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CATALYTIC PROPERTIES OF IMMOBILIZED TANNASE PRODUCED FROM *Aspergillus aculeatus* COMPARED WITH THE FREE ENZYME

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Abstract - *Aspergillus aculeatus* tannase was immobilized on several carriers by entrapment and covalent binding with cross-linking. Tannase immobilized on gelatin with cross-linking agent showed the highest activity and immobilization yield. The optimum pH of the immobilized enzyme was shifted to a more acidic range compared with the free enzyme (from pH 5.5 to pH 5.0). The optimum temperature of the reaction was determined to be 50°C for the free enzyme and 60°C for the immobilized form. The thermal stability, as well as stability over a wide range of pH, was significantly improved by the immobilization process. The calculated K_m of the immobilized tannase (11.8 mg ml^{-1}) is higher than that of the free tannase (6.5 mg ml^{-1}), while V_{max} of the immobilized enzyme (0.32 U (µg protein)$^{-1}$) is lower than that of the free tannase (2.7 U (µg protein)$^{-1}$). The immobilized enzyme was able to retain 84 % of the initial catalytic activity after 5.0 cycles.
Keywords: Tannase; Enzyme immobilization; SSF, Green tea (*Camellia sinensis* L.).

INTRODUCTION

The hydrolysis of tannic acid by tannase results in the liberation of glucose, gallic acid and other galloyl esters of glucose (Van de Lagemaat and Pyle, 2006). Bacteria (Kumar *et al*., 2010), yeasts (Zhong *et al*., 2004) and filamentous fungi (Battestin and Macedo, 2007; Hamdy, 2008) are able to degrade hydrolysable tannins and produce tannase. However, *Aspergillus* and *Penicillum* are the best tannase producers during submerged (SmF) and solid state fermentation (Aguilar *et al*., 2007; Kumar *et al*., 2007). These fungi tolerate the higher concentrations of tannic acid and other tannery effluents without deleterious effects on either growth or enzyme production (Belmare *et al*., 2004). Further studies reported that tannase is mainly intracellular at the beginning, but is then secreted to the culture medium due to the proteolytic activities in the culture system (Aguilar *et al*., 2007).

Depend on the catalytic efficiency of gallic acid production, tannase has wide industrial applications. Gallic acid is used in pharmaceutical industry for synthesis of antibacterial and anti-malarial drugs (Trimethoprim) (Bajpai and Patil, 1997; Yu *et al*., 2004). In the food industry, gallic acid is also used as the substrate for the chemical synthesis of food preservatives antioxidants such as pyrogallol and gallates (Belmare *et al*., 2004; Vaquero *et al*., 2004). These antioxidants are also employed in cosmetics, hair products, adhesives and lubricant industry (Yu and Li, 2006). The applications of tannase in the food industry also include the removal of the undesirable effects of tannins as in instant tea (Boadi and Neufeld, 2001). Treatment of tea with tannase inhibits the carcinogenic and mutagenic effects of

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N-nitrosamines Lu and Chen, 2007). Furthermore, improvement of the color and organoleptic properties of tea occurred when it was treated with tannase (Lu *et al*., 2009). Tannase is also used in clarification to remove the unwanted bitterness in many fruits juices, lowering haze without deteriorating juice quality (Rout and Banerjee, 2006; Srivastava and Kar, 2010). In animal feed, tannase is used to reduce the anti-nutritional effects of tannins and improve animal digestibility (Graminha *et al.,* 2008; Murugan and Al-Sohaibani, 2010).

There are several methods for tannase immobilization. Entrapment inside a semi-permeable membrane capsule using sodium alginate is very common and cost effective and shows good performance in industrial applications. As anticipated, Abdel-Naby *et al*. (1999) observed a higher activity of tannase when cross-linked to the surface of chitosan beads than when entrapped in polyacrylamide or calcium alginate. Tannase could be bioaffinity immobilized on concanavalin A–Sepharose CL-4B beads (Sharma *et al*., 2002) and microencapsulated in a coacervate calcium alginate membrane (Yu *et al*., 2004), with higher conversion of methyl gallate/tannic acid into gallic acid than with the free enzyme.

