Asian Journal of Bio Science, **Vol. 3 No. 2 : 275-278 (October, 2008)**

Immobilization of tannase for commercial use in food industries

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(Accepted : July, 2008)

Immobilization of tannase from *Aspergillus heteromorphus* was studied on DEAE-Sephadex A-50, Chitin and Ca-alginate. Among the three methods studied, DEAE-Sephadex A-50 was observed the best support to immobilize *A. heteromorphus* tannase with 91.5% immobilization. On the other hand Ca-alginate and Chitin supported 79.3% and 34.5% immobilization, respectively. When the immobilized enzyme was studied for its repeated use, a constant decline in the enzyme activity was observed. However, at the end of seventh cycle, operational stability of the immobilized enzyme was 88 %, 61.7 % and 55.7 % for Chitin, Ca-alginate and DEAE-Cellulose, respectively.

Key words : Tannase, Immobilization, Food industries, *Aspergillus heteromophus.*

INTRODUCTION

Tannin acyl hydrolase (E.C.3.1.1.20) commonly called as tannase, is a hydrolytic enzyme that acts on tannins (Lekha and Lonsane, 1997). Tannins are water soluble polyphenolic compounds with different molecular mass, which form hydrogen bonds in solution that results in the formation of tannin protein complexes and they are thus considered effective anti nutritional compounds. Tannase catalyse the hydrolysis of ester and depside linkages in hydrolysable tannins such as tannic acid, releasing gallic acid and glucose. (Haworth *et al.*,1985). The products of hydrolysis are glucose and gallic acid. Gallic acid is used in the synthesis of propyl gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages. Tannase is also extensively used for the preparation of instant tea, acorn wine, coffee-flavoured soft drinks, clarification of beer and fruit juices, and detannification of foods (Rodríguez *et al.*, 2008; Aguilar *et al.*, 2007 and Lekha and Lonsane, 1997).

Despite the several advantages of tannase in food industries, the commercial use has been limited because (a) most of them are relatively unstable; (b) the cost of enzyme isolation and purification is still very high, and (c) it is technically expensive to recover active enzyme from the reaction mixture after completion of the catalytic process (Kennedy *et al.*, 1987 and Lekha and Lonsane, 1997).These problems can be circumvented by immobilizing the enzyme. Owing to this, the objective of the present study was to immobilize tannase from *Aspergillus heteromorphus*.

MATERIALS AND METHODS

Microorganism and maintenance of culture:
A tannase producing fungus was isolated from the soil of G.J.U.S.&T. campus and identified as *Aspergillus heteromorphus*. The strain was sub-cultured at an interval of 4–5 weeks and maintained routinely on potato dextrose agar (PDA) slants. Freshly grown slant cultures (30^0C) were then used for further work or stored at 4^0C.

Preparation of spore inoculum:
Fungal spore inoculum was prepared by adding 2.5 ml of sterile distilled water containing 0.1 % Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and the number of spores in the suspension was determined using the Neubauer chamber. The volume of 1 mL of the prepared spore suspension was used as the inoculum, with concentration of $5x10^9$ spores.

Fermentation of medium:
For the fermentation process, a 250 mL Erlenmeyer flask with 50 ml of Czapek Dox minimal medium (Bradoo *et al.*, 1996) containing (g/L): $NaNO_3$, 6; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4$. $7H_2O$, 0.52; $FeSO_4$. $7H_2O$, 0.01; $ZnSO_4$. $7H_2O$, 0.01 was employed. The medium was adjusted to pH 5.0 and then sterilized at 121°C for 15 min. Tannic acid solution was prepared separately and the solution was adjusted to pH 4.5 with 0.1 M NaOH, then sterilized by filtering through a sterile membrane (pore size 0.2 mm) and added to the sterilized medium afterward to adjust the final tannic acid concentration of 1%. Flasks were incubated at 30^0C in an incubator shaker at 150

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rpm for 48 h. After the desired incubation period, biomass was harvested by filtration through Whatman No.1 filter paper and the cell free culture broth was assayed for extracellular tannase activity.

Tannase assay:

Tannase activity was determined colorimetrically using the modified method of Mondal *et al.* (2001). The reaction mixture contained 0.6 ml of tannic acid (0.5% in 0.2M sodium acetate buffer, pH 5.5), 0.2 g of enzyme and was incubated at 30^0C for 20 min followed by centrifugation (3000xg 10 min), 0.3ml of supernatant was taken and 3ml of BSA solution was added, which resulted in precipitation of tannic acid. The tubes were centrifuged (5000xg 10 min) and the resultant precipitate was dissolved in 3ml SDS-triethanolamine solution. One ml of $FeCl_3$ reagent was added to each tube and was kept for 15 min at room temperature for stabilization of the color. The absorbance was read at 530nm against the blank (*i.e.* without tannic acid). One unit enzyme activity is defined as the amount of enzyme required to hydrolyze 1 mmol of tannic acid in 1 min.

