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Immobilization of Aspergillus niger GH1 Tannase for the Production of the Antioxidant Gallic Acid

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***Abstract*— In the present research work, a immobilized tannase system was used to produce gallic acid from two sources, methyl gallate and tannic acid. Tannase was obtained by solid-state culture (SSC) using the strain of A. niger GH1. The tannase extract was concentrated with polyethylene glycol 6000 at 4°C for 12 h. Tannase activity was assayed by a HPLC method. The tannase activity detected in the concentrated extract was almost 40 times higher than the activity in the extract crude. The results obtained in the degradation of tannic acid by the immobilized tannase showed that the concentration of gallic acid was higher (0,387 g/L) than that obtained with methyl gallate (0,199 g /L). Production of gallic acid with an enzymatic process is an attractive alternative that permits the reuse of the biocatalyst.**

***Index Terms*—Tannase, Gallic Acid, Immobilization.**

I. INTRODUCTION

Gallic acid (3,4,5-trihydroxibenzoic acid) is a potent phenolic antioxidant which can be applied in a great diversity of active and intelligent foods. This acid is mainly produced under fermentation conditions.). Tannins are high molecular weight polyphenolic compounds that exist in a variety of plant species. Tannase or tannin acyl esterase (EC 3.1.1.20) is the enzyme responsible for the decomposition of gallotannins and complex tannins to glucose and gallic acid and derivates of catechin and gallic acid, respectively (Aguilar et al., 2007).

Applications of tannase are concentrated in the leather-processing, food and pharmaceulitcal industries (Belmares et al., 2004). For industrial application, the immobilized form of enzyme offers, several advantages, including use on the enzyme, case of product separation, improvement of enzyme stability and continuous operation in packer-bed reactors. However, there are few reports on immobilized tannases (Abdel-Naby et al., 1999).

This work describes the immobilization of *Aspergillus niger* GH1 and its application in the production of gallic acid.

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The properties of the immobilized enzyme were compared with those of the free enzyme.

II. MATERIALS AND METHODS

A. Microorganism

Aspergillus niger GH1 (DIA/UAdeC-Collection) was the fungal strain used as producer of tannase. Spores cryo-conserved at -70ºC were activated by inoculation on PDA (potato dextrose agar) plates. Petri dishes were incubated at 30ºC during 3 days. Spores were harvested using a solution of 0.01% Tween 80. Then, spores were counted in a Neubauer chamber.

B. Solid-State Culture

Polyurethane foam microparticles were impregnated (at 70% of moisture) with the culture broth previously inoculated with the spores of *A. niger* GH1 (inoculation level of $2x10^6$ spores per gram of support). Culture conditions were: tray reactor, 30ºC of temperature, a initial pH of 5.5, initial humidity 70% and 20 h of culture time. Composition of culture broth was that reported previously by Rivas-Martinez *et al*., (2008), using tannic acid as carbon source and sodium nitrate as nitrogen source. Crude extract of tannase was produced in triplicates.

C. Recovery of tannase

Each content of bioreactor was introduced in a mechanical plastic compressor and the liquid extract was recovered and conserved in plastics vials.

D. Concentration of tannase

Crude extract was dialyzed using a Sgima dialysis tubing with a pore size diameter of 12,000 Da. Then, the filled membrane was collocated on a bed of polyethylenglycol (PEG) 6000. Obtained extract was considered and concentrated extract and it was used for the studies of immobilization. Protein content was measured by the Bradford method and the tannase activity by the HPLC method (Rivas-Martinez, 2008).

E. Immobilization of tannase

Alginate at 2% was used to entrap the tannase enzyme. A enzyme:carrier ratio of 1:10 was used. A solution of 0.06M $CaCl_2$ was used to form the corresponding microparticules.

F. Production of gallic acid

Gallic acid was produced from two substrates, tannic acid and methyl gallate. Reaction conditions were: per each liter

of substrate, 1500 microparticles were used to produce the antioxidant, gallic acid, a reaction temperature of 60ºC, agitation of 300 rpm during 48 h of reaction. This experimental section was evaluated with triplicates.

