid culture media at low water activities (Jin *et al* 1991). Manipulation of this culture parameter is easily achieved in solid substrate cultivation (SSC) technique. Hence, the latter has been reported to be more promising cultivation system to produce *Trichoderma harzianum* conidiospores for biological control purposes. Furthermore, higher spore production yields have been reported with SSC technology (Papavizas, 1985). However, the role of the culture system on other key properties of the spores, which must be met by a successful biocontrol agent, such as field efficacy or sustainability of the resulting propagules, have not yet been investigated. Indeed, biocontrol agents must withstand environmental variations in temperature, desiccation, radiation and relative humidity, to survive and establish active populations, both in the soil and phylloplane (Elad and Kirsshner, 1993).

It has been suggested that outerwall components are necessary in establishing the biocontrol agent for successful biocontrolling. The mucilage of *Colletrotricum musae* spores has been shown to have a role both in adherence to the substrate and detoxification of the interface (Mondal and Parbery, 1992). Hydrophobins, the recently discovered small hydrophobic proteins, have been isolated from cell surfaces of several aerial fungal structures (Wosten *et al*, 1993). These have shown their capabilities in pathogenicity (Stringer and Timberlake, 1993) and importance in adherence (St Leger *et al*, 1992).

The present communication reports the quantitative and qualitative differences between conidiospores of *T. harzianum*, obtained under solid substrate and submerged culture conditions. The consequences of these differences regarding the successful control of phytopathogens, are also discussed.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

Trichoderma harzianum P1 (ATCC 74058) was used throughout this study. Aerial spores were obtained from YMI plates. spores in submerged cultivation were produced in a defined liquid culture medium, with a carbon : nitrogen ratio of 14 at pH 7.0 (Volpe *et al*, 1995).

Mass production of spores by solid substrate cultivation was carried out in columns (Raimbault and Alazard, 1980), containing 3 g vermiculite or 6 g wheat bran-based culture medium. Columns were incubated at 28°C and continuously flushed with humidified air, at a flow rate of 100 ml/min.

SPORE RESISTANCE ASSAYS

Shelf-life of the spores was evaluated, by comparing their viability at harvest and after storage (Volpe *et al,* 1995). During storage, the spore samples were placed in a humidity chamber, previously equilibriated at 25°C and 75% constant relative humidity (RH). After 45 days storage, the spore pellets were resuspended in sterile water and the viability was evaluated, by comparing the total number of spores and the corresponding colony forming units (cfu).

UV-resistance assays were carried out, by preparing a suspension of both types of the spores in deionized water and adjusting the final concentration to $1x10^7$ spores/mL. Five mL of each suspension was placed in a sterile Petri dish, irradiated for different exposure times, using a UV lamp (Mineralight, Biorad), placed 21 cm above the plate, and the cfu were then determined.

ELECTRON MICROSCOPY

TEM analyses of ultra-thin sectioned spores samples were carried out, as previously described (Volpe *et al,* 1995). Samples used for scanning electron microscopy were prepared, by the method of Porter *et al* (1972) and examined with a Jeol JSM-25-SII scanning electron microscope.

EXTRACTION AND ANALYSES OF THE SPORE PROTEINS

To obtain cell wall proteins, 200 mg freeze-dried spores (both types) were suspended in 30 ml water, using a glass-teflon homogenizer. Dried supernatants were resuspended in 100% trifluoroacetic acid (TFA) and the TFA-soluble fraction was evaporated to dryness in a nitrogen stream (Worsten *et al,* 1993). The final material was resuspended directly in SDS-sample buffer and subjected to a 12.5% SDS-PAGE analysis (Laemmli, 1970).

RESULTS AND DISCUSSION

DIFFERENCES IN PRODUCTION PARAMETERS OF *T. HARZIANUM* CONIDIOSPORES OBTAINED BY SOLID SUBSTRATE OR SUBMERGED CULTIVATION

Conidiospore production yields in solid substrate cultivation were much higher - over 100 times - than those obtained by submerged cultivation (Table 1). Even though cultivation times were longer in SSC, it still allowed significantly higher mass productivities, than those in submerged cultures.

Table 1: Influence of the culture system on conidiospore production parameters for *Trichoderma harzianum*.

	Culture system	
Parameter	**Solid**	**Liquid**
Production (log spores/ml)	10.0	8.0
Incubation time (h)	76.0	48.0
Productivity (log spores/ml/h)	8.1	6.3

MORPHOLOGICAL DIFFERENCES BETWEEN AERIAL AND SUBMERGED SPORES

Aerial spores from SCC were covered with extracellular matter and clustered (Fig. 1A). The presence of dense cytoplasm, with few organelles, mainly electrodense bodies, suggests a reduced metabolic activity (Fig. 1C).

Spores produced by submerged fermentation were mostly collapsed, non-aggregated and showed no extracellular matter (Fig. 1B). They contained vacuoles and a high number of mitochondria (Fig. 1D). These characteristics are normally found in metabolically active cells, such as germinating spores (Rosen *et al*, 1974).

Another major difference between aerial and submerged spores was the wall thickness. In *T. harzianum* spore, the walls are formed by two layers, the outermost wall (W2) being more electrondense, than the inner wall (W1) (Fig. 1). Wall thickness of the aerial spores produced in SSC was nearly twice that of the spores produced by submerged fermentation and this is mainly attributed to a thicker W2 layer in former (Table 2, Fig. 1). W2 is spore specific. Indeed, vegetative hyphae do

not contain this layer, but it is formed during the first steps of spore development (Muñoz and Agosin, unpublished). This layer is the first barrier of the spore, against external conditions, and may has a role in spore viability and its shelf-life.

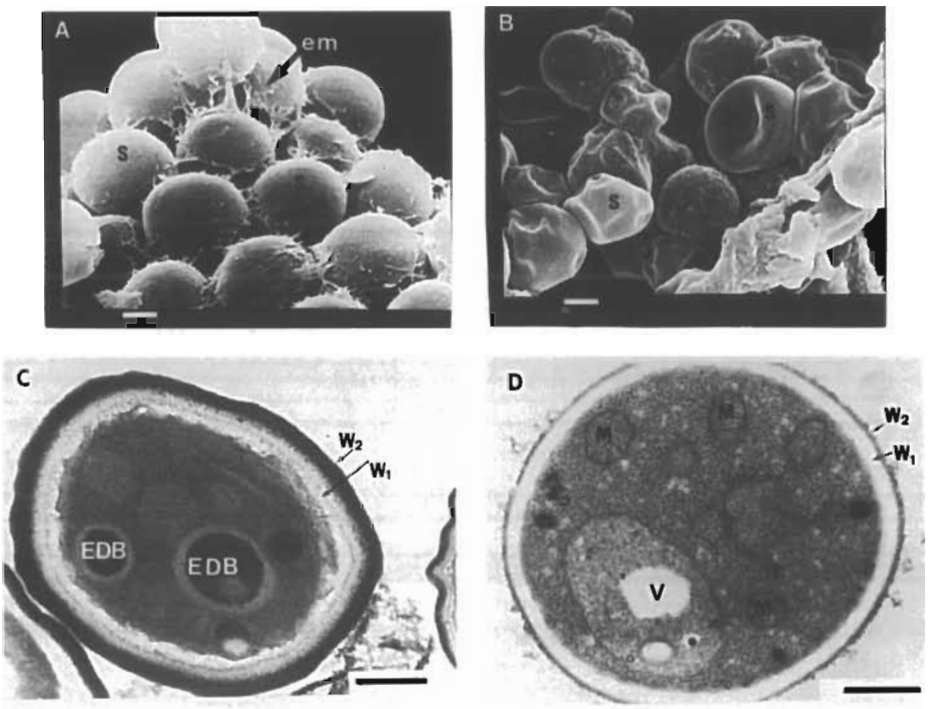

Fig. 1. Scanning electron micrographs (A, B) and transmission electron micrographs (C, D) of the spores of *T. harzianum* produced by SSC (A, C) and submerged fermentation (B, D) techniques. Symbols: **em**, mucilage; **W1**, inner wall layer; **W2**, outer wall layer; **V**, vesicles; **M**, mitochondria; **EDB**, electrondense bodies. Bars represent 0.5 μm.

DIFFERENCES IN CHEMICAL COMPOSITION BETWEEN SPORES PRODUCED BY SSC AND SUBMERGED FERMENTATION

Spores produced by aerial mycelium in SSC showed higher resistance, than those produced under submerged culture conditions. Viability of aerial spores was fully maintained after 45 days storage at 75% relative humidity (Table 2), while it decreased to less than 15% over the same time period in case of the spores produced by the submerged fermentation technique.

Table 2. Properties of *Trichoderma harzianum* spores produced by SSC and submerged cultivation techniques.

Property	Spores produced by	
	SSC[1]	Submerged cultivation[2]
UV-resistance[3] (LD50, min)	4.0	2.4
Longevity (%)[3]	100.0	15.0
Hydrophobicity	++	-
Wall thickness (nm)[4]		
W2	119.0	30.0
W1	116.0	150.0
Total	235.0	180.0

[1] obtained from YMI Petri plates.

[2] harvested at 24 h after the maximun spore yield was reached in a medium with a C/N ratio of 14 and pH 7.0 [8].

[3] % cfu, determined after 45 days storage at 25°C and 75% RH

[4] Results from Figure 1

Moreover, aerial spores produced by SSC were able to resist much higher doses of UV-radiation (Table 2). This could be related to the darker-green surface of aerial spores, which, in turn, may be due to their thicker outer wall layer. Although the role of these surface pigments has not yet been investigated, it could be attributed to the reducing power at the spore´s surface, as a defence mechanism against photosensitizers (Cooperman Sollod *et al*, 1992).

Only aerial spores exhibited hydrophobic properties (Table 2). Because of this hydrophobic nature, the only way to obtain homogeneous suspensions of aerial spores is by using a glass-teflon homogenizer. Consequently, this character is lost during homogenization, due to release of the hydrophobic component. Hydrophobicity has been identified as a key parameter in the establishment of micro-organisms in the phylloplane (Doss *et al*, 1992), as it allows rapid and effective adhesion of *T. harzianum* aerial spores to leaves and other host surfaces.

The hydrophobic contribution of surface proteins was evaluated. SDS-PAGE analysis of the water-extractable material from aerial spore walls, produced in SSC, showed a single major band, corresponding to a protein of low molecular weight (lower than 14 KDa) (Fig. 2).

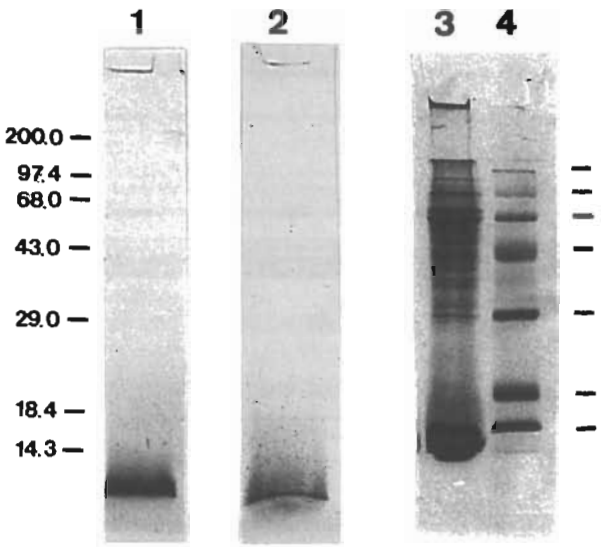

Fig. 2. SDS-PAGE analysis of extractable material from *T. harzianum* spore cell walls. Lanes: Trifluoroacetic acid soluble proteins in spore cell walls produced in SSC (1) and in liquid culture (2). Total extracellular proteins of *T. harzianum* obtained from a liquid culture medium (3). Molecular weight markers (kDa) (4).

This protein was the only one obtained from aerial spores produced in SSC, after dissolving in TFA. When spores produced by submerged cultivation were analysed under the same conditions, the content of this low molecular weight protein was much lower. A similar low molecular weight protein was precipitated from the supernatant of the liquid medium, in which spores were produced (Fig. 2). It is assumed that this protein, arising from spores produced in submerged cultivation, is the same hydrophobin, as above in case of SSC, in accordance with the recently proposed model of Wosten *et al* (1993) for hydrophobin production in fungal hyphae.

Spores produced in the aerial mycelium in SSC would be able to persist longer in the harsher environmental field conditions. This higher resistance could be related with the apparent resting state of aerial spores. In this respect, the hydrophobicity of phyaloconidia of *T. harzianum* might contribute to the maintenance of the dormant state of aerial spores. Spores produced in submerged conditions might facilitate interactions with the external medium, such as quick water or nutrient uptake. This will result in rapid responses to external conditions, allowing a shorter germination time than for aerial spores. However, they will also be more susceptible to deterioration reactions.

CONCLUSIONS

The results show that the culture system selected for the mass production of fungal spores will be a determining factor in the quantity, quality and biocontrol efficacy of the resulting spores. Aerial spores of *T. harzianum*, produced in SSC, will be more durable, than those produced in submerged condition, even to the extent that they will be able to be delivered in the field, without the necessity to provide them in sophisticated formulations. The presence of hydrophobins on the spore surface appears to be a key factor enabling adhesion to the host, increased resistance to environmental variations, and preservation of the structural integrity of the spore.

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Use of agro-industrial residues for bioinsecticidal endotoxin production by *Bacillus thuringiensis* var. *israelensis* or *kurstaki* in solid state fermentation

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SUMMARY

The feasibility of biological control, against agricultural pests and disease vectors, depends on the production of these bioagents at reduced costs, in order to compete successfully with harmful chemical insecticides. Among the entomopathogens that have been studied for biocontrol purposes, *Bacillus thuringiensis* is produced by submerged fermentation processes. Laboratory scale solid state fermentation processes have demonstrated effective *B. thuringiensis* endotoxins production, which could be adopted for small scale local production in the near future. Several factors make its local production highly desirable for pest control in developing nations. It can be cheaply produced on a wide variety of low cost, organic substrates. Local production would result in considerable monetary savings of heavy expenditure on import of chemical and biological insecticides.

Keywords : *Bacillus thuringiensis*, solid state fermentation, bioinsecticide, biocontrol, agroindustrial residues, efficacy, legislature.

RESUME

Utilisation des résidus agro-industriels pour la production d'endotoxines bioinsecticides par *Bacillus thuringiensis* var. *israelensis* or *kurstaki* en fermentation en milieu solide

CAPALBO D.M.F. ET MORAES I. O.

La faisabilité de la lutte biologique contre les parasites des cultures et les vecteurs de maladie, dépend essentiellement des techniques de production. Le but de ces études étant d'obtenir des organismes à coût réduit et compétitif par rapport aux agents chimiques. *Bacillus thuringiensis*, est probablement l'agent enthomopathogène le plus étudié pour la lutte biologique et le plus facilement disponible commercialement. Il est produit industriellement par fermentation submergée. La fermentation en milieu solide a prouvé son intérêt dans la production d'endotoxines de *B. thuringiensis* et on peut envisager son utilisation locale, pour la production à petite échelle. Plusieurs facteurs jouent en la faveur de l'utilisation de cette technique dans les pays en voie de développement : le coût de production est réduit et la fermentation peut être réalisée sur divers substrats organiques bon marché. La production locale permet d'économiser les devises dépensées dans l'import d'insecticides chimiques et biologiques.

Mots clés : *Bacillus thuringiensis*, fermentation en milieu solide, bioinsecticides, lutte biologique, résidus agro-industriels, efficacité, législation.

INTRODUCTION

Under current agricultural practices, it has been estimated that more than 40% losses of crops in Latin America are due to insect pests. In spite of the heavy and extensive use of chemical pesticides, these losses are occurring annually. Similar concerns could be pointed out for the utilization of chemical pesticides against mosquitoes, which are vectors of human diseases.

Chemical pesticides, especially the insecticides, are usually toxic to non-target species (including many beneficial insects), domestic animals and have been steadily loosing effectiveness, due to the development of resistance.

Biological control has been widely studied and used in many countries throughout the world, as an effective alternative for chemical control. The micro-organism or its

component have shown the practicability and effective action of the biological control against pest problems (Burges, 1981; Moraes, 1991; Entwistle et al, 1993).

Bacillus thuringiensis (Bt) has many attributes, such as specificity, potency and efficacy, against several insects that are usually considered pests, either to agriculture or to human health, which are essential for a successful microbial pesticide. Its toxicity is primarily due to the parasporal crystal formed within the mother cell during the sporulation, which is believed to be a protoxin, that is converted into a toxin by specific proteolytic enzymes located at some insects' midgut. Usually, a mixture of spores and crystals are commercially marketed.

Great success has been obtained with the development of new Bt strains. Among its 40-50 varieties already identified, two have been well studied: the variety *kurstaki* (Btk) (strain HD-1, specially toxic to Lepidoptera normally associated with agricultural damage), and variety *israelensis* (Bti strain H-14, usually toxic for Diptera, which is considered as a vector of human diseases).

FERMENTATION PROCESS FOR BT PRODUCTION

The practical use of micro-organisms for pest control is only possible, if an industrial scale production is developed. This was understood even by the early workers, who attempted to industrialize their cultivation process for Bt in the mid sixties (Burges, 1981). Dulmage (1971), Dulmage and Rhodes (1973) sucessfully used agroindustrial by-products in submerged fermentation of Bt.

In Brazil, studies were initiated in 1970, at the Faculty of Food Engineering of the State University of Campinas (UNICAMP), to explore the feasibility of producing the endotoxins preparations of Btk, using submerged fermentation, with cheap liquid by-products as components of the media (Moraes, 1973, 1976a, 1976b; Moraes and Capalbo, 1985; Moraes *et al*, 1994*a*).

Based on the promising results obtained in laboratory and pilot scale production at UNICAMP, and on previous tests with solid state fermentation techniques (SSF) for bacterium (Salama, 1984), attempts were made to apply SSF process, in order to arrive to a less expensive and more feasible method for Bt production.

SOLID STATE FERMENTATION FOR BT

Btk

The selection of the components for the medium was made, based on the availability of the agro-industrial solid by-product in the region where the work was conducted, consistency of the composition of the by-product and low cost of this by-product (Capalbo and Moraes, 1988; Capalbo, 1989).

Preliminary experiments were developed with selected residues as substrates, utilizing small flasks as reactors. The range of incubation temperature and growth conditions, as well as some SSF parameters were taken into account, i.e., periodic humidification was accomplished in order to avoid dryness of the culture medium for a long term fermentation (Moraes *et al*, 1989).

For the simplest fermentation development analysis, viable-spore counts, pH and substrate humidity were analyzed (Capalbo, 1989). Some typical results for Btk are summarized in Table 1.

Table 1. Production of Btk spores by solid state fermentation process on solid residue from pulp and paper industry, with periodic humidification (Typical results).

Harvest time (h)	pH	Moisture (%)	CFU_g
0	5.5	50.1	-
48	6.3	46.2	$1.8x10^5$
72	8.0	-	$7.5x10^9$
168	8.1	54.6	$>10^{10}$

CFU = colony forming unit

The bioassay indicates the activity of the fermentation product. Typical results attained with Btk, produced by SSF process, against *Anticarsia gemmatalis* (soybean catterpilar) are presented in Table 2.

Bti

For Bti, the substrate tested was rice (commercially available, low-cost type), and small plastic bags were used as reactors. The procedures for preparation, inoculation and fermentation control were the same, as applied to Btk production. The following analysis was carried out: pH, viable-spore counts, and humidity analysis. Typical results are shown in Table 3.

Table 2. Bioassay of Btk, obtained by solid state fermentation, against *Anticarsia gemmatalis.*

CFU/ml applied on the leaf	Mortality (%)
$1.9x10^9$	100
$1.9x10^6$	75
$2.3x10^9$ b	100
control c	0

CFU = colony forming unit. Standard: commercial product. Control = only water

Table 3. Production of Bti spores by solid-state fermentation process on rice in plastic bags (Typical results).

Harvest time (h)	pH	Moisture (%)	CFU/g
0	5.5	56.1	-
16	5.9	57.5	$9.3x10^6$
88	6.2	58.6	$1.8x10^8$
112	6.4	55.8	$2.5x10^8$
144	6.4	56.4	$1.4x10^8$

CFU = colony forming unit

The bioassays were developed with larvae of *Culex* sp, reared in special containers. The results obtained in laboratory showed that it is necessary to use 143 mg of the whole biomass (100 h fermentation, dried in oven at 60°C) to achieve 100% kill of the larvae, when spread over an area of 1 m^2 of clear water. Additional work on drying procedures to determine thermobacteriological parameters (Moraes *et al*, 1994*b*), design parameters for laboratory scale reactor (Capalbo *et al*, 1994) and tests for efficient formulations (Medugno *et al*, 1994) are still under study.

DISCUSSION AND CONCLUSIONS

Brazil is internationally known for the recent intensive activities in the area of biological control. A large portion of those activities refer to the mass production and use of bioagents for pest control. It is recognized that Bt is a desirable agent for insect pest control, since it could be developed at low cost and with high specificity. The results presented in Tables 1-3 indicated that the laboratory scale models used in this study are effective in producing Btk or Bti endotoxins on low cost substrates.

The SSF process showed some advantages over the conventional stirred or aerated liquid fermentations, because a) the medium is relatively simple, since only meal plus small amounts of water are needed, b) the required space occupied by the fermentation equipment is relatively small, as compared to the yield of the product, c) aeration is easily obtained, since there are air spaces between each particle of the substrate, and d) the product may be dried at low cost for storage.

Some problems, such as a) the selection of Bt strain that do not produce exotoxins on SSF, and b) the need for monitoring methods and devices for small scale SSF process, were encountered. These topics are now under study.

There are many groups in Brazil that produce Bt at laboratory scale by means of SSF and submerged fermentations. Many problems will arise, when they decide to produce Bt at a commercial scale. These include quality control of the product, standardization, formulation, cost/benefit ratio, and registration. Besides, Brazil does not have a specific legislation for registration and/or experimental use of such biological control products. This has certainly negatively affected the greater use of these products. Effective actions are being taken by CNPMA, in collaboration with the legislation makers, in order to overcome this gap (Capalbo, 1995).

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Production of mycelial cell inoculum of *Pleurotus opuntiae* on natural support in solid state fermentation

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SUMMARY

A new culture technique was developed for studying the physiology of *Pleurotus* mycelium in solid state fermentation (SSF) system. This method consists of utilization sugar cane bagasse, a by-product of sugar industry, as solid support for absorbing a liquid culture medium. Compared with classical techniques, it offers the advantage of better control of liquid medium composition as well as culture conditions. Fermenters were coupled with on-line gas analysis by using a gas chromatograph equipped with thermal conductivity detector. Solid state culture were aerated at a very low flow air rate (0.04 ml/g moist solids). Concentrations of CO_2 and O_2 in the exhaust air from fermenters were continuously analysed. By means of this technique, culture conditions were optimized respect to the ratio urea/ammonium sulphate, the particle size of bagasse and inoculation rate. Under best conditions, nearly 90% of substrate was consumed in 6 days. This culture technique offers, excellent practicability for the development of mycelium inocula for commercial exploitation of *Pleurotus* spp.

Keywords: Solid state fermentation, natural support, sugarcane bagasse, on-line gas analysis, gas chromatograph, mycelium, *Pleurotus opuntiae*, respirometry, physiology, endogenous pH control, inoculum production.

RESUME

Production de mycelium de *Pleurotus opuntiae* sur support naturel en fermentation en milieu solide.

ROUSSOS S., BRESSON E., SAUCEDO-CASTANEDA G., MARTINEZ P., GUINBERTEAU J. ET OLIVIER J.M.

Une nouvelle technique a été mise au point pour étudier la physiologie de croissance mycélienne de *Pleurotus opuntiae* cultivé en milieu solide. Cette méthode consiste à utiliser la bagasse de canne à sucre comme support solide pour y absorber une solution nutritive. Comparée à des techniques classiques, cette méthode offre des avantages pour un meilleur contrôle de la composition des solutions nutritives tout en respectant les conditions définies de fermentation en milieu solide. Les cultures en milieu solide ont été aérées avec un très faible débit d'air (0.04 ml/g de milieu humidifié). Les concentrations en CO_2 et en O_2 ont été analysées à partir des prélèments automatiques des effluents gazeux à la sortie des fermenteurs en utilisant un chromatographe de gaz équipé d'un détecteur à conductivité thermique. Par cette technique, les conditions de culture ont été optimisées en particulier le rapport urée/sulfate d'ammonium, la taille des particules du support et de l'inoculum. Sous ces conditions, environ 90% du substrat carboné a été consommé en 6 jours. Cette technique de culture offre des perspectives interessantes pour la production de mycélium de Pleurotes et peut être utilisée pour une exploitation commerciale.

Mots clés: Fermentation en milieu solide, support naturel, bagasse de canne à sucre, analyse en ligne, effluents gazeux, chromatographie en phase gazeuse, mycelium, *Pleurotus opuntiae*, respirometrie, physiologie, contrôle endogène du pH, production d'inoculum.

INTRODUCTION

Production of mushrooms (*Agaricus, Pleurotus, Lentinus, Auricularia*, etc) has witnessed significant increase throughout the world, the increase being from $2182x10^3$ to $3763x10^3$ tonnes/annum in the last ten years (Chang and Miles, 1991). *Pleurotus* is cultivated extensively in tropical areas and the increase in its production is highest in last ten years (169 to 909 tonnes/annum) among the different cultivated mushrooms. Even, the yield of *Pleurotus* is also higher.