IMMOBILIZATION OF TANNASE :

Tannase enzyme was immobilized on various carriers by different methods:

Ionic binding:

Anion exchanger (DEAE-Sephadex A-50, 0.1g) equilibrated with 0.2 M acetate buffer (pH 5.5), was incubated with four different volumes (5 ml, 10ml, 20ml and 30ml) of the enzyme (*Aspergillus heteromorphus* tannase having enzyme activity 1.7 units/ml) solution dissolved in same acetate buffer, for 12 hours at 4ºC.The unbound enzyme was removed by filtering the mixture and washing with acetate buffer until no enzyme or soluble protein was detected in the filtrate. The immobilized enzyme was assayed for enzyme activity.

Physical adsorption

Chitin (0.5 g) was incubated with the different volumes (2.5ml,5ml,10ml and 15ml) of enzyme solution (*Aspergillus heteromorphus* tannase) dissolved in 0.2 M acetate buffer (pH 5.5) at 4ºC overnight. The unbound enzyme was removed by filtering the mixture and washing with same acetate buffer until no enzyme activity or soluble protein was detected in filtrate. The immobilized enzyme was assayed for enzyme activity.

Entrapment in Ca-alginate:

5 ml of 5 %(w/v) Na-alginate was mixed with different volumes (2.5ml, 5ml, 10ml and 15ml) of *Aspergillus heteromorphus* tannase. This mixture was dropped into 100 mM Calcium chloride solution so as to form beads. The beads were collected and washed with acetate buffer to remove unbound enzyme. The immobilized enzyme was assayed for enzyme activity.

Operational stability:

Immobilized *Aspergillus heteromorphus* tannase was assayed using modified method of Mondal *et al.* (2001). At the end of reaction, the immobilized tannase was collected by centrifugation at 5000 rev min^{-1} for 20 min followed by washing with distilled water and was further incubated with 0.6ml of tannic acid to start a new reaction

RESULTS AND DISCUSSION

Immobilization of tannase:

Immobilization by ionic, entraptment and physical adsorption are the simple and cheap methods to prepare an immobilized enzyme. Table 1 shows the results of immobilization of tannase from *Aspergillus heteromorphus* on DEAE-Sephadex A-50, Chitin and Ca-alginate. Among the three methods studied, DEAE-Sephadex A-50 was observed the best support to immobilize *A. heteromorphus* tannase with 91.5% immobilization. However, Ca-alginate and Chitin supported 79.3% and 34.5% immobilization, respectively. The possible reason for comparatively lower level of immobilization by Chitin may be that the adsorbed enzymes were rather susceptible to desorption from the carrier (Rua and Ballesteros, 1994). While in case of Ca-alginate the rate of mass transfer of the substrate and products to and from immobilized enzymes presents problems not found with free enzymes. The percentage of immobilization in the present study on DEAE-Sephadex A-50 (91.5%) and Ca-alginate (79.3%) were found to be higher than that on Amberlite IR(25%) and DEAE-Cellulose(46%) as reported by Sharma *et al.* (2002) for the immobilization of tannase from Aspergillus niger. Earlier Abdel-Naby *et al.* (1999) also reported immobilization of tannase from Aspergillus niger on various carriers by different methods of immobilization including; physical adsorption on AS-alumina and colloidal chitin; ionic binding onto Dowex 50W and DEAE-Sephadex A-25; covalent binding on chitosan and chitin; and entrapment on polyacrylamide and ca-alginate. They found that the immobilized enzyme prepared by covalent binding to chitosan had the highest immobilized activity (107u\g carrier) and the highest immobilization yield (26.6%). The decrease in specific activity of immobilized enzyme as compared to free enzyme may be due to

Table 1: Immobilization of *Aspergillus heteromorphus* tannase

Carrier	Volume of enzyme loaded (ml)	Total enzyme Loaded	Unbound enzyme	Immobilized enzyme
		(U/5ml gel)	(U/5ml gel)	(U/5ml gel)
Calcium alginate	2.5	4.25424	2.11054	2.14361
	5	8.50849	3.04199	5.46659
	10	17.0169	5.15265	11.8642
	15	25.5255	5.34938	20.1762
Chitin		(U/2.5g carrier)	(U/2.5g carrier)	(U/2.5g carrier)
	2.5	4.25424	4.21052	-
	5	8.50849	8.6106	-
	10	7.0169	3.33189	3.68509
	15	25.5255	16.6914	8.83346
		(U/0.2g carrier)	(U/0.2g carrier)	(U/0.2g carrier)
DEAE Sephadex	5	8.508449	1.96122	6.547227036
	10	17.01688	2.39664	14.62023397
	20	34.03375	3.37305	30.66074523
	30	51.05063	4.33812	46.71252166

diffusional limitation *i.e.* resistance of the substrate to diffuse into the immobilization matrix and resistance of the product to diffuse out. These diffusional processes often result in a lower concentration of substrate and a higher concentration of product at the enzyme site than in the bulk solution. With increasing enzyme activity, the diffusional problems become more significant. Immobilization of an enzyme on the surface of a carrier usually changes the Km of that enzyme. Some apparent Km values of immobilized enzymes are larger than the values for soluble enzymes, and others are smaller. Kinetics of immobilized enzymes are generally affected by carrier and substrate charges, pore diameter of the carrier, bulk and pore diffusion rates, and many other parameters (Bissett and Sternmerg,1977).

