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Evaluation of orange peel and green soybean as a substrate for the production of α-galactosidase by a soil isolated *Aspergillus oryzae* in solid state fermentation

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Orange peel and green soybean were used as substrate in Solid State Fermentation by a native strain of *Aspergillus oryzae* to produce α-galactosidase. During four culture days was evaluated the pH and incubation temperature effect on the enzyme production. Results displayed the green soybean like a better substrate than orange peel, but both can be used as potential substrates with considerable enzyme activity 6.48±1.24 U g^{-1} and 2.19±0.30 U g^{-1}, respectively. For both substrates the best culture condition obtained for the enzyme production was at 30ºC and pH 5.00. Use of green soybean and orange peel as a substrate could be of great commercial significance in agro-industrial countries like Colombia giving value to agriculture wastes. The process of α-galactosidase production using the isolated strain and the green soybean as substrate in laboratory scale may have the potential to scale-up.

1. Introduction

α-Galactosidase (α-D-galactopyranoside galactohydrolase EC 3.2.1.22) is widely distributed in the domain eukarya. α-galactosidase catalyzes the cleavage of terminal α-1,6 linked galactosyl residues from a wide range of organic and synthetic substrates like linear and branched oligosaccharides, polysaccharides and synthetic substrates as substituted phenyl α-D-galactosides. (Li *et al.*, 2006; Yip and Withers, 2004; Ademark *et al.*, 2001; Varbanets *et al.*, 2001; Dey and Pridham, 1972). Filamentous fungi like *Aspergillus oryzae* and *Aspergillus niger* are an attractive source for enzyme production because their hability to produce extracellular enzymes, especially those that hydrolyses a wide variety of complex polysaccharides (Kobayashi *et al.*, 2007).

α-Galactosidase has several industrial applications. In food industry can be used in the sugar beet preparation hydrolyzing rafinosse, this action avoid the inhibition of normal sucrose crystallization (Shankar and Mulimani, 2006; Li *et al.*, 2006; Ademark *et al.*, 2001; Arora, 1991). It is also used in the cleavage of galacto-oligosaccharides present in soybean milk and other food obtained from legumes, and is used to improve the gelling properties of galactomannans commonly used as food thickeners (Pandey *et al.*, 2005; Ademark *et al.*, 2001). In paper industry α-Galactosidase hydrolyze hemicelluloses main groups (galactomannanes and galactoglucomannanes) present in soft wood to obtain paper pulp (Golubev *et al.*, 2004; Shabalin *et al.*, 2002). In the pharmaceutical

industry is used to avoid the discomfort sensation of indigestibility caused by the presence of non digestible sugars like rafinosse and stachyose present in various leguminous and fruits (Donkor *et al.*, 2007; Li *et al.*, 2006). α-galactosidase is also used in the treatment of Fabry Disease, which is caused by a lysosomal enzyme deficiency of α-galactosidase, this results in the storage of globotriaosylceramide (Gb_3) in a variety of organs and cells. Also, it is used in medicine in blood group transformation (Bodary *et al.*, 2007; Khan *et al.*, 2006; Varbanets *et al.*, 2001; Maranville and Zhu, 2000).

Solid state fermentation (SSF) is a traditional way for the production of food and alcoholic beverages in oriental countries (miso and sake) (Pandey *et al.*, 2005). In SSF, microorganisms grow in a solid environment, this cause them to feel in their natural environment, and because of this can produce metabolites that can not produce in an artificial environment (Ramana *et al.*, 1993). SSF offer some advantages over the classic process. These include less waste of energy, less space per substrate unit, higher concentrations of products, simple fermentation equipment and no effluent problems. Also SSF has some disadvantages, for example the control in the system and the scale up process presents problems because of heat accumulation (Li *et al.*, 2006; Pandey *et al.*, 2005; Ramana *et al.*, 1993; Arora, 1991). SSF is a useful technique because it uses agro-industrial wastes without a pretreatment of the substrate, this make SSF a very feasible and economic operation (Yovita *et al.*, 2006). Filamentous fungi are most exploited in this technique of fermentation, due to their ability to grow on complete solid substrates, producing a wide range of extracellular enzymes (Li *et al.*, 2006; Bennett, 1992).