Latin American countries, like Mexico, produce mainly *Agaricus*, but the production of *Pleurotus* is also on increase (Martinez-Carrera *et al.* 1989). The inoculum, popularly referred as spawn, for mushroom production is generally of the mycelial inoculum in the substrate used for mushroom cultivation (Fritsche, 1981; Yang and Jong, 1987). Straw of different agro-industrial residues can also be used for spawn production, but it poses problems in uniform distribution of the inoculum. Moreover, the cereal grains are costly and appropriate grains, such as those of wheat or sorghum, are not sufficiently available for spawn production, as these are consumed as stable food in many countries (Goltapeh and Kapoor, 1987). In addition, these are not produced in a number of tropical countries.

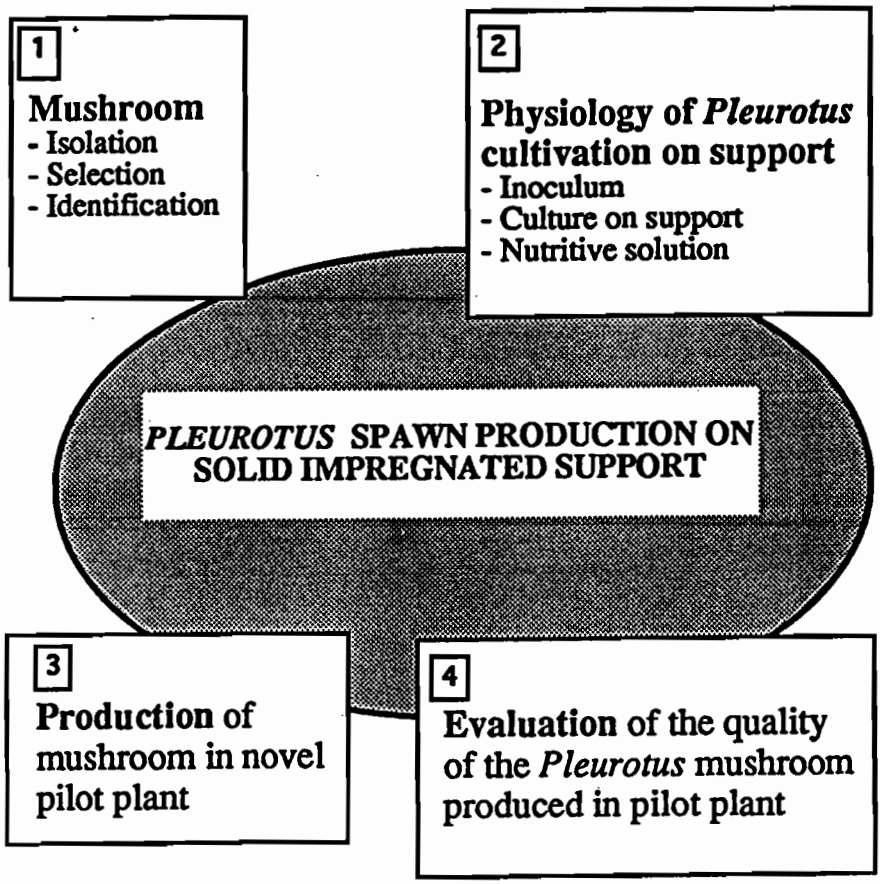
In their programme to initiate large scale production of *Pleurotus* Mexican industries faced problems in economic production of spawns of *Pleurotus* on wheat grains at industrial scale. Hence a need was felt for developing a simpler, cost-efficient and newer solid state fermentation process, based on substrate other than wheat or cereal grains (Roussos, *et al.* 1990). Sugar cane pith bagasse has been successfully used in ORSTOM, Montpellier, France, as an inert support in solid state fermentation for production of various fungal and yeast metabolites (Oriol *et al*, 1987; Trejo-Hernandez *et al.* 1993; Saucedo-Castañeda *et al*, 1993) as well as biomass (Saucedo-Castañeda *et al.* 1992). Its use for spawn production, after absorption of nutritive medium, may also prove potential and provide advantage of economy.

A team was, therefore, formulated involving l'Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Montpellier, France; Institut National de la Recherche Agronomique (INRA), Bordeaux, France; and Universidad Autonoma de Tlaxcala (UAT), Tlaxcala, México, with a goal of the production of *Pleurotus* spawn on inert natural solid support impregnated with nutritive medium. The work was carried out in four stages, as shown in Fig. 1.

The aim of the work, reported in the present paper, was to use chemically defined synthetic medium, absorb it on inert support and study the utilization of soluble sugars, pH evolution and support colonization during the mycelial growth in column fermenter, as well as under the influence of specific physico-chemical factors. In addition, attempt has been made to relate on-line measurement of oxygen utilization and carbon dioxide evolution with physiological state of the culture.

It is emphasized that a lot of work has been carried out on production of spawn in liquid and solid culture for use in the production of *Pleurotus*. Most of it concerns with delignification (Sannia *et al.* 1991; Valsameda *et al.* 1991; Martinez *et al.* 1993), bioconversion of lignocellulosic agro-residues (Martinez-Carrera *et al.* 1985; 1986; Guzman and Martinez-Carrera, 1986; Bisaria *et al.* 1987; Gupta and Langar,1988) and concomitant formation of enzymes (Kannan and Oblisami 1990;) during the growth of the spawn on cereal grains (Aslam Azizi *et al.* 1990, Guillen F.

et al. 1990). In all these cases, molasses, starchy substrates or cereal grains were used as substrate in polyethylene bags, bottles or Petri dishes. The column fermenter was never used earlier, nor the medium was aerated during the cultivation. Similarly, no work has been carried out yet on the physiology and metabolism of *Pleurotus* mycelial cell growth in solid state fermentation system.



Organisms participants :

1- UAT, INRA Bordeaux ; 2- ORSTOM Montpellier
3 - UAT, Mexico 4- INRA, Bordeaux

Fig. 1 : Stages of the collaborative projet.

MATERIALS AND METHODS

MICROORGANISMS

A total of 6 strains each of *Pleurotus* were isolated at the Universidad Autonoma de Tlaxcala, Mexico, and INRA, Bordeaux, France. These were subjected to preliminary sceening and *P. opuntiae* C73, the isolate from Tlaxcala, Mexico, was selected for further studies. It was maintained on potato dextrose agar plates at 4°C and subcultured every two months.

INOCULUM DEVELOPMENT

Two different media, potato dextrose agar and sugar cane pith bagasse medium, were used for inoculum development for use in physiological studies. The inoculum in each case was developed first in Petri dishes, using above media. The plates were incubated at 25°C in the absence of light and without any aeration for 5 days. This served as a freshly grown culture for futher development of inoculum in culture bottles of 250 ml total capacity in case of potato dextrose agar or column fermenter of 100 ml total capacity in case of bagasse medium.

The sugar cane pith bagasse was washed repeatedly to remove all the nutritive substances, sieved to obtain a particle size of 0.8 to 2.0 mm length, dried and sterilized as per the procedure of Saucedo-Castañeda *et al* (1992). Such dried bagasse was used throughout this study to eliminate any influence of the variation in the composition of sugar cane pith bagasse and its processing method. It was hydrated to 70% initial moisture content with a nutritive solution containing (g/L) soluble starch 70.0, malt extract 10.0, urea 2.9, ammonium sulphate 3.5, KH_2PO_4 4.0 and distilled water 1000. The moist bagasse medium was autoclaved at 121°C for 30 min, cooled to 25°C, inoculated and 40 g moist medium was charged in column fermenter.

In case of potato dextrose agar medium, the autoclaving was at 121°C for 15 min in the culture bottle itself. It was used in such a way that the flat surface of the 1 L solidified medium occupies about 50% of the total legth of 8O cm of the bottle.

The inoculation of these two media was done by using 1 cm^2 units of the mycelial mat from Petri dishes. In each case, three such units were used and these were placed at equal distance from each other, one unit being at the bottom side of the medium, while another at the top side of the medium. The growth of the mycelial cells was allowed to takes place at 25°C under darkness and without any aeration for 5 days. The mycelial mat formed on potato dextrose agar served as source of final inoculum

in case of culture bottle. The mycelial cells grown in the column fermenter were mixed thoroughly with the bagasse medium for use as inoculum. This latter inoculum is referred as activated bagasse hereafter.

Comparison of Inoculum Grown on Potato Dextrose Agar in Culture Bottles and Bagasse Grown Column Fermenter

The inocula grown as above in two media were tested for comparative performance in column fermenter, using sugar cane pith bagasse, after impregnation with nutritive solution. In case of inoculum from potato dextrose agar, three inoculum units were used to inoculate the medium in the column. Thoroughly mixed activated bagasse was used at 10% (w/w) level, based on the moist weight of the inoculum as well as the fresh bagasse medium. In the latter case, the inoculum was mixed thorougly with the fresh medium and the inoculated medium was charged in the column. In case of inoculum from potato dextrose agar, the fresh bagasse medium was charged in the column and the inoculum units were placed appropriately during the charging procedure. The inoculation was done in laminar air flow unit in all the cases.

The column fermenter unit, consisting of 24 columns each of 100 ml total capacity and as developed by Raimbault and Alazard (1980) was adopted for all studies on growth and physiology of *P. opuntiae*. The fermentation was carried out at 25°C for 74 h in darkness at the aeration rate of 0.15 ml air/min/g dry matter. Samples were removed at specific intervals. The contents of each column served as a sample. The growth of the mycelial cells is expressed as the velocity of the growth/100 g bagasse support medium. The cultures were also observed every day macroscopically to note the mycelial growth, especially the compactness of the substrate due to the mycelial growth.

Effect of Light on Growth and Metabolism

The column fermenters were wrapped in aluminium foil for fermentation in darkness. In case of fermentation under light, the natural light cycle was used, i.e. the fermentation was no light in the night. All other conditions were as described above.

Effect of Ratio of Urea and Ammonium Nitrogen

Three different ratios were used. In medium A, the ratio of urea to ammonium nitrogen was 1:3, while media B and C contained 1:2 and 3:1 ratios (Table 1). All other conditions were same as specified earlier. In all the case the C/N ratio was maintained constant at 14 (Khanna and Garcha, 1985).

Table 1 : Composition of the media containing different ratios of urea and ammonium nitrogen.

Composition	Medium		
	A	**B**	**C**
Sugar cane pith bagasse	80	80	80
Soluble starch	20	20	20
Malt extract	2.9	2.9	2.9
Nutritive solution (g/100 g starch)			
Urea	2.4	6.0	8.4
$(NH_4)_2SO_4$	9.7	6.1	3.7
KH_2PO_4	5.0	5.0	5.0
Distilled water	1000	1000	1000
pH	6.1	6.1	6.2

SAMPLE TREATMENT AND ANALYSES

Dry matter content was determined in each sample by drying it at 105°C overnight and the data are expressed as % dry matter loss. These results were also used to calculate moisture content of the fermentation medium. For measurement of total soluble sugar and pH of the medium, 100 ml distilled water was added to 5 g sample, mixed for 2 min in Ultraturrax and the clear filtrate was subjected to analyses. Total soluble sugars were estimated by the method of Dubois *et al.* (1956) while pH of the clear filtrate was measured using pH meter. The exhaust gas from each column was subjected to on-line analysis of carbon dioxide during whole of the fermentation period, as per the methodology of Saucedo-Castañeda *et al.* (1993).

RESULTS AND DISCUSSION

COMPARISON OF INOCULA

In solid state fermentation, the size of inoculum is often given as number of spores/cells per g moist medium or initial dry matter content of the medium (Raimbault and Alazard, 1980; Oriol *et al*, 1987; Roussos *et al*, 1991; Soccol *et al*,

1994). In case of *Pleurotus*, this strategy can not be used. In another work (Martinez-Ortiz and Roussos, 1991), it was been clearly demonstrated that the apical growth of *Pleurotus* is linear. It is therefore, necessary to augment the inoculum units to obtain a rapid and homogeneous colonization of the substrate. For this reason, three units of inoculum were used in case of potato dextrose agar medium, while 10% of the fermented bagasse support medium was used in column fermenter.

Table 2 : Comparative performance of the inoculum developed on potato dextrose agar and on bagasse support medium.

Inoculum source	Fementation time (h)	pH of the medium	Consumption of soluble sugar (%)	content of the medium (%)	Loss of dry matter (%)
PDA*	0	6.1	0.0	77.6	0.0
	70		30.0	76.3	12.0
	80	6.2	43.0	77.8	19.5
	103	6.2	72.0	78.3	22.0
	143	6.5	82.0	79.3	28.7
BSM**	0	6.2	0.0	76.7	0.0
	20	5.9	5.2	76.4	2.0
	42	5.1	32.0	78.0	9.4
	50	5.0	43.0	78.0	12.0
	74	4.8	62.0	78.0	27.0

PDA*: from Potato dextrose agar ; BSM** : from Bagasse support medium

The data on the comparative performances of these two types of inocula are presented in Table 2. The data indicated that the start of the growth of *P. opuntiae* C73 is directly related to the source of inoculum. Another industrially and economically important observation is the significant reduction in the lag phase of the culture, with the use of inoculum of activated bagasse. On the contrary, the start of the growth of the mycelial cells is very long and the growth is very slow in case of the use of inoculum from potato dextrose agar (PDA). This prolongs the process time in case of inoculm from PDA, though the velocity of the production of carbon dioxide is higher (Fig. 2). The results demonstrate that the total carbon dioxide production is affected by the source of the inoculum. In case of activated bagasse inoculum, the rate of total dioxide production is 30 ml/g initial dry matter (DM) after 74 h, as

against that of 151 ml/g in case of the inoculum from PDA. Moreover, the total production of carbon dioxide after 140 h in case of inoculum from PDA is comparatively much higher , through its production is slower.

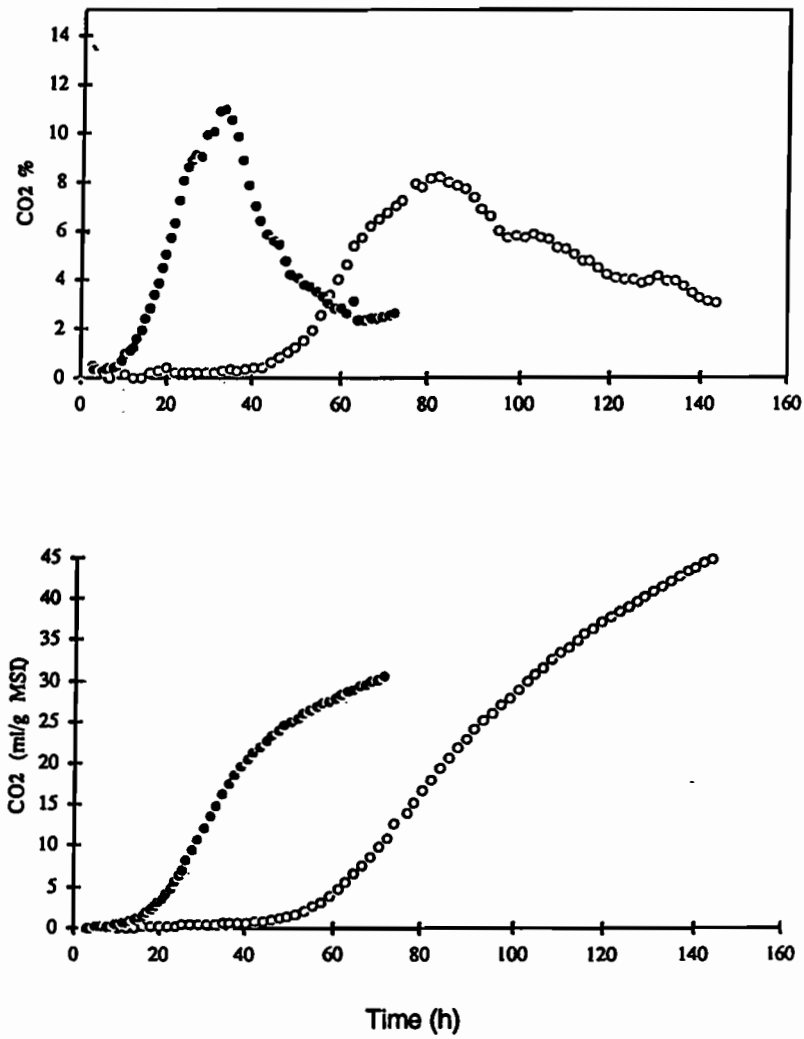


Fig. 2 : Influence of the source of inoculum on evolution of carbon dioxide during growth of *P. opuntiae* C73 in bagasse support medium in column fermenter. MSI = Initial Dry Matter. Inoculum from bagasse support medium (●), and from Potato Dextrose Agar (○).

In both cases, the moisture content of the medium during the fermentation is fairly constant and varies between 76.3 to 79.3%. At the end of both the fermentation (143 h in case of inoculum from PDA and 73 h in case of activated bagasse inoculum), the solubles sugars consumption was 82% in the former case, as against that of 62% in the latter case. In addition to this difference, another critically important difference was observed with respect to the change in the pH of the medium during the course of fermentation. In case of inoculum from PDA, the pH of the medium remained stable and varied between 6.1 to 6.5 during the course of 143 h fermentation. However, the pH of the medium was reduced rapidly from initial value of 6.2 to that of 4.8 by the end of 74 h fermentation. It is clearly apparent that this reduction in the pH of the medium stopped the respiration of the culture and, consequently, the consumption of the carbon substrate (solubles sugars).

In addition, the % dry matter loss is more rapid and higher in case of activated bagasse inoculum. The value at 74 h fermentation was 27%, as against that of 12% at 70 h in case of inoculum from potato dextrose agar. It is of interest to note that the % dry matter loss is nearly same at 74 and 143 h in case of activated bagasse and potato dextrose agar inocula, respectively.

On the whole, the above data indicated that the best source of inoculum is the activated bagasse. With its use, the number of inoculum points in the inoculated media are numerous and consequently very homogeneous growth is achieved and the start of the growth is also rapid. In addition, the utilization of soluble sugars is rapid, but it leads to the acidification of the medium. This problem can be overcome by using strongly buffered medium and with initial pH near to neutrality for favoring mycelial growth.

EFFECT ON LIGHT ON MYCELIAL GROWTH

Literature survey indicated the effect of light on the growth of *Pleurotus*. For example, Olivier *et al* (1991) have reported that the exposure of the culture to light during mycelial development accelerates the fructification of *Pleurotus* by reducing the lag phase and consequently, the time of cultivation. Hence, effect of light during mycelial growth of *P. opuntiae* C73 was studied.

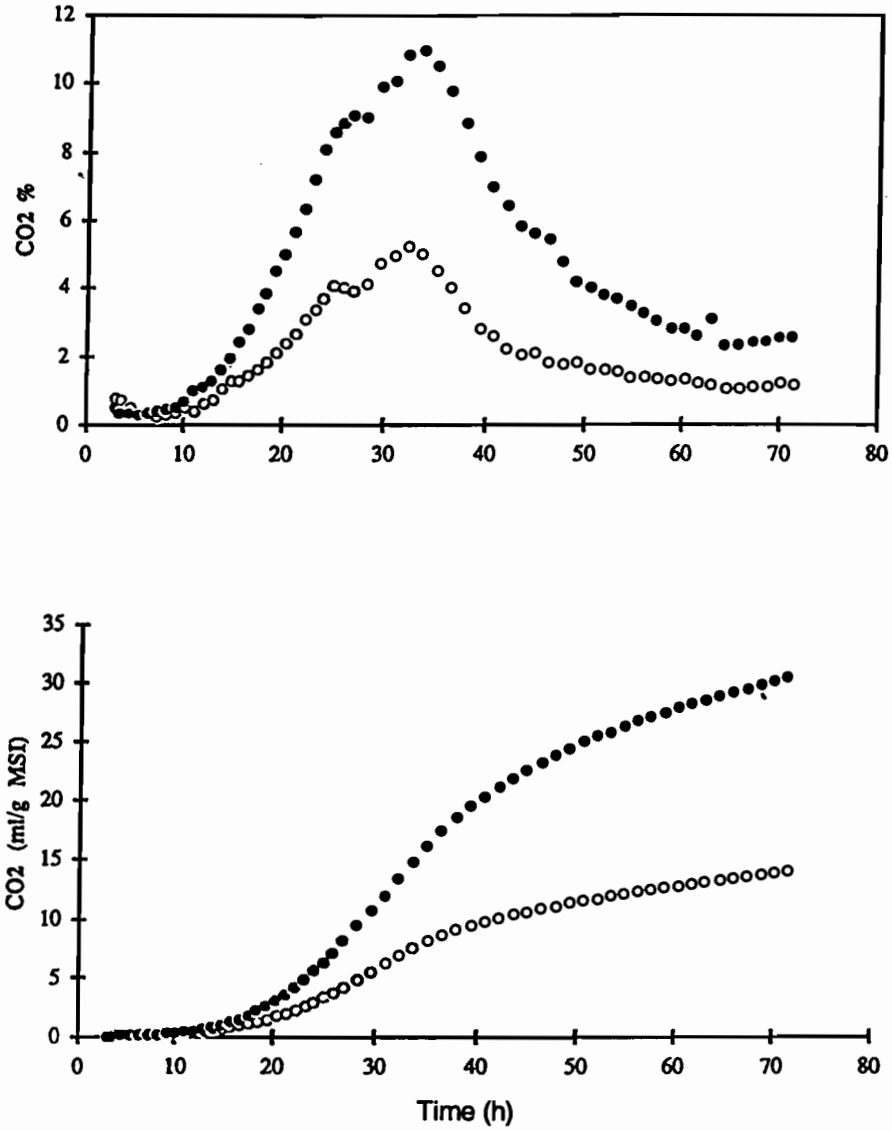


Fig. 3 : Influence of light (○) and darkness (●) on the evolution of carbon dioxide during the growth of *P. opuntiae* C73 on bagasse support medium in column fermenter. MSI = Initial Dry Matter.

The results demonstrate that the exposure of the culture to light acts negatively on the growth of the mycelium and the consumption of soluble sugars (Table 3). In particular, the maximum velocity of carbon dioxide production was 2 times lower in case of exposure to light, as compared to the cultivation in darkness (Fig. 3). The data, thus, confirm that the exposure of the culture to light during mycelial development is an important factor to control the growth and metabolism of *P.opuntiae* C73. The velocity of apical growth, the biomass formation, % loss of dry matter and consumption of soluble sugars decreased in the absence of light. However, the changes in pH and moisture content of the medium were nearly same in both the cases.

Table 3 : Influence of light and darkness on the growth of mycelium in column fermenter.

Attribute	Fermentation time (h)	pH of the medium	Consuption of soluble starch (%)	Moisture content of the medium (%)	Loss in dry matter (%)
Natural light	0	6.2	0.0	76.7	0.0
	20	6.0	0.4	77.8	7.3
	42	5.1	35.3	77.4	6.6
	50	5.0	42.5	78.3	10.5
	74	4.8	57.5	78.2	11.3
Darkness	0	6.2	0.0	76.7	0.0
	20	5.9	5.2	76.4	1.8
	42	5.1	32.0	78.1	9.2
	50	5.0	43.3	78.0	12.0
	74	4.8	62.0	78.0	26.7

It is of interest to note the profile of carbon dioxide production between 24 to 30 h in Fig. 2. At above 24 h, the carbon dioxide % in the exhaust air became stationary for few hours and then again sturted rising, but also for few hours, before declining in the further period of fermentation. It probably indicates the change in the metabolic pattern of the culture during these periods. This is also corroborated by a very large increase in the consumption of soluble sugars between 20 and 42 h (Table 3).

EFFECT OF RATIO OF UREA AND AMMONIUM NITROGEN ON THE pH CHANGES IN THE MEDIUM

Data in Tables 2 and 3 and Figs. 2 and 3 indicate that the acidification of the medium during the growth of mycelial cells results in arresting the growth and metabolism of *P. opuntiae* C73. By overcoming this problem, it may be possible to continue the growth of the culture beyond 74 h.

To avoid changes in the pH of the medium during mycelial growth in bagasse support medium and also to prevent diminution of the growth, an endogenous strategy was formulated and verified experimentally. It involves the use of different ratios of urea and ammonium nitrogen, without changing the C/N ratio of the medium (Table 1). This can prevent the changes in pH of the medium, as these will be no H^+ ions liberation during the assimilation of urea, in contrary to that in case of ammonium nitrogen. Thus, the use of more of urea nitrogen can prevent the acidification of the medium during mycelial growth.

Media A, B and C, containing three different ratios of urea and ammonium nitrogen (Table 1) as described by Saucedo-Castañeda *et al* (1992), were evaluated. Literature survey indicated the positive effect of CO_2 on the growth of *Pleurotus* (Zadrazil, 1975) and hence the rate of air flow was reduced by 10 times (10^{-2} in place of 10^{-1} ml of carbon dioxide/g initial dry matter).

The results showed that the pH values changed negligibly up to 70 h fermentation in all the three media. Thereafter, the medium A was progressively and gradually acidified, but no such change took place in media B and C, in spite of better soluble sugar utilization to a tune of more than 80%. The lower consumption of dry matter in medium A confirm that *P. opuntiae* C73 does not normally grow in medium whose pH is less than 6.0.

Data on the analysis of carbon dioxide in exhaust gases indicated different phases of mycelial growth in media B and C (Fig. 4). Total carbon dioxide production was nearly equal in these two media. The respirometric data (Fig. 4) also demonstrated very poor growth in media A.

