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Production of hydrolytic enzymes from grape pomace and orange peels mixed substrate fermentation by *Aspergillus awamori*

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Many microorganisms that decompose lignocellulosic material are being studied as producers of enzymes to perform enzymatic hydrolysis of the lignocellulosic material present in residues from the agroindustries. Grape pomace is the residue left after juice extraction from the grapes in the wine making industry, but it is under-exploited and most of it is generally disposed in open areas, leading to serious environmental problems. Citrus peels are the main solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight. The disposal of the fresh peels is also becoming a major problem in many factories. As an alternative to the disposal of both residues, in this work the production of xylanase, exo-polygalacturonase and CMCase has been studied by using a mixture 1:1 (w/w) of grape pomace and orange peels as solid substrate for the fermentation of the fungus *Aspergillus awamori*. A solid state glass-column reactor with forced air at lab scale was used for the experiments and the effects of different airflow rates on enzymes production were evaluated.

1. Introduction

Solid State Fermentation (SSF) is an attractive method for fungal enzymes production because it simulates the natural growth of microorganisms on a moist insoluble substrate in the absence or near absence of free liquid (Pandey, 2002). This cultivation technique is acquiring a special relevancy in the field of the biotechnological processes, as an alternative to the traditional submerged fermentation, because has lower energy requirements, produces less wastewater, gives high product concentrations, avoids the foaming and has lower risks of contamination (Gessesse and Mamo, 1999; Suryanarayan, 2003). In addition, SSF allows the use of different agricultural and agro-industrial residues as substrates such as wheat bran (Castilho *et al*., 1999; Soares *et al*., 1999; Singh *et al*., 1999), soy bran (Castilho *et al*., 2000), sugar cane bagasse (Acuña-Agüelles *et al*., 1995), lemon and orange peels (Garzón and Hours, 1992; Ismail, 1996, Martins *et al*., 2002, Silva *et al*., 2002 and 2005), etc.
In every fermentation process, the fermentor (bioreactor) provides the environment for growth and activity for the microorganisms, which cause the biological reaction. There are several parameters (aeration, pH, humidity, agitation, temperature) relevant for the

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selection of the suitable bioreactor for each particular fermentation process. SSF employs a great variety of matrices, which vary in composition, mechanical resistance, porosity and water holding capacity. All this factors also affect the reactor design and the control strategy for the parameters. In addition, the morphology of the fungus and its resistance to mechanical agitation and the necessity or not to have a sterile process should be considered in the election of the bioreactor configuration (Rodriguez Couto *el al*., 2005).

The bioreactors used in SSF processes can be classified by the type of aeration or the mixed system employed in: (A) immersion (humidified air; mechanical agitation); (B) packed-bed (humidified air; static); (C) rotating drum (humidified air; mechanical agitation); (D) tray (passive aeration; static). Our research group has studied the production of xylanase, exo-polygalacturonase and CMCase in three different configurations: rotating drum (Díaz *et al*., 2009), plates and packed bed bioreactor. In this work, results obtained in the production of above enzymes by *Aspergillus awamori* on a mixture of grape pomace and orange peels source has been evaluated in the last configuration of reactor. Moreover, the effect of different air flow rates in the enzyme production was evaluated. Packed-bed bioreactor was selected for these studies because it is one of the most commonly fermentor employed for SSF processes. It consisted on a glass column filled with the bioparticle system (support-fungus) in which humidified air was supplied in a continuous way.

2. Materials and Methods

2.1 Microorganism and solid substrates

A. awamori NRRL 3312 was propagated and stored on slants containing a medium composed by (gL^{-1}): 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan and 1 pectin.

