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Immobilization of *Penicillium notatum* NCIM 923 tannase and properties of immobilized tannase compared with the free enzyme

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Tannase was effectively immobilized on alginate by the method of cross linking entrapment with a high activity recovery of 75.59%. The properties of immobilized tannase were investigated. Bead size of 0.70x32mm and 1% glutaraldehyde for cross linking were found to be optimum. Its optimum temperature was determined to be 45^{0}C, increasing 5^{0}C compared with that of free enzyme, whereas the optimum pH of 5.0 did not change. The thermal and pH stabilities of immobilized tannase increased to some degree. Glutaraldehyde cross linked beads showed more operational as well as storage stability compared with non cross linked beads. The kinetic parameters, Km, for immobilized tannase was estimated to be 0.66x10-2M and Vmax was 16.9U/mg.

Key words: Alginate, fermentation, glutaraldehyde, immobilization, tannase

INTRODUCTION

A biocatalyst is termed immobilized, if its mobility has been restricted by chemical means. Immobilization of enzymes refers to techniques which represent variety of advantages over free enzyme catalysis including increased stability of enzyme, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme which will ultimately save the enzyme, labor and overhead costs (Gerhartz, 1990). Immobilized enzymes have been widely used for many years in different industrial processes. Usually, immobilization of enzymes is carried out by three principal means, matrix assisted entrapment of enzyme, adsorption on a solid support, ionic or covalent binding (Swaisgood, 1985; Zoborsky, 1973). The use of gel entrapment method (entrapping the enzyme within the interstitial spaces of cross-linked water insoluble polymer gels) is very common and the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, protects enzyme from microbial contamination (Kennedy, 1987). Alginate beads are reported to be one of the most popular carriers for enzyme immobilization (Fujii *et al*. 1999). The salts of sodium are water-soluble, whereas those of polyvalent cations, like calcium are water insoluble and can be used for the purpose of immobilization. This process of gelation simply involves the exchange of calcium ions with sodium ions under relatively mild conditions (Hota *et al*. 2007). The nature of the solid support or matrix plays an important role in retaining the actual confirmation and activity of enzyme in the processes that utilized immobilized biocatalysts (Riaz *et a*l. 2009).

Tannase (E.C. 3.1.1.20, tannin acyl hydrolase) is an extracellular enzyme produced by several microorganisms and plant cells (Pourrat *et al*. 1982). It is the enzyme responsible for the decomposition of hydrolysable tannins to glucose and gallic acid (Lbuchi *et al.* 1972). Tannase is one of the most important and widely used enzymes whose spectrum of applications are concentrated in the leather processing, food and pharmaceutical industries (Giovanelli, 1989; Majumdar and Moudgal, 1994). These industries would find their boosted economy if tannase can be re-used which is possible by their immobilization. Therefore, the present study is attempted to immobilize tannase produced by *Penicillium notatum* NCIM 923 in sodium alginate using glutaraldehyde as a coupling agent. The

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properties of the immobilized enzyme were compared with those of the free enzyme in order to explore the benefits of immobilization of enzymes.

MATERIALS AND METHODS

Microorganism

Penicillium notatum NCIM 923 was collected from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune (India) and was maintained on Czapek Dox agar slants of composition - Glucose 5%, $NaNO_3$ 0.2%, KCl 0.05%, $MgSO_4.7H_2O$ 0.05%, $FeSO_4.7H_2O$ 0.001%, KH_2PO_4 0.1%, Agar 3% pH 5. The fungal strains were cultured periodically, grown at 30^{0}C for 6 days and were stored at 4^{0}C.

Chemicals and glass wares

All the chemicals used were of analytical grade and the glass wares were from Borosil Ltd., Mumbai, India.

Substrates

Wheat bran was collected from local market and then dried at 60^{0}C in Tray drier (ICT, India) for 6 hrs. Used marigold flower (orange variety) was also collected from market, sorted, washed and then dried at 60^{0}C in Tray drier (ICT, India) for 6 hrs. Both the dried materials were milled (jx 5, Bajaj Electronics Ltd, India) to pass through 0.25 mm sieve and stored for future use.

