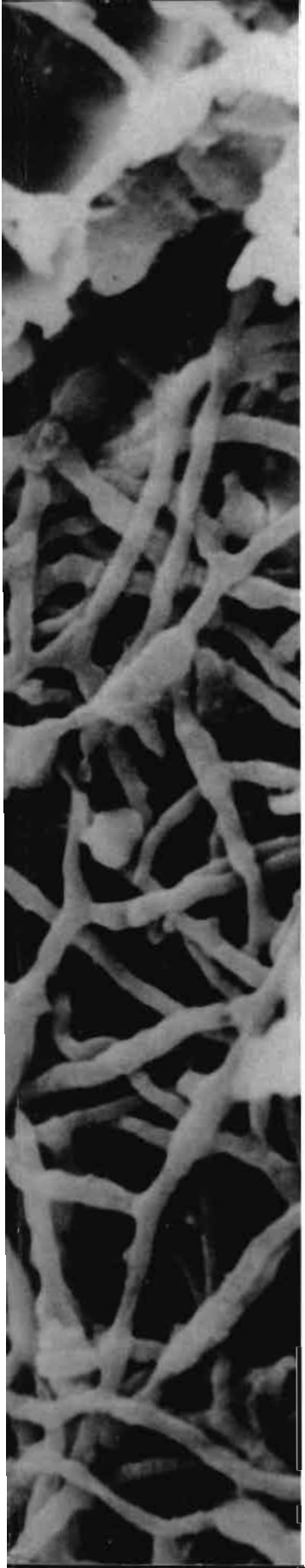

SOLID STATE FERMENTATION IN BIOCONVERSION OF AGRO-INDUSTRIAL RAW MATERIALS

Proceedings of the Seminar
ORSTOM - MONTPELLIER (France)
25, 26 and 27 July 1988

Edited by Maurice RAIMBAULT - ORSTOM

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PREFACE

Solid state fermentation is an old technique to be transformed for new purposes, using new approaches of microbiology, biochemistry and biochemical engineering.

This volume gathers the papers presented in a workshop organized by the Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) and Universidad Autonoma Metropolitana, Mexico (UAM) with the financial support of the Economic European Community (EEC) which was celebrated in Montpellier, France on July 25-th to 26-th, 1989.

The purpose of such workshop was to communicate the main results of the joint research done in Mexico and France by UAM and ORSTOM to a selected group of international research workers in order to criticism, exchange points of view and make an appraisal of the perspectives and limitations of this subject of research and developement.

The title "Solid state fermentation in bioconversion of agro-industrial raw materials" indicates the approach for research and applications and the type of technology to be presented.

In the first place, the use of agro-industrial raw materials calls for technology applied on bulky substrates wich are difficult to sterilize and should be handled in large quantities, for example, sugar cane bagasse, beet pulp, cassava meal.

In the second place, the notion of bioconversion indicates the use of traditional or modern techniques of fermentation mainly by microorganisms that are well adapted to solid substrates, such as, molds to be grown on porous materials in the presence of oxygen, or yeasts and bacteria to be grown in wet pastes in the abscence of such gas. It also implies the posibility if changing the economical and commercial value of products, transforming waste or low value substrates into fine chemicals i.e., enzymes, antibiotics, biopesticides of phytohormones or, improving the feed value of cheap raw materials. It could also involve the upgrading of traditional processes such as indigenous lactic fermentations or mushroom production with local strains and materials.

The proceedings comprise sixteen papers in a wide range of subjects from very traditional subjects : from the description of some indigenous fermentation such as pozol or the use of *Pleurotus cornucopiae* a traditional mushroom from central Mexico to, new subjects such as the production of penicillin, cheese flavours, giberellin, enzymes, microbial protein and alcohol, including also fundamental subject including the importance and measurement of water activity and engineering subjects such as scale-up procedures, reactor design and operation and practical matters such as the economics and commercial perspectives.

The authors came from various continents, a large proportion of them came from Mexico and met French colleagues which have worked together for more than five years as part of the cooperative agreement UAM-ORSTOM but it was also interesting to compare results with other French research groups from the Institut Nationale de la Recherche Agronomique (INRA), Université Technique de Compiègne (UTC), Université de Languedoc, Université de Bourgogne and other groups around the world : Guatemala (ICAITI), India (CFTRI), United States of America (Vanderbilt University) and Israel (Technion).

The main thrust of the workshop was the review that many new applications can be developped for solid state fermentation and some of them do not seem to need complex techniques of substrate conditioning such as sterilization, stirring and liquid waste management.

There were important questions on the future of this type of fermentation. The major one is the difficulty to transfer the potential applications to industrial developments mainly because there is little experience in this area for reactor design and the engineering principles of conventional submerged fermentation.

It was refreshing and at the same time puzzling to know that penicillin production was tried by solid fermentation many years ago, according to the report of Ziffer and Shelef from Israel. In a way, it seemed accidental that the major consulting engineers in the Nothern Research Laboratories of Peoria, Illinois had no feeling for solid fermentation during the early stage of penicillin development in USA in the 40's but a better understanding for stirred tanks and homogeneous reactors. Since then, solid fermentation remained a speciality for oriental food fermentations with very little dispersion of industrial "kow how" in western countries. New attempts for industrial development of solid fermentation in France have found economical limitations, specially in the area of feed production from beet pulp and also in the mexican attempt for enriching cassava by solid fermentation.

The main trend for future developments seem to concentrate on high added value products such as food enzymes and additives and fines pharmaceutical and agrochemical products. Here the competition with conventional liquid fermentation could be overcome by reduction in capital investment, simplification in equipment and decreasing energy costs for up and down stream processing.

Participation of Third World countries in this subject of research is well represented by contributors from Latin America, India and Guatemala showing that this line of work offers interesting opportunities for last comers to biotechnology. In this sense, the joint effort UAM-ORSTOM supported by the EEC could be an interesting example of internacional cooperation for developping new technologies and finding local applications in both sides of the Atlantic Ocean. It should be noted that the pioneering vision of Professor J. Senez in the future of novel applications for solid fermentation has played a significant role in the integration of this type of cooperation including the fundamental work developed by Dr M. Raimbault in Senegal, France and Mexico.

The hospitality of ORSTOM Laboratories at Montpellier was an important ingredient for the cellebration of this workshop.

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ENZYMES PRODUCTION BY SOLID STATE FERMENTATION.

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B.P. 5045, 34032 MONTPELLIER cédex.

AMYLASES

Solid state fermentation are mainly used for traditional food processing and for fungal enzymes production.

Enzyme production by solid fermentation is known for a long time, mainly for glucoamylase production derivated from the traditional koji process described by Takamine in 1914 as the Mouldy Bran Process using Aspergillus oryzae cultivated on mixture of wheat bran and rice. It was the first industrial process for enzyme production by solid substrate fermentation. This process was developped in Japan. Now, several processes are described in the litterature to produce fungal enzymes.

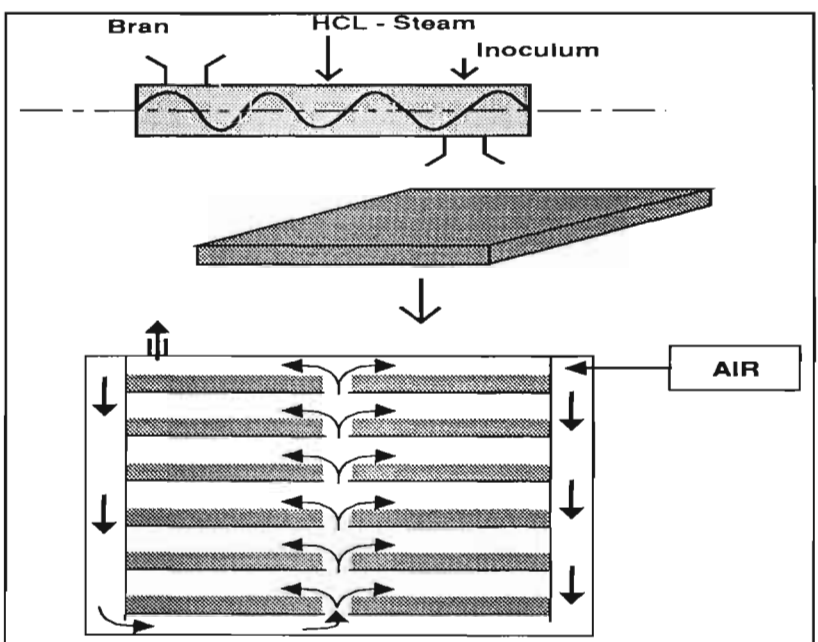

Figure 1 AMYLASE PRODUCTION BY THE MOULDY BRAN SYSTEM

The Mouldy Bran Process consist in growing *A. oryzae* on a mixture of fibrous and starchy solid substrate pretreated by acid and steam, then inoculated by spores. The inoculated substrate is disposed on perforated trays on 4-5 cm layers. Trays are placed in an incubating cabinet with control of aeration, relative humidity and temperature during several days. An intense mycelium growth, then sporulation occure. The total mass is cropped , air dried and grounded for current uses. An alternative consists in water extraction and precipitation of amylases for food purposes.

The product is generally known as "Takadiastase" and is yet practiced in Japan.

A modification of the process was proposed by Takamine to simplify the manipulation of trays.For that it was designed a slow rotative cylinder (1-2 rpm) on an horizontal axis allowing mixing and homogeneization of the mash.

More recently, other workers studied amylases and glucoamylases production by the Mouldly Bran Process, especially in the view of the optimization of environmental conditions of aeration, temperature and relative humidity.

Mudgett and co-workers reported an increase in production when partial presure of oxygene is maintained at a high level, and a decrease of yield when CO_2 partial pressure arise. This result confirms the importance of an efficient aeration device for amylase production.

On the other hand, several authors consider the importance of the water activity (Aw) and reported a decreasing of the *A. oryzae* growth rate, corelated with the decreasing of the Aw. In this case, the growth stopped when the water activity was less than 0.9. They report a maximum production of amylase at 35% water content, 2% CO_2 and 38°C.

The attention of authors was focused on kinetic and biochemical properties of amylases production by solid state cultivation in the Mouldy Bran Process.

Mitsue and coworkers reported 3 forms of glucoamylases with different moleculars weights and biochemical characteristics.

For Ueda, glucoamylases II and III were the products of attack of proteins by hydrolases synthetized during the secondary metabolism. Also, it could be due to a modification of the glycosidic portion of the form I of the enzyme.

Other research works attempted to select enzyme, hydrolysing crude starch and, for that, the absorption Enzyme/Substrate capacity and cutting chain power are actively investigated. For Sato, the use of such enzymes could avoid the gelatinization step in the saccharification process of starchy substrates.

In our group glucoamylases produced during solid state fermentation of cassava by *A. niger* indicated differences between properties when produced by liquid or solid cultivation. The major part of enzyme production in solid cultivation was produced during the stage corresponding to the secondary metabolism. In the case of liquid fermentation we observed an autolysis with quick decrease of the glucoamylase activity. It would be necessary to add more substrate to maintain alive the mycelial biomass.

More important is the difference observed about physical and biochemical characteristics.

A major point for amylase production is the water content of the substrates. All the results confirm the importance of the initial water content.

So, it is important to improve methods of water control and measurement of the Aw in the solid state fermentation.

Recently Oriol studied the importance of water activity in solid state fermentation`. All the results confirm that enzymes produced in solid fermentation can differ significatively than enzymes produced in liquid conditions. Particularly the good performances of resistance to acidic conditions or thermic treatment for enzymes solid cultivation are important to point out.

Ghildyal in 1985 published an economic analysis comparing solid and liquid processes. Due to the low cost of investements and the high concentration in enzyme, he concluded that it would be more profitable to produce glucoamylase by solid cultivations, in spite of considering the most pessimistic estimations for the solid fermentation process. However, that is not sufficient, because commercial amylases are produced in industrial manufactures by liquid process, industrial operators will not change entirely their technology.

For this reason, more than a pure commercial competition, it would be preferable to consider production of enzymes with different characteristics which couldn't be obtained by liquid process.

PROTEOLYTIC ENZYMES.

Generally, this kind of enzyme is produced only by liquid fermentation process. Litterature is very poor about fungal proteases produced by solid cultivation.

- Fukushima in 1982 described preciselythe proteolytic complexe present in the *koji*; he demonstrated that a great number of proteases and peptidases produced by *A. oryzae* in solid state cultivation were not present in liquid cultivation.

- Hesseltine in 1977 and Aldoo in 1982 reported about an industrial process of protease production by *Mucor pusillus* through a technique similar to the *koji* one. However, we have no further information.

It would be important to investigate new fungal protease activity in relation to the production of flavour or aroma for food purpose. It is possible that properties of fungal proteases obtained from solid cultivation during the secondary metabolism would be different of proteases produced in liquid culture and more comparable to natural flavors.

CELLULASES.

On the contrary, in the case of cellulases productions by solid or liquid fermentation, the litterature is so rich that it is difficult to make the list of all groups working on the subject. However no significative industrial production of cellulase at the commercial level is known. Probably the cost is to high for potential and applications in feed are or for saccharification of ligno cellulosic wastes. In this last case, it was calculated by an european group, that for enzymatic saccharification, the cost of enzyme represent like 60% of the production cost. The

necessity to produce cellulases at low costs incited various groups to perform studies in view of developing solid fermentation process.

was the first to report cellulases production of *T. reseI* by an adapted koji process. After that, a lot of studies was reported in the litterature for the optimization of culture conditions, using mainly *Trichoderma* genus but also with *Talaromyces* or *Pestaliotopis* and *Sporotrichum*: Chamal in 1985 working with *T. resei* on wheat straw, reported superior yields in solid than in liquid cultivation. Deschamps in 1984 reported interesting results for producing ß-glucosidase (a limiting step in cellulose attack) through a solid state cultivation with *Aspergillus phoenicis*.

In all solid fermentation systems described in litterature, cellulase were obtained from fermented mash after their extraction. For this purpose, the solid sample is mixed with 2-4 volume of water, following a lag time of maceration and the liquid containing cellulases is filtrated or centrifugated. This method induces a high dilution of enzymes which have to be reconcentrated, by precipitation or ultra-filtration, loosing advantage of the solid state culture for obtention of concentrated enzyme.

Roussos working with selected strain of *T. harzianum* cultivated on steam vapor pretreated material (sugar cane bagasse), developped a new static solid process with direct extraction of cellulase by pressing the material, using spongious effect of the bagasse allowing to recover more than 80% of the activity of synthetized cellulases in a concentrated juice.(Roussos, 1985).

From 100 g dry matter of bagasse, we obtained after 48 hours of incubation:

- 2000 FPA Unit international and 20000 CMC Unit international. The final concentration in the liquid was 8 FPA/Units/ml and 100 CMC/Units/ml respectively.

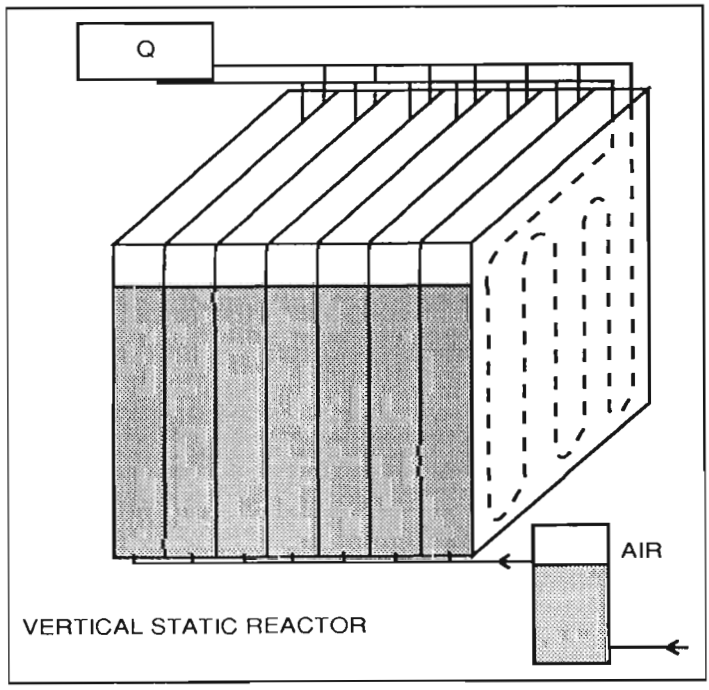

Figure 2:

For this cultivation we designed a vertical static reactor, which consists in a cubic cabinet containing exchanger for temperature control and device for forced aeration with humidified air. The capacity of this reactor is about 50 kg of wet product (about 10 kg of dry matter).

On the figure 3, the results for cellulases production on sugar cane bagasse indicate that maximum production was obtained after 48 hours, for a production of 16 international units of FPA/g of bagasse.

HOURS	% WATER	FPA (IU/100G)	CMC (IU/100G
0	68,3	29	5
20	70,7	220	675
24	71,0	145	639
28	71,4	408	2214
44	72,8	1466	17889
48	**73,5**	**1644**	**20942**
52	72,7	1570	21052
68	73,9	1340	21036

Figure 3: Cellulase production by *Trichoderma harzianum* in static solid cultivation on sugar cane bagasse.

The different steps of this process consist in pretreatment, inoculation, incubation, pressing and alternative evaporation, ultrafiltration or lyophilisation. The composition of the medium and the environmental conditions are described on figure 13. Typically, we get crude juice containing 8 FPA/ml, that is twice or three times more than the concentration obtained by liquid cultivation.

The process is not yet optimized; it was tested at the small scale lab pilote (50 kg wet matter, about 10 kg dry matter), with no significative difference regarding to yield, kinetic and concentration.

The most interesting in this work, consisted in designing a new concept for solid substrate fermentations. So, we applied the same process using sugar cane bagasse as "solid support" and not as "solid substrate" for cultivating other fungi without attack of cellulose. For that, we realize an impregnation of the pretreated bagasse with a liquid culture medium (the same used in liquid process).

The sugar cane bagasse acts as a tank of water and liquid medium for the fungi, but allows to maintain the conditions required to realize a solid state cultivation. The process was applied with success in our ORSTOM/UAM group in Mexico and allowed to study degradation of very concentrated glucose media by *A. niger*, amylase and pectinase production.

PECTINASES

Previous reports on pectinases production by solid state fermentation have been reviewed by Mushikova (1981) and Hildyalin (1981) using respectively *A. awamori* and *A. carbonarius*. But these works seem not continued. Pectinolytic enzymes are mainly endo-enzyme depolymerase. This type of enzyme is not of high interest in fruit juice manufacturing.

In the industrial processing of food, enzyme utilizations are quickly increasing. It is estimated at about 15 millions dollars per year.

For food industry purpose, pectinase are mainly extracted from fungi (especially from *A. niger, ventii* and *orizae*), because, from a commercial point of view, fungal pectinases are prefered to bacterial for 3 reasons.

- They are extracellular enzymes and simple to extract.

- mixture of pectinase ("cocktail") can very quickly reduce viscosity of juce.

- Fungal pectic enzymes characteristics like pH, temperature are compatible with conditions of process used in juice manufactures.

However essential information about fungal pectinolytic enzymes were obtained from liquid cultivation.

A comparative study for pectinase production by solid state process was developped in our joint group ORSTOM/UAM in the Mexican University. The process was the same than explained with pretreated sugar cane bagasse, impregnated with liquid culture medium containing saccharose and pectin. Trials on the concentrated enzyme after ultrafiltration proved that the protein could replace the commercial pectinase for extraction of coconut oil.

An attempt of optimization at the small pilote level realized by Dufour in our group of Mexico resulted in improving significatively the process with production of crude juce containing pectinolytic enzymes, 15 times more concentrated than in the case of liquid cultivation.

FUTURE AND PERSPECTIVES.

Initially, we tried to develop solid substrate fermentation in the view of protein enrichment for animal feeding. The low cost of soybean protein was the most evident problem.

Solid substrate fermentation for producing fungal enzymes is a new field of investigation which could be more promising than protein production. Cellulases can be easily produced through solid state process. The new results obtained in our group tend to demonstrate that we can use same culture medium optimized with same substrates (soluble or not soluble), including inducer for synthesis of specific enzymes.

Solid state culture conditions are particularly efficient for fungal growth and metabolites production, we specially refer to the following advantages:

. Great surface contact allows rapid transfer of O_2 and nutrients.

. Substances synthetized are not diluted in a great volume of water and can attack more efficiently insoluble substrates.

. Natural conditions for fungal development are solid state conditions in the nature.

. Physiology and metabolism of fungi differ when they are cultivated in liquid or solid conditions.

. We can synthetize substances that are not produced in liquid conditions.

For the future, we have to investigate more specifically in the fields of:

. Selection of strains for specific cultivation in solid culture.

. Physiological studies of the specific reaction of fungi in solid state cultivation.

. Work about new solid inert supports to diversify the sugar cane bagasse support.

. Develop researches about secondary metabolism of fungi and substances synthetized in liquid and solid conditions.

We are confident that all the progress realized in the last ten years will be profitable at short time and will be soon applied in the food or feed industry.

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COFFEE PULP DETOXIFICATION BY SOLID STATE FERMENTATION : Isolation, Identification and physiological studies.

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ABSTRACT :

Coffee pulp is a greatly abundant agroindustrial waste with a limited use as feed, due to its high contents of toxic compounds such as caffeine, tannines and polyphenols. An alternative to increase its utilization could be a detoxification process through Solid State Fermentation, using filamentous fungi. These microorganisms are able to degrade caffeine when it is used as nitrogen source. Some degradation pathways are known, and urea is one of the ultimate products of this degradation.

Soil, leaves and coffee fruits samples was recolected from Xalapa, Ver. and Soconusco, Chis., Mexico; the isolation was done by inoculating samples on 3 semisynthetic culture media contening coffee extract (A), coffee extract with sucrose (B) and coffee pulp extract (C) with mineral salts and streptomycine (30 ppm). Cultures were incubated at 25°C and 35°C.

The purification was made through succesive replication with the same isolation culture medium. In this form, 280 pure strains were isolated and identified by macroscopic and microscopic observations on standard culture media plates and microculture.

Subsequently, a physiologic study of these strains was made in liquid medium with caffeine and other controled conditions. Strains were selected for their caffeine degradation capacity in this selective liquid medium.

After 70 hours of incubation, strains with nearly 80% of caffeine degradation were found and in some cases correlated with a notable increase in pH. Most of the isolated microorganisms were *Aspergillus, Penicillium, Trichoderma and*

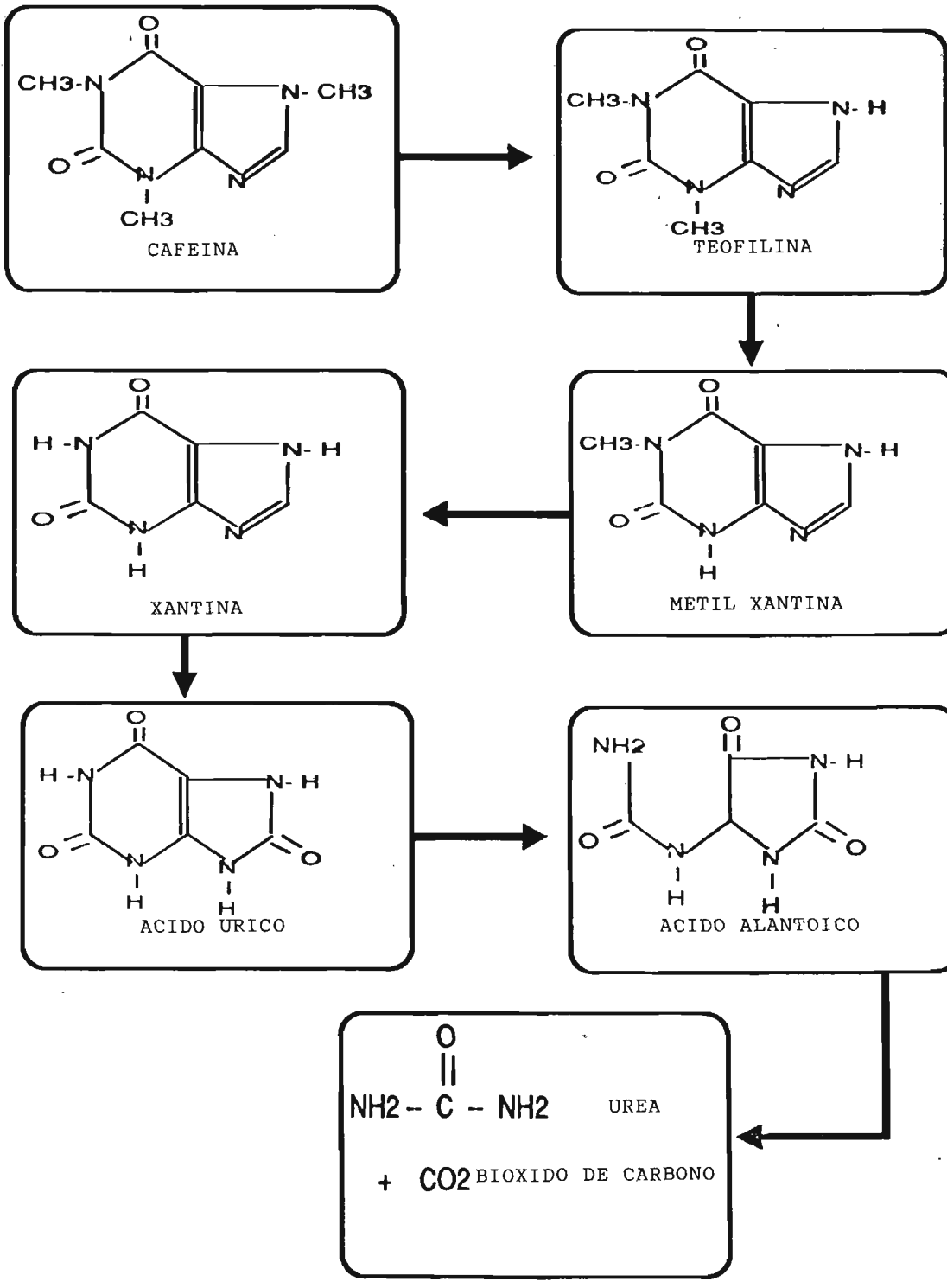

fig. 1 : Biodegradation of caffeine

Fusarium strains, which correspond to Kurtzman y Schwimmer,(1971) who considered biological alternatives to decaffeination with *Penicillium crustosum* strain NRRL 5452 with coffee infusions.

INTRODUCTION :

Coffee pulp represents the most abundant waste produced during the pulping operation of the coffee cherry needed to separate the coffee grain (Bressani, 1979). In Mexico as well as in other coffee producing regions, coffee pulp is barely used and, therefore, it is considered to be the most abundant polluting factor for rivers and lakes located near the coffee processing sites.

The utilization of coffee pulp as an animal feed has been mentioned as an atractive possibility. However, such utilization is limited by antiphysiological factors naturally ocurring in the material such as caffeine, tannins, chlorogenic acid, caffeic acid, and an excess of potassium (Adams and Dougan, 1981; Bressani, 1979).

Many works were performed for elimination of the antiphysiological compounds of coffee pulp using decaffeination (Molina, 1974), silage, as well as treatments of the material with calcium hydroxide or potassium bisulfite, water, and the combination of these compounds with physical treatment: grinding, extrusion, heating and drying (Gomez, 1979). But all methods were either inefficient in reducing the toxicity or too expensives.

Some reports indicate that the biological methods could be successfuly used in detoxification of coffee pulp. Bergmann and al. (1962) reported a *Pseudomonas aeruginosa* strain which oxidize 2-aminopurine as well the 2-methylamino purine and 2-dimethylamino purine derivative at position 8, with a xanthine oxidase system. This bacteria degraded caffeine via theobromine. Schwimmer and Kurtzman (1972) isolated and studied the metabolism of a *Penicillium crustosum* strain, which efficiently utilized caffeine via theophyline as source of nitrogen in clearly defined growth media with coffee infusions.

Recently a cytochrome P-450, a flavoprotein cytochrome mediated oxidase systems, has been detected in enzyme extracts of a caffeine-degrading yeast (Sauer and al. 1982) which implies that caffeine metabolism in yeast might be similar to the human one. As it could be expected from the literature on purine metabolism in both fungi and in higher organisms, xanthine was found to be dehydrogenated to uric acid, which was further metabolized to allantoin, then to allantoic acid, and finally to urea and carbon dioxide. The metabolism of methylated xanthines such as caffeine may involve a direct oxidation to methylated allantoine (Franke, W, 1955) or demethylation, following the catabolic pathway shown in Fig.1.

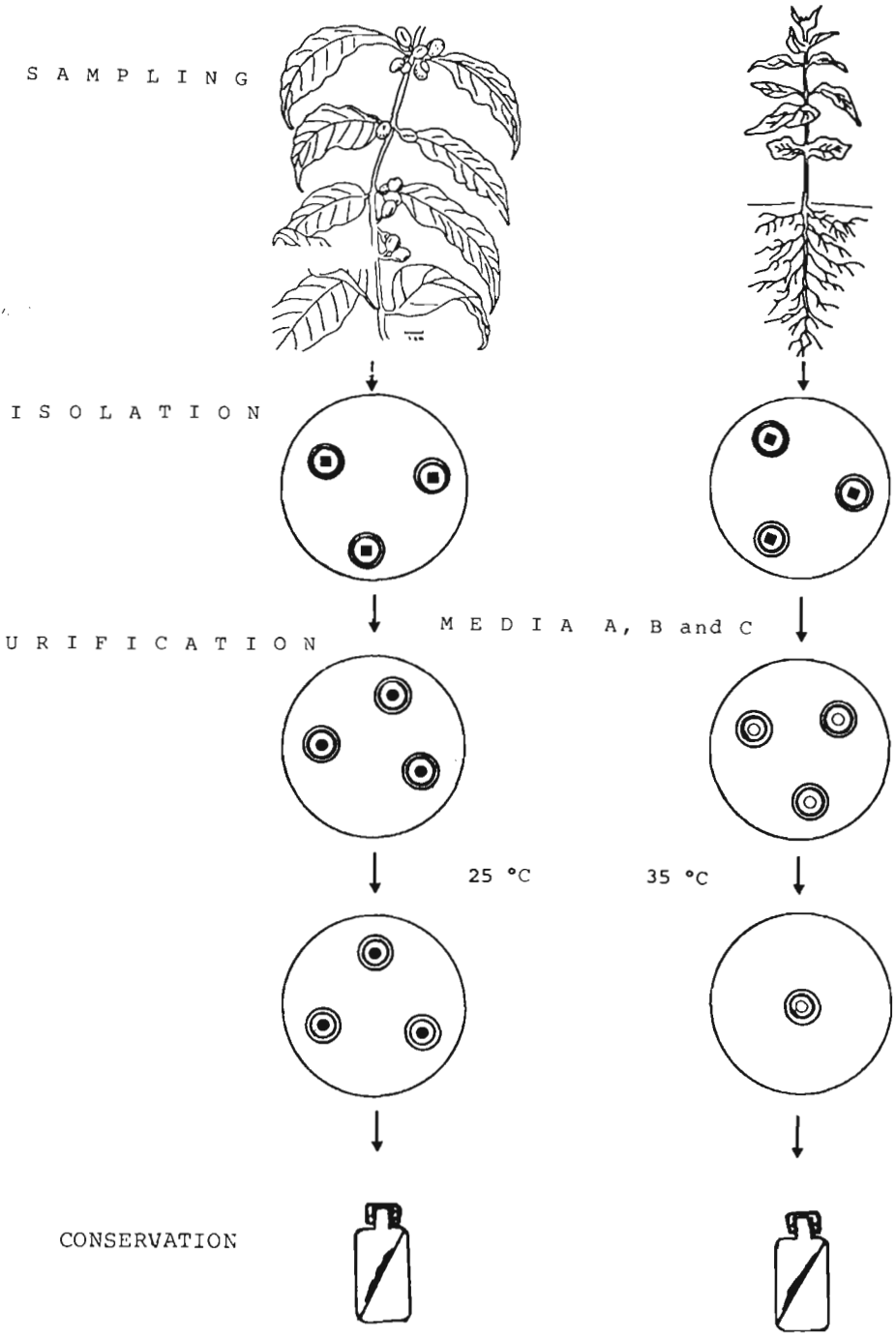

Fig. 2. Isolation, Purification and Conservation of filamentous fungi Diagra

The present work was undertaken to obtain new filamentous fungi strains with ability for caffeine degradation. The isolation, purification and physiological characterization of microorganisms was also necessary. Some 280 strains were screened, 8 of them were chosen for their high capacity for caffeine degradation. The best one was assayed in solid state fermentation using coffee pulp as solid substrate.

MATERIAL AND METHOD :

Isolation and purification of fungi strains.

Leaves, fruits and coffe pulp were sampled from coffee industry in Jalapa, Veracruz and Soconusco, Chiapas in Mexico. Strain isolation and purification was made in a basic medium containing: 1.3 g KH_2PO_4, 0.12 g Na_2HPO_4, 0.3 g $MgSO_4$, 0.3 g $CaCl_2$, in 1000 ml of water (pH 5.6) with coffee extract (medium A), coffee extract and sucrose (medium B), or coffe pulp extract (medium C). Each media were supplemented with 30 mg/l of streptomycin and cultured at 25 and 35°C. The isolation flow sheet is shown in fig. 2.

Identification

The strains identification was made in plate and microculture on Potato-Dextrose Agar Medium, and cultured at same isolation temperature. The conservation strains was made in slants containing the same isolation media and maintained at 4°C

Screening of caffeine degrading strains

The screening of caffeine degrading strains was carried out in 250 ml. shaker flasks containing 50 ml of liquid medium with mineral salts with the same composition as the isolation medium; caffeine (1.2 mg/ml) was the nitrogen source; the incubation was 2-3 days at 25 and 35°C (fig.3).

Solid state fermention

Solid State Fermentation (SSF) was carried out at a laboratory scale, using the unit device described by Raimbault and Alazard (1980), in which a constant temperature can be obtained through forced convection water bath. Regulation of the air flow in each fermenting column could be set by independent valves. Fermentations were run at 25°C and moistened saturated air flow rate was 4 liters/min/device.Each device contained 20 g of the moist coffee pulp (60 mesh). The substrate was mixed with the same mineral salts solution than used in the isolation medium. Inoculation was performed with a suspension of

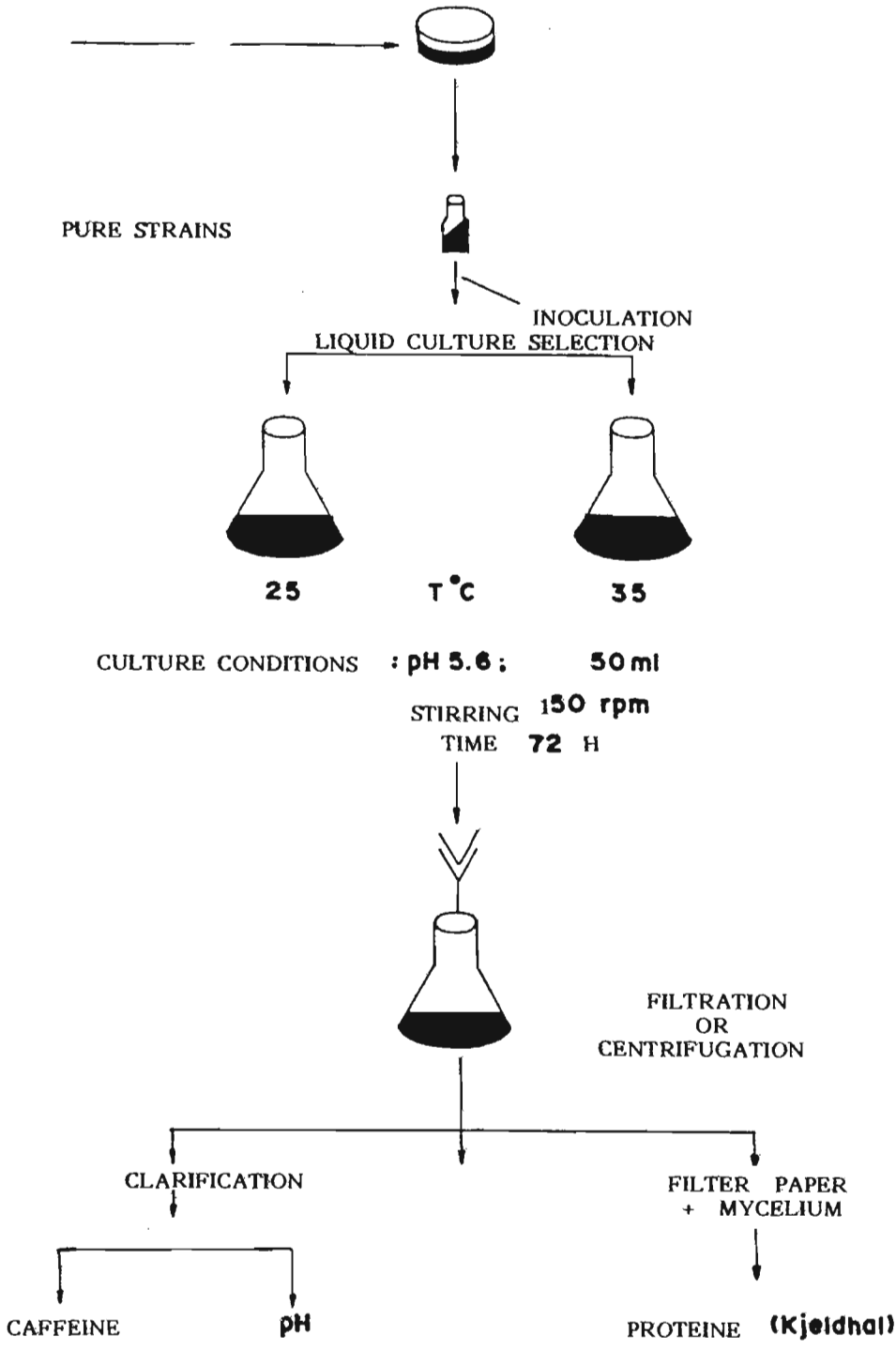

Fig. 3 ISOLATES TREATMENT

conidiospores of the selected strain (2.107 106 spores per g of dried coffee pulp), coffee pulp initial moisture was adjusted to 68-70% and the pH to 4.4 with diluted HCl solution.

