CONTINUOUS ENZYMES AND FUNGAL METABOLITES PRODUCTION IN SOLID STATE FERMENTATION USING A COUNTER-CURRENT REACTOR

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Abstract:

This work presents the continuous production of fungal biomass and enzymes by solid state fermentation (SSF) in a counter-current reactor adapted for this purpose. Pre-germinated conidia of *Aspergillus niger* were used as an inoculum and sugarcane bagasse, embedded with a nutritive solution, was the solid support. The Solids residence time distribution (RTD) was carried out by feeding one impulse of blue-coloured humidified bagasse and this RTD was fixed at 20 hours. This study demonstrated that the values of the measured parameters (pH, moisture, biomass, glucoamylases production) were similar to those reported for batch SSF using the same solid support and micro-organism. A marked increase in biomass occurred from the progressive compartment (from compartment no.1 to no.9) into the reactor and the enzyme production was important (40 IU/g dry exit solids). No mycelium damage or sporulation was observed. The above results confirmed that the continuous production of enzymes by SSF under no sterile conditions was successful. Inoculation with pre-germinated conidia shortened the processing time and allowed control of the age of the mycelium in each compartment. Aeration was accomplished by natural convection and moisture content had to be controlled. This process can be applied to the continuous production of fungal biomass and metabolites in SSF with industrial applications using environmentally friendly biotechnology.

Key words: Solid State fermentation, Continuous enzyme production, glucoamylases, *A.niger*, pre-germinated conidia, counter-current reactor, fungal metabolite.

Definition of SSF: Solid State Fermentation (SSF) is a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients (Viniegra Gonzalez 1997). The solid materials could other be biodegradable or not. For example, starch and cellulose are solid materials of the first type, whereas, amberlite or polyurethane belong to the second type (Alazard and Raimbault 1981; Moo-Young *et al.* 1983, Barrios Gonzalez *et al.* 1988, Oriol *et al.* 1988a, Auria *et al.* 1990, Gonzalez-Blanco *et al.* 1990, Roussos *et al.* 1991).

Advantages and disadvantages of SSF: Solid state fermentation offers various advantages in comparison with submerged ones (Aido *et al.* 1982, Lonsane *et al.* 1992). Aeration is facilitated through the spaces between the substrate (Lambraki *et al.* 1994, Soccol *et al.* 1994). Substrate agitation, when necessary, is discontinued (Senez *et al.* 1980, Deschamps *et al.* 1982). The absence of a liquid phase and a low water content permit a) reduction of fermentor volume of liquid effluents from the process, b) reagents saving during metabolites recovery, c)

reduction of bacteria contamination and d) use of no sterile solid substrate in some cases. Culture media are simple mainly composed of agro-industrial residues (Lonsane *et al.* 1985, Roussos *et al.* 1991). Culture growth conditions are close to those in the natural environment (Roussos *et al.* 1997). Its main disadvantages are the following: a) risks of high temperature rise (Saucedo-Castañeda *et al.* 1990, Rodriguez et al. 1991, Saucedo *et al.* 1992a), b) difficulty in parameter regulation (Durand *et* Cherau 1988), c) need of pre-treatment of solid material (Raimbault *et al.* 1985) d) high loss of humidity in fermentations lasting of long, e) necessity for high inoculation when natural microflora is not used (Roussos *et al.* 1991), and f) critical role of water and water activity (Oriol *et al.* 1988b, Gervais and Bensoussan 1995).

Metabolites production in SSF: There has been a considerable amount of attention given to the physiology of the micro-organisms involved and the characteristics of the metabolites produced (Trejo -Hernandez *et al.* 1993, Gutierrez-Rojas *et al.* 1995). Culture of filamentous fungi on solid supports has been applied to the production of enzymes, primary and secondary metabolites (Oriol *et al.* 1988b; Saucedo-Castañeda *et al.* 1992b; Trejo-Hernandez *et al.* 1992; Christen *et al.* 1995). It has also been used for the detoxification of a wide variety of materials (Aquiahuatl *et al.* 1988).