Production of tannase under SSF

Fermentation medium used for tannase production contained 10g of substrate (wheat bran and marigold flower mixed in the ratio 4:1) (Gayen and Ghosh, 2008) in a 500 ml Erlenmeyer flask moistened with (1:1 w/v) distilled water. The contents were sterilized by autoclaving and after cooling the sterilized solid substrate was inoculated with spore suspension (1 x 10^7 spores/ml).The contents were mixed properly and incubated at 30^{0}C for 96 hrs under stationary condition (Gayen and Ghosh, 2008).

Extraction and analysis of crude enzyme

Mouldy substrate produced by SSF was mixed with distilled water (1:5 w/v) and agitated for 2 hrs at 90 rpm in a shaker incubator and filtered through cheese cloth followed by centrifugation at 10,000 rpm for 20 mins. The clear supernatant was used as crude enzyme. Tannase activity was estimated by a protein precipitation method (Iibuchi *et al*. 1966).The reaction mixture contained 1ml 1% tannic acid (in citrate phosphate buffer, pH 5.0), 2ml of citrate phosphate buffer (pH 5.0) and 1ml of the culture filtrate. The mixture was incubated at 37^{0}C for 30 mins in a water bath. The reaction was stopped by adding 4ml 2% BSA solution. In the control BSA was added in the incubation mixture prior to incubation. All tubes were left for 20mins at room temperature to precipitate residual tannins and were centrifuged at 3000xg for 20mins. The tannase activity in the supernatant was estimated after appropriate dilution and reading O.D. at 260nm (this wavelength corresponds to the optimal absorption of gallic acid) against double distilled water as blank. One enzyme unit is the amount of enzyme that liberates 1µ mol gallic acid per ml per min under standard assay conditions. Total soluble protein was determined by the method of (Lowry *et al*. 1951) and was expressed in mg/ml.

Partial purification of tannase

The cell free filtrate was then precipitated with 75% ammonium sulphate. Precipitated protein was collected by centrifugation at 10,000 rpm for 15 mins at 4^{0}C. The precipitate obtained after ammonium sulphate precipitation was dialyzed against 0.1M citrate phosphate buffer (pH 5) in order to remove the ammonium sulphate from the precipitate. Dialysis was carried out for 24 hrs with several changes of 0.1M citrate phosphate buffer (pH 5). The enzyme activity and protein content of the dialyzed fraction was measured.

Immobilization of partially purified and immobilized enzyme assay

Partially purified and dialyzed tannase was immobilized by entrapment method using calcium alginate gel. 10 ml dialyzed enzyme solution was added to 20 ml sodium alginate solution (1-4 %) w/v and mixed thoroughly. Then the solution was added to calcium chloride solution (0-5 %) w/v drop by drop using hypodermic syringe (0.70 x 32mm). The beads were allowed to cure at 30^{0}C in calcium chloride solution for 2 hrs (Mahendran *et al*. 2006). The beads were collected and washed twice with

distilled water and once with 0.1M citrate phosphate buffer to remove the unbound enzyme and then air dried. Beads were then ready for use. Beads were stored at 4^0C till further use.

The immobilized beads (4g) were added to 100 ml of 1 % tannic acid solution in 250 ml Erlenmeyer flasks and incubated for 30mins for the assay. Reaction samples were taken for analysis of hydrolytic product. The control experiments were also performed using only calcium alginate beads without tannase immobilization (Mahendran *et al*. 2006). Immobilization efficiency was calculated from the following formula (Riaz *et al*. 2009).

$$\text{Immobilization efficiency (\%)} = \frac{\text{Specific activity of enzyme after immobilization}}{\text{Specific activity of enzyme before immobilization}} \times 100$$

Alginate concentration on immobilization

The concentration of alginate was varied from 1-4% (w/v) and enzyme immobilization was done as stated above.

Calcium chloride solution on immobilization

The concentration of calcium chloride was varied from 0-5% (w/v) during preparation of beads. Assay of immobilized beads was done and immobilization efficiency was calculated.

Time of glutaraldehyde treatment on immobilization

Glutaraldehyde (1 % v/v) cross linking was done for 30 mins during beads formation (added to sodium alginate and enzyme solution) and after beads formation (beads treated for 30 mins) and assay of immobilized enzyme was done in each case.

Concentration of glutaraldehyde on immobilization

Immobilization was carried with different concentrations of glutaraldehyde (0.5 %. 1 %, 1.5 % and 2 % v/v) during beads formation.