Analysis

In selection trials, biomass was measured as crude protein by using standard Kjeldahl method (Bremer, J.M. 1960). Caffeine was measured spectrophotometrically after decoloration with magnesium oxide (Isler, 1948).

For caffeine analysis in solid state fermented products, 5 g of sample were mixed with 75 ml of water and homogenized with Ultra-turrax (IKA, W.Germany) during 2 min at 5000 rpm then the pH was measured, heated to boiling temperature during 10 min and finally filtered, rinsed and adjusted to 100 ml with water (Smyly, Dan S., 1976).

Moisture content was determined in an oven at 100°C during 24 h.

RESULTS AND DISCUSSION :

According to the isolation protocole used, 280 strains of filamentous fungi in pure culture were isolated from Veracruz and Chiapas samples.The table I summarizes the results of isolated and identificated strains: *Aspergillus* and *Penicillium* strains were predominant, but other genera could be identified as *Fusarium*, *Trichoderma*, *Geotrichum* and some Zygomycetes. These results agree with litterature report of Schwimmer and Kurtzman (1972).

The list of filamentous fungi with high capacity to degradate caffeine in liquid medium are reported in tables II and III. This experiment was made with caffeine as sole nitrogen source in mineral salts of basic media containing sucrose as carbon source. The best caffeine degrading strain was Penicillium roquefortii with 95.25% efficiency and a rate of 0.224 mg/ml/day. Other strains such *Aspergillus* and *Penicillium* species also degraded caffeine with similar efficiency and rate. In some cases it should be noted that the growth was associated with an increase in pH.

Figures 4, 5 and 6 show the pH, caffeine depletion and moisture evolution with *Penicillium roquefortii* in solid state fermentation. The coffee pulp required a higher moisture content (70%) than the 50% reported for A. niger grown on starchy substrates by Raimbault (1980). That indicates that the constituents of the pulp bound a larger amount of moisture than the starchy materials and, need more water to allow growth of *Penicillium roquefortii*. Since coffee pulp has been reported to contain 6-8% mucilagenous content, it is possible that these

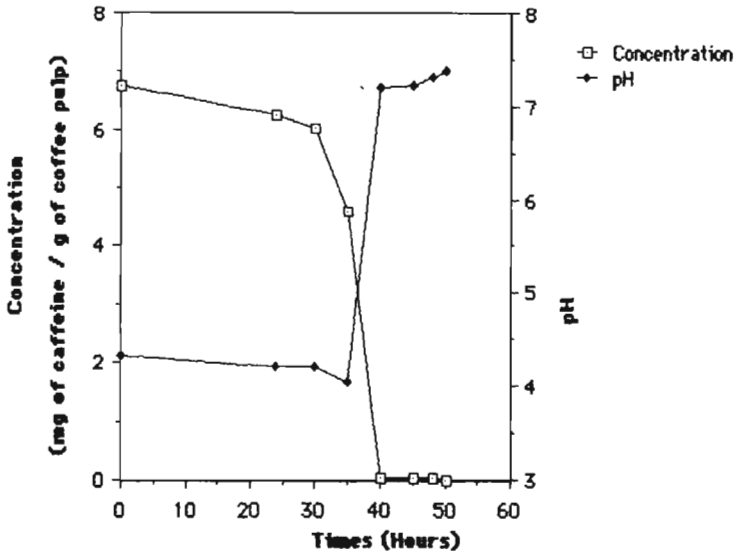

Fig 4 : Kinetics of caffeine degradation and pH evolution during coffee pulp Solid State Fermentation

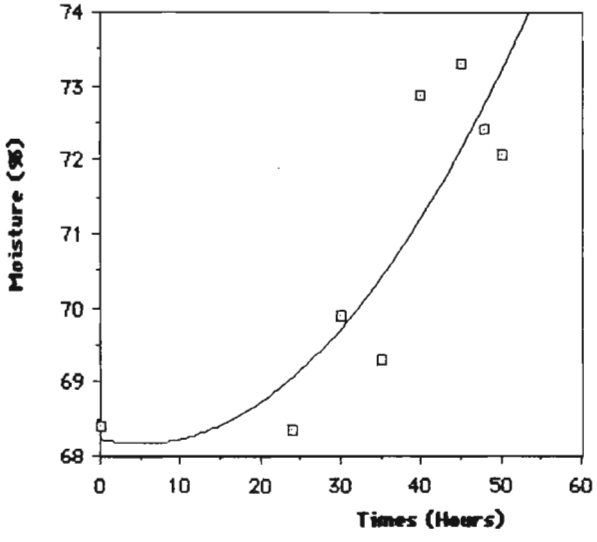

Fig. 5 : Moisture evolution during coffee pulp SSF

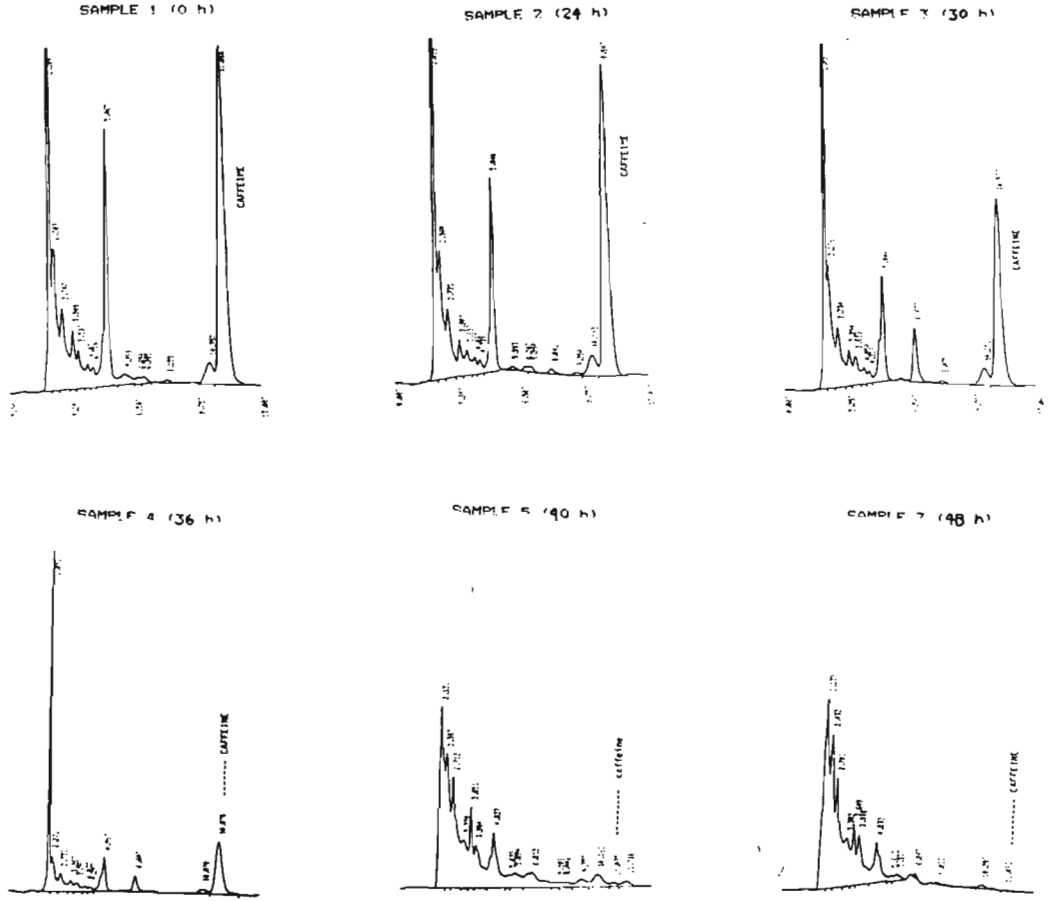

fig. 6 : Determination of caffeine by HPLC Method

TABLE I Isolation and identification of filamentous fungi growed on media A, B y C in 25º C. (% isolated strains).

	VERACRUZ			CHIAPAS		
	A	B	C	A	B	C
Aspergillus	15.2	2.1	2.1	--	14.2	3.2
Penicillium	10.8	2.1	--	4.7	--	--
Fusarium	8.6	--	--	7.9	17.4	11.0
Trichoderma	--	--	--	--	1.6	3.2
Geotrichum	4.3	2.1	13.0	--	1.6	3.2
Zygomycetes	15.2	23.5	--	9.5	3.2	14.2

TABLE II LIST OF FILAMENTOUS FUNGI WITH HIGH CAPACITY TO DEGRADATE CAFFEINE IN LIQUID MEDIUM. STIRRED CULTURES AT 25ºC, pH=5.6,150 RPM AND CAFFEINE AS NITROGEN SOURCE (1.2 g/l).

STRAIN	NAME	% CAFFEINE CONSUMPTION	FINAL pH	DEGRADATION VELOCITY mg/ ml/ days
V12A25	Aspergillus oryzae	77.75	6.7	0.157
V26A25	Penicillium sp.	62.13	7.2	0.126
V33A25	Penicillium roquefortii	95.25	6.5	0.126
C16A25	Penicillium sp.	61.66	2.5	0.123
C28B25	Aspergillus fumigatus	69.60	6.1	0.119
C11B25	Aspergillus sp.	70.66	6.3	0.120
C23B25	Aspergillus niger	63.58	3.4	0.108
C17B25	Aspergillus fumigatus	60.50	2.4	0.103

TABLE III Quantitative selection of strams of filamentous fungi, with hability to deplete caffeine in liquid media, by the use of stirred flasks at 35ºC during 4 days.

STRAIN No.	% OF DEPLETED CAFFEIN	pH FINAL	GROWTH	
V20A35	18.25	6.56	+++	(pellets)
V25A35	17.08	6.63	+++	pellets
V3A35	10.83	6.27	+++	pellets
V2A35	6.66	6.34	++	pellets
V12A35	4.58	3.65	++	pellets
V29B35	18.66	6.28	+++	pellets
V31B35	16.25	6.34	+++	pellets
V20B35	14.33	6.30	++	pellets
V12B35	4.16	3.06	++	pellets
V33B35	2.5	6.3	+	difuso
V29C35	26.5	6.49	+++	pellets
V25C35	25.16	6.31	+++	pellets
V26C35	21.0	6.16	+++	pellets
V13C35	19.83	6.16	+++	pellets
V10C35	14.83	6.3	+++	pellets
V15C35	12.50	6.23	++	pellets
V16C35	11.64	6.35	++	pellets
V23C35	4.41	6.15	+++	pellets

(+) low growth
(++) medium growth
(+++) high growth

Conditions: pH=5.6, 2,00 rpm.

Caffein concentration: 1.2 g/l.

components are mainly responsible for the difference in binding water with the starchy materials (Penaloza,W and all, 1985).

Solid state fermentation process caused an increase of pH after from 35 h correlated with caffeine decrease initiation just in the same way than observed in liquid culture. This could be due to by-products of the degradation such as area, according to the proposed metabolic pathway (Fig. 1). After a fermentation period of 45 hr, the caffeine was completely consumed (Fig.6).

From the evidence presented here, we may conclude that this isolation, purification and selection methodology allowed to find highly degrading strains of caffeine (80-100%).

The present investigation established that caffeine can be removed from coffee infusions as well as synthetic growth media mainly by Aspergillus, Penicillium and Trichoderma strains.

Also results proved that filamentous fungi could be used to degrade caffeine by solid state fermentation processe vith the objective of improving nutritional value of coffee pulp for animal feed.

ACKNOWLEDGMENTS :

This work was carried out as part of the cooperation agreenment between the Consejo Nacional de Ciencia y Tecnologia (CONACYT, México) and the Institut Francais de Coopération Scientifique pour le Développement en Coopération (ORSTOM, France) with a specific research program agreed between the Universidad Autonoma Metropolitana, Mexico and ORSTOM. The authors wish to thank CEE for financial supports (projects n° TSD-A-106 and TSD-A-418).

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ISOLATION, GROWTH IN ARTIFICIAL CULTURE MEDIA, AND FRUITING BODY PRODUCTION OF *Pleurotus cornucopiae* IN THE AGAVE CACTUS LEAVES.

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The true fungiae of the genus *Pleurotus* are economically attractive due to its nutritional value, fine taste and possibility of industrialization. In Mexico, some efforts have been realized in order to achieve the *Pleurotus ostreatus* strain adaptation for sporocarpe production over different ligno-cellulose residue. These works are now used as a basis for the preliminar application of *Pleurotus cornucopioides* in Mexico, as well as in other countries.

In the Tlaxcala and Puebla States, at the center of Mexico, *P. cornucopioides* is harvested and sold in the town market in rainy seasons. The fresh mushrooms are used to prepare several sausages and traditional foods, while dry it is used as seasoning.

Preliminar observation realized by the authors, suggest that the moisture and sun exposure are very important bioclimatic factors for the fungi mushroom development over agave leaves. The agave leaves are the carbon and nitrogen sources as well as other nutritional metabolites. These preliminar results allows to establish an experimental model for the study of vegetative mycelium and sporocarpe production during the sexual phase, as has been done with other fungi species. This laboratory model allows a scaling up process to industrial production.

This work was conducted on the development of *P. cornucopioides* in agaves *sp* leaves, with the following objectives:

1. Mushroom identification and classification
2. Effect of climatic factors
3. Isolation of vegetative mycellium.
4. Simulation of natural conditions for sporocarpes production.

Thus, the work hypothese was: if the *P. cornucopioides* is cultivated under similar conditions to natural ones, it is able to produce sporocarpes.

The study phases were as following:

a) isolation of vegetative mycelium in Malt extract agar (MEA)

b) propagation tests in several culture media

c) preparation of cultures seed or "Spraw" in the classic procedure using wheat seeds

d) inoculation in composted agave leave residues, previously fermented and

dried.

The methodology contributions were:

- Agar-Agave culture media (peel, peel and pulp) with sucrose added as main carbon source.

- Seed or Spraw production with *Agave sp.* (peel, peel and pulp) biodegradable.

First phase: *P. cornucopioides* isolation (after harvested and clasified) is realized by taking a portion of basal mycelium in MEA, PDA and Agave Peel Agar (APA).

The MEA and PEA culture media were prepared as usual. In the APA case, the peel is milled to obtain an extract, which is used to prepare the solid culture media. The MEA culture can be used either to isolate or to propagate the mushroom medium.

In our laboratory a mycelial biomass comparative study has been realized among different native strains of *P. cornucopiae* and *P. ostreatus* taking in account its development and efficiency, while varying pH, temperature, moisture and light intensity.

At room temperature, the optimum pH is 5 for PDA and MAE, while 6.5 for APA, in the latter the rate growth was as well as using MEA, thus it was adequate for mushroom isolation and propagation.

The agave leaves substrate for seed production of sporocarpes, was prepared by milling the agave leaves, adding 30% of water and allowing to proceed in wood cases, after 20 days it is dried. This procedure was the most convenient as compared with dried or natural degraded agave.

The mushroom seed was prepared with moistened sterilized wheat, in a similar way, agave biodegraded seed were prepared at different particle sizes.

The substrate pH was around 6-8, it was moistened, pasteurized, inoculated and incubated at 20-25°C, and 18-22°C for the sporocarpes production using a high humidified atmosphere.

Different kind of substrate were tested as:

Peel, pulp, peel and dried pulp, at pH 5-6 with 0.35% nitrogen and incubated at 20-25°C during 60 days.

Peel and fermented pulp, peel and harvested dried pulp, at pH 7-8 with 0.42% nitrogen and 80 days of incubation, which as been reduce to 60 days.

The mushroom *P. cornucopioides* composition was as follows: Moisture 72-75%, Total protein 24.5% and fiber 0.2%.

The total nitrogen substrate concentration was: 0.35-0.42% after 20 days degradation and is: 0.52-0.54% after mushroom growth.

MEXICAN TRADITIONAL SOLID STATE FERMENTATIONS

CHEMICAL AND MICROBIOLOGICAL STUDY OF LACTIC ACID FERMENTATION IN POZOL (A MEXICAN CORN -BASED BEVERAGE)

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INTRODUCTION :

Pozol (from Nahuat language, "pozzoli", foamy) is a fermented corn - based beverage, which has been consumed in some states located in the south and southeast of Mexico, since before the Spanish conquest.

According with the traditional procedure (4), white or yellow corn (*Zea mais*) grains are boiled in milk of lime and let standing overnight. Afterwards, grains are rinsed and husked. Additional boiling in water may also be performed. Grains are then ground. Small pieces of corn-dough are then formed by hand, covered with banana leaves and let standing at room temperature during 4-5 days. When dough becomes sour and flavourfull, it is dispersed and beaten in cold water. Non fermented pozol is also consumed in some communities as a soft drink.

Some nutritional studies performed by Cravioto et al. (4) on pozol dough, showed that the fermentation process enhanced the content of protein, tryptophan, lysin and vitamins contents.

The atmospheric nitrogen fixation has been observed by Ulloa et al. (14), during pozol fermentation.

Some lactic acid bacteria, such as *Lactobacillus brevis* and *Lactobacillus fermentum* have been isolated and identified from fermented pozol by Siva-Villarreal et al (13).

Material and methods.

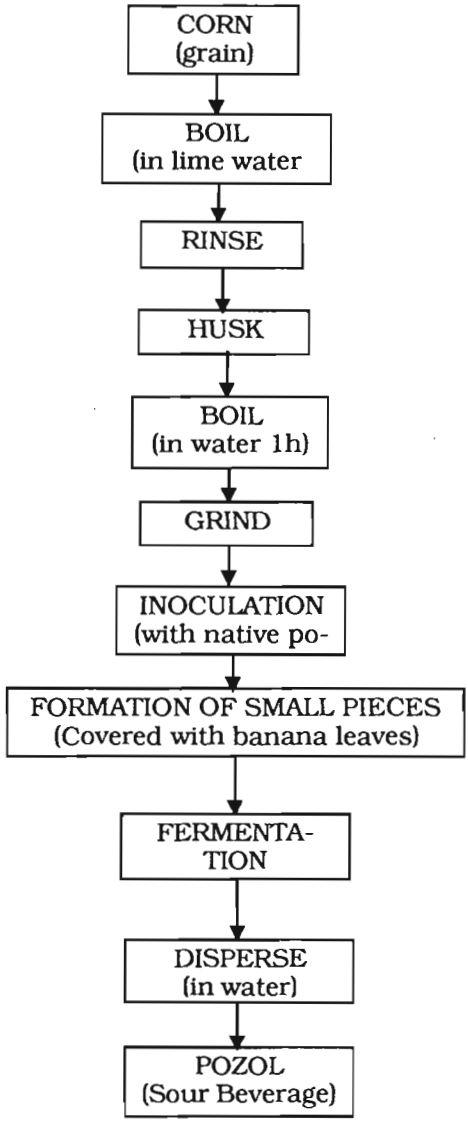
Fermented pozol has been prepared in the laboratory, following the traditional procedure. Process outline is shown in figure 1. Native pozol dough was obtained from Chiapas state, and it was used as a fermentation starter. It was mixed with 50 g pieces of corn dough in a proportion of 3%. Pieces were then covered with cleaned banana leaves and then incubed. Process conditions are shown also in figure 1.

-Sampling

Two pieces of pozol dough were randomly removed from the incubator every 4 hours during the first two days; and every 6-8 hours during the other days. From every piece, 1g was taken aseptically, sampling from several sections. Pro-

FIGURE 1

Pozol preparation procedure



per dilutions were prepared from this sample with isotonic phosphate solution. Microbiological analysis were performed on the dilutions. The rest of the dough was freezed at -17°C. Samples were then thrawed and homogenized before performing physicochemical analysis.

-Microbiological methods

Total and differential viable counts where carried out by plating 0.1 ml aliquots of sample dilutions on the surface of modified Microinoculum-agar plates (6). Medium composition is shown in Table 1. Plates were incubated at 28°C during 24-48h. Colonial morphology and plate color change were observed directly, and Gram stained preparations under optical microscope. Strains whose morphology fitted with those of lactic acid bacteria, were isolated in Microinoculum-agar plates.

-Analytical Method for Amylolytic Activity (3)

Starch agar plates were inoculated with light loops of isolated lactic acid strains. Medium composition is shown in table 2. Inoculated plates were incubated at 30°C during 24-72 hours. Plates surfaces with colonies development was exposed on iodine vapor. Clear surface surrounding the isolated colonies was considered as an evidence of amylolytic activity. It was assigned a scale to the clear surface magnitude, from 0 to 4, where 4=10cm diameter.

-Methods for Methyl Red / Voges Proskauer (MR/VP) tests (8)

Acid (MR test) and acetoin or diacetil formation ability from glucose (VP test) of isolated strains were tested. Light loops of those strains were inoculated in MR/VP broth, and treated according to the usual methods.

-Physicochemical methods

Humidity. Samples were dried in an oven at 90°C to constant weight. Humidity content was calculated by difference on weight (1).

Total carbohydrates. 2g samples were soaked in 50 ml of distilled water at 4°C during 8 hours. An acid hydrolysis was then carried out by adding 5 ml of concentrated hydrochloric acid and warmed at 60°C, during 30 min. The suspension was then neutralized by adding enough 30% NaOH solution. Volume was made up to 100 ml with distilled water. Reducing sugars were analysed from this suspension by the Nelson-Somogy method (9).

Total protein. 2g samples were dispersed in 50 ml of distilled water. Volume was then made up to 100 ml. Protein was precipitated in 5ml of this suspension with trichloroacetic acid. It was then centrifuged and solubilized according to the method reported by Lowry (10). From the resulting solution, soluble protein was analysed following the Folin-Denis method (7).

Total nitrogen. Samples were dehydrated in an oven at 90°C to constant weight. Total nitrogen was analysed in 100 mg of the dehydrated sample by micro-Kjeldhal method (11,12).

Lactate. 2g of sample were dispersed in 50 ml of distilled water and volume was made to 100 ml. Lactate content was analysed from this suspension by the spectrophotometric method reported by Barker (2).

Titrable acidity. 5g fresh corn dough samples were dispersed in 10 ml of distilled water. Titrable activity was determined with 0.1 N NaOH, using phenolphtalein as indicator (1).

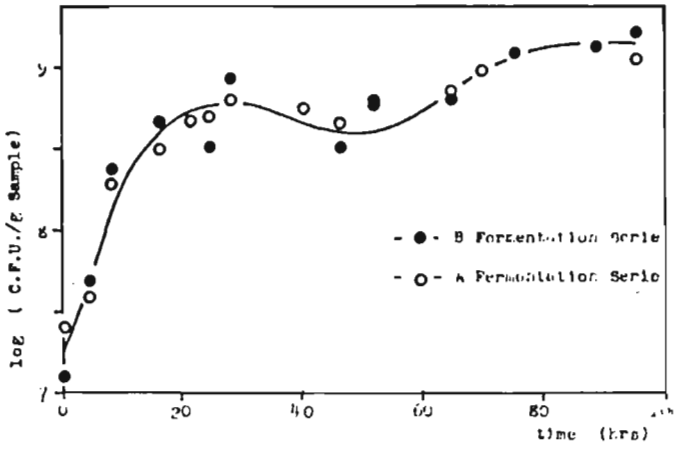


Fig. # 2 TOTAL VIABLE COUNT OF MICROORGANISMS IN POZOL FERMENTATION (IN MICROINOCULUM AGAR PLATES)

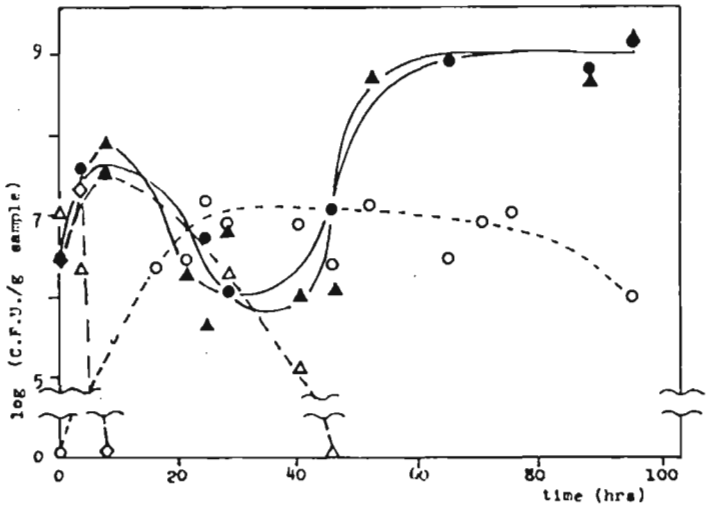


Fig # 3 DIFFERENTIAL COUNT OF MICROORGANISMS IN THE POZOL FERMENTATION (IN MICROINOCULUM AGAR PLATES)

- ● - Lactobacilli
- ▲ - Streptococci
- ○ - Yeasts
- △ - Gram (-) bacteria
- ◇ - Staphylococci

pH. pH was mesured in fresh samples with a pH-meter. Measurements were carried out in different points in corn dough samples.

RESULTS AND DISCUSSION :

A diauxic growth pattern was observed when total viable count was plotted in figure 2.

All the microbial groups grew substantially during the first 6-12 hours, according to the graphs shown in figure 3 (differential counts). Few hours later count of gram negative cocci and bacilli in single arrangement, and gram positive cocci in closter arrengement were zero. During this period, all bacterial group counts decreased; however, oval yeasts increased rapidly. Antagonist effect has been demonstrated frequently in several cultures between fermentative yeast and lactic acid bacteria (17), when environmental conditions favour one of these microbial groups. From 36 hours, to the end of the fermentation period, Lactobacilli and spectrococci became the main microbial groups in pozol fermentation.

Based on their morphological characteristics and ability to acidify the microinoculum-agar medium, 26 lactic acids were isolated. 11 of those were considered to belong to *Streptococcus* genus, and 15 to *Lactobacillus*. The methyl-red and Voges Proskauer tests used were positive to all the isolated strains, indicating that selected strains were able to acidify the glucose medium and able to produce acetoin or dyacetile. The last compound might be related to the pleasant fermented-milk like flavour developed during pozol fermentation. Figure 4 shows that all isolated strains had amylolytic activity when growing on starch agar plates. Two streptococci and three lactobacilli strains showed a severe activity of starch hydrolysis. Further biochemical analysis has to be performed in order to rich a proper taxonomic classification.

Throughout the fermentation period, no changes on water content of corn dough were observed (Hum.= 68.5 + 1%). Reducing sugar presence was not detected.

Total carbohydrates were scarcely consumed during the first period of the fermentation process (0-18 hours), whereas lactate formation was slowly initiated (figure 5). High carbohydrate utilization rate has been observed to start at 24 hours of fermentation. Since reducing sugars were not detected, it was assumed that hydrolyzed products were immediatly assimiled by the microbial population. Starch is so supposed to be the growth limitant substrate. An intense lactate formation has been observed to start at 38 hours of fermentation, causing a pH decrease (figure 6). This observation is an agreement with the active lactic acid bacterial growth observed in figure 3.

During the first fermentation period, the protein content of corn dough decreases, probably due to the utilization of protein as a main carbon source. However, during the active lactic fermentation period, protein content increases notablely (figure 7).

Total nitrogen and raw protein (N x 6.25) contents of several samples are shown on table 3. According to these results, and due that no gram negative bacteria were observed after 12 hours of fermentation, nitrogen fixation phenomenom reported by Ulloa et al. to occur in pozol from Tabasco State (15), was not observed here. This may be due to the hazardous mixture of microorganisms present in pozol, brought from natural environments. However, no matter the origin of native inoculum, after some hours, lactic acid fermentation predominated, as

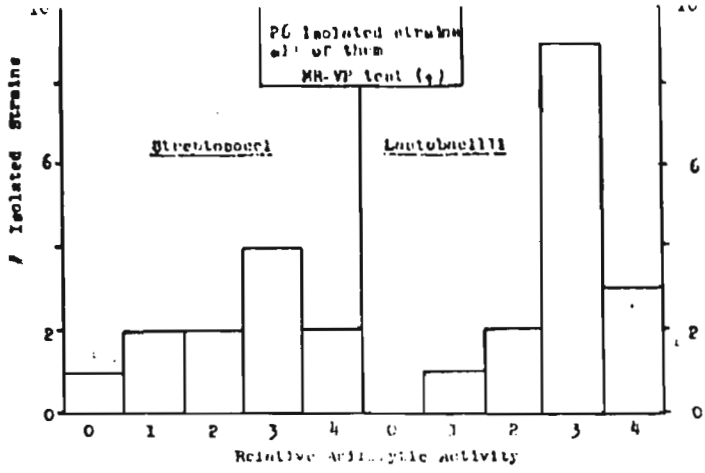


Fig.#4 RELATIVE AMYLOLYTIC ACTIVITY OF POZOL ISOLATED STRAINS

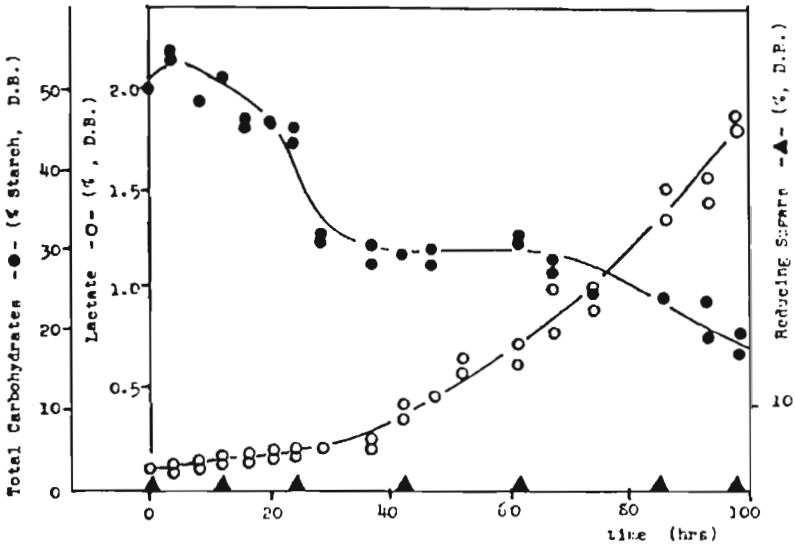


Fig. # 5 TOTAL CARBOHYDRATES, REDUCING SUGARS AND LACTATE IN POZOL FERMENTATION

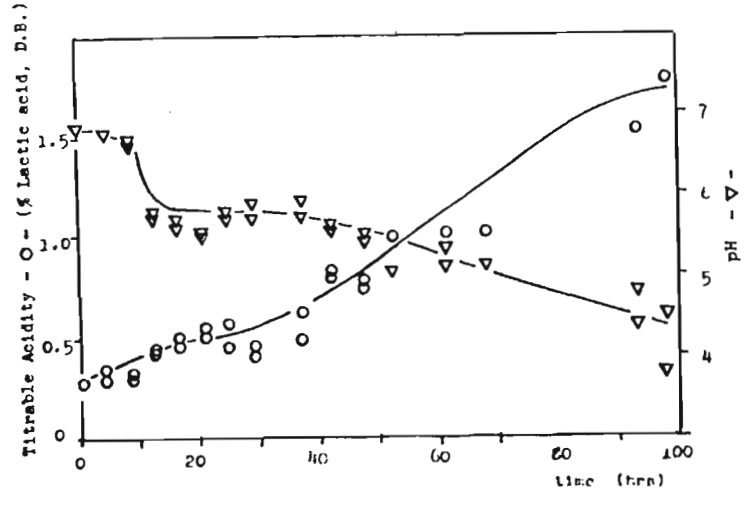


Fig. # 6 TITRABLE ACIDITY AND pH IN POZOL FERMENTATION

TABLE I

Total nitrogen and raw protein contents in pozol

Fermentation period (hours)	Total nitrogen (% D.B.)	Raw protein (N x 6.25 % D.B.)
0 (initial)	1.49	9.32
8.5	1.47	9.21
37.5	1.60	9.21
61.5	1.68	10.52
98.0	1.65	10.30
(Final-Initial)	0.14	0.89

TABLE II

Concentration levels of some components in POZOL FERMENTATION

Fermentation period (hours)	Humidity (%)	Total carbohy-drates (% D.B.)	Lactate (% D.B.)	Titrable acidity (% D.B.)	Total protein (% D.B.)	pH
0 (Initial)	68.75	50.11	0.11	0.27	0.57	6.87
24.5(Main lactic fermentation initial time)	68.86	44.41	0.19	0.51	7.77	5.75
98.0 (Final)	68.33	17.71	1.83	1.50	8.40	3.80
(Final - Initial)	-0.42	-32.40	1.72	1.23	3.83	3.07

observed by several authors (13,16).

A summary of compounds present is shown on table 4. Initial and final values, together with those corresponding to the start of active lactic acid fermentation were presented.

CONCLUSIONS :

A diauxic growth pattern was observed during the pozol fermentation.

Main microbial groups growing in pozol fermentation belonged to the *Streptococcus* and to the *Lactobacillus* genera. Oval yeast counts were lower, but present during all the fermentation period. Other microbial groups disappeared during the first 12 hours. No pathogenic groups were observed after this time.

After 5 days of fermentation, surface mould growth was observed.

Isolated lactic acid bacteria were able to hydrolyse starch, producing lactate and diacetile/acetoin.

Nitrogen fixation phenomenom was not observed to occur throughout the fermentation period.

During the first period of fermentation, protein was assimilated as main carbon source. Pleasant fermented milk-like flavors were also developed during this fermentation period.

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PENICILLIN PRODUCTION BY SOLID STATE FERMENTATION

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SUMMARY

Penicillin was produced by a non-sterile solid state fermentation (SSF) on bagasse impregnated with culture medium. The use of concentrated media greatly enhanced the antibiotic production in this system. It was observed that adecuate initial moisture content (70%) of the impregnated solid medium results in higher production. A comparison between solid and liquid fermentation showed superior yield and productivity.

INTRODUCTION

Solid state fermentation (SSF) is an ancient culture method that has been revaluated and modernized lately to produce protein and enzymes (Aidoo, et al, 1982). Regarding the production of secondary metabolite production, several mycotoxins have been produced in very high quantities by SSF on grains and other agricultural products (Hesseltine, 1972). Recently, a solid fermentation process for gibberellic acid production was reported (Kumar & Lonsane, 1987).

In 1980 Raimbault & Alazard developped a method to study fungal growth in solid fermentation, which allows relatively high control on culture conditions. This technique has been used by our group in the development of a process for protein enrichment of cassava by SSF (Raimbault *et al*, 1985). It has also been applied in the development of similar processes to produce: celulases (Roussos, 1985), pectinases (Trejo, 1985) and aflatoxins (Barrios-Gonzalez *et al.*, 1986). Some of these results have been scaled up to 30 Kg in reactors with different configuration (Huerta *& al.*, 1986).

Recent studies explored different SSF systems, particulary the use of inert supports impregnated with liquid media. This system has been patented (Barrios-Gonzalez *et al*, 1988) and characterized by (Oriol *et al*, 1988).

The objectives of this work were to evaluate the posibility of producing penicillin by this SSF system and determine its advantages, if any, over the conventional

LF process.