When amount of enzyme loaded was studied with

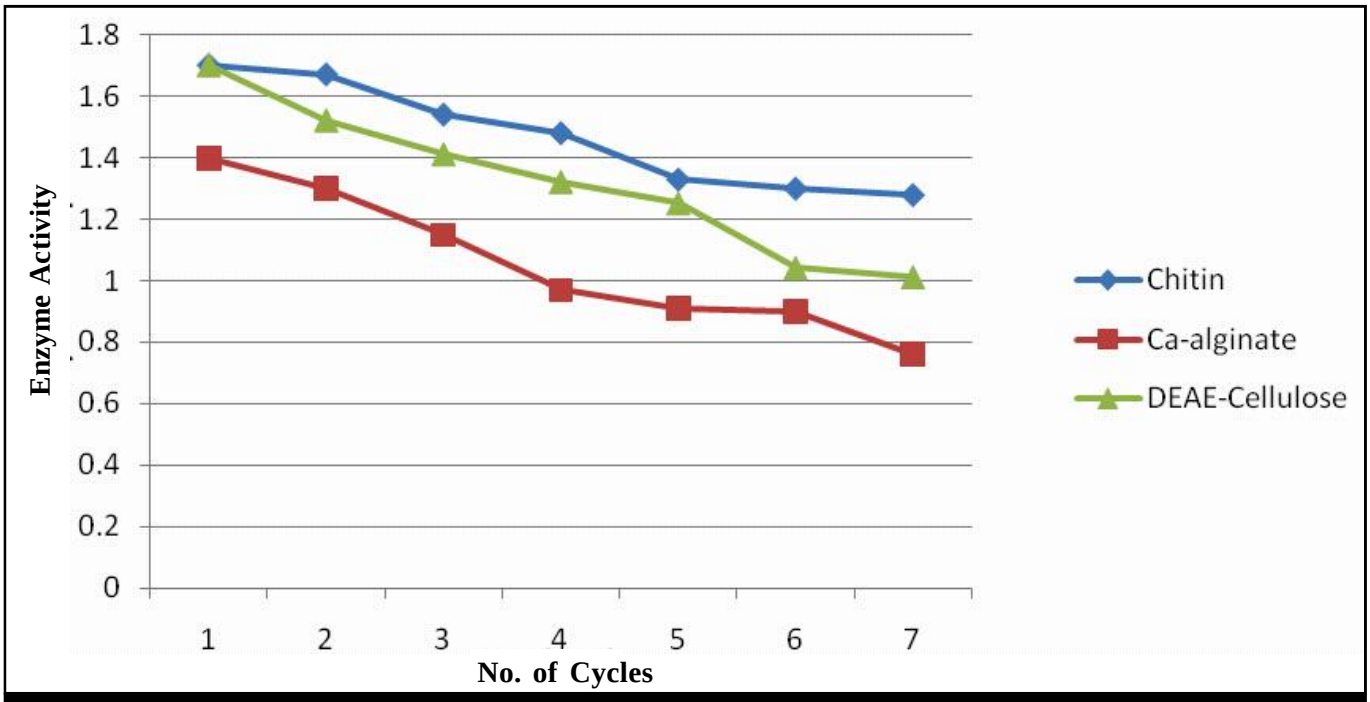

Fig. 1 : Operational stability of chitin, Ca-alginate and DEAE Sephadex immobilize tannase after repeated use

respect of enzyme immobilization, it was found that percentage of immobilized enzyme was increasing with the increase in total enzyme loaded but in case of physical adsorption by chitin, enzyme was not immobilized when lower concentration of the crude enzyme was loaded.

Operational stability of the immobilized tannase:
When the immobilized enzyme was studied for its repeated use, it was found that immobilized enzyme preparation could be recycled at least seven times without appreciable loss of activity. The activity at the seventh cycle was still 88%, 61.7 %and 55.7% of the activity of the starting preparation in case of enzyme immobilized by Chitin, DEAE-Sephadex A-50 and Ca-alginate, respectively. Abdel-Naby *et al.* (1999) evaluated the operational stability of the immobilized Aspergillus oryzae tannase in repeated batch process. They reported that the immobilized enzyme was durable under repeated use, producing a good yield of hydrolysis products, with as high as 85% of the initial catalytic activity after 17 runs

It may be concluded from the present study that the overall performance of immobilized *A. heteromorphus* tannase catalytic activity and its possible reuse are more promising than for the free enzyme. Accordingly it is suggested that *A. heteromorphus* tannase immobilized on DEAE-Sephadex A-50 by ionic bonding is suitable for practical applications.

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