White grape pomace from the Xerez-Sheres-Sherry area in Spain (Palomino Fino variety) was obtained from a local wine cellar. Orange peels (Washington Navel variety) were obtained after juice extraction from oranges collected at a local market. Before their use in SSF experiments, both solids were washed several times with distilled water, dried in an oven (60◦C for 48h), milled and sieved (56.3 % and 62.8% of the total weight of particles of grape pomace and orange peels were over 1 mm in diameter, respectively). Finally, the solids were mixed in a 1:1 proportion and sterilised in an autoclave for 20 min at 120 ◦C and 1.2 atm.

2.2 Packed bed reactor

The fermentor consisted of a glass column (Ø 2.5 cm, length 40 cm) connected to a filtered-air supply. The air flow was measured by a rotameter and then was sterilized by passing through a 0.45 µm cellulose filter. The humidifier system was based on a glass column filled with glass beads (3mm) and sterilized distilled water. Glass beads were used to increase hydraulic retention time of air into the water.

The effect of aeration in the production of xylanase, exo-polygalacturonase (exo-PG) and carboximetil cellulase (CMC-ase) was evaluated in the packed bed bioreactor. For this purpose, different air flow rates were tested: 0, 6, 60, 90, 120, 180 and 300 mL/min.

2.3 Solid state fermentation and enzymes extraction conditions

The column was filled with 10 g of pre-inoculated solid substrate with $4.5 \cdot 10^8$ spores/g. The bed reached a length of 13 cm. Initial moisture content of the solid substrate was adjusted to 70% with a nutrient solution composed of (g/L): 2.4 urea, 9.8 $(NH_4)_2SO4$, 5.0 KH_2PO_4, 0.001 $FeSO_4 \cdot 7H_2O$, 0.0008 $ZnSO_4 \cdot 7H_2O$, 0.004 $MgSO_4 \cdot 7H_2O$, 0.001 $CuSO_4 \cdot 5H_2O$, 11.5 pectin and 11.5 sacarose. The pH of this solution was adjusted to 5. The reactor was then incubated at 27°C for 5 days.

After fermentation, the whole solid was extracted with 70 mL of Tween 80 (0.01%) and then introduced in a rotary shaker for 30 min at 4ºC with a stirring rate of 150 rpm. The suspension resulting after the extraction was centrifuged at 10000 rpm for 10 min at 4ºC to discard the solids and the supernatant obtained was used as crude enzyme solution.

2.4 Enzyme activity assays

Xylanase activity was measured by incubating 0.1 mL of crude enzyme solution with 0.9 mL of substrate solution at 50 ºC for 10 minutes. The substrate solution was made of 0.5% birchwood xylan (Sigma) in 0.1 M sodium acetate buffer (pH 5.0). CMC-ase activity was assayed by the same procedure described for xylanase, but using carboxymethyl-cellulose (Panreac) as the substrate. Exo-polygalacturonase activity (PG) was assayed by incubating 0.8 mL of solution of 0.5% apple pectin (Fluka) in 0.2 M acetate-NaOH buffer pH 5.0 and 0.2 mL of crude enzyme solution. The reaction mixture was incubated at 45ºC for 10 min.

For all the enzymes assays, the reducing sugars released were measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using D-xylose, D-glucose or D-galacturonic acid as the standard. A unit of activity (IU) was defined as the amount of enzyme that produced 1 μmol of reducing sugars per minute under the conditions described above. All the measurements were made in triplicate and the results expressed as reduced sugars using a calibration curve.

3. Results and Discussion

The concentration of reducing sugar, pH and xylanase, exo-PG and CMC-ase activities were analysed in the extracts obtained from the bioreactor after five days of fermentation. Different air flow rates were evaluated in order to identify the best aeration conditions for the highest enzyme activities production. Results obtained are included in Figure 1 and as every experiment was made in duplicate, the average values and the confidence limits for a probability of 95% are also shown.

The concentration of reducing sugars measured after the utilization of different air flow rates was very similar (data not shown), with values ranging from 0.021 ± 0.001 to 0.028 ± 0.002 mmol/gds (grams of dried solid). An important consumption of sugars was produced by the fungus with all the flow rates tested, even when no aeration was used. The pH values analyzed in the extracts at all the flow rates assayed were also quite similar (between 4.66 and 5.31), with the exception of the experiment carried out without aeration, in which a pH value of 6.82 was measured (data not shown).