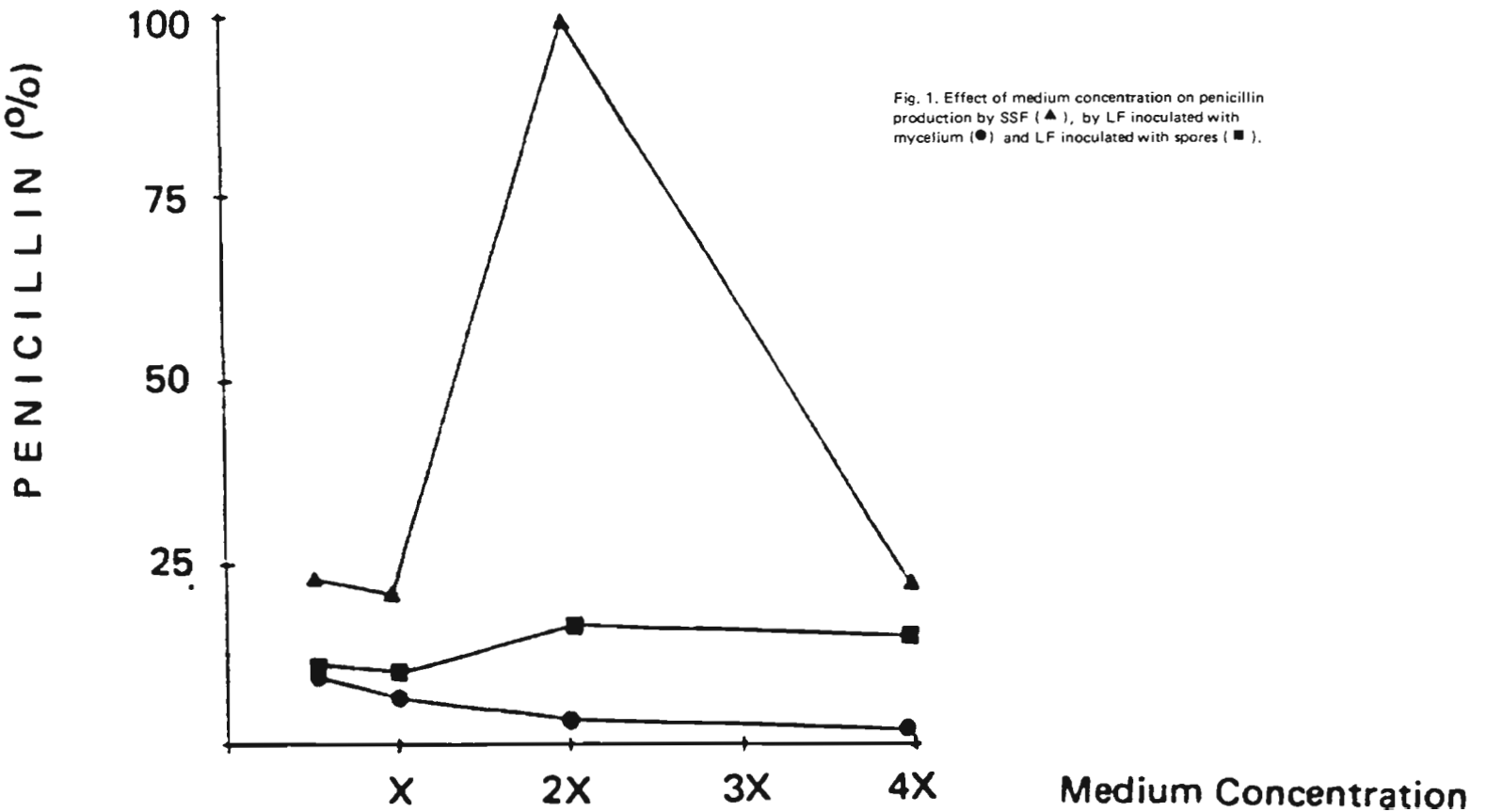

Fig. 1. Effect of medium concentration on penicillin production by SSF (▲), by LF inoculated with mycelium (●) and LF inoculated with spores (■).

METHODS

Microorganisms : *Penicillium chrysogenum* NRRL 1951 was used in initial experiments and a reisolate of *P. chrysogenum* Wis 54-1255 which was called Wis 54-1255 N, was used in the rest of the study. Strains were maintained in soil cultures.

Pretreatment of raw material : Sugarcane bagasse, free from sugar was obtained and prepared as previously described (Oriol *et al*, 1988b).

Preparation of spore inoculum. Spores were obtained from flasks with PDA medium incubated at 27°C for a week and suspended in sterile water. An inoculum size of 0.5 $x10^6$/ml was used in all experiments.

Liquid fermentation. Submerged culture was performed in 250 ml Elenmeyer flasks with 50 ml of production medium (in g/l) reported by Sylvester & Coghill (1954) : corn steep liquor 30, lactose 30, glucose 5.0, $CaCO_3$ 3.0, lard oil 1.87, phenyl-acetamide, $NaNO_3$, 3.0, $ZnSO_4$ 0.044, $MgSO_4$ 0.25, pH adjusted at 5.5. This medium was inoculated with spores or with mycelium and incubated at 26°C in a rotary shaker at 250 rpm. Mycelium was obtained from a 3 days shake flask culture with the following medium: glucose 20 g/l, sucrose 6.8 g/l, $(NH_4)_2SO_4$ 15 g/l, KH_2PO_4 9.08 g/l, $CaCO_3$ 3.0 g/l, $ZnSO_4$ 0.02g/l, $CuSO_4$ 0.005 g/l, $MgSO_4$ 0.02 g/l, pH adjusted at 5.0. In both cases, two flasks were collected at every sampling time.

Solid state fermentation.The culture was achieved under non-aseptic conditions as previously described (Raimbault & Alazard, 1980). Column fermentors containing 11 g of moistened and inoculated support were incubated in a 26°C water bath. Several fermentors wereused simultaneously with individual areation of 2 l/h.

The production medium solids were solubilized in the rest of the water needed to achieve the desired moisture content (usually 70%). In this stage, pH was adjusted to 3.5 and sterilized 15 min at 1 bar. Sterile medium was inoculated and mixed with the bagasse which resulted with a pH value of 5.5. In some experiments the concentration of solids (except lard oil and phenoxyacetamide) was multiplied by 2 or by 4, etc. and these media were respectively named 2X, 4X, etc.

Sample analysis. Samples from the LF were filtered, rinsed and the biomass dried in a convection oven at 60°C for 48 hrs. Penicillin, reducing sugars and pH were determined in the filtrate.

Two complete columns of SSF were collected at each sample time and 1.0 g of each was used for pH determination (by mixing with 10 ml of distilled water, stiring for 5 min and using a pH in the solution), 0.5 g for penicillin assay and the rest was dried at 60°C for 48 hrs. The dry sample was used afterwards to determine moisture content (weight loss), reducing sugars and biomass as nucleic acids.

Penicillin was extracted from SSF samples by mixing with 3 ml of ethyl acetate and separated by centrifugation (10 min) at 5000 rpm. Twenty microliters of the

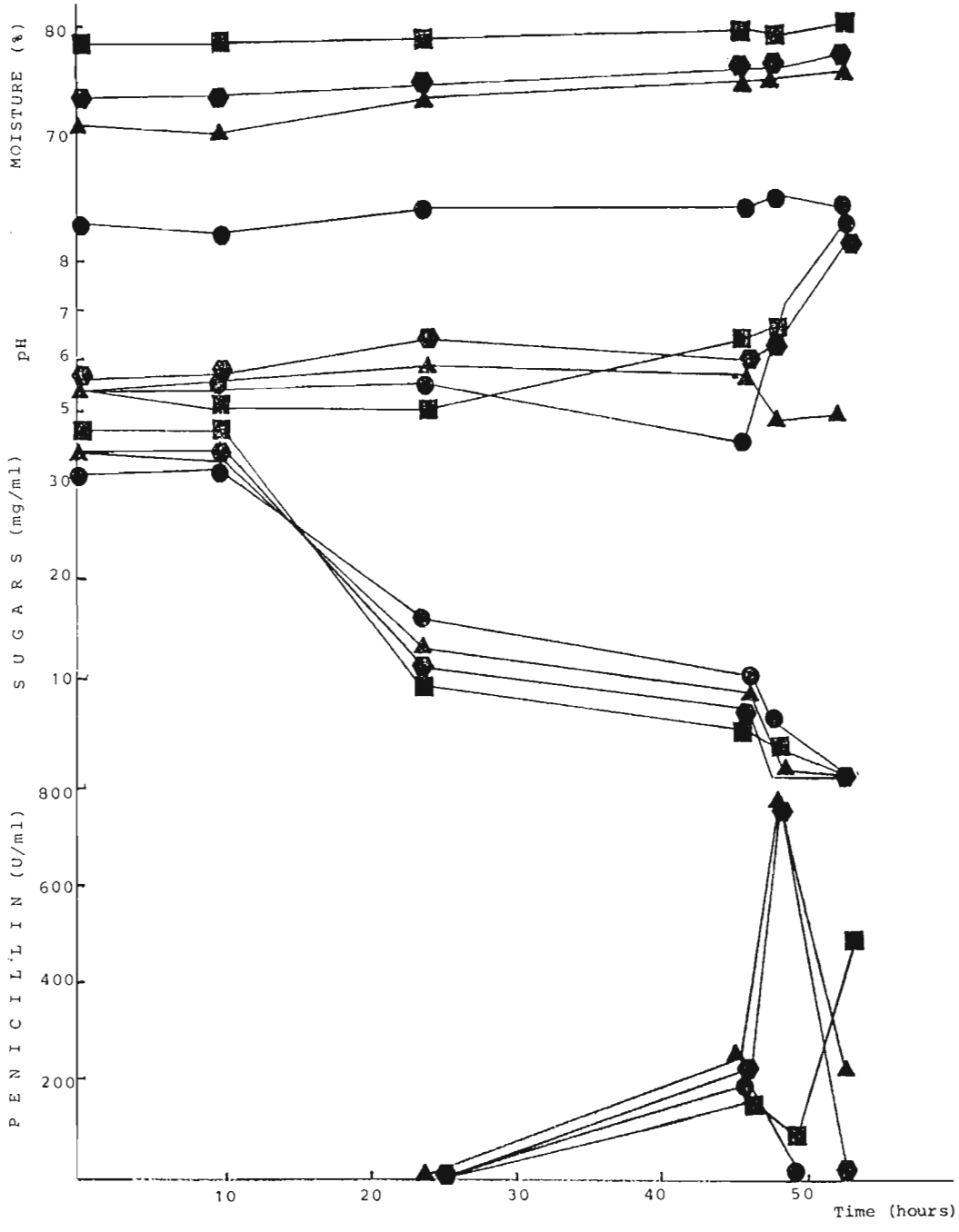

Fig.2 Time course of penicillin solid state fermentation using 2X medium and different initial moisture content: 60% (●), 70% (▲), 73% (⬢) and 78% (■). All fermentation were inoculated with spores of P. chrysogenum Wis 55-1255 N.

solvent were used to determine penicillin concentration in bioassay. In LF, the bioassay was performed directly with the filtered fermentation broth.

0.5 g of the dry bagasse were homogenized in 45 ml of distilled water with an Ultra-Turrax. A 5 ml aliquote was centrifuged (5min at 5 000 rpm) and reducing sugars measured in the supernatant by the Miller method (1959). Nucleic acids were extracted from the pellet with 5 ml of 0.7 M $HClO_4$ and absorbance determined at 260 nm.

RESULTS

Two solid fermentations were performed simultaneously : one was inoculated with spores of *Penicillum chrysogenum* NRRL 1951, and the other one with mycelium. A weak production of 6 U/ml was obtained in the first case, while no production was obtained in the last case. The same results were obtained in a second experiment, so spore inoculum was used throughout the study.

Medium Concentration

The effect of medium concentration in SSF was studied using *P. chrysogenum* Wisconsin 54-1255 N. To keep initial moisture content (IMC) constant (70%) in these experiments, nutrient increase was balanced by a decrease in bagasse. That resulted in great differences in medium tonicity but very small differences in bagasse/H_2O ratios.

Fig.1 shows maximal production obtained in fermentations using different medium concentrations. One can see that in SSF, the production increased with medium concentration, and that medium 2X supported a production 5 times higher than other. In LF inoculated with mycelium, increasing medium concentration had an adverse effect on production, while no significant effect was observed in LF inoculated with spores. In SSF penicillin, the production started after 24 h in all cases except for 4X medium(48 hrs). When lard oil and precursor concentrations were also multiplied, production did not increase and best performance was obtained with X medium.

Four simultaneous fermentations were performed with 2X medium and different initial moisture contents (IMC). To adjust IMC at different levels and keep nutrient content of the impregnated solid medium constant in 2X, water increase was compensated with bagasse decrease. This resulted in great differences in IMC (60, 70, 73, 78%) and bagasse/H_2O ratios (0.58, 0.35, 0.3, 0.22 respectively) but slight differences in medium tonicity.

Fig.2 shows that moisture level increases slightly during the fermentations but within the range of the initial IMC of the case. In the range studied (60 - 78 %), IMC did not affect the time at which production started, but had an important influence on the penicillin production and its stability. A maximum production of 800 U/ml was obtained after 46 hrs of incubation with 70 and 73% IMC.

Sugar consumption kinetics showed similar form and 5 stages could be pointed out : a) reducing sugar concentration increase; probably caused by hydrolysis of residual sucrose in the bagasse; b) rapid sugar consumption; c) slow consumption rate; probably represents lactose consumption and correlates with low rate production phase; d) fast sugar consumption in 70 and 73% IMC, practically exhausting sugars, correlating with high production rate; moderate consumption in 60 and 78% IMC with total penicillin degradation in the former and partial degradation in the latter; e) fairly rapid consumption in 78% joined by a rapid production rate while rapid consumption rate but no production was

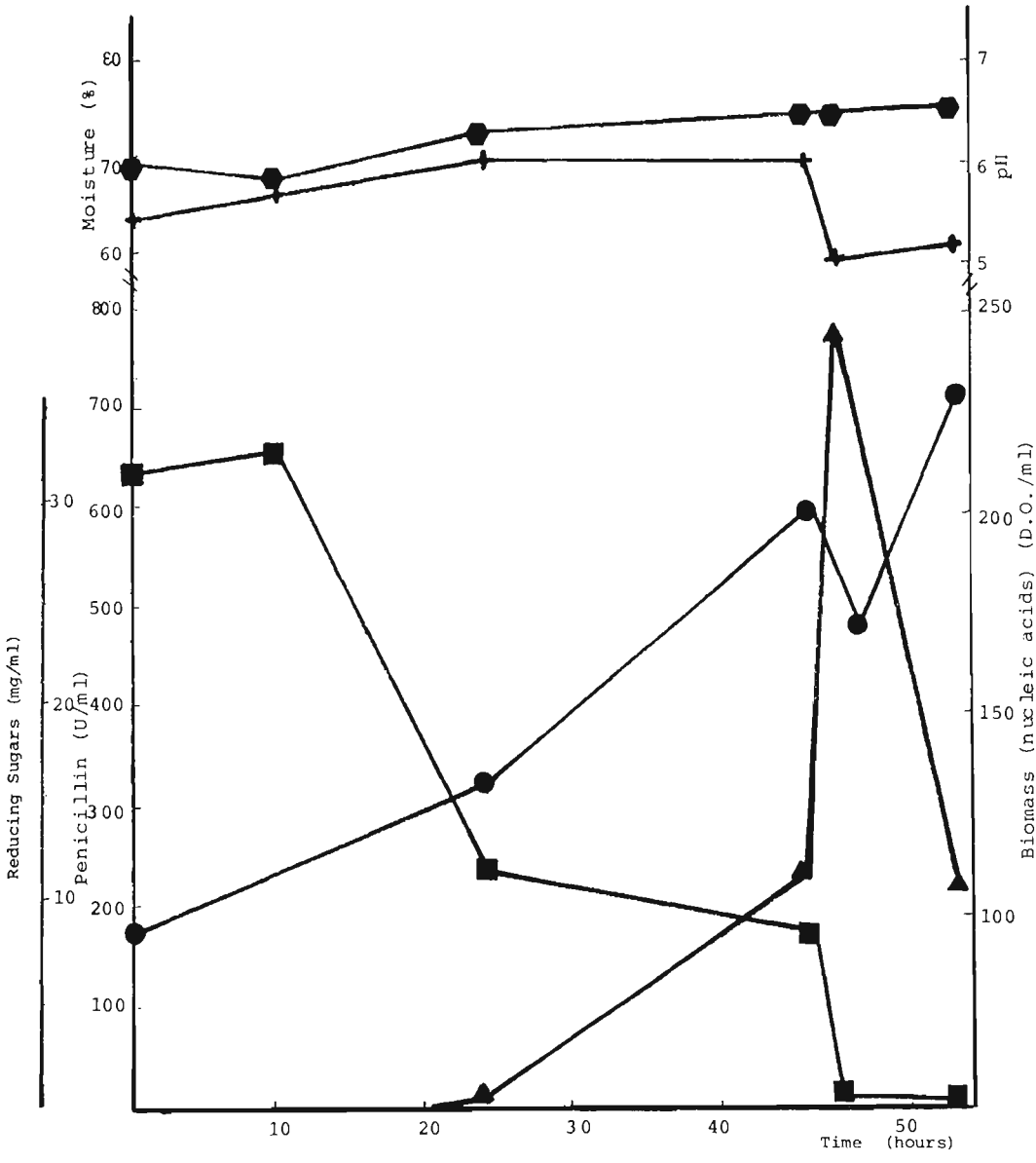

Fig. 3 Penicillin solid state fermentation on bagasse impregnated with double strength (2 X) Sylvester & Coghill (1954) medium. The solid medium with a moisture content of 70 %, was inoculated with spores of P. chrysogenum Wis. 54-125 N. Moisture content (⬢), pH (+), biomass as nucleic acids (●) and penicillin in the liquid phase (▲).

observed in 60%.

The performance of several solid and liquid fermentations was compared (Table 1). It can be observed that average production by SSF was 17 times higher than the one obtained by LF, and that achieved in one third of the time. An aproximate calculation revealed that the efficiency of sugar utilization to produce the antibiotic (Y p/s) was 7 times higher in SSF. Finally, SSF showed 8.7 times greater volumetric productivity.

System	P max (a) (U/ml)	Time (Hrs)	Pmax (U/mg)	Y p/s = Productivity (U/r-ml Hr)
SSF	686	49	10.77	2.01
LF	38.5	166	1.5	0.23

Table 1. Average performance of five penicillin solid and liquid fermentations with *P. chrysogenum* Wis. 54-1255 N. The medium reported by Sylvester & Coghil (1954) (X medium) was use in shake flask culture or (2X medium) in solid state fermentations (SSF) carried out at 70% initial moisture. (a) maximum penicillin production.

DISCUSSION

The present work shows that it is possible to produce penicillin by SSF. The system employed solid fermentation on impregnated bagasse, which has been developped and characterized previously by our group (Oriol *et al*, 1988). In this system, liquid media developped for LF can be employed and the fermentation products is recovered by extraction with solvents or by pressing the bagasse. In this way, accurate comparisons can be established between solid and liquid fermentations.

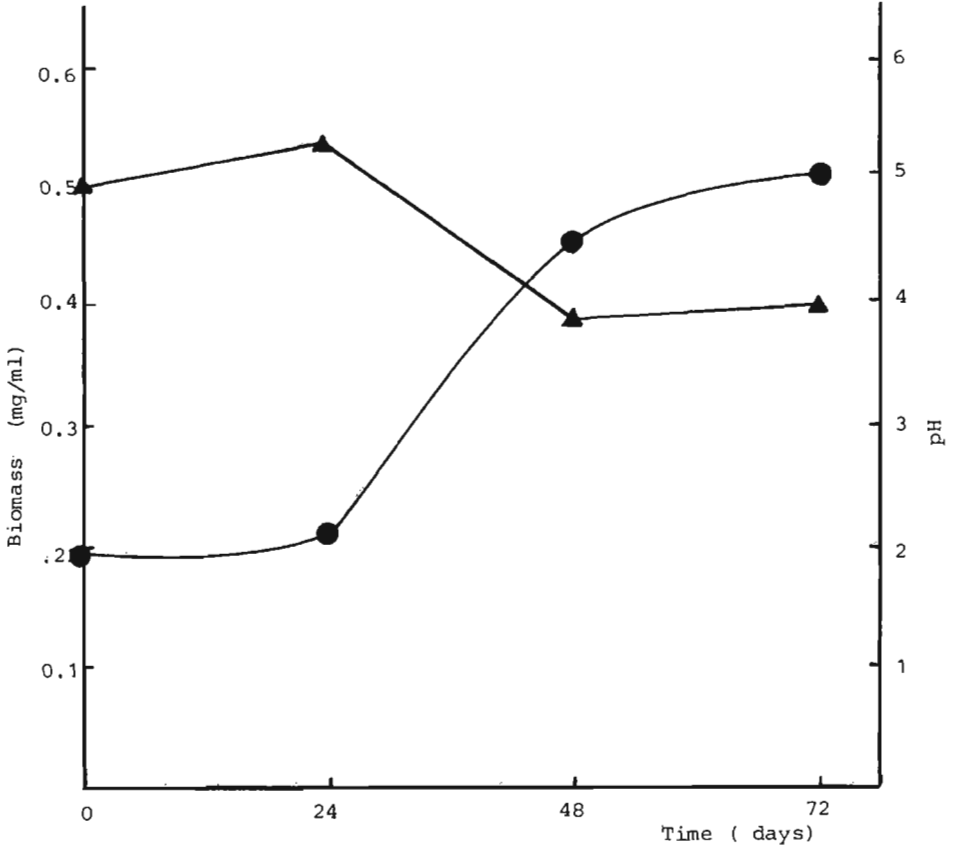

Fig 4 Growth stage ,in submerged culture, of P. chrysogenum Wis. 54-125 N. Biomass as dry weight (●) and pH (▲).

Results showed important differences between both culture methods. An important feature of the penicillin SSF is that the process is carried out under non-sterile conditions without contamination or product degradation problems.This means that the culture technique allows the setting of environmental conditions that give ecological advantages to the fungus.

P. chrysogenum showed different physiology in SSF since it was proved that relatively concentrated media are needed in this solid fermentation system to reach adequate growth and production and that this effect is not observed in LF. These findings agrees with previous studies (Oriol *et al.*, 1988 b) which indicated that, in this system, *A. niger* can efficiently use high glucose concentrations that would be inhibitory in submerged culture.

It is considered that in cassava SSF (and similar systems), growth is limited by water availability, i.e. high tonicity of the residual free water (Raimbault, 1980 ; Oriol *et al*, 1988 a). In SSF on impregnated bagasse the support is inert, so it does not dissolve in the residual free water as the fermentation proceeds. Therefore growth must be limited by a different cause in this system.

In the present study, sugar consumption and penicillin production kinetics indicate that a first and partial limitation of the growth is caused by a change of the substrate (lactose) that is beeing consumed. At the end of the culture a second and complete limitation seems to be caused by sugar exhaustion.

Previous work (Oriol *et al*, 1988 b) showed that water content (IMC and bagasse/H_2O ratio) does not have an important effect on the growth phase of *Aspergillus niger* in this SSF system. The authors observed that growth and germination were affected by Aw (tonicity) of the liquid phase.

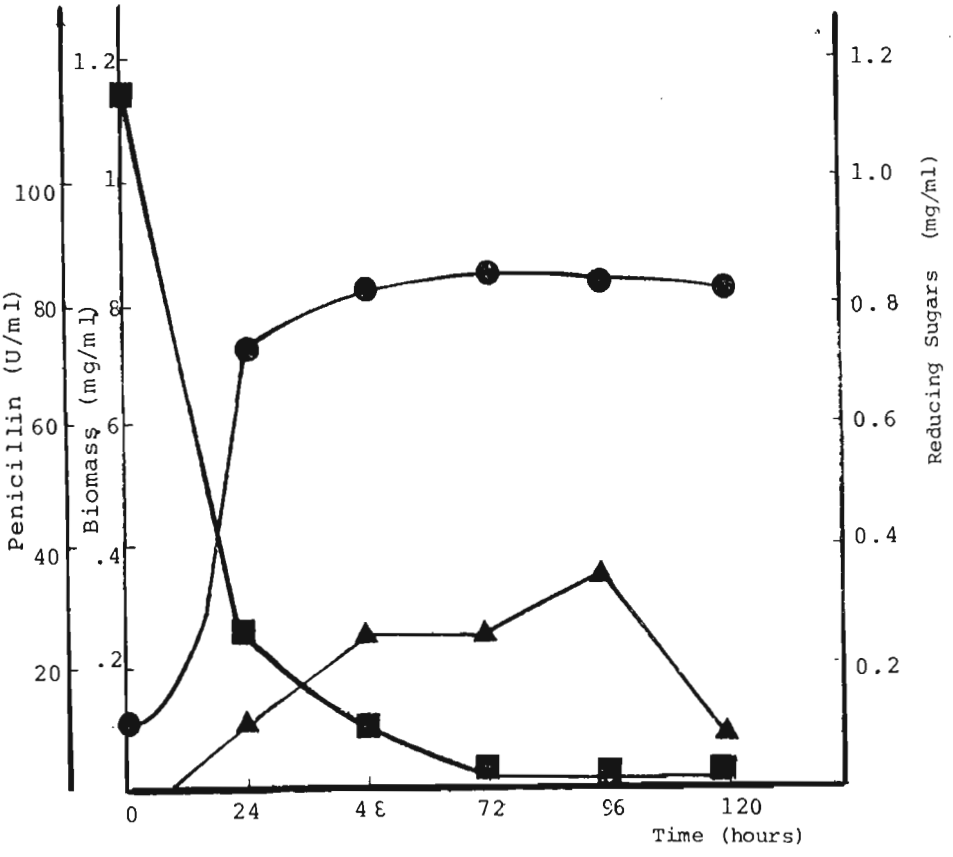

Fig. 5 Penicillin fermentation in submerged culture using the production medium reported by Sylvester &Coghill (1954). This medium was inoculated with mycelium of P. chrysogenum Wis. 54-125 N, grown in a previous stage. Biomass as dry weight (●), reducing sugars (■) and penicillin (▲).

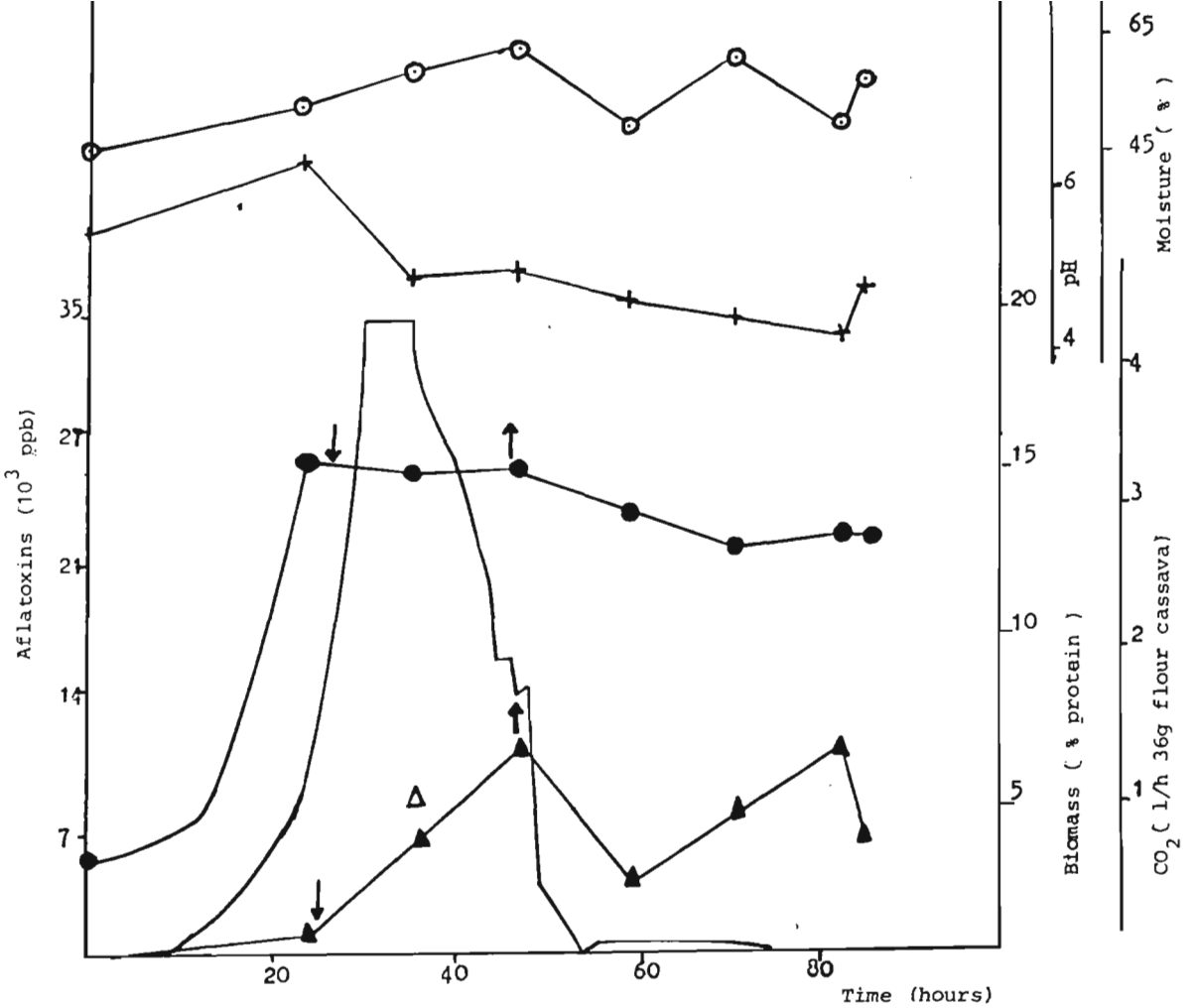

Fig. 6 Time course of an aflatoxin SSF on cassava with decreased aeration (0.3 l/H column) between 24 and 48 hours. The organism used was A. parasiticus NRRL 2999. Moisture content (⊙), pH (+), respiration as CO2 production (__), biomass as protein (●) and aflatoxin concentration (▲).

Results of the present work showed that IMC strongly influenced idiophase. During the last hours of the culture, fermentations with IMC of 70% (bagasse /H_2O ratios near 0.35) presented very high production rates while the fermentation with lower IMC (60%) presented fast antibiotic degradation rate.

From an applied point of view, we must admit that, in the penicillin SSF, higher production was obtained and in a shorter time than in LF. Higher penicillin yields and volumetric productivity were also observed in this culture method.

These advantages together with the low energy costs of the process (sterilization, areation, agitation) indicate that this system has an important industrial potential. It is also seen that physiology in solid medium can be very different from the one observed in liquid medium, so further studies at his level are necessary to explore the system's full potential.

AKNOWLEDGEMENTS

The authors wish to thank CONACYT, Mexico, for financial support (Grant No. PVT/AI/NAL/85/2759). This work was performed as part of the agreement UAM (Mexico)-ORSTOM (France).

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CHEESE FLAVOURS BY SOLID STATE FERMENTATION.

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INTRODUCTION :

Cheese flavor concentrates are produced at industrial level by enzymatic (lipases and proteases) and microbial treatment of young cheese which is diluted and homogeneized (1,2). These products, known as Enzyme Modified Cheese (EMC), require precisely controlled reaction and stabilization conditions. Certains flavours, (such as blue cheese), can also be produced by liquid fermentation with caseine and fat or even directly from cream.

Flavor and texture formation during cheese ripening is produced by a complex system of reactions (chemical, enzymatic and metabolic) that occurr with rates depending on temperature, pH, composition and water activity. Furthermore, the majority of cheeses present spatial heterogeneity, so the rates depend also on the position. As a consequence, substrate, product and rate regulator gradients are present. In cheese technology, ripening is controlled by using low temperatures, (sub optimal for microbial and enzymatic activity), which allow a desired balance between diffusional and reaction rates. The EMC approach consist in forming slurries of high dry matter content (ie 35-45%) that can be agitated allowing the control of certain parameters such as pH and the possibility of sequential addition of enzymes or microorganisms.

An alternative to the slurry process consist in ripening the cheese with a granular structure (granular curds of 2-6 mm diameter). This alternative would allow reduced diffusional limitations and thus an improved control of the fermentation.

MATERIAL AND METHODS :

All the microorganisms studied here are commonly used in cheese production and were obtained from collections or from starter suppliers (3). Raw materials were fluid or powdered milk. Methods for the preparation of the granular curd for the different experiments and the analytical methods used are described elsewhere (3-6).

RESULTS AND DISCUSSION :

Flavours produced by *Brevibacterium linens*. This microorganism is associated with cheeses such as Pont L'évêque, Limburger and Camembert. It grows on the surface of the cheese and requires relatively high pH for its development. The granular curds in these experiments were not acidified by chemical or microbial means. The evolution of the nitrogen solubilization (due to the protease activity) and the pH is depicted in the figure 1, the arrows indicate the day

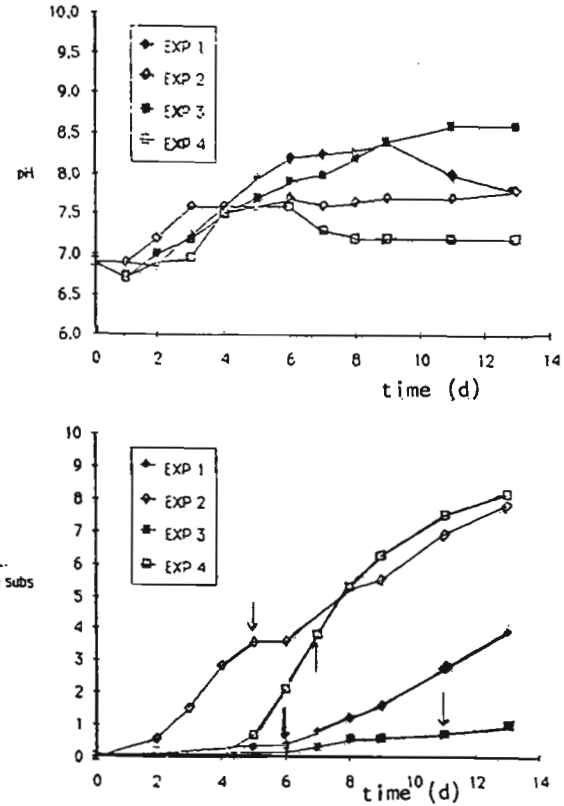


Figure 1. Evolution of the fermentation of B. lines on granular curds. Exp. 1 - Agitated, Exp. 2 Agitated with Sodium lactate, yeast extract and Casamino acids (4% each), Exp. 3 agitated with salt 3.6%, Exp. 4 static.

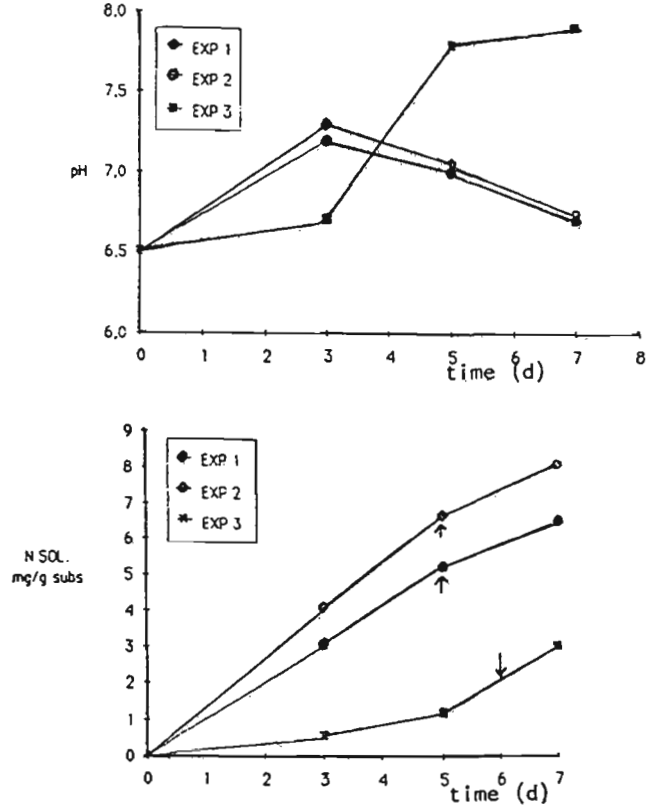


Fig. 2 Evolution of the static fermentation of P. camemberti on granular curds. Exp. 1 non-sterile, Exp. 2 non sterile with rennet 0.075%, Exp. 3 sterile curds.