The results (Table 4 and Fig. 4) confirm that *P. opuntiae* C73 has a optimum pH around neutrality for growth. This was also confirmed by the results in media B and C, which have a strong buffering action. The balance between these two sources of nitrogen (urea and ammonium sulphate) or a little excess of urea, permitted to maintain the pH value of the medium at near neutrality.

Table 4 : Effect of different ratios of urea and ammonium nitrogen on the growth and metabolism of *P. opuntiae* C23 in bagasse support medium in column fermenter.

Parameters Evolution	Fermentation time (h)	Culture media		
		A	B	C
pH of the medium	0	6.02	6.06	6.14
		5.98	6.10	6.17
	80	5.68	6.24	5.99
	103	5.76	6.24	6.68
	143	5.56	6.50	6.78
Consumption of soluble sugars (%)	0	0.0	0.0	0.0
	70	16.5	30.5	50.9
	80	31.0	42.7	52.4
	103	11.4	71.8	74.0
	143	39.0	81.7	85.7
Loss of dry matter (%)	0	0.0	0.0	0.0
	70	3.7	11.9	13.4
	80	10.6	19.5	21.8
	103	9.8	21.9	25.0
	143	13.7	28.7	25.6
Moisture content of the medium (%)	0	78.1	77.6	78.7
	70	77.8	76.3	77.3
	80	78.0	77.8	78.0
	103	79.4	78.3	79.5
	143	78.9	79.3	80.1

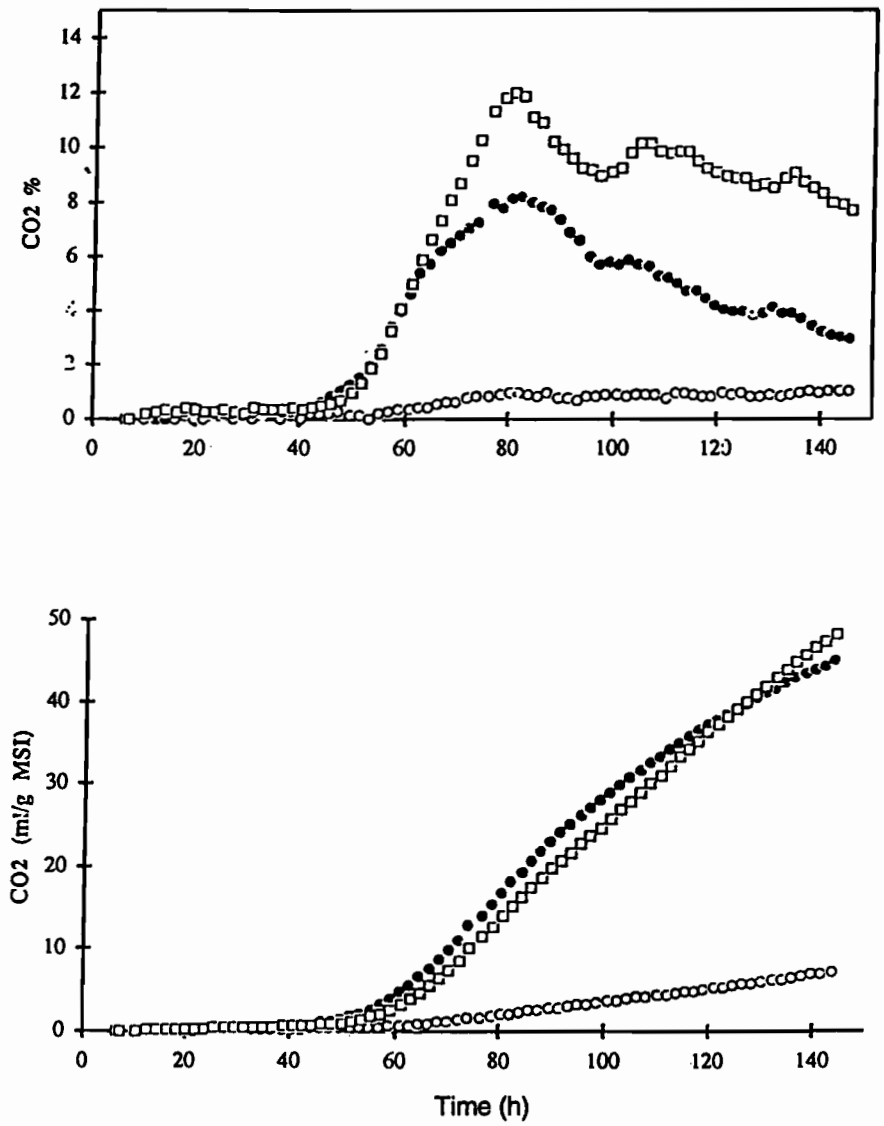


Fig. 4 : Influence of the ratio of urea and ammonium nitrogen on the evolution of carbon dioxide by *P. opuntiae* C73 on bagasse support media (**A**: ○ ; **B**: ● and **C**: □) in column fermenter. MSI = Initial Dry Matter.

CONCLUSIONS

Sugar cane pith bagasse as inert support, after impregnation with nutritive solution, seves as a better source of inoculum of *P. opuntiae* C73, as compared to the conventional potato dextrose agar medium. The changes in the pH of the inoculum medium can be overcome by using appropriate ratios of urea and ammonium nitrogen in the medium. Activated bagasse may prove as more efficient spawn and avoid reliance on cereal grains. Work on these aspects is in progress. This culture technique can be used for study physiology and metabolism of different mushrooms and ectomycorrhizal fungi mycelial growth.

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Cultivation of *Lentinula edodes* on mixture of cassava bagasse and sugarcane bagasse

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SUMMARY

Potential of the production of *Lentinula edodes* (shiitake) mushrooms on agro-industrial residues, such as cassava bagasse and sugarcane bagasse, is evaluated under solid state fermentation. Visual observations in flaskes and also plastic bags indicated good growth on these substrates. The fructification and biological efficiency of the process was found to be good. Best results for basiodiocarp production were evident in the medium containing 80 % cassava bagasse + 20 % sugarcane bagasse (w/w). Data on kinetics of starch consumption and protein synthesis showed the consumption of 77.42 % starch during the biotransformation process, with three times increase in protein content. Fructification was achieved after 60 days of inoculation and it amounts to decrease by 7 times, as compared to the conventional process of the production on oak tree logs. The results provide a novel alternate technology for shiitake production on the mixture of cassava bagasse and sugarcane bagasse.

Keywords: *Lentinula edodes* , shiitake , solid state fermentation, cassava bagasse, sugarcane bagasse, fructification, basidiocarp production, process cycle, kinetics, biological efficiency.

RESUME

Culture de *Lentinula edodes* sur un mélange de résidus fibreux de manioc et de bagasse de canne à sucre.

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La possibilité de produire des *Lentinula edodes* (shiitake) sur des résidus agro-industriels, comme la bagasse de manioc et la bagasse de canne à sucre, par fermentation en milieu solide, est explorée. Les observations morphologiques effectuées sur des cultures en flacon ou en sac en plastique permettent d'estimer une bonne croissance sur ce type de substrat. La fructification et l'efficacité biologique du procédé sont déclarés satisfaisants. Les meilleurs résultats pour la production de basidiocarpes sont obtenus en employant un milieu contenant 80% de bagasse de manioc et 20% de bagasse de canne à sucre. Des résultats concernent la cinétique de la consommation de l'amidon contenu dans la bagasse de manioc parallèlement à celle de la synthèse de protéines. Pendant la biotransformation, une consommation d'amidon, estimée à 77,42%, est associée à une synthèse de protéines trois fois plus abondante que celle couramment décrite. La fructification est achevée au bout de 60 jours d'incubation et a été diminué de 7 fois si l'on compare au procédé conventionel de production sur des troncs de chênes. Ce temps de fructification s'avère singulièrement réduit. Sa durée s'avère le septième de celle couramment décrite pour la culture faite sur résidus de chênes. Ces résultats ouvrent une nouvelle voie pour la production de *Lentinula* en développant une nouvelle stratégie de culture sur un milieu innovant, un mélange de bagasse de manioc et de bagasse de canne à sucre.

Mots-clés : Shiitake, *Lentinula edodes*, fermentation en milieu solide, bagasse de manioc, bagasse de canne à sucre, fructification, production de basidiocarpes, cycle de production, cinétique, efficacité biologique.

INTRODUCTION

The goal of this present paper will be the elaboration of a new alternative for the solid waste from raw cassava and sugarcane residues, agro-industrial residues produced in Paraná State, Brazil, to allow the edible mushroom *Lentinula edodes* (shiitake) cultivation.

In nature, the shiitake mushroom grows on trunks of the Fagaceae family, mainly *Quercus acutissima* and *Quercus errata* (oak tree) (Lee, 1980). This fungus is the most important edible mushroom cultivated in Japan (Tokimoto and Tomatsu, 1978). It is popular, for its excellent organoleptical properties and nutritional quality as well as presence of all the essential aminoacids. It also possesses therapeutical properties due to the production of specific polysacharides which have antitumoral activities (Chiyokichi, 1990). Some adenine derivatives that are able to reduce the cholesterol and some substances capable of stimulating the immunological system are also reported (Chiyokichi, 1990).

The traditional cultivation of this mushroom is on the trunks of the trees specified above and the fructification occurs after about sixteen months (Lee, 1980).

In Brazil, oak trees do not grow and, therefore, searching for an altenative substrates and also to reduce the cultivation period may constitute a good opportunity to upgrade some of the potential agro-industrial residues of Paraná State. It is estimated that the cassava crop increased by 55.86 % and the sugarcane crop increased 25.81 % during the 91/92 and 92/93 harvests, respectively, when compared with the late five harvests. Currently, 770,458 tons/year of solid wastes from raw cassava and 3,840,197 tons/year of sugarcane residue is generated in Brazil (Seab, 1993). The upgradation of these agro-industrial residues in to mushroom with economical, nutritional and therapeutical properties justifies development of process concerning large scale production of *Lentinula edodes*.

In this paper, the results concerning the inocula production, substrate preparation, fructification and the biological efficiency are reported.

MATERIALS AND METHODS

MICRORGANISM AND SUBSTRATES

Lentinula edodes LPB 031 from the Biotechnological Processing Laboratory of the University was used. In this paper, two by-products were i.e. cassava bagasse (solid fibrous waste) and sugarcane bagasse. Wheat grain was used for comparative purposes.

INOCULA PRODUCTION

It was carried out in plastic bags containing 5 g (or 15 g, when necessary) of wheat grain, moistened until 60 %. The plastic bags were autoclaved for 15 min. at 121° C (Tan and Chang, 1989) and, after cooling, these were inoculated with discs (6-mm diam) of wheat extract agar containing sufficient mycelium of *Lentinula edodes* LPB 031 in an advanced growth stage. The plastic bags were incubated at 23°C for 15 days.

SUBSTRATE PREPARATION

Before using, the cassava bagasse and the sugarcane bagasse were dried in an air oven at 65° C for 24 h. After drying, these were sieved and, the fraction between 2 and 0.8 mm was used (Soccol, 1994). For visual evaluation of the mycelium, the wet substrate (approximately with a moisture content of 70 %) was weighted to 50 g each, placed in glass flasks, and autoclaved at 121°C for 1 h.

PLASTIC BAG PREPARATION

To evaluate the biological efficiency, the cassava bagasse was used alone or in combination with sugarcane bagasse at a level of 20 to 80 % proportionally. It was placed (150 g) in each autoclavable plastic bag. They were moistened with distilled water until 70 % moisture and autoclaved for 1 h at 121° C.

INOCULATION, INCUBATION AND FRUCTIFICATION

Inoculation was at 10 % for each substrate type and was performed in a aseptic chamber. The inoculated flasks were incubated at 23°C for 40 days. The plastic bags containing different proportions of solid wastes were incubated at 23° C for 60 days and, after this period, these were transferred and opened in a cultivation chamber, maintained at 18°C and 80 and 90 % relative humidity. The basidiocarps were removed at the opening time and were weighted using a semi-analytical scale balance.

ANALYTICAL ASPECTS

Moisture content, ash, fats, starch, proteins, fibres and total caloric values of the mushroom were determined according to Nadial (1990).

RESULTS AND DISCUSSION

VISUAL EVALUATION OF THE MYCELIUM GROWTH IN FLASKS

The mycelium growth was followed during 40 days after the inoculation (Fig. 1). After 30 days, the mycelium had spread over the substrates totally. The mycelium mass formed on the cassava fibrous residue was more dense than that on the sugarcane bagasse. On the 40th day, the solid fibrous wastes from cassava began to change its color to brown, thereby reflecting the basidiocarpus development of *Lentinula edodes* (Olivier *et al*, 1991). Consequently, the ability of filamentous fungi to growth can be measured visualy and the degree of colour change (due to the growth of fungi) can be used as an indicator.

VISUAL EVALUATION OF THE MYCELIUM GROWTH IN PLASTIC BAGS

The exact moment to remove the plastic bags from the culture room was determined by the mycelium characteristics. This was found to vary with the substrate.

A

a b c d

B

Fig. 1. A comparison of the mycelial growth of *Lentinula edodes*, cultivated during 40 days, on different supports. A : cassava fibrous waste and B : sugar cane bagasse (a : 0 day; b : 10 days; c : 20 days and d : 40 days).

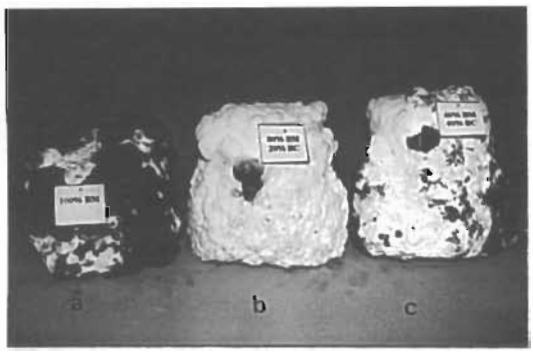

Fig. 2 - Mycelium of *Lentinula edodes* on the substrate surface of different solid support after 60 days inoculation (a : 100 % cassava bagasse; b : 80 % cassava bagasse + 20 % sugar cane bagasse; c : 60 % cassava bagasse + 40 % sugar cane bagasse).

After 60 days of inoculation and incubation, it was observed that the dense white mat on the substrate surface started turning progressively brown. Until today, this fact constituted the best indicator to remove the plastic bags from the culture room (Fig. 2).

FRUCTIFICATION AND BIOLOGICAL EFFICIENCY

The early basidiocarps appeared 65 days after the inoculation start (5 days after the plastic bags removal). But, the time varied between 70 to 90 days based on the substrate used and their combination. The composition of the substrates is given in Table 1.

Table 1. Chemical composition of cassava and sugar cane bagasse (g/100 g).

	Cassava bagasse	Sugarcane bagasse
Humidity	9.03	5.89
Ashes	1.70	1.80
Fats	0.21	0.62
proteins	1.26	0.70
Fibres	12.61	34.81
Carbohydrates	75.61	56.19
Total caloric value*	307.84	233.10

*Kcal/100 g

According to Soccol (1994), the substrate texture has an influence on the fructification. It was observed that the substrate prepared with cassava bagasse only was toot dense and thick, thereby resulting in a reduction in the yield of *Pleurotus ostreatus* 22 basidiocarps. In order to optimize the texture, it was mixed with different quantities of sugarcane bagasse as it has high fibre content and is available abundantly in the State of Parana.

Fig. 3. Basidiocarps of *Lentinula edodes* grown on 100 % cassava bagasse.

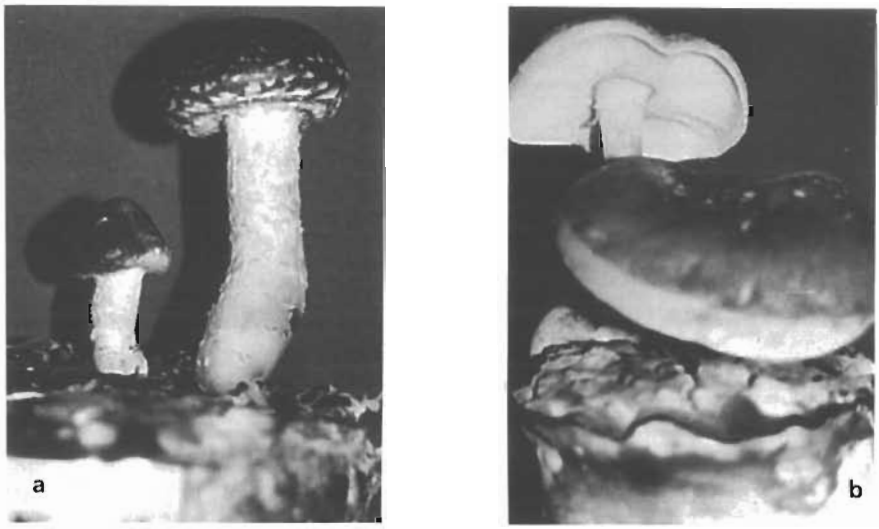

Fig. 4. Basidiocarps of *Lentinula edodes* developed on the medium containing 80 % cassava bagasse (a : first crop; b : second crop).

The fructification occurred in 100 % cassava bagasse medium (Fig. 3), medium containing 80 % cassava bagasse + 20 % sugarcane bagasse (Fig. 4) and, the medium containing 60 % cassava bagasse + 40 % sugarcane bagasse (Fig. 5). Contrastly, there was no trace of any basidiocarpus development in the medium containing 40 and 20 % of cassava bagasse (results not shown).

Fig. 5. Basidiocarps of *Lentinula edodes* grown on medium containing 60 % cassava bagasse.

Table 2. Biological efficiency of the process.

Dried substrate (g)					
Cassava bagasse (%)	Cassava bagasse (g)	Sugar cane bagasse (g)	Inoculum (g)	Total weight (g)	B.E. (g)
100	150	-	15	59.11	39
80	120	30	15	109.37	72
60	90	60	15	57.36	38
40	60	90	15	-	-
20	30	120	15	-	-

B.E. : Fresh weigth of the fructification bodies/Dry weigth of the substrate used

Better biological efficiency was obtained when the substrate used was composed of 80 % cassava bagasse and 20 % sugar cane bagasse (Table 2). Fig. 6 shows the kinetic evolution of starch consumption and protein synthesis before and during the 60 days incubation of the fructification substrate containing 80 % cassava bagasse and 20 % sugar cane bagasse.

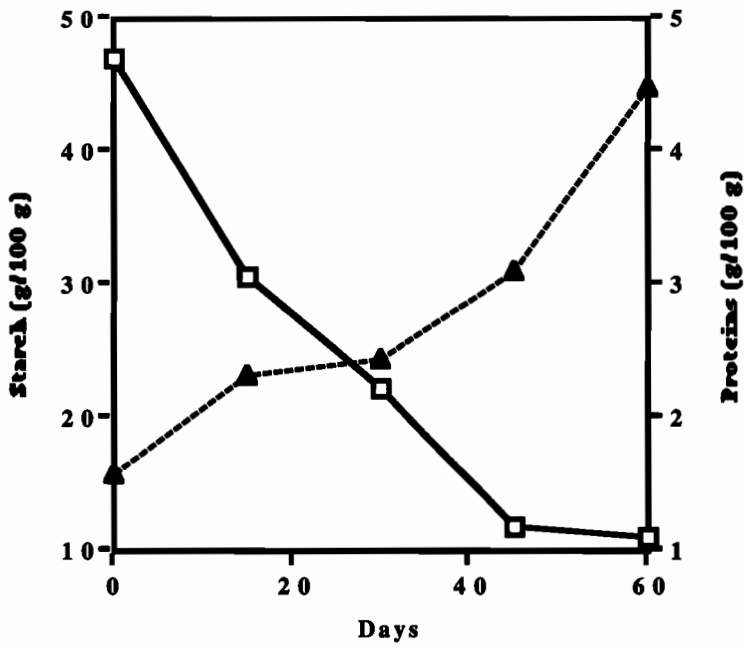

Fig. 6. Evolution of starch consumption and protein synthesis of the fructification substrate, containing 80 % cassava bagasse and 20 % sugar cane bagasse.

The results show that 77.42 % of the starch contained in the substrate has been consumed during the biotransformation. After 60 days, the protein content was nearly tripled.

According to Moyson and Verachter (1991), though shiitake grows on logs, the lignine degradation is considered as a secondary metabolism, beginning only on the 42nd day after the mycelium inoculation, when the fungus is inoculated into substrate containing a large quantity of polysaccharids. According to Leatham (1986), the enzymatic degradation of starch, through amylase liberation by the

mycelium, is stronger in the beginning of the incubation, and *Lentinula edodes* is an effective starch degrading agent. Data obtained confirmed the results of these authors.

After 60 days from inoculation, the fructification started. The fructification starts when a limit in nutrients has been reached, and it happens faster on substrates containing easily-degradable carbohydrates than on logs.

The fructification time on this substrate has been reduced by about 7 times as compared to the growth on logs.

CONCLUSION

It is possible to use cassava bagasse and sugarcane bagasse to efficiently cultivate *Lentinula edodes.* Change in colour during the fungal growth provides an interesting technological alternative to use these by-products. When compared with the traditional method, the most effective medium contained 80 % cassava + 20 % sugarcane bagasse. It reduced the cultivation cycle of *Lentinula edodes* by seven times. This method could be more economic than the presently followed method.

ACKNOWLEDGEMENTS

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Prospect for production of *Pleurotus sajor - caju* from cassava fibrous waste

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SUMMARY

Cassava fibrous waste, alone or in combination with sugar cane bagasse, was studied for its potential for production of *Pleurotus sajor-caju* LPB 19 in solid state fermentation. Various physico-chemical parameters such as fermentation temperature, initial pH of the medium, enrichment of the medium with glucose and the level of NaCl in the medium were studied, along with production of fruit bodies. The data indicated high potential of cassava fibrous waste in the production of *Pleurotus* mushroom, as the average production of fresh fruit bodies and the biological efficiency of the process involving 80 : 20 ratio of cassava bagasses : sugar cane bagasse medium are excellent. The work provides a novel approach for upgradation of cassava fibrous waste.

Keywords : *Pleurotus sajor-caju*, solid state fermentation, cassava fibrous waste, sugar cane bagasse, fermentation temperature, initial pH of medium, glucose enrichment, sodium chloride, fruit bodies, biological efficiency.

RESUME

Prospectives de production de *Pleurotus sajor-caju* sur des résidus fibreux de manioc.

BARBOSA M. C. S., SOCCOL C. R., MARIN B., TODESCHINI M. L., TONIAL T., ET FLORES V.

La bagasse de manioc, un produit fibreux, utilisée seule ou mélangée avec de la bagasse de canne à sucre, est étudiée dans le but de connaître sa possibilité d'être employée comme support pour la production de *Pleurotus sajor-caju* LPB 19 par fermentation en milieu solide. De nombreux facteurs sont ainsi évalués pour optimiser la production de carpophores : la température de fermentation, le pH initial et la composition du milieu d'imprégnation (glucose et NaCl). Les résultats obtenus indiquent que la bagasse de manioc est un excellent support pour la production de carpophores de *Pleurotus sajor-caju*. Les meilleurs résultats sont obtenus avec un milieu contenant un mélange de bagasse de manioc et de canne à sucre avec un rapport respectivement de 80% et 20%. Ce travail décrit une nouvelle approche pour la production de ce type de champignons avec ce support.

Mots clés : *Pleurotus sajor-caju*, fermentation en milieu solide, écarts fibreux de manioc, bagasse de manioc, bagasse de canne à sucre, température de fermentation, pH initial de fermentation, enrichissement eu milieu en glucose, addition de NaCl, carpophores, efficacité biologique.

INTRODUCTION

Microbial conversion of cassava fibrous wastes has been extensively worked out in the past. But, the digestibility of cellulose and hemicellulose was considerably reduced due to the presence of lignin (Fan *et al*, 1987). *Pleurotus* is one of the most efficient lignin degrading edible fungi (Bisaria and Madan, 1983). According to several authors, *Pleurotus* species actively degraded lignin of agricultural wastes by secretion of laccase and peroxidase (Bisaria and Madan, 1983; Gujral *et al*, 1987; Hadar *et al*, 1993; Kerem *et al*, 1992). Consequently, agro-industrial wastes could be converted into sugars, fuels (alcohol, methane), solvents (acetone, butanol), animal feeds and organic fertilizers. The cassava fibrous solid residue and especially, the starch present in it, could be used for cultivation and production of these edible

mushrooms (Soccol, 1994). Thus, the cultivation of this fungus on cassava fibrous wastes could be a excellent biotechnological aternative to upgrade this byproduct produced by the various milling companies of Parana in Brazil.

Pleurotus can be considered as a good alternative for production of protein-rich food in tropical countries (Bisaria and Madan, 1983; Martínez-Carrera *et al*, 1992). These edible mushrooms are rich in proteins, carbohydrates, lipids, vitamins, organic acids and minerals (Bisaria and Madan, 1983). In addition, it has a therapeutical anti-tumoral value (Martínez-Carrera *et al*, 1992 ; Soccol, 1994). According to Macaya - Lizano (1988), *Pleurotus* mushrooms are resistant to diseases and can be cultivated in short time and at low costs.

The objective of the present work was to define the factors influencing growth of *Pleurotus* on cassava solid wastes. The results described could generate some innovative biotechnological alternatives and better utilization of cassava solid wastes.

