

Solid state fermentation in a rotating drum bioreactor for the production of hydrolytic enzymes

A.B. Díaz, I. de Ory, I. Caro and A. Blandino

Dpt. of Chemical Engineering, Food Technology and Environmental Technologies
Faculty of Sciences, Pol Rio S Pedro S/N. Puerto Real-Cádiz (Spain)

Previous studies demonstrated that *Aspergillus awamori* produces pectinases, xylanases and cellulases by solid state fermentation. In order to evaluate the production of these enzymes in a laboratory-scale bioreactor, a rotating drum fermentor was used. Different air flow rates were tested demonstrating a positive effect of this parameter on enzyme activity. The production of the highest enzyme activity level was attained working in static or with an agitation of 1 min/day, using an aeration of 120 mL/min.

1. Introduction

Solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of visible water between the substrate particles (Pandey et al., 2001). Compared to submerged fermentation, the solid media used in SSF contain less water but an important gas phase exists between the particles (Durand, 2003). This feature is of great importance because of the poor thermal conductivity of the air compared to the water. As a result of the low A_w in SSF bioreactors, smaller fermenters are required and more concentrated products are produced, simultaneously reducing energy requirements for downstream processing (Robinson et al., 2001; Robinson and Nigam, 2003). However, this technique shows several disadvantages over submerged fermentation (SmF), which have discouraged its use for industrial production (Hölker et al., 2004). One of the major obstacles is the limited knowledge related to the design and operation of large-scale bioreactors (Ashley et al., 1999; Durand and Chereau, 1987). Difficulties in controlling important culture parameters, such as mass transfer and heat removal, have not been overcome completely (Fujian et al. 2002). The low moisture and poor thermal conductivity of the substrate make heat transfer and temperature control difficult in SSF (Bhargav et al. 2008). Many bioreactors have been traditionally used in SSF processes. These can be mainly classified in two groups: the ones which show an agitation system and the ones which work in static conditions. The first category comprises rotating drums, gas-solid fluidized beds, rocking drums, horizontal paddle mixer, etc, while the second one includes the packed-bed and the trays bioreactor. Static beds are required when the substrate bed must remain static throughout the growth phase or when the substrate particles have to be knitted together by the fungal mycelium, such as in the production of fermented foods like Tempe (Mitchell and von Meien, 2000). On the other hand, the use of mixed bioreactors improves the

homogeneity of the bed and ensures an effective heat and mass transfer (Bhargav et al. 2008). However, the shear forces caused by rotation and agitation damage or disrupt fungal mycelia and reduce the porosity of the substrates (Fujian et al., 2002; Ramesh and Lonsane, 1990; Fernandez et al., 1996).

Our research group has previously demonstrated that enzymatic extracts obtained from the fungus *Aspergillus awamori* grown on grape pomace as the sole solid substrate produces pectinases, xylanases and cellulases by SSF. We are now interested in the study of the mentioned enzymes in a laboratory scale bioreactor, using a rotating drum with this purpose. In spite of this configuration is considered to be better and more uniform than other fermenters, fungal cultures can be damaged as a consequence of the shear forces during mixing (Pandey et al., 2001). For this reason, the effect of the type of agitation on the enzyme production at different air-flow rates was evaluated.

2. Materials and Methods

2.1 Spore production

Aspergillus awamori 2B.361 U2/1, classified by the Commonwealth Mycological Institute as *Aspergillus niger* complex, was propagated and stored on slants which contained a synthetic medium for fungal growth composed of (g/L): 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan and 1 pectin. This medium contains xylan and pectin as sole carbon sources to induce the production of xylanase and exo-polygalacturonase.

Spores stored on synthetic medium slants were washed with 10 ml of 0.9 % NaCl. The spore solution (0.5 ml) was spread on the surface of 100 ml synthetic medium in Erlenmeyer flasks (500 ml), and incubated at 30°C for 5 days. After the incubation period, 10 ml of 0.9% NaCl solution was added to the flasks and the spores were suspended by gentle shaking. The number of spores was counted in an Improved Neubauer Counting Chamber. Inoculum concentrations were adjusted to $4.5 \cdot 10^8$ spores/g solid substrate.