In the present work orange peel and green soybean were studied as a substrate for the production of *α*-galactosidase by a native strain of *Aspergillus oryzae*, and the pH and incubation temperature effect was evaluated for the enzyme production.

2. Materials and methods

2.1 Chemicals and reagents

All chemicals were analytical grade. Orange peel and green soybean were obtained in Cundinamarca and Meta (Colombia), respectively. Enzyme substrate *p*-nitrophenyl-α-D-galactopyranoside (PNPG) was purchased from Sigma chemicals (USA).

2.2 Culture medium and conditions

The native strain *Aspergillus oryzae* used in this work was isolated from soil. It was grown on malt extract agar (MEA) for 14 days at 30±1ºC. To prepare spore suspensions, spores were scrapped down from the MEA plates with a sterilized tensoactive solution (15% $^{w}/_{v}$, glycerol; 0.1% $^{w}/_{v}$, Tween 80 and acetate buffer 0.1 M (pH 6.0) q.s.f. 100 mL). Spores were counted in a Neubauern chamber. The final solution contained 1×10^6 spores ml^{-1}. The spore suspensions were kept at –20±1ºC and subcultured once a month.

2.3 Fermentation media

Ten grams of chopped dry substrate were taken into 250 mL shake flask, separately. Each flask was moistened with a mineral salt solution in a ratio of 2:1($^{w}/_{v}$) (containing in g L^{-1} K_2HPO_4 (6.3), KH_2PO_4 (1.8), $(NH_4)_2SO_4$ (1), $MgSO_4 \cdot 7H_2O$ (1), $CaCl_2 \cdot 2H_2O$ (0.1), $FeSO_4$ (0.1)). The sterilized medium was inoculated with 1.0 mL of spore suspension. To evaluate the pH and temperature effect, the inoculated mediums were incubated at 30±1ºC and 25±1ºC, for each temperature three pH values were studied (3.00, 5.00 and 8.00) for four days. pH media was adjusted using 0.1M HCl or 0.1M NaOH. Each assay was made by triplicate.

2.4 Enzyme extraction

Enzyme extraction was carried out by mixing 1 g of fermented mass with 10 mL sodium acetate buffer 0.2 M (pH 4.8) for 1 h in an orbital shaker at 150 rpm. Contents of the flask were filtered using a vacuum pump and a muslin cloth, the filtrate was centrifuged at 2100 g for 10 min. The supernatant obtained was used as a crude extract of α-galactosidase and used for enzyme assay (Shankar and Mulimani, 2007).

2.5 Assay of α-galactosidase

α-Galactosidase activity was assayed according to the method proposed by Dey and Pridham (1972). Reaction mixture contained: 0.1 mL of enzyme crude extract, 0.8 mL of 0.2 M acetate buffer (pH 4.8) and 0.1 mL of 0.2 mM PNPG. The mixture was incubated at 50±1ºC for 15 min. Reaction was stopped by adding 3 ml of 0.2 M Na_2CO_3 solution. Absorbance of the mixture was measured at 405 nm (Thermospectronic GENESYS 5) to determine the amount of *p*-nitrophenol (PNP) released in the reaction.

One unit of enzyme activity was defined as the amount of enzyme that produces 1μmole of PNP min^{-1} under assay conditions. α-Galactosidase production was expressed as U g^{-1} of dry fermented mass.

3. Results and discussion

Fermentations were carried out at different incubation temperatures 25 and 30±1ºC and different pHs (3.00, 5.00 and 8.00), using orange peel and green soybean in order to find the best conditions for α-galactosidase production.

3.1 α-galactosidase production by *A. oryzae* in orange peel

The obtained results for orange peel at the conditions mentioned above are shown in Figure 1. The results of the enzyme activity evaluation at 25±1ºC were: pH 3 (1.07±0.22 U g^{-1}), pH 5 (2.17±0.22 U g^{-1}) and at pH 8 (0.9±0.21 U g^{-1}), while in the evaluation at 30±1ºC they were: pH 3 (0.9±0.09 U g^{-1}), pH 5 (2.19±0.30 U g^{-1}) and at pH 8 (0.95±0.25 U g^{-1}).

