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Evaluation of Agro-industrial By-products for α-Galactosidase Production by *Aspergillus oryzae* in Submerged Culture

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Several agro-industrial by-products (orange peel, soybean husk, bean husk and corn husk) were evaluated as carbon source to produce α-galactosidase by *Aspergillus oryzae* in submerged fermentation. Fermentations were carried out during 10 days at two temperatures (25 and 30°C) and three pHs (3.0, 5.0 and 8.0) to evaluate their effect on enzyme activity. For substrates that showed greater enzyme activity, the agitation effect on enzyme production was evaluated. The highest enzyme activity was obtained at 30 ºC and pH 5.0 with soybean husk (33.96 U g^{-1} protein) followed by corn husk (23.83 U g^{-1} protein), Orange peel (10.68 U g^{-1} protein) and bean husk (9.01 U g^{-1} protein). Agitation showed an improvement in α-galactosidase production, giving an enzyme activity 1.4-fold higher than obtained with static soybean and corn husk culture.

1. Introduction

α-Galactosidase (α-D-galactopyrinoside galactohydrolase EC 3.2.1.22) is widely produced in plants and animals. It can hydrolyse simple and complex α-D-galactosides (Dey and Pridham, 1972). α-Galactosidase has been used in several industrial applications. Zeilinger *et al.* (1993) reported the use of this enzyme in the paper industry, supported by Szendefy *et al.* (2006) who used it in the hydrolysis of galactomanose, one of the main wood hemicellulose components. α-Galactosidase has been used in the sugar beet refining, in the improvement of gellifying properties of thickners, and galacto-oligosacharide reduction in soybean milk and vegetable products (Ademark *et al.*, 2001). Moreover, it is used in enzyme replacement therapy for Fabry disease (Germain, 2007; Bodary *et al.*, 2007; Clarke, 2007).
Many filamentous fungi have been reported to produce hydrolytic enzymes that are used in several food industries. The fungus *Aspergillus oryzae* is the source of the most used enzymes in food and feed because of its advantages over many other microbial sources and its acceptance as GRAS (generally regarded as Safe) (Kobayashi, 2007). *A. oryzae* has been identify as a source of α-galactosidase (Shankar and Mulimani, 2007).
Although solid state fermentations is one of the best process for fungi culture, it can be successfully control. For that reason, submerged culture is a common process in the enzyme production industry. It has several advantages such as easy process control and

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enzyme and biomass collection. However, it must be taken into account the fact that products are diluted and in some cases the enzyme extract might be less stable than in solid state fermentation (Sandhya, 2005).

Due to the wide industrial application of α-galactosidase, their production process has had a great development and capital investment. For that reason is important the search of new substrates and microorganism able to produce it in a great proportion. The aim of this project is to evaluate different agro-industrial by-products (orange peel, soybean husk, bean husk and corn husk) as carbon source for the production of α-galactosidase by *A. oryzae* in submerged culture, and the effect of temperature, pH and agitation on the enzyme activity.

2. Materials and Method

2.1 Chemicals

p-Nitrophenyl-α-D-galactopiranoside (PNPG) was purchased from Sigma Chemicals (USA). All other chemicals used were of analytical grade. Orange peels, bean husks and corn husks were obtained at a local market in Bogotá (Cundinamarca, Colombia), and soybean husks were obtained from a soybean crop in Villavicencio (Meta, Colombia).

3.2 Microorganism culture and spore production

The strain *Aspergillus oryzae* was a soil isolated obtained by the Microbioly Department, Universidad de Los Andes. The strain was grown on malt extract agar (MEA) plates at 30±1 ◦C for 7 days. To prepare spore suspensions, spores were scraped down from the MEA plates with a sterilized tensoactive solution (15%, w/v glycerol, 0.1%, w/v Tween 80 and acetate buffer 0.1M (pH 6.0) q.s.f. 100 mL) and diluted to a concentration of about 1×10^6 spores mL^{-1} with sterilized water. The spore suspensions were kept at −20±1 ◦C and subcultured once a month.

3.3 Submerged culture

Cultures were carried out in 250 mL flasks containing 40mL of culture medium (K_2HPO_4, 6.3 g L^{-1}; KH_2PO_4, 1.8 g L^{-1}; $(NH_4)_2SO_4$, 1.0 g L^{-1}; $MgSO_4$, 1.0 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}; $FeSO_4$, 0.1 g L^{-1}; and 0.8 g of the respective agro-industrial byproduct, pH was adjust with dilute HNO_3 or NaOH solution according to the assay) inoculated with 1 mL of spore suspension (Shankar and Mulimani, 2007). To evaluate the temperature and pH effect on enzyme production, independent cultures were adjust to pH 3.0, 5.0 and 8.0, and incubated at 25 or 30 °C during 10 days. Samples were removed every 24 h to evaluate the enzyme activity. Assays were carried out without agitation, however for substrates that showed the greatest enzyme activity, cultures at 30°C and 150 for the tested pHs were carried out. Each trial was made in triplicate.

