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Plates bioreactor for the production of hydrolytic enzymes by solid state fermentation

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Solid State Fermentation (SSF) is an attractive method for fungal enzymes production because it permits the use of different agricultural and agro-industrial residues as substrates. In this work, a novel bioreactor for the production of hydrolytic enzymes (xylanase, exoPG and cellulase) using mixes of grape pomace and orange bagasse as solid raw materials for SSF is presented. The reactor was built with sterile roux flasks interconnected for aeration with an air supply. For the fermentations, pre-inoculated wet solid was added to each plate. Fermentations in plates were carried at 27°C for 5 days and the activities of the three mentioned enzymes, pH and reducing sugars as well as the influence of aeration flow rate were evaluated. The results clearly show that the flow rate plays an important role in the fermentation yields. Maximum production for xylanase (45 IU/gds), exoPG (6 IU/gds) and cellulase (2.5 IU/gds) were obtained.

1. Introduction

In nature, solid organic substrates such as animal and plant residues, wood, crop residues, fruits, etc. undergo complex microbial degradation and transformation by various microbiological processes. Solid state fermentation (SSF) stimulates the natural growth of microorganisms on a moist insoluble solid support in the absence (or near absence) of free water (Pandey, 2003). SSF offers several opportunities to processes with agro-industrial residues, increasing the interest on their applications. For example, the reutilization of agro-industrial wastes for enzymes production using SSF minimizes the pollution and allows obtaining high added-value products using an economical technology. In many industrial processes, enzymes provide a viable alternative to chemical hydrolysis due to its high specificity, besides being environmental friendly (Kuhad et al., 1997). Among enzymes, hydrolytic enzymes have prevalent applications in textile industry, the production of juices and fruit extracts, the pulp and paper and animal feed industries etc. These enzymes degrade polysaccharides in the plant cellular walls like celluloses, hemicelluloses and pectins.

In most cases, agroindustrial wastes used to produce these enzymes by SSF do not posses all the necessary nutrients for this purpose, or maybe they are available in sub-optimal concentrations. In these cases, the substrate must be supplemented to stimulate or improve the enzyme production by adding extra carbon sources or extra nitrogen

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sources (Galiotou-Panayotou and Kapantai, 1993). Supplementation can also be carried out with the adjustment of the initial moisture content of the residue using a solution containing mineral salts or mixing the solid with another residue (Martin et al., 2007).
Grape pomace is the residue left alter juice extraction from the grapes in the wine making industry. Only in Spain, over 250 million kg of this by-product (constituted by seeds, skin and stem) are generated. This material is under-exploited and most of it is generally disposed in open areas, leading to serious environmental problems. In contrast, the potential utility of this waste for value-added products by SSF is promising (high carbohydrate content with the fibre representing about 50% of the total mass). Given this composition, it has been used as substrate for the production of hydrolytic enzymes such as xylanase, exo-polygalacturonase (a type of pectinase), cellulase, etc. (Botella et al., 2007). However, this composition changes according to season, types of grapes, weather conditions, etc., and, therefore, no reproducible enzymatic productivities use to be achieved. In order to enhance the optimum production of some hydrolytic enzymes, orange peels have been selected as a natural source of nutrients to mix with grape pomace. Citrus peels are the main solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight. They are rich in pectin, cellulose and hemicellulose and, in addition, constitute a waste abundantly available. The disposal of the fresh peels is becoming a major problem in many factories. As for other food processing wastes, various microbial transformations have been proposed for citrus peels, producing valuable products like biogas, ethanol, citric acid, chemicals, various enzymes, volatile flavouring compounds, fatty acids and microbial biomass (Mamma et al., 2008).
Bibliography reports many examples of Solid State Fermentations developed at laboratory scale, mainly with petri dishes. However, there is a limited knowledge related to the design and operation of large-scale SSF bioreactors. Difficulties in controlling important culture parameters, such as mass transfer and heat removal, have not been overcome completely (Ashley et al., 1999). The low moisture and poor thermal conductivity of the substrate make heat transfer and temperature control difficult in SSF. The SSF bioreactors can be mainly classified in two groups: agitation systems and static reactors. The first category comprises rotating drums, gas-solid fluidized beds, rocking drums, horizontal paddle mixer, etc, while the second includes packed-bed and trays bioreactor (Bhargav et al., 2008). The aim of this study is to evaluate the production of hydrolytic enzymes by *Aspergillus awamori* through SSF in a tray (plates) bioreactor, using a mixture of grape pomace and orange peels as the carbon source. Moreover, the effect of different air flow rates in the enzyme production was analyzed. This work is related with that one entitled: *Solid state fermentation in a rotating drum bioreactor for the production of hydrolytic enzymes* (Díaz et al.), which is going to be presented in ICheaP-9.