The results show a higher activity of α-galactosidase activity (2.19±0.30 U g^{-1}) compared with the study made by Shankar and Mulimani (2007), they obtained an enzyme activity of 0.810±0.04 using the same substrate at 30±1ºC and pH 5.5, this value of pH was the optimum for α-galactosidase production by *A. oryzae* (Shankar and

Mulimani, 2007), this shows that we get an activity increase of aproximately 2.5 times the previosly reported.

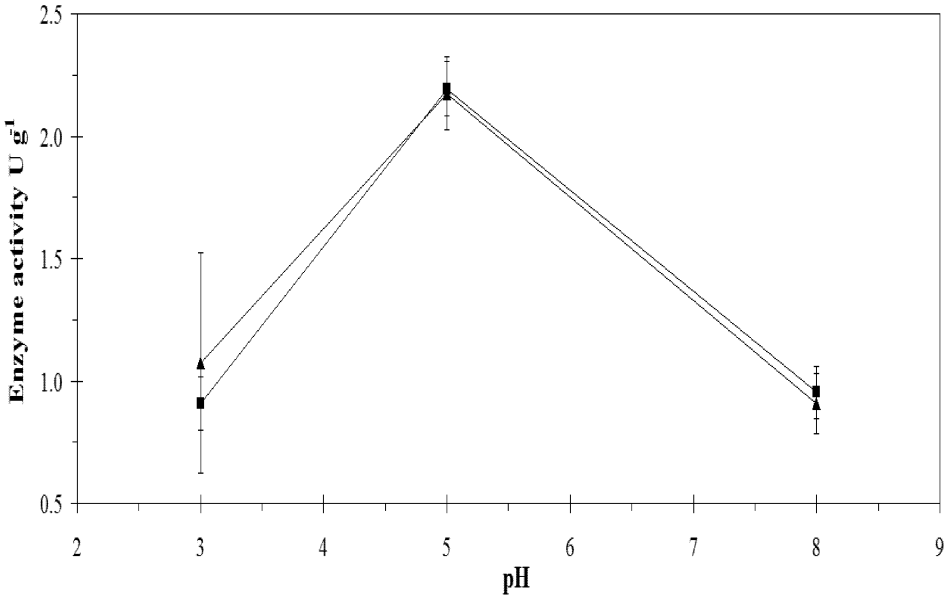

Figure 1. *pH and incubation temperature (25-▲- and* 30ºC-■*-) effect on the* α-*galactosidase activity in SSF using orange peel as substrate.*

The differences could be atributted to external conditions as the orange peels composition (carbohydrates, proteins, salts, acids and moist content) or process conditions as the particle size of the substrate and the fungal strain. Shankar and Mulimani(2007) report a particle size of 1200µm, while we did not make further transformations about this condition, however this is an important variable in SSF because it determines the mass and heat transfer regimes in the process as encountered by Annunziato *et al.* (1986), who evaluate different particle sizes as a process condition in the production of α-galactosidase by *A. oryzae.* The results obtained by Annuziato, showed a high enzyme activity when the particle size of solid substrate passed trought mesh 6 obtaining an enzyme activity of 9.32 U g^{-1}. Changing the particule size (mesh 8 and 20) resulted in a decrease of enzyme activity obtaininig 6.05 U g^{-1} and 5.54 U g^{-1} respectively. (Annunziato *et al.*, 1986)

3.2 α-galactosidase production by *A. oryzae* in green soybean

The obtained results for green soybean at the conditions mentioned above are shown in Figure 2. The results of the enzyme activity evaluation at 25±1ºC were: pH 3.00 (3.28±0,91 U g^{-1}), pH 5.00 (6.31±1.88 U g^{-1}) and at pH 8.00 (4.56±0,26 U g^{-1}), while in the evaluation at 30±1ºC they were: pH 3.00 (3.67±0,24 U g^{-1}), pH 5.00 (6.48±1.24 U g^{-1}) and at pH 8.00 (3.66±0,92 U g^{-1}).