Beads size on immobilization

The needle size was varied from (0.55 x 25 mm), (0.70 x 32 mm) and (0.80 x 38 mm) during preparation of beads.

Repeated use of immobilized tannase

To test the stability of immobilized tannase, the beads (glutaraldehyde coated and uncoated) were used several times for the hydrolysis reaction. Each cycle was conducted for 30mins. After every cycle, the used solution was analyzed to check formation of gallic acid. Beads were then collected, washed twice with 0.1 M citrate phosphate buffer and used for subsequent reaction experiments with fresh tannic acid (Mahendran *et al.* 2006). Residual activity was estimated under standard conditions and expressed as percentage of the relative tannase activity.

Storage stability of immobilized tannase

The storage stability of immobilized tannase was studied by using the beads (glutaraldehyde coated and uncoated beads) after every 7 days up to 28 days. Residual activity was estimated under standard conditions and expressed as percentage of the relative tannase activity.

Temperature activity profile

To determine the temperature activity profile the enzyme substrate reaction was carried out at various temperatures (30, 35, 40, 45, 50, 55 and 60)^{0}C using the partially purified free and immobilized enzyme in 0.1M citrate phosphate buffer (pH 5) for 30 mins with tannic acid as substrate and enzyme activity was measured (Sivashanmugam and Jayaraman, 2011).

Thermal stability

Thermal stability of the partially purified free and immobilized enzyme was determined by 60 mins incubation over the temperature range (30, 35, 40, 45, 50, 55 and 60)^{0}C at (pH 5). Residual activity was estimated under standard conditions and expressed as percentage of the relative tannase activity (Burnecka-Kasiezka *et al*. 2007).

pH activity profile

The pH activity profile for partially purified free and immobilized enzyme was determined at 30^{0}C by incubating the enzyme with substrate at different

pH ranges from 3 to 8. The pH of the reaction mixture was varied using different buffers (0.1M citrate phosphate buffer for pH 3-7 and tris HCl buffer for pH 8) and enzyme activity was measured.

pH stability

The stability of the partially purified free and immobilized enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3-8 (0.1M citrate phosphate buffer for pH 3-7 and tris HCl buffer for pH 8) for 12hrs at 30^0C. Residual activity was estimated under standard conditions and expressed as percentage of the relative tannase activity (Battestin and Macedo, 2007).

Kinetic parameters

The effect of substrate concentration on the activity of partially purified free and immobilized tannase was determined by using different concentrations of tannic acid (0.5, 0.8, 1, 1.5, and 2) %, in the reaction mixture. The enzyme activity was determined after 30 mins incubation at 40^0C for free enzyme and 45^0C for immobilized enzyme. Km and Vmax were determined by plotting the reaction velocity against the substrate-tannic acid-concentration in the Lineweaver- Burk plot (Lineweaver and Burk, 1934).

Scanning Electron Microscope (SEM)

Scanning electron microscope was used to view the surface texture of beads without enzyme immobilization, beads with enzyme immobilization but without glutaraldehyde cross linking and beads with enzyme immobilization as well as glutaraldehyde cross linked.

All the experiments were done in triplicate and the mean values with standard errors are reported.