III. RESULTS AND DISCUSSION

In this study a xerophilic strain of Aspergillus niger was used to produce under a solid state culture and immobilize the tannase enzyme using an alginate system. Figure 1 shows a picture the solid state culture bioreactor employed to produce the tannase activity.

Figure 1. Microparticules of polyurethane foam impregnated with the culture broth (right) and tray reactor used to produce the tannase (left)

The tannase activity detected in the extract crude was 109.92 U/L, while in the extract concentrated the activity was 4268.26 U/L almost 40 times higher that the activity in the extract crude (Table 1).

Table 1. Concentration of tannase by dyalisis on PEG

	Crude extract	Dialyzed extract	Concetrated extract
Volume (L)	0.40	0.36	0.03
Proteín (mg/L)	0.49	0.12	2.82
Volumétric activity (U/L)	109.92	1650.40	4268.26
Specific Activity (U/mg)	225.71	13203.22	1510.89
Total activity (U)	43.97	594.14	132.32

During the entrapment there was not residual activity, which indicates 100% encapsulation of the tannase in alginate. The results obtained in the degradation of tannic acid showed that the concentration of gallic acid was higher (0,387 g/L) than that obtained with methyl gallate (0,199 g /L).

Table 2 presents the kinetic results of the use of immobilized tannase to produce gallic acid. Km and Vmax values were determined under similar reaction conditions.

Table 2. Kinetic constants of immobilized tannase.

Substrate	K_M (M)	V_{max} (mM/min)
Tannic acid	$2.3*10^{-7}$	$1.3*10^{-9}$
Methyl gallate	$1.3*10^{-6}$	$1.81*10^{-10}$

Respect to uses of tannase, one of the major applications of tannase is in the manufacturing of instantaneous tea. Tannase applications in food and beverage industrial products contribute to remove the undesirable effects of tannins. Enzymatic treatment of fruit juices to reduce the bitterness has got advantages such as the higher quality of juice due to the lower haze and non-deterioration of juice quality. New fruit juices (pomegranate, cranberry, raspberry, cold tea, etc) have recently been acclaimed for their health benefits, in particular, for its disease-fighting antioxidant potential. Presence of high tannin content in those fruits is responsible for haze and sediment formation as well as for colour, bitterness and astringency of the juice upon storage. Due to the inability of conventional fruit juice debittering processes for removing the bitterness effectively, enzymatic debittering should be preferred (Aguilar et al 2007).

Tannase has been also immobilized to reuse its catalytic action in agarose, chitosan, alginate, ceolite and different derivatives of silicious materials can be used for tannase immobilization. Abdel-Naby et al. (1999) immobilized tannase from *Aspergillus oryzae* on various carriers. However, the enzyme immobilized on chitosan - glutaraldeheyde showed the highest activity. The bound enzyme retained 20.3% of original specific activity. On the other hand, Sharma et al. (2002) immobilized tannase from *A. niger* on concavalin A-Sepharose via bioaffinity interaction. The immobilized preparation was quite stable to reuse, there was no loss of enzyme activity after three cycles and it retained 81% activity even after the sixth cycle. Ester hydrolysis using the immobilized enzyme led to a 40% conversion into gallic acid as compared with 30% obtained with the free enzyme. The gallic acid is used in the pharmaceutical industry for the synthesis of antibacterial drugs and in the food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates.

IV. CONCLUSION

The operational stability of the immobilized *A. niger* GH1 tannase was evaluated in repeated batch process. The results indicated that the calatytic activity of the immobilized enzyme was durable under repeated use. Thus the immobilized enzyme was able to keep producing a good yield of hydrolysis products, with as high 90% of the initial catalytic activity after 15 runs. The overall performance on the immobilized tannase is promising. Production of gallic acid with an enzymatic process is an attractive alternative that permits the reuse of the biocatalyst.

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