Up to now no further studies have been published for the production of α-galactosidase in SSF using green soybean. However, there are many studies of the production of this enzyme using soy products as soybean (ground), soy flour, other studies use wheat bran enriched with soybean carbohydrate solution.

The results obtained by Annunziato *et al.* for the production of α-galactosidase in soy bean ground (8.63 U g^{-1}) and soybean flour (10.4 U g^{-1}) show higher enzyme activity, but there are significant differences between both process conditions. Cruz and Park (1892) obtained an enzyme activity of 2.58U g^{-1} using wheat bran as a substrate enriched with soybean carbohydrate solution, the soybean carbohydrate solution increases enzyme production 2,5 times the activity in wheat bran without soybean carbohydrate solution. Because of this, we can presume that the presence of soybean products in the fermentation cause a higher yield in enzyme production, making soy products specially green soybean a potential substrate in SSF for the production of α-galactosidase.

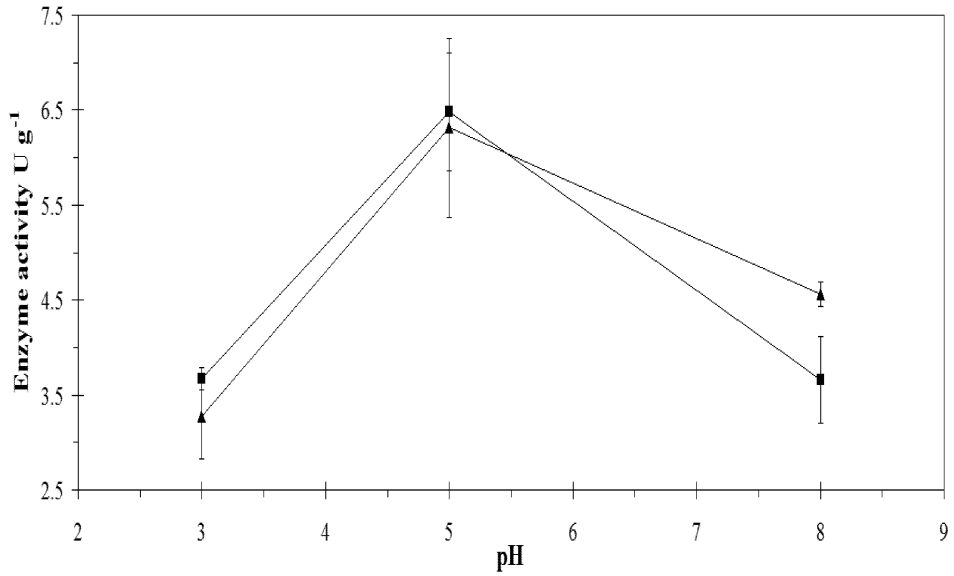

Figure 2. *pH and incubation temperature (25-▲- and* 30ºC-■*-) effect on the* α*-galactosidase activity in SSF using green soybean as substrate.*

3.3 Effect of pH and temperature in α-galactosidase production

The results obtained show that the best condition for α-galactosidase production is pH 5.00 and a culture temperature 30±1ºC when evaluated in both substrates. Likewise results reported Shankar and Mulimani (2007) who obtained an optimal pH 5.5 for the enzyme production. Changes in medium pH have important impact, at pH values of 3.00 and 8.00 enzyme activity decreases about a 50% in the evaluated substrates. This

result is valid just for SSF, for submerged cultivation, more acidic pH can be obtained as the optimal (Shankar and Mulimani, 2007).

The best culture temperature for α-galactosidase production was 30±1ºC, which is similar to reported by Shankar and Mulimrani (2007). Incubation tempeature is an important factor in SSF; due to the absence of water, heat is acumulated in the bed this affect the fungus metabolism, decreasing enzyme production (Raghavarao *et al.*, 2003). In the present study, for both substrates no significant difference (about 1%) was observed in the enzyme activity for the best pH (5.00) at 25±1ºC and 30±1ºC, because of this, fermentations could be carried at the lower temperature 25ºC, avoiding metabolic inactivation of the microorganism because of heat acumulation.