2. Materials and Methods

2.1 Inoculum

Aspergillus awamori was kept in a synthetic medium for fungal growth composed of (g/L): 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan and 1 pectin at 4ºC. After 5 days of incubation at 30ºC, the spores were suspended in NaCl (0.9%).

2.2 Fermentation media

The solid used for the fermentation was a mixture 1:1 of washed white grape pomace and orange peels.

White grape pomace from the Xerez-Sheres-Sherry area in Spain was used as natural substrate for the SSF experiments. For any given series of experiments, sub-samples 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 (60ºC for 48 h), milled and sieved (56.3 % of the total weight of particles was over 1 mm in diameter).

Orange peels (Washington Navel variety) were obtained after juice extraction and frosted. 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 60ºC for 48 h. and then milled (62.8% of its weight was constituted of particles over 1 mm in diameter).

Both solids were mixed (1:1) and sterilised in an autoclave (120ºC, 20min). The initial moisture content of the solid substrate was adjusted to 70% with a nutrient solution constituted (g/L) of 2.4 urea, 9.8 $(NH_4)_2SO_4$, 5.0 KH_2PO_4, 0.001 $FeSO_4{\bullet}7H_2O$, 0.0008 $ZnSO_4{\bullet}7H_2O$, 0.004 $MgSO_4{\bullet}7H_2O$ and 0.001 $CuSO_4{\bullet}5H_2O$. The pH was adjusted at 5.

2.3 Plates bioreactor

A SSF plate-type bioreactor was built with ten sterile 250 mL roux flasks (henceforth referred to as ‘plates’ for convenience) interconnected for water saturated aeration. The reactor was connected to a filtered-air supply, entering sterile air into the first plate and living the reactor at the tenth plate after passing through the whole reactor. 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 (see Figure 1).

For the fermentations, 10 g of pre-inoculated solid substrate with $4.5{\bullet}10^7$ spores/g were added to each plate. The system was incubated at 27°C for 5 days. The effect of aeration in the production of xylanase, exo-PG and CMC-ase was evaluated; for this purpose, different air flow rates were tested: 9, 120, 200 and 300 mL/min.

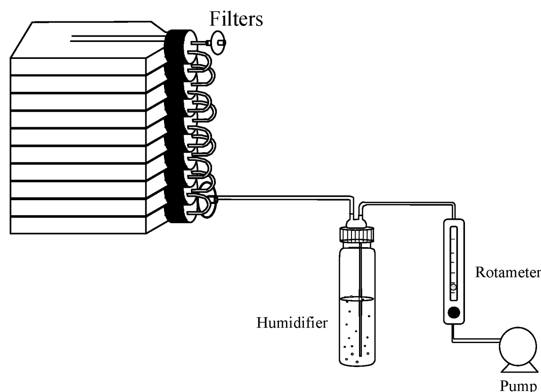

Figure 1. Plate-type bioreactor for solid state fermentations.

2.4 Extraction conditions

After five days of fermentation, the content of each plate was spilled in Erlenmeyer flasks containing 70 mL of Tween 80 (0.01%) and then stirred in a rotary shaker (150 rpm, 30 min, 4ºC). These conditions of extraction were optimized in a previous work (Díaz et al., 2007). The suspensions resulting after each the extraction (10 susp.) were centrifuged at 10000 rpm for 10 minutes at 4ºC. The supernatant obtained –the enzymatic extract- was stored at -20ºC 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 assays

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 50ºC 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). 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 adding 0.2 ml of enzymatic extract to 0.8 ml pectin solution (0.5% pectin in 0.1M acetate buffer, pH 5.0). Samples were incubated at 45ºC 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 expressed as reducing sugars using a calibration curve. A unit of enzyme activity (IU) was defined as the amount of enzyme producing 1 μmol 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 (95% probability) are also shown. Moreover, different air flow rates were evaluated in order to identify the best aeration for the production of the highest enzyme activities in the bioreactor described above.