RESULTS AND DISCUSSION

Tannase from *P.notatum* NCIM 923 was immobilized by entrapment in calcium alginate beads. The immobilization was carried out by varying the concentration of sodium alginate (1-4% w/v) and calcium chloride (0-5% w/v) respectively (Table1a). Among the various concentrations of sodium alginate and calcium chloride used maximum immobilization efficiency of 75.59% (Table 1b) was obtained with 3% sodium alginate as well as calcium chloride and it was therefore used in the succeeding part of this work. The specific activity exhibited by free tannase was 11.43 U/mg. However the specific activity of immobilized tannase was 8.64 U/mg. This drop in the specific activity after immobilization may be due to diffusion limitation (i.e., resistance to diffusion of the substrate into the immobilization matrix and resistance to diffusion out of the products) (El-Tanash *et al*. 2011). A decrease in specific activity after tannase immobilization has been previously reported (Abdel-Naby *et al*. 1999). There are two counteracting factors affecting the efficiency of the immobilization by entrapment, the diffusion efficiency and enzyme leak out. Higher concentrations of alginate reduce the pore size and consequently reduce the leakage of enzyme. A lower percentage of alginate on the other hand increases the pore size which leads to increased leakage of enzyme but reduces the limitations of substrate diffusion (El-Tanash *et al*. 2011). The concentration of cationic solution (calcium chloride used here) also has a significant effect on the stability and pore size of the bead (Kumaravel and Rajakumar, 2010). So, optimization of the concentration of sodium alginate and calcium chloride is essential for successful immobilization of any enzyme. However 3% sodium alginate and 2% calcium chloride solution was reported for tannase immobilization from *Rhizopus oryzae* (Hota *et al.* 2007) and 4% sodium alginate and 2% calcium chloride was needed for tannase immobilization from *Aspergillus niger* ITCC 6514.07 (Srivastava and Kar, 2010). Mahendran *et al*.2006 used 2.5% sodium alginate and 0.6 M calcium chloride for immobilization of tannase from *Paecilomyces variotii*.

Use of a cross-linking agent (glutaraldehyde) during immobilization probably increases the local surface area, which contributes to minimizing the steric affect around the active site of the immobilized enzyme and also hardens and improves the beads texture to prevent leakage of the enzyme (El-Tanash *et al*. 2011; Tanriseven and Dogan, 2002). Addition of glutaraldehyde (1%) during immobilization results in better cross linking (9.55 ±0.035 U/mg) than treating the beads with glutaraldehyde (1%) after their curing (8.83 ±0.055 U/mg) (Fig. 1). Hydroxyl groups of alginate can react better with the aldehyde groups of glutaraldehyde during beads formation than after beads have formed (Yeom and Lee, 1998). Abdel- Naby *et*

*al.(*1999) added glutaraldehyde during the formation of beads with chitosan for immobilization of tannase. Li *et al*.(2007) added glutaraldehyde during the formation of beads by sodium alginate for immobilization of pectinase. Ortega *et al*.(2009) added glutaraldehyde during the formation of beads with sodium alginate for immobilization of neutrase. Various concentrations of glutaraldehyde (0-2% v/v) were also used during the process of immobilization to achieve optimum immobilization efficiency (Fig.2). From the figure it is clear that better specific activity (9.03±0.073 U/mg) was obtained with 0.5% concentration of glutaraldehyde than that of control (8.65 ±0.135 U/mg) with no glutaraldehyde. However, 1% glutaraldehyde concentration was optimum (9.53 ±0.178 U/mg) for the crosslinking process. There are reports of addition of glutaraldehyde (2.5% v/v) during formation of beads with chitosan for tannase (Li *et al*. 2007), 2% v/v with sodium alginate for pectinase (El-Tanash *et al*. 2011) and 6.2% v/v for neutrase (Ortega *et al*. 2009).

Size of the beads has great importance in the immobilization process. Decrease in bead diameter increases enzyme leakage and too much increase in bead size affect mass transfer barrier (Kumaravel and Rajakumar, 2010; Srinivasulu *et al*. 2004). From Fig. 3 it is clear that the bead size of 0.72 x 32 mm is optimum (8.71 ±0.053) for the immobilization process. Increasing or decreasing the bead size beyond this level reduces immobilization efficiency.

The operational stability of immobilized tannase is the most important factor affecting the release of gallic acid in tannin bioconversion and in other industrial applications in food, beverage and juices to remove the undesirable effects of tannins such as astringency, bitterness and deterioration of juice quality (Srivastava and Kar, 2010; Belur and Mugeraya, 2011;Cruz-Aldaco *et al*. 2009).The operational stability of immobilized tannase (glutaraldehyde treated and untreated) was evaluated in repeated batch processes. After each run, the immobilized tannase was washed and reused at optimum conditions for another reaction. The results in Fig.4 indicate that tannase cross linked with glutaraldehyde retains the tannase enzyme on the beads more effectively than when glutaraldehyde is not used. It was observed that after 6 cycles about 85.21% of the original activity was retained by glutaraldehyde treated beads whereas about 77.01% of the original activity was retained by glutaraldehyde untreated beads. So, this process of immobilization with sodium alginate cross linked with glutaraldehyde may be effective industrially. Brodi and Neufled (2001) reported that there is an increase in the retention of tannase in alginate beads cross linked with glutaraldehyde. Abdel- Naby *et al*.(1999) reported an operational stability of 85% after 17 cycles with chitosan beads cross linked with glutaraldehyde for tannase. *A. niger* tannase immobilized on sodium alginate beads without cross linking with glutaraldehyde was used repeatedly for 7 cycles with 77% relative activity (Srivastava and Kar, 2010). There was 55% loss in activity after 4 cycles for pectinase entrapped in alginate without glutaraldehyde cross linking (Ipsita *et al*. 2003).