To date, there are no publications focusing on immobilized *Aspergillus aculeatus* tannase. However, other immobilized tannases have been used for tannin degradation in tea extracts for instant tea manufacture and tea cream removal. This application has many advantages, such as avoiding heating of the tea, improved flavor and reduced costs (Boadi and Neufeld, 2002). For industrial applications, immobilized enzymes offer several advantages relative to the soluble form, including; repeated use of the enzyme, convenience of product separation, improvement of enzyme stability, and continuous operation in packed-bed reactors. Furthermore, other tannases could be immobilized for gallic acid production through bioconversion of tannin-rich agro/forest-residues (Hota *et al*., 2007; Cruz-Aldaco *et al*., 2009). Despite of its importance, more work on different forms of tannase is needed to increase its application and clearly established its optimum temperature, pH conditions, thermal stability and operational stability. This research is an extension of a study on tannase production from some *Aspergillus* species under solid state fermentation using commercial green tea (*Camellia sinensis* L) as solid substrate (Sherief *et al*., 2011).

The objective of this paper was to immobilize tannase to improve its thermal, pH and operational stabilities compared with the free enzyme.

MATERIALS AND METHODS

Microorganism and Culture Maintenance

The fungal strain used in the present study was isolated from a tannery soil sample and identified as *Aspergillus aculeatus* by the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt. The strain was sub-cultured on modified Czapek's agar media containing 0.5% tannic acid as sole carbon source at 30°C for 7.0 days and maintained at 4°C. Induced slants of *Aspergillus aculeatus* were mixed with 10 mL of basal medium to prepare the spore suspension. The spore count in the suspension was 2.0×10^7 spore mL^{-1}.

Raw Materials for Solid State Fermentation

Commercial green tea (*Camellia sinensis* L.) was collected from the local market in Al-Mansoura, Egypt, dried at 70°C and used as tannin substrate under solid state fermentation.

Mode of Fermentation and Tannase Extraction

Tannase production by *Aspergillus aculeatus* was performed through batch solid state fermentation using green tea as the source of tannins (tannic acid). One gram of solid substrate was transferred to a 250 mL Erlenmeyer flask and mixed with 3.0 mL of basal medium (0.3% peptone; 0.1% $CaCl_2$-$2H_2O$, 0.1% KH_2PO_4 and 0.05% $MgSO_4$-$7H_2O$); then the mixture was autoclaved at 121°C at 15 lbs for 20 min. After cooling, the medium was inoculated under aseptic conditions by 1.0 mL of spore suspension (2.0×10^7spores) and incubated at 30°C under static conditions. The final moisture content was 80%. After 4.0 days of incubation, the fermented substrates were mixed by adding 30 mL of 0.1 M acetate buffer (pH 5.5) to the fermented medium. The flasks were then kept on a rotating shaker for 1.0 h at 10°C and centrifuged at 5000 rpm for 10 min to remove all fungal cells and residue of the substrate. The clarified extract was collected and stored in a freezer (-20°C) until it was used as crude tannase.

Assay of Tannase Activity

Tannase activity was estimated by detection of the gallic acid liberated from the used substrate (tannic acid) through two steps. The first step was the precipitation of unhydrolyzed tannins with quinine HCl solution, as reported by Nishira and

Mugibayashi (1958). The second step was to estimate the released gallic acid as reported by Deschamps *et al*. (1983). One unit of tannase activity (U) was defined as the amount of enzyme that liberates 1.0 μmol gallic acid per min under assay conditions.

Protein Determination

Soluble protein was determined according to Bradford (1976) by measuring the optical density of the colour developed at 595 nm using a Spectro UV-VIS RS spectrophotometer (Labomed Inc. U.S.A). The μg of protein was estimated using bovine serum albumin (BSA) as standard.

Immobilization Methods of Tannase

Covalent Binding with Cross-Linking

Chitin (1.0 g) was shaken with 10 ml of 2·5% glutaraldehyde. Chitin was then collected by filtration using a sintered glass funnel and washed with distilled water to remove the excess glutaraldehyde. The wet chitin was mixed with 4.0 ml of the enzyme solution (300 U; *A. aculeatus* tannase) for 1.0 h at room temperature (23-25°C). The unbound tannase were removed by washing with 0.1M acetate buffer pH 5.5 (Abdel-Naby *et al*., 1999).

One gram of gelatin/BSA was dissolved by heating (at 50°C) and treated with 0.7 mL (50% v/v) of glutaraldehyde. The final concentration of gelatin was kept at 10% (w/v) and cooled to room temperature. Then 4.0 ml *A. aculeatus* tannase solution (containing 300 U) was added to the mixture and incubated overnight at 4.0°C. The resulting gel was washed and then cut into small cubes (0.2 cm^3) (Ghosh and Nanda, 1993).