2.2. Solid substrate for fermentations

White grape pomace from the Xerez-Sheres-Sherry area in Spain (*Palomino Fino* variety) was used as natural substrate for the SSF experiments. Different samples of white virgin pomace were collected. The pomace was obtained from a local wine cellar after pressing the above mentioned variety of grape and stored at -24 °C until use. For any given series of experiments, sub-samples (250 g) were taken and defrosted to ambient temperature. The solid was then washed several times with distilled water to reduce its high reducing sugars content. After this, it was dried in an oven (60 °C for 48 h), milled and sieved (56.3 % of the total weight of particles was over 1 mm in diameter). Finally, the solid was sterilised in an autoclave for 20 min at 120 °C and 1.2 atm. Orange peels (*Washington Navel* variety) were obtained after juice extraction from oranges collected at a local market. Samples were stored at -24°C. Before their use in SSF experiments, orange peels were defrosted and extensively washed in order to remove all water soluble compounds. Solid was dried at 60°C for 48 h., then milled (62.8% of its weight was constituted of particles over 1 mm in diameter) and finally sterilized in an autoclave (20 min, 120°C, 1.2 atm). When mixtures of grape pomace and orange peels were used, both residues were conditioned separately as it has been previously described, mixed in a 1:1 proportion and sterilised in an autoclave.

2.3 Rotating drum

The fermentor consists of a 250 mL glass roller bottle (\varnothing 7 cm) connected to a filtered-air supply (Figure 1). Air flow is measured by a rotameter and then is sterilized by passing through a 0.45 μ m cellulose filter. The humidifier system is based on a glass column filled with glass beads (3mm) and sterilized distilled water. The air is introduced in the roller bottle through a syringe, which is assembled to a barbed wire to remove the solid stuck in the bottle.

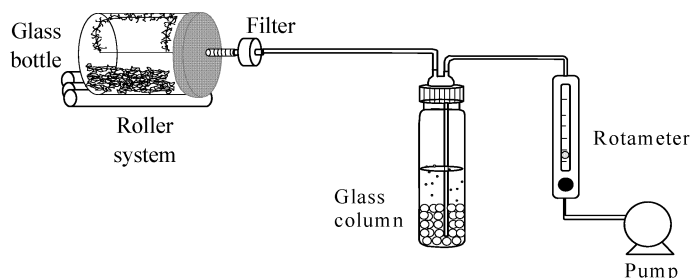


Figure 1: Laboratory scale rotating drum bioreactor

For the experiments carried out in agitation, the bottle is placed in a roller system composed of 5 cylinders which rotate continuously. In this way, the mixture of the media of fermentation is produced as consequence of the movement of the bottle.

For the fermentations, 10 g of pre-inoculated solid substrate with $4.5 \cdot 10^8$ spores/g were added to the bottle. The bed reached a length of 2.5 cm leaving enough space to obtain good agitation. The system was incubated at 27°C for 5 days.

The effects of aeration and agitation in the production of xylanase, exo-PG and CMC-ase were evaluated in the rotating drum. For this purpose, the air flow rates of 0, 9, 120 and 200 mL/min in static were tested, studying different kind of agitation: constant and intermittent (one agitation of 1 min/day and 2 agitations of 10 min/day).

2.4 Extraction conditions

After the fermentation, the media was spilled in Erlenmeyer flasks containing 70 mL of Tween 80 (0.01%) and then stirred in a rotary shaker (150 rpm, 30 min, 4°C). These conditions of extraction were optimized in a previous work (Díaz et al., 2007).

The suspension resulting after the extraction was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant obtained –the enzymatic extract– was stored at -20°C until required for analysis.

In each sample, the concentration of reducing sugar, the pH and xylanase, exo-PG and CMC-ase activities were evaluated.

2.5 Enzyme assay

The enzymatic activities of xylanase (EC 3.2.1.8), exo-polygalacturonase (EC 3.2.1.67) and CMC-ase (EC 3.2.1.4) in the different extracts obtained were assayed. For the xylanase, the reaction mixture containing 0.1 mL of enzymatic extract and 0.9 mL of xylan suspension (0.5%w/w Birchwood xylan in 0.05M citrate buffer, pH 5.4) was incubated at 50°C for 10 min. The reducing sugars produced were measured by a modification of the dinitrosalicylic acid method (DNS) using D-xylose as the standard (Miller, 1959). They are given in mmol per gram of dried solid (mmol/gds). CMC-ase activity was determined by the same procedure described for xylanase, but

carboxymethyl-cellulose (Panreac) was used as substrate. Exo-polygalacturonase (Exo-PG) activity was evaluated by adding 0.2 mL of enzymatic extract to 0.8 mL of pectin solution (0.5% pectin in 0.1M acetate buffer, pH 5.0). Samples were incubated at 45°C for 10 min and the reducing groups in the enzymatic extract were determined by the DNS method. All the measurements were made in duplicate and the results are expressed as reducing sugars using a calibration curve. A unit of enzyme activity (IU) was defined as the amount of enzyme producing 1 μ mol of reducing sugars per minute at the specified conditions.