Bioreactors: Considering to all these aspects mentioned above, bioreactors have been developed traditionally for different purposes and SSF has been carried out as a batch process in laboratory scale (Raimbault and Alazard 1980; Lepilleur *et al.* 1997), in pilot plant scale (Deschamps *et al.* 1985; Lonsane *et al.* 1984; Durand *et all.* 1985; Pandey 1991, Roussos *et al.* 1993) and in industrial scale (Deschamps *et al.* 1982; Lonsane *et al.* 1992; Bandoor *et al.* 1997; Durand *et al.* 1997). However, continuous production of biomass and metabolites in SSF has not been reported yet.

What is a CCR? In the early 1980s, a new CCR has been developed by the Commonwealth Scientific and Industrial Research Organization (CSIRO) of Australia in cooperation with Bioquip Australia Pty Limited (Casimir, 1983). It has been demonstrated that this unit has a potential for high yields of soluble solids, flavours and colours. Leach (1993) assessed the effect of processing variables on the performance of this reactor in the extraction of apple juice. The performance was strongly influenced by temperature. The effects of draft ratio, screw speed and addition of pectinase enzyme were also investigated. More recently, this extractor has been successfully studied by Gutierrez-López *et al.* (1996) based on a chemical reaction engineering theory, where the extractor was divided into three different zones, according to the flow patterns present. The counter-current reactor has also been used as a solid-liquid extractor for processing fermented products such as enzymes, organic acids, antibiotics, phytohormones and salts (Greve and Kula 1991; Johansson *et al.* 1985; Klyueva and Zakharevich 1985; Kumar and Lonsane 1987; Likidis *et al.* 1989; Schwartzberg 1980; Srikanta *et al.* 1987).

Objective: The objective of this work is to describe the continuous production of glucoamylases by *Aspergillus niger*, a Gras fungus (Samson *et al.* 1997) grown in SSF using a CCR adapted for this purpose because there are not any bioreactors reported in the literature, capable of working in a continuous process for the production of fungal biomass or enzymes in SSF. Before the onset of fermentations it was necessary to study the flow of the solid

support (sugarcane pith bagasse) in the extractor in conditions similar to those used later during the fungal growth in SSF. It was important to know precisely the dependence of the mean residence time of the solid on the different possible programs of the extractor. It was also necessary to know the degree of mixing in different experimental conditions. It was desirable that during the fermentation two solid particles fed at the same time came out of the extractor approximately at the same time too. Otherwise, in each point of the bioreactor there would co-exist micro-organisms at different stages of development.

Residence time distribution

The counter-current extractor consists of a stainless steel ribbon flighted screw situated in a U-shaped stainless steel trough. The screw transports the solid material in both forward and backward directions. It is driven by an external motor which can work intermittently in both directions. In this way, the screw reverses its direction of rotation and that is the distinctive characteristic of the reactor. This reversing movement results in a very efficient solid-solid contact because the solid is constantly in movement between both sides of the CCR.

The reversing movement is controlled by four programmable timer switches located on the control panel of the extractor. One timer selects the desired time of forward movement and another timer, the time of backward movement. The other two timers control the stopping interval between the forward and backward motions. The screw is operated with a much more forward movement a backward. The solids are thereby given a net forward movement.

The screw speed of rotation is also selected in the control panel. Obviously the combination of a variable screw speed and variable forward/backward cycles enable a considerable flexibility in the control of the solid phase residence times[1] and mixing parameters.

The ideal unmixed flow pattern of the solid particles in a CCR is known as plug-flow. In this ideal flow no solid particle overtakes any other particle ahead or behind. Thus, all the particles take exactly the same time to go through the extractor. However, real reactors never fully follow this flow pattern. Usually each particle may take different routes through the reactor and, as a result, different lengths of time are required to reach the exit. In some cases, the deviation from ideality is considerable. This deviation always lowers the performance of the unit.

To determine the extent of deviation from the ideal flow we usually just need to know how long the individual particles stayed in the equipment or, more precisely, the residence time distribution (RTD) of the flowing stream. The RTD can be obtained experimentally using a stimulus-response technique. The system in study is disturbed somehow and the way the system responds to this stimulus gives us the desired information.