Entrapment in Ca-Alginate

According to Abdel-Naby *et al*. (1999), *A. aculeatus* tannase solution (4.0 mL, containing 300 U) was mixed with different concentrations of sodium alginate (Pharmacia chemicals) to final concentrations of 3.0, 5.0, and 7.0%. The entrapment was carried out by dropping alginate solution in 0.1 M $CaCl_2$ solution. The resulting beads were collected, washed with acetate buffer (0.1 M; pH 5.5) and kept in the same buffer at room temperature for 2.0 hours to remove the unbound enzyme.

Properties of the Free and Immobilized Tannase

Optimum pH

The optimum pH for free and immobilized tannase was determined by incubating each enzyme at different pH values (0.1 M acetate buffer was used for the range from pH 3.5 to 5.5 and 0.1 M phosphate buffer for pH 6.0 to 8.0) at 45°C for 20 min using 2.0% tannic acid as substrate with different controls. Tannase activity was then assayed as described above.

Optimum Temperature

The effect of temperature was studied by incubating both soluble and immobilized tannase in their respective optimum pH at different temperatures (ranging from 25 to 70°C) with different controls for 20 min using 2.0 % tannic acid as substrate.

Activation Energy (E_a)

The activation Energy was determined from the slope of a linear plot of the log of the enzyme activity (v) versus 1/T, according to the Arrhenius law:

$$\log(v) = Cte - E_a/(RT) \quad (1)$$

The enzyme activity (v) was expressed in U (μg protein)$^{-1}$, the temperature (T) in Kelvin (K), the gas constant (R=1.987 cal K^{-1} mol^{-1}) and the activation energy (E_a) in kcal mol^{-1}.

pH Stability

The pH stability of the free and immobilized enzyme was examined after pre-incubating enzyme samples at 25°C for 30 min at different pH values (0.1 M acetate buffer was used for the range from pH 3.5 to 5.5 and 0.1 M phosphate buffer for pH 6.0 to 8.0), followed by adjusting the pH to the value of the standard assay system. The residual activity was assayed under the standard conditions for free (pH 5.5 and 50°C) and immobilized tannase (pH 5.0 and 60°C).

Thermal Stability

The enzyme samples were incubated in 0.1 M acetate buffer at designated temperatures of 60, 70 and 80°C for times ranging from 5.0 to 90 min. The residual activity was assayed under the standard conditions for free (pH 5.5 and 50°C) and immobilized tannase (pH 5.0 and 60°C).

Determination of the Half-Life ($t_{1/2}$)

The half-life of the enzyme activity ($t_{1/2}$), which corresponds to the time necessary for the residual enzyme activity to decrease to 50% of its initial value, can be calculated from the equation:

$$t_{1/2} = 0.693 / K_d \quad (2)$$

Determination of the Deactivation Energy (E_d)

The deactivation energies of free and immobilized tannase were determined by plotting the activity data [log of the ratio of Ar (residual activity)/A_0 (initial activity)] as a function of time to obtain the deactivation rate constant (K_d) at each temperature. From Arrhenius equation:

$$K_d = K_{do}\exp\left(-E_d / RT\right) \quad (3)$$

plotting the log of K_d as a function of the inverse of the absolute temperature, the energy of deactivation (E_d) is obtained as the product of the slope of the resultant straight line times R, the universal gas constant.

Kinetic Values (K_m and V_{max})

Following Abdel-Naby *et al*. (1999) different concentrations of pure tannic acid (Sigma) (2.0-50 mg mL^{-1}) were prepared for tannase assay. The enzyme activity was determined after 30 min incubation at 50°C for free enzyme and at 60°C for immobilized tannase. The kinetic values of the enzyme (K_m, and V_{max}) were investigated through Hanes–Woolf plots of the different substrate concentrations [S] against [S/V] using Graph-Pad Prism 5 software.

Effect of Different Metal Ions

This was determined by pre-incubating the enzyme solution or the immobilized form with the test substance (1.0%, w/v) for 30 min at room temperature. Then, the residual activity was assayed at the optimum conditions.