3.4 α-Galactosidase assay

α-Galactosidase activity was measured according to Dey and Pridham method in which p-nitrophenol (PNP) is produced by the enzymatic transformation of PNPG (Dey and Pridham, 1972). PNP was photospectrometrically quantify at λ 405 nm (Varian UV-Vis Cary Spectrophotometer). Reaction mixture contained supernatant (0.1 mL), acetate buffer 0.2 M (0.8 mL, pH 4.8) and PNPG 2 mM (0.1 mL), and was incubated at 37ºC for 15 min. The reaction was stopped by adding 3 mL of Na_2CO_3 0.2MOne unit of

enzyme activity is defined as the amount of enzyme which is able to produce 1μmol of PNP per minute. The specific activity was defined as units of enzyme activity per gram of protein.

3. Results

Figure 1(a-d) shows for the static culture the effect of pH, temperature, culture time and agro-industrial by-product source on specific α-galactosidase. It is observed that the highest enzymatic activity was obtained in the 8th culture day, but using bean husk it was in the 7th culture day. Also, it is noticed an increase in enzyme activity during the second day for all substrates, however, after the third day there is a drop and then it starts to increase gradually. For all the substrates the highest enzyme activity was obtained at pH 5.0±0.1 and 30±1°C. Nevertheless, cultures with soybean husk presented the highest α-galactosidase activity, obtaining a specific activity of 33.96 U g^{-1} protein. This high activity probably is owing to raffinose oligosaccharides present in this seed that could be in present in the husk, and that favor the production of α-galactosidase (Viana *et al.,* 2004). For corn husk, orange peel and bean husk the maximum specific enzyme activity obtained was 23.83, 10.68 and 9.01 U g^{-1} of protein, respectively.

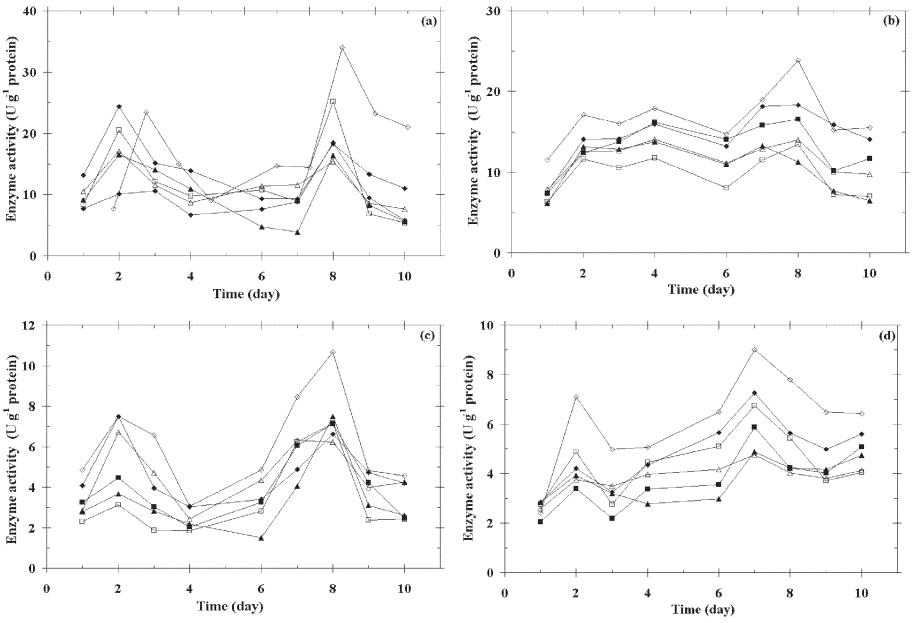

Figure 1. Effect of agro-industrial by-product (soybean husk ***(a)****, corn husk* ***(b)****, orange peel* ***(c)*** *and dean husk* ***(d)****), pH, temperature and culture time on specific α-galactosidase activity. Nomenclature: pH 3.0 (□), 5.0 (◇), and 8.0 (⇔); 25 (filled symbols) and 30°C (empty symbols).*

Contrary to the other substrates, as shown in Figure 1-b, the specific enzyme activity using corn husk as substrate shows an almost constant performance along the culture

with no abrupt decrease of the activity, there is a small increment towards the eighth day corresponding to the highest activity. However this behavior is useful to induce high production of α-galactosidase in short periods of time.