The nominal mean solids residence time is given by equation 1.

$$\tau = \frac{N}{n} \times \frac{t_F + t_B + 2.t_S}{t_F - t_B} \qquad (1)$$

where τ = nominal mean residence time (in minutes)

N = number of flights of the screw

n = screw speed (in revolutions per minute, rpm)

t_F = time for which the screw is set to move forward

t_B = time for which the screw is set to move backward

[1] The residence time is the time necessary for the feed to travel from the feed-end to the discharge- end.

t_S = time for which the screw is set to be stationary.

In each fermentation the CCR was initially filled with the amount of non-inoculated bagasse corresponding to the hold-up. This bagasse was uniformly distributed throughout the compartments. Then, the screw movement was started at 1 rpm under the conditions presented in Table 1.

Table 1. Screw movement program for the continuous glucoamylases production in SSF using a CCR. Screw speed = 1 rpm.

	Screw Movement			
Movement	Forward	Stopping	Backward	Stopping
Time (sec)	36.0	870.3	23.4	870.3

We knew from the results of the preliminary experiment that the dispersion should be low if there were 1.2 Kg per compartment. This corresponds approximately to a feeding rate of 0.5 Kg / h and that is the rate used in the first run. In the other two runs higher rates were tested. The residence time distributions obtained in these 3 runs are represented in Figures 1, 2 and 3. In this experiment, 3 runs with tracer were carried out in which we tested the effect of the feeding rate on the dispersion using the program of 36.0 seconds forward / 23.4 seconds backward [2].

The time needed to recover 90 % of the tracer fed gave us an idea of the dispersion. This value was calculated for each one of the runs and is given in Table 2. The variances of the residence time distributions were also calculated for each one of the runs and are presented in the same

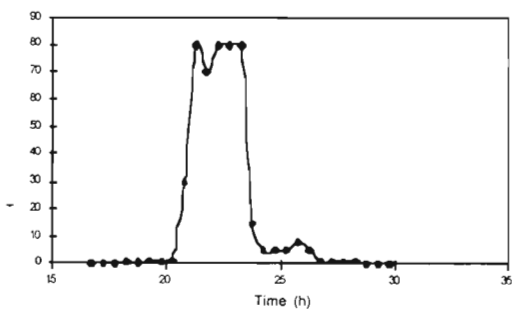

Figure 1.: RTD of the tracer in the first run. Feeding rate = 0.50 Kg / h.

[2] The objective was to work with exactly the same program used later in the fermentations. For practical reasons we were interested in having cycles of exactly half an hour during the fermentation. For this reason, it was necessary, to use a program in which the backward movement lasted 23.4 second in order to have a mean residence time of 20 h.

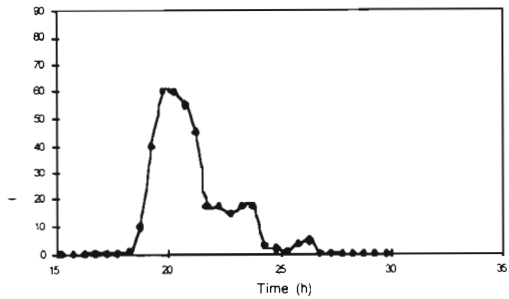

Figure 2 : RTD of the tracer in the second run. Feeding rate = 0.75 Kg / h.

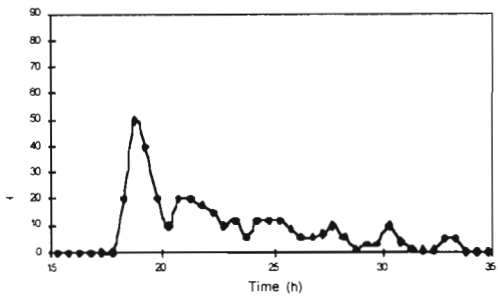

Figure 3 : RTD of the tracer in the third run. Feeding rate = 1.00 Kg / h.

table. These values were obtained using Equation 3. The experimental hold-up is also presented, as well as the hold-up calculated by multiplying the feeding rate by the residence time (Equation 1) .