3. Results and discussion

The concentration of reducing sugar, pH and xylanase, exo-PG and CMC-ase activities were assayed in the extracts obtained from the bioreactor in the different conditions tested. As every experiment was made in duplicate, the average values and the confidence limits for a probability of 95% are also shown.

Different air flow rates were evaluated in order to identify the best aeration for the production of the highest enzyme activities. The effect of the type of agitation was also tested. The concentration of reducing sugars analysed in all the extracts was very low, around 0.020 mmol/gds, with the exception of the experiments carried out without force aeration in static and with constant agitation (data not shown). In these situations, the reducing sugars were not consumed because the fungus didn't grow properly. When the media was not agitated neither aerated it could be explained considering that there wasn't enough oxygen to obtain good growth. In relation to the experiment carried out without aeration in constant agitation, the mycelium and the substrate particles were agglomerated. In these conditions, the heat, mass and oxygen transfers were greatly reduced, affecting the fungus growth.

The pH values of the extracts agreed with the results of the reducing sugars (data not shown). The experiments carried out without force aeration, in static and with constant agitation, showed a pH of 3.5, which is the typical value of the substrate before the fermentation. This fact confirms that good growth had not been produced in these conditions. Enzyme activities measured for the studied enzymes are shown in Figure 2.

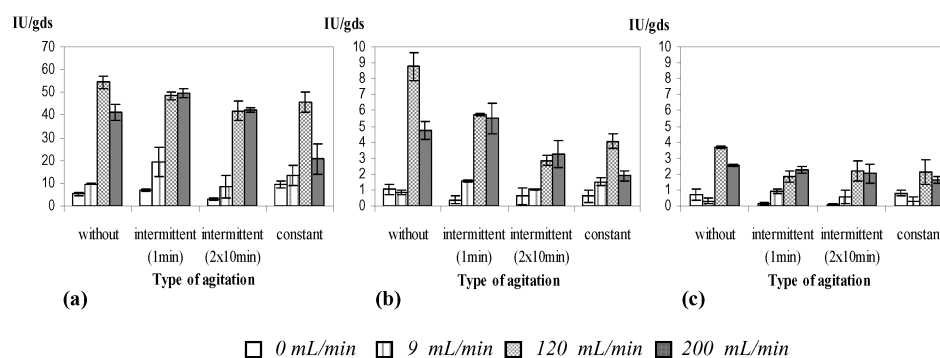


Figure 2: Effect of the type of agitation at different air flow rates in enzyme production (a. xylanase, b. exo-PG and c. CMC-ase) using the rotating drum

It can be observed that the air flow rate influenced in xylanase production, analysing the highest activities with 120 and 200 mL/min. On the other hand, the effect of agitation depended on the air flow rate used. When force aeration wasn't applied, the xylanase activities measured were very low and practically the same with all the types of agitation used. In these conditions the fungus didn't possessed enough oxygen to grow properly and produce enzymes.

The maximum xylanase activity obtained with 9 mL/min (19.52 ± 6.39 IU/gds) was attained with an intermittent agitation of 1 min/day. At low flow rates, a soft aeration could improve O₂ and CO₂ transfer; however, a more frequent agitation could damage fungal cultures as a consequence of the shear forces during mixing. A similar effect was observed with 120 mL/min, analysing the lowest xylanase activity when the system was agitated twice a day and continuously. However, this flow rate provided enough oxygen for the metabolism of the fungus and, in consequence, for xylanase production because it wasn't necessary to agitate the system to attain the maximum activity of 54.42 ± 2.88 IU/gds. This value is 10 times higher than the one obtained in the same conditions without force aeration. Moreover, it was of the same order of magnitude as the maximum attained with the flow rate of 200 mL/min. Thus, a flow rate of 120 mL/min was enough to produce the highest xylanase activity.