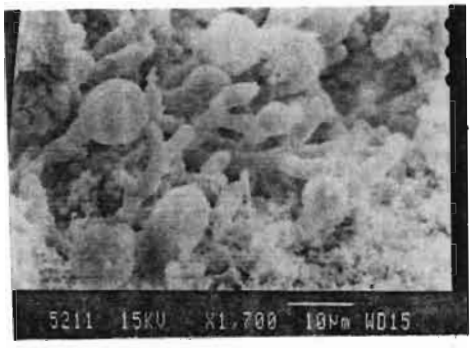
when the product aquired its best sensory properties. Growth occurs within the first for days while the pH is below 7.5. There is a slow initial flavor production that accelerates with time and a very fast degradation, this is associated to the ammonia produced by the deamination of the aminoacids. The granular substrate maintains its structure in the beginning of the fermentation,which allows a correct intragranular aeration. Advancement of the proteolysis, which is independant of growth beyond the fourth day, and an increased residual protein solubilization, due to the pH, provoke the formation of a paste. At this point, the product is over ripened.

Agitation of the granules accelerated the paste formation which limited gas transfer and flavor development. Best results were found with static incubation.

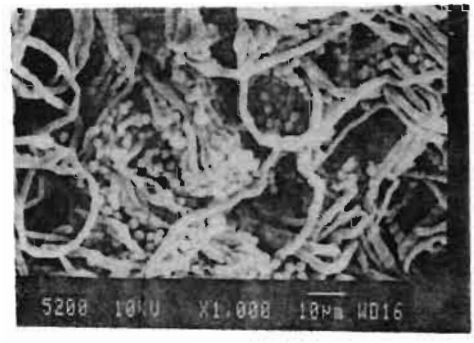
Flavours produced by *Penicillium camemberti.* This mold is associated with cheeses such as Camembert and Brie. In this experiments (Figure 2), with unacidified curds, the mold grows on the surface of the granules. In contrast with *B. linens*, the mold growth limits granule fusion and the paste formation. The relation of mold grow to the substrate is much more important than in the cheese which reduces the ripening time, 5vs. 45 days. Best results were obtained with static fermentation.

Flavours produced by *Penicillium roquefortii.* In this experiment with microbial acidification (mesophilic lactic bacteria) characteristic flavours were produced in four days. Spores of the mold are incorporated to the milk and retained during the curd formation. The spores germinate in the first day and start to colonize the granule from the second day (Figure 3 a,b). Important growth is observed on the third day (Fig. 3c) and in the fourth day the surface is totally covered (Fig 3d,e). The growth may be compared with the evolution of some of the components of the cheese as seen in Figure 4. Figure 4a shows the evolution of the pH which decreases as lactic acid is produced by the lactic acid bacteria, pH increases from the fourth day due to the lactic acid consumption by the mold and the ammonia produced by proteolysis induced aminoacid deamination, (Fig 4b). The activity of the lipases is observed by the increase of the free fatty acids (Fig 4c) which in turn serve as precursors for the formation of carbonyl compounds, mainly methyl ketones, which are the main components in the characteristic blue cheese aroma. The flavours produced with the granular curds in fiva days are fully typical of a ninety days old blue cheese. More concentrated flavours were obtained with lipase or lipolyzed cream addition (6). The final concentration of methyl ketones in the granular curd is highly dependent on the total fat content. Fat serves as a precursor of fatty acids as well as a solvent for the non polar and volatile aroma compounds.

Flavours produced by *Propionibacterium freudenreichi* sbsp. *shermanii.* This bacterium is responsible for the flavours production of Swiss-cheese (Emmental, Gruyere). In the cheese case, it transforms the acid produced by the lactic acid bacteria into carbon dioxide, which forms the characteristic "eyes", and propionic and acetics acids. This process is anaerobic and is strongly dependent on pH because *P. freudenreichi* is pH sensible (it will not grow below pH of 5.1). For this fermentation, growth occurs in the mass of the granules instead of the surface as in the previous example. The evolution of the fermentation can be seen in the Figure 5. Typical Swiss-cheese flavours are developed from the fifth day. Figure 5a represents the lactose, lactic acid and soluble nitrogen evolution, on the fifth day almost all the acid has been converted from C_2 to C_4 fatty acids. Nitrogen solubilization in this system is much less important than in the pre-



day 2 (x 1900)



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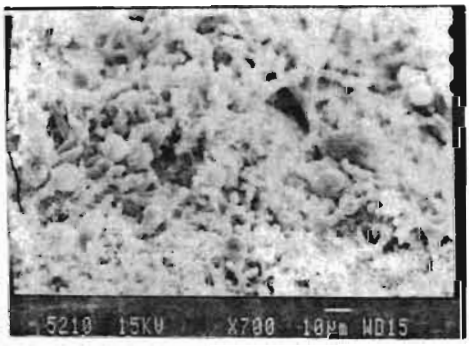

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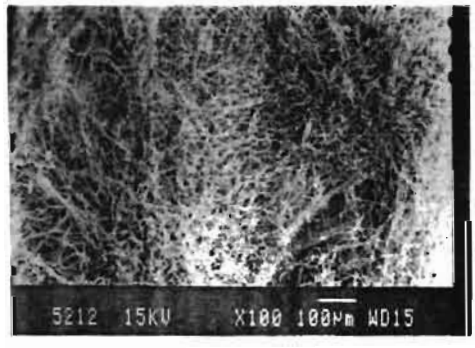
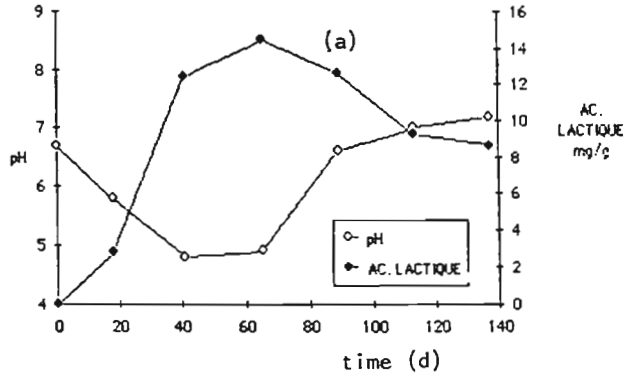
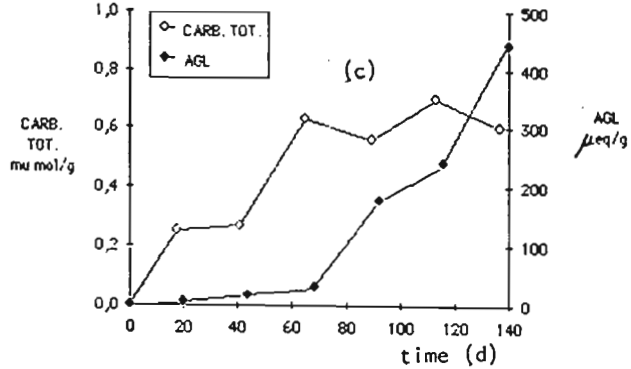


day 4 (x 1000)

day 3 (x 1000)

Fig. 3 Evolution of the fermentation of P. roqueforti on granular curds.
Electronic microscope photographs.





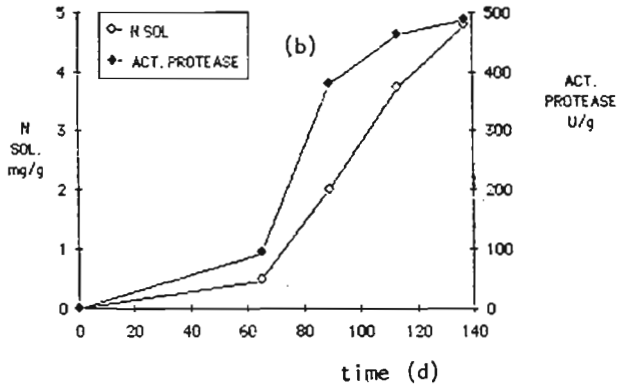
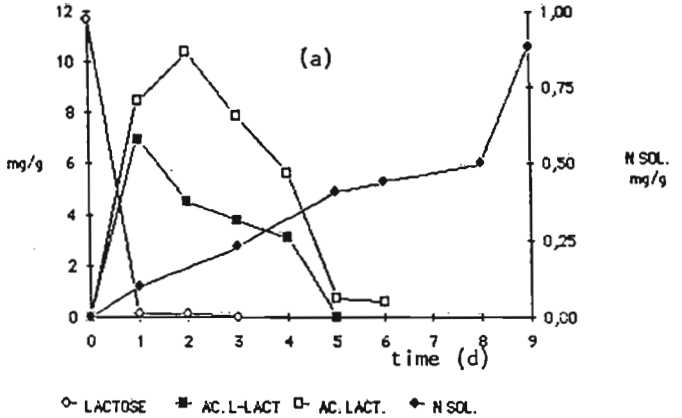
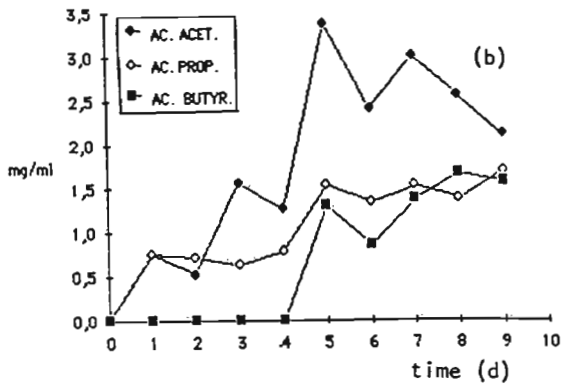


Fig. 4 Evolution the fermentation of P. roqueforti on granular curds





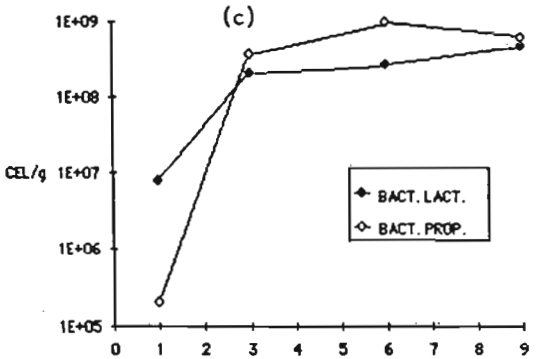


Fig. 5 Evolution of the fermentation of P. freudenreichii on granular curds.

vious examples, due to the limited proteolytic activities of the propionic and lactic bacteria. The observed increase in soluble nitrogen is due to the secondary activity of the rennet and to the peptidases liberated by both types of bacteria due to cellular lysis. The action of the propionic bacteria peptidases are thought to be important in the generation of the typical swiss cheese-flavour. Butyric acid (Fig 5b) is produced by *Clostridium tyrobutyricum*, which resists to pasteurization and competes with *P. freudenreichi* for the lactic acids. The evolution of the lactic and propionic bacteria is seen in Figure 5c. The results with granular curd are similar to those obtained in slurry (5).

CONCLUSION.

Our results point out that different cheese flavours can be produced by fermentation of curd granules. The results were obtained with temperatures close to the optimal for the microbial species involved. This provokes an acceleration of the overall processes (diffusion and enzymatic, microbial and chemical reactions) including degradation, which is a technological problem to be solved for these systems. The application of these products would be in process cheese or flavoring for sauces, soups etc...

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SOLID STATE FERMENTATION : SCALING UP PROTOTYPES AND STRATEGIES.

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SUMMARY :

Some scaling up considerations in solid state fermentations (SSF) and those dealing with heat transfert are specially discussed. Operating conditions for protein production by SSF of cooked cassava meal (*Manihot esculenta*) with *Aspergillus niger*, were determined at laboratory level (0.1-1.0 kg of dry matter). These results were succesffully scaled up to pilot plant (10-20 kg) under non sterile conditions. The control strategies and prototypes selection based on our results on constant dimensionless numbers as criteria for scalling up in SSF.

INTRODUCTION :

Since 1981, at the Biotechnology department of the Universitad Autonoma Metropolitana Iztapalapa in Mexico, part of the group of microbiology in collaboration with the ORSTOM-France, has performed an complex task: to developed a solid state fermentation (SSF) process of protein enrichment of agricultural raw materials by growing filamentous fungi on this substrates. The final product was aimed for feed purposes. The smallpacked columns technics for SSF was already developped in Africa by Raimbault (1981). The same methodology was optimized and carried out at laboratory level in Mexico, with two new objectives: on one hand to scale up the results, on the other hand to lead a research on new products which could have potential for industrial applications.

Scaling up SSF presents serious problems: many are due to impaired heat and mass transfer between the laboratory and pilot plant scale. The importance of these problems mainly depends on three aspects:

1. the heterogeneity of these substrates,
2. the scale factor which is taken up or down
3. the lack of basal prototypes on which scale-up strategies could lay on.

Let us discuss something about these aspects.

1. HETEROGENEOUS SUBSTRATES :

Solid substrates always presents the difficulty of being heterogeneous. They have a poor thermal conductivity due to their proper texture, i.e. porosity and lack of free water. Furthermore, their rheological properties always have a

tendency to change during the fermentation time. The presence of more microorganisms and metabolites as well as reducing sugars turn, an almost free flowing material into a sticky one. This physical changes during the fermentation decreased the heat and mass transfer coefficients. The problem get worse when the scale is increased.

2. SCALE FACTOR :

In going from a SSF using grams to one using kilograms, it is necessary to scale-up a fermentor of constant geometry by a factor of about 1000 in volume. This means that on average, a microorganism is for cylinders or columns 10 times more distant from the bioreactor wall in the big equipment than it was in the laboratory unit and therefore, heat transfer area per unit of reactor volume is decreased by a factor of 10. Thus an exergonic biochemical reaction, such as microbial growth, that is barely perceptible at the small scale could be catastrophic at the kilograms or larger scale (Marynowski, 1987). Thus, an effective strategy in order to select and design bioreactor prototypes and the control is recommanded.

3. PROTOTYPES AND STRATEGIES :

The liquid fermentation (LF) method has been extensively used in fermentation industries since the antibiotic production became commercially attractive. Physiological and technical aspects are now well understood. Early experience gained in reactor design and control in the chemical industry was used to LF's advantages. Control and new bioreactor design has been well developped and laboratory results successffully scaled-up to almost any operation scale. Beside this progress, the SSF has shown no expansion of application (Lonsane et al., 1984). In japan, this technology is day after day much more sophisticated, specially for koji production at commercial scale (Okazaky et al., 1980 and Sato et al., 1982), nevertheless there is no reactor prototype which has been characterized enough to be unique on its kind such as in LF was the well known STIRRED tank. Recent research dealing with SSF has been trying to adapt new bioreactor designs to a non-well understood physical and physiological phenomena (Aldoo et al., 1982 and Moo-Young et al., 1983). Exactly the contrary which happened before in the LF development. Thus, the fermentor characterization and the implementation of the control systems are two important steps in the development of SSF technologies (Huerta-Ochoa, 1984). For our group, it was necessary to know first, most of the main technical responses for several types of fermentors in order to evaluate their potential, and choose the most adequate for a specific process, and then scale down the choosen equipment in order to find some specific criterion for further scales. The objective of this work is to discuss the advances on the scale-up from a laboratory level (10-20g of dry matter) to the pilot plant (10-20 kg) performed by our group and the future strategies for commercial production level based upon fixed constant dimensionless numbers as scaling-up criterion.

MATERIAL AND METHODS :

1. Microorganisms. The *Aspergillus niger* strain reported by Raimbault (1981), was used for amylaceous materials and *Trichoderma harzianum* reported by Roussos (1985), for cellulosic substrates.

2. Substrates. Two kind of substrates were used: Cassava meal and sugar cane bagasse, both of them were heat pretreated by using the dynamic fermentor 2. See point 7.

3. Spore production. In all cases spores were produced in large amounts with the giratory horizontal sporulator reported by Raimbault and Roussos (1985).

4. Laboratory columns. Isothermal columns packed up to 0.01 Kg of dry matter or less. The same technic and culture media developed by Raimbault and Alazard (1980) was used..

5. Jacketed columns. Glass columns 60 mm ø and 450 mm height packed with 0.6-0.8 kg of dry matter. The temperature of the medium was measured with thermocouples.

6. Bench top fermentors. Several types of fermentors (10 kg of dry matter) were designed and adapted: two dynamic and one static.

Dynamic fermentor

1. Medium was agitated with fixed baffles. 2. Medium was agitated with an horizontal control led speed stirrer. Static fermentor. 3. Temperature was controlled with vertical heat exchange plates (Zymotis).

These three prototypes were areated by forced means. The air was previously saturated by passing through a 0.18 m ø and 1.4 m height column packed with rashing rings.

RESULTS AND DISCUSSION :

Under isothermal conditions at the lab level, the productivity was 0.995 kg of protein / cubic meter of reactor / hr. However, under non isothermal conditions such as jacketed columns, metabolic heat removal was found to be one of the limitant steps of the process. Different temperature control systems were implemented for each of the pilot plant prototypes. In the dynamic fermentors, heat removal was improved when medium was gently agitated (1-1.5 rpm), although agitation had to be intermitent: without temperature control 5 min periods at 1 rpm and with control as long as the temperature of the mass increased agitation started on. In the static fermentor the metabolic heat was removed by either conduction (heat exchange plates) or convection (forced dry air) means, the optimal geometry of packed media for heat transfer was found to be 0.04 m width and 0.6 m height.

The analysis of the principal technical data demonstrated that yield and productivity were similar in magnitude for the three fermentors, with all the fermentors tested it was possible to obtain productivity levels only 22% lower than those obtained at the laboratory. An adequate temperature control was an important factor to reach these productivities. The pilot plant prototypes were also tested for spores, cellulases and pectinases production with the same results.

In spite of these encouraging results and having no answers for further scaling up levels, we have initiated a new phase of development: the mathematical modeling of the kinetics and transport phenomena involved (Saucedo-Castaneda, 1987). In order to avoid the scale factor problems, we first scale down the above mentioned static prototype (Zymotis) and studied the heat transfer behaviour of an intermediate size prototype: jacketed columns packed with 0.6 kg of dry matter. Radial temperature gradients were found to be very important, up to 15°C between the wall and the center of the column. However, axial gradients were negligible. Heat balances coupled to kinetic terms were mathematicaly solved until experimental and predicted results correlated properly. Three parameters in the form of dimensionless groups were resulted: the Damköller, Peclet and Biot numbers. The first two numbers deal with bioreaction and axial heat dispersion respectively and the third one is seen to be the ratio of the conductive resistance to heat transfer to the convective resistance. The magnitude of Biot thus has some physical significance in relating were the greater resistance to heat transfer occurs. A large value of Biot indicates that the conductive resistance controls, that is, there is more capacity for heat to leave the fermented surface by convection than to reach it by conduction. A small value for Biot represents the case were internal resistance is negligibly small and there is more capacity to transfer heat by conduction than there is by convection. The evaluation of the three dimensionless was the first thing done. The values for the Biot modulus (5-10) were greater than those expected, confirm and an unsteady-state *conduction* situation. In other words, our SSF static system is always controlled by conduction and metabolic heat must be removed by convection.

The importance of these observations for scaling-up SSF process is very clear. To set constant dimensionless numbers such as Biot modulus as a relevant criterion could lead us to predict the new operation conditions for fermentors geometricaly similars but in differnt scale.

CONCLUSIONS :

During the scale up research, it was possible to obtain high productivity levels due to the development of an adecuate temperature control. According to the three fermentors characteristics and their different temperature control systems, it may be assumed that each one should be used to obtaine different specific products such as: micellial proteins, spores, enzymes, giberellic acids and antibiotics. Scale up criteria such as the dimensionless numbers obtained from heat and mass balances is recommended.

ACKNOWLEDGEMENTS :

Authors want to thank to the Consejo Nacional de Ciencia y Technologia CONACyT/MEXICO, the American States Organization and the European Economical Community for their financial support and to B. Schettino for her technical assistance. This work was part of the agreement between ORSTOM-FRANCE and UAM I-MEXICO.

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PERSPECTIVES AND LIMITATIONS OF SOLID FERMENTATION IN MEXICO

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WHAT IS SOLID FERMENTATION ?

Aerobic microbial transformation of solid materials or "Solid Fermentation" (S.F.) can be defined in terms of the following properties of the substrate to be transformed, as be made of:

- A solid porous matrix which can be biodegradable or not but with a large surface area per unit volume, in the range of 10^3 to 10^6 m^2/l for a ready microbial growth on the solid/gas interphase.

- The matrix should absorbe water once or several times its dry weight with a relatively high water activity on the solid/gas interphase in order to allow fast rates of biochemical processes.

- Air mixture of oxygen with other gases and aerosols should flow under a relatively low pressure fead and mix evenly througout the fermenting mash.

- The solid/gas interphase should be a good habitat for the fast development of specific cultures of molds, yeasts or bacteria, either by isolated or mixtures of species.

- The mechanical properties of the solid matrix should stand compression or gentle stirring as required for a given fermentation process. This requires small granular or fibrous particles which do not tend to breack or stick to each other.

- The solid matrix should not be contaminated by inhibitors of desired microbial activities and should be able to absorb or contain available microbial foodstuffs such as carbohydrates (cellulose, starch, sugars, etc...) nitrogen sources (ammonia, urea, peptides, etc...) and mineral salts.

CURRENT APPLICATION OF SOLID FERMENTATION :

Typical exemples of S.F. are traditional fermentations such as:

- Japanese "koji" which used steamed rice as solid substrate inoculated with solid strains of the mold *Aspergillus orizae.*

- Indonesian "tempeh" or Indian "ragi" which use steamed and cracked legume seeds as solid substrate and a variety of non toxic molds as microbial seed.

- French "blue cheese" which uses perforated fresh cheese as substrate and selected molds, such as *Penicillium roquefortii,* as inoculant.

- Composting of lignocellulosic fibers, naturally contaminated by a large variety of organisms including cellulolytic bacteria, molds and *Streptomices sp..*

- In addition to traditional fermentations new versions of S.F. have been invented. For example, it is estimated (Nagai, personal communication) that nearly a third of industrial enzyme production in Japan which is made by S.F. process and koji fermentation has been modernized for large scale production of citric and itaconic acids.

Furthermore, new applications of S.F. have been suggested for the production of antibiotics (Barrios & al., 1988), enriched foodstuffs (Senez, 1979).

- Presently S.F. has been applied to large scale industrial processes mainly in Japan. Traditional *koji* manufactured in small wooden and bamboo cases has evolved gradually to fixed bed room fermentations, rotating drum processes and automated stainless steel chambers or trays with microprocessors, electronics sensors and servomechanical stirring, loading and discharging. The usual scale in *sake* or *miso* factories is around 1 or 2 metric tons per batch but reactors can be made and delivered by engineering firms to a capacity as large as 20 tons (Fujiwara, Ltd.).

- Outside Japan, Kumar (1987) has reported medium scale production of enzymes, such as pectinases, in India. *Koji* type processes are widely used in small factories of the Far East (Hesseltine, 1972) and *koji* fermentation as been adapted to local conditions of United States and other Western countries, including Cuba (III A).

- Blue cheese production in France is being modernized with improvements on the mechanical conditioning of cheeses, production of mold spores and control of environment conditions.

- Composting which was produced for small scale production of mushrooms has been modernized and scaled up in Europe and United States.

New versions for S.F. reactors have been developed in France (Blachere, 1988), Cuba (Cabello, 1985; Enriquez, 1983 and Rodriguez, 1984) and fundamental studies on process engineering are being conducted in Mexico (Saucedo, 1987).

- S.F. is usually a batch process in order to avoid heterogenous materials with various ages, (Raimbault, 1980 and Tengerdy, 1985), giberellic acid (Kumar, 1987), pectinases (Kumar, 1987; Oriol, 1988), cellulases (Roussos, 1985), spores as biopesticides, flavors and frangancies and feed detoxification.

Generally, most of the recent research activity on S.F. is being done in developing nations as a possible alternative for conventional submerged cultures which are the main process for pharmaceutical and food industries in industrialized nations.

COMPARISON BETWEEN SUBMERGED (LIQUID) AND SOLID FERMENTATIONS :

Present industrial fermentations use submerged liquid cultures (L.F.) for producing biomass, enzymes and metabolites. This kind of process was developed in order to speed up the time consuming procedure of surface fermentation.

- The medium contains mostly water (90/95%) which later has to be separated from very low amounts of microbial products, generally at concentrations lower than 50 g/l (5%).

- Residual water has to be treated sometimes as costly as the fermentation plant

itself.

- Bacterial and yeast contamination have to be avoided by expensive sterilization techniques of wort and air because many interesting metabolites, such as antibiotics, are produced by slow growing molds in rich media that would be readly use by contaminant microorganisms.

- Reactant and product concentrations are rather low, making recovery processes expensive and a key factor in the over all process economy.

- Oxygen solubility in water is very low, making necessary to use complex and expensive machinery for agitation and forced aeration with rather high energy expenses.

On the other hand, S.F. seems to have theorical advantages over L.F. such as,

- The fermented mass usually contains less water (60 to 80%) than in L.F..

- A large fraction of residual water can be evaporated in rotating drums yielding smaller volumes for waste treatment plants.

- Lower water activity in S.F. gives ecological advantages for slow growing molds over bacteria and yeasts, reducing the needs for sterile operations.

- Molds can use and transform sugars impregnated in solid materials at rather low concentrations, well above 50 g/l (up to 400 g/l, according to Oriol, 1988) and can produce higher concentrations of valuable products, such as giberellic acid (Kumar, 1987), penicillin (Barrios, 1988), pectinase (Trejo, 1987). By this way, recovery costs can be reduced.

- Oxygen is not a significant limiting factor because it is readly soluble in air which is the mixing fluid of S.F.. Therefore, S.F. would have lower energy costs than L.F..

Nevertheless, S.F. has several important limitations.

- Aerobic microorganisms are limited to grow as thin films of aerated surfaces leaving interstitial air (30 to 50% of reactor volume) and intraparticular volumes without use (Raimbault, 1980). Thus, microbial concentration is of a similar level as in L.F. (lower life cycle). In general, batch processes are less productive than continuous ones.

- Process control is more dificult in S.F. because of the heterogenous quality of the fermented material and because mixing is very slow or absent.

- Product recovery can be difficult if the desired products are absorbed or contaminated by solid residues.

- There is little experience of the use of S.F. for large scale production of elaborated metabolites and few Companies, mostly in Japan for food industries, have "know how" for building and operation of S.F. plants.

POTENTIAL APPLICATIONS OF SOLID FERMENTATION IN MEXICO :

Mexican Government in 1983 in its National Plan of Development outlined three major subjects for biotechnological development: Agroalimentary industries, Pharmaceutical production and Environmental protection. Within this framework of reference, S.F. is mostly related to the first to lines of research and development for future applications.

1. AGROALIMENTARY INDUSTRIES :

Mexican situation has changed during the last six years and priorities for R&D have to be adjusted to such changes. Before 1982 there was an increasing demand for animal products such as meat, dairy and poultry derivatives. Annual consumption was increasing at a rate of 8 or 9% per year, and such increase resulted in large imports of sorghum and soy beans. But after 1982 salaries were reduced in their purchasing power to less than half of previous years and net consumption of meat and dairy derivatives fall using non sterile S.F. for penicillin production. ICAITI in Guatemala is considering the feasability for rumendown to less than 40% of 1982 levels. As a consequence, feed grain consumption decreased and packing and cheese making industries reduced their operations.

The new economical and marketing situation has led to several companies to developed new extended products using vegetable materials as raw materials and biotechnological derivatives such as glutamate, yeasts, gluconic and lactic acids as food additives. Those food additives, combined with soy bean and cereal flours or casein and lactose powders had served to produce new extended products with similar biochemical composition but reduced price.

Beyong the legal and ethical problems for defining food adulteration vs. food extension, there is some rationale for supporting partial substitution of animal products: it is an economical use for food ressources since animal production is an ineficient transformation of scarce feed grains. It can be shown that for each kilogram of substituted meat (dry bases) there is a potential saving of 20 to 40 kilograms of feed grains (sorghum/soy beans). Fortunately, the technology for flavoring vegetable raw materials is advancing very fast. Enzyme and microbial transformations make possible to develop many new flavours from a wide variety of starting materials, without many of the legal and commercial limitations for chemical food additives.

A general estimation of the potential for microbial and biochemical food additives (microbial biomass, organic acids, enzymes and special flavor compounds) in Mexico is of the order of 10,000 to 20,000 tons per year, based in the large volume of meat (sausages) and dairy products (cheeses and yoghurts). The interesting aspect of this field is the relatively higher price of microbial products (800 to 1,500 US dollars per ton) as compared to the lower price of soy beans (250 to 300 US dollars per ton). As an indication of the importance of this market it is worth recalling that Mexico has been importing yearly nearly 100,000 tons of dried milk during the last five years which could be partially substituted by extenders in dairy industries.

Another interesting application of fermentation technology is the production of cheap enzyme preparations for increasing the digestibility of feedstuffs, for example, cellulase and protease production in order to increase feed grain digestion, especially by delicate animals such as calves or little chickens. This is the field of the so called "probiotics" which are materials used in less than 3% amount of the whole ration but which increase the feed conversion and/or intake for more than 5% of previous levels. Here the potential market is in the order of few thousands of ton per year.

The use of S.F. for inexpensive food and feed products or for speciallized microbial biomass to be grown better on the surface of solid substrates is a promising possibility.

Food production can also be increased by reduction of agricultural costs

using more effective and ecological biopesticides. A very specific line of interest for S.F. would be large scale production of mold spores that help to control plant diseases.

2. PHARMACEUTICAL AND FINE BIOCHEMICAL PRODUCTS :

S.F. was considered during the first stages of antibiotic production but was discarded by large scale liquid fermentation systems (Ziffer, personal communication). There is, however room for improvment in the field of process and product development for new techniques of production of secondary metabolites, specially for materials that tollerate slight bacterial or yeast contamination. Barrios (1988) have indicated the possivility of sin production., an antibiotic produced by Streptomycete and used as feed additive in cattle production. Kumar (1987) and Barrios (1988) have proposed the production of giberellin using S.F. techniques.

Enzymes for pharmaceutical and chemical industries could be produced by S.F. especially if they can be used without purification. In this sense, an interesting application is the use of hydrolases for improving separation processes, such as, the use of pectinase for improving apple and mango juice extraction.

CONCLUSIONS :

Solid fermentation is a traditional processes which can be revaluated as an alternative for the production of food (flavor) and feed (probiotics) additives, biopesticides, enzymes and fine biochemical (antibiotics, giberellin, etc...).

Best uses of solid fermentation are in processes not requiring sterilisation of fermentable mashes and using crude products directly or having much higher productivity than in conventional submerged cultures.

Research and development is necessary for product development and scale up of S.F. products since there are very few experiences, outside Japan, for the construction and operation of industrial plants using S.F..

ACKNOWLEDGEMENTS :

The author wishes to thank Dr. Maurice Raimbault from ORSTOM for a long standing collaboration and many fruitful discussion related to the subject of this paper. He also wishes to acknowledge the close collaboration and trust of his colleagues in the Universitad Autonoma Metropolitana. Financial support was obtained from Organization of American States and European Community Comission and the Ministery of Foreign Affaires.

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THEORETICAL AND PRACTICAL ASPECTS OF WATER ACTIVITY MEASUREMENTS IN INTERMEDIATE MOISTURE MEDIA

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INTRODUCTION :

Notion of water "availability"

The development of microorganisms and the diffusion and reactivity of solutes which have an effect on the stability of agricultural produce and foods, are the direct consequence of water availability.

From a practical point of view, SCOTT (1,2) determined one of the most useful ways of determining water availability, by measuring water activity (aw).

This makes it possible to establish a practical correlation in many cases between the aw and the microbiological and physiochemical stability. With a low aw (3,4,5,6,7,8,9,10,11), some microbiological, enzymatic and chemical processes are generally observed to be lower. Only the oxydation reaction of lipids or the reactions involved in a non-aqueous liquid phase (e.g. enzymatic hydrolysis of oils) are not necessarily correlated with the aw .

Water activity of a liquid or solid media is thus, along with water content, temperature, pH and the oxydo-reduction potential, an important and acute criterium for the control of microbial growth and metabolic production of microorganisms or biocatalysed systems.

Although the aw concept is very useful, over-generalizations must be avoided because of the theoretical limits to this concept (12,13,14,15,16,17) related on the one hand to non-ideal reactions and states of "pseudo-equilibrium" frequetly observed in agricultural produce and food systems, and on the other hand to the fundamental consideration of some factors which, along with water, are responsible for reactant mobility or for the properties of microorganisms's membranes (17,18,19). As we know, a given microorganism is not intrinsically aw sensitive. GOULD (19) suggests that product stability with regard to microorganisms should be indicated with a criterium or a group of criteria

including the following factors : environmental osmolarity, membrane permeability for the main solutes, the nature of these solutes, and their eventual physiological or toxic effects.

Therefore, water activity is not the only property which can indicate the relation between physiochemical and micro-biological medium stability and hydration. Other properties such as the functional, structural or dynamic properties of water may also be used to indicate the state of water in agricultural and food products (6,20).

Measuring activity levels and thermodynamic equilibrium

Water activity measuring techniques are generally divided into two categories : the "direct" methods when the measurement provides an "absolute" value which, by applying physics laws, can be used to deduce the value of aw ; the "indirect" methods when the measurement is only significant when compared with those obtained with standards whose aws should be well-known.

Aw measuring methods have been reviewed by many authors (21, 22, 23,6,10,24,25,26). The recent development of new methods and improvement of the performances and reliability of aw measuring techniques make it necessary to confront the practical limits of these methods (aleatory deviation) with the predictable theoretical limitations (systematic error) by studying the thermodynamics of the aw concept and the principles applied when measuring. The theoretical aspects of aw measuring techniques were discussed by GAL (27), REID (12), VAN DEN BERG (28), and MORIN (29).

This paper aims at the review of aw measuring techniques and at the development of theoretical or practical criticisms for each case.

We have divided these techniques into five groups : 1) Direct manometry, 2) Techniques derived from manometry, which measure relative humidity in equilibrium with samples, 3) Measurement fo capillary pressure, 4) Measurement of osmotic pressure, 5) Calorimetry.

The practical methods for measuring aw are based on a property directly or indirecty related to water activity. To be validated by traditional thermodynamic standards, an operation must concern an isolated system in equilibrium with zero flow rates. This is hard to obtain with small-sized samples, when the aw is low, with heterogeneous foods or agricultural produce, with emulsions, etc (6,12,13,30,31). When equilibrium (essentially calorific and hydrous) is reached between a sample and a measuring system, as well as inside the sample itself, chances of aleatory deviation are lessened.

For some products, the thermodynamic equilibrium necessary for measuring water activity is long to be reached. Furthermore, there is generally a hysteresis

in the sorption-desorption curves of agricultural produce and foods which means that the same water content may lead to two different aw values. This hysteresis may actually represent two different equilibriums. In the case of native potato starch, desorption is apparently an unstable equilibrium (32). However, BRYAN (33) and CEROFOLINI and CEROFOLINI (34) suggest that there may be two real equilibriums for proteins because chain structures may differ according to the way the products are prepared (ad- or de-sorption).

Moreover, the structure and/or composition of most products is heterogeneous, for example : products whose surfaces are harder and drier than their inner content, or whose structures are more or less porous, emulsified or gelled. This is also true for products with "chunks" or a "filling" or with barrier layers (most often lipids), or else for products with some unstable components (supercooling, supersaturation). How the ingredients (for instance fats, flour) are composed may also vary depending on incorporation order or on the preparation techniques involved.

Cases of products which may vary in time (even during the lapse necessary for measuring) are also very delicate. These products undergo a physico-chemical evolution (recrystallization of supersaturated sweet solutions (35), appearance of crystalline hydrates (36) or structural rearrangements of macromolecules (36,37), or else an enzymatic or microbiological evolution.

For good reproducibility, the mode of operation corresponding both to the measuring technique and to the nature of the sample must be strictly respected. Despite precautions, the measurement obtained can not always be considered an actual thermodynamic activity, even though it obviously remains of technological and practical interest.

The CNERNA "Intermediate moisture foods" committee (38,25) provides some general recommendations for measuring aw. Samples must be pre-balanced at measurement temperature, and eventually broken into chunks (never mashed) before being introduced (in large enough amounts) into the measuring system. The temperature (usually 25° C) must be controlled and homogenized with less than 1/2° C deviation, and if possible with a 1/10° C deviation. Sensors must be standardized regularly, if possible before and after each measurement (in order to distinguish an eventual influence of volatile contaminants such as glycerol or aromatic substances (39,40)). Samples should be measured (except for dynamic methods or when measuring surface aw) for as long as possible (generally at least two hours), but measuring should be stopped before any sample evolution becomes perceptible. The most widely used standard solutions are sulfuric acid, glycerol or salt solutions (22,41,42,43,44). These must be carefully prepared (25,44). Saturated salt solutions are reliable standards, but laboratories disagree on some of the aw values (42,44).