Table 1a: Optimal concentration of sodium alginate and calcium chloride for immobilization of tannase

	Enzyme specific activity (U/mg) % of Sodium alginate			
% of Calcium chloride	1	2	3	4
0	0.06±0.013	0.11±0.013	0.13±0.036	0.09±0.048
1	2.02±0.057	3.64±0.025	4.34±0.073	2.91±0.068
2	3.64±0.023	6.49±0.048	7.08±0.036	4.64±0.063
3	4.72±0.017	7.13±0.086	8.64±0.075	6.14±0.048
4	4.18±0.036	5.74±0.037	6.46±0.048	4.73±0.056
5	2.54±0.085	4.91±0.068	5.45±0.076	3.50±0.058

Table 1b: Immobilization efficiency of tannase by varying the concentrations of sodium alginate and calcium chloride

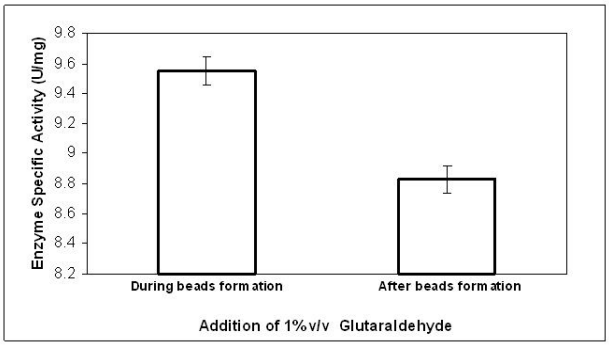
	Immobilization efficiency (%) % of Sodium alginate			
% of Calcium chloride	1	2	3	4
0	0.52	0.96	1.14	0.79
1	17.67	31.85	37.97	25.46
2	31.85	56.78	61.94	40.60
3	41.29	62.38	75.59	53.72
4	36.56	50.22	56.52	41.38
5	22.22	42.96	47.68	30.62

* Specific activity of partially purified free enzyme was 11.43 U/mg

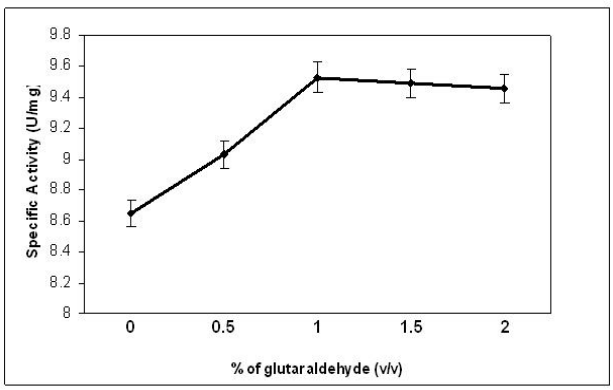
The stability of the enzymes might be expected to either increase or decrease on immobilization depending on whether the carrier provides a microenvironment capable of denaturing the enzymic protein or stabilizing it. Stability to denaturing agents may also be changed upon immobilization. Fig.5 represents the effect of storage time of immobilized tannase with or without glutaraldehyde. From the figure it is seen that sodium alginate beads cross linked with glutaraldehyde is quite stable and it retains 67.22% relative activity after 28 days of storage. Tannase immobilized on sodium alginate

beads without cross linking retained 52.75% relative activity after 28 days. â-galactosidase immobilized on alginate- gelatin fibre treated with glutaraldehyde was active for 35 days (Tanriseven and Dogan, 2002).