Operational Stability of the Immobilized Tannase

1.0 g of wetted gelatin-immobilized *A. aculeatus* tannase was incubated with 5.0 mL of 2.0% (w/v) tannic acid in acetate buffer (0.1 M, pH 5.0) at 60°C for 30 min. At the end of the reaction, the immobilized enzyme was collected by filtration, washed with distilled water, and re-suspended in 5.0 mL of freshly prepared substrate to start a new run. The supernatant fluid was assayed for gallic acid.

Reproducibility

All the experiments were repeated at least four times and the results were reproducible. The data points represent the mean values within ±5.0% of the individual values.

RESULTS AND DISCUSSION

Immobilization Process

Tannase from *A. aculeatus* was immobilized by two methods, including: 1) covalent binding with cross-linker on chitin, bovine serum albumin (BSA) and gelatin; and 2) entrapment in Ca-alginate (Table 1). The immobilized enzyme prepared by covalent binding with cross-linker to gelatin had the highest specific activity (0.19 U (μ*g* protein)$^{-1}$) and the highest immobilization yield (28.4%) and it was therefore used in the succeeding part of this work. In this connection, Cao (2005) suggested that immobilization by covalent binding using a cross-linking agent (glutaraldehyde) probably increases the local surface area, which contributes to minimizing the steric effect around the active site of the immobilized enzyme. In addition, these results are similar to those reported by Abel-Naby *et al*. (1999).

Table 1: Immobilization of *A. aculeatus* tannase on different carriers

Immobilization method	Carriers	Enzyme units			Immobilization Yield I/(A-B)%	Specific activity U (μg protein)$^{-1}$
		Add (A)	Unbound (B)	Immobilized (I)		
Covalent bonding	Chitin	300	90	50	23.8	0.176
	Gelatin	300	40	74	28.4	0.190
	BSA	300	50	63	25.2	0.182
Entrapment	Alginate (3%)	300	70	40	17.4	0.152
	Alginate (5%)	300	60	30	12.5	0.140
	Alginate (7%)	300	50	21	08.4	0.132

The immobilization yields of the immobilized enzyme by entrapment on 3.0, 5.0, and 7.0% Ca-alginate were 17.4, 12.5 and 8.4%, respectively. This lower immobilization efficiency with entrapment may be due to enzyme leakage, as explained by Cao (2005). There are two counteracting factors affecting the efficiency of the immobilization by entrapment, the diffusion efficiency and enzyme leak out. Higher concentrations of alginate reduced the pore size of beads and, consequently, reduced leakage of enzyme. However, the diffusion efficiency of the substrate and internal transport was reduced. A lower percentage of alginate increased the pore size, which leads to increased leakage of enzyme, but reduced the limitations on substrate diffusion. Similar observations were previously reported for other immobilized tannases (Yu *et al*., 2004).

The specific activity exhibited by free tannase was 2.6 U (μg protein)$^{-1}$. However the specific activity of immobilized tannase on gelatin was 0.19 U (μg protein)$^{-1}$. 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), as reflected by the lower apparent activation energy for immobilized tannase (7.6 kcal mole^{-1} vs. 9.8 kcal mole^{-1}, see below). Lower activation energy for the immobilized enzyme has been reported to be an indication of diffusional limitations (Kitano *et al*., 1982). On the other hand, Gottschalk and Jaenicke (1991) reported that the immobilization of the enzyme by covalent binding could lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in catalytic activity. A decrease in specific activity after tannase immobilization has been previously reported (Abdel-Nabey *et al*., 1999).

Optimum pH

The optimum pH of immobilized tannase was shifted to a more acidic value than free tannase, reaching the maximal activity at pH 5.0 as compared to pH 5.5 as the optimum for free tannase (Figure 1). This may be attributed to an ionic change around the enzyme active site as result of the immobilization process (Krajewska *et al*., 1990). The shift of pH optima to more acidic pH values has been previously reported for other immobilized tannases (Abel-Naby *et al*., 1999, Srivastava and Kar, 2010). In contrast, the optimal pH of the *A. niger* tannase entrapped in Ca-alginate was shifted to a more alkaline range (Yu *et al*., 2004). Furthermore, both immobilized and free *Aspergillus niger* tannase have the same optimum pH (Sharma *et al*., 2008).

pH Stability

The profile of pH stability (Figure 2) showed that immobilized tannase was more stable in a wider range of pH (4.5-8.0) compared with the free enzyme (5.0- 6.0), where it retained 80% residual activity. This result means that immobilized tannase would be more resistant to pH changes and could be used industrially. This effect may have been caused by the micro-environmental pH of the gelatin matrix. At high H^+ concentrations, the amino groups of gelatin would be protonated, thereby attracting hydroxide ions, which would maintain a higher micro-environmental pH than in the bulk solution and thus stabilize the immobilized enzyme (Cao, 2005). These results are in agreement with those obtained for other immobilized tannases (Abdel-Naby *et al*., 1999; Yu *et al*., 2004).