MANOMETRY :

The basic systematic error involved in measuring water vapor pressure is very low. The aw is underestimated by one amplitude less than 2.103 in relative value (45).

The direct and absolute measurement of vapor pressure in equilibrium with the sample is obtained with a mercury or oil manometer made of glass. In general, the sample is frozen before being vacuum-pressed (approximately equal to 1 Pascal) so as to evacuate various components of the atmosphere which are uncondensable at low temperatures (46). After having heated the sample back up to the desired temperature, the vapor pressure can be directly read on the manometer (equilibrium time : 2-3 hours). This is probably the most precise method known for measuring aw since the products contain no volatile components. The CNERNA's IMF committee considers this method, developed by BIZOT and MULTON (46), to be the reference method. It can be applied to aws ranging from 0 to 0,99. Its precision (aleat ory deviation estimated with standard deviation) is better than = 0,005 units of aw and its accuracy,estimated with standard saturated salt solutions (whose aws are taken from the compilation of the most reliable bibliographical sources), is highly satisfactory. However, this method is slightly difficult to apply and is used mainly for research.

METHODS DERIVED FROM MANOMETRY :

Direct methods 1

(1Remark : these methods are theoretically "direct" but as some of them involve implementation difficulties, standardization is necessary to improve their performances).

Dew points measurements can be used to determine relative humidity, and thus aw (see, for ex. (47,48)). The measuring spectrum of this method ranges from aw = 0 to 0,98. Numerous errors may be made (mirrors not clean enough, temperature gradient, convection currents between mirrors and samples...). Its precision (estimated by standard deviation) varies between more or less 0,005 and more or less 0,01 according to the device used and the measurement spectrum (10,24,25). The accuracy of this method, estimated with standard salt solutions, is mediocre. It is possible to improve these performances by standardization with solutions already known.

Results of aw measurements by titration of water vapor in the air with chromatography during the gaseous phase do not seem to be satisfactory (49). It is also possible to measure E.H.R. (Equilibrium Relative Humidity) with volumetry by observing how the volume varies in correspondance with the desiccation of air humidity (50).

It is very difficult to use psychrometry humidity measurements (moist thermometer method) for measuring aws. However, it is possible to measure high aws, between aw = 0,93 and 1, correctly (precision around 0,01 aw) with psychrometry in static diffusion by using a Peltier effect micro-psychrometer with the precaution of standardization (51).

The bi-calorific equilibrium method used for measuring aw in a reference solution was first described by STOKES (52). It is based on the equilibrium of vapor phases between the sample (solution at 25° C) and pure water at a lower temperature (Ti). At equilibrium, the relation between vapor pressures at temperature Ti and at 25° C (on tables) gives the solution's aw. The concentration of the solution at equilibrium is determined by dessiccation or by water titration. This method can only be applied to liquids. At present, no data is available concerning the precision of this method. Furthermore, the verification of its accuracy seems useless since the reference values generally used were determined by STOKES with this method.

Methods using equilibrium with a substance or with reference air

The most widely used isopiestic method consist in establishing sample equilibrium (3,6, or 24 hours) in a closed cell with a reference substance previously dehydrated (usually a micro-crystalline cellulose (53), a protein (54) or a paper filter (55). The aw is determined by comparing water titration in this reference substance with its standard adsorption isotherm.

LANDROCK and PROCTOR's interpolation method (56) (or the micro-climatic method) consists in the determination of the environment's relative humidity in which sample mass does not vary at all. This method, said to be "dynamic", differs from most methods because an atmosphere/sample equilibrium is not necessary. This allows for quicker measuring. After a standard exchange time, ranging from 1/2 hour to 24 hours in cells with different E.R.H.s, the mass variation of each test sample is indicated according to its E.R.H. The zero-variation graphic interpolation determines the sample's aw. BOUSQUET-RICARD et al (57) use miniature cells (Conway cells) which permit very quick measurements (1/2 hour). MULTON et al (58) use air-conditionned chambers which make it possible to obtain measurements in only 5 minutes.

aw can also be determined with solvent extraction (benzene) of sample water(59). This method is based on the fact that, once equilibrium is reached, absolute activity is the same in both the aqueous and benzenic phases.

All three of these methods are easy, inexpensive and fast. They can be used for aws ranging from O to 1. In general, their precision is better than more or less 0,005 % (more or less 0,02 % for the fastest) and their accuracy is very satisfactory, even for the spectrum of high aws (aw > 0,90).

Standardized hygrometric sensors

These hygrometers are composed of a small moisture and air-proof measuring cell equiped with a sensor whose mechanical or electric properties are directly

connected to the E.R.H. of the cell's atmosphere. Fibre hygrometers (57,60) are based on the stretching of synthetic fibres (polyamides). Electric hygrometers (57,61) measure either the electric conductance of a thin layer of gelled liquid electrolyte (essentially lithium chloride) or the capacity or conductance of a thin layer of a hygroscopic material (anodized alumina or synthetic polymers). These hygrometers are often used for research purposes or for routine measurements.

Results with these methods are very good for aws ranging from 0,4 to 0,99 (fibre hygrometers) or from 0,1 to 0,99 (electric hygrometers) as well as for frequent standardizations when approaching a sample's supposed aw. Their precision is more or less 0,01 aw for the former (47,57,60,61) and more or less 0,005 aw for the latter (57,61,62). Accuracy expressed in comparison with standard solutions is at least equal to more or less 0,01 aw (57). Pre-measurement equilibrium time must be at least 30 minutes long and must often be 2 to 4 hours long for fibre hygrometers. However, these devices may be affected by some of the sample's volatile components, by an evenutal hysteresis phenomenon of the sensor (which is why it is generally better to measure water adsorption with the sensor) or by a bad calorific equilibrium (temperatures must be regulated at more or less 0,01° C, especially for electric hygrometers which have a very high temperature coefficient). The sensors are usually fragile and must be protected from splatterings or volatile contaminants with filters (which are not equally efficient, sometimes inducing longer equilibrium times). These sensors can not be used for measuring aws between 0,99 and 1, and their use in such cases may even damage the sensors.

Recently Gervais (63) has proposed a new sensor for on-line continuous water activity measurement during submerged or solid substrate fermentations.This sterilisable sensor allows the measurement of the relative humidity of the atmosphere in a small chamber by means of a capacitive element separated from the medium by a thin ethylenepolytetrafluoride membrane..A sequencial circulation of dried gaz in the chamber is needed to prevent the sensor saturation .The precision and the response time of this sensor are compatible whith liquid fermentation media in which the aw variations are extremely slow..Slower and less accurate measurements are obtained whith solid fermentation media.

CAPILLARITY :

This technique is described by MULTON et al. (26), MULTON (38) and LABUZA and LEWICKI (64). A sample is placed on a porous sheet. Then the capillary suction pressure of the water present in the sample is pressed by external means until equilibrium is reached. This method can only be applied to samples with an aw higher than 0,90 (signifying that there is still a liquid capillary phase). This method is limited by the use of very high pressure as well as by the time it takes (at least 24 hours). Standardization is also necessary

OSMOTIC PRESSURE :

Osmotic pressure can only be used to determine aw for liquids which contain only non-electrolyte solutions and which have e molar mass high enough to eliminate diffusion through the dialysis membrane.

Results of this method are satisfactory for aw values higher than 0,99.

CRYOSCOPY, EBULLIOMETRY :

Basically, the idea is to compare sample and reference transitional temperatures (boiling or freezing) and to deduce the water activity.

Aw values determined with this method are obviously relative to the temperature used for measuring.

If there is no correction, there is a systematic error in comparison with an absolute method. Nevertheless, it may be used as a relative method with standard solutions.

The measuring method which consists in lowering the freezing point to determine aw is recent and results are satisfactory (accuracy estimated in comparison with standard solutions to be better than 0,01 aw). This method can only be applied to liquid samples.

It seems highly recommendable when measuring very high aws (65,66). Results are also very good for solutions containing a volatile solvent (ex : ethanol) for which conventional methods can not be used. Furthermore, although tha aw obtained is given for the freezing point, this value appears to differ little from the value at room temperature (67).

CONCLUSIONS :

As thermodynamic equilibrium, temperature and pressure homogeneity and stability are never perfectly reached, the activities measured are actually only "pseudo-activities". Moreover, the principles on which the methods used to measure water activity are based lead to certain fundamental errors. The aleatory deviations involved in measuring must also be considered.

In actual fact, the values of the "pseudo-activities" are not that different from the thermodynamic water activities, except for products which undergo a microbiological evolution or a very slow physiochemical evolution (e.g. recrystallization of supersaturated sweet solutions (9) or else for products with

fats or surface-active agents (for which the hydrous equilibrium is very long to be reached).

Direct manometry is an absolute direct method for measuring aw ; it should be considered a reference method. All the other methods which we have discussed must be standardized with "reference" solutions. However, the aw values of these solutions are not always known with precision.

Some of the systems currently available can not be used to measure high aws correctly above 0,99 (standardized hygrometric sensors, for instance).

The highest limits of fundamental systematic errors due to the methods' principles vary between 0,002 and 0,07 according to the method. These can be considered insignificant after standardization, in comparison with the margin of aleatory error which, in the present technological state, is rarely lower than 0,01.

Many practical parameters, such as cell instability or contamination, or else the imperfect control of temperatures, may lead to incorrect values.

Comparisons between the different methods are often unsatisfactory : the different comparative studies of measuring techniques or laboratories (25,62,23,68) reveal deviations up to 0,15. With a better comprehension of the basic thermodynamic principles involved in each technique, these differences may be clarified.

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SOLID STATE FERMENTATION

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SUMMARY

Solid state fermentation may be defined as a microorganism growing technique on and inside humidified particles (solid substrate). The liquid content, bound with this solid matrix, maintained at the level corresponding to the water activity assuring correct growth and metabolism of cells, but not exceeded the maximal water holding capacity of the solid matrix.

This process, although very old, has not be developed industrially in the West despiteof many important advantages such as; the use of unpretreated solid waste materials or by-products; production of concentrated metabolites; production of some spores impossible to obtain in submerged cultures, etc...

Our laboratory is working on Solid State Fermentation with 3 principal aims:

- Studies on fungi metabolism related to:

  -the water activity and the water content of the media;

  -the gaseous environment, the agitation;

  -the nature, the structure of the solid matrix (organic and mineral matrix).

-Conception of different reactors (laboratory scale and pilot plant), transfert studies, elaboration of automatic controls, optimization of processes;

-Collaborations with different compagnies for the applications.

Engineering aspects of Solid State Fermentation, design of fermentors, control systems for maintaining parameters are very important for the research in this area. Laboratory scale reactors (3kg dry matter capacity) were constructed to allow control of the temperature and moisture level of the substrate without agitation. (Figure 1).

Forced aeration is carried out by mean of thermostated air injected at the bottom of the reactor. By bubling in a water bath and after heating, the thermostated air allows the regulation of the temperature and moisture content of the medium during cultivation (Figure 2 & 3).

As shown on Fig. 1 a unit is made of two reactors having strictly the same conditions for temperature and relative humidity of the inlet air. This system presents the great advantage to allow experiments in duplicate, and on the other hand to study some parameters without variations of the environment conditions.

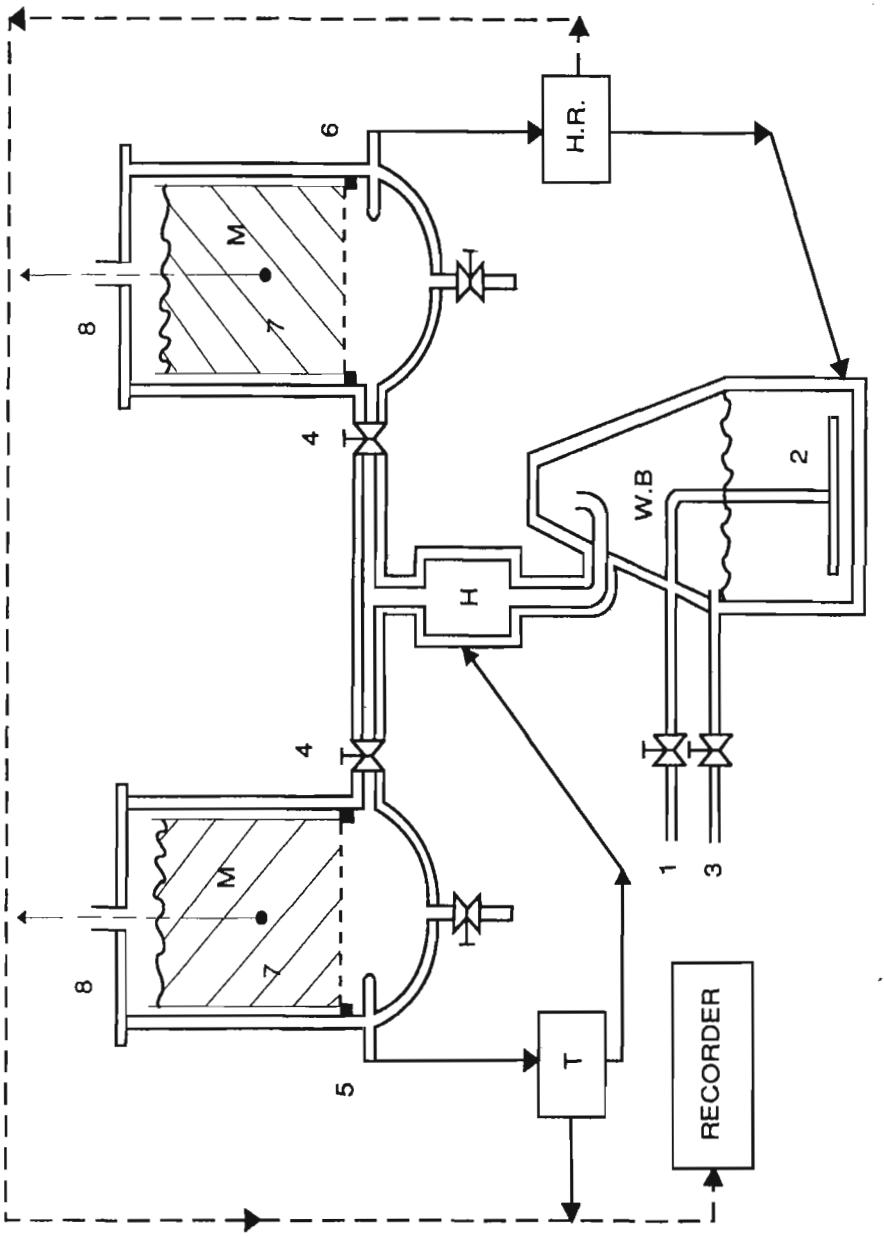


FIGURE 1

Figure 2

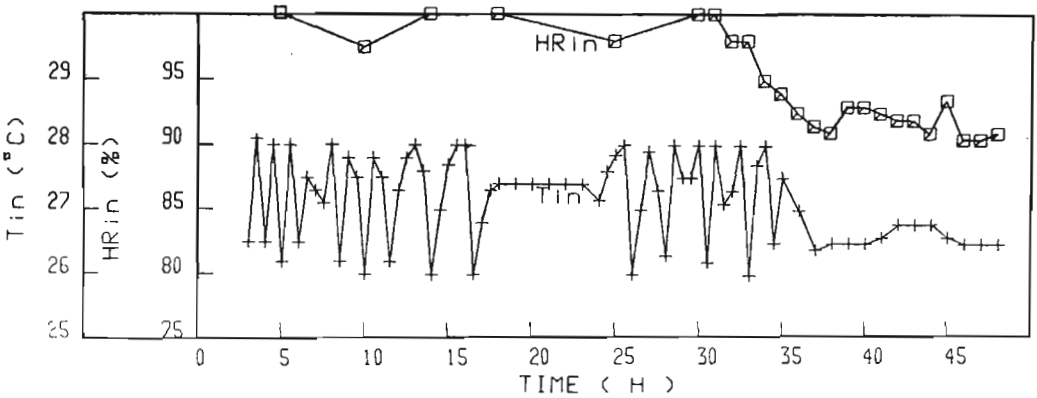
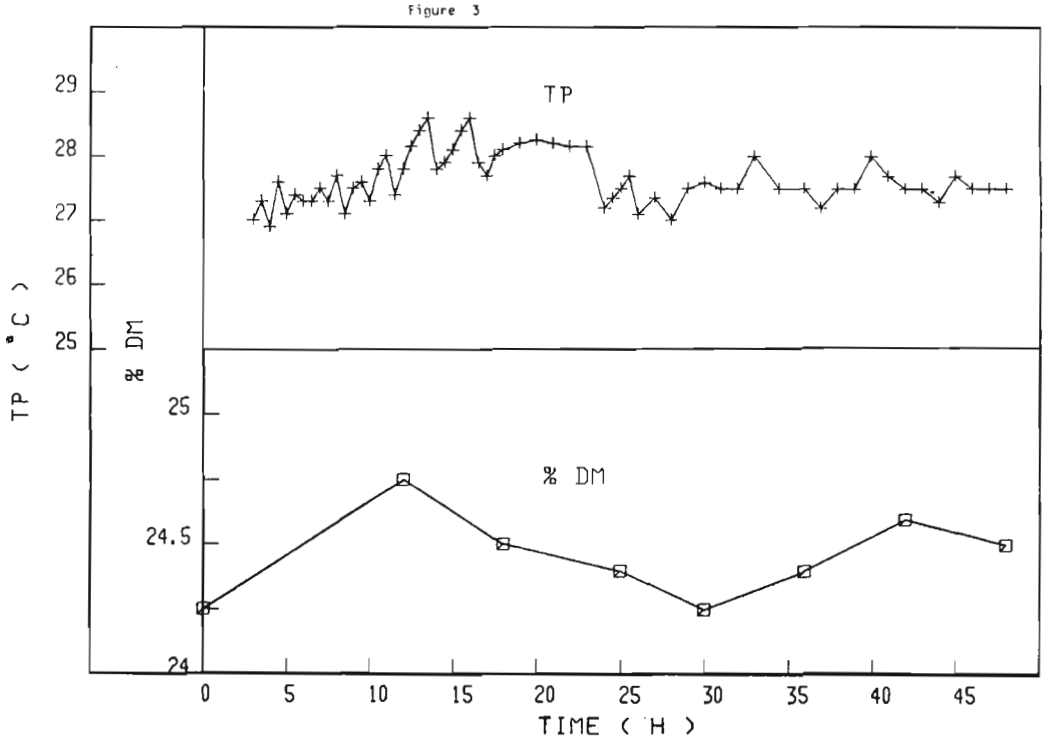


Figure 3



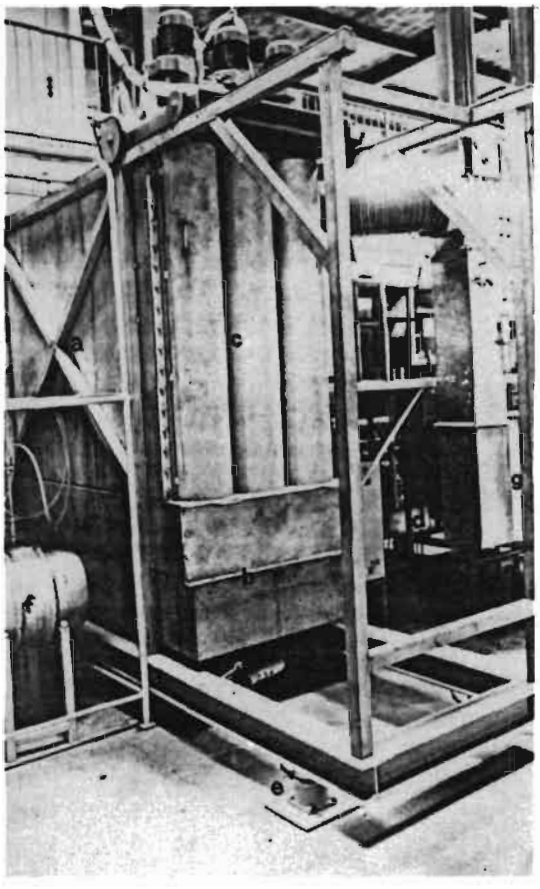


Figure 4.

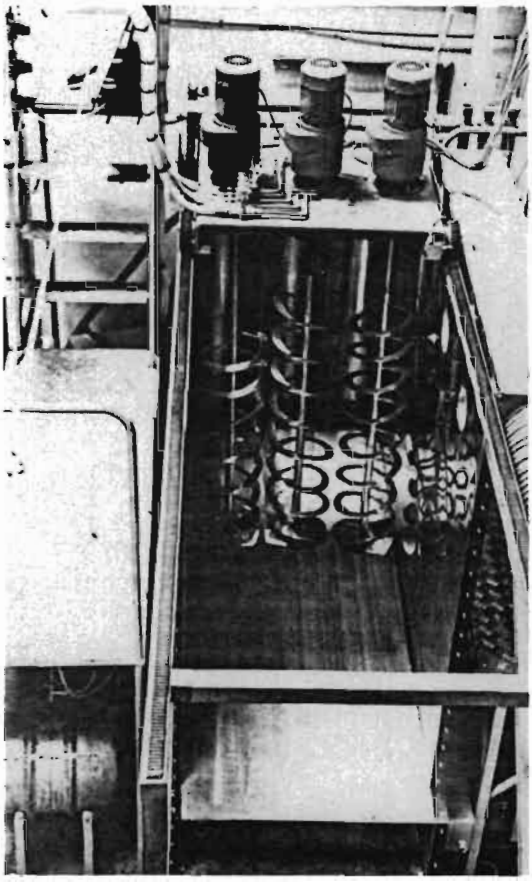
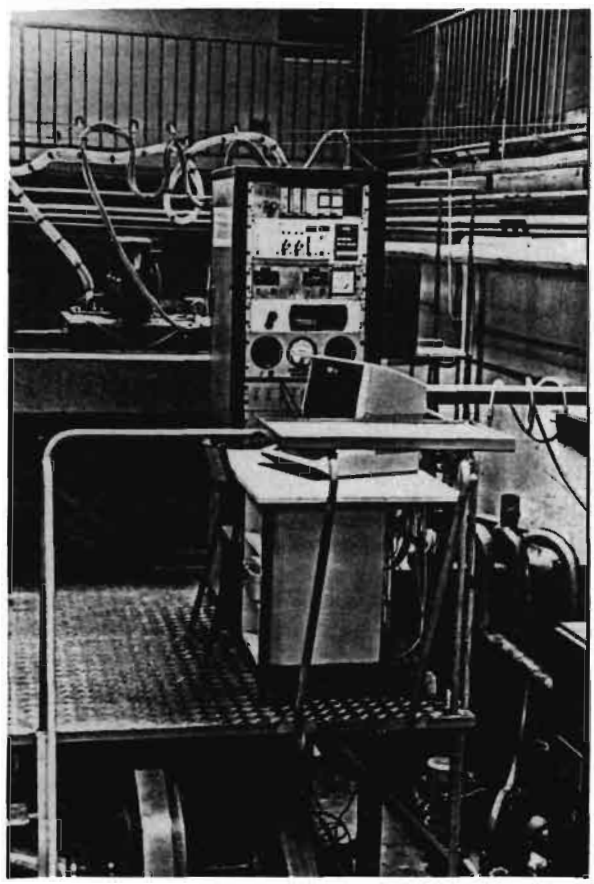


Figure 5

Figure 6



In collaboration with the GENERALE SUCRIERE and the NORDON Company, a 1 ton prototype reactor (Figures 4 to 6) and a growing technique using sugar beet pulp as a substrate and a filamentous fungus *Trichoderma viride* T. S. (French patent N° 7717449) were developed. The studies enabled a protein-enriched fermented food product of excellent quality to be obtained (about 21% protein with respect to dry matter in 48 hours). This food product, tested as a substitute for soybean cake, showed excellent performances when used with slaughter lambs and rabbits. Unfortunately, taking into account the context of the sugar industry (seasonal industry) and the price of soya-bean, this fermented food product does not appear to be economically competitive. Nevertheless, this process and the technology has found a more attractive industrial outlet. The GENERALE SUCRIERE built an industrial reactor in one of its sugar factories, and applied our process in the production ofseveral metabolites from sugar beet pulp.

Now, studies on engineering aspects are in progress, in order to elaborate a new sterile reactor (100 l capacity) for research needs and industrial applications.

In Solid State Fermentation, the gaseous phase is very important because it plays three parts:

- the regulation of substrate temperature

- the regulation of moisture level of the medium during the course of the fermentation.

- the maintainance of aerobic conditions.

We developed methods to evaluate the aeration efficiency. One method is based on heat transfert measurements. The kinetics of temperature evolution when heated airpasses through the medium layer are recorded at different points.

An other convenient method for measuring Kla in a solid-state medium has been proposed. Due to particular nature of the substrate used, different modifications of the sulfite oxidation method have been necessary. This first approach allows to study the influence of air inflow rate and dry-matter percentage of the medium on the oxygen volumetric mass transfer coefficient.

The results obtained with these methods allow to understand and build an optimal aeration and agitation device for a reactor in Solid State Fermentation. This reactor, operational in Autumn, will be able to perform sterile processes on different substrates and it will be totaly controled by computer with the utilisation of an Expert System.

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LEGEND OF FIGURES :

Figure 1: Schematic diagram of the solid substrate reactor.

WB: Water bath where humidification occures

H: Heating box

M: Basket with perforated bottom

HR: Relative humidity regulator

T: Temperature regulator

1: air inlet

2: sparger

3: water input

4: valves for airflow adjustement

5: temperature probe

6: relative humidity probe

7: temperature probe in the medium

8: air output

Figure 2: Evolution of temperature (Tin) and relative humidity (HRin) of the inlet air.

Figure 3: Evolution of the temperature (TP) and the dry-matter percentage (%DM) in the culture medium.

Figure 4: Details of the 1-ton prototype: (a) culture room, (b) aeration room, (c) removable end with semi-circular alveoles, (d) ventilation shaft, (e) weight gauges, (f) barrel of urea, and (g) air conditioning system.

Figure 5: Inside of reactor with the agitation device

Figure 6: View of the control board and microcomputer.

STRATEGIES FOR SPORE PRODUCTION OF *PENICILLIUM ROQUEFORTI* BY SOLID STATE FERMENTATION TECHNIQUES

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SUMMARY :

Three strategies of spore production of *Penicillium roquefortii* by solid state fermentation (SSF) techniques are presented. The fungus may be cultivated on a natural starchy substrate, buckwheat seeds, using a rotating fermentor or a fixed-bed column reactor. Development changes of the microorganism grown on buckwheat grains may be achieved without modifying the water activity of the substrate.A high total spore production is obtained by means of Casamino acids addition to the standart (A) medium. A good productivity along with an increase of the external sporulation are achieved with higher water content of the grains and a better initial hydrolysis of the buckwheat starch. Cultivations on porous particles (pozzolano) impregnated and continously fed with a liquid medium enable direct estimation of the biomass, the total spore production is easly recovered from the packed-bed reactor. Ca-alginat-coated buckwheat grains give rise to a high internal spore content of the particles. Semi-continousfermentations, carried out with buckwheat seeds, may be performed using a rotating fermentor.

INTRODUCTION :

The solid state fermentations (SSF) are widely used for spore production of filamentous fungi (Bartet et al. 1981; Cuero et al., 1987; Goettler, 1984; Hussong et Hammer, 1935; Lewis and Papavizas, 1983; Lotong and Suwanarit, 1983; Sansing and Ciegler, 1973; Singh et al., 1978). This method presents several advantages over submerged culture techniques because it gives rise to better yields of homogenous and pure spores (Vezina and Singh, 1975).

The spores obtained may be usedfor strain conservation and dissemination, or for a larger scale fermentor inoculation. They may also serve as biocatalyst in bioconversion reactions since they generally exhibit a high catalytic activity related to their dry matter (Vezina, 1987). The lack of mycelium proliferation during the process may lead to an easier product recovery (Moskowitz, 1979).

In this paper, we report an overview of results dealing with the grow and sporulation behaviour of *Penicillium roquefortii* in several SSF techniques. Cultivation on a natural, starchy substrate which consist of buckwheat grains are presented. A culture technique, based on the development of the microorganism on po-

Medium	Water content (g/gDM or IDM)	a_w (adim)	Total net[a] protein synthesis (mg/g DM or IDM)	Mycelium yield[b]	Total spores (10^9/gDM or IDM)	Productivity total spores 10^7 / h.g DM or IDM	Sporulation efficiency[c] (adim)	Spore yield[d] (%)	% external spores
Standard (A)	0.67	0.989	35.6	65	8.05	1.87	0.79	28.7	23.8
MCA	0.89	0.991	34.8	69	14.5	2.42	1.46	49.4	35.2
B	1.50	0.991	60	91	8.5	2.36	0.49	17.9	60

a: This value correspond to the maximal protein content of the medium minus the initial protein content

b: Correspond to the ratio of the total mycelium produced (deduced from protein synthesis assuming that the biomass contains 29.3% proteins, Desfarges et al., 1987) versus the reducing sugars consumed.

c: Defined as the ratio of spore dry weight (one spore: 11.93 10^{-9} mg) versus the maximal mycelium dry weight.

d: Dry weight of spores obtained (see above) versus sugars consumed.

TABLE I : Summary of diffrerent parameters value concerning the growth and the sporulation of *P. roquefortii* on various buckwheat - based substrates

rous particles (pozzolano) impregnated and continously fed with a concentrated substrate, is also described. Preliminary results obtained with alginate-coated buckwheat grains are given in the last part of this report. These cultivation techniques have been considered to follow two distinct strategies wich are summarized in the following diagram.

MATERIAL AND METHODS :

Microorganism

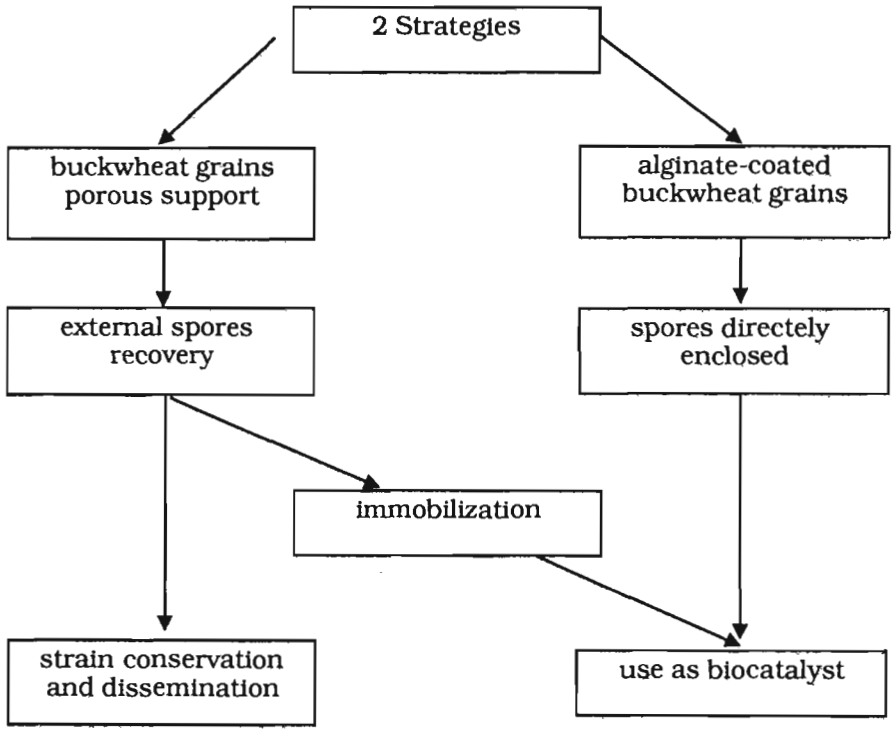
Penicillium roquefortii ATCC 64383 (Thom, 1930; Pitt, 1979) was first isolated by the firm Lactolabo from french blue cheeses. It was conserved by replicating on a Czaped-type gelosed medium (Meyers and Knight, 1958).

Cultivations on buckwheat seeds

Preparation of the substrate

Method A.

Buckwheat seeds, sorted so as to obtain an homogenous sample, were soaked in tap water or in an aquaeous solution of Casamino-acids 0.15% for 36 hours, and drained. The medium prepared in this way was steam sterilized for 20 min at 120 °C.



Supports	Glass beads 3mm	Wood shavings	Biogrod N [a] 2 - 3 mm	Pozzolano 2 - 3.15 mm
Density (Kg/l)	1.51	0.048	1.19	0.67
Medium content: 0h (ml/lf) 140h	30 150	74 217.5	59 194	128 360
Mycelium (g/lf)	6.6	b)	58	37.5
Spore production (10^{11}/lf)	0.92	9.5	5.5	6.65
Spore yield [c] (%)	2	22	21	27
Average productivity (10^9 spores/h.lf)	0.66	2.5	3.93	4.75
Sporulation [d] efficiency	0.15	b)	0.11	0.203

a: Biogrod N particles are ceramics manufactured by Argiles et Mineraux, Clerac, France.

b:The biomass could not be extracted from wood shaving.

c: The spore yield is calculated on the basis of the substrate (sugars) consumed.

d: See table I for explanations.

TABLE II: Influence of various supports on the development of *P. roquefortii.*

The parameters are expressed per liter of fermentor (lf) because of the disparity in the materials densities.

t° = 25°C, aeration rate 1 l/h, feed rate 4 ml/h, 500 ml Pyrex column fermentor.

Method B.

Buckwheat seeds were cooked at 100 °C for 30 min in the presence of an excess of a 0.013% chloramphenicol solution in water and without preliminary impregnation. This medium was cooled for 15 min, drained for 15 min and then sterilized for 20 min at 120 °C.

Coating of the buckwheat grains.

Buckwheat seeds (method B) were cultivated for 4 days and coated with a 3% dearated, pasteurized (60°C, 3 hours) alginate SG 800 (Mero-Rousselot-Satia, France) solution. The alginate layer reticulation was performed by pouring the particles in a 0.1 M $CaCl_2$ solution, the hardening being allowed to continue 12 hours at 4°C. The supernatant was then drained and the beeds were washed twice with sterile distilled water. When a double coating was performed, the first hardening occured for only 30 min before being rinsing. The second reticulation procedure was the same above.

Inoculation.

From a culture grown on a Petri dish, aged 9 to 12 days (possibly congelated) a suspension of spores was prepared and the media inoculated at 10^5-10^6 spores/g of dry matter (DM). When necessary 0.05 ml/g DM of an enzyme solution containing 1% a -amylase (Sigma n° A-2771) and 6 mM NaCl was added.

Analysis

Sugar determination

The sample (1 g DM) was homogenized using an Ultre Turrax blender, and dilute as necessary.

For search determination, an aliquote of the homogenized sample was kept at 100°C for 15 min; an enzymatic (amyloglucosidase) hydrolysis (Thivend et al., 1972) was followed by reducing sugars determination by dinitrosalicylate method of Sumner (1925).

Free reducing sugars were determinated after baryum hydroxyde deproteinization (Slein, 1965) of the sample by means of the dinitrosalicylate method of Miller (1959).

Protein content

The protein of the sample were extracted with a 2% SDS (Sodium Dodecyl Sulfate) solution by heating at 100°C for 5 min and assayed using the spectrophoretic method of Ehresmann et al. (1973). Bovine Serum Albumin (BSA) standart solution were used for calibration.

Chitin content.

The method of Ride and Drysdale (1972) modified by Whipps and Lewis (1980) was used.

CO_2 evolution and O_2 consumption.

The effluent gas was dried on P_2O_5 and periodically injected into a gaz chromatograph (Delsi Instruments, IGC 121 MB) fitted with an automatic sampling port and a commutation valve. A Porapak Q column was used for CO_2 determination and a molecular sieves (5 Å) for O_2 determination (Ramstack et al, 1973). A titrimetric method (Vogel, 1961) could also be used for total CO_2 evolution measurement.

Substrate	Inoculation	Water content (g/gDM)	a_w (adim)	Maximum protein synthesis (mg/g DM)	Biomass yield (%)	Total spores (10^9/g DM)	Internal spores / Total spores	Sporulation efficiency (adim)
A	Control	0.67	0.989	54	65	8.05	0.68	0.79
	External	3.17	1.000	83	63.0	7.05	0.26	0.30
	Alginate	3.16	1.000	61	57.8	7.6	0.33	0.44
	Precultivation	4.00	1.000	39	53.2	6.7	0.35	0.60
B	Control	1.50	0.991	60	91	8.5	0.40	0.49
	External	5.25	1.000	35	34.1	4	0.25	0.40
	Alginate	3.35	1.000	80	86.7	7	0.39	0.31
	Precultivation	4.56	1.000	53	72.4	10	0.25	0.66

TABLE III: Summary of some parameters dealing with the growth and the sporulation of *P. roquefortii* on alginated-coated buckwheat grains. See table I legend for definitions.