MATERIALS AND METHODS

MICROORGANISM

Pleurotus sajor-caju LPB 19 was obtained from Laboratório de Processos Biotecnológicos (U.P.R., Curitiba, Brasil). The strain is maintained on rye-yeast agar medium at 5°C.

SUBSTRATES

The substrates used were cassava (*Manihot esculenta* Crantz) fibrous waste and sugarcane (*Saccharum officinarum L.*) bagasse. Table 1 summarizes the composition of cassava waste (Cereda, 1994). Solid residues were mixed. Five different compositions of mixture were tested (cassava fibrous waste : sugar cane bagasse) : 20 : 80 ; 40 : 60 ; 60 : 40 ; 80 : 20 and 100 : 0

Table 1. Composition of cassava fibrous waste (on basis of dry substrate).

Component	Concentration (% weight/weight)
Moisture	9.52
Ash	0.66
Starch	63.85
Fat	0.83
Nitrogen (x 6.25)	0.32
Fibers	14.88

GROWTH OF *PLEUROTUS SAJOR -CAJU* LPB 19 ON CASSAVA FIBROUS WASTE AGAR MEDIUM

Incubation of fungi was carried out on cassava fibrous waste agar medium, which contained (g / L) cassava waste, 40.0 and agar 15.0 in Petri plates (8.5 cm diam). The pH was adjusted to 7.0 with 1.0 N $CaCO_3$. Disks (5 mm diam) were cut from the central zone of *Pleurotus sajor-caju* LPB 19 cultivated on rye - yeast medium. These disks were placed in the centre of the test plate, containing the appropriate medium. Test plates were incubated at 25°C for 6 days.

Radial growth measurements were made daily during the entire incubation period (Soccol *et al*, 1994). In some cases, dry biomass was weighted. For this, the medium in the plates was heated and the mycelium was separated from the melted culture medium in a Buchner funnel equipped with a 8 mm filter paper, which was previously weighted. Mycelial cells were washed several times with boiling water for the total elimination of medium that could remain on the paper filter. Then, the filter was dried at 105°C for 24 h and the mycelial biomass was calculated by the difference in the weights. Each determination was carried out in triplicate.

SPAWN PRODUCTION OF *PLEUROTUS SAJOR-CAJU*

Spawn production was made in polyethylene bags containing 100 g wheat grains, supplemented with 1% $CaCO_3$ at the moisture content of 50 %. The bags were sterilized by autoclaving for 1 h at 121°C. After cooling to 25°C, the bags were

inoculated with 5 disks of rye-yeast agar and incubated at 25°C for 15 days (Soccol, 1994).

SPAWN RUN AND FRUITING IN CASSAVA FIBROUS WASTE AND SUGARCANE BAGASSE

Substrate mixtures were prepared in polyethylene bags, with addition of 1% $CaCO_3$, moistened to 75 % with distilled water and autoclaved at 121°C for 1 h, according to Manu-Tawiah and Martin (1986) and Royse (1992). After sterilization and cooling, the bags were inoculated with 10 % spawn and incubated in the dark at 24°C until the mycelium has completely covered the medium (Gujral *et al*, 1987). After completion of the spawn run, the environmental conditions were changed to induce fruiting body formation. The bags were perforated and incubated in a chamber with 70 % relative humidity and ambiant temperature of 24 - 28°C for 60 days.

RESULTS AND DISCUSSION

PHYSIOLOGICAL STUDIES OF *PLEUROTUS SAJOR - CAJU* GROWTH IN CASSAVA WASTE AGAR

Influence of temperature

Cultures were carried out at different temperatures, i.e., 5, 22, 24, 28, 32, 35 and 37°C. For each temperature, the growth rate was measured in the exponential phase of growth. As shown in Fig. 1, the growth was highest between 24 and 28°C. There was a sharp reduction in growth at higher temperatures. The metabolism of fungus generates heat. Unless this heat is removed, it may raise the temperature of the fermenting material and leads to growth inhibition or even killing of the fungus at higher temperatures.

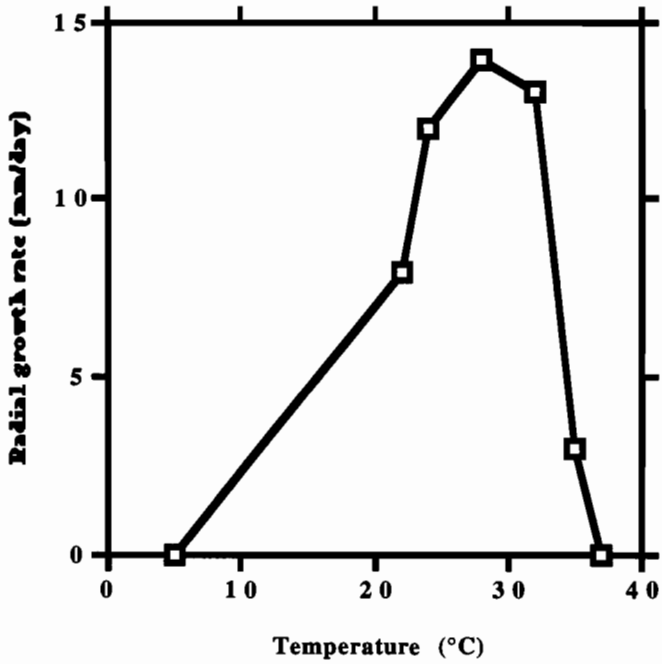

Fig. 1 . Temperature effect on the radial growth of *Pleurotus sajor-caju* LPB 19.

The fungal growth was evaluated by the measurement of colony diam, after six days cultivation at 25°C.

Influence of pH

Data showed that the initial pH of the medium strongly influences the rate and extent of fungal growth (Fig. 2).

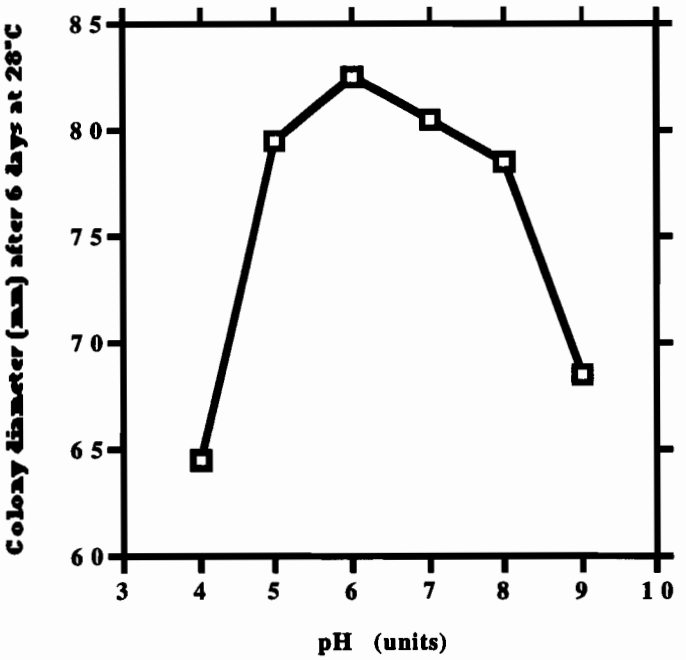

Fig. 2. Effect of initial pH of the medium on the radial growth of *Pleurotus sajor-caju* LPB 19.

The maximal radial growth was observed at the initial pH values in the range of 5.0-8.0, the best being at initial pH of 6.0. It was lower when the medium was too acidic (4.0) or too alkaline (9.0). Most of white-rot fungi grow best at slightly acidic pH values between 4 and 5. Nevertheless, some alkaliphilic species are also reported to grow on such media.

An initial pH of the medium at 6.0 was, therefore, selected for further work.

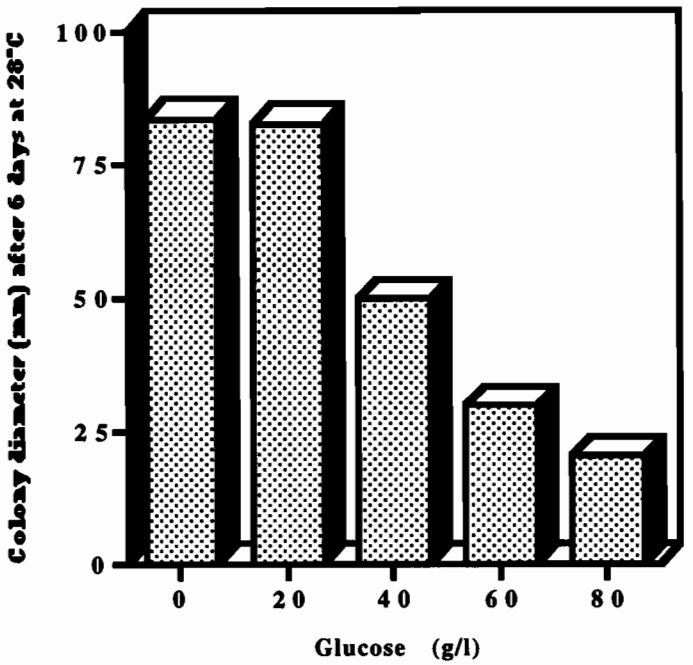

Fig. 3. Effect of glucose addition to the medium on the radial growth of *Pleurotus sajor-caju* LPB 19.

Influence of glucose

Fungal growth was not significantly altered with an increase in the concentration of glucose up to 20 g/L in the medium (Fig. 3). However, glucose concentration over 20 g/L inhibited the growth. This effect could be due to an elevation of osmotic pressure of the medium. Other explanation could be reported in relation with the fungal metabolism of glucose. The catabolism repression induced by the presence of glucose was often reported.

Influence of NaCl

Fig. 4 illustrates the effect of the addition of NaCl on the radial growth of *Pleurotus sajor - caju* under the conditions as described for the above experiments. In the range of concentrations tested, the effect of NaCl was significant.

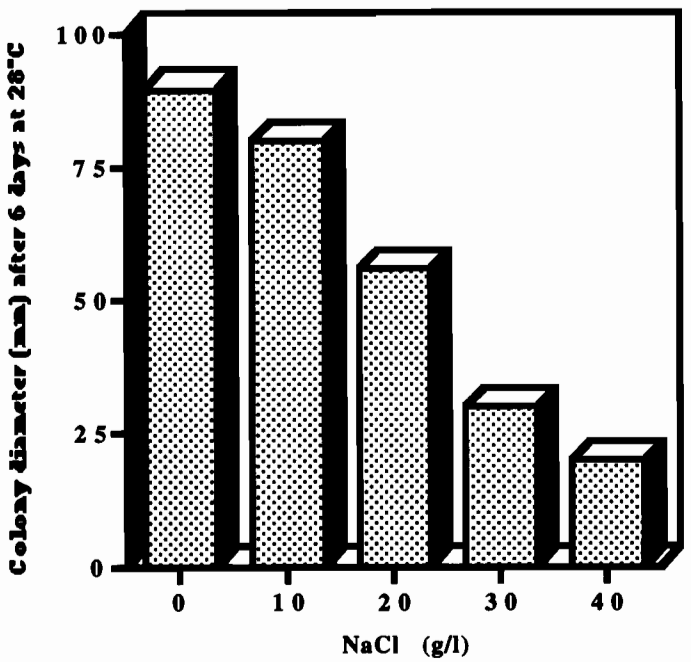

Fig. 4. Effect of NaCl on the radial growth of Pleurotus sajor-caju LPB 19.

The decrease in fungal growth was proportional to the increase in the concentration of NaCl in the medium. With the use of NaCl at 40 g/L, the fungal growth decreased by 76.27 %. Such effect could be related to the sensitivity of membrane translocators to Cl. This is the case of the plasmalemma ATPase. The role of Na^+ could also be suggested since this ion modifies the electrical properties of the plasmalemma and, consequently, strongly influences the plasmalemma electrochemical potential.

Influence of addition of yeast extract addition

Addition of yeast extract to the cassava fibrous waste medium showed a positive effect on the fungal growth, until a concentration of 0.8 g/L (Table 2). When supplemented, the production of fungal biomass was significantly higher than the control one (more than 51.4 % of the control). Yeast extract stimulated growth probably, because of its proteins, amino-acids and vitamin content (Manu-Tawiah *et al*, 1990; El-Kattan *et al*, 1990; Fasidi and Olorunmaiye, 1994).

Production of fruit bodies of Pleurotus sajor-caju on cassava waste and sugarcane bagasse media

The first crop was harvested on 36 days, after the inoculation, and the second on, 49 days, after the opening of bags. Major yield of mushrooms was in the first flush.

Table 2. Effect of addition of yeast extract on the growth of *Pleurotus sajor-caju.*

Yeast extract (g/L)	Colony diameter (mm)	Biomass produced in Petri dishes (mg)
0.0	9.6 ± 0.4	87.2 ± 19.3
0.4	10.2 ± 0.6	103.0 ± 5.9
0.8	10.4 ± 0.6	132.0 ± 22.8
1.2	10.2 ± 0.4	106.0 ± 0.6
1.4	10.4 ± 0.4	73.6 ± 9.4
2.0	9.7 ± 0.1	73.6 ± 9.4

The best results were obtained with the use of solid support containing 80 % (w/w) cassava bagasse and 20 % (w/w) sugarcane bagasse (Table 3). Parallely, the biological efficiency of *Pleurotus sajor-caju* was estimated to be 30.6 %, as per the methodology reported by Gujral *et al.* (1987) and Ortega *et al* (1992).

Table 3. Average production of fresh fruit bodies of *Pleurotus sajor-caju* LPB 19 and its biological efficiency on cassava and sugar cane wastes.

Substrates					
Cassava bagasse (g)	Sugarcane bagasse (g)	Spawn (g)	Starch (g/100 g)	Crude fiber (g/100 g)	B . E. (%)[a*]
20	80	10	12.77	30.15	22.89 ± 1.56
40	60	10	25.54	26.33	24.31 ± 3.67
60	40	10	38.31	22.50	27.78 ± 6.85
80	20	10	51.08	18.69	30.60 ± 2.82
100	-	10	63.85	14.88	25.89 ± 6.38

a) B.E.% = Biological efficiency % = weight fresh mushrooms /dry substrate x 100

* Average of three determinations

The cassava and sugar cane bagasse mixture were characterized by their specific nutritional equilibrium in starch, and crude fibres. The results are shown in Table 3. Consequently, these substrates permitted the production of fruit bodies of *Pleurotus sajor - caju*. (Fig. 5).

Fig. 5 . Fruit bodies of *Pleurotus sajor-caju* cultivated on cassava and sugar cane bagasse media.

CONCLUSION

The ability of *Pleurotus sajor-caju* to grow on cassava fibrous wastes is an important result. It grew well at a temperature between 24 to 32°C, and a pH range between 5.0 to 8.0. The radial growth was not significantly affected when addition of glucose was not excessive (up to 20 g/L). When the medium was supplemented by yeast extract, up to 0.8 g/L, fungi grew with higher production of biomass.

These results are encouraging and demonstrate the feasibility of the use of cassava fibrous waste as a substrate in solid state fermentation substrate for the production of *Pleurotus*.

Thus, the residues of the starch milling process constitutes a good alternative for the production of edible mushrooms. Nevertheless, further researches is needed in order to improve the biological efficiency of the process, which could be more economically attractive.

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Solid state fermentation of wheat straw for paper production

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SUMMARY

The white-rot basidiomycete, *Lentinus edodes*, was grown axenically on wheat straw in a tumbling drum bioreactor. The effects of the gas phase composition and the mixing conditions on some aspects of the mycelial metabolism are discussed briefly. Moreover, some results concerning the development of a biologically-aided pulping process are reported.

Keywords: *Lentinus edodes,* white-rot fungi, solid state fermentation, biodelignification, biopulping, paper production, annual plants, wheat straw, trumbling drum fermentor, pilot scale bioreactor.

RESUME

Fermentation solide de la paille de blé pour la production de papier.

GIOVANNOZZI-SERMANNI G., D'ANNIBALE A. ET CRESTINI C.

Le basidiomycete *Lentinus edodes* a été cultivé en milieu solide sur de la paille de blé dans un fermenteur cylidrique en conditions axéniqes. L'effet de la composition de la phase gazeuse et des conditions d'agitation sur quelques aspects du métabolisme du champignon sont discutés brièvement. Les résultats obtenus concernant la mise au point d'un procédé biologique pour le dépulpage de papier sont présentés.

Mots clés: *Lentinus edodes*, fermentation solide, biodelignification, biodépulpage, production de papier, plantes annuelles, paille de blé, fermenteur cylindrique, bioreacteur échelle pilote.

INTRODUCTION

Significant amounts of agricultural residues of nonwoody plants, such as cereal straws, are produced worldwide annually. These agro-residues could constitute an attractive long-term alternative to the well established wood fiber feed-stocks for manufacture of the papers (Kaldor, 1992; Sabharwal *et al,* 1994). The increasing demand of the global market and environmental considerations, will probably lead to an increasing use of fast growing annual fibers. In fact, a strategy based on the exploitation of annual plants would not require long-term capital commitments and, furthermore, the areas under cultivation could be modulated on an annual basis, depending on the demand.

Recently, the advantage of using fungal or enzymic pretreatments of annual plant materials, prior to alkaline pulping, has been reported (Giovannozzi-Sermanni *et al,* 1994a,b; Martinez *et al,* 1994). In fact, considerable improvements of paper strength properties were realized by using enzymic preparations, obtained from solid state cultures of *Lentinus edodes* (Giovannozzi-Sermanni *et al,* 1994b). In addition,

comparable technical results with the control, were obtained by reducing the times of refining by 30% (Giovannozzi-Sermanni *et al,* 1994a).

In spite of the increasing interest in solid state fermentation (SSF) processes, their extensive use in current industrial applications is not yet completely realized (Ramana Murthy *et al,* 1994). This situation can be ascribed to the difficulties in performing a reliable environmental control and the lack of efficient bioreactor design (Ryoo *et al,* 1991). As a consequence, several problems of oxygen, heat and mass transfer, as well as the development of concentration gradients inside the colonized biomass, affect the reproducibility and the performance of the bioconversions and, therefore, the economic results. As alternative to traditional stationary layer trays, various reactor types have been proposed in order to reduce the aforementioned problems by either mixing the substrate in rotating reactors (plowing, raking and tumbling motion) or percolating nutrients in packed tower reactors (Giovannozzi-Sermanni *et al,* 1993; Barstow *et al,* 1988; Ramana Murthy *et al,* 1994).

This paper points out the effects of some key-factors, including the internal gas phase composition and the mixing rate of the biomass, which significantly affect the performance of a solid state fermentation (Itavaara *et al,* 1994; Saucedo-Castañeda *et al,* 1992), with the white-rot fungus, *Lentinus edodes,* in a tumbling drum bioreactor. In addition, few main results concerning the upgrading of the biopulping of wheat straw for the paper production are briefly reported.

MATERIALS AND METHODS

BIOREACTOR DESIGN

Significant attention has been devoted to bioreactors design for SSF, especially for controlling and recording of the growth conditions. Two kinds of bioreactors have been developed (Giovannozzi- Sermanni and Perani, 1987; Giovannozi-Shermani *et al,* 1994a), i.e., a 25 L lab-bioreactor, involving a glass cylinder leaned on two rolling axes with rubber rings, which permit the rotation of the glass cylinder at different speeds. The bioreactor is covered with a polystyrene box and the temperature can be maintained at 28°C, using resistors controlled by a vertex thermometer. The exhaust air coming out from the bioreactor is periodically sampled and monitored, through an infra-red detector for CO_2 determination and a

paramagnetic device for oxygen determination (Fig. 1). The values are recorded every 30 min and CO_2 concentration is maintained constant around a prefixed value by a pump, driven via a RS-232 serial interface by a control software.

For a pre-industrial simulation, a pilot bioreactor consists of a stainless-steel cylinder (150 cm i. d., 400 cm long), closed at both ends by two stainless-steel lids with o-rings, to ensure a perfect sealing (internal volume about 3.4 cubic meter), which can be charged with 700-800 kg lignocellulose mass (Giovannozzi-Sermanni *et al,* 1994a). The temperature is maintained through an external thermostatted water jacket.

MICROORGANISM AND INOCULUM PRODUCTION

Lentinus edodes Berk Peg., strain SC-495, was used for this study because of its selective lignin-degrading capability (Giovannozzi-Sermanni *et al,* 1994b). The inoculum was produced, as described previously (Giovannozzi-Sermanni *et al,* 1990).

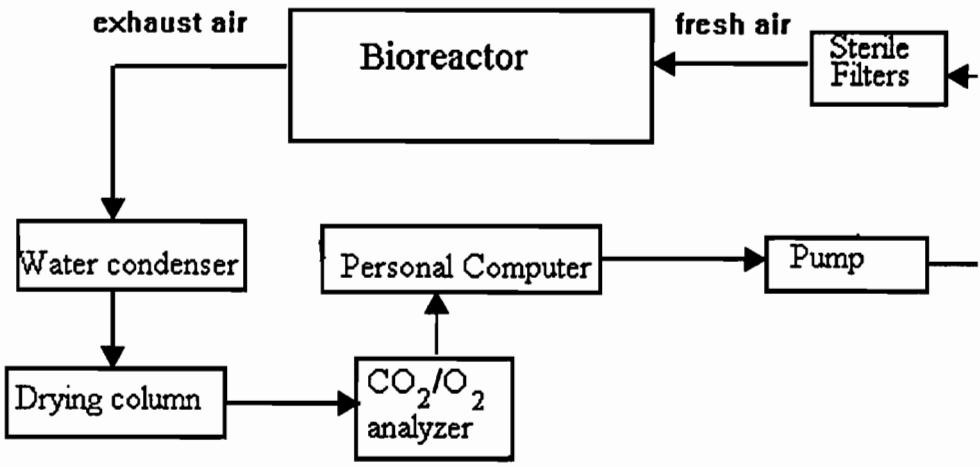

Fig. 1. Schematic representation of the system developed for on-line automated monitoring/control of exhaust gases in aerobic solid-state cultures. Pump is activated by a PC via RS-232 serial interface, when the CO_2 concentration in exhaust air is higher than a prefixed level.

SOLID STATE FERMENTATION CONDITIONS

A liquid salt medium (Giovannozzi-Sermanni *et al*, 1994a), supplemented with 2% malt extract, was added to wheat straw (cut into 5 cm pieces), to obtain a final moisture content of 75%. After 1 h steam-sterilization, the substrate was inoculated aseptically and incubated at 30°C for 10 days. Some growth experiments were performed at different CO_2 concentrations (3, 5 and 9%), which were kept constant by using the above described monitoring/control system, whereas oxygen concentration was allowed to fluctuate freely. The O_2 consumption was determined every 30 min, by calculating the differences of its concentration in fresh and the exhaust air. The respiratory quotient was obtained by dividing CO_2 production by O_2 consumption.

A prefixed CO_2 concentration of 5% was selected, to confirm the effects of different mixing rates of the solid substrate (4, 8, 16, 32 rev.day-1). At the end of the selected fermentation period, a representative sample of the colonized biomass was used for chemical analysis, and a sufficiently enough aliquot was squeezed by a hydraulic press at 350 atm, for obtaining crude enzyme solution.

CHEMICAL AND BIOCHEMICAL ANALYSIS

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined, according to the procedure of Van Soest (1963). Hemicellulose and cellulose amounts were calculated from the fractions obtained by this procedure, i. e., NDF-ADF and ADF-ADL; respectively. The determination of phenolic hydroxyl group content was performed by UV and 1H-NMR spectroscopy, as described by Faix *et al* (1992). The chlorine number was estimated by TAPPI 611 method (TAPPI, 1991).

The crude enzyme solution, after filtration through glass wool and centrifugation at 11,000 g for 30 min, was tested for the following enzyme activities: laccase (E.C. 1.10.3.2), according to Haars and Hutterman (1980), endo-cellulase (E.C. 3.2.1.4) and exo-cellulase (E.C. 3.2.1.91) according to Shewale and Sadana (1978), endo-xylanase (E.C. 3.2.1.8), according to Mc Carthy *et al* (1985). Mn-dependent peroxidase was assayed by the method of Lundell *et al* (1990). Boiled enzyme solution was used as control.

ENZYMATIC PRETREATMENTS

The enzymic preparations from *Lentinus edodes* solid state cultures, were added to the raw plant material at a 5:1 (v/w) ratio, in order to have a free liquid phase in equilibrium with the solid material. The enzymatic charge, referred to 1 kg dry weight, was as follows: 5000 units of phenoloxidase, 125 units of endo-xylanase, 75 units of endo-cellulase, 12 units of exo-cellulase and 3 units of Mn-dependent peroxidase. No hydrogen peroxide and Mn(II) additions were made, taking into account the low levels of Mn-peroxidase. The enzymic pretreatments were carried out at 40°C, in the above described lab-bioreactor, which was turned at 64 $rev.day^{-1}$, in order to obtain homogeneous conditions. Furthermore, air was purged in the bioreactor, in order to support phenoloxidase-catalyzed oxidative reactions. Appropriate controls were performed with heat-inactivated enzyme preparations.

PULP AND PAPER PREPARATIONS

Control and biotreated samples of durum wheat straw (*Triticum durum* L.) were cooked at 120°C for 30 min,. in a laboratory rotating digester (Lorentzen and Wettre, Sweden) in the presence of 16% NaOH (related to the pulp weight) and then beaten in a conical refiner (Toniolo, Italy) for 10, 20, and 30 min. The paper handsheets were obtained by the TAPPI 205 om-88 method, the freeness was determined by the TAPPI 227 om-85 method, while the breaking length and the burst index by the TAPPI methods 404 om-87 and 403 om-85 (TAPPI, 1991),respectively.