Table 2. Time necessary to recover 90 % of the tracer, variance of the RTD and experimental and calculated hold-ups.

Run	$t_{90\%}$ (h)	σ^2	Hold-up (Kg)	
			experimental	calculated
1	3	1.5	10.7	10
2	4.5	2.6	15.1	15
3	12	16	21.8	20

It can be seen from Figures 1, 2 and 3 and from the values of $t_{90\%}$ and σ^2 in Table 2 that, the dispersion increases proportionally with the feeding rate. This was expected and as explained before, this happened because as the feeding rate increased, the hold-up increased too and

hence so, the accumulation of bagasse on the ribbon was higher and it was easier for the bagasse to go over the ribbon, pass over the axis of the screw and finally fall in another compartment. Comparing the experimental with the calculated hold-ups a similarity was observed. Therefore, it was confirmed that the mean residence time was 20 h.

It is important to note that, although the hold-up and the feeding rate were expressed in units of mass, it was not the weight of the solid inside the equipment that influenced the dispersion. The dispersion occurred when the volume of the solids was so high that the solid passed over the axis to another compartment. In our case, as all the experiments were made with the same solid, we could always work just with the weight. However, if, for instance, the water content of the bagasse was changed, its density would change too and the results would be different. It would be interesting to carry out similar experiments with different solids and observe if experiments done with similar volumetric flow rates but different solids gave similar results in terms of dispersion.

Continuous glucoamylase production in SSF

Previously sterilised solid material was inoculated with $2x10^7$ conidia of *A. niger* per gram of dry bagasse and all this material was incubated at 37 °C for 8 hours. Following that, this pre-germinated material was stored at 4°C for a few hours. At two hours intervals, one plastic bag was taken from the cold room to the incubator at 37°C and left for an hour before feed of the CCR. 28 feedings (0.5 Kg each) of the pre-germinated material were carried out each hour. Non-inoculated solid material was fed in the subsequent ten feedings.

The CCR screw movement was monitored as shown in Table 1. In this way the mean residence time of the solids in the reactor was 20 hours. During the fermentation no sterile conditions was followed. No special precautions have been taken to avoid the exchange of micro-organisms with the exterior of the reactor. Despite the metallic covers being used, there was contact at the discharge-end and at the feeder. No forced aeration has been used. The natural movement of the air would probably be sufficient enough to supply the necessary oxygen and remove the carbon dioxide produced during fermentation.

Samples from all the compartments (1 to 9) of CCR were collected at 16, 20, 24, 30 and 38 hours of fermentation. At the end of SSF in the CCR, analysis of the samples was carried out to observe the evolution of the main fermentation parameters (water content, pH, biomass, reducing sugars and glucoamylases). The results of these analysis are presented in Figure 4.

Water content: Kinetics of water content in the fermented solid material for each compartment of the CCR show that the humidity of the material decreased from 75 to 65 % from compartments 1 to 9. All kinetics present similar patterns (Figure 4.A). Only at 20 (compartment 9) and 38 hours (compartments 2 and 3) kinetics were different.

pH: The pH evolution was the same for all kinetics. At the beginning (compartments 1 and 2) the pH was stable and around 5.5. In compartments 3-6 it decreased and a value of 3 was reached. This acidic conditions were maintained in compartments 7-9 (Figure 4.B). Only at 38 a different pattern was shown. In compartments 1-6 the pH was always higher than the other kinetics. From compartment 7 onwards the pH changes were similar to the other kinetics.

Biomass: Biomass has only been analysed at 20, 24 and 30 hours of fermentation. Kinetics at 20 and 30 hours presented a similar pattern. At 24 hours kinetic was different. In this study, the change in biomass concentration pattern 20 and 30 hours only is described. At compartments 1-3 the biomass concentration was very low. Compartments 3-7 showed an exponential growth. The growth slowed down after compartment 7 and there was even a decrease in biomass (Figure 4.C)

The biomass change ranged between approximately 1 mg/ g of dry matter at the first compartment and 40 mg/g of dry matter at the 9th compartment, reaching a maximum of 55 mg/g of dry matter at compartments 7 and 8.