The type of agitation didn't show an important effect in exo-PG and CMC-ase production with 0 and 9 mL/min, moreover, low activities were measured under these conditions of aeration. As it happened with xylanase, the maximum exo-PG and CMC-ase activities (8.77 ± 0.88 and 3.69 ± 0.05 IU/gds, respectively) were reached using a flow rate of 120 mL/min. This aeration provided enough oxygen for the metabolism of the fungus and, in consequence, for the enzyme production being unnecessary the agitation of the system. The application of a higher flow rate didn't increase exo-PG and CMC-ase production. The highest exo-PG and CMC-ase activities, obtained with 200 mL/min were assayed when the system was not agitated or it was done 1 min/day. A more frequent agitation could damage fungal cultures as a consequence of the shear forces during mixing.

The production of enzymes using a rotating drum has been reported for other authors. For example, Kalogeris et al. used a laboratory-scale rotating drum to study the effect of the air flow in the production of cellulases and hemicellulases (Kalogeris et al., 1999; Kalogeris et al., 2003). The results revealed that high aeration favoured both biomass and enzyme production. However, there was a maximum air flow rate above which the enzyme recovery didn't enhance.

4. Conclusions

A laboratory rotating drum bioreactor was designed and operated for the production of hydrolytic enzymes by solid state fermentation on a mixture 1:1 (w/w) of grape pomace and orange peels. In this reactor, the air flow rate shows a significant effect on enzyme activity, which increases when aeration is used rather than a static environment. The maximum enzyme activities were attained in static conditions using an air flow rate of 120 mL/min. Although this reactor was designed to work in agitation, the best results were reached in static or with low agitation (1min/day).

References

- Ashley, V., Mitchell, D., Howes, T., 1999. Evaluating strategies for overcoming overheating problems during solid-state fermentation in packed bed bioreactors. *Biochemical Engineering Journal*, 3, 141–150.
- Bhargav, S., Panda, B., Ali, M., 2008. Solid-state Fermentation: An Overview. *Chemical and Biochemical Engineering*, 22, 49–70.
- Díaz, A., Caro, I., de Ory, I., Blandino, A., 2007. Evaluation of the conditions for the extraction of hydrolytic enzymes obtained by solid state fermentation from grape pomace. *Enzyme and Microbial Technology*, 41, 302–306.
- Durand, A., Chereau, D., 1987. A new pilot reactor for solid-state fermentation: Application to the protein enrichment of sugar beet pulp. *Biotechnology and Bioengineering*, 31, 5, 476–486.
- Durand, A., 2003. Bioreactor designs for solid state fermentation. *Biochemical Engineering Journal*, 13, 2, 113–125.
- Fernandez, M., Perez-Correa, J., Solar, I., Agosin, E., 1996. Automation of a solid-state substrate cultivation pilot reactor. *Bioprocess Engineering*, 16, 1–4.
- Fujian, X., Hongzhang, C., Zuohu, L., 2002. Effect of periodically dynamic changes of air on cellulase production in solid-state fermentation. *Enzyme and Microbial Technology*, 30, 1, 45–48.
- Hölker, U., Höfer, M., Lenz, J., 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology*, 64, 175–186.
- Kalogeris, E., Fountoukides, G., Kekos, D., Macris, B., 1999. Design of a solid-state bioreactor for thermophilic microorganisms. *Bioresource Technology*, 67, 313–315.
- Kalogeris, E., Iniotaki, F., Topakas, E., Christakopoulos, P., Kekos, D., Macris, B., 2003. Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. *Bioresource Technology*, 86, 3, 207–213.
- Miller, G., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*, 31, 426–8.
- Mitchell, D., von Meien, O., 2000. Mathematical Modeling as a Tool to Investigate the Design and Operation of the Zymotis Packed-Bed Bioreactor for Solid-State Fermentation. *Biotechnology and Bioengineering*, 68, 2, 127–135.
- Pandey, A.; Soccol, C.; Rodriguez-Leon, J.; Nigam, P., 2001. *Solid-State Fermentation in Biotechnology*, Asiatech Publishers Inc., New Delhi, India.
- Ramesh, M., Lonsane, B., 1990. Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus Licheniformis* in a solid-state fermentation system. *Applied Microbiology and Biotechnology*, 33, 501–505.
- Robinson, T., Singh, D., Nigam, P., 2001. Solid state fermentation: a promising microbial technology for secondary metabolite production. *Applied Microbiology and Biotechnology*, 55, 284–289.
- Robinson, T., Nigam, P., 2003. Bioreactor design for protein enrichment of agricultural residues by solid state fermentation. *Biochemical Engineering Journal*, 13, 197–203