RESULTS AND DISCUSSION

MYCELIAL GROWTH

CO_2 concentration significantly affected the respiratory quotients (Fig. 2). In addition, the extent of plant cell wall degradation also appeared to be heavily affected.

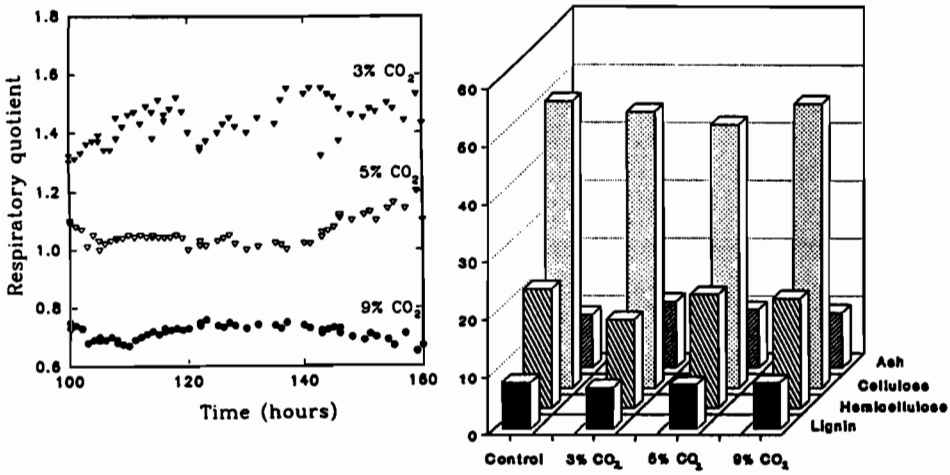

Fig. 2. Respiratory quotients of *Lentinus edodes*, grown under SSF conditons on wheat straw, at different CO_2 concentrations (3, 6, 9%) (left panel). Cell wall composition of wheat straw was determined after 10 days of SSF (right panel). The values for hemicellulose, cellulose and lignin are for 100 g dry weight.

In fact, the reduced mycelial growth, which was observed visually at 9% CO_2, has also reflected in a decreased biodegradative ability. The best results were obtained in the presence of the lowest level of this gas, i.e, at 3% (Fig. 2). The results observed could be due to the fact that the automated monitoring/control system of CO_2 and O_2 can maintain the culture under non-limiting conditions, with respect to O_2 concentration. Furthermore, this contributes to reduction in biomass and temperature gradients, in adition to the elimination of volatile products generated during the fermentation (Saucedo-Castañeda *et al*, 1994). Apart from these considerations, the organism under study appeared to be rather sensitive to carbon dioxide (White and Biddy, 1992; Zadrazil, 1975), taking also into account that the growth of other species, such as *Pleurotus* spp., appeared to be directly stimulated by concentrations up to 30% CO_2 (Zadrazil, 1975). Moreover, other fungi, exposed to high CO_2 concentrations (up to 60%), appeared to be rather tolerant to CO_2 (Levonen-Munoz and Bone, 1985). An interesting correlation between the ecological roles of these fungi and their distinct tolerances to environmental factors, including CO_2, has been suggested, (White and Boddy, 1992). In fact, *P. rufa*, a primary colonizer, was more tolerant to CO_2, than *P. radiata* and *C. versicolor*, which are ubiquitous invaders of already attacked decomposing wood. Therefore, the poorer

tolerance of *Lentinus edodes* could be explained by its later appearance in fungal communities decaying wood (Han *et al,* 1981).

One common feature of SSF is the gradients in several parameters (temperature, biomass, moisture) within the colonized substrate. It has been suggested that the mixing of the substrate could somehow contribute to reduce the occurrence of these gradients (Giovannozzi-Sermanni and Perani, 1987; Ramana Murthy *et al,* 1994). At the same time, the use of filamentous fungi impose serious probems, especially in the rotating conditions (Giovannozzi-Sermanni *et al,* 1990).

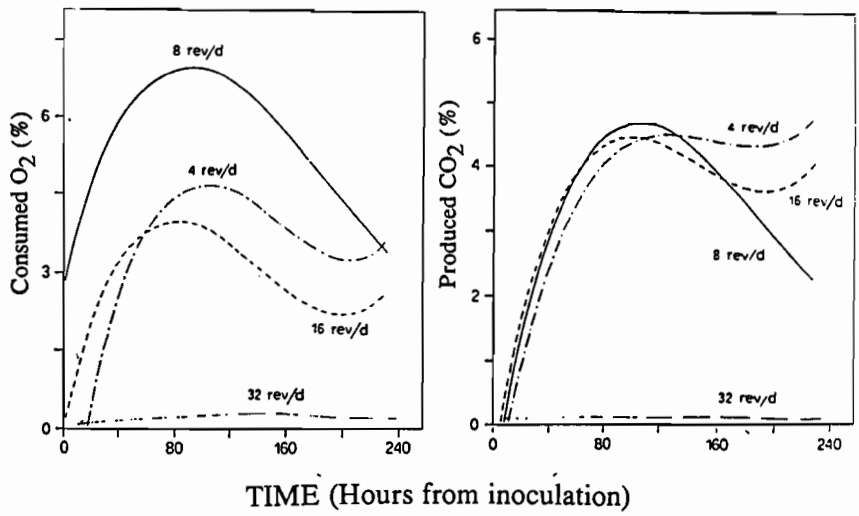

Fig. 3. CO_2 production and O_2 consumption by *Lentinus edodes* during SSF of wheat straw at different mixing rates of the solid substrate (4, 8, 16 and 32 rev·day^{-1}).

In fact, the abrasion, due to the tumbling of the substrate, can damage the hyphal structures of the mycelium. The damaging effect, due to the tumbling, appeared to be dependent on the microorganism employed (Giovannozzi-Sermanni *et al,* 1990, 1991). Results in the above described bioreactors revealed that only slow mixing rates allowed mycelial growth. In fact, the growth of *Lentinus edodes* appeared to be blocked with the use of rotation at 32 rev.day^{-1}, (Fig. 3). Different revoltion rates also seriously affected the metabolism of the cells, as well as phenol-oxidase isoenzymes release (Giovannozzi-Sermanni *et al,* 1993). Recently, a novel type of solid-state bioreactor, called rocking drum reactor, has been proposed (Ryoo *et al,* 1991), where the rocking motion in the reactor allows an even distribution of air and

water in the susbtrate, without disturbing the growing mycelium. This fermentor, equipped with a computerized temperature/moisture control, seems to reduce the drawbacks ascribed to hyphal damages, which often occur in conventional rotating bioreactors. However, some perplexities have been posed to its economic feasibility, since it involves sophisticated reactor fabrication and complex control systems (Ramana Murthy *et al,* 1994).

BIOLOGICAL PULPING AND CHARACTERISTICS OF WHEAT STRAW PAPER

Enzymic treatments of wheat straw, prior to pulping, were emphasized, since these are less time-consuming, than those based on the mycelial colonization. In addition, the dry weight losses were obviously of a lower magnitude in the former case. Such pretreatments, apparently did not produce remarkable effects in terms of lignin degradation, whereas a consistent attack on the hemicellulosic fraction was observed (Fig. 4).

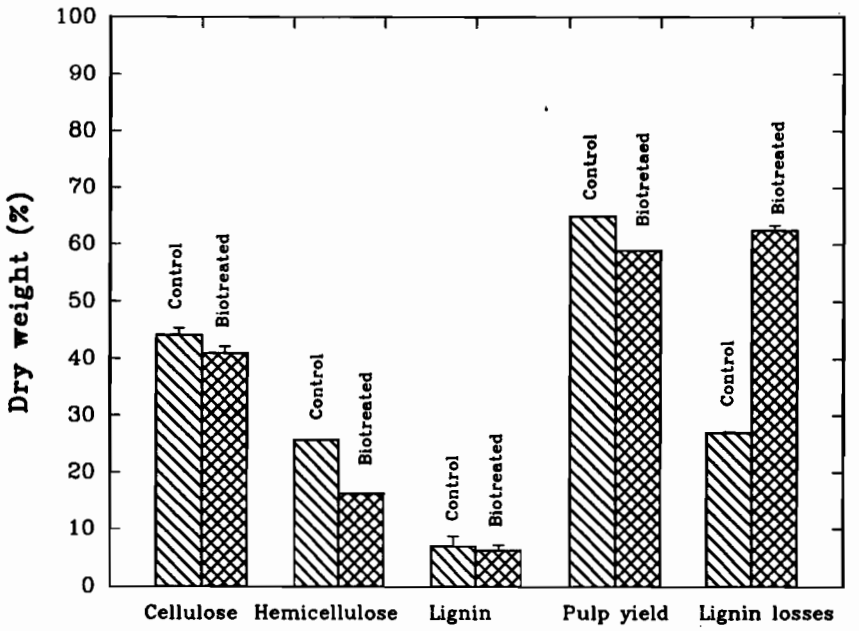

Fig. 4. Cell wall composition of control and enzyme treated wheat straw. Pulp yield and lignin extractability were determined, after alkaline cooking. All the values are for 100 g dry weight and the means of three determinations.

However, enzymic treatment of straw resulted in several positive effects. In fact, an enhanced lignin extractability was observed in the treated samples, after alkaline cooking, probably due to an increase in porosity of the material, which in turn improved the accessibility of the cooking liquor. Furthermore, such effect could also be ascribed to the increase in the phenolic hydroxyl content, which was observed in the treated samples. In fact, the phenolic hydroxyl-methoxyl ratio increased in treated samples by about 40%, thereby resulting in an enhanced alkaline delignification rate (Evstignevev *et al*, 1992). Such incorporation of the hydroxyl group in the aromatic ring is not surprising, as the phenol-oxidizing enzymes of *Lentinus edodes* also include tyrosinase and methoxyl cleaving activities (Leatham and Stahmann, 1981; Crestini and Giovannozzi, 1994). Significant improvements of some technical properties, including breaking length, burst index and tear index, were also observed in the enzyme-treated samples (Fig. 5).

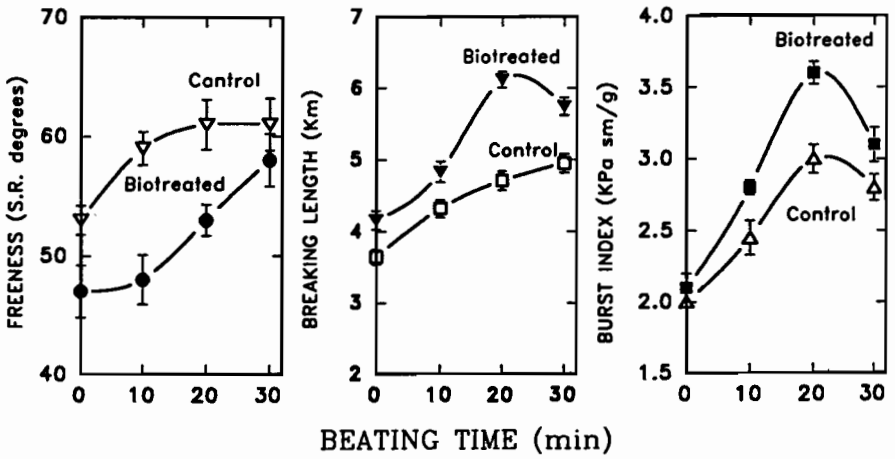

Fig. 5. Freeness and technical characteristics of paper handsheets, obtained from control and biotreated wheat straw. The data of breaking length, burst index and freeness are referred to different lengths of refining time (0, 10, 20 and 30 min). The data are the means of three determinations and the vertical error bars represent standard deviation.

Hence, the biotreatments appear to improve the fiber-bonding characteristics, which occur in chemical pulping. The greater extent of delignification observed, after alkaline cooking in the biotreated samples, could have resulted in an enhanced

cellulose to cellulose bonding and, therefore, in significant improvements in terms of paper strength properties. In addition, it has been suggested that an increase in carboxyl and hydroxyl groups, could be a further reason for the increased strength parameters of fungal treated materials (Setcliff *et al,* 1990). Fig. 5 also shows that the freeness of pulps reduced to that of the biologically untreated controls. Such reduction, which was also observed with corn straw (Giovannozzi-Sermanni *et al,* 1994b), could allow a substantial gain in drainage of pulp, and therefore, in the paper machine speed. Pommier *et al* (1989) claimed that the enzymic mixtures, containing cellulases and hemicellulases, can improve the drainage properties, which are essential in case of recycled paper. They suggested that the enzymes act on the surface of the fibers, thus producing the so-called peeling effect. It results in the removal of components, with very high affinity for water, which do not contribute to the hydrogen bonding potential of the fibers (Sabharwal *et al,* 1994).

CONCLUSIONS

The main points, which are of interest in the development of the biopulping methodology of wheat straw, include the design of special SSF bioreactors, the possibility to turn the lignocellulosic mass at different rates, and the effects of CO_2 content during the bioconversion process. *Lentinus edodes* appears to be rather sensitive to CO_2 concentration and this was particularly evident at 9% of this gas, due to the low substrate colonization, the reduced biodegradative ability and the low R.Q. values. The mixing of the substrate, which helps in homogeneous fermentation conditions, can be easily performed in a tumbling drum bioreactor, but a careful choice of the mixing rate should be made, in order to avoid growth inhibition phenomena, mainly due to mechanical damage to the hyphae. The combination of enzymatic pretreatments with conventional alkaline pulping resulted in several positive effects on some technical properties (freeness, breaking length, burst index) of pulps and paper handsheets. It is worth noting that such effects were not predictable on the basis of the cell wall composition, but the biotreatments determined physical and chemical variations, which were evident after chemical pulping.

ACKNOWLEDGEMENTS

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Use of earthworms for reintroducing of organic matter from towns: A restoration of natural cycle by a new ecotechnology Features and efficiency of the first industrial unit

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ABSTRACT

By exploiting ecosystems, the human activity globaly results in garbage, i. e. waste organic matter mixed with inert components and various pollutants.This causes.environmental problems and dictates the restoration of the natural cycles of organic matter. Earthworms have the capacity to ingest organic components and to defecate calibrated matter. In synergy with micro-organisms acting before ingestion, during digestion and after defecation, earthworms allow an advanced maturation of the organic matter (C/N < 15) within a rather short time (< 3 months).Therefore, a new technology, using *Eisenia andrei*, has been developed for industrial application. It consists 1) in an opening of garbage bags, 2) a grading of the matter, 3) various mechanical and manual sortings, 4) a sanitization process by an aerobic thermophilic fermentation at *circa* 75° C, killing weed seeds, most pathogenics and grubs, 5) the vermicomposting phase and 6) the final refining phase. It allows efficient sortings and refinings. The various vermicomposts produced by this way for agricultural, horticultural or domestic uses provide a real improvement to soil fertility. On these principles, the first treatment unit making use of soil animals was built in 1991 and is in operation. Operational results are presented here.

Keywords: Vermicomposting, earthworms, *Eisenia andrei*, solid state fermentation, house hold waste, sanitization, microorganism, recycled material, compost, pollution, waste disposal.

RESUME

Utilisation des lombriciens, pour valoriser la matière organique issue des villes. Un rétablissement ces cycles naturels par une nouvelle ecotechnologie. Caractéristiques et rendements de la première réalisation industrielle.

BOUCHE M.B. ET NOUGARET M.

Les activités humaines, utilisants des matières premières issues d'écosystèmes, produisant *in fine* des déchets sous forme de mélanges variables de matière organiques et inertes additionnées de divers contaminants. Ces déchets causent différents problèmes environnementaux et obligent à rétalbir les cycles naturels des matières. En co-action avec les microorganismes agissant avant ingestion, en cours de digestion et arpès défécation, les lombriciens permettent une rapide (< 3 mois) bonne maturation (C/N < 15) de la matière organique. L'application industrielle utilise *Eisenia andrei* et porte sur six étapes: 1- ouverture des sacs d'ordures, 2- tamisage puis 3- triage physiques complétés namuellement des déchets, 4- hygiénisation par fermentation thermophile (= 75°C) réduisant les maivaises graines, insectes et germes pathogènes, 5-lombriculture formant le lombricompostage et 6- affinage des produits. La production de diverses qualités de lombricomposts à usages horticoles et agricoles contribue à la fertilité des sols. L'article présente les résultats opérationnels de la première unité industrielle construite en 1991 et utilisant les lombriciens en biotechnologie.

Mots clés : Lombricompostage, lombriciens, *Eisenia andrei,* vers de terre, fermentation en milieu solide, ordure ménagère, hygiénisation, microorganismes thermophiles, matériaux recyclés, compost, lombricompost, pollution, traitement des déchets.

INTRODUCTION

Generally speaking, humans, in the exploitation of forest, meadow or cultivated ecosystems, remove organic matter (wood, meat, plants) in huge quantities. As it is insufficiently recycled back into soils, this organic matter soon becomes deficient in the ecosystem and this leads to leaching, slatching, a decrease in water-holding capacity as well as erosion.

In order to restore the natural cycle of organic matter, it is necessary to improve the transfer back into ecosystems. This cycle restoration allows the reintroduction of biogenic elements (N, P, K, Mg, etc.), the biological properties of organic matter (carbon compounds), which are essential for microbial and animal life in soils, and the resulting soil physical qualities.

The raw materials removed from fields by humans are transformed into food, packaging or technical products, such as paper. Most of these products and the leftovers are thrown away after use as urban waste, which, contains large quantities of inert components (glass, scrap iron, plastics, rubble) and various pollutants (inks, batteries, pesticides, medecines, etc.) (Table 1).

Table 1. Composition of household waste in France (Anred, 1991)

Category	Gross Weight (%)
Decomposable material	25
Paper and cardboard	30
Rags	2
Plastics	10
Metals	6
Glass	12
Miscellaneous (incl. medecines, batteries)	15

The great diversity in this rather complex mixture makes it difficult to recycle the organic part of domestic waste back into ecosystems. The traditional methods of treatment (landfilling, incineration and composting) do not allow for a complete recycling. These also do not take into account, or do so only partially, the environmental requirements of all types, such as a) physical : volumes of landfill tips, b)chemical : carcinogenetic chemicals, heavy metals, emissions of methane gas and carbon dioxide which contribute to the greenhouse effect, offensive-smelling gases, c) biological : pathogenic bacteria, insects, weed seeds, and d) social : implantation, costs.

In such a substrates, which are extremely diverse in nature, earthworms have the capacity to sort out and consume the organic part (peelings, leftovers, paper and cardboard). Amongst epigeous species of earthworms living in almost pure organic

milieus, forest litter or decaying bark of dead trees are the best candidates. For practical and empirical reason, *Eisenia andrei* Bouché 1972 was the most efficient species and finally selected. In synergy with microorganisms, they ingest, digest and produce an advanced maturation of organic matter (C/N < 15) in a relatively short time (< three months). Inert elements, which are not destroyed, are easily separated from the graded earthworm faeces and can then be mostly recycled.

Taking this observation as a starting point, and in order to make the best possible use of these properties, it was necessary to design a completely new technology, which 1) avoids any sort of crushing (this makes the inert matter unreclaimable and contaminates the organic matter) and 2) allows for a continuous control of contaminants. The end result was the NATURBA process, which is in operation at present. A description of the process, including its environmental assessment as well as material balance are presented in this paper.

EARTHWORMS, ORGANIC MATTER AND WASTE

The important role played by earthworms in ecosystems, as the main protagonist in the health of soils, through their physical activity in breaking up soils and their biophysicochemical activity in recycling organic matter, was highlighted at a very early date (White, 1777, as quoted by Russel, 1910; and Darwin, 1837). The use of earthworms for the processing of organic matter was advocated (Barret, 1949) for manure heaps and various types of plant waste, while the high-quality of matter obtained was demonstrated by Anstett (1952). This technique was, however, not suggested for use on domestic waste before 1976 (Bouché, 1979). The enhancing of organic matter is what is sought after for this type of substrate (Bouché, 1982, 1987; Fayolle, 1982) containing contaminants (Table 1), as opposed to the production of earthworms (Chaudonneret, 1977) which comes up against the problem of contamination (Bouché, 1979).

The fundamental technical aspects were rapidly determined (Bouché, 1979, 1982, 1987; Fayolle, 1982; Chaudonneret, 1977), but their application to small units (Straumann, 1988) could not be adapted on an industrial scale, which must be simultaneously of scientific, technical, social and environmental types (Bouché, 1987).

All these aspects led to the perfecting of the NATURBA process, developed by the SOVADEC Company. It was tested and experimented during 1988-1991, commercially launched in 1990 and took shape with the building of the first unit in La Voulte-sur-Rhône (France) in 1991.

The NATURBA process uses both physical and biological methods. The requirements of -microorganisms and earthworms, the biological actors- determine the general plan of operation and the biological methods used.

THE BIOLOGICAL METHODS

The direct treatment of fresh waste, using earthworms, turned out to be negative as these creatures do not stand up to the heat given off by the aerobic fermentation. Earthworms require living conditions, which are incompatible with the destruction of undesirable organisms (insects, pathogenic bacteria or weed seeds). Therefore, these two stages had to be separated both in terms of in time and space into bisanitization and vermicomposting.

BISANITIZATION

The conditioned waste, resulting from the initial stages of sorting, undergoes a process using thermophilic aerobic microorganisms. By breaking down the most heat-labile organic parts, these microorganisms ensure a significant rise in temperature to 70-75°C, if good ventilation is ensured in heaps. This destroys most pathogenic microorganisms, weed seeds and grubs.

Simustaneously, microorganisms produce different antibiotics, which act on plant and animal pathogens, during the succession of psychrophilic, mesophilic and thermophilic microflorae. Hence, the term bisanitization has been derived for this association of two mechanisms of sanitization, through temperature and antibiotics. The bisanitization consumes oxygen and water to produce carbon dioxide and steam. A constant control of the water and oxygen supplies is absolutely essential.

As the heat-labile organic part in the matter decreases, both microbial activity and the temperature are reduced. Simultaneously, the quality of the organic matter is modified and it becomes, after 4 to 6 weeks, soft and partially composted. It, however, remains insufficiently mature for agronomic use and hence, earthworms must be brought into action.

BIOLOGICAL SORTING AND ORGANIC MATURATION THROUGH THE USE OF EARTHWORMS

After bisanitization, the matter can be ingested, digested and rejected as graded faeces by *Eisenia andrei.* These creatures require good aeration and plenty of moisture (pF = 2.7), temperatures which are compatible with their requirements (25--30°C) and a constant food supply.

The earthworm's primary function is, by selection of organic food only, to allow a complete separation of organic matter from inert matter, thereby making there fractions cleaner and more acceptable. Their second function is their digestive actions. Breaking up, together with the complex action of the enzymes of the digestive tract and microorganisms, leads to the production of a mature, stable, homogeneous organic matter.

The separation of inert matter from the organic part involves, both before and after the biological treatment, physical sorting without any crushing, which otherwise would pollute the organic part and would make the inert matter unreclaimable.

GENERAL PLAN OF OPERATION AND METHODS USED

The NATURBA process is structured around four successive blocks (Fig. 1), as described below:

After reception from collecting trucks, the urban waste from selective or non-selective collection goes through a specific patented machine, the Autoselecteur, which ensures 1) the opening of plastic bags by thermofusion without any sort of crushing or breaking up and 2) sifting of the articles smaller than 160 mm, thereby separating the rubbish in two fractions.

Large articles (over 160 mm), approximately 17% of the arriving tonnage, are sorted manually, so as to take out rags, clean papers and cardboards, plastics and metals for enhancing their value. This sorting aims to eliminate certain cumbersome articles such as tyres or car batteries. Other elements (branches, large cardboard boxes) are broken up mechanically (= the brocken fraction).

The graded part (< 160 mm) is subjected to a magnetic field to eliminate scrap iron. Then rolling objects, as bottles, are eliminated by automatic sorting using a stick-bounce machine. These rolling objects are sorted manually in order to recover glass, PVC and PET bottles and aluminium. The main part constitutes, with brocken

fraction, the conditioned waste (about 72% of the initial tonnage). It is transferred into the bisanitization block.

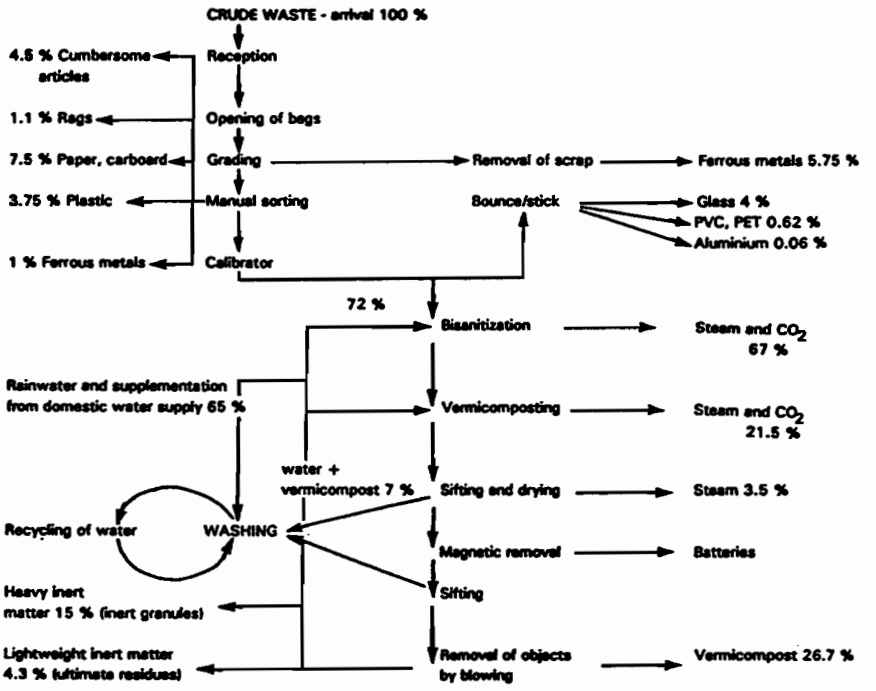

Fig.1 : General plan of operations and weight balance of the process. Notice they are two entries (crude waste 100 % and water supply 66 %). All values are expressed as % of the crude waste processed.