3.4 Effect of incubation time in α-galactosidase production

Figure 3 shows the fermentation profiles for the best conditions of α-galactosidase production in the evaluated substrates.

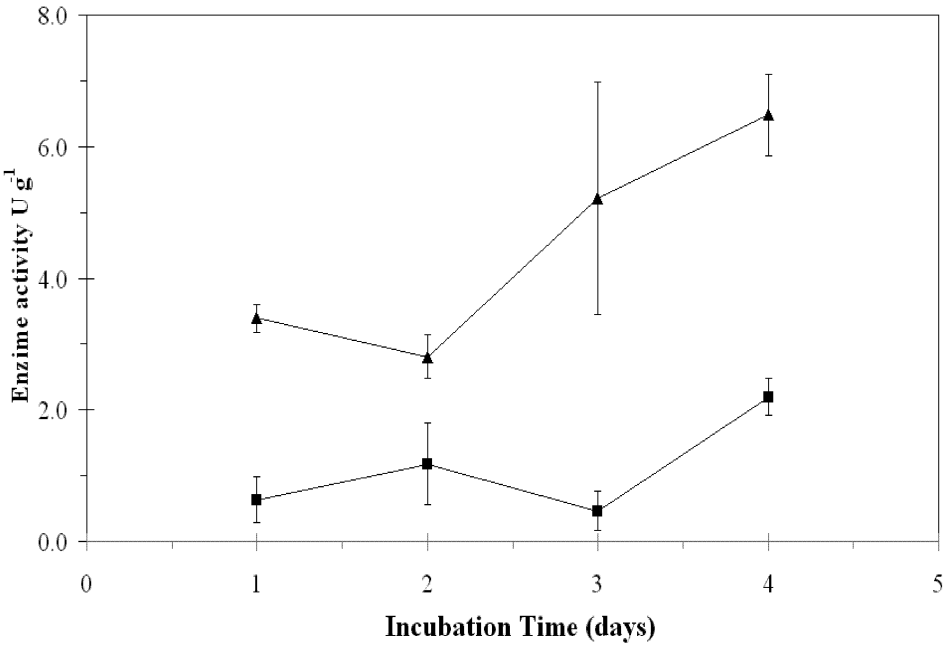

Figure 3. *Effect of incubation time in the α-galactosidase production by A. oryzae in SSF. (green soybean-▲- and orange peels-■-)*

For the fermentation in orange peels, activity didn't show considerable changes during the first 3 days, and in the fourth day activity increased about 100%, this suggest that in the first days of fermentation, the microorganism was on its lag phase, and in the last day it begins its exponential phase increasing the enzyme production, suggesting that growth of the mycelium is nedded for the production of extracellular proteins. (Ramachandran *et al.*, 2004)

For the fermentation in green soybean, the increase in enzyme activity began in the second day. This posibly is due to the composition of green soybean, which could be a better carbon source for α-galactosidase production than orange peels.

Profiles show a better enzyme activity in the last day of incubation, this suggest that fermentations could be carried out for more than 4 days obtaining a better enzyme activity, this is supported by Cruz and Park (1982) and Mulimani *et al.*(1986), who reported a fermentation time of 5 days. Further assays should be made to find the greater enzyme activity.

4. Conclusions

Orange peel and green soybean can be used as a potential substrate for α-galactosidase production under solid state fermentation by *A. oryzae,* with higher enzyme production in the fermentation using green soybean producing 6.48±1.22. The enzyme activity in orange peels is higher than the reported previously by Shankar and Mulimani (2007). The best pH and incubation temperature obtained were 5.00 and 30±1°C. The difference between evaluated temperatures at best pH is not significant, because of this, fermentations can take place at lower temperatures, which result in energy saving.

The use of green soybean as a substrate for the production of α-galactosidase under SSF could be of great commercial value, especially in agroindustrial countries like Colombia. The employed native strain is a potential source for α-galactosidase production; further assays should be made for the scale-up of the solid state fermentation process in order to increase the enzyme production.

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