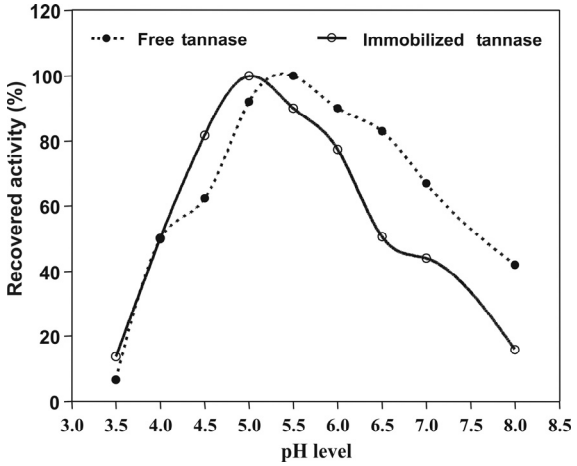


Figure 1: Effect of different pH levels on free and immobilized *A. aculeatus* tannase

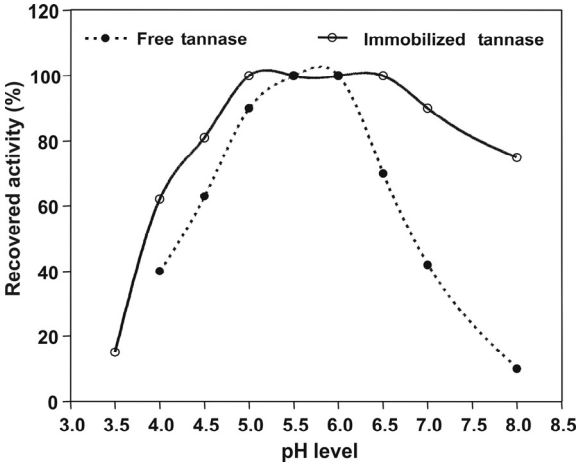


Figure 2: pH stability of free and immobilized *A. aculeatus* tannase

Optimum Temperature

The effect of temperature on the activity of the immobilized and free tannase (Figure 3) shows that the optimum temperature is shifted from 50ºC for free tannase to 60ºC for immobilized tannase. This increase of the optimum temperature is probably a consequence of enhanced thermal stability. The shift of optimum temperature to higher values after the immobilization of tannase was previously reported by Sharma *et al*. (2008), Su *et al*. (2010) and Srivastava and Kar (2010).

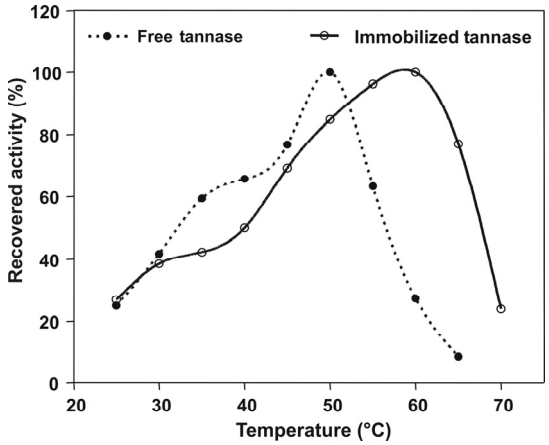


Figure 3: Effect of different temperatures on free and immobilized *A. aculeatus* tannase

The temperature data are replotted in the form of Arrhenius plots (Figure 4). The plots of both free and immobilized tannase were linear and the activation energy of immobilized tannase was lower (7.6 kcal mole^{-1}) than the activation energy of free enzyme (9.8 kcal mole^{-1}). These results are similar to those reported for immobilized and free *A. oryzae* tannase (Abdel-Naby *et al*., 1999). This decrease in the activation energy may be due to the diffusion limitations (Yu *et al*., 2007).