BISANITIZATION

The conditions required by aerobic microorganisms led to the conception of mechanical methods which allow oxidation of the substrate above the threshold at which anaerobiosis occurs. An automatic grab carries out a daily ventilation of the matter by turning over the heaps as they are moved along for 40 days. A sprinkler system compensates for the loss of water as steam due to the temperature rise.

Enforced ventilation of the building brings in the oxygen necessary for the respiration.

The daily turning over and ventilation are carried out according to a sequence which has been decided upon empirically. Each heap is moved along in small successive quantities and turned over in such a way as to bring what is at the bottom to the top. Each dose is allowed a pause so that it can be in air contact for at least five minutes. This daily turning over also prevents excessive compaction of the matter, which would reduce air circulation.

Each tonne of conditioned waste uses between 650 and 800 litres of water (depending on the season and the composition of the domestic waste) according to the Table 2. The water is added by sprinkling so that it has time sufficient enough to get absorbed.

Table 2. Water consumption *versus* time, during the bisanitization (for a total of 750 litres)

Age (days)	Water Supply (L/ton)
0	90
4	100
9	120
14	120
17	85
21	75
26	60
31	50
36	50

Measurements have enabled to establish that 25% of the mass is lost in the form of CO_2 ; two thirds of this during the bisanitization phase. This necessitates, in theory, to bring in 3 cubic meters of air per hour per tonne of dry matter to keep the building's CO_2 content in the air to below 5% (the equivalent of human respiration). In practice, a safety margin has been adopted and the ventilation carries 15 cubic metres of air per hour per tonne of dry matter.

Under these conditions, thermic evolution (Fig. 2) ensures sanitization of the matter by a rise in temperature to 70-75°C (155-170°F). After several days, a drop temperature is needed for efficient functioning of the next stage of vermicomposting.

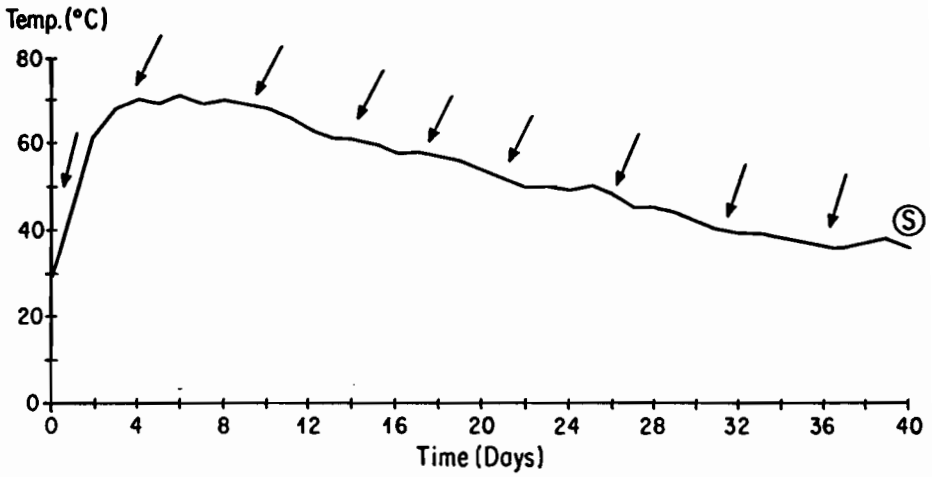

Fig. 2 : Evolution of the mass temperature during the bisanitization phase. Arrows indicate water supply to compensate evaporation. S : steeping.

BIOLOGICAL SORTING BY VERMICOMPOSTING

Different species of earthworms belonging to the ecological category of epigeics (Bouché, 1971) were tested. They were already known to be particularly well-adapted to this type of substratum. *Eisenia fetida* (Savigny, 1826), *Eisenia andrei*, (Bouché, 1972) and *Eudrilus eugeniae* (Kinberg, 1867) were found to be the most efficient. Although their biological requirements were closely related, *Eisenia andrei*, the most prolific, was chosen as it allowed a greater flexibility in use. Its vital requirements concerning moisture and heat were defined as per the methodologies used by (Fayolle, 1982) and (Reinecke and Venter, 1987).The means of acquiring them were studied and these studies led to the perfecting of the lombricontrôleur.

The vermicomposting is conducted in a building in which temperature, relative humidity and ventilation are controlled so as to obtain the best earthworm activity. Ventilation is 7 cubic metres of air per hour per tonne of dry matter and relative humidity is maintained between 65 and 100%. The temperature of the air has, in fact, only an indirect influence, as the temperature of the matter is very important and must not exceed 29°C, the upper temperature tolerance limit of *Eisenia andrei*.

The waste is managed in quantities of equal volume, moistened and cooled by soaking, when it comes out of the bisanitization block and then spread in successive layers in separate lombricubateurs, where vermicomposting takes place. The

lombricubateurs are metallic containers operating continuously, the matter is poured in at the top and emptied from the bottom as per the patented mechanical extractor. The matter remains in the lombricubateurs for two months, moving slowly from top to bottom.

The earthworms reproduce, colonize and ingest this matter in lombricubateurs (Reinecke and Viljoen, 1990). They move against the current of the matter, moving endlessly up towards the freshly-added layers at top. The earthworms tend to over-reproduce but population growth is limited by food supply and remains well-balanced. After two years of the operation, it has not become necessary to introduce an extra supply of earthworms. The living requirements of earthworms have determined the size of the lombricubateurs, a width of 85 cm should not be exceeded if problems of asphyxia and increase in temperature in the centre of the lombricubateurs are to be avoided. On the other hand, their excellent capacity to move upwards allows the use of units which are 4.50 m high and 4 m long. Each lombricubateur is made up of four compartments of a total volume of 31 cubic metres.

This method of operation in separate units enables to cope up with possible problems of accidental contamination, which would only affect one unit without upsetting the others. Removal, after the two biological phases (bisanitization and vermicomposting) totalling to about 100 days, allows to obtain an unrefined vermicompost (vermicompost plus a quantity of small inert elements) which is then dried and refined.

SORTING AND REFINING

After drying at a moderate temperature (< 60°C), the unrefined vermicompost undergoes refining by different physical process, thereby permitting the separation of vermicomposts from batteries (and in particular all button batteries) and final waste.

A double sifting operation (50 mm and 7 mm) produces three different parts, i. e., a) inert matter contaminated by organic traces (> 50 mm), b) inert matter containing heavy metals (in the form of batteries) of between 50 mm and 7 mm, and c) organic matter vermicompost with inert matter (< 7 mm).

Part b) is subjected to a high magnetic field, which is highly efficient in its action on the graded substrates, all batteries, including button batteries are removed intact as they have not undergone crushing or breaking.

The rest of part b) rejoins part a) and then undergoes washing and decanting. This draws off the residual organic matter (in the form of sludge), thereby leaving behind the heavy inert matter which is recycled in granule form and lightweight inert

matter. The latter, together with the lightweight inert matter, which has been blown out of part c), make up the final residue.

The quality of vermicomposts obtained is constantly monitored, especially concerning maturity (C/N), physico-chemical and agronomic characteristics and content of macro-elements and oligo-elements (Mg, Mn, Ca, etc) as well as heavy metal, in order to comply with the strictest acceptable levels pescribed by of North European countries (Table 3).

Table 3. Characteristics of vermicompost and compost regulations.

	Physical Characteristics	Standard (France)		
pH	7.5	none		
Organic matter *	31.8	>10		
Water content *	80.8	none		
Water capacity *	150.0	none		
Macroelements *				
C	15.9	none		
N	1.2	<2		
C/N	13.2	<20		
P(P_2O_5)	0.9	none		
K(K_2O)	1.5	none		
Ca(CaO)	11.9	none		
Oligoelements		Limits in compost		
Heavy metals (ppm DM)		F	B	CH
Na	2781	none	none	none
Mg	5392	none	none	none
Mn	314	none	none	none
Zn	391	none	1000	500
Cu	102	none	100	150
Pb	150	800	600	150
Hg	1.9	8	5	3
Cd	3.0	8	5	3
Ni	32.5	200	50	50

* % of dry matter ,F : France ; B : Belgium ; CH: Switzerland, DM: dry matter

ASSESSMENT

MATERIAL ASSESSMENT

The factory in La Voulte-sur-Rhône has been in operation for more than two years. The values reported are the results of the first eleven months of operation (Table 4) during which the unit treated a total of 8200 tonnes.

Table 4. Material assessment (percentages of the initial gross tonnage).

RECYCLED RAW MATERIALS	
Ferrous metal (scrap iron)	6.7
Non ferrous metals	0.1
PVC, PET	0.6
Paper and cardboard	7.5
Rags	1.1
Glass	4.0
Plastics	3.7
Total	23.7
VERMICOMPOSTS	26.7
GRANULES FROM RECYCLING	15.0
BATTERIES	0.03
ULTIMATE WASTE	
Cumbersome articles	4,5
Residues	4,3
LOSS IN WEIGHT (through respiration)	25.5

ENVIRONMENTAL ASSESSMENT

The unit as a whole uses up water and so there is no liquid effluent (Fig.3) The gaseous exchanges are those of a biological respiration, i.e., using oxygen and producing steam and carbon dioxide. This CO_2 is given off in quantities and at a rhythm similar to that of natural humification in meadows or forests. There is, therefore, no excessive contribution to the greenhouse effect.

The contaminants are monitored by dosing the earthworm concentrations. Batteries of all types, the main source of heavy metals, are removed from contact with all other products.

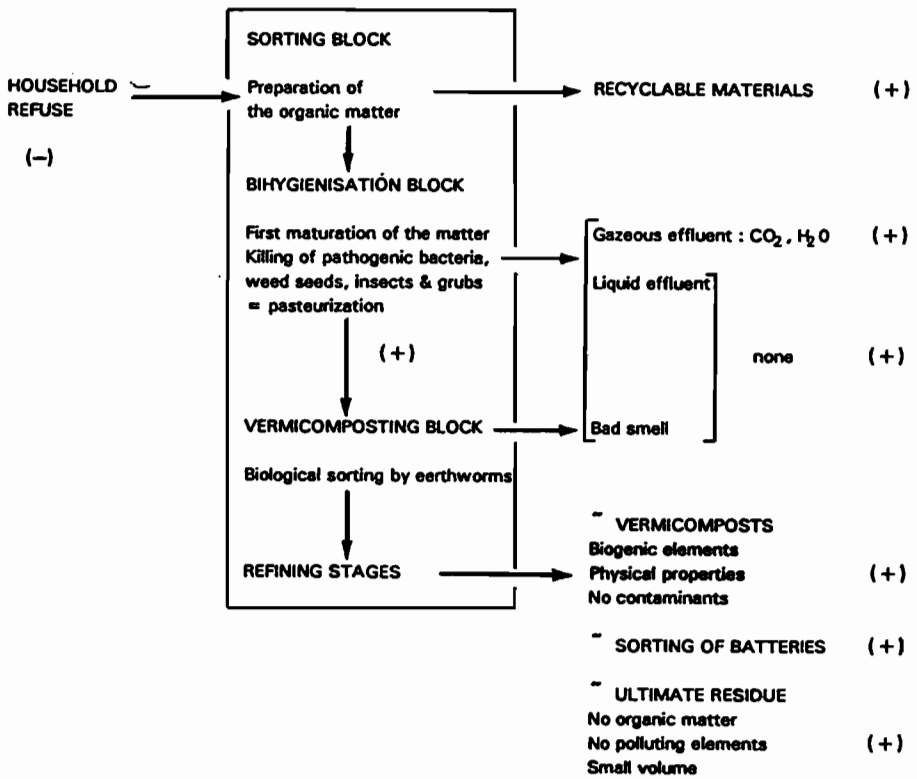

Fig. 3 : Environmental balance of the process : (-) negative aspects and (+) positive aspects

The ultimate remaining waste has to be treated in the traditional way (*e.g.* land filling), and only represents 20 % of initial tonnage and 3).% of volume. An additional fraction of granulated inert materials (15%) could be re-used by public works. The total balance is presented Table 4.

CONCLUSION

Social, commercial and industrial organisation of developed society has led to a break in the natural recycling of organic matter and biogenic elements. Consequently, it has been necessary to make up for)the lack of biogenic elements by using chemical fertilisers which very often turn out to be pollutant in use, and organic matter obtained by the exploiting of peat bogs, which often have to be closed down to preserve these fragile milieus. This has not prevented a deficiency of organic matter in soils, and leading to leaching, slatching, decrease in water-holding capacity and soil erosion.

The fact that earthworms have been replaced in their ecosystemic place within an industrial process conceived to that end, allows us to take advantage of the properties of these creatures, i. e., their capacity to sort and digest organic matter. These properties have made it much easier to find a solution to environmental management of problems associated with urban and ordinary industrial waste.

This very first industrial implementation in the world, using a soil-based creature, paves the way for other uses, especially for the recycling of agro-industrial waste.

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Biotreatment of wastewater sludge by solid state fermentation and its reuse : A case study in Spain

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SUMMARY

Wastewater sludge (biosolids) disposal involves high cost of sludge treatment, and its use in agricultural fields encounters risks, because of the high content of pathogens and lack of stabilization. Biosolids can be composted using several methods that improve its quality and reduce the risks. A channel-type semi-industrial plant for composting sludge with different materials available in the area was built in Felanitx (Majorca, Balearic Islands, Spain). Three-years results, obtained using different materials, the characteristics of the final product, and the profitability of the process are described. Sawdust, grinded straw and almond shells were the more convenient support materials. A market survey of the final product was also made during the time of operations. The plant was modified by covering the piling channel, because of occasional heavy summertime and autumn rains, which lowered the temperature of the pile and stopped the composting reactions.

Keywords: Wastewater sludge, biotreatment, composting, channel type composter, semi-industrial plant, solid state fermentation, support material, reuse, agriculture, regulations.

RESUME

Biotraitement des boues par fermentation en milieu solide : un exemple de compostage étudié en Espagne.

SALGOT M., FOLCH M., AMENGUAL A., CAÑELLAS N. ET CAUS J.M.

Un des principaux problèmes des stations d'épuration est la production de boues (biosolides) : le traitement des boues est onéreux et leur devenir pose problème. Une des solutions pour réduire à la fois le coût de traitement et l'impact environnemental des boues est de les valoriser. L'épandage agricole des boues peut provoquer des nuisances, notamment à cause des fortes concentrations en pathogènes et de leur manque de stabilisation. Pour augmenter leur qualité et réduire les risques et les nuisances on peut composter ces biosolides selon différentes techniques. A Felanitx (Majorque, Iles Baléares, Espagne) une unité de compostage semi-industrielle de type "tunnel" a été construite avec les matériaux-support disponibles localement. Après trois années de fonctionnement, nous présentons les résultats obtenus avec différents matériaux (paille, copeaux, écorce...) et les caractéristiques du produit final. Des améliorations ont été faites et notamment la construction d'un toit au dessus de l'unité de compostage. Il protège le compost des fortes chaleurs estivales et des pluies abondantes de l'automne qui provoquent une baisse de la température de fermentation et par conséquent son arrêt.

Mots clés : boues des stations d'épuration, biotraitement, compostage, compostage type tunnel, fermentation en milieu solide, pailles, copeaux, agriculture, station d'épuration.

INTRODUCTION

Among the problems related to the operation of wastewater treatment plants in Spain, biosolids or wastewater sludge management is of critical importance, because of a) the treatment difficulties inherent to the material, b) disposal problems, mainly due to presence of pathogenic microorganisms and c) cost of the operation.

The regulations allow the disposal of the biosolids, if these meet the specific requirements, into the sea, or on land (land application/reuse or sanitary landfills) and by incineration. Some of the possibilities for land disposal are as follows:

Wastewater sludge could be disposed of, with or without transformation, i.e., without modifications, after leaving the wastewater treatment plant (usually digested/stabilized sludge) or composting the product. The existing regulations for sludge's land disposal, without modification, for application to agriculture in Spain, provide the legal frame for sludge production, its use on land and the effective protection of agricultural environment. Main features of the regulations deal with heavy metal content and the soils and types of cultures, where sludge could or could not be applied. Land application problems have been posed, because of technical inexactness of the law regarding sampling, extraction procedures or analytical methods for soils and sludges, and the fact that other important parameters, such as CEC (cation exchange capacity), electrical conductivity, or Fe, Al, and Mn contents, are not taken into account (Cortés *et al*, 1992).

Regulations for composted material (biosolids) from wastewater state that a) the compost material must be obtained by controlled fermentation of organic wastes, and b) contain a minimum amount of organic N (1%) and total organic matter (75%); and a maximum amount of water (40%) and heavy metals, as shown in Table 1.

Table 1. Maximum amount (mg/kg) of heavy metals allowed in compost.

Metal	Level	Metal	Level
Cd	40	Zn	4000
Cu	1750	Hg	25
Ni	400	Cr	750
Pb	1200		

Regulations are far less restrictive for compost than for sludge. This allows a bigger market for compost, but it is advisable to combine the use of both materials, because of the transformation cost. This combination must be done, according to health protection criteria and economic considerations (Cabré and Salgot, 1990).

THE CASE OF MAJORCA ISLAND

Many (40) wastewater treatment plants were built during the last decade in Majorca island. As a result, an increasing amount of wastewater sludge is being produced. The island includes many important tourist resorts (Sampol and Salgot, 1993), which makes the production of sludge non-uniform along the time and the production especially concentrated in the vicinity of the coastline.

The possibility to compost sludge in the part of the island where the amount of support material is limited, has been studied. The little town of Felanitx (11,000 inhabitants plus 5,000 during weekends and in summertime), was chosen to construct a channel-type semi-industrial plant for composting sludge with different materials available in the area: grinded and ungrinded straw, sawdust, bark, dried *Posidonia*, yardwaste, woodchips, almond peels and urban solid waste. The three-years results, obtained using some of these different materials and the characteristics of the end product, are discussed in this paper.

MATERIALS AND METHODS

PROCESS

The composting was done using the channel-pile method, with a purposedly-built turning machine, equipped with a scooped-drum. The residence time of the material in the pile was established between 45 and 80 days, depending on the climate, the proportion of support material, the time of the year, and the need for selling the compost earlier. Twenty-five to 40 days composting was considered enough for fermentation and at least 20 for maturation.The machine turned the pile twice or three times a week, alternatively. The pile was aerated using air blowers. The aeration was programmed accordingly to the pile conditions (usually 15 min/h).During the passage of the mixture through the pile, temperature reached 60°C for at least 10 days. As occassional heavy rains were the hindrance to the composting process, the channel was covered, thus allowing a better control of the system.

SLUDGE

The activated sludge generated at the wastewater treatment plant of Felanitx was used. It was digested aerobically, thickened using a gravity device and dried using a belt press. Its composition is presented in Table 2. The plant processes 2,500 m^3/day of wastewater and produces around 900 m^3/year of sludge (20% dry matter).

Table 2. Composition of untreated wastewater sludge and compost

Parameters	Raw sludge[a]	Semi-composted. sludge[b]	Compost (sup. 1)[c]	Compost (sup. 2)[d]
pH	6.93	6.33	7.13	6.66
E.C. (dS/m)	1.80	0.92	0.60	1.52
Humidity, (%)	81	70	60	65
Organic matter, (%)	78	84	71	68
N_2 , (mg/kg)	45,000	21,300	35,900	21,446
Na_2O, (mg/kg)	2,336	2,665	1,631	4,142
K_2O, (mg/kg)	1,047	3,471	877	1,560
Fe, (mg/kg)	2,475	-	3,550	-
Cu, (mg/kg)	250	-	355	-
Zn, (mg/kg)	850	-	1,575	-
Mn, (mg/kg)	55	-	80	-
Cd, (mg/kg)	3	-	3,75.	-
Pb, (mg/kg)	160	-	240	-
Ni, (mg/kg)	15	-	28	-
Cr, (mg/kg)	25	-	73	-

[a] Raw sludge: Sludge obtained from the wastewater treatment plant. [b]Semicomposted sludge: Sample obtained in the middle of the pile. [c] Compost (support material 1): Sludge composted with wood chips. [d] Compost (support material 2): Sludge composted with straw. - : Not determined

BULKING AGENTS

Best composting procedure was obtained using grinded straw, sawdust, and grinded almond shell as bulking agents, either alone or in combination. Their physical characteristics are reported in Table 3. Other materials presented various problems. Dried *Posidonia* reached the end of the pile practically without transformation (this material was used without cleaning and grinding). Ungrinded straw did not mix with the sludge and the composting process did not start. Wood chips needed to be added in a relatively high proportion, which made the process more expensive. Some difficulties of material availability were experienced and the material did not degrade through the process. Yard waste needed a previous transformation with a crushing machine, not available at the plant neither on the island. Pine bark was not easily available on the island, due to a strong competition with other users, such as greenhouse growers. Urban solid waste was not useful, because of the bad quality of the end product, due to an inadequate separation during the process of selection.

However, the above cited efficient materials had also some disadvantages. Grinded straw performed well but needed to be processed in the plant. Sawdust was relatively expensive. Almond shells performed really well, giving excellent colour, homogeneity, and texture to the final product. The main problem was the seasonal availability of the product.

Table 3. Physical characteristics of bulking materials

	Density, g/cm^3	Humidity, %	Organic matter, %
Wood chips	0.06	17.5	98.9
Posidonia	0.08	59.8	69.6
Sawdust	0.20	30.0	98.0
Almond shells*	0.15-0.30	48.0-58.0	87.0

* Characteristics depend upon the time elapsed since collection and the season.

The second problem was the determination of the right proportion among sludge, bulking agent and humidity. Sludge:support ratios tested were between 1:1 and 1:5 (sludge:support). Mixtures were also used as a bulking agent and good results were obtained using 0.25+0.25+0.50 mixture of sawdust+almond shells+sludge.

COMPOST QUALITY

Results obtained are summarized in Table 2, together with the composition of raw sludge.

MARKETING

The first material produced was offered free of charge to local farmers, tree and flower nurseries, neighbours and to people who were interested in it. Later on, the compost was transported and offered free of charge to farmers in an agricultural area located 10 km away from the production plant. All the users were satisfied with the material and asked for more. The initial objective was to prospect the market and determine if the compost was competitive against similar products sold in the area, apart from introducing the material to the potential users.

During the second year the compost was sold at a nominal fee (2,000 ptas/tm = 15 US$/ton). The price was increased to 5,000 ptas/tm (38 US$/ton) during the fertilizing season. At present, it is sold at prices varying with the season.

Because the composting plant is experimental, the costs are extremely high (between 8,000 and 9,000 ptas/tm = between 60 and 70 US$/ton). Calculations show that, with an increase in the size of the plant by 4-5 times, the current production prices could decrease to 3,000-4,000 ptas/tm = 23-30 US$/ton).

Studies were conducted to determine if a market was available for a large amount of the product, and if it was advisable to increase the plant production and to establish an industrial type of composting facility. The result of this study was not conclusive, and showed that some marketing difficulties would be experienced, when the production is increased by 10 times.

CONCLUSIONS

The composting process in a limited environment, such as a Mediterranean island, presents different problems, such as the difficulty to find a material to maintain the process all year-round, a strong competition among materials used for land improvement, and a rapidly decreasing agriculture due to the competition with tourism. A market for the compost for use in gardening was found, although it is not clear yet, if it will absorb large amounts of the product. From a practical point of view, the more adequate support materials in the environment of Mallorca will be

sawdust, grinded straw and almond shells. There is some future for yard waste, if an adequate grinding machine is available. The initial results are promising and an improvement of the process as well as an enlargement of the facility are foreseen.

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Use of composted biosolids for tropical orchids growing : preliminary results

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SUMMARY

The majority of orchids with commercial value are epiphytic, and require growing media. The substrate needs to be highly aerative and supply a high degree of humidity to the roots. The material currently used is pine bark, but it has major draw-backs due to the necessity to change it every two years. A more long lasting substrate is of importance due to the slow growing rate of these plants and requirement of more than two years before the first flower appears. Hence, several mixtures of pine bark, zeolites and composted biosolids (wastewater sludge) were used as alternative growing media. The results of two years experiments using these media for the growing of *Phalaenopsis sp.* (a commercial orchid) are presented. The best results were obtained with the exclusive biosolids or use of a mixture of zeolite and biosolids.

Keywords : Solid state fermentation, activated sludge (biosolids), biosolids composting, use of composted biosolids, tropical orchids growing, *Phalaenopsis* sp., commercial orchid.

RESUME

Utilisation des biosolides compostés pour la culture d'orchidées tropicales: résultats préliminaires.