Reducing Sugars: The change in reducing sugars is heterogeneous for the different kinetics. However, in general an initial increase in their concentration could be observed, a maximum of 70 mg/g of dry matter being reached around the 4th compartment. Following that, a decrease occurred until compartment no.7 (Figure 4.D). Only at 38 hours the kinetics was clearly different.

Glucoamylases: Glucoamylases production was only analysed at 20 hours fermentation in the CCR. Compartments 1-4 presented a very low enzymatic activity (approx. 10 IU/g of dry matter). Then an increase in activity was predominant (compartments 5-9) until a maximum of 40 IU/g of dry matter was reached in the last compartment (Figure 4.E).

Fungal aspect during a continuous SSF process: Microscopically observations have also been carried out with samples at 20 and 30 hours. From each sample a very small particle of fermented solid material was taken and stained with a "blue Cotton" dye. This preparation was observed under 100x, 200x or 400x magnification.

At the 1 st compartment the conidia had already germinated and the germinative tube length was approx. 3 times the diameter of the conidia. At the 2nd compartment the germinative tubes were 5-10 times longer than the conidia diameter and at the 3rd compartment around 100 times. From compartment no.3 onwards the mycelium was vigorous, thick and presented ramifications.

At compartment no. 4 the mycelium was very well grown. There was a strong ramification of the hyphae and the surface of the solid material had been invaded. Compartments no. 4 showed a strong evolution. The surface of the solid material had been completely covered and the starch particles were degraded.

At compartment no.6 the mycelium replaced the hydrolysed starchy solid and offered a solid structure for the fermented substrate. Compartments no.7, 8 and 9 presented similar change to that of compartment no.6. The only difference was that in compartments no.8 and 9 the formation of a few asexual reproductive forms was detected. Phialides were formed however, these forms were still very young and no conidia were produced.

This first *A. niger* solid state fermentation in the CCR, a good fungal growth was present and a reasonable amount of glucoamylases was produced. The water content decreased significantly from the first to the last compartment. Hence, a second SSF was carried out (results not shown in this paper) with a higher initial water content and with no use of the heating jacket of the CCR. The feeding rate increased to 0.8 Kg/h because, with the feeding rate of 0.5 Kg/h used in the first fermentation, just a small part of the capacity of the reactor was in use.

The mycelium in each compartment was at a similar physiological state. At the first compartment, only germinated conidia were present. Successive compartments showed an increase in the colonisation of the substrate and at compartment no.5 the substrate was covered in dense mycelium. A marked increase in biomass occurred from compartment no.5 onwards. No mycelium damage or sporulation was observed.

In Table 1, the change of the measured parameters is shown for each compartment of the CCR. These values were similar to those reported for batch SSF using the same substrate and micro-organism (Oriol *et al.* 1987). However, moisture content dropped from about 74% in compartment no.1 to 64% in compartment no.9 due to the water evaporation effect caused by the high temperature of the fermented material.

In solid state fermentations several kinetic parameters have been analysed and, in general, all of them presented a similar behaviour. To facilitate the discussion of the results a representative kinetics has been calculated, from different values of the fermentation (Table 3). However, reference to the other kinetics was also made whenever necessary.

Parameters evolution in each CCR compartment during SSF

In this fermentation, 20 hours was selected as the most representative one. In Table 3, changes in water content, pH, biomass, reducing sugars and glucoamylases are shown at 20 hours fermentation.

Table 3: Parameters changes in each CCR compartment over 22 hours fermentation. Sugarcane bagasse moistened with a nutritive solution and containing 10 hours old pre-germinated conidia of *A. niger* was used for feeding in the continuous glucoamylases production under non-aseptic conditions during 38 hour SSF.