The evolution of the reducing sugars through the column was different depending on the air flow rate used (data not shown). The flow rate of 9 didn't provide enough aeration to all the trays because a high concentration of reducing sugars was detected in the last plates. The concentration of reducing sugars registered with 120 mL/min and higher was very low in all the trays, which showed that fungus growth had been produced in the whole column. A similar situation was observed for the pH (data not shown). In the experiment carried out at 9 mL/min, the last plates showed a value below 3.7 which is the typical pH of a substrate before the fermentation.

Figure 2 shows the different enzyme activities (xylanase, exo-PG and CMC-ase) registered in every tray with the applied flow rates.

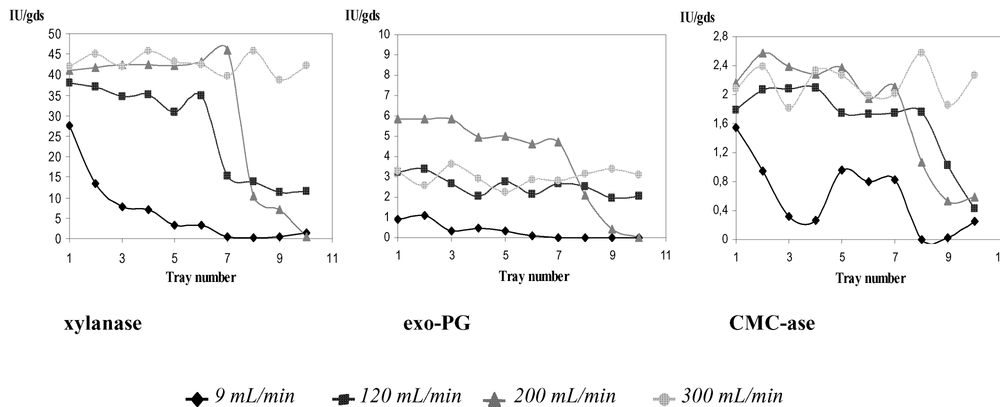

Figure 2: Enzyme activity evolution (xylanase, exo-PG and CMC-ase) through the trays bioreactor with the different air flow rates used.

Xylanase activity was not detected in all the plates when the flow rates of 9, 120 and 200 mL/min were used (Figure 2 left). These results were expected for the aeration of 9 mL/min, since a high concentration of reducing sugars was registered in the last plates. For the flow rates of 120 and 200 mL/min, the blockage of the pipes with the evaporated water from the solid was observed in the last plates. The maximum xylanase activity (45.73 ± 9.93 IU/gds) was registered using an air flow rate of 300 mL/min, keeping practically constant through the column.

The same trend was observed with CMC-ase, being necessary to apply a flow rate of 300 mL/min to obtain enzyme activity in all the plates (Figure 2 right). Moreover, this aeration provided the maximum activity of 2.58 ± 0.64 IU/gds.

The highest exo-PG activities were attained with 200 mL/min, however, activity wasn't observed in all the plates for the same reason described for the other enzymes (Figure 2 center). The application of an air flow rate of 120 or 300 mL/min leaded to the production of exo-PG activity in all the trays. The blockage of the pipe observed with 120 mL/min was produced after the fungus growth and exo-PG release. This fact demonstrates that in these conditions exo-PG was produced before xylanase and CMC-ase. The highest exo-PG activity of 3.62 ± 1.06 IU/gds was measured with 300 mL/min.

In conclusion, aeration showed an important effect on enzyme production. In this way, an increase on flow rate from 9 to 300 mL/min resulted in increases of maximum xylanase, exo-PG and CMC-ase activities on 39.49%, 69.06%, and 39.53%, respectively. These maximum activities can be considered high in comparison with those obtained with other agroindustrial wastes and other configurations of reactor, showing that a mix of grape pomace and orange bagasse is a potentially good natural medium for the production of hydrolytic enzymes with solid state fermentation.

4. Conclusions

- The mix of grape pomace and orange bagasse is a potentially good natural medium for the production of hydrolytic enzymes with solid state fermentation in a plates bioreactor, showing maximum activities that can be considered high in comparison with those obtained with other agroindustrial wastes and other configurations.

- Aeration has an important effect on enzyme production in the plates bioreactor. Increasing flow rates from 9 to 300 mL/min resulted in increases of maximum xylanase, exo-PG and CMC-ase activities on 39.49%, 69.06%, and 39.53%, respectively. Aeration of 200 ml/min and below entails an insufficient aeration of the whole column.

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