Results in Figure 1-c demonstrate low enzymatic activity for orange peel. This agro-industrial by-product presents a different composition from the husks, and contains other components that might affect the fungus growth and metabolism. However, under these conditions the medium increased its viscosity probably by the α-galactosidase gelifying properties.

The poorest results were obtained for bean husk mediums at pH 8.0 incubated at both 25°C and 30°C obtaining a specific enzymatic activity of 4.8U g^{-1} protein and 4.71 U g^{-1} protein, respectively. Highest enzyme activity obtained for this substrate was 9.01 U g^{-1} protein, which indeed is very low compared to the other substrates. Therefore, it is not recommended for α-galactosidase production.

Different authors like Shankar and Mulimani (2007), and Prashanth and Mulimani (2004) have reported the α-galactosidase production in submerged cultures at pH 5.0 and 30±1ºC. Also, stability studies of α-galactosidase have shown that it is stable at pH 5.0-6.0 (Ademark *et al.*, 2001). Although the effect of temperature on enzyme production at 25±1ºC and 30±1ºC is not significant, it must be considered in an economical evaluation.

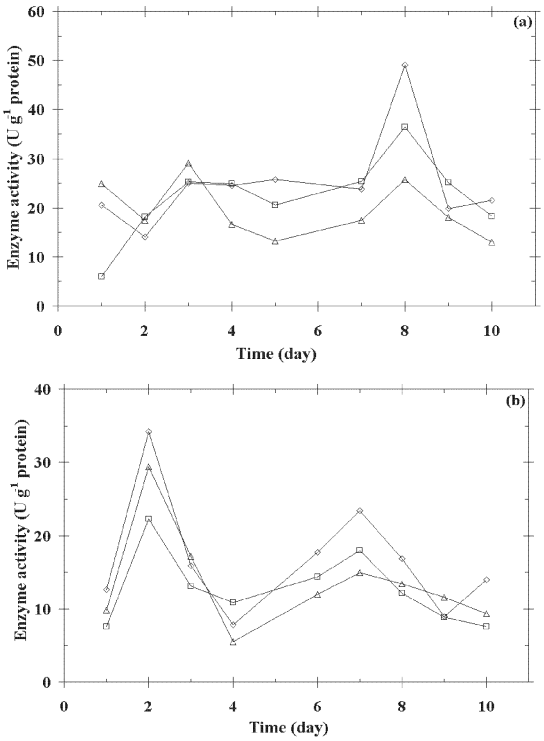

Figure 2. Effect of agitaciónand pH on specific α-galactosidase activity for soybean husk ***(a)*** *and corn husk* ***(b)****. Nomenclature: pH 3.0 (□), 5.0 (◇), and 8.0 (⇔).*

The effect of agitation was evaluated for soybean husk and corn husk since these showed the highest specific enzyme activities (Figure 2a-b). The specific enzyme activity kept a similar performance regarded to pH, obtaining at pH 5.0 the highest activity. For soybean husk was observed in the 8th day of culture the maximum activity (49.10 U g^{-1} protein, Figure 2-a). Nevertheless the maximum enzyme production was observed in a different lap of time for cultures with the corn husk, obtaining in the second day an activity of 34.14 U g^{-1} protein (Figure 2-b). Agitation was favorable for enzyme production increasing in 1.4-fold the enzyme activity for both substrates.
It is important to take into account, that enzyme activity results obtained for agitated corn husk during the second day is approximately the same magnitude as non agitated soybean husk (which showed the best enzyme production for non agitated systems). This increase in enzyme activity may be occurs by a major availability of nutrients in the medium that facilitates fungal growth, and therefore, enzyme production.

4. Conclusions

The production of α-galactosidase by *A. oryzae* using agro-industrial by-products as substrate in submerged culture showed to be a feasible process for obtaining the enzyme. For the different substrates tested, the highest activities were obtained at 30±1ºC and pH 5.0. Nevertheless, the soybean husk presented the highest enzyme activity, and agitation improved enzyme production.
Using agro-industrial by-products as substrate for this process is considered as an advantage because it has economical benefits, and not much energy and economical investments are required to process this kind of raw materials. For countries like Colombia, this type of projects can be of great commercial value due to low prices of the substrates in the agricultural sector. Moreover it will have a positive environmental impact because these materials will no longer be consider as residues, and can have a new function in other process.

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