The effect of temperature on the activity of the immobilized and partially purified free tannase (Fig.6) shows that the optimum temperature is shifted from 40^{0}C for free tannase to 45^{0}C for immobilized tannase. This increase of the optimum temperature is probably a consequence of enhanced thermal stability (El-Tanash *et al*. 2011). Both the forms of enzyme were active in 30-60^{0}C temperature range. Abdel-Naby *et al.*(1999) reported that the optimum temperature of tannase from *A. oryzae* was determined to be 40^{0}C for the free enzyme and 55^{0}C for the immobilized form. The shift of optimum temperature to higher values after the immobilization of tannase was also reported by El-Tanash *et al*.(2011), Su *et al*.(2010), Sharma *et al*. (2008)and Srivastava and Kar(2010).



All the results were expressed in mean ±SD from n = 3
Fig. 1: Effect of glutaraldehyde treatment on immobilization of tannase

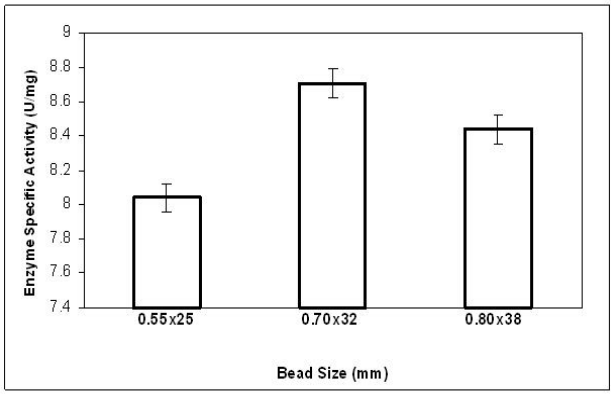


All the results were expressed in mean ±SD from n = 3
Fig.2 : Effect of various concentrations of glutaraldehyde on immobilization of tannase

The results in Fig.7 indicate that the immobilization process significantly improves the thermal stability of immobilized tannase relative to the partially purified free tannase. The immobilized enzyme had a higher thermal stability and retained 80.15% relative activity after 1hr at 60^{0}C, while the free enzyme retained 62.17% relative active under the same conditions. El-Tanash *et al*.(2011) reported that immobilized tannase retained 83% of its original activity after heating for 90 mins at 60^{0}C, while 76.2% of the activity was retained by the free enzyme after the same treatment.

The optimum pH of both immobilized and free tannase was found to be 5 (Fig.8). Curiel *et al*.(2010) reported a pH optimum of 5.0 for immobilized tannase from *Lactobacillus plantarum*. Sharma *et al*.(2008) reported that both immobilized and free *Aspergillus niger* tannase have the same optimum pH.

The profile of pH stability (Fig. 9) showed that immobilized tannase was more stable in a wider range of pH (3-8) compared with the free enzyme where it retained more than 60% relative activity after 12hrs. This result means that immobilized tannase would be more resistant to pH changes and could



All the results were expressed in mean ±SD from n = 3
Fig.3: Effect of beads size on immobilization of tannase

be used industrially. Enemuor and Odibo(2011) reported that the immobilized tannase had its maximum stability at pH 4, for 1hr and retained over 60% of its activity between pH 3.0 and 6.0. El-Tanash *et al*.(2011) reported that immobilized tannase was stable in the range of pH 4.5-8 where it retained 80% residual activity.

The plot of the rate of hydrolysis of tannic acid catalyzed by the free and immobilized enzyme against substrate concentration showed saturation kinet-