Spore counting

The externalspores were extracted by vigorous agitation of the sample (1g) in a 1% Tween 80 solution and counted using an hematimeter (Malassez cell). Total spores were estimated after homogenization of the sample using an Ultra Turrax blender.

Water content

The water content of the medium was determinated by heating at 110°C for 24 hours in an oven.

Water activity

It was measured by means of a Thermoconstanter TH2 BS RTD33 Novasina apparatus at 25°C.

Cultivation on porous supports (pozzolano)

Culture medium

The medium used had the following content (g/l): sucrose 43.75, Malt Extract 50, $NaNO_2$ 2.5, KCl 1.25, $MgSO_4$ 1.25, $FeSO_4$ 0.025, Chloramphenicol 0.125.

Support preparation.

The particles were washed with water, dried in an oven (110°C, 48 h), and steam sterilized (120°C, 20 min).

Inoculation

The culture medium was inoculated with about $3 .10^5$ spores/ml and poured into a column fermentor (see latter) previously filled with the support. After 1 hour, the excess liquid was removed and the aeration was started. This was taken as the zero time of cultivation. The substrate feed was usually started at t = 4-5 hours.

Analysis

Sugar determination

The sample (usually 1 g) was poured into 5 ml distilled water, vortexed and centrifuged (10,000 g, 10 min). Glucose was determinated in the supernatant by means of the enzymatic glucose oxydase (GOD) - peroxydase (POD) system with ABTS as chromogen reagent (Bergmeyer and Bernt, 1974). Sucrose was determinated as the amount of glucose liberated (GOD-POD) after invertase hydrolysis. The same method was employed for maltose (from malt extract) after amyloglucosidase hydrolysis.

Protein assay

The protein assay of the supernatant (proteins of the medium) or of the total medium (liquid plus biomass) were solubilized by alkaline hydrolysis (Herbert et al, 1971) and determined by means of the Bicinchoninic acid (BCA, Pierce) colorimetric method (Smith et al., 1985) using BSA (Bovine Serum Albumine) solutions as reference.

Biomass estimation.

The sample poured into water was vortexed and filtred on a Millipore prefilter AP 32 type. The filtrate was then passed through a Millipore Filter (0.45 µ), the biomass being measured by drying this filter (110°C, 24 hours). The same treatment was applied to "clean" pozzolano particles in order to take into account the weight of small particles which could be released when vortexing the pozzolano.

TABLE IV: Comparison of the results achieved with the three main *P. roquefortii* spore production process. (lf) means liter of fermentor.

Substrate or support	Spores (10^{12} / lf)		Productivity (10^9 spores / h.lf)	
	Total	Directly	Total	Usuable spores
Buckwheat (B)	3.08	1.85 a)	8.56	5.14 a)
Pozzolano	1.04	1.04	4.72	4.72
Two-layer coated buckwheat	1.35	1.01 b)	3.38	2.53 b)

a: External spores b: Internal spores

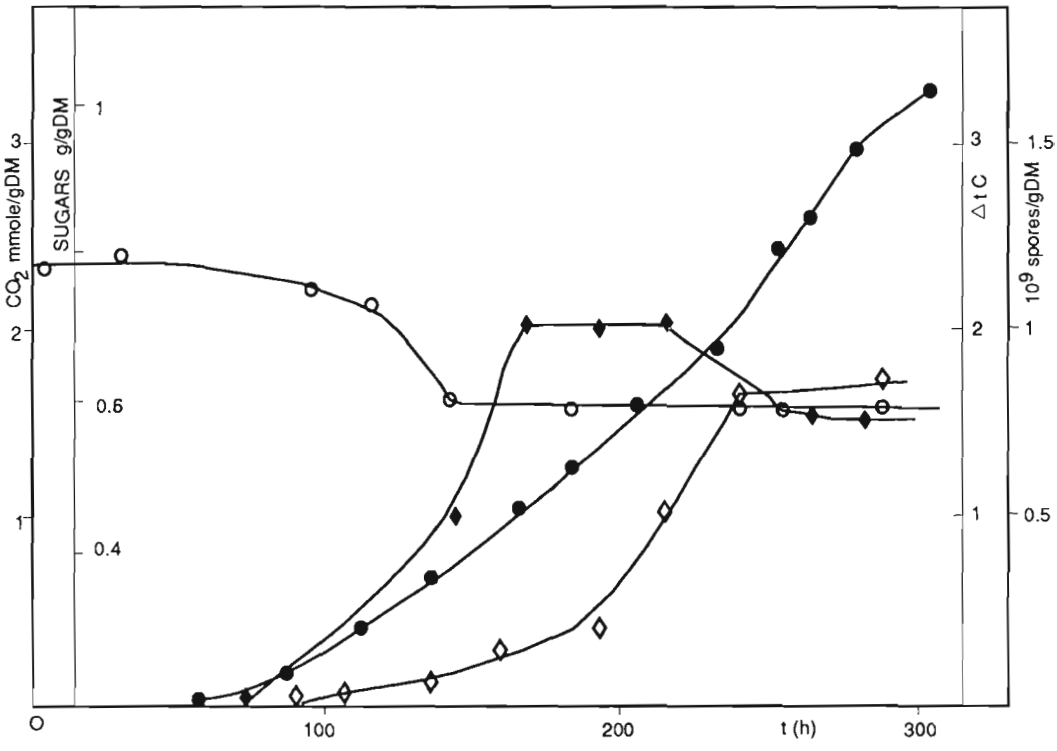


Figure 1: Kinetic evolution of a culture of *Penicillium roquefortii* on a A-buckwheat seeds in the rotating fermentor. •—•, CO_2 evolution; o —o ,total reducing sugars; ◊ ◊, external spores; ® ®, temperature elevation.
1.34 kg DM, t° = 23.5°C, aeration rate 7.5 l/h, 1.5 revolutions per 24 hours.

Other parameters

CO_2 evolution, O_2 consumption and spore counting were performed using the same methodology as described in the paragraph "Cultivation on buckwheat seeds".

Fermentors

A rotating fermentor of total volume 30 l, allowing the use of 1 to 5 kg DM of buckwheat (Larroche and Gros, 1986) was used along with jacketed fixed bed column reactors ranging from 0.5 to 2.5 liters. They all were fed with CO_2 free air by passing it either through a KOH solution or a KOH pellet column.

In the case of pozzolano cultivations, the fixed-bed reactor was fed with air and liquid medium at its top.

Expression of the results

The data obtained during buckwheat cultivations were expressed per unit mass of either dry matter (DM) or initial dry matter (IDM).

For support fermentations, the results were expressed either per unit of liquid medium volume remaining in the particles or per unit mass of support.

RESULTS :

Buckwheat cultivations

A preliminary study of various starchy substrates including oats, sorghum, maize, triticale, wheat, rice, barley allowed to retain buckwheat grains for *Penicillium roquefortii* cultivations (Maheva et al., 1984). This choice was made due to the excellent mechanical properties of the particles (retention of structure, lack of agglomeration) along with their high external sporulation yield.

Use of rotating fermentor

A drum fermentor has been assayed for external spore production of *P. roquefortii* on buckwheat seeds prepared according to the procedure A (see Material and Methods section)(Larroche and Gros, 1986). A typical time-course evolution of cultivation parameterd is given in figure 1.

The main problem encountered in the use of this fermentor is the metabolic liquor (mainly water) produced during a cultivation. The free water causes the grain to stick together and on the fermentor walls; the aeration and the homogenization of the substrate rapidly become difficult. Finally, bacterial and fungal contaminations appear. This problem can overcome by inclination of the reactor in order to allow the free water to be removed from the vessel.

The importance of the rotation effect on the growth and the sporulation of *P. roquefortii* must be underlined. Two parameters have to be taken into account. The time at which the first rotation appears after the inoculation of the medium is of critical importance. If this time duration is less than the lag period for spores germination, the spores are displaced from one grain to another, giving a delayed substrate consumption. If the rotation frequency during the overall fermentation is maintained at a low value (3 revolutions per day in the case of *P. roquefortii*), the mycelium is scarcely injured and a good sporulation occurs, the substrate

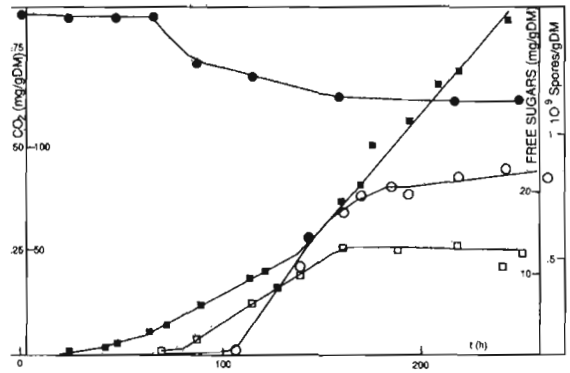


Figure 2: Kinetic evolution of a cultivation in a column fermentor filled with A-buckwheat seeds. ●—●, total reducing sugars; ⊙—⊙, CO_2 evolution; ◇—◇, free sugars accumulation; ○—○, external spores.
900 g DM, t° = 25°C, aeration rate 15 l/h.

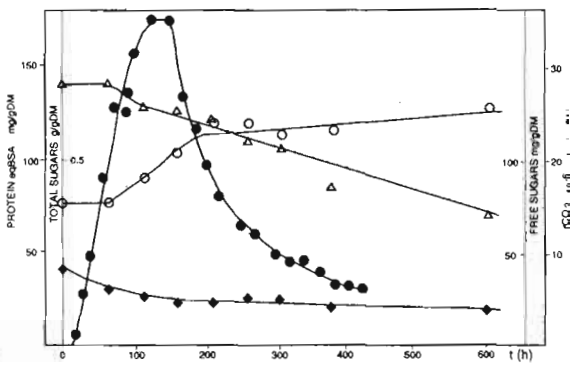


Figure 5: Time course of growth parameters during a cultivation on the MCA medium.
○—○, protein content; △—△, total reducing sugars; ⊙—⊙, free reducing sugars; ●—●, CO_2 evolution rate.
The other other experimental conditions are these of figure 3 legend.

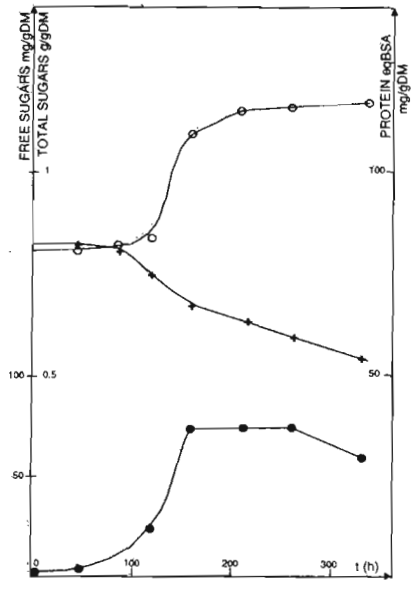


Figure 3: Time-course of growth parameters on A-buckwheat grains as substrate.
○—○, protein content; ●—●, free reducing sugars; + − +, total reducing sugars.
70 g DM, t° = 25°C, aeration rate 1 l/h.

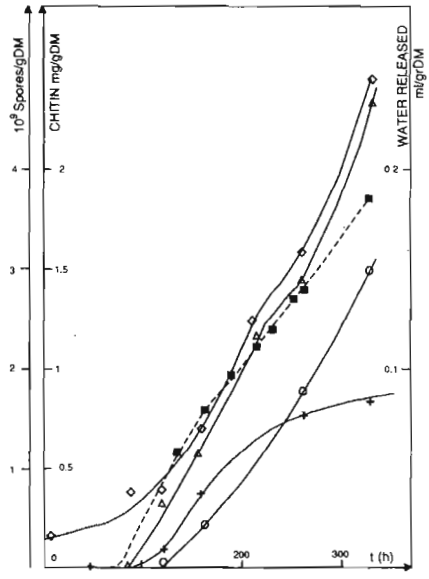


Figure 4: Time-course of sporulation parameters on A-buckwheat grains as substrate.
○—○, internal spores; + − +, external spores; △—△, total spores; ◇—◇, chitin content; ⊙—⊙, water released.
The experimental conditions are the same as in figure 3 legend.

colonization may even be promoted. When the medium is frequently mixed throughout the cultivation, e.g. 3 revolutions every 5 hours, the mycelium is damaged and the spore content of the medium is reduced.

Use of a column fermentor

A typical time-course of parameters obtained using a 2.5 l volume column reactor containing 900 g DN (dry matter) of buckwheat grains prepard according to the procedure A is shown in figure 2.

The slowness of the growth of *P. roquefortii* means that the thermal effect is small, thus allowing cultivations of this fungus in a fixed-bed column reactor exhibiting a diameter of c.a. 10 cm without any internal cooling.

About 0.2 ml/g DM water is produced during such a cultivation; this water ie readily removed from the medium at the bottom of the reactor.

The axial homogeneity of the spore content of the packed bed has been established.

No agregates (or blocks) are observed in the fermentation medium. These block could come from interparticular bridges resulting from the growth of fungal hyphae from a substrate particle to another (Moo-Young et al., 1983).

This behaviour make the packed-bed column reactor an easy-to-handle tool to carry out *P. roquefortii* cultivations and it is then used for further more detailled studies.

Optimization of the spore production.

The direct observation of *P. roquefortii* shows that the development of the fungus on buckwheat seeds proceeds roughly into four steps, involving a lag phase of about 10 h and three growth phases. At first, the spore germination is followed by uniformly external colonization of the grains by the mycelium. Then mainly external sporulation and internal colonization of the seeds occur, and finally, internal sporulation takes place; The first internal spores appears between the hulls and the endosperm. The development of *P. roquefortii* on buckwheat seeds seems then to exhibit a behaviour close to a colony growth and differentiation of a filamentous fungus (Bull and Trinci, 1977). We have first at the periphery of the colony, a young sterile mycelium, and then older mycelium which may be sporulating. Th e spore production then should not be considered as growth-associated.

A kinetic study performed on type A buckwheat (Desfarges et al., 1987) shows that the biomass production, expressed as protein content of the medium, occurs with an active growth phase ($\mu max = 0.03\ h^{-1}$) followed by a stationary period corresponding to a maximum protein synthesis of 35.61 mg/g DM (Figure 3). A lysis of the mycelium takes place after this plateau. The chitin content of the medium increases when the protein content (biomass) remain contant and the sporulation continue (figure 4). The spore formation occurs when without overall protein synthesis, but with formation of walls, which contain chitin. The chitin content of the medium is then a good sporulation indicator after the growth has stopped. Another rather good sporulation indicator is the amount of metabolic liquor (water) released from the fermentation medium during a cultivation (figure 4).

The stoichiometry of the growth and the sporulation of *P. roquefortii* on this substrate may be written as (Desfarges et al., 1987):

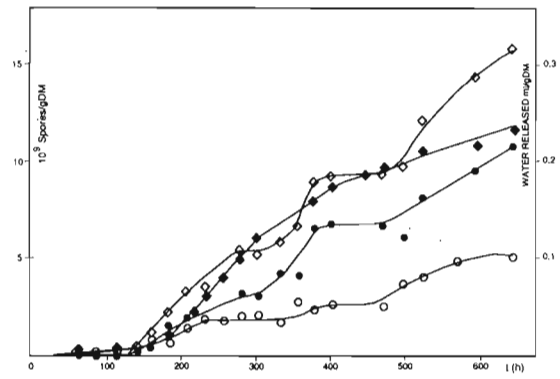


Figure 6: Time course of sporulation and water released during a cultivation on the MCA medium. o—o, external spores; ●—●, internal spores; ◇—◇, total spores; ⊙—⊙, water released.
The experimental conditions are given in figure 3.

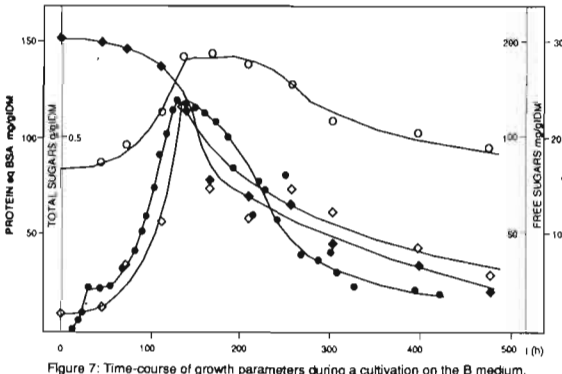


Figure 7: Time-course of growth parameters during a cultivation on the B medium.
The legend symbols and the experimental conditions are those of figure 5.

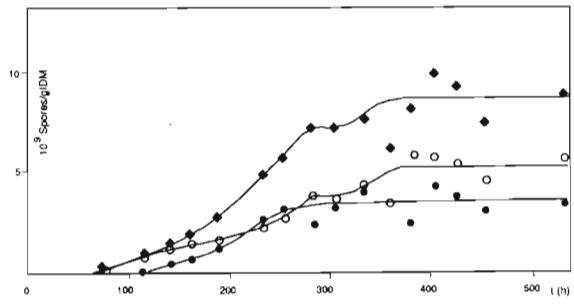


Figure 8: Sporulation of *P. roqueforti* on the B medium
See figure 6 legend for symbols explanation and experimental conditions.

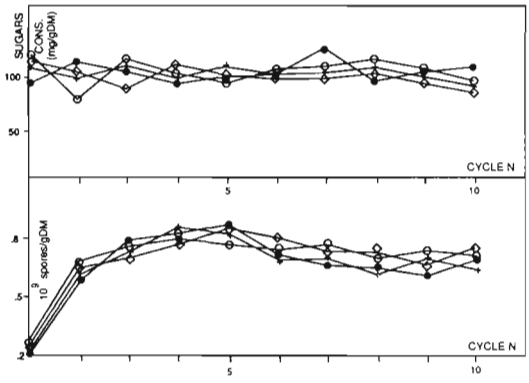


Figure 9: External spore content and sugar consumption at each emptying-filling sequence during a semi-continous cultivation in 1 l bottles (one cycle duration is 120 h).
Agitated cultures: ●—●, inoculum 2.5%; + – +, inoculum 5%.
Non agited cultures: o—o, inoculum 2.5%; ⊙—⊙, inoculum 5%.
t° = 25°C.

Biomass synthesis:

$$CH_{1.862}\ O_{0.768}\ N_{0.059} + 0.289\ O_2 + 3.436\ H_2O$$

$$0.678\ (CH_{1.882}\ O_{0.603}\ N_{0.087}\ ;\ 5.5\ H_2O) + 0.322\ CO_2$$

Spore formation from mycelium:

$$(CH_{1.882}\ O_{0.603}\ N_{0.087}\ ;\ 5.5\ H_2O) + 0.073\ O_2$$

$$(CH_{1.832}\ O_{0.724}\ N_{0.087}\ ;\ 4.085\ H_2O) + 1.44\ H_2O$$

Finally, the growth of the fungus stops when only 20% sugars are consumed, which is a feature often encountered in SSF (Raimbault, 1981; Baldensperger et al., 1985). This behaviour cannot be attributed to a bad digestibylity of the remaining sugars, since an important maintenance consumption is observed after the growth has stopped (Figure 3).

This high maintenance activity can be related to the response of a microorganism grown on a nutrient-limited medium (Richelato, 1975). An optimization of the sporulation of *P. roquefortii* on buckwheat grains has then been performed with two objectives. The first was an improvement in the overall productivity of the spore production. An increase in the external spore content was also a target because the spore located in this way do not need any grinding of the medium for their recovery.

Two kinds of modified buckwheat substrates were found to give a significant sporulation improvement in comparison to the A-type medium (standart), with respect to these two objectives (Larroche et al., 1988a).

The first consist of a A-type buckwheat grains impregnated with a 0.15% solution of Casamino acids (Difco) and supplemented with an a-amylase solution, and is called the MCA medium. The time-course of the growth parameters are given in the figure 5, while the sporulation patterns are plotted in the figure 6. The substrate allows the production of 14.5 10^9 spores/gDM obtained with the A (standart) one. About 35.2% of the spores produced are external. This sporulation enhance is achieved without any increase in the total biomass synthetized, giving rise to better sporulation efficiency any yield (table I).

The second substrate is prepared using the procedure B (see Materiel and Methods section). This medium, which exhibits a higher water content (1.50 g water/g IDM against O.67 g/g DM for the standart one) allows a lowest cultivation time (figure 7) and the production of 8.5 10^9 spores/gIDM , including 60% external spores. In this case, the biomass production is higher and the sporulation efficiency lower than for the standart medium (table I). This result indicates an effect of the water content of the medium; a high level induces a mycelial proliferation which gives rise to a decrease in the sporulation efficiency.

Semi-continous operation

A semi-continous cultivation using sequential emptying-filling has been performed in 1l bottles with the standart medium. The interval between two operations was made so that no free water could appear in the medium. The inoculation of each cultivation was made with 2.5% or 5% of the previous fermentation medium, allowing the lag phase, corresponding to the spore germination, to be suppressed. The first emptying was made 140 h after spore inoculation, and 120 h separated two cycles.

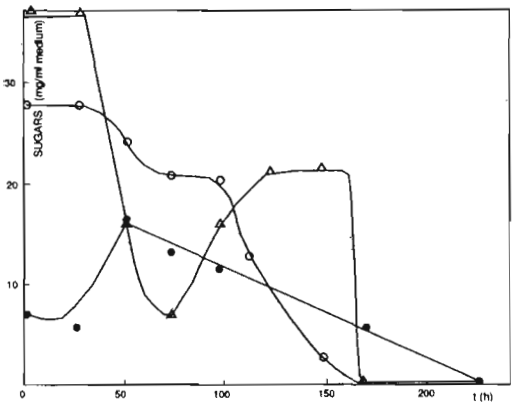


Figure 10: Substrate concentrations in a pozzolano packed-bed during a cultivation. △—△, sucrose; ○—○, maltose; ●—●, glucose.
350 g pozzolano, t° = 25°C, aeration rate 1l/h, medium feed rate 4,25 ml/h. The arrows indicate the beginning and the end of the medium inlet.

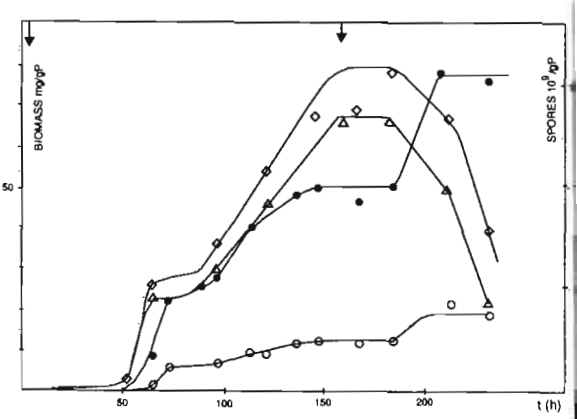


Figure 11: Kinetic evolution of biomass and spore production during a cultivation on pozzolano. ◇—◇, total biomass; △—△, mycelium; ○—○, spore dry weight; ●—●, spore number.
The parameters are expressed related to gram of pozzolano (gP).

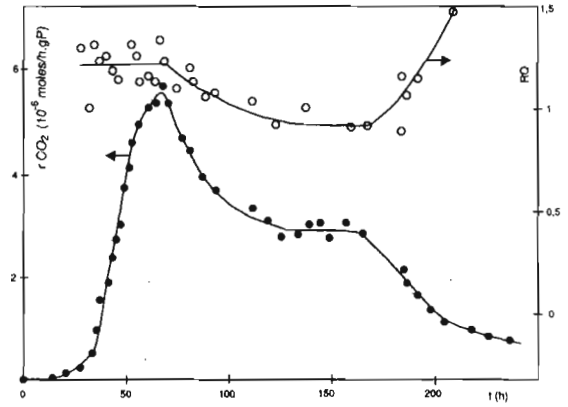


Figure 12: Time-course of CO_2 evolution rate r CO_2 and of the respiratory quotient, RQ = rCO_2 / CO_2. ●—●, r CO_2; ○—○, RQ.
gP means gram of pozzolano; the experimental conditions are those of figure 10 legend.

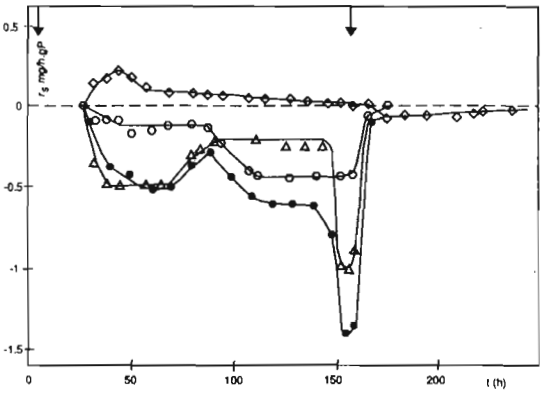


Figure 13: Sugar consumption rates, r_s, plotted against the time.
◇—◇, glucose; △—△, sucrose; ○—○, maltose; ●—●, total sugars.
gP means gram of pozzolano; the experimental conditions are those of figure 10 legend.

This kind of procedure allows a steady state to be established during at least ten cycles, i.e about two months (figure 9).

Such a cultivation may be considered as a plug flow with a recycle process. The best recycle ratio allowing to get the maximum spore concentration in the medium may be found according to Levenspiel (1979). In this case, a maximal spore content of 1.1 10^9 external spores/g DM is attainable for a productivity close to 3.87 10^7 total spores/h.g. DM, which is lower than the one achieved in batch cultivations with standart medium (see tableI).

The use of a slightly inclined drum fermentor, allowing the water produced to be removed, should permit an increase in the residence time, thus giving rise to a much better sporulation of the fungus.

Cultivation on inert porous particles

Experimental conditions

A preliminary study (Larroche et al., 1986) showed that a continous substrate feeding was required to ensure good cultivation conditions on porous particles which exhibit a small liquid retention. The experimental procedure retained allows an homogenous development of *P. roquefortii* through the packed bed. The low value of the feedf rated used, 4 ml/h for a 500 ml total volum reactor (8 ml/h.l fermentor) permitted a small retention of liquid in the grow in solid state-like cultivation conditions.

The behaviour of *P. roquefortii* on several supports has been investigated (table II). The need of a porous texture of the particle is brought to the fore by the results obtained with the glass beads as fermentation support.

Pozzolano particles were selected for further studies due to their good suitability for spore production of *P. roquefortii*.

Pozzolano particles colonization

The direct observation with a magnifying glass shows that the fungus develops at the surface of the particles and in the cavities connected with the outside. At the end of a cultivation, the spores completely invade the attainable external surface of the pozzolano grains and a very small residual myceliuym is still present. No sporulation occurs inside the particles.

A physical study of the porous texture of pozzolano particles was performed by the Laboratopire Central des Ponts et Chaussées, Paris, France (Guelin-Desfarges, 1988). The porometry tests show a great total porosity (35%) and a lower open porosity, close to 10%. The latter corresponds mainly to a pore radius in the range of 3 to 10 μm, value allowing a spore penetration, since the *P. roquefortii* spores exhibit an average diameter of 4.4 μm (Botton et al., 1985). An additional calculation, performed assuming a bilayer spore accumulation in these open pores leads to a maximal "internal" spore loading of the support close to 1.4 10^8 spores/g support. The experimental spore production (1.5 10^9 spores/g pozzolano, from table II) shows that 90.5% of the spores obtained are located at the periphery of the particles. These results demonstrate the suitability of pozzolano for the obtaining of easy to extract spores of *P. roquefortii*.

Time-course of a cultivation on pozzolano particles

The curves of the figures 10, 11, 12 and 13 shows the occurence of four phases during the development of *P. roquefortii* grown on pozzolano particles in a Pyrex column reactor.

The active growth proceeds through a diauxic growth. Sucrose is the first sub-

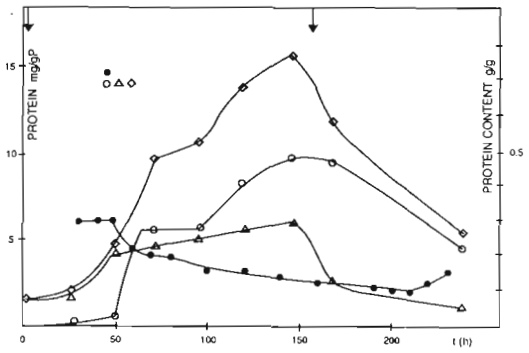


Figure 14: Kinetic evolution of the protein content of the medium.
o—o ,total protein; ◇—◇, soluble proteins; o—o, biomass proteins; ●—●, protein content of the biomass.
gP means gram of pozzolano; see figure 10 legends for experimental conditions

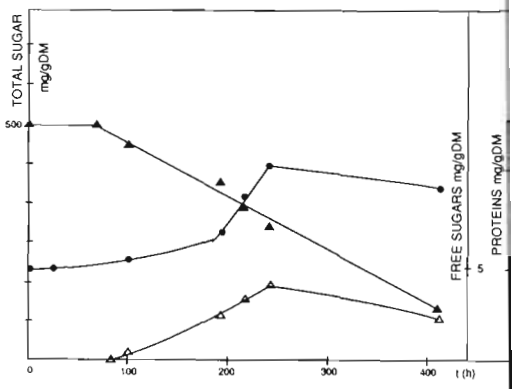


Figure 15: Kinetic evolution of growth parameters during a cultivation on one layer al
nate-coated B-buckwheat grains previously cultured for 4 days. ⊙—⊙, total reducing s
gars; ◇—◇, free reducing sugars; ●—●, protein content of the medium.
Cultivation in 250 ml Erlenmeyer flasks, t° = 25°C.

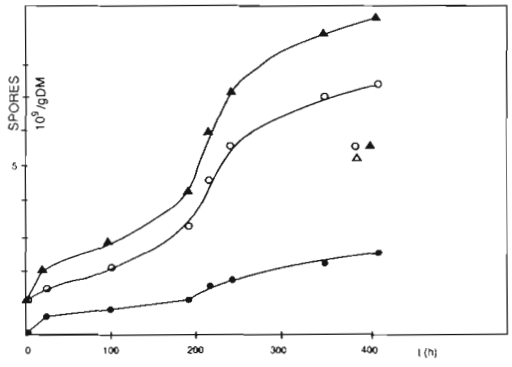


Figure 16: Sporulation pattern of *P. roquefortii* on coated buckwheat grains. ⊙—⊙, total spores, ◇—◇, external spores; ●—●, internal spores.
The experimental conditions are given in figure 15 legend.

strate consumed, associated with glucose accumulation in the medium. A material balance indicates that the accumulation mainly comes from maltose (from malt extract) hydrolysis. This period is connected with a little sporulation. The second growth phase correspond to a rapide maltose degradation (in all likelihood metabolised as glucose), and the sucrose consumption rate decreases (figure 13). The growth occurs within this time interval with a linear pattern exhibiting a biomass synthesis rate close to 0.56 mg/h gP (P = pozzolano). A second wave of sporulation is observed (figure 11).

When the substrate feeding is stopped (t = 160 h), the growth stops immediatly, and a plateau takes place. The residual sugars of the medium are rapidly exhausted (t = 180 h) and a lysis appears in connection with a resipratory activity decrease and a third sporulation wave. As the end of the fermentation, the biomass content of the reactor mainly consists of spores (figure 11).

The pozzolano-based cultivations allow a direct biomass determination. The protein content of the fermentation medium may be separated into a soluble and a non soluble form, the latter being related to the fungal protein (figure 14). The analysis show that the protein content of the biomass vary during the course of a fermentation. At the beginning of the process (0-50h), the microorganism contains about 30% proteins. This value exhibits a continous decrease after this time until a minimal value close to 10% is achieved.

When the substrate is fed into the reactor for a longer period, a stationary phase starts from 180 h, the final spore content may be slightly enhanced, but the productivity and the yield are affected. A shorter feed (e.g. 140 h) gives rise to a mycelial lysis without any plateau, and the final spore content of the reactor is lowered. The typical curves shown are related to a cultivation carried out in near optimal conditions.

This procedure allows a strong decrease of the fermentation duration with respect to the buckwheat-substrate ones. It gives rise to *P. roquefortii* spores readily recovered by shaking of the support. The particles should be reusable in further cultivations.

Alginate-coated buckwheat grains

In this paragraph, preliminary studies dealing with the production of entrapped *P. roquefortii* spores are presented.

When a substrate made of buckwheat flour and Ca-alginate is used, the development of the fungus occurs without any sporulation (Guelin-Desfarges, 1988). This behaviour may be attributed to a too high water content of the medium (5.67 g/g DM) giving rise to a nutrient dilution. A drying procedure is able to re-establish a good sporulation; the addition of a well-known water activity depressor, namely glycerol, to non-dried particles also allows a sporulation to occur. However, the fungus entirely develops outside the beads, making this kind of support unsuitable for our purpose.

A method allowing the coating of buckwheat grains with an alginate layer has thus been designed.

The mode of inoculation of these particles is of great importance. The results of table III show that an external inoculation by spores gives an important lag phase and finally lower sporulation yield, especially with the B-type buckwheat. This phenomenon may be understood by the distance of the spores from the substrate (buckwheat grain).

When the inoculation is performed by an entrapment of the spores within the

alginate, the mycelium formation is favoured against sporulation.

If pre-fermented grains are used, that is, a cultivation is preformed on the sole buckwheat seeds for 4-5 days before the coating procedure, a final spore content of the medium similar to the one obtained with the non-coated grains is achieved with the B-buckwheat (table III).

When a cultivation is performed with this last medium, we observe a lenghtened lag phase since the protein synthesis becomes significant only after 200 h of cultivation (figure 15). Moreover, the protein content of the medium at the beginning of the cultivation is not higher than on non-grown supports. As the initial substrate concentration is lowered, one can assume that a growth could realy take place before the coating. Hence, this coating procedure causes a linkage of external hyphae, giving rise to a fungal protein release. This phenomenon leads, as a response of the microorganis, to an improved sporulation (figure 16).

70% of the spores obtained on these coated grains are easily extracted from the medium; these spores cannot thus be considered as immobilized. Furthermore, the initial spores are all "external" when the culture medium is coated (figure 16). These considerations allow us to conclude that the "external" spores have a double origin. At first, the buckwheat grains are not located at the center of the alginate particle, giving rise to an alginate layer presenting a non homogenous thickness. Secondly, it may be seen a development of the fungus at the periphery of the particle, especially at the end of the fermentation.

A double coating procedure has thus been carried out. A great increase of the spores entrapped occurs during a cultivation, since only 25% of the spores are external in this case. Further experiments in this area are under treatment.

DISCUSSION :

The cultivation of *P. roquefortii* on a natural starchy substrate, buckwheat seeds, may be performed using a fixed bed column reactor. This design has already been used in SSF (Golueke, 1977; Spohn, 1977; Deschamps and Huet, 1984). During the course of a fermentation, three sporulation phases occur. The first appears in connection to the active growth and is of minor importance; it is not growth-associated but correspond to the maturation of the older mycelium which becomes competent to sporulation (Dahlberg and Van Etten, 1982). The second wave arises when the mycelium is in the stationnary phase and the third is related to the mycelial lysis.

The same feature appears when the fungus is grown on pozzolano particles, but the use of malt extract, which is a sporulation activator (Watkinson, 1975), gives rise to a faster sporulation of the fungus.

The colonization of the buckwheat grains leads to an internal and an external spore production. It is possible to modify the external/internal ratio of the spore content by varying the substrate pretreatment. An addition of a small quantity of Casamino acids allows a great increase in the spore production; this improvement concerns the total sporulation. The enhance of the water content of the medium, in connection with a better pre-hydrolysis of the starch leads to a more rapid cultivation. Despite of the lower sporulation efficiency, the spore content remains of the same order of magnitude than on the standart (A) medium; the external/internal spores ratiois enhanced. All these modifications could be achieved without any change in the water activity of the media used.

Drum fermentors are often considered as ehibiting a negative effect in SSF conditions, due to damage of fungal hyphae occuring during the rotation (Moo-Young et al., 1983; Silman, 1980). Our results show that if the rotation frequency is maintained at a low value, a good sporulation of *P. roquefortii* can be achieved. This vessel may be used in semi-continous operations. This last kind of cultivation becomes of interest in SSF (Abdullah et al., 1985; Kumar and Lonsane, 1988).