The enzyme activities are presented in Figure 1. A significant effect of the air flow rate on the production of all studied enzymes was observed. In the case of xylanase, when air flow rate increased from 0 to 60 mL/min, enzyme production improved largely, reaching a maximum of 48.70 ± 3.78 IU/gds at 60 mL/min. When higher air flow rates were used, xylanase activity was kept almost constant. A similar situation was observed

for exo-PG and CMC-ase activities, which increased with air flow rate and reached a peak at 90 mL/min and after this decreased slightly. The highest exo-PG and CMC-ase activities measured were 6.2 and 5.3 times higher, respectively, than the ones analyzed without forced aeration.

The effect of aeration on enzymes synthesis could be explained considering that an increase in the aeration rate causes a decrease in the temperature of the culture medium and, consequently, possible enzymes denaturalization can be reduced. However, when too high flow rates are employed, the moisture content of the solid substrate is extremely reduced and, therefore, enzymes production is reduced. In general, for SSF processes, too low moisture levels in the solid lead to reduced diffusion of the nutrients in it, lower degree of swelling and higher water tension.

For experiments with no aeration, reducing sugars consumption by the fungus was detected but low enzymes activities were assayed. These results can be explained considering that in these conditions, the heat and CO_2 removal is more impeded and therefore, the fungus growth and the enzymes production are affected.

Similar results were obtained for Milagres *et al.* (2004), who established an important effect of the air flow rate on xylanase production using a packed bed fermentor. The highest enzyme activity was attained with an intermediate flow rate, however with higher aeration the activity decreased. In these conditions, the reduction of the initial moisture content from 80% to 50% after 48 h of fermentation was produced, affecting xylanase production.

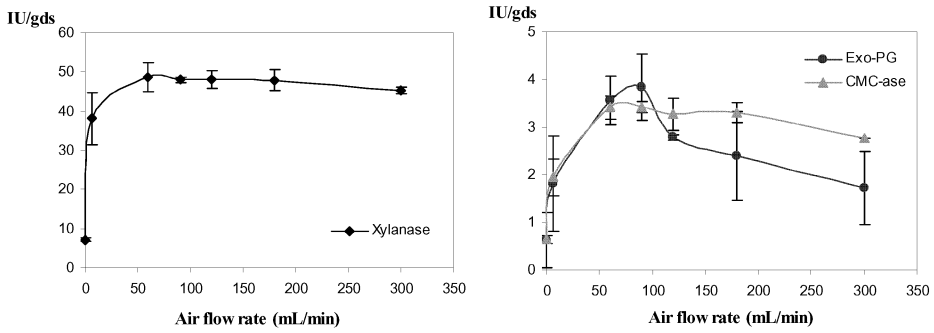

Figure 1. Effect of different air flow rates in the production of xylanase (a), exo-PG and CMC-ase (b) in the packed bed bioreactor.

4. Conclusions

The production of some hydrolytic enzymes –xylanase, exo-polygalacturonase and CMCase- was investigated in SSF using a packed-bed bioreactor. The solid medium consisted of grape pomace mixed with orange peels (1:1 dry weight basis), with 70% initial moisture content. Cultivation was carried out in a glass column, which was packed with pre-inoculated mixed substrate and with forced aeration for 5 days. Increase in air flow rate showed a beneficial effect on enzymes production. However,

there was an optimum air flow rate value for the synthesis of each enzyme: 60 mL/min for xylanase and 90 mL/min for exo-PG and CMCase. When air flow rate was higher than the optimum value, no increase in enzyme productivity was observed. Moreover, the results proved the potential of the two agro-industrial residues as substrates as well as the column bioreactor for the selected enzymes production.

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