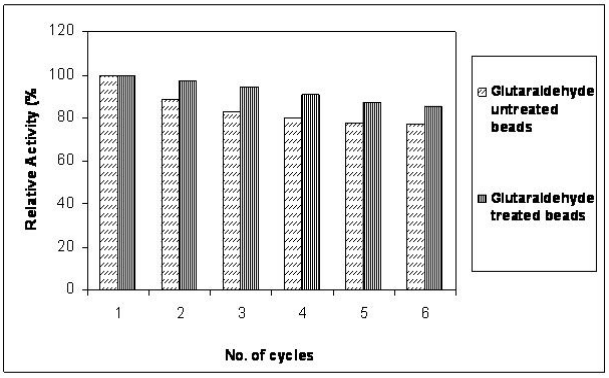


Fig. 4: Effect of repeated use of immobilized tannase

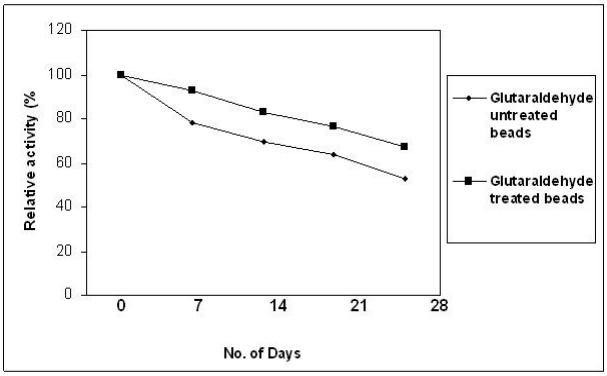
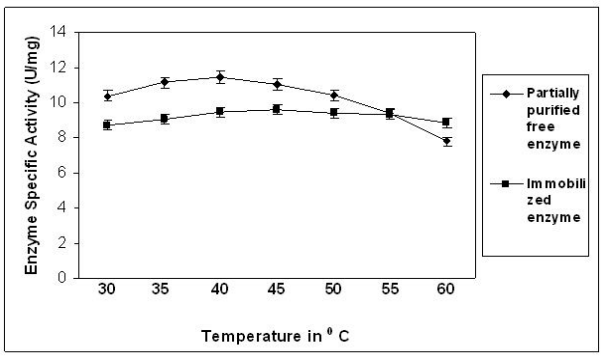


Fig. 5: Effect of storage stability on immobilized tannase



All the results were expressed in mean ±SD from n = 3
Fig.6: Effect of temperature on immobilized tannase activity

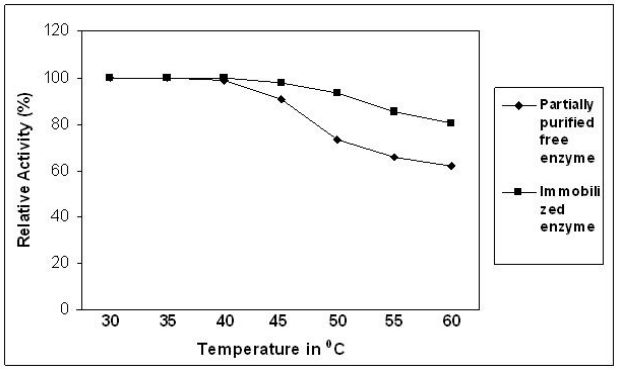
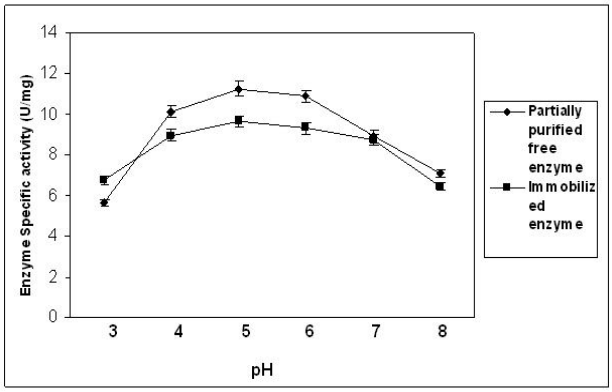


Fig. 7: Temperature stability of immobilized tannase



All the results were expressed in mean ±SD from n = 3
Fig.8 : Effect of pH on immobilized tannase activity