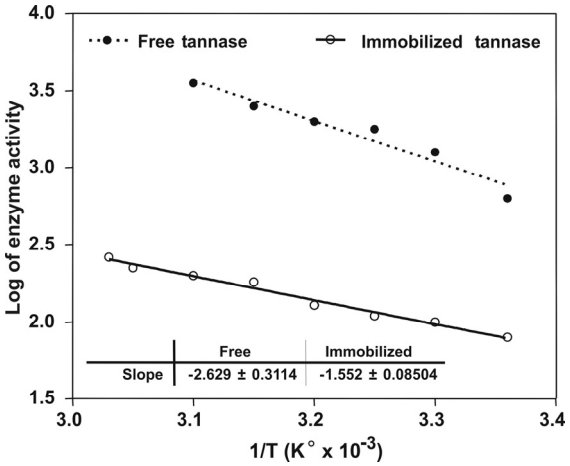


Figure 4: Arrhenius plots for the activation energy of free and immobilized *A. aculeatus* tannase

Thermal Stability

The results in Figure 5 indicate that the immobilization process significantly improves the thermal stability of immobilized tannase relative to free enzyme. For example, the immobilized tannase retained 83 % of its original activity after heating for 90 min at 60ºC, while 76.2% of the activity was retained by the free enzyme after the same treatment. In addition, the calculated half-lives (Table 2) of the immobilized enzyme at 60, 70 and 80ºC were 312, 47.4, and 27.5 min, respectively, whereas in the same conditions the free enzyme was less stable (half-lives of 195.9, 32.4, and 17.5 min respectively). Furthermore, the deactivation rate constant of the immobilized *A. aculeatus* tannase at 80ºC was 2.51 x10^{-2} min, which is lower than that of the free enzyme (3.94 x10^{-2} min). The Arrhenius plot of deactivation energy (Figure 6) indicates that immobilization process is increase the thermal stability to tannase. The calculated value of deactivation energy (Table 2) for free tannase (45.8 kcal mol^{-1}) is lower than its value of immobilized tannase (57.3 kcal mol^{-1}).

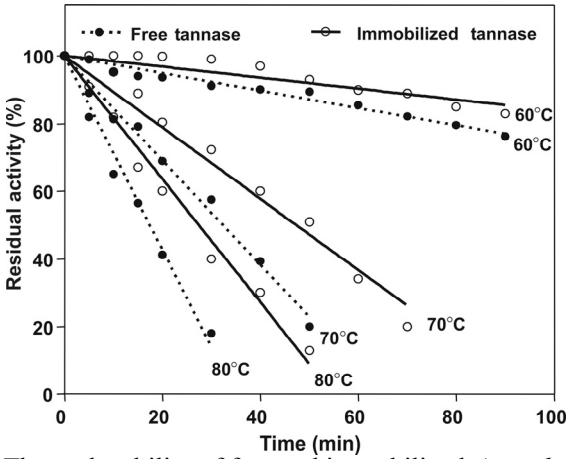


Figure 5: Thermal stability of free and immobilized *A. aculeatus* tannase

Table 2: Comparison between the thermal stabilities of free and immobilized tannase

	Free tannase	Immobilized tannase
Optimum pH	5.5	5.0
Optimum temperature	50°C	60°C
Activation energy (E_a; kcal mole^{-1})	9.8	7.6
Half life time (min)		
60ºC	195.9	312.3
70ºC	32.4	47.4
80ºC	17.5	27.5
Deactivation rate constant K_d (min^{-1})		
60ºC	0.35 x10^{-2}	0.22 x10^{-2}
70ºC	2.13 x10^{-2}	1.46 x10^{-2}
80ºC	3.94 x10^{-2}	2.51 x10^{-2}
Deactivation energy (dE_a; kcal mole^{-1})	45.8	57.3
K_m (mg ml^{-1})	6.5	11.8
V_{max}	2.70	0.32

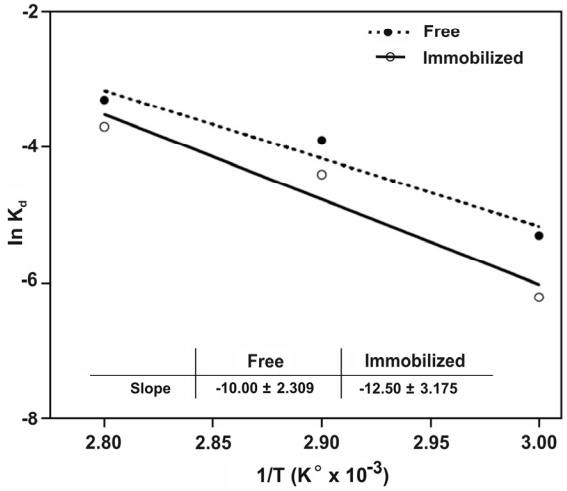


Figure 6: Arrhenius plots for the deactivation energy of free and immobilized *A. aculeatus* tannase