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La majorité des orchidées ayant une valeur commerciale sont des épiphytes ce qui rend leur culture difficile à cause du milieu nutritif dont elles ont besoin. Le substrat doit être bien aéré et doit apporter aux racines de la plante une humidité élevée.

Le substrat le plus communément utilisé est composé d'écorces de pin. Il montre certains inconvénients : il faut le changer tous les deux ans, pour éviter le pourrissement et parce que l'on ne peut pas y ajouter les nutriments nécéssaires à la plante. Une seconde raison pour laquelle il faut augmenter la durée de vie du substrat est que la croissance des orchidées est lente et plus de deux années sont nécéssaires pour obtenir des fleurs.

Nous avons utilisé différents mélanges d'écorces de pin, de zéolite et de biosolides compostés (boues de station d'épuration) comme milieu de culture. Nous présentons ici les résultats de deux années d'étude sur la croissance de *Phaelaenopsis sp.* (une orchidée commerciale). Les meilleurs résultats ont été obtenus en utilisant exclusivement des biosolides et un mélange de zéolite et de biosolides.

Mots Clés : Fermentation en milieu solide, boues activées (biosolides), biosolides compostés, utilisation des biosolides, culture d'orchidées exotiques, *Phalaenopsis* sp., orchidées commerciales.

INTRODUCTION

The family *Orchidaceae* is one of the most widespread in the vegetal kingdom, as more than 25,000 species are already known and new ones are being discovered every year. In addition, the growers are still obtaining large number of hybrids (Lecoufle, 1981; Williams and Kramer, 1983). The biggest and most beautiful flowers, the widest variety of genera and the maximum number of plants, appear in the tropical and subtropical ecosystems.

The tropical orchids are mainly epiphytes or litophytes, i.e., they live using other plants or stones as support, always with the root system exposed to the air. This is why the roots show special structures called velamen. These structures are formed by

thick, spongy layers of dead cells, which surround the roots and allow a more effective retention of air humidity, rainwater and its nutrients. Nevertheless, there is a group of terrestrial tropical orchids capable of growing in well aerated and non compacted soils.

The tropical orchids have two typical growth patterns. The sympodial plants, like *Cattleya*, are characterized by the production of individual buds of finite growth. The monopodial orchids, like *Phalaenopsis*, have a central stem, with never-ending growth and alternating leaves. The floral stems develop at the base of the leaves. These plants are adapted to areas where humidity and temperature are high during the whole year.

In the epiphyte plants, the substrate has a really important role in the orchid life, especially taking into account that the root system is changing its living media from the air into a new medium. It is necessary to use media with number of voids, which allow a high ventilation to the root system. The substrate must have both a high degree of humidity and a good drainage, in order to prevent the pot from being flooded, and so the roots from rotting (Anon., 1990).

A number of substrates are used, namely pine bark, rock wool, vegetal coal, coconut hairs, almond husks, expanded clay, porexpan, and many others. The most used substrates are pine bark and rock wool, either alone or mixed with the other media (Batchelor, 1981; Nagel, 1965; Penningsfeld, 1990; Thomas, 1989). None of the existing substrate fulfills all the required needs (Hanger, 1986). Pine bark and rock wool, have draw backs. The pine bark has a strong tendency to liberate resins and phenols which affect the plant. Due to its decay, pine bark has a tendency to fix too much water, thereby causing root. For this reason it is necessary to change the bark every year. The rock wool has a strong tendency to become compacted, retaining too much water and causing the roots asphyxia (Chen *et al*, 1988; Inbar *et al*, 1988).

The efforts in the present studies were focused on two new substrates (zeolites and wastewater sludge compost) capable of having a strong influence on the nutrition pattern of the plant. Zeolite helps in retaining the mineral salts of the fertilizers and then liberating these slowly, when the irrigation water enters into the substrate, thereby improving the use of fertilizers. From the physical point of view, zeolites have the advantage of being resistent to the degradation, in addition to the drainability of media.

The wastewater sludge also improves water retention in the media, thereby facilitating a good degree of humidity in the roots area.

It is even possible to combine these advantages by using a mixture of these coumpounds.

Composted domestic wastewater sludge, has a high organic matter as well as nutrient content and allows the plant to have nutrients over during a long period of time.

The aim of these studies is to achieve a higher growth rate, as the orchid cultivation is a conventional slow process.

MATERIAL AND METHODS

PLANT MATERIAL

Phalaenopsis genus hybrids is one of the orchids with more commercial possibilities, both as a indoor plant or as a selling flower. Moreover, it has a good growing rate among the orchids and hence, the results can be obtained in an acceptable period of time.

Three year old plants with one previous flowering were used as a working material. The plants did not have any earlier growing media and dead leaves as well as roots were also eliminated. Later on, plants were weighed, the leaves counted as well as measured and the orchid was planted in the new media. Different combinations of substrates were used, with 10 plants in each combination.

EXPERIMENTAL CONDITIONS

The temperatures were between 20 and 25°C during the day and between 12 and 15°C during night. Good humidity, light and aeration were also ensured.

SUBSTRATES

Pine bark, zeolite and composted wastewater sludge were used. Pine bark and zeolites were passed through sieves to obtain the materials of 1 to 1.5 cm diameter.

Clinoptilolite was used as zeolite, with the composition indicated in Table 1.

Table 1. Composition of the zeolite (clinoptilolite) used in the experiment.

Components	Quantity
SiO2, %	66.03
Al2O3, %	10.47
CaO, %	1.55
MgO, %	0.58
TiO2, %	0.13
Na2O, %	3.25
K2O, %	1.81
Fe2O3, %	1.12
MnO, %	0.027
Density	1.603
Pore volume, %	15
Specific surface	40
C.I.C.	1.65

The composition of the compost used is described in Table 2.

Table 2. Composition of compost used (dry matter).

Humidity, %	38.2
Organic matter, %	60.1
Total nitrogen, %	1.5
P_2O_5, %	1.5
K_2O, %	0.4
Total porosity, %	80
pH	6.5

The substrates were mixed as indicated in Table 3.

Table 3. Mixtures used as a growing media for orchids.

Pine bark 100%			Referred as Control
Sludge compost 90%	+	Zeolite 10%	referred as 90-10
Sludge compost 75%	+	Zeolite 25%	referred as 75-25
Sludge compost 50%	+	Zeolite 50%	referred as 50-50
Sludge compost 25%	+	Zeolite 75%	referred as 25-75

Pine bark was used as a control because it is the medium usually employed by commercial growers.

RESULTS

The experiment was performed during 2 years, and the results obtained for weight increase, number of leaves, died plants, size of leaves and external aspect were as follows :

WEIGHT INCREASE

Best results were obtained in 90-10 growing media, with an increase of 54.6 g/plant (81.4%), followed by the control with 49.0 g/plant (73.2%). With the rest of mixtures there were obtained reduced increases, and it was observed the following relationship: less amount of compost less weight increase.

NUMBER OF LEAVES

The best results were also obtained with 90-10, with a 23.1% of increase in the number of leaves, followed with 50-50 and 75-25 mixtures with increases of 19.2 and 18.8%.

The control was giving an increase of 11.1% and 25-75 a decrease of 8.9% in the number of leaves.

DEAD PLANTS

1 or 2 plants for each combination, including control.

SIZE OF LEAVES

The control gave the higher increase on leaves' size: 36.0%. 90-10 and 50-50 followed with increases of 34.8% being the rest around 20%.

Table 4. Results of *Phalenopsis* development using different mixtures.

Mixture	Control	90-10	75-25	50-50	25-75
Weight increase	49.0	54.6	26.9	17.8	6.1
Weight increase in %	73.2	81.4	38.4		8.8
Leaves number incr.	0.4	0.7	0.5	0.6	-0.3
Leaves nr. incr. %	11.2	23.1	19.2	18.8	-8.8
Leaves size incr.cm	5.0	4.0	2.6	4.6	0.9
Leaves size incr. %	36.0	29.2	17.7	34.8	6.8

Every figure is the media of the surviving plants (8-9) See also figure 1.

OTHER

All plants conserved a good root system. The flowering pattern was good for all mixtures and for control.

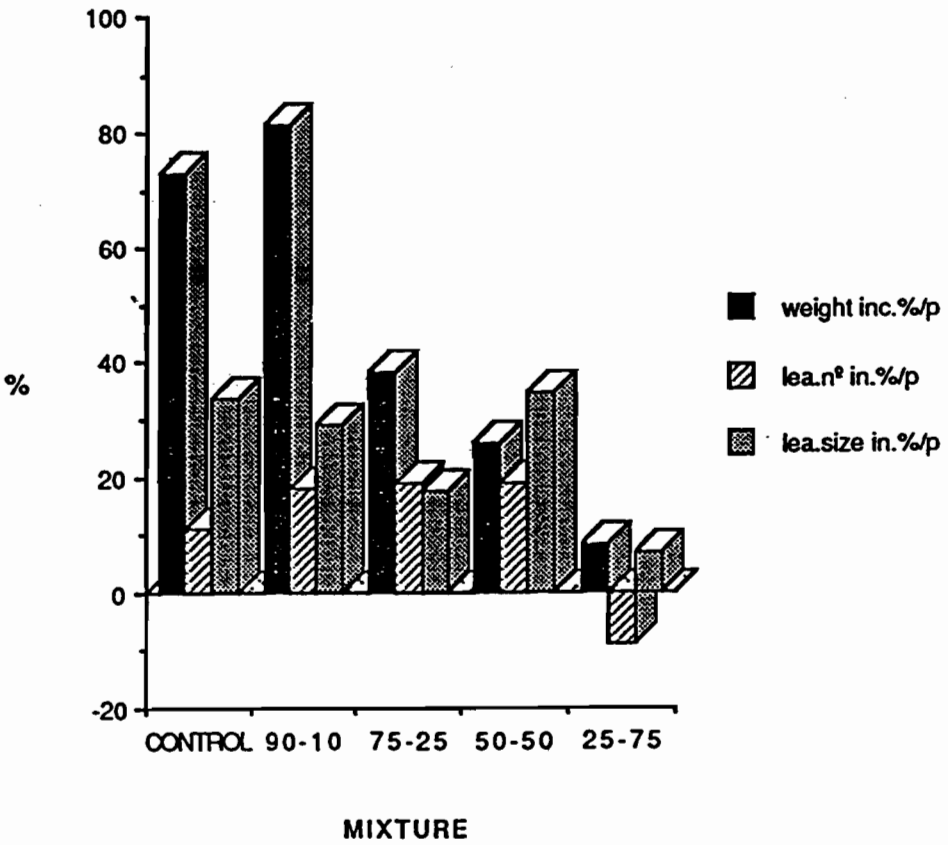

Figure 1. *Phalaenopsis* development. Mean values.

CONCLUSIONS

We observed a good possibility of using a new substratum for growth of orchids, based on zeolites and composted wastewater sludge, but preparing a mixture with an adequate relationship of zeolites and composted sludge.

Substratum 90-10 (cws-sludge) showed the best results taking into account the maximum number of parameters followed by the control. Nevertheless, results with other substrata were not bad.

A further development of the study of this substrata is suggested, working with proportions around 90-10.

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Solid state fermentation at ORSTOM: Evolution and perspectives

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SUMMARY

An attempt is made to formulate the general global history of solid state fermentation right from ancient times to the present day, with emphasis on the resurgence of interest in the technique throughout the world. ORSTOM, Institut Français de Recherche Scientifique pour le Développement en Coopération, France, understood the utility value of solid state fermentation as early as 1975 and has put intensive efforts from different angles. The history and evolution of solid state fermentation research at ORSTOM are described, along with the perspectives. The ORSTOM activities on cassava protein enrichment, lignocellulosics waste upgradation, enzymes production, spores for inoculation, physiological aspects, support cultures, bioremediation, and biopesticides are reviewed critically, along with the perspective for next few years.

Keywords: Solid state fermentation, global history, history in ORSTOM, evolution, cassava, sugar cane, protein enrichment, filamentous fungi, mushrooms, physiology, enzymes, spores, support cultures, bioremediation, biopesticides, perspectives.

RESUME

Fermentation en milieu solide à l'ORSTOM: Evolution et perspectives.

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L'historique du programme de recherche mené à l'ORSTOM depuis vingt ans sur les fermentations en milieu solide a été brossé dans le but de faire une synthèse de nos travaux de recherche, au moment même où apparait un regain d'interêt international sur cette technique originale de culture de microorganismes. L'ORSTOM, Institut Français de Recherche Scientifique pour le Développement en Coopération, avait compris dès 1975 l'intérêt scientifique et les applications potentielles des fermentations en milieu solide (FMS). L'historique et l'évolution des feremententations en milieu solide ont été développées tout au long de cette présentation. Les activités de l'ORSTOM dans le domaine de l'enrichissement en protéines du manioc, du tourteau de coprah, de la valorisation des substrats lignocellulosiques, la production d'enzymes, des spores pour l'inoculation, les aspects physiologiques, les cultures sur support, la bioremédiation, les biopesticides, les métabolites secondaires, ont été analysées d'une manière critique afin de présenter les perpectives de cette technique de culture dans le domaine de la recherche pour les années à venir.

Mots clés: Fermentation en milieu solide, historique global, historique à l'ORSTOM, évolution, manioc, bagasse de canne à sucre, enichissement protéique, champignons filamenteux, champignons comestibles, physiologie, enzymes, spores, culture sur support, bioremédiation, biopesticides, perspectives.

INTRODUCTION

It is really interesting to derive the general global history of solid state fermentation, right from ancient times to the present period. One has to let the imagination loose and even assume that a number of things or events might have occurred, with a view to come to a stage from where the history is documented. The present exercise for global history is no exception and some wild guesses might not have taken place at all.

Obviously, the first such natural activity of micro-organisms, observed by the man in ancient time, was the process of solid state fermentation. Hence, it is not surprising that the initial biotechnological processes exploited by man from ancient times are those based on solid state fermentation. A plenty of examples can be and have been cited on this aspect by many researchers interested in solid state fermentation.

Similar phenomena in liquid state fermentation was also evident. Probably, it was more visible, amenable to mixing and resulted in more homogenous product. Consequently, some groups of our ancestors preferred this technique over the solid state fermentation processes. Such tribes increased day-by-day and then started neglecting solid state fermentation processes.

Luckily, both techniques were intensively studied up to 1940, but then the leaders of science from many countries took decision to use only liquid state fermentation and to completely ignore solid state fermentation. The decision was more or less arbitrary, as it was neither taken on scientific grounds nor were the two techniques compared effectively. The reasons considered were probably the amenability of liquid state culture for manipulation, monitoring and control. The validity of this decision was recently debated by Ramesh and Lonsane (1991), as they observed that the production of alpha-amylase by *Bacillus licheniformis* in liquid state culture is lower, unless the medium is agitated, as against no need of agitation when solid state fermentation is employed. Similarly, Viniegra-Gonzalez (1996) has commented that the disadvantage, which were attributed to solid state fermentation, have, in fact, been recognised as its advantages in recent years. It has been even suggested that this neglect of SSF in Western and European countries is responsible for the slow growth of fermentation industries, as compared to those in Japan (Lonsane *et al* 1985).

Fortunately, a few groups, scattered in different parts of the world, have not subscribed to the view that liquid state fermentation is superior to solid state fermentation and continued their R&D efforts in the latter technique, even after 1940. They were in a very small minority, but slowly and steadily they proved that solid state fermentation cannot be ignored. It took 40-45 years for them to prove this point and to convince the others. These efforts have ultimately borne fruit and a resurgence of interest in solid state fermentation was generated throughout the world. Ultimately, even those who considered solid state fermentation as a crude or inferior technique, started researching it with an intense vigour. Consequently, the star was reborn and this is of industrial and economic advantage.

The biotechnology group in ORSTOM, France, is one such group which realised the value of solid state fermentation as early as 1975 and invested intensive efforts

on the technique. The present paper describes the history, evolution and perspectives of solid state fermentation in ORSTOM, France.

HISTORICAL HIGHLIGHTS OF SSF IN ORSTOM

The work on SSF in ORSTOM was initiated in 1975 at its Senegal centre by Raimbault (1980), in collaboration with Jacques SENEZ, reverently referred to as the father of industrial fermentation in France (Senez, 1979; Senez *et al.* 1979). The work involved upgradation of cassava for its protein content by SSF, using a column fermentor at laboratory scale and *Aspergillus niger* 10 as the filamentous fungus (Raimbault and Germon, 1976). During 1978-1982, the work mainly focused on development of agitated pilot and industrial scale reactors and initiation of the work on cellulases production (Deschamps *et al.* 1979; Senez *et al.* 1980; Deschamps *et al* 1985. The need for inoculum in large quantity for pilot scale work led to the development of an efficient sporulator (Raimbault and Roussos, 1985), and to the design and fabrication of 50 kg capacity fermentor, Zymotis (Prebois *et al.* 1985). The importance of physiology of the culture during fermentation was also realised and culminated in the development of efficient methods and analysis systems for respirometric studies on column fermentors (Raimbault and Alazard, 1980; Aufeuvre and Raimbault, 1982).

The period of 1982-1985 witnessed expansion of the activities on tropical waste upgradation, cellulases production on an inert support (sugar cane pith bagasse), development of efficient methods for recovery of the product from fermented solids and formulation of a simpler strategy for disposal of spent solids by ensiling (Roussos, 1985). The conversion of waste banana for its upgradation and use as animal feed was also realised (Baldensperger *et al.* 1985). Teaching of SSF at Universidad Autonoma Metropolitana (UAM-Iztapalapa), Mexico was also initiated by ORSTOM during this period and this activity was extended to many universities (Viniegra-Gonzalez *et al.* 1991).

During 1981, collaboration with UAM-I, Mexico, was established for upgradation of different tropical agro-industrial wastes (cassava, bagasse and coffee pulp). It continues to the present day and has yielded many useful results (Table 1). Engineering studies, along with designs of static/agitated reactors, were initiated and studies were made on heat and mass transfer, energy conservation, water activity, isolation of about 500 tropical fungi and evaluation of these fungi for production of different enzyme as well as microbial metabolites (Oriol *et al.* 1988; Aquiahuatl *et al.* 1988, Saucedo-Castañeda *et al.* 1990). Different fermentation systems were

successfully computerised for efficient monitoring as well as control of different parameters, in addition to the development of appropriate sensors (Gutierrez-Rojas *et al.* 1988).

From 1990, the attention under the above collaborative project was also focused on genetic improvements of the cultures and molecular approaches were extended to explain different behaviour of the micro-organism in liquid and solid state cultures (Antier *et al.* 1993a and b; Augur and Viniegra-Gonzalez, 1995). Efficient mathematical models were developed and physiological studies were extended to the processes involving cell growth and metabolism (Viniegra-Gonzalez, 1988; Saucedo-Castañeda *et al.* 1992; Gutierrez-Rojas *et al.* 1995). Recently, bioremediation of solid waste and air purification were conducted through SSF (Perraud-Gaime, 1995; Morales *et al.* 1994; Revah *et al.* 1995).

Collaboration was subsequently developed with a number of institutions in France, other European countries and also with a wide spectrum institutions from around the globe (Deschamps *et al.* 1982; Lonsane *et al.* 1991; Marakis *et al.* 1995; Roussos *et al.* 1994; Viniegra-Gonzalez *et al.* 1991).

At ORSTOM, Montpellier, intensive efforts have been devoted, right from 1987, on the physiology, metabolism and respirometry of fungi in SSF processes (Dufour, 1990; Saucedo-Castañeda, 1991; Soccol, 1992; Trejo-Hernandez, 1992; Perraud-Gaime, 1995; Denis, 1996). New areas, such as biopesticides, ectomycorrhiza and mushrooms mycelium physiology as well as secondary metabolites (alkaloids and aroma) were also initiated for intensive R&D efforts (Montero *et al.* 1989; Roussos *et al.* 1989; Roussos *et al.* 1996; Trejo-Hernandez *et al.* 1992 and 1993). As it stands now, ORSTOM has achieved an international recognition for its efforts on SSF and it has a number of planned prospects for intensive inputs on this fermentation technique of industrial importance (Raimbault, 1988).

SSF BASED ON NATURAL ORGANIC SUBSTRATES

As mentioned earlier, natural organic substrates were the first on which work was initiated in 1975 in Dakar, Senegal (Raimbault *et al.* 1979). The efforts were initially taken up with a view to obtain upgraded agro-industrial products for use as human food (Senez, 1979). However, the later work was for developing upgraded agro-industrial products/residues/wastes for animal feeding (Senez *et al.* 1980). The details of the efforts on the natural organic substrates are presented in Table 1, with respect to the substrates, micro-organisms used in fermentation and the end products. A glance at Table 1 indicates that the efforts were studies on diverse natural organic

substrates as well as fungal/yeast species for production of a large number of primary and secondary metabolites.

FERMENTATION BASED ON INERT SUPPORT

The efforts on natural organic substrates indicated a severe problem of variation in the composition of substrates and consequent difficulties in obtaining end-product of uniform quality. Moreover, many of these natural substrates are available in a limited period in the year and may not be not be available in many countries. Their import from other countries is impracticable from the economic point of view (Raimbault *et al.* 1989). The efforts on inert supports are illustrated in Table 2.

Table 1. Solid State Fermentation based on natural organic substrates.

Substrates	End products	Microorganisms studied	References
Cassava	Biomass,	*A. niger 10*	Raimbault and Alazard (1980) Raimbault *et al.* (1985)
	Amylases,	*A. niger 10*	Alazard and Baldensperger, 1982
		R. arrhizus	Soccol (1992)
	Biopesticides,	*B. bassiana*	Roussos *et al*, (1989)
	Conidiospores	*T. harzianum*	Roussos *et al*, (1991)
	Lactic acid	*R. oryzae*	Soccol *et al*, (1994)
	Ethanol	*Sw. castellii*	Saucedo-Castañeda *et al*, (1993)
	Alkaloids	*C. purpurea*	Trejo *et al*, (1993)
Sugar cane bagasse	Biomass	*A. terreus*	Gonzalez-Blanco *et al* (1990)
	Cellulases, Animal feed, Biopesticides, Conidiospore	*T. harzianum* *B.bassiana*	Montero et al. (1989); Roussos *et al*, (1991a, 1992, 1993); Tapia *et al.* (1988)
Carob waste	Tannase	*A. carbonarius*	Lambraki *et al*, (1994)
Wheat straw	Cellulases	*T. harzianum*	Deschamps *et al*, (1985)
Sugar beet cosset	Biomass, cellulases	*T. harzianum*	Roussos *et al*, (1982)
Wheat straw+ wheat bran	Cellulases, Biomass,	*T. harzianum*	Roussos *et al*, (1991b)
Coprah cake	Biomass, Probiotics	*A. niger*	Roussos *et al*, (1994); Ramirez-Islas *et al*, (1996)
Coffee pulp	Pectinase,	*Aspergillus*	Antier *et al.* (1993); Boccas *et*
	Decaffinated	*sp.Penicillium*	*al*, (1994) Roussos *et al*, (1994)
	Substrate, Silage	*sp.*	Perraud and Roussos (1996)
Banana	Biomass	*A. niger 10*	Baldensperger *et al*, (1982)
Potato waste	Biomass	*A. niger 10*	Raimbault *et al*, (1979)

Table 2. Solid state fermentation based on inert support.

Inert supports	End products	Microorganisms studied	References
Sugar cane pith bagasse	Amylases	*A. niger 10*	Oriol *et al*, (1988)
	Pectinases	*A. niger CH4*	Trejo *et al*, (1991)
	Ethanol	*S. castellii*	Saucedo *et al*, (1992)
	Lactic acid	*R. oryzae*	Soccol *et al*, (1994)
	Alkaloïdes	*C. purpurea*	Trejo *et al*, (1993)
	Aroma	*T. harzianum*	Sarhy-Bagnon *et al*, (1996)
		C. fimbriata	Christen and Raimbault, (1991)
		M. esculenta	Kabbaj *et al*, (1996)
	Biomass	*S. castellii*	Saucedo *et al*, (1992)
	Conidia	*T. harzianum*	Roussos *et al*, (1991)
		A. niger	Roussos *et al*, (1991)
	Citric acid	*A. niger*	Gutierrez-Rojas *et al*, (1995)
	Penicillin	*P. chrysogenum*	Barrios-Gonzalez *et al*, (1988)
	Gibberellins	*G. funjikuroi*	Barrios-Gonzalez *et al*, (1989)
	Lipases	*R. oligosporus*	Christen *et al*, (1993, 1994)
	Ectomycorrhizes	*Suillus collinitus*	Roussos *et al*, (1995)
		Lactarius deliciosus	Roussos *et al*, (1995)
	Aflatoxin	*A. flavus*	Barrios-Gonzalez *et al*, (1988)
	Probiotics	*A. niger*	Tapia *et al*, (1988 and1989)
	Mushroom	*P. cornucopiae*	Roussos *et al*, (1996)
	Biomass	*A. niger 10*	Oriol *et al*, (1990)
Polyurethan	Penicillin	*P. chrysogenum*	Barrios-Gonzalez *et al*, (1988)
Vermiculite	Biomass	*Suillus collinitus*	Roussos *et al*, (1995)
		Pisolithus tinctorius	Roussos *et al*, (1995)
Amberlite	Ethanol	*C. utilis*	Christen *et al*. (1994)
	Biomass	*A. niger*	Auria *et al*, (1990, 1992, 1993)

Sugar cane pith bagasse has been extensively used in ORSTOM, because it offers many advantages (Dufour, 1990, Raimbault *et al*. 1989; Roussos, 1985; Saucedo-Castañeda, 1991 and Trejo-Hernandez, 1992). It does not find any worth-while use and is available in plenty in sugar cane processing countries. A method was also developed for making it inert and also store over a long duration (Oriol *et al*, 1987; Raimbault *et al*, 1989; Saucedo-Castañeda *et al*, 1992).