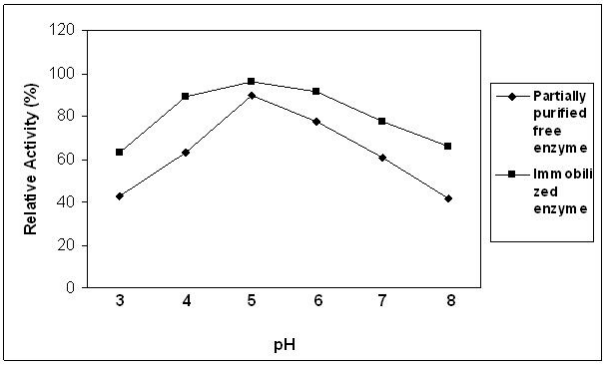


Fig. 9: pH stability of immobilized tannase

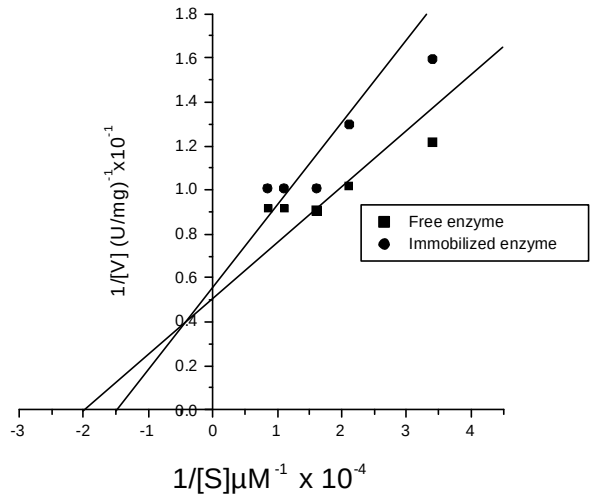


Fig. 10 : Kinetic parameters of free and immobilized tannase

ics, thus obeying Michaelis-Menten Kinetics (Fig.10). Lineweaver-Burk plots of the free and immobilized tannase gave Km (Michaelis constant) of 0.5 x 10-2 M and 0.66 x 10-2 M, respectively, with tannic acid. The Vmax (the maximum reaction rate) of the free and immobilized tannase were 17.9 U/mg and 16.9 U/mg respectively. This increase in Km value after immobilization may be

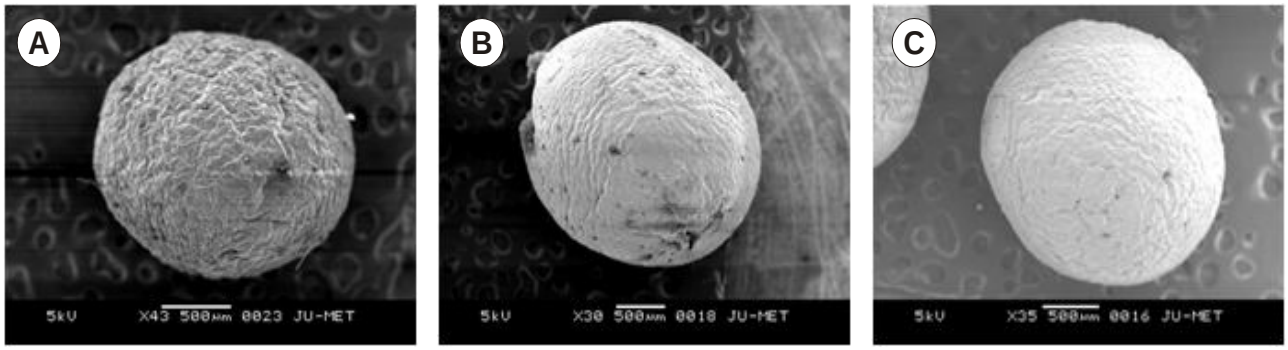


Fig. 11: A - Whole bead morphology of calcium alginate bead without immobilization with tannase
B - Whole bead morphology of calcium alginate bead with immobilization with tannase but without glutaraldehyde cross linking
C - Whole bead morphology of calcium alginate bead with immobilization with tannase and with glutaraldehyde cross linking

partially due to mass transfer resistance of the substrate into the alginate immobilization medium. Also, it could explain why the substrate had low accessibility to the enzyme –active sites (Abdel-Naby *et al*. 1999). However, multiple fixations due to covalent binding may lead to a decrease in the flexibility of the enzyme molecule, thus decreasing the catalytic activity (Gottschalk and Jaenicke, 1991). The increase in Km value and decrease in Vmax after enzyme immobilization have also been reported by other investigators (Abdel-Naby *et al*. 1999; El-Tanash *et al*. 2011; Su *et al*. 2010;Yu *et al*. 2004;Yu *et al*. 2007).

The whole bead morphology was studied using scanning electron microscopy (SEM). Clear difference can be seen between enzyme coated, uncoated and glutaraldehyde coated beads [Fig.11 (A, B, C)]. The SEM micrograph of glutardehyde coated bead showed significant surface texture changes compared with the untreated bead. This change in surface morphology may be somehow related to the increase in immobilization efficiency on cross linking with glutaraldehyde.

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