ENZYMES PRODUCTION BY SOLID STATE FERMENTATION.

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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.

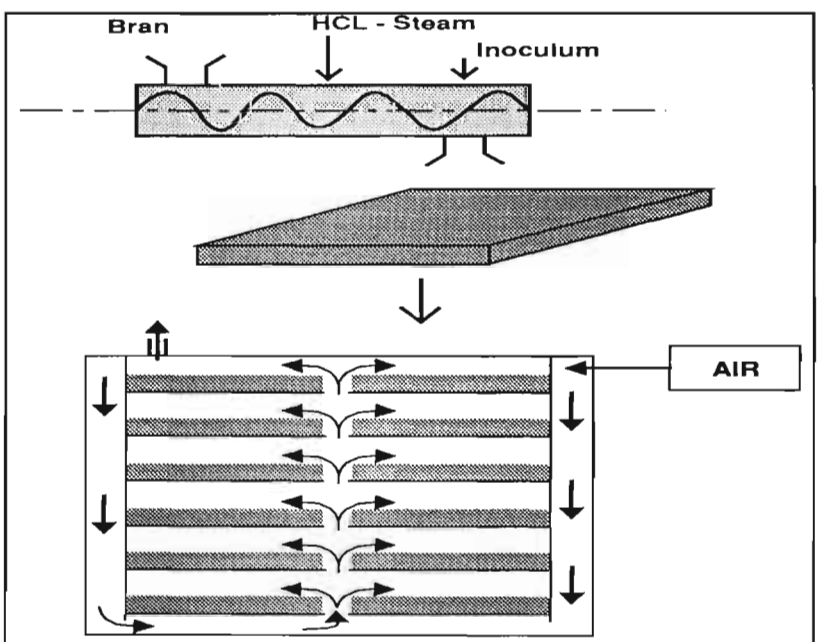

Figure 1 AMYLASE PRODUCTION BY THE MOULDY BRAN SYSTEM

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.

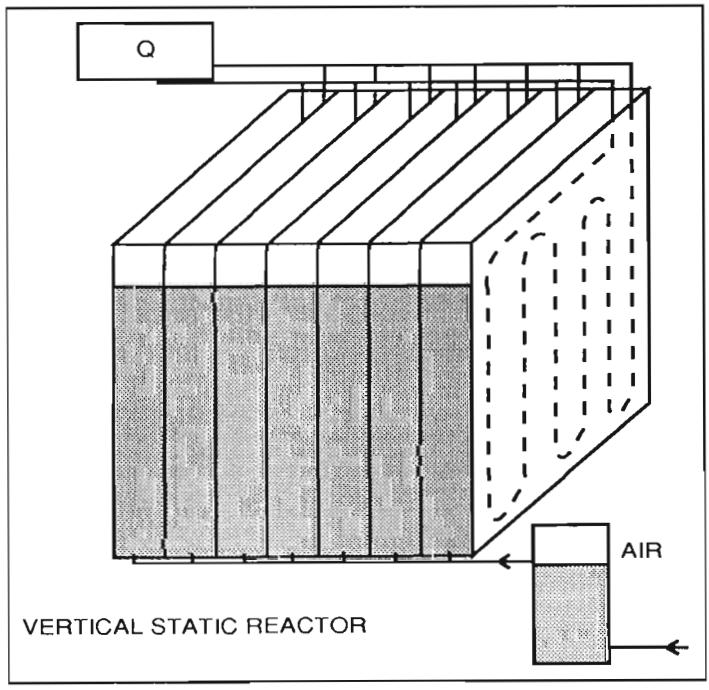

Figure 2:

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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