A number of chemical or mineral compounds can serve as inert support in solid state fermentation (Auria *et al.* 1992; 1993; 1994; 1995; Barrios-Gonzalez *et al*, 1988; Christen *et al.* 1995). These compounds, such as polyurethane, alginates, amberlite and vermiculite, were also investigated (Table 2).

DESIGN AND DEVELOPMENT OF BIOREACTORS

Researchers have always faced difficulties in obtaining bioreactors from commercial sources, as laboratory scale bioreactors for solid state fermentation are not produced industrially. Consequently, a number of different types of bioreactors have been used by various workers (Lonsane *et al,* 1985, Gutierrez-Rojas *et al,* 1988) and the choice is determined mainly by the infrastructure available in the laboratory. The efficiency of the fermentation also depends on the type of the bioreactor, parameters control and reliability of parameter control strategies. Consequently, comparison of literature data is not possible.

With the above in view, ORSTOM has developed a number of bioreactors for laboratory, pilot plant and industrial scale operation (Raimbault and Alazard, 1980; Prebois et al. 1985; Raimbault and Roussos, 1985 and Lepilleur *et al,* 1997). These are described in Tables 3a, b and c, along with details of their specific features and uses in different projects in ORSTOM. Different reactor designs have also been developed for static and agitated fermentation as well as for pilot scale inoculum production (Gutierrez-Rojas *et al.* 1988; Roussos *et al.* 1993, Auria *et al.* 1992).

Table 3a: Design and development of laboratory solid state fermentation bioreactors for study fungal physiology, respirometry, sporulation and large scale cultivation.

Bioreactor	Agitation status	Working capacity, kg DM	Distinct features	Uses	References
Column fermenter (20 columns	static	0.01-0.1 /column	Control of temperature, moisture, aeration ; on-line respirometry; sterility maintenance ; suitable or all kinds of microorganisms	Screening of strains, optimization, kinetics, metabolic patterns, respirometry	Raimbault and Alazard (1980) ; Saucedo *et al*, (1993)
Column fermenter (16 columns)	static	0.01-0.1	Available commercially from Gauthier, Montpellier		
Modulated, multiple-column verticle fermenter with double jacket	static	0.1-2	Control of temperature, moisture, aeration, on-line respirometry;Double jacket ;Increase or devrease in bed height ; Sterility maintenance comparative of different bed height ; recycle of air; Suitable for all kinds of microorganims	Scale-up fo process; Collection of engineering data	Auria et al (1992) Saucedo *et al*, (1992, 1993)

Table 3b: Design and development of disk fermentor for study fungal physiology, respirometry, sporulation and large scale inoculum production.

Bioreactor	Agitation status	Working capacity, kg DM	Distinct features	Uses	References
Disk fermenter	Agitated or static	0.2-2.0	Control of temperature, aeration and moisture; on-line respirometry; sterility maintenance; adjustible distance between two plates; Uniform conidiation; Overcoming of fungal competition during growth and sporulation; suitable for filamentous fungi	Pilot scale spore inoculum production in absolutely sterile conditions; Optimization of sporulation; Physiological studies of fungal sporulation; In situ inoculation and recovery of spore inoculum in liquid in concentrated form and without agar piece	Raimbault and Roussos (1985) Montero *et al* (1989) Roussos *et al* (1991)

Table 3c: Design and development of pilot solid state fermentation bioreactors for study fungal physiology, respirometry, sporulation and large scale cultivation.

Bioreactor	Agitation status	Working capacity, kg DM	Distinct features	Uses	References
Zymotis	Static	10-100	Control of temperature, one-line respirometry, moisture, aeration, and weight, recycle air, In pith driying of medium; modulated compartments; Sterility maintenance; Suitable for all kinds of microorganism	Scale-up of process; Pilot plant production; Industrial conidia production; Data for design of industrial bioreactor;	Prebois *et al*, (1985); Roussos *et al*, (1994); Gonzalez-Blanco *et al*, (1990); Roussos *et al*, (1991)
IRCHA-ORSTOM Pilot scale reactor	Agitated	5-20	Control of temperature, agitation, pH, aeration, nitrogen and weight; Efficient heat and mass transfers; In situ drying of medium, inoculation, homogénization, and substrate treatment	Agitation effect on mycelial growth; Pilot scale studies; Engineering data collection; protein enrichment of starchy materials	Senez *et al*, (1980) Deschamps *et al* (1979, 1985) Raimbault (1980)
IRCHA-ORSTOM industrial reactor	Agitated	100-500	" "	Feed production	Deschamps *et al* (1982)

COMPUTERISATION OF BIOREACTORS

Is it well known that the heat and mass transfer are poor in many types of bioreactors for solid state fermentation (Lonsane *et al*, 1992; Saucedo-Castañeda *et al*, 1993, Roussos *et al*, 1993). Consequently, it is difficult to control temperature and maintain desired moisture levels in most bioreactors (Gonzalez-Blanco *et al.* 1990). Similarly, carbon dioxide level in the fermenting mass is of critical importance in many processes (Lonsane *et al*, 1985, ; Saucedo-Castañeda *et al*, 1992). In order to have accurate control of these parameters, bioreactors have been coupled to computer for efficient control. The details are presented in Table 4.

Table 4. : Computerisation of reactors for solid state fermentation.

Reactors	Computerised regulation for	References
Lab. scale column fermentor	Temperature, oxygen carbon dioxide, pressure drop, humidity, aeration rate	Saucedo-Castañeda *et al*, (1990, 1992, 1993, 1994); Auria *et al*, (1993, 1994, 1995)
Double jacketed multi-column vertical fermentor	Temperature, carbon dioxide, aeration rate	Saucedo-Castañeda *et al*, (1992) Auria *et al*, (1990, 1992)
IRCHA-ORSTOM Pilot agitated reactor	Temperature, pH, weight	Deschamps *et al*, (1979, 1981, 1985)
Zymotis, static pilot reactor	Temperature, aeration rate	Gutierrez-Rojas *et al*, (1988) Gonzalez-Blanco *et al.* (1990)

PHYSIOLOGICAL STUDIES

Physiology of filamentous fungi during growth in solid state fermentation has been a speciality of ORSTOM group. A number of improvements in process productivity were possible through the physiological studies.

Table 5. Physiological studies on filamentous fungi.

Physiological attribute	Microorganisms studied	End products	References
Spore germination	*T. harzianum*	Cellulases	Roussos and Raimbault (1982)
	A. niger	Biomass	Barrios-Gonzalez *et al*, (1989)
	Penicillium sp. *Aspergillus* sp.	Detoxified coffee pulp	Perraud and Roussos (1996), Denis (1996)
	A. carbonarius	Tannase	Gaitis and Marakis (1994)
	C. purpurea	Alkaloid	Trejo *et al*, (1993)
	R. oryzae	lactic acid	Soccol *et al*, (1994)
	A. niger	Amylases	Roussos *et al*, (1989)
Spore viability during preservation	*T. harzianum*	Cellulases	Roussos *et al*, (1989)
	B. bassiana	Biopesticide	Montero *et al*, (1989)

The contributions of ORSTOM in this specific area of vital importance are presented in Table 5. It is emphasised that physiological aspects of filamentous fungi in solid state fermentation have been largely neglected by most researchers studies (Oriol, 1987; Perraud-Gaime, 1995; Roussos, 1985; Saucedo-Castañeda, 1991; Soccol, 1992, Trejo-Hernandez, 1992).

MATHEMATICAL MODELS

A number of mathematical models have been developed for every aspect of submerged fermentation and these proved highly useful in understanding the intrinsic details of the process, optimisation of parameters, scale-up of the process and designing industrial technology. In contrast, mathematical models for solid state fermentation were rare before 1990 and these started appearing in the literature subsequently. Formulating efficient mathematical model for solid state fermentation is difficult because of the non-homogeneity of the system (Gutierrez-Rojas, 1995).

ORSTOM and its team of collaborators from Mexico and Cuba have put intensive efforts into developing mathematical models for solid state fermentation. The types of the mathematical models developed are given in Table 6.

Table 6. : Mathematical models for solid state fermentation.

- Growth of *A. niger* 10 on cassava for protein enrichment in static column fermentor (Raimbault, 1980).
- Solid State Fermentation limitation and monitoring Viniegra-Gonzalez (1996).
- CO_2 balance in solid state fermentation of cassava for protein enrichment (Rodriguez-Leon *et al*, 1991).
- Mycelia fungal growth on inert support (Gutierrez *et al*, 1996)
- Energy activation in cassava silage (Saucedo-Castañeda *et al*, 1990).
- Heat transfers in solid state fermentation (Saucedo-Castañeda *et al*, 1990).
- Biomass formation in solid state fermentation involving alginate as inert support (Auria *et al*, 1994)

UPGRADATION OF AGRO-INDUSTRIAL MATERIALS

Protein-rich food for humans was the goal of the ORSTOM team in initiating work on solid state fermentation in 1975. The philosophy behind this approach was the fact that plants make carbohydrates easily and abundantly, but not proteins. These plant materials, therefore, can be made nutritionally balanced by upgrading their protein content, via the fermentation route (Senez, 1997). The initial efforts were concentrated on cassava to a large extent and on banana, potato waste as well as sugar beet waste to a limited extent (Table 7).

Table 7. Upgradation of agro-industrial products/wastes/residues.

- Banana (Baldensperger *et al*, 1982)
- Carob waste (Lambraki *et al*, 1994)
- Cassava (Raimbault and Alazard 1980; Raimbault *et al.* 1985)
- Coffee pulp (Perraud-Gaime and Roussos 1995; Roussos *et al*, 1989)
- Copra cake (Ramirez-Islas *et al*, 1995; Roussos *et al*, 1994)
- Potato waste (Raimbault *et al*, 1979, 1981)
- Sugar beet waste (Roussos *et al*, 1982)
- Sugar cane bagasse (Gonzalez-Blanco *et al*, 1990; Roussos *et al*, 1991)
- Wheat straw + wheat bran (Roussos *et al*, 1991b; Deschamps *et al*, 1985)

Attention was subsequently focused from 1980, on upgrading the agro-industrial residues/wastes with a view to produce efficient animal feed (Senez, 1979; Senez et al. 1981; Deschamps et al. 1982). A number of residues/wastes were investigated as specified in Table 7.

ENZYME PRODUCTION

Among the microbial products in international and national markets, enzymes occupy a prominent place and large monetary turn-over. A number of enzymes have been economically manufactured in Japan and other Oriental countries by using solid state fermentation, though submerged fermentation is relied on in European and Western countries. This was mainly due to the neglect of solid state fermentation in these latter countries.

Table 8. : Studies on enzyme production in solid state fermentation system.

Enzymes	References
Amylases	Alazard and Baldensperger (1981) ; Raimbault and Alazard (1980) ; Soccol *et al*, (1993a and b) ; Raimbault (1989)
Glucoamylases	Oriol *et al* (1988) ; Soccol *et al*, (1993)
Cellulases	Roussos *et al* (1991) ; Soccol *et al*, (1994)
Pectinases	Boccas *et al*, (1994) ; Favela *et al*, (1989) ; Antier *et al*, 1(993a, b) ; Solis *et al*, (1994) ; Cordova *et al*, (1996)
Decaffeinases	Denis, (1996)
Tannase	Lambraki *et al*, (1994; 1996)
Lipase	Martinez-Cruz *et al.* (1993); Christen *et al*, (1994; 1995)
Protease	Villegas *et al*, (1993)

Intensive efforts have been made at ORSTOM and by its collaborators to investigate solid state fermentation processes for the production of different enzymes as specified in Table 8.

PRODUCTION OF METABOLITES OTHER THAN ENZYME

In addition to the production of fermented foods and enzymes by solid state fermentation, a number of other primary and secondary metabolites can be produced, with a number of advantages (Lonsane *et al*, 1985, 1992). It is of interest to note that penicillin was produced industrially in USA during 1940s by solid state fermentation (Zeifer et al. 1988).

Table 9. : Studies on production of metabolites other than enzymes by solid state fermentation.

Metabolites	References
Alkaloids	Trejo-Hernandez *et al*, (1992, 1993a, 1993b)
Aflatoxin	Barrios-Gonzalez *et al*, (1990)
Penicillin	Barrios-Gonzalez *et al*, (1988, 1990, 1996)
Gibberellic acid	Barrios-Gonzalez *et al*, (1988)
Ethanol	Saucedo-Castañeda *et al*, (1991, 1992, 1993, 1994); Christen *et al*. (1994)
Lactic acid	Soccol *et al*, (1994)
Fumaric acid	Soccol (1992)
Citric acid	Gutierrez-Rojas *et al*, (1995)
Alpha-pentyl pyrone	Lozano *et al*, (1995), Sarhy-Bagnon *et al*, (1996)
Octane-1-ol	Kabbaj *et al*, (1996)

A number of industrially important metabolites were identified by ORSTOM and efforts were put to study their production by solid state fermentation (Table 9). In all the cases, the process was optimised and a number of interesting facts were discovered. The metabolites studied are diverse in nature and include organic acids, antibiotics, plants growth hormones, alkaloids, toxins and aroma compounds (Table 9).

BIOMASS WITH SPECIFIC CHARACTERISTICS

It is well known that chemically synthesised pesticides are problematic, mainly because of their difficult biodegradation and persistent residues in foods and even in human bodies. Biopesticides offer efficient alternatives and are being used increasingly throughout the world. Mainly filamentous fungi have antagonistic properties against a number of plant pathogens, in addition to specificity of action. ORSTOM has invested efforts on production of the biomass of such fungi for their use as biopesticides (Montero *et al*, 1989). The fungi studied include *Trichoderma harzianum* and *Beauveria bassiana*. (Table 10).

Table 10. Biomass production in solid state fermentation with specific characteristics.

Products	References
Probiotics	Tapia *et al*, (1989) ; Ramirez-Islas *et al*, (1996); Herrera-Saltaña *et al*, (1990) ; Campos *et al*, (1994)
Biopesticides	Roussos *et al*, (1989) ; Montero *et al*, (1989)
Mushroom spawn	Roussos *et al*, (1993; 1996)
Ectomycorrhiza mycelial cells	Roussos *et al*, (1995)

Mushroom production is one of the major examples of the exploitation of solid state fermentation and the process involves SSF at three stages, i.e. composting of substrate for use in mushroom production, spawn production and the cultivation process of mushrooms itself (Lonsane *et al.* 1985). Cereal grains are mainly used in spawn production but these are either costly, not sufficiently available for spawn production or not produced in many countries. The efforts in ORSTOM were, therefore, directed to studies on production of spawn on sugar cane pith bagasse as an inert support, after impregnation with nutritive solution (Roussos *et al.* 1996).

Studies have also been initiated on production of ectomycorrhiza mycelial cells by solid state fermentation (Table 10) and economically useful results have been obtained (Roussos *et al*, 1995).

BIOREMEDIATON

The chemical methods for remediation are either elaborate, cost-intensive or are non efficient. Bioremediation overcomes these problems to a large extent. Hence, efforts were put in ORSTOM to develop solid state fermentation processes for bioremediation of commodities, such as coffee pulp and carob pod for detoxification, decaffeination, and degradation of polyphenol as well as tannin (Table 11). Solid state fermentation process was also relied up on for degradation of volatile contaminants in air for its purification (Morales *et al*, 1994; Revah, 1995).

Table 11. Bioremediation through solid state fermentation.

Bioremediation	References
Detoxification of coffee pulp through polyphenol degradation	Gaime -Perraud *et al.* 1991; Perraud-Gaime (1995); Perraud-Gaime *et al.* (1996)
Decaffeination of coffee pulp for its use in animal feed	Roussos *et al*, (1989, 1991, 1993, 1994, 1995) ; Perraud -Gaime and Roussos, (1996)
Polyphenol and tannin degradation in carob waste for detoxification	Lambraki *et al,* (1992, 1993, 1994, 1995, 1996)
Degradation of volatile contaminants in air for its purification	Morales *et al*, (1994); Revah *et al*, (1995)

NATIONAL AND INTERNATIONAL COLLABORATIONS

Collaboration with developing and developed countries is one of the goals of ORSTOM and it has strived hard to achieve it. The example of collaboration with Mexico can be cited as a best collaboration that could be established between two countries. Similar collaboration exists with a number of other developing and developed countries in most part of the world (Table 12). The output from these collaborative efforts is commendable and found recognition internationally.

Table 12. National and International collaborations.

Institution	Country	Area of co-operation
UAM-Iztapalapa	Mexico	Physiology and metabolism of fungi, proteins enrichment of cassava and sugar cane bagasse, coffee pulp detoxification, collection of micro-organisms, bioreactors, pilot scale, mathematical models, solid state fermentation, genetic of filamentous fungi, molecular biochemistry and academic formation.
UASP	Mexico	Solid state fermentation of agricultural wastes.
UNAM	Mexico	Pectinases, academic formation.
INMECAFE	Mexico	Coffee pulp upgradation.
ICIDCA	Cuba	Solid state fermentation, sugar cane bagasse upgradation, mathematical models, physiology of filamentous fungi sporulation.
UNIVALLE	Columbia	Cassava rot protein enrichment, solid state fermentation, sugar cane bagasse upgradation, lactic acid bacteria, academic formation.
CFTRI	India	Sugar cane bagasse upgradation, cellulases and amylases production, coffee pulp detoxification, solid state fermentation and new bioreactors.
Athens University	Greece	Carob pod protein enrichment, polyphenols degradation, solid state fermentation, physiology of *Aspergillus carbonarius*.
Montpellier university	France	Academic formation, solid state fermentation.
Provence university	France	Academic formation, solid state fermentation.
CIRAD	France	Cassava and copra cake, protein enrichment, Coffee pulp upgradation, lactic acid bacteria.
UTC	France	Academic formation, solid state fermentation.
ENSBANA	France	Academic formation, aroma production by fungi.
CNRS	France	Biodegradation of new plastics.
INRA	France	Mushrooms spawn physiology; aroma production, ectomycorhyzal mycelium, biopesticides.

PATENTS

The efforts of ORSTOM on solid state fermentation were always centred on topics of economic and industrial importance. In many cases, the resulting technology was either novel or with larger economic benefits. It is, therefore, not surprising that ORSTOM obtained 9 patents on its solid state fermentation processes or bioreactor design (Table 13).

Table 13 : Solid State Fermentation patents developed by ORSTOM

Patent	Authors	References
Protein enrichment process	Raimbault and Germont	Fr 76.06.67
Spores production process	Raimbault and Roussos	Fr 85.08.555 Eur 0223.809
Zymotis, static bioreactor	Prebois *et al*, 1985	Fr 85.17.934
Coconut aroma production	Lozano, Pioch and Roussos	Fr 95.01.713
Moulds control system	Roussos and Bossis	Fr 95.02.204
Fungal metabolites production	Barrios-Gonzalez *et al*, 1988	Mex. Secofi n° 17.184
Fungal enzymes production	Roussos *et al*, 1988	Mex. Secofi n° 17.185
Fungal spores production	Gutierrez-Rojas *et al*, 1988	Mex. Secofi n° 17.187

Table 14: Doctoral theses presented to different universities in the aera of SSF.

Autors	Aerea	University	Year	Page
Raimbault Maurice	*Aspergillus* growth in starchy substrates	Toulouse (Fr)	1980	291
Huerta-Ochoa Sergio	Heat and mass transfert on cassava SSF	UAM-I (Mex)	1984	147
Roussos Sevastianos	*Trichoderma* growth on cellulosic materials	Marseille-I (Fr)	1985	193
Oriol Eric	*A. niger* growth on natural support	Toulouse (Fr)	1987	133
Trejo-Hernandez Maria	Pectinase production in natural support	UNAM-I (Mex)	1986	106
Saucedo-Castaneda Gerardo	Cassava protein enrichment and silage	UAM-I (Mex)	1987	169
Dufour Dominique	Pectinase production on natural support	UTC (Fr)	1990	262
Saucedo-Castaneda Gerardo	Yeast growth and metabolism on support	Montpellier II (Fr)	1991	197
Soccol Carlos	Cassava protein enrichment by *Rhizopus*	UTC (Fr)	1992	218
Trejo-Hernandez Maria	Alcaloïds production by *Claviceps*	Marseille-I (Fr)	1992	163
Aquiahuatl Maria	Isolation of caffeine degrading fungi	UNAM- (Mex)	1992	72
Perraud-Gaime Isabelle	Coffee pulp silage and decaffeination	Montpellier II (Fr)	1995	209
Gutierrez-Rojas Mariano	Mathematical models for SSF on inert support	UAM-I (Mex)	1995	

DOCTORAL THESES

A number of students from the collaborating countries have realised their doctoral theses at ORSTOM, under the fellowship from France government. The staff of ORSTOM also worked for their doctoral thesis. A total of 14 doctoral theses have emerged from these efforts during 1980-1995 and these were accepted for doctoral degree by a number of different universities in France (Table 14).

PUBLICATIONS

The research and development efforts on solid state fermentation at ORSTOM found place in a number of national and international, peer-reviewed standard journals. The information on publication of research as well as review papers and papers presented at various national and international symposia/seminar/convention/meetings is given in Table 15.

Table 15: Publications and papers presented at national and international symposium.

Publications	Number
Research papers	102
papers	8
Papers present at symposium	148

TECHNOLOGY TRANSFER

Some of the technologies developed at ORSTOM have been successfully transferred to industries for commercial exploitation. For example, the process for conidiospore production of *Beauveria bassiana* and *T. harzianum*, for use as biopesticide, has been utilised by two French companies Calliope and Letellier. In addition, the technology for production of *Pleurotus* spawn by solid state fermentation based on the use of inert support, after impregnation with nutritive solution, has been commercially exploited by Ayusa, Mexico. The laboratory scale column fermentor unit, consisting of 16 columns, has been successfully manufactured by Gauthier, Montpellier, France, for international marketing.

SYMPOSIA / SEMINARS ORGANISED

ORSTOM has successfully organised a number of national and international symposia/seminars on solid state fermentation (Table 16). The organisation of these events have been commended by the scientific community involved in solid state fermentation. Proceedings of all these seminars/symposia have been published, except for FMS-89 in Mexico.

Table 16. : Symposia/Seminars organised.

Year	Evenment	References
1988	FMS-88	Montpellier, France
1989	FMS-89	Mexico D.F., Mexico
1989	SIBAC-I	Xalapa, Ver., Mexico
1991	SIBAC-II	Manizales, Columbia
1995	FMS-95	Montpellier, France
1995	Vème Entretiens Agropolis	Montpellier, France

TEACHING AND TRAINING

As a part of co-operation, in Mexico ORSTOM participated in teaching of biotechnology, with specific emphasis on solid state fermentation, at graduate, post-graduate and post-doctoral levels in the Universidad Autonoma Metropolitana - Iztapalapa, Mexico. A course of 4 weeks duration, entitled "Ecole Chercheur Orstom en Fermentation en milieu Solide, ECO-FMS" is conducted once in a year at ORSTOM, Montpellier, France. It involves theory, practical and research in solid state fermentation, along with computer application.

LITERATURE BANK

ORSTOM, at its Montpellier centre, has developed a large literature bank on solid state fermentation. It includes 90 theses and 7.000 reprints on filamentous fungi and solid state fermentation.

STAFF STRENGTH

The staff strength at ORSTOM, Montpellier is around 10 and their specialisation is in microbiology, biochemistry, physics and engineering. In addition, about 10 graduate students from various universities are trained for a period of 2-6 months, at any given time. The work on solid state fermentation is also carried out by 6 doctoral students and 2-3 post-doctoral students/annum at any given time.

PERSPECTIVES

The experience on different aspects of solid state fermentation and its exploitation for production of biomass and primary as well as secondary metabolites over the past 20 years have provided an efficient in-sight in this fermentation technique to ORSTOM and its collaborating teams. This formed an efficient base for chalking out the perspectives in solid state fermentation at ORSTOM for the next years:

- Isolation of new fungal strains adapted in SSF
- Molecular Biology and genetic engineering of fungi involved in SSF
- Mixed Cultures with bacteria, yeast and fungi
- Production of fungal biomass and metabolites
- Engineering of bioreactors and sensors
- Solid state fermentation in continuous system
- Bioremediation of commodities and soil detoxification
- Degradation of volatile contaminants in air for its purification

As can be seen, the perspectives are diverse and more focused on specific areas of economical and industrial aspects. These perspectives may also lead to extensive commercial exploitation of solid state fermentation processes.

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Advances in Solid State Fermentation

S. Roussos, B. K. Lonsane, M. Raimbault and G. Viniegra-Gonzalez (Eds.)

This book covers a wide range of studies in the field of Solid State Fermentation (SSF). The Work begins with a collection of useful definitions followed by contributions on biomass estimation and the kinetics of fungal growth on solid substrates. Key articles are included on engineering and SSF reactor design and agro-industrial waste upgrading, followed by papers on enzyme technology by SSF processes and secondary metabolites and biopesticides. The book concludes by considering the latest SSF applications.

This book will be useful to academic and commercial researchers and students interested in a wide variety of aspects of SSF. We hope that readers will be stimulated by those questions raised and will discover enough answers to consider SSF processes as one of the many alternatives in the rapidly developing biotechnology sector.

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