Kinetic Values (K_m and V_{max})

A plot of the activity of free and immobilized tannase on pure tannic acid is illustrated in Figure 7 (a) and (b), showing that K_m of the immobilized tannase (11.8 mg ml^{-1}) is higher than the value of free tannase (6.5 mg ml^{-1}), while V_{max} of the immobilized enzyme (0.32 U (μg protein)$^{-1}$) is lower than that of free tannase (2.7 U (μg protein)$^{-1}$).

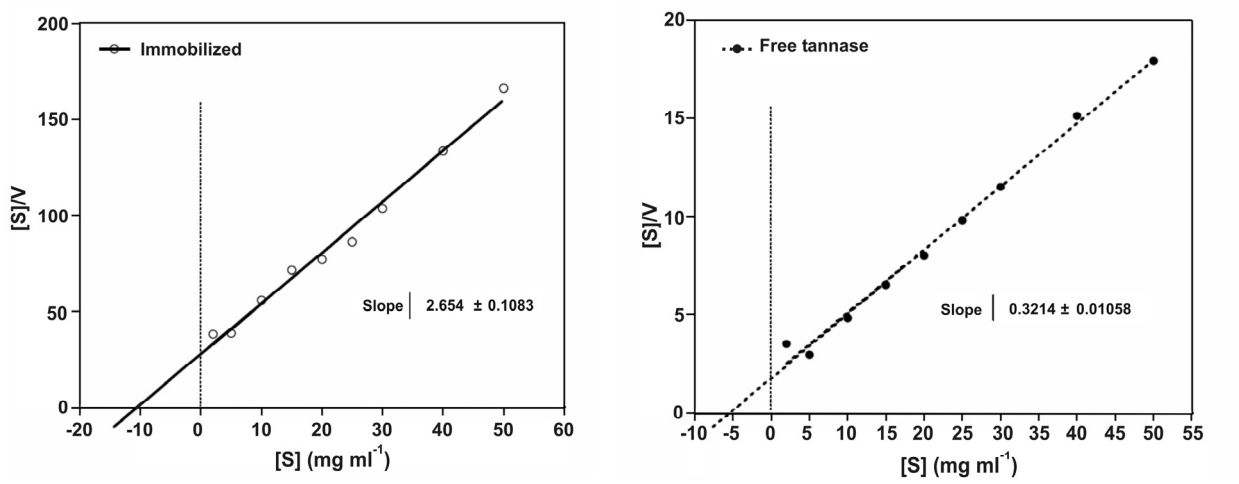
This increase of the K_m value after the immobilization may be partially due to mass transfer resistance to diffusion into the immobilization matrices and/or to low substrate accessibility to the enzyme active site. On the other hand, fixation of the enzyme on the immobilization matrix could lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in the catalytic activity (Cao, 2005). Consequently, the maximum rate of the reaction catalyzed by the immobilized enzymes was lower than that of the free enzyme. Several researchers reported an increase of K_m and decrease of V_{max} for tannase due to immobilization (Abdel-Naby *et al*., 1999; Yu *et al*., 2004, 2007; Su, *et al*., 2010).

Effect of Different Metal Ions

In this experiment, free and immobilized enzymes were incubated with different metal ions in solution at room temperature for 30 minutes. Then the residual activity was measured at optimum conditions. The results in Table 3 show that Ca^{2+} ion is significantly activating for both free and immobilized tannase (residual activity is 118% and

112.4% for immobilized and free tannase, respectively), while Mg^{2+} ion is only activating for the immobilized enzyme (residual activity is 107%). Gelatin as a carrier appears to protect tannase against the inhibitory effect of other metal ions; hence, it was generally observed that the inhibitory effects of the ions were less pronounced in immobilized tannase compared with the free enzyme. This protection may be due to the following: 1) structureal changes in the enzyme molecule introduced by the immobilization procedure, lower the accessibility of inhibiting ions to the active site of the enzyme; and 2) the chelating effect of gelatin, which is known to be a very powerful chelating agent, especially when cross-linked with glutaraldehyde forming glutaraldehyde- cross-linked- gelatin particles (Kennedy, and Kalogerakis, 1980).