	Compartment								
Parameter	1	2	3	4	5	6	7	8	9
Temperature (°C)	30	35	37	39	40	38	39	39	36
Water content (% w.b.)	74	74	73	72	73	72	71	66	64
pH	5.1	5.3	4.9	4.4	3.1	3.0	2.9	2.8	2.9
Biomass (mg/g)	0.4	2.4	11	22	41	45	47	55	42
Reducing sugars (mg/g)	8.0	22	45	63	38	36	39	47	32
Glucoamylases (IU/g)	5.0	9.0	12	9.0	22	23	25	39	40

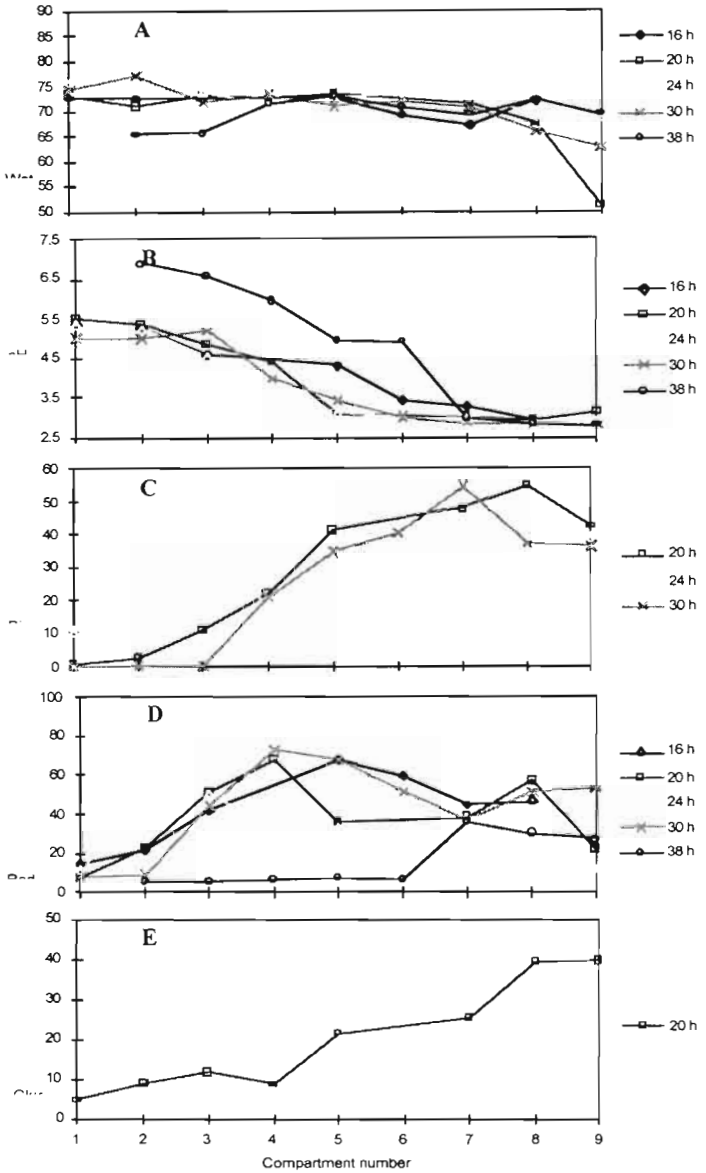

Figure 4 : Water content (A), pH (B), biomass (C), reducing sugars (D) and glucoamylases (E) evolution in each compartment of the CCR during the fermentation. Sugarcane bagasse media containing pre-germinated *A. niger* conidia was continuously fed. Residence time = 20 hours.

Water Content Evolution

Before culture solid medium sterilisation, the inert solid support was moistened to 50% with a nutritive solution.Then, the water content was adjusted to 74% with the spores suspension. The change in water content for the 40 hour SSF in a CCR is represented in Table 3.

A decrease from 74% in compartment no.1 to 64 % in compartment no.9, however is evident this amount of water is enough to ensure good conidia germination and mycelium growth. The last compartments were characterised by a low water content, but it is believed that it did not affect the enzymes production because other studies confirmed the production of glucoamylases in SSF with water content lower than 60 % (Raimbault and Alazard 1980), using starch material at 50% water content and had good glucoamylases production).