The inert porous particles fermentations allow the recovery of the total spores produced. If the production of a metabolite was the aim of a cultivation, the product could be easily extracted from the liquid effluent of the reactor (Hoschke et al., 1988). This system enables a fast cultivation to be performed and a direct biomass determination, which is not the case in classic SSF conditions. It is of great interest because of its versatility in the use of any synthetic medium, thus allowing reliable studies of the development of the fungi on solid state fermentations. For example, the measurement of the protein content of *P. roquefortii* leads to demonstrate a protein content variation of the fungus through the process when the mycelium is aging. We must point out that designs allowing the performance of "artificial" SSF meet an increasing interest (Mitchell et al., 1986; Oriol et al., 1987; Oriol et al., 1988; Sakurai et al., 1985).

The use of alginate-coated buckwheat grains allows the production of directely immobilized spores which could be used in bioconversion reaction without any further treatment. Buckwheat has been shown to be compatible with the 2-heptanone synthesis from octanoic acid by *P. roquefortii* spores (Larroche et al., 1988b).

A summary of the results achieved with the three batch cultivations proposed is shown in the table IV. It appears that buckwheat and pozzolano methods give similar results with regards to the external spore production, which is the sole parameter of interest for a bioconversion reaction. The apparent decrease in the spore content and in the spore productivity obtained with the coated grains is mainly due to a "dilution" of the substrate by the alginate layer. This pattern is counterbalanced by the very much simpler use of the medium for bioconversion purposes.

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LABORATORY AND PILOT SCALE PRODUCTION OF ENZYMES AND BIOCHEMICALS BY SOLID STATE FERMENTATION AT C.F.T.R.I., MYSORE

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ABSTRACT :

While solid state fermentation (SSF) has been used from ancient times for production of fermented foods and of late for the manufacture of some industrial enzymes, its application has remained at a modest level. This situation is changing now and the potential of SSF for the efficient production of valuable biological products like enzymes is increasingly being recognised. Central Food Technological Research Institute, Mysore, India, has been engaged in research and development in this area for last tqo decades and this paper gives an account of the work carried out, knwledge generated and processes developed.

INTRODUCTION :

The demand of industrial enzymes and biochemicals, traditionally produced by fermentation, is usually low in developing coountries mainly due to limited scope for such industries. Their production by submerged fermentation, a technique of choice in the Western Countries, is uneconomical at lower production scale due to higher capital and recurring expenses (1). Solid state fermentation (SSF), on the other hand, is a technique which overcome these problems. Therefore, intensive efforts were put-up in the last two decades at Central Food Technological Reseasrch Institute (CFTRI), Mysore, India on this technique. The initiation of the work was necessitated by the need to meet the slowly increasing demand of food enzymes in the country.

The efforts in the first decade were of exploratory nature at laboratory scale (2-5) and also involved investigations on the parameter standardization of the product in industrial application (6-8). Subsequently, the challenges involved in the scale-up of SSF technique and the need for pilot plant studies were recognized and solved (9). In recent years, the SSF technique was also extended to the production of yeast and bacterial metabolites which were little studied earlier for production by this technique (10-13).

LABORATORY SCALE STUDIES :

During the early efforts, thee need for special attention in formulating laboratory scale SSF technique was recognized mainly due to the absence of such information. Selection of strategies for standardization of varioous parameters and unit operations at the laboratory level were dictated by the problems and difficulties involved in scale-up of SSF processes. A comparison of various features, advantages and disadvantages of different large scale solid state fermentors were made and the tray fermentor was selected (14). Based on the above considerations, an efficient laboratory scale SSF ttechnique was formulated using a

well established scale-down approach available in the submerged fermentation process (15), and was found to yield excellent results.

A typical SSF process evolved consists of the following unit operations :

a) moistening of wheat bran (WB) with mineral salts-acid solution with or without supplementary nutrients.

b) distribution of 25-40 g moist WB medium in 500 ml capacity Erlenmeyer flasks.

c) cooking-cum-sterilization of the medium at 121 C for 60 min.

d) cooling to about 30 C.

e) inoculation of the medium with spore suspension.

f) through mixing of the inoculum in the medium by gently tapping the flask repeatedly.

g) incubation at desired temperature in slanting position.

h) continuation of fermentation till a peak in the product formation is achieved.

i) drying of the mouldy WB medium at predetermined time and temperature.

j) extraction of the product from dry mouldy bran (DMB) using standardized conditions.

Alternatively, the product can also be extracted from moist WB medium at the end of the fermentation, without resorting to ,the drying of the material. The spore inoculum was obtained by suspending the spores from potato-dextrose agar slant in sterile water containing emulsifier such as Tween-80.

Recently, fed-batch culture method, a useful technique in submerged fermentation process, was successfully extended to SSF technique to overcome substrate inhibition in the production of gibberellic acid (16,17).

LARGE-SCALE FERMENTATION :

A typical pilot scale technique evolved at CFTRI, Mysore, is essentially similar to that used at laboratory level and is based upon geometric similarity for scale-up. The differences are however, limited to the use of large scale tray fermentor and the mechanisation of some unit operations. It involves the use of a planetary mixer for preparation of moist WB medium and mixing dry spore inoculum with sterilized medium. Perforated shallow trays are used for holding the medium and are stacked 5 cm apart in trollies in the large tray fermentor. Temperature and relative humidity are controlled, along with efficient heat removal. At the end of fermentation, moist fermented bran is dried in a forced air circulation drier. The product is then extracted from DMB. The dry spore inoculum was raised in trays in WB medium incubated in tray fermentor for a longer time to obtain a large crop of active spores.

The large-scale cultivation technique evolved was found to give the same yield of the product as in the laboratory scale cultivation. In fact, the yields were better in many cases due to the control of relative humidity in pilot scale operation. The use of higher inoculum have also resulted in a reduction of the fermentation batch time at the pilot scale.

DESIGN AND EVALUATION OF LARGE TRAY FERMENTORS :

In the initial trials, a batch scale prototype of tray fermentor of size 46 x 46 x 92 cm was manufactured using teak wood borders and wire-mesh bottom were used along with white long cloth for holding and covering the fermenting bran while humid atmosphere was created by placing a tray containing sterile water at the bottom of the unit. The heat generated dissipates slowly through the wire-mesh faces. The unit was found to be deficient with respect to temperature control and occasional contamination problems. However, it provides an easy and quick means for evaluating the feasibility of the microbial culture selected at the laboratory scale process in flasks for its succssful growth and product formation in the trays.

A commercially available bench scale humidity-cum-temperature controlled incubator of 5 trays capacity was unable to cope with efficient removal of large-scale production of pectinases, an industrially available forced-air circulation tray drier of 96 tray capacity was converted into a tray fermentor (20).The modifications effected include installation of humidifier, humidostat, precision temperature controller, humidity and temperature recorders. The unit with 96 kg commercial WB/batch functioned efficiently in the production of pectinases. The fermentaation ba
tch time was reduced to 18-20 h in this unit as compared to about 48 h in flasks. The chemical sterilization of the interior of the fermentor by formaldehyde before charging the trays in the trollies, the use of dry spore inoculum ratio and the shorter fermentation batch time prevented any contaminating microorganism growing in the fermenting mass. The unit was used successfully for producing pectinase concentrate over a period of nearly 2 years.

However, the above unit posed serious problems in the scale-up studies for production of fungal amyloglucosidase mainly due to the higher fermentation time of 30-32 h. The problems encountered include :

a) partial drying of the fermenting solids as well as dehumidifiction of the chamber due to frequent automatic running of the large circulation fans for effective heat removal.

b) condensation of water drops on the inner surfaces of the chamber and at the outer bottom side of the trays due to the existence of narrow confined path at one corner for escape of hea
t as well as the existence of negligible free-space in the chamber.

c) contamination at localized spots where these condensed water droplets fell on the fermenting solids (19).

These problems were largely overcome by designing a koji room of 150 tray capacity (19). It consists of a room of size2,5 x2,5 x 3,5 m with an air-tight glass paned door and two vents of 0,3 m dia in opposite walls with louvres. An air blower was fixed at the bottom side vent and ywo circulation fans were mounted at 2,5 m height. A small humidifier of 9 L/h capacity was suspended from the center of the ceiling and a large humidifier of 31.5 L/h capacity was placed at the floor level. A humidostat was fitted at the rear of the room. A slotted angle structure held 150 trays. The temperature probe was inserted in the fermenting solids and the control panel which housed main switch gear, starters for humidifiers, temperature indicator/controller and humidity-cum-temperature recorder was located outside the room.

The comparative evaluation of the koji room against other bench and large scale fermentors developed showed superiority of the former in performance and higher yields in the production of pectinases, amyloglucosidase and gibberellic

TABLE 1. Technology development for production of enzymes and biochemicals by SSF process at CFTRI, Mysore.

Product	Microorganism employed	Scale of operation	References
Pectinases	*Aspergillus carbonarius* CFTRI 1048	Laboratory and pilot	20,26,27
Amyloglucosidase	*Aspergillus niger* CFTRI 1105	-do-	22,28
Rennet	*Rhizopus oligosporus* CFTRI 1104	-do-	29
	Mucor miehei	-do-	30
Fungal Alpha-amylase	*Aspergillus oryzae* CFTRI 1048	-do-	31
Catalase	*Rhizopus niveus* CFTRI 1053	-do-	32
Bacterial Alpha-amylase	*Bacillus megaterium* 16M *Bacillus lichneiformis* M27	laboratory scale	11 33
Gibberellic	*Gibberella fujikuroi* P-3	-do-	10,16,17
Acid			24,25
Acid protease	*Aspergillus niger* CFTRI 1071	-do-	34
Ribonuclease	*Aspergillus candidus* M16a	-do-	35
Cellulase and D-xylanase	*Aspergillus ustus* *Botrytis* sp., *Trichoderma* sp. *Trichoderma viride* QM 6a	-do- -do-	36,37,38
Citric acid	*Aspergillus niger* 16	-do-	39
Fats and lipids	*Acremonium terricola* CFA-5 *Rhodotorula gracilis* CFR-1	-do- -do-	13 12

acid (19). The cost of the unit also works out to be reasonably small. Consequently, it was used for production of large quantities of amyloglucosidase concentrate as well as in scale-up studies on other products.

DEVELOPMENT OF PRODUCT EXTRACTION STRATEGY :

The presence of the product in concentrated form in SSF processes and the consequent lower expenses on down-stream processing as well as effluent disposal have resulted in a surge of interest in SSF processes in the recent years (21). The use of percolation technique for extraction of the product from DMB, requires the use of high volume of solvent for achieving acceptable extraction efficiency (22). The resulting extract thus is too diluted and demands energy and cost intensive vacuum concentration.

The multiple-contact counter-current leaching technique was, therefore, used to extract the product in concentrated form from DMB and to obtain 1 L extract from 1 Kg DMB, as against 10 L/kg in the percolation method (22). Various parameters such as the selection of the solvent, number of contact stages, the ratio of DMB : solvent, contact time, pH and temperature of extraction were found to affect the degree of extraction. The technique was used successfully for the extraction of various products such as pectinases, amyloglucosidase, alpha-amylase and gibberellic acid from the fermented solids (20-22-24).

The super critical fluid extraction of DMB, with carbon dioxide and athanol as entrainer, was also worked out to extract undesirable product such as sterol which is coproduced by Gibberella fujikuroi P-3 along with gibberellic acid (25). This technique is technically feasible but its application is limited to high-value products due to the high cost of the equipment as well as recurring expenses.

COMPARATIVE ECONOMICS OF SUBMERGED AND SOLID STATE FERMENTATIONS :

Due to the lack of information on comparative economics of submerged and SSF processes (14), studies were undertaken to compare scaled-up technologies for the production of amyloglucosidase at the production capacity of 9, 30 and 150 KL enzyme/annum (1). The production of the enzyme is 10 times higher by SSF process as compared to submerged fermentation when the enzyme titre per L both obtained by submerged fermentation was compared to that in 1 L extract obtainable from 1 kg DMB by SSF technique. Two hypothetical cases where in the product titre is assumed as equal and three times more by SSF technique as compared to submerged fermentation were also analyzed (1).

The results showed superiority of SSF in an economical respect. The greatest advantage of SSF technique was a lower investment of 17, 14 and 10% on plant, machinery and equipment, as compared to submerged fermentation, for plants of 9, 30 and 150 KL capacity/annum (1).

TECHNOLOGY, DEVELOPMENT AND TRANSFER :

A number of technologies based on SSF technique were developed over a period of two decades and these are presented in Table 1. Some of these technologies were scaled-up to industrial level. Notable among these are the technologies for pectinases, amyglucosidase, rennet and catalase while scale-up studies are in progress for gibberellic acid. The selection of these technologies for scale-up was based on the demand of the product in the country. The scale-up processes have been successfully transferred to industry.

One of the notable technologies under development is the process for production of gibberellic acid by SSF technique. The yield of 1.0 g gibberellic acid/kg DMB as compared to 1.0 g/l obtained under submerged fermentation with the use of precursors, indicates that the SSF technique for production of gibberellic acid can be competitive in comparison with the submerged fermentation process (40).

Another industrially important development is the extension of SSF technique for production of bacterial thermostable alpha-amylase (11,33). The enzyme titre produced by SSF process is much higher than in submerged fermentation (33). The use of SSF process for production of fats and lipids by *Rhodotorula gracilis* or *Acremonium terricola* are also being explored (12,13).

COLLABORATION WITH OTHER INDUSTRIAL R&D ORGANISATIONS :

A laboratory scale SSF process developed by Indian Jute Industries Research Association (IJIRA), Calcutta, for production of enzyme complex by *Aspergillus terreus* IJIRA -6, for use in jute industry was recently scaled-up by CFTRI; The major enzymes present in the complex are cellulases, xylanases and proteases and these are used by the jute industry for up-gradation of jute fibres as well as in the biomodification of tamarind kernel powder used in sizing of jute fibres. A plant to produce 100 kg DMB/day was also designed and is under erection.

Preliminary scale-up trials were also jointly conducted with Central Leather Research Institute (CLRI), Madras, on the laboratory scale process developed by them for the production of alkaline proteases by *Aspergillus flavus* under SSF techniques. The enzyme is of industrial use in dehairing of hides and skins as well as bating of leather.

MATHEMATICAL MODELING OF SSF PROCESSES :

Negligible information on mathematical models is available in the literature (14) except for the recent kinetic models for relationship between biomass, substrate and heat generation (41,42). The work on development of mathematical models to show the relationship between enrichment of WB medium with starch and production of gibberellic acid is in progress. It quantifies the enhancement of product formation up to certain degree of enrichment as well as inhibition of product formation at higher level of enrichment by starch.

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WHEAT BRAN CULTURE PROCESS FOR FUNGAL AMYLASE AND PENICILLIN PRODUCTION

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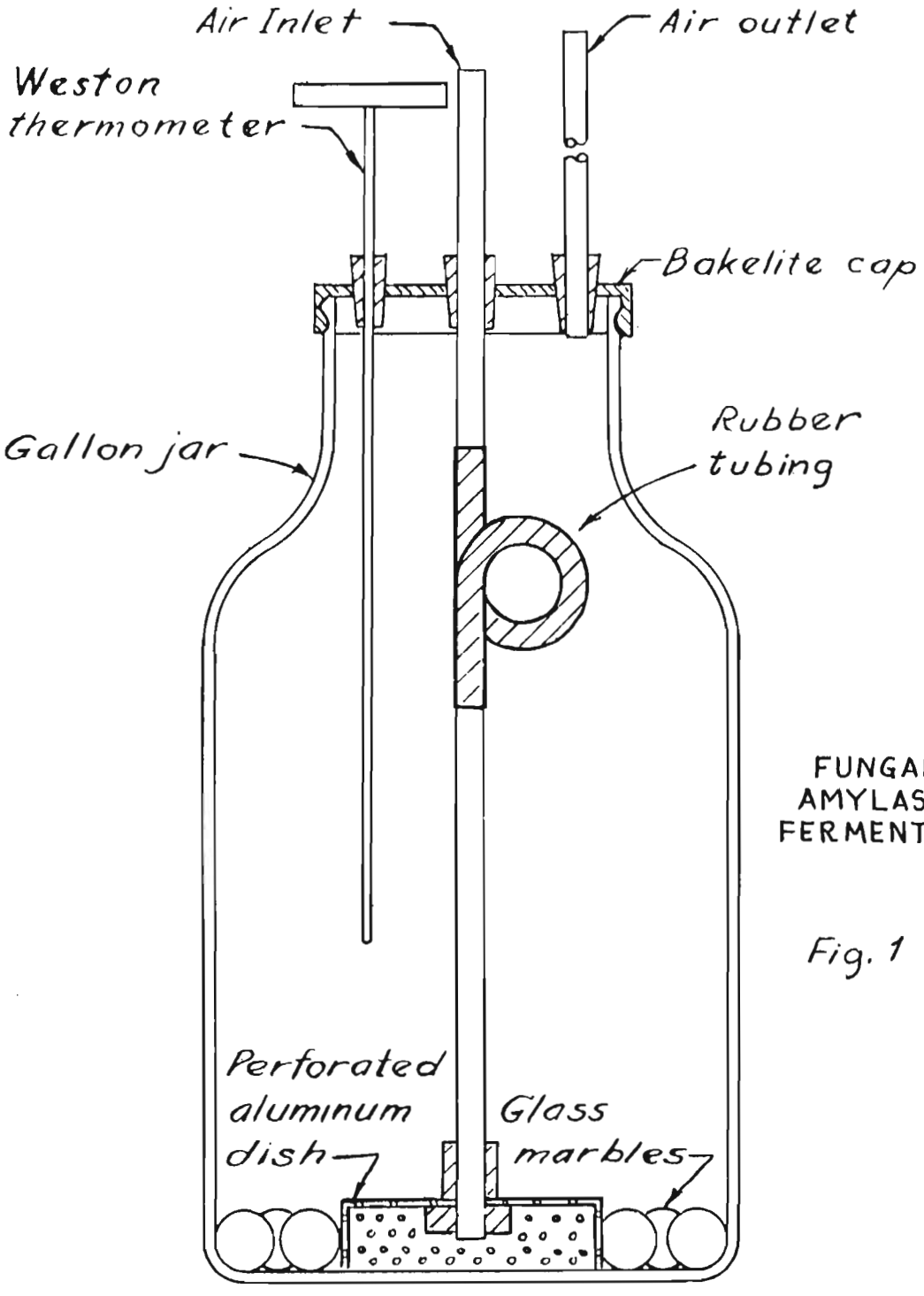
1 FUNGAL AMYLASE SEMI-SOLID WHEAT BRAN CULTURE

During WWII, the Schenley Research Institute of the Schenley Distillers Corporation, at Lawrenceburg, Indiana, was involved in the development of semi-solid fermentation processes for the production of fungal amylase and penicillin. Due to secrecy restrictions during this period, the results of these investigations were never published, and the following covers stidies which were carried out with *Aspergillus oryzae* for the production of fungal amylase by semi-solidf wheat bran culture.

In the spring of 1942, there was as increased wartime demand for industrial alcohol in the U.S. and the Schenley Distillers Corporation was one of the principal companies to be involved in this effort. The increased production of ethanol, resulted in a critical shortage of the diastatic malt normally used for the saccharification of the starchy grain fermentation media. As a consequence, there was an urgent need to develop a suitable microbial alternative. The obvious choice for this purpose was *Aspergillus oryzae* fungal amylase, since this enzyme complex had historically been used for the production of sake in the Far East, and had been the subject of extensive studies in the U.S. as early as 1894 by Takamine (1) and in the 1930's by Underkofler and associates (2,3,4,).

Takamine was one of the earliest investigators in the U.S. to explore the use of *A. oryzae* fungal amylase for use in the beverage ethanol fermentation. While the process in the Orient consisted of growing the fungus on rice, spread out thinly on concrete or wooden floors, or on trays, Takamine developed procedures for the growth of the fungus on wheat bran, initially on trays, and later in rotating drum fermentors. Although Takamine was successful in producing good amylase preparations by this process, with confirmatory alcohol plant trials, the process was not accepted by the beverage alcohol industry.

After Takamine's pioneering investigations, there were very few fungal amylase studies until Underkofler and associates at Iowa State College re-activated the project, this time for the production of industrial alcohol. The studies at Iowa State College confirmed Takamine's workn and were important in promoting renewed interest in the fungal amylase process. In their earlier studies, Underkofler, Fulmer and Schoene (2) produced *A. oryzae* fungal amylase on wheat bran in laboratory rotating drum fermentors. In later studies, Hao, Fulmer and Underkofler (4) described the use of modifications of the aeration pot fermentor, developed earlier by Beresford and Christensen (cited in 4). Hao, et al reported that mold growth in these aluminium pot fermentors was more rapid and uniform and the amyolitic activities higher than those obtained in the rotating drum fermentor. While the aluminium pot fermentors restricted the number of units



FUNGAL AMYLASE FERMENTORS

Fig. 1

that could be conveniently handled in the laboratory. More importantly, it was not easy to maintain fermentation temperature control with these fermentors since the temperature during fungal growth, which normally rises to 40° - 50°C, could only be controlled over a range by increasing the aeration rate. In addition, it was not possible to observe the growth of the fungus in the fermentors during the fermentation period.

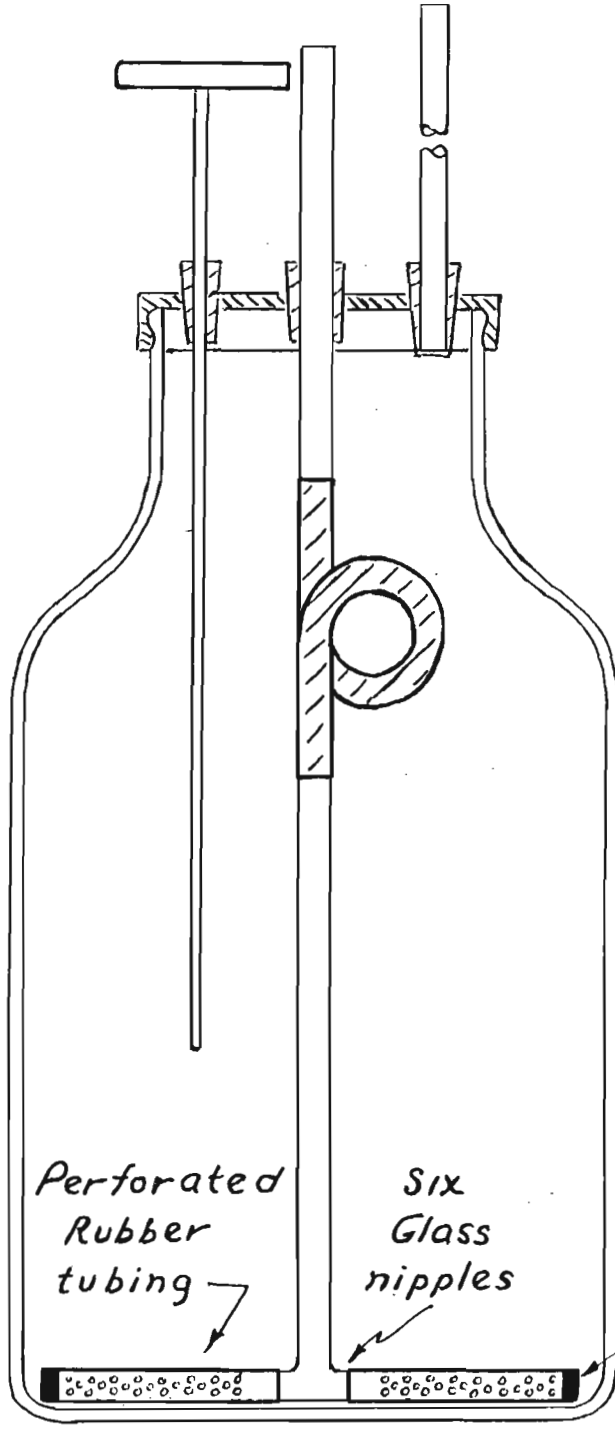
The author, who had been a graduate student of Prof. Underkofler, came to the Schenley Research Institute in 1942 to initiate an *A. oryzae* fungal amylase project, and in the subsequent laboratory studies devised one-gallon glass fermentors, with overhead aeration lines, permitting the use of waterbaths for accurate temperature control. In addition, the use of glass jars facilitated the observation of fungal growth during the entire fermentation period. Two types of aeration designs were devised (Fig. 1 and 2) and the fungal amylase preparations from both types of fermentors gave experimentally equivalent results in saccharified corn mash ethanol fermentation tests.

METHODS :

A strain of *A. oryzae*, isolated from pineapple juice and designated N°.10, was used throughout this study. The mold was maintained on malt extract agar slants 10% (w/v) malt extract, 1% (w/v) glycerol and 2% (w/v) agar, transferred monthly and just before use. Wheat bran koji inoculum was prepared (per 250 Erlenmeyer : wheat bran 10 g., 0.3N HC1 10 ml : no pH adjustment : sterilized for 30 min. at 121°C) by inoculating from a sporulated agar slant culture and incubating 5 days at 30°C.

The *A. oryzae* wheat bran enzyme samples were prepared in these one-gallon glass fermentors. Wheat bran medium was prepared by mixing 400 g. of wheat bran with 400 ml. 0.3N HC1 solution, transferring the mixture to an individual fermentor, and sterilizing for one hour at 121°C. The fermentors were then allowed to cool to 35°C and contents of one 250 ml. Wheat bran koji inoculum Erlenmeyer added to each fermentor. After mixing the inoculum into the bran medium by means of a sterile spatula, the fermentors were fitted with Weston thermometers (externally sterilized with 70% ethanol) and the fermentors placed in the waterbath at 30°C. The fermentors were then aerated using sterile filtered, humidified air. The fermentation temperature was maintained in the range of 30°-33°C by means of the waterbath and aeration. The observed mold growth in these fermentors was very rapid and uniform. The fermentations were continued for 48 hours and the mold brans then removed and used as such, or dried in a forced-draught cabinet at 25°-35°C for 36-40 hours.

Corn ethanol fermentation tests were used to evaluate the *A. oryzae* enzyme preparations. Corn mash media were prepared by adding 40 g. ground corn meal (Mikre pulverizer, 3/32 mesh screen) to 200 ml. 0.04N HC1 solution in 500 ml. Erlenmeyers and the mixtures heated in a boiling waterbath until gelatinization occurred. The corn media were then sterilized for 15 min. at 121°C. After cooling to 30°C, the media were adjusted asceptically to pH 5 with 20% NaOH. The *A. oryzae* enzyme preparations (equivalent to 10 g., dry basis) were the added to the individual Erlenmeyer and the flasks held at 300C, with occasional shaking, for one hour. The flasks were then inoculated with *Saccharomyces cerevisia* (distillery yeast inoculum ; 10 ml., 200×10^6 cells/ml. (av.)), and incubated at 30° for 72 hours. At the completion of the fermentation, the individual fermented mashes were neutralized by the addition of $CaCO_3$ and transferred to one-liter round bottom flasks. The mixtures were then distilled, collecting 200



FUNGAL
AMYLASE
FERMENTORS

Fig. 2

ml. distilate in volumetric flasks. The distillates were refrigerated overnight, filtered, and the ethanol concentrations determined by specific gravity determination at 20°C.

In a typical alcohol fermentation using yellow corn, grade 2 (67.5% starch ; 7.55% moisture), 17.06 ml. of ethanol were obtained, equivalent to a yield of 94.96% of theoritical.

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2 PRODUCTION OF PENICILLIN BY SEMI-SOLID WHEAT BRAN CULTURE IN ROTATING FERMENTORS

The discovery of penicillin and its commercial development has been one of the major achievements of recent industrial biotechnology. Despite the initial discovery by Alexander Fleming (1) in 1929 of the presence of an antibacterial substance in the filtrates of Penicillin notatum, and the subsequent cultural and chemical investigations by Harold Raistrick and associates (2), it was not until Howard Florey's group at Oxford University undertook chemical trials in 1939-1940, that the antibacterial importance of penicillin was realized and production initiated. It was soon obvious, however, that due to the wartime bombing of England and the shortage of manpower, it would be necessary to carry out the required production in the United States. Plans were then formulated in 1941 to transfer the penecillin project to the U.S.

During this period, the author was a graduate student at Iowa State College, working under Prof. Leland Underkofler, and in the summer of 1941, came to the U.S. Department of Agriculture, Northern Regional Research Laboratory (N.R.R.L.) at Peoria, Illinois, as a volunteer research assistant in the Fermentation Department. The N.R.R.L. was one of four U.S.D.A. research centers which had been established in 1941 to work on regional agricultural projects. The Fermentation group, originally located in Arlington, Virginia, had been transferred to the Peoria research center in the spring of 1941. The head of the Fermentation Department, at that time, was Robert D. Coghill.

The author arrived at the N.R.R.L. on Wednesday, July 23, 1941 and this turned out to be a fortuitous date, as Prof. Howard Florey and Dr. Norman Heatley arrived the next day, Thursday, July 24, 1941, bringing with them the Fleming *P. notatum* culture and samples of penicillin. Prof. Florey and Dr. Heatley had

been sent to the Peoria research center by the U.S.D.A. Washington, D.C. office. Prof. Florey was a medical pathologist and only stayed in Peoria for one day, leaving the next day to return to England. Dr. Heatley, who was a biochemist and had been working with the organic chemist Dr. Ernst Chain, in Florey's group, stayed on to assist Dr. Andy Moyer, who was one of the senior fermentation microbiologists in the department, initiate the penicillin project. The author had been assigned to work with Dr. Moyer, and during this period, assisted him and Dr. Heatley in carrying out the first penicillin fermentation experiments in the United States. Dr. Heatley was at the N.R.R.L. for about two months, during which time we had an opportunity to work closely with him, using the techniques developed at Oxford. Dr. Heatley was the inventor of the elegant cup-plate antibiotic assay, which was of critical importance in the overall success of the penicillin program.

By early 1943, the U.S. penicillin project had become an extensive program, involving a number of pharmaceutical companies an was being conducted under wartime secrecy restrictions. The author, who by this time was at the Schenley Research Institute in Lawrenceburg, Indiana, working on the *Aspergillus oryzae* fungal amylase semi-solid wheat bran koji process for the production of industrial alcohol, brought the penicillin project to the attention of the Schenley organization. The decision was quickly made to work on the penicillin project and permission was requested from Washington to participate in this program. This was granted, and the fungal amylase work was stopped in order to concentrate on the penicillin project.

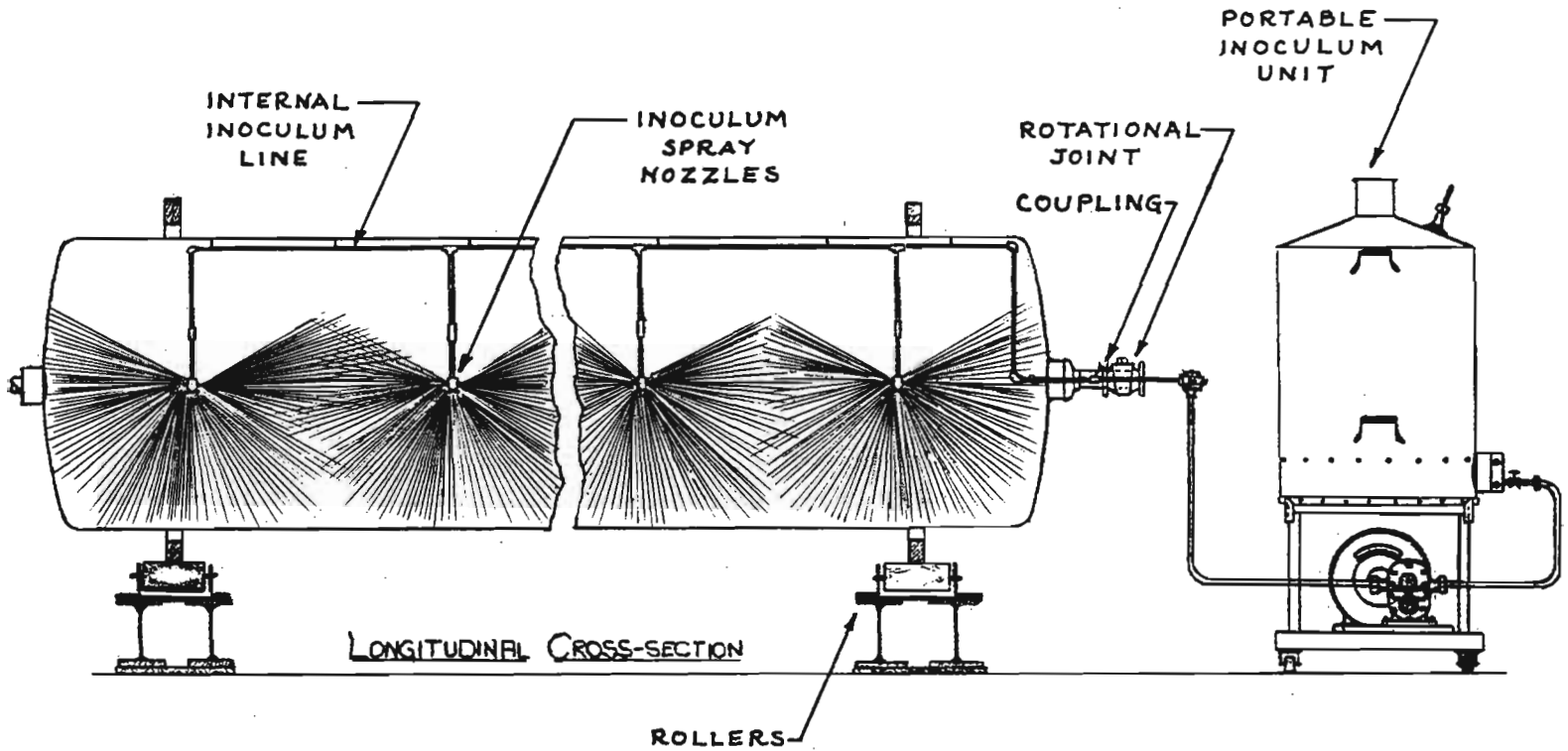
Penicillin production during this period primarily involved surface culture fermentation, although submerged culture studies were already being investigated at N.R.R.L. Surface culture penicillin potencies, initially in the range of 2-5 Oxford units per ml., had gradually been increased to approximately 50-100 units per ml., mainly through the pioneering studies carried out by Andrew Moyer.

Since we had already been using the semi-solid wheat bran process for A. oryzae fungal amylase at Schenley, and had developed considerable expertise, we decided to use this method in our exploratory penicillin experiments. We were able to achieve penicillin potencies of 200-300 units per g. bran (as is basis) in our initial stationery culture experiments and decided to continue using the semi-solid wheat bran procedure. It was soon apparent, however, that we would have to carry out the production process under sterile conditions, and this precluded the possibility os using asceptic surface culture methods. We then decided to investigate the use of wheat bran in rotating fermentors. We initially tried this with rotating 5-gallon glass carboys(3) and were fortunate in that the rotating fermentor concept was not only feasible, but we were able to achieve penicillin yields in excess of 500 Oxford units per g. (as is basis).

The pilot plant had been in operation for only a few months, when the parent Schenley Distillers Corporation decided to build a commercial plant. To expedite the construction involved, a bonded whiskey warehouse of the adjacent Schenley Old Quaker distillery was taken over and, in a relatively short period of time, the fermentation plant, containing 40 rotating fermentors (Fig. 1 ; 1.22 m. dia.; 11.28 m.l.) was built. Thus, in less than ten months, under the pressure of the utmost wartime need, and with the magnificant cooperation of the parent Schenley organization, the penicillin project was carried from the laboratory and pilot plant, through the plant, with full production by December, 1943.

Our achievements during this period were remarkable in view of the general

FIG. 1 PENICILLIN PLANT ROTATING FERMENTORS



lack of knowledge and experience in industrial biotechnology, and especially in indeustrial procedures involving semi-solid fermentations in rotating fermentors. Even more remarkable was the fact that, with concurrent process improvements, the plant was able to consistently achieve penicillin yields in the range of 1500-2000 Oxford units per g. bran (as is basis), equivalent to approximately 6008000 units per g., on a dry weight basis. This was during the period when penicillin yields in industry were only in the range of a few hundred units per ml. Unfortunately, it soon became apparent that the submerged culture process would eventually be the more flexible method to use, with the potential to achieve much larger capacities. Accordingly, after less that $1^{1/2}$ years of operation, a decision was made to convert the penicillin plant to the submerged culture process.

METHODS :

The initial penicillin wheat bran semi-solid fermentation studies were carried out in the stationery one-gallon glass jar fermentors. The major development studies, however, were conducted in 5-gallon pyrex glass carboy rotating fermentors. The fermentations in the plant were carried out in rotating steel fermentors.