Enzyme inactivation by heavy metals, including mercury (Hg^{2+}), proceeds by the reduction of the thiol group in cysteine residues, with the formation of mercaptides, or the reduction of disulfide bridges, leading to S-Hg-S bonds (Vallee and Ulmer, 1972). These results are in agreement with those obtained for immobilized tannase by Abdel-Naby *et al*. (1999). Furthermore, Su *et al*. (2010) reported that Fe^{2+} and Mn^{2+} could enhance the activity of immobilized tannase when used for tannin degradation in extracts of green tea, black tea and oolong tea.



(a) (b)

Figure 7: Hanes–Woolf plots of immobilized and free *A. aculeatus* tannase acting on pure tannic acid

Table 3: Effect of different metal ions on the activity of immobilized and free tannase

Metal ions	Relative tannase activity (%)	
	Free tannase	Immobilized tannase
Na^{+}	53.8 ± 2.2	66.0 ± 3.1
Hg^{+2}	13.2 ± 0.9	22.0 ± 2.2
Cu^{+2}	52.2 ± 1.6	68.0 ± 3.1
Fe^{+2}	52.6 ± 2.1	61.0 ± 2.0
Zn^{+2}	72.9 ± 2.2	85.0 ± 2.2
Ca^{+2}	112.4 ± 3.0	118.0 ± 3.1
Mg^{+2}	96.7 ± 2.1	107.0 ± 2.1
Mn^{+2}	69.0 ± 2.2	71.0 ± 3.0
K^{+}	53.2 ± 1.1	73.0 ± 1.7
EDTA	29.0 ± 1.2	35.0 ± 2.1
Control*	100	100

* Without mineral ions

Operational Stability of Immobilized Tannase

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 (Cruz-Aldaco *et al*., 2009; Srivastava, and Kar, 2010; Belur and Mugeraya, 2011). The operational stability of immobilized tannase is evaluated in repeated batch processes. After each run, the immobilized tannase was washed and reused at optimum conditions for another reaction. The percent of residual tannase activity was determined for 8 cycles. The results in Figure 8 indicate that immobilized tannase retains 84 % of its original activity after 5 cycles. This is an industrially promising result similar to that obtained by Sharma *et al*. (2002) and Yu *et al*. (2007). In addition, *A. niger* tannase immobilized on sodium alginate beads was used repeatedly for 7 cycles with 77% efficiency (Srivastava and Kar, 2010).

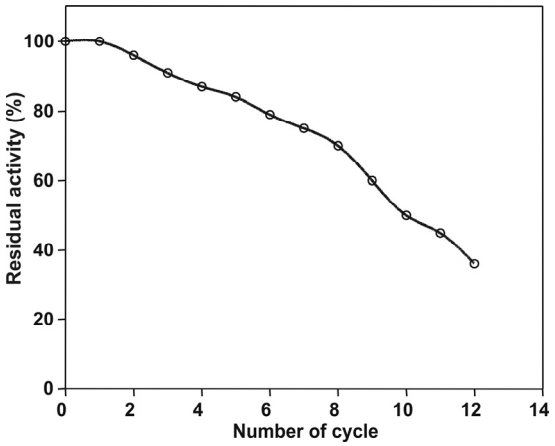


Figure 8: Operational stability of immobilized *A. aculeatus* tannase

CONCLUSIONS

In this study, *A. aculeatus* tannase produced by solid sate fermentation on green tea substrate was efficiently immobilized by two methods of entrapment and covalent bonding with cross-linking. Cross-linking within gelatin in the presence of glutaraldehyde gave the highest immobilization yield (28.4%) compared with the other carriers used. The immobilized tannase remained stable for longer periods of time and also at higher temperatures as compared to the free enzyme. In addition, pH studies indicated that the enzyme remained significantly active over a broader pH range (4.5-8.0) compared to the free enzyme in solution. The kinetic properties of tannase revealed a lower affinity of the immobilized enzyme with a higher K_m (11.8 mg ml^{-1}) compared to free tannase. In addition, after five operational cycles, it was observed that the immobilized tannase retained 84% of its original activity. The properties of the immobilized tannase described here suggest its value for industrial applications that would not be feasible with the free enzyme system. Further studies are needed of its use in the continuous removal of undesirable tannin molecules in the food and juice industries and in the production of the pharmaceutical antioxidants gallic acid or ellagic acid.

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