As long as it does not affect the fungal metabolism, a low water content is good because it avoids bacterial contaminations. On the other hand, when there is need to implement the process at an industrial scale, a high water content in the final fermented product could be helpful in the recovery of metabolites. For example, if this product had a water content of 75% ,80% of the fermented juice could be recovered by just hydraulically pressing the fermented solid material (Roussos *et al.*, 1992). Thesis one of the simplest and cheapest methods available to recover fungal metabolites.

Glucoamylases Continuous Production

Initially at 20 hours there was a slow increase in the concentration of glucoamylases between compartments no.1 and no.3. From compartment no.3 to no.4 a small decrease in concentration was observed but after the 4th compartment a share increase was evident.

We think that the glucoamylases present at the first four compartments are the glucoamylases that were contained inside the conidia and were liberated during germination. These glucoamylases were responsible for the initial increase in the reducing sugars concentration described above.

The biosynthesis of new glucoamylases started immediately after the 4th compartment and was clearly associated with the growth of mycelium. During this phase, a decrease in the reducing sugars concentration was observed because, although the glucoamylases production was high, it was not sufficient to compensate the reducing sugars consumption by the micro-organism. The micro-organism was on the exponential phase of growth and, as soon as the reducing sugars were produced, they were consumed by the mould.

It is very interesting to note that, although the reducing sugars concentration was high at compartments no.4-8, the biosynthesis of glucoamylases was not repressed. On the contrary, in submerged fermentations a strong catabolic repression in the biosynthesis of glucoamylases was observed when the reducing sugars concentration was high. This fact could be explained thinking of what happened in the surroundings of the mycelium in the solid state fermentation. The mycelium was surrounded by a thin layer of water. The glucoamylases produced by *A. niger* in SSF had to move toward this layer in order to reach the starch material. When the reducing sugars were liberated from the starch, they had to diffuse back through the water layer until they reach the micro-organism. As this diffusion process was

low, as soon as the sugars reached the micro-organism they were consumed by it. In this way, the concentration of sugars close to the micro-organism was very low and the micro-organism continued the production of glucoamylases in SSF, even if this concentration away from the mould was high.

In submerged fermentations, a layer of stagnant solution is also found around the micro-organism. However, due to the agitation of the fermentation broth, its thickness is much smaller and the micro-organism is better informed about the concentrations away from it (Favella-Torres *et al.* 1997).

Spore Germination and Mycelium Development.

Microscopy observations showed that the conidia were uniformly distributed throughout the solid material and the inoculation had been homogeneous. The amount of conidia used ($2x10^7$ conidia/g of dry bagasse) was enough to invade sugarcane bagasse during the mycelium growth, without being excessive. A high percentage of conidia was germinated.

However, when the pre-germinated solid material was fed to the CCR, the spores were still at the beginning of germination. Probably the temperature and time of incubation were not the most adequate.

At compartment no.3 the ramification of the mycelium was evident. At this phase, the exponential growth started (Figure 4.C). There was a clear association between the exponential increase of the biomass and the ramification and strong growth of the mycelium. After compartment no.8 the formation of some reproductive forms was observed and this corresponded to the slight decrease observed in biomass from compartment no.8 to no.9.

Conclusions

The continuous production of enzymes by SSF has been demonstrated. Inoculation with pre-germinated conidia shortened the processing time and allowed control of the age of the mycelium in each compartment. Aeration was accomplished by natural convection and moisture content was controlled. The fungal growth occurred in good conditions, and the movement of the screw no had caused any damage for the mycelium. The sugarcane bagasse was an excellent solid support used in this equipment. Similarities between batch fermentation and fermentation in CCR were observed. This process could be applied to the continuous production of fungal biomass and other metabolites (enzyme, organic acids, antibiotics), spores or biomass. However it is necessary to investigate thoroughly different aspects such as: a) The development of systems to measure and control fermentation parameters in SSF. b) The increase of capacity in the reactor, c) The use of the same biomass in successive cultures, d) The monitoring and control of the humidity , pH, temperature, oxygen transfer during the SSF in a CCR.

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