The *Penicillium* culture used throughout most of this period was *P. notatum* Westling N.R.R.L. 1249.B21, although other strains were also used. The same basic bran medium, with some modifications, was used for inoculum development and penicillin production, and was prepared by mixing equal weights of wheat bran and the following nutrient solution ; per liter : $NaNO_3$, 3 g., KH_2PO_4, 0.5g., $MgSO_4.7H_2O$, 0.25 g., $ZnSO_4.7H_2O$, 0.034g., and corn steep liquor (as is) 50 ml. For 250 ml. Erlenmeyers, 10 ml. of solution were used for 130 g. wheat bran ; for Fernbach flasks, 130 ml. of solution were one liter of solution was used for one kilogram of wheat bran ; for the plant fermentors, a proportional amount of nutrients were used for 636 kg wheat bran, with the liquid volume adjusted to compensate for stream condensation during sterilization. The Erlenmeyers were sterilized for 45 minutes at 121° C, while the pilot and plant fermentors were sterilized for 2 hours at 121° C.

The *P. notatum* master culture was maintained as a soil culture and stored in the refrigerator (5°- 6°C) until required. Transfers were made weekly from the master culture to malt extract agar slants which were incubated at 28°C for 6 days and then refrigerated until used. Primary 250 ml. Erlenmeyer bran cultures were prepared by inoculation from the refrigerated sporulated slant cultures, using approximately one cm. square of mold sporulated growth per 250 ml. Erlenmeyer. After mixing well, the Erlenmeyers were incubated at 24°C for four days, at which time, the *Penicillium* culture was heavily sporulated. Fresh primary 250 ml. Erlenmeyer cultures were prepared for each inoculation series.

Primary Fernbach Erlenmeyer cultures were prepared by inoculating the Fernbach Erlenmeyers using the 4-day old 250 ml. Erlenmeyer cultures. One 250 ml. Erlenmeyer was used to inoculate three Fernbach flasks. The Fernbach flasks were well mixed and then incubated at 24°C for 4 days, at which time, the culture was heavily sporulated. Secondary Fernbach Erlenmeyer cultures were preparted in a similar manner, except that primary Fernbach culture was used to inoculate the secondary Fernbach Erlenmeyers. A total of 200 secondary Fernbach Erlenmeyer cultures were prepared daily for plant fermentations ; the

pilot plant inoculum schedule was as required.

The 5-gal. glass carboy fermentors, after sterilization, were individually inoculated in the sterile room with the contents of one Fernbach secondary inoculum flask. The glass carboys were then shaken to achieve uniform mixing and the sterile cotton plug replaced by a sterile aeration assembly. The inoculated glass fermentors were then placed on the rotating units and the fermentor aeration assemblies connected to a humidified sterile air source. The 5-gal. glass fermentors were rotated at a standard speed of 1 rpm during the subsequent 5-6 day incubation period, during which time the fermentation temperature was controlled by maintaining the room temperature at 24°C.

The plant fermentors had five manholes spaced evenly along its length, to facilitate the uniform charging of the bran medium. The wheat bran was mixed with the nutrient solution in a "day mixer" and transferred tot the individual fermentord by an overhead monorail. The manholes were then closed and the fermentors sterilized for 2 hours by the direct injection of steam (15 psi ; 121°C). During the srerilization period, the fermentors were rotated at 24 rpm. At the end of the sterilization period, the steam line was closed, and the fermentor pressure slowly released, during which time the fermentors were not rotated. When the fermentor pressures had decreased to 3-5 psi, aeration (30 cfm) was started.

The individual fermentors were equipped with an external water spray system for cooling and temperature control. This water system was not turned on during the cooling period until the medium temperature reached 70°C. When the temperature reached 70°C, the water system was turned on and the fermentors again rotated (24 rpm). When the medium temperature reched 24°C, the inoculation temperature, the external water system was stopped.

With the fermentors rotating at 24 rpm and the air volume reduced to 10 cfm, the fermentors were inoculated by means of an internal inoculum spray system, using concentrated P. notatum spore suspension, prepared from 30 Fernbach inoculum secondary flasks, per fermentor. After inoculation, the fermentor air flow was maintained at 10-15 cfm until the 30th hour, when it was slowly increased to 40 cfm. The rotational speed was maintained at 24 rpm for the first 6 hours, and then slowly reduced to 5 rpm until the 30th hour, when it was increased again to 24 rpm. The fermentor was maintained at an air flow of 40 cfm and a rotational speed of 24 rpm for remainder of the fermentation. The fermentation temperature was maintained at 24°C during this period by the periodic use of the external cooling water system.

At the end of the fermentaion period (112 hours), the fermentors were stopped with the manholes at the top of the rotational cycle. The manholes were then opened, and the fermentors emptied by means of a pneumatic vacuum system. The penicillin bran was transferred over to the recovery section for extraction and recovery.

All penicillin assays were carried out by the Heatley cylinder-plate method (4), as modified by Schidt and Moyer (5).

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SOLID STATE FERMENTATION OF SUGAR BEET

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The aimof this paper is to present the work carried out at the University of Compiegne in the Laboratory of Pr. Lebeault.

To present these laboratories, I must present several of the maintopics which were initiated :

- optimization of sauerkraut production.
- cellulases by *T. reesei* and cellulose hydrolysis.
- S.C.P. from cassava.
- proteases from *A. niger.*
- cheese flavours production.

Two of these projects deal with aroma production by fermentation: cheese flavour and sauerkraut.

The work I herewith present is related to cellulase projects, which were divided in three parts:

- production of cellulases by *T. reesei.*
- hydrolysis of sugar beet pulp by these cellulases,
- the third part is related to the subject of this congress:

  Solid state fermentation of sugar beet.

Solid state cultures were adapted to ethanol production from sugar beet. In this case, the advantage of this type of culture are due to the combination of sugar extraction and fermentation in one step.

Alcoholic solid state fermentation has already been tested on various substrates: sorghum, sugar cane, grape rape and sugar beet. The main work published in this area was the Ex-Ferm process developed by Rolz in 1980.

Our work was based on the adaptation of the results published by Rolz to the sugar beet substrate.

On one hand, I am going to explain the favourable action of solid state fermentation on the sugar extraction and to determine the best conditions of fermentation/diffusion in flasks.

On the other hand, I will describe a batch fermentation in a tubular reactor.

INFLUENCE OF FERMENTATIVE ACTIVITY ON SUGAR DIFFUSION :

Cossettes depletion is evaluated in two sets of assays carried out in flasks for different values of L/S (Ratio liquid medium (in ml) / wet weight of cossettes in gr).

First set is conducted without inoculum on a shaker at 60°C; second set

with $2.6.10^8$ cells/flask is incubated at 30°C.

On the figure, it seems that yeast growth favours sugar extraction.

FIGURE 1

Influence of initial pH of the medium.

Depletion of cossettes and sugar concentration in the liquid phase are measured for different initial pH values. Results are reported on this figure.

FIGURE 2

Optimal value of initial pH is 4.5.

Influence of inoculum size

This is also an important parameter. We expressed biomass load as number of cells/g of cossettes wet weight.

Sugar content which remains in the cossettes, and sugar consumption in the liquid are measured for various biomass load values.

FIGURE 3

The figure shows a drop of residual concentration in the liquid, when the inoculum size increase.

On the other hand, the influence of inoculum size of cossettes depletion is well established until 10^8 cells/g. When the biomass load exceeds this value, cossettes depletion remains constant.

Ethanol formation is evaluated with a set of experiments carried out in one liter flasks.

Sugar and ethanol concentration are measured over 24 hours.

The maximal value of ethanol concentration not exceed 2.8% beeing reduced afterwards due to evaporation.

In 10 hours, the cossettes depletion is almost achieved. The sugar diffusion slowly continued from 10 to 44 hours.

At 10 hours, the sugar concentration in the liquid phase is maximum. From 10 to 44 hours, we observe the drop of sugar concentration resulting of yeast consumption.

From these experiments, we evaluated the yield values:

Y_D (24h) = 92%

Y_C (24h) = 99.3%

$Y_{P/S}$ (24h) = 0.28

were S_O = 12.9

$S_1 = (1.72 \times 60) / 100$

$S_L = (0.88 \times 84) / 100$

$P = [(2.52 \times 84) / 100] + [(2.52 \times 48) / 100]$

These low values may be explained by :

- conditions of aeration.
- L/S to high
- uncontrolled evaporation.

In order to favour fermentative metabolism, we have considered the use of a tubular reactor with L/S O.2.

SHEME OF REACTOR

The results obtained are showed here: after 28 hours of fermentation-diffusion.

From these measurement, it is possible to determine the process assessment

COSSETTES	LIQUID MEDIUM	
Residual sugar concentration (%)	Sugar concentration (g/l)	Ethanol concentration (%w/v)
0.99	3.87	8.3

and to calculate the yield values:

Y_D (24h) = 95.4%

Y_S (24h) = 99.6%

$Y_{P/S}$ (24h) = 0.407

were S_O = 94.6

S_1 = (0.95 x 440) / 100

S_L = (3.87 x 89) / 100

P = [(8.3 x 89) / 100] + [(8.3 x 352) / 100]

- diffusion yield is better than in flasks
- consumption yield is almost the same
- fermentation metabolism increased with 8.3% ethanol concentration.

CONCLUSION

This study realized on a laboratory scale was undertaken to demonstrate the feasability of a SSF on sugar beet cossettes.

Concerning sugar diffusion out of the cossettes, these results could be im-

proved. We have shown the positive influence of fermentation yeast activity on sugar transfer to the liquid phase.

Sugar consumption during batch fermentation in the tubular reactor is estimated by the residual sugar concentration in the liquid phase. The value obtained has to be reduced by a gentle agitation of the column.

During this experiment, $Y_{P/S}$ reaches 0.407, which is comparable to the 0.3 and 0.4 achieved by Roltz. The traditional value of 0.48 cannot be reached. It may be explained by the impossibility of measuring and controlling yeast growth. In fact, sugar beet juice gives all the necessary nutrients for yeast growth.

But the disadvantages presented by this system must be pointed out. Continuous work with SSF needs the development of an endless screw reactor, the yeast biomass must be recycled by centrifugation, filtration or sedimentation with flocculent cells, and pressing cossettes to extract the ethanol solution is an obligate step.

However, this system presents several technological advantages: supression of the diffusion step, reduction of reactor capacity and ease of use.

PARAMETER CALCULATION :

Diffusion yield

$$Y_D = \frac{100(S_o - S_1)}{S_o}$$

Consumption yield

$$Y_S = \frac{100[S_o - (S_1 + S_L)]}{S_d}$$

Production yield : $Y_{P/S} = P/(S_o - (S_1 + S_L))$

Where : P = ethanol in liquid and solid phase (g)

S_o = initial sugar in cossettes (g)

S_1 = residual sugar in cossettes (g)

S_L = residual sugar in liquid (g)

S_d = diffused sugars (g)

$S_d = S_o - S_1$

BAKERS YEAST GROWTH AND RECOVERY OF EXTRACELLULAR PROTEINS USING SEMISOLID-STATE FERMENTATION IN AN AIR FLUIDIZED BED FERMENTOR.

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ABSTRACT :

An air-fluidized bed of semisolid mash was fermented by baker's yeast producing microbial cells, ethanol and extracellular proteins. The mash in this fermentation process is comprised of only about 80% water, hence has only about one twentieth of the reaction volume of a typical submerged culture system. On the other hand, it is about twice the volume compared to a traditional solid substrate process in which fungi grow on distinct solid particlates (at the 40% water level). This 80% moisture level, however, allows for the growth of yeast and/or bacteria, relatively easy mixing, heat removal, and moisture control. An additional advantage of this semi-solid process is that it has the potential of selective and immediate recovery (in the effluent air phase) of extracellular proteins produced by the fermentation process.

INTRODUCTION :

In three recent papers (1,2,3), a method for growing baker's yeast in a low water environment (80% moisture potato solids) has been described. The potato substrate was supplemented with defined mineral and vitamin media to obviate nutrient limitations, other than oxygen. Unlike other recent studies on low water substrates supporting microbial growth, fluidized by air or other gases, control and reproductivity was easily achieved. The key to this simple control scheme is that the water in the fermentation media was regulated only by the water vapor in the incoming air stream, not the traditional, but awkward direct liquid water feed to the substrate. Figure 1 depicts the apparatus. Typical cell growth and extracellular protein time profiles in this batch system are shown in figures 2 and 3. Since the semisolid substrate (a thick or high solid mash) could be easily fluidized, like a viscous liquid, as shown in figure 4, no mechanical stirring was required as in other contemporary studies.

RESULTS AND DISCUSSION :

In the semisolid air fluidized bed, the growth rate of cells following the lag phase is about double than in a typical baker's yeast system. As shown in Figure 1, cells achieve a level of about 45 g/l (when corrected for the 80% water level) between 4 and 7 hours, versus the typical 10-15 g/l concentration in submerged cultures under similar conditions. This presumably means that the k_La for oxygen transfer may be doubled (but this needs direct verification using a sensitive oxygen measuring device in the effluent gas). Complicating this possible oxygen enhancement, however, is the cell level (that normally achieved after 5 batch sequential fermentations in a commercial baker's yeast process). This

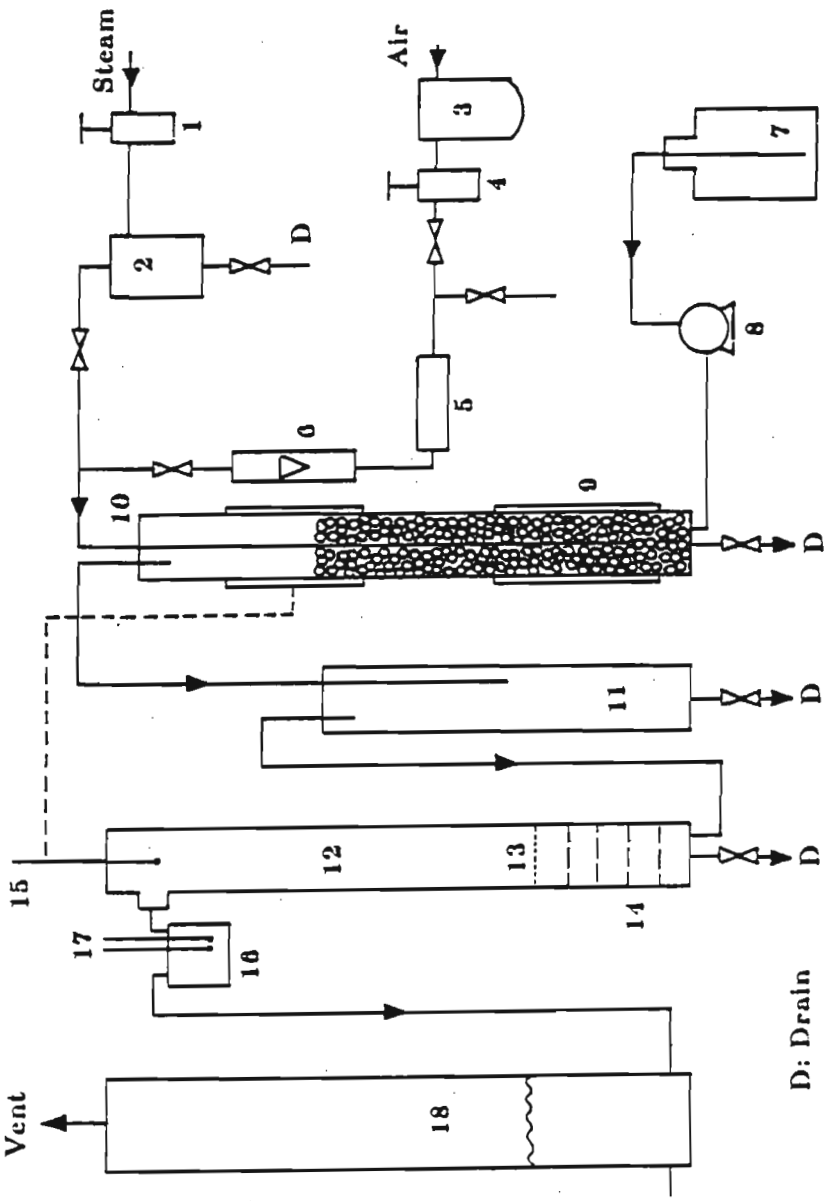

Figure 1. Schematic diagram of the air-fluidized bed fermentor apparatus.

1. Steam regulmator. 2. Steam trap. 3. Air filter. 4. Air regulator. 5. Air sterilization filter. 6. Air flowmeter. 7. Water reservoir. 8. Water pump. 9. Heating tape. 10. Air saturation column. 11. Air cooling column. 12. Fermentation column. 13. Fine grid supporting plates. 14. Large hole flow distributor. 15. Thermoregulator. 16. Humidity measuring chamber. 17. Wet and dry bulb thermometers. 18. Overhead collector. Figure taken from reference 1.

high cell level is apparently due to the fact that a growth limiting factor has been reduced, since all of essential nutrients are present in both the liquid and semi-solid fermentations. One candidate for this limiting factor is a-pheromone which inhibits yeast budding in the sexual cycle. In the semisolid case it may be either absorbed on the potato dextrins or even carried out of the system by the high flow rate effluent air stream. Oxygen indeed becomes limiting (either directly or by this carry-over mechanism) at a level of about 40 l/min air flow rate, as shown in figure 1. Note that proteins in the effluent gas stream seem to approach an equilibrium with the extracellular proteins in the bed as the air flow rate is reduced. (see Figure 3).

GAZIFICATION RATE :

It is interesting to compare the numerical value of the VVM (variable volume of air sparged to the fermentation broth per volume of broth per minute) in the air-fluidized bed to that in the comparable submerged culture.

Considering the wet broth as the basis for comparison, the conventional submerged culture process is typically around 1 VVM (we usally use 1.4 VVM in our work). On the other hand, for the air-fluidized bed with such a small potato volume (say, 0,15 liter), the specific gasification rate is in the range of 200 to 467 VVM, using the air flow rates given in figure 1, some 2 orders of magnitude greater than in the submerged culture. With such a large difference in VVM, but not in actual cell growth, it seems that the comparison should be redefined to be on a per total solids basis. Therefore, if we assume that the solids in a typical liquid batch fermentor are 1%, with ca. 10 g/l of wet yeast cells and liquified nutrients, on a wet solid basis, this becomes 100 VVM for the former 1 VVM case. Here, when the VVM is underlined it indicates a total (average) solids basis and the solids density is assumed to be 1 g. For the air-fluidized bed case, with an average solids level over the course of the fermentation of .08 liter wet potato and wet yeast cells, the redefined average specific gasification rate becomes 375 to 875 VVM, for a 4 to 9 fold difference. Finally, defining the gasification rate on a per cell basis only, gives 100 VVM for submerged culture, and 700-1500 VVM (for a 45 g/l cell level) for the air-fluidized bed. Italic and underlining the VVM's denotes this per cell basis. The comparison between the two systems shows a 7 to 15 fold difference, placing the magnitude measure of aeration between the per total volume case and the per wet solids case. Since the cost associated with both processes is centered about the cell production either directly or proportionally through protein production, it seems reasonable to compare the aeration rates according to the cell levels. Using 10 fold as the basis of comparison, the energy of mixing and cooling viscous materials by stirring and heat exchange must be 1/10 that in the fluidized bed vs. the submerged culture in order to compensate for ten-fold additional costs of air compression and sterilization (without recycle). This energy cost comparison assumes equal protein productivity. However, recent japanese studies indicate that air-fluidized bed can produce 5 times the desired extracellular enzyme levels compared with liquid systems, so that the costs may in certain cases become comparable. The ability to pursue further work refining the air-fluidized bed parameters experimentally and by use of models (3), offers additional encouragement for closing the possible gap in costs between the two procedures.

PRODUCT RECOVERY :

There is no need to refine the substrate for the air fluidized bed, both costs of such semisolid and solid substrate systems can be reduced. An additional advantage to fluidized beds is that when cells themselves are the product, the product recovery costs become negligible for solid and semisolid systems, further

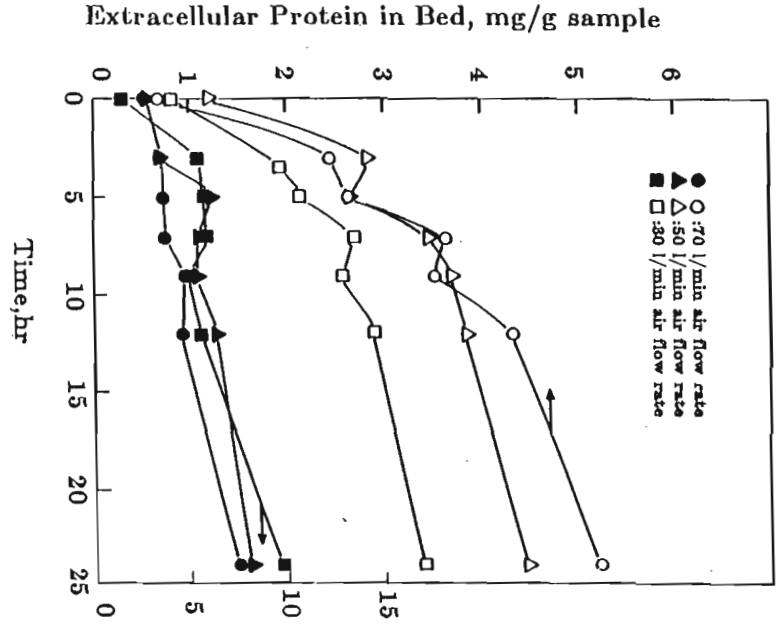

Figure 3. Typical flow behavior of the semisolid substrate in the air-fluidized bed fermentor. Figure taken from reference 3.

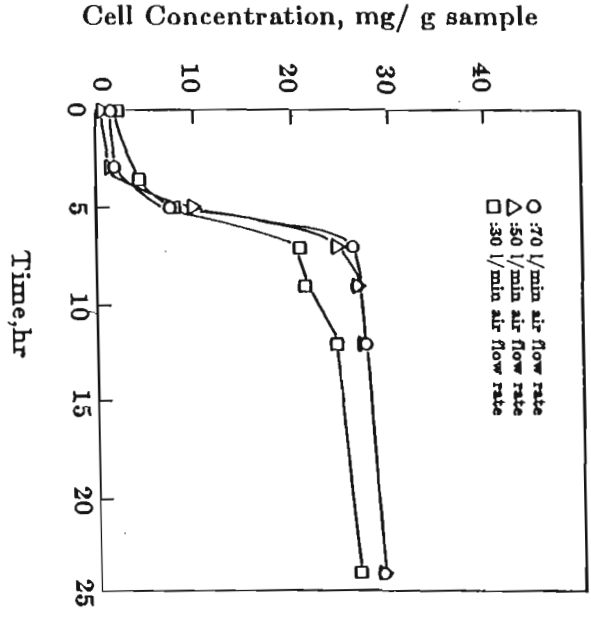

Figure 2. The effect of air flow rate on the cell grown. Figure taken from reference 2.

strengthening the possibility for developing an economical fluidized bed process.

An other advantage to microbial fluidized beds is that the high gas throughputs may offer a new route for selective extracellular protein recovery (2). Removing protease sensitive extracellular proteins in-situ and on-line may offer the only means at present in wich to recover such proteins before they are destroyed in the broth. With no comparable process for those enzymes/proteins made on semisolid substrates, the economics of gasification becomes secondary to feasability.

SCALE UP :

Two problems seem to be important in scalling-up air or inert gas solid/semisolid fluidized beds: humidity control and heat removal. In fact, the two problem areas are coupled. As the cell level increases significant amounts of water generated by respiration and metabolism must be removed. In a nearly 100% humid isothermal gas stream, however, there is no capacity for removal. Raising the temperature of the effluent gas would increase that capacity and, fortuitously, this coincides with the need for removal from the rapidly respiring cells. It seems reasonable, therefore, that temperature gradient along the axis of the fluidized bed, coordinated with these two process needs, could be designed for optimal system performance.

SUMMARY :

Air and other gas fluidized beds offer much promise as alternative systems to conventional submerged microbial culture process. To date, control and reproductibility have been established and cost effectiveness now needs to be demonstrated. Novel applications for a semisolid/solid substrate fluidized bed may be provided by difficult-to-produce fermentation products such as protease-labile estracellular enzymes. With such a challenge between this new system and a needed product, a potentially new tool may be introduced to the collection of bioreactors.

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BRIEF DESCRIPTION OF SSF PROCESSES AT ICAITI

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GREEN BANANA MEAL FERMENTATION :

The production of banana represents an important source of hard currency for centralamerican countries. This is particulary true for Honduras and Costa Rica. For Guatemala it means around US $ 30 million a year roughly less than 1% of total export.

However, for Honduras and Costa-Rica where annual production figures are around one million tons per year, there is an important amount of banana rejected because they do not comply with standards of quality based on color shape and size. Value for internal commercialization when possible decreases from US $ 400 for export to US $ 20 internal price. Also a great amount is lost due to insuficient demand.

A solution to this problem has been searched by countries like Colombia and Ecuador where alarge number of experimental trials have been performed using green or unripe bananas for animal feeding. Ecuador started a large production of green bananas meal for this purpose.

Chemical composition of this meal is as follows : 65% starch, 10% reducing sugars, 7% fibers, 6% crude proteins and 5% minerals. Solid state fermentation was visualized as a mean of increasing the low protein content and improve nutritional value.

The IRCHA-ORSTOM process, as developed to use cassava, was adapted in this case under a special contract with the government of Honduras. Results are similar to those obtained by Baldensperger et al. (1985) at the laboratory of ORSTOM in Martinique. ICAITI was able to use a reactor furnished by IRCHA of 15 kg dry meal capacity to produce close to 200 kg of final product of an average of 16 % protein content with 21% solids lost to respiration and fermentation time of 44h. The microorganism used was *Aspergillus niger* A-10.

Poulty feeding evaluation was carried out at the Panamerican School of Agriculture in Honduras with good results. Economic evaluation had to be done by using quotes for equipment from japanese Koji manufacturing factories since there was no available industrial equivalents in France at that time.

BALDENSPERGER J., LE MER J., HANNIBAL L., QUINTO P.J., (1985). Solid state fermentation of banana wastes. *Biotechnol. Lett.*, 7, 743-748.

PRODUCTION OF FOOD AND FEED FROM WHEAT STRAW BY *PLEUROTUS SOJOR-CAJU* :

The use of cereal straw as feed material has been known for centuries. Among many suggestions to improve its digestibility, theuse of white rot fungi has attracted attention, due to the possible delignification. Among fungi able to grow at tropical temperatures and with good growth ability, the *Pleurotus* genus pre-

sents good rates of growth and yields. In this case, the left and spent residue after harvest constitutes the feed material.

The raw wheat straw was packed in bales (.38 m x .35 m x .20 m). The bales were pasteurized by submerging in hot water (75°C or more for 15 min). Groups of six bales were packed in bags of black polyethylene plastic, forming a mattress of 0.20-0.25 m deep and allowed to cool to 35°C before inoculation. The inoculum was prepared on sorghum (Chang, 1982) and added on top of the bales : 5.6% inoculum by dry straw weight. The bags were loosely closed and kept for 30 days, then opened and the cultured bales were stacked in shelves in which air an light were available. The first fruiting bodies appeared in five days and the harvesting was extended for 30 days.

The spend straw was sun dried in open yards and chopped at 0.15 m pieces. This material was used to feed seven growing lambs, against a control of seven lambs fed raw chopped straw. Only straw, minerals and water were fed to the individually penned animals. Manure was collected and analyzed. The weight was recorded daily.

Eleven batches of straw were processed during a three month period. The average yield was 0.28 kg of fresh mushroom/kg raw straw (0.11 kg dry mushroom/kg of dry straw lost or 3.46 kg of dry mushrooms per 100 kg of original draw straw). Results are very closed to those reported by Zadrazil (1980) : 3.5 kg dry mushrooms per 100 kg dry straw, 31% losses in weight and 0.11 kg dry mushrooms/kg dry straw lost. Intakes in the feeding trials were 0.27 and 0.41 kg/animal/day for the raw and spent straw, respectively. Dry matter digestibilities: 52 and 55% and the organic matter digestibilities: 55 and 58%, for the same material. The spent straw also showed improved characteristics as a vehicle for liquid nutritional elements. One kilogram of spent straw was able to retain 2.71 kg of water, while raw straw was able to retain 1.98 kg of water.

ZADRAZIL F., 1980. Influence of ammonium nitrate and organic supplements on the yield of *Pleurotus sajo-caju* (fr) sing. *European Journal of Applied Microbiology & Biotechnology, 9,* 31-35.

CALZADA J.F., E. de PORRES, R. de LEON, ROLZ C., FRANCO L.F., 1987. Production of food and feed from wheat straw by *Pleurotus sajor-caju. Mush. J. Tropics. 7,* 45-46.

PRODUCTION OF *PLEUROTUS CAJOR-CAJU* ON COFFEE PULP :

There has been for many years, a strong interest in valorization of coffee pulp. A great number of research works is available, concerning ensiling, composting, biogas production, caffeine extraction, use as a substrate for protein production by microbial fermentation of larvae growth.

From early experiments in 1980 as published by de Leon et al. (1983) and also shared by the mexican group in Veracruz (Martinez, Carrera *et al.*, 198), the use of coffee pulp as substrate for *Pleurotus* sp production has been proposed, either alone or mixed with other materials.

In this particular experiment, we have tried to solve the problem of coffee pulp availability for year long production. A first idea was to use fresh coffee pulp for *Pleurotus* production, while the remaining pulp is ensiled for preservation; when

the coffee processing season is over, then the ensiled coffee pulp could be used. To show that, experiment was carried out using ensiled coffee pulp for more than a year, and comparing the results with fresh coffee pulp.

A first experiment was carried out by using ensiled and pressed coffee pulp. To avoid the problems caused by water extraction, it was subjected to a short time of heating (about 20 min at 85°C) then cooled to 30°C and packed into plastic bags containing 6-8 kg wet weight each one, and then inoculated with mycelium grown on sorghum grain at the rate of 5% by weight. For several weeks, the mycelium growth was visually monitored and it was very weak. Until two months later the plastic bags were shredded and the fruiting bodies appeared. Yields were low, the structure of the pulp was very loose. The bad results were blamed on poor ensiling, particularly the presence of organic acids other than lactic.

A second set of experiments included ensiled, pressed coffee pulp treated with steam. Results indicated that it took nine months for the mycelium to cover the substrate, yields were estimated as 2.55 kg dry mushrooms per 100 kg of dry initial pulp weight, as 7.66 kg dry mushrooms per 100 kg lost dry pulp and as 6.38 kg dry mushrooms per 100 kg fresh weight.

On the ensiled, pressed pulp, but treated with hot water four times, it took five weeks for the mycelium to cover the pulp. Yields were of 1.92 kg per 100 kg dry initial pulp, 2.89 kg dry mushrooms per 100 kg lost dry pulp, and 4.42 kg dry mushrooms per 100 kg fresh initial weight of pulp.

For comparison, fresh coffee pulp was used in the experiments and treated with hot water. Growth was faster than the other types of pulp and it took only two and a half weeks for the mycelium to cover the substrate. Yields were of 4.3 kg dry mushrooms per 100 kg dry lost pulp, and 8.6 kg dry mushrooms per 100 kg fresh initial weight of pulp.

Ensiling coffee pulp seems to be a cheaper way to store the material, but it takes longer for the mushroom to grow, since it needs at first to consume and neutralize acids. Yields were lower than those obtained with fresh coffee pulp.

FILAMENTOUS FUNGI GROWTH ON COFFEE PULP :

Objective :

The reduction of toxic compounds (Caffeine and Polyphenols) and increase of protein content of coffee pulp by fungal solid state fermentation with the following strains: *Aspergillus niger* A-10, *Aspergillus oryzae* and *Sporotrichum pulverulentum*.

Methodology:

The inoculum was prepared by grinding 150 g of sun dried fresh coffee pulp, and humidifying them with 750 ml water, and adding 11,32 gr of ammonium sulfate as nitrogen source. The ingredients were homogenized and the final material distributed in six pyrex glass pans, covered with aluminium sheets and sterilized by 15 minutes at 120 °C. Afterwards, the pans were inoculated with the fungi strains (*Aspergillus niger* A-10, *Aspergillus oryzae* and *Sporotrichum pulverulentum*) and incubated for 3 to 4 days at 35°C. All the coffee pulp samples in inoculum preparation and solid state fermentations were first mechanically pressed in a continuous screw press. Solid state fermentations on fresh or ensiled coffee pulp were carried out taking 15 kg of fresh material and pasteurizing

with live steam and continuous mixing by 15 min. The material was cooled and the moisture content was determined, portions of 5 kg were taken and 75.47 g of ammonium sulfate per kg of dry material were added. The initial dry weight and pH were recorded.

The prepared material was innoculated with the fungus strain, transfered to metallic pans (0.26 M wide, 0.4 long and 0.12 height) and incubated in a contolled temperature chamber at 35°C.

The material was mixed every 12 hours and the addition of an air-flow during the fermentation process was not required. The growth of fungi was monitored by taking samples of the fermented material and observing them at the microscope; the process was stopped when sporulation occured.

The fermented product was analized for: dry weight, moisture content, caffeine, polyphenols, nitrogen, total sugars, acidity, soluble solids, ash and *in vitro* dry matter and organic matter enzymatic digestivilities.

Conclusions

1. It is technically possible to transform coffee pulp (fresh or ensiled) to a final product containing higher protein and lower toxic compound contents than the initial material, using solid state fermentation.

2. The fermented fresh coffee pulp with different fungi strains presented the following true protein (Lowry) contents: 23.6% with *A. niger* A-10, 23.53% with *A. orizae* and 19.7% with *S. pulverulentum.*

3. The reductions in caffeine rates between the fermented material and freshed and pressed coffee pulp, with the different strains ranged from 13.2% (*A. niger* A-10) to 46% (*S. pulverulentum*). The reductions in polyphenols ranged from 3.1% (*A. niger* A-10) to 54% (*S. pulverulentum*).

4. Solid state fermentation of ensiled and pressed coffee pulp presented the following reductions: caffeine 1.8% and polyphenols 75%. The final content of true protein (Lowry) was 24.1%; furthermore it is important to note that the kjeldhal protein of fresh coffee pulp varies between 9-12.

PRODUCTION OF AN ANTIBIOTIC SUPPLEMENT FOR FEED USE :

The production of feed from agricultural wastes through biotechnological means is still suffering from the lack of an adequate process able of yielding a low cost product competitive in the market. However, if one looks to the use and especially to the price of what is usually called supplements such as antibiotics, then the objective is to give a final product containing the antibiotic without the expenses of a purification by valorising agro-industrial residues by solid state fermentation. The final product can have a higher price and it will compete with similar premixes.

The initial experiments were carried out on banana wastes with poor results. A strain of *Streptomyces cinnamonensis* wasexperimented at laboratory level, in flasks on particles of banana wastes. Later experiments were tried on grains such as sorghum, barley oats and coffee pulp. On substrates rich in nutrients, growth was better and also production of monensin. The useful concentration of monensin is around 1 to 50 ppm or mg/kg; at this moment, the concentration

reached (in sorghum for instance) has been much inferior and in coffee pulp only 20 µg per g. It is obvious that an optimization of production is required ; at this moment, no pretreatments other than sterilization were performed and taking into consideration the shape and size of particles, a considerable improvement is yet to be obtained.

FERMENTATION OF SUGAR CANE IN A SSF EX-FERM :

The EX-FERM process was setted up in early 1980's due to the increasing need of ethanol for fuel purposes. At this time, because of oil prices, the only alternative seemed to be the standard system for ethanol fermentation based on the submerged fermentation of sugars from starch, sucrose, mixtures etc, and the distillation step to produce an azeotropic mixture of 95% ethanol. Because of the energy requirement in hydrolysing and crushing cane, most of these processes, (which gave ethanol that must be converted into absolute or anhydrous alcool) were not competitive against gasoline. The advantage of using it relied in a positive balance of international trade for those countries devoid of oil.

The EX-FERM system applied to sugar cane circunvented the use of high energy in the crushing of cane, extraction and later on evaporation of the juice into virgin syrup. The process however needed water to perform the fermentation which carried on chips of sugar cane and this contributed to dilute the ethanol.

A new idea was to decrease the amount of water to give a more concentrated liquor in ethanol. Different levels of water were added from 0 to 125% of the sugar cane weight. Two mechanical systems to cut the cane were also evaluated, and two levels of inoculum. A cylindrical reactor was designed to work in different stages. The research work is still going on, and data are being collected; a detailed publication will be made later on.

Achevé d'imprimer sur les presses
de l'Imprimerie ARTI à Nîmes
Juin 1989

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