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Immobilization and Characterization of Tannase from a Metagenomic Library and Its Use for Removal of Tannins from Green Tea Infusion

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Tannase (Tan410) from a soil metagenomic library was immobilized on different supports, including mesoporous silica SBA-15, chitosan, calcium alginate, and amberlite IRC 50. Entrapment in calcium alginate beads was comparatively found to be the best method and was further characterized. The optimum pH of the immobilized Tan410 was shifted toward neutrality compared with the free enzyme (from pH 6.4 to pH 7.0). The optimum temperature was determined to be 45°C for the immobilized enzyme and 30°C for the free enzyme, respectively. The immobilized enzyme had no loss of activity after 10 cycles, and retained more than 90% of its original activity after storage for 30 days. After immobilization, the enzyme activity was only slightly affected by Hg^{2+}, which completely inhibited the activity of the free enzyme. The immobilized tannase was used to remove 80% of tannins from a green tea infusion on the first treatment. The beads were used for six successive runs resulting in overall hydrolysis of 56% of the tannins.

Keywords: Calcium alginate, immobilization, tannase, tea infusion

Introduction

Tannins comprise a large group of polyphenolic compounds that are widely distributed in the plant kingdom, and high tannin concentrations are found in nearly every part of the plant, such as the wood, leaf, fruit, root, and seed. They also widely occur in some foodstuffs such as banana, strawberry, mango, grape, blackberry, and cashew nut [9]. Tannins are considered to be secondary metabolic compounds of plants because they play no direct role in any plant metabolism. They are different from other phenolics as they can precipitate proteins from solutions to form strong complexes and react with other macromolecules such as starch, cellulose, and minerals [20]. Because of their ability to precipitate proteins, tannins are responsible for several phenomena, including protein haze formation in tea and alcoholic beverages [18].

Tannase (tannin acylhydrolase, E.C.3.1.1.20) is an enzyme that catalyzes the hydrolysis of ester and depside linkages in hydrolysable tannins such as tannic acid to release gallic acid and glucose [23]. The main commercial applications of tannase are as a clarifying agent in beer, wine, and fruit juices [2, 4]. It is also used in the manufacture of instant tea, food, feed, and gallic acid [22]. Gallic acid is an important intermediary compound in the synthesis of the antibacterial drug trimethroprim, used in the pharmaceutical industry, and propyl gallate, a very important food antioxidant. Despite these important applications of tannase, its use on a large scale is restricted because of high production costs and insufficient knowledge of the enzyme. Many methods have been implemented in an effort to improve the production and recovery of tannase as well as broaden its applications and identify novel sources of the enzyme [3, 12, 21, 29].

Immobilization of enzymes is one method used to overcome limitations of employing free enzymes and several attempts have been made to immobilize tannase on a suitable matrix [1, 6, 10, 14, 17]. In this study, a novel tannase (Tan410) from a soil metagenomic library was immobilized on Ca-alginate beads and the properties of free and immobilized Tan410 were studied.

Materials and Methods

Bacterial Growth Conditions and Production of Recombinant Tannase

Escherichia coli BL21 (DE3) containing pET28-*tan410* used for tannase production was obtained from a metagenomic library as described in a previous study [29]. *E. coli* cells were grown in a 250 ml flask containing 50 ml of LB (containing 50 mg kanamycin/ml) at 37°C until the cell concentration reached OD_{600} of 0.7, and then the cells were induced with 0.5 mM IPTG. After incubation at 25°C with shaking at 220 rpm, cells were harvested by centrifugation at 6,000 ×*g* for 10 min at 4°C. The cells were sonicated and the supernatant was collected by centrifugation (16,000 ×*g*, 20 min) at 4°C. The purification of tannase was carried out by using a Ni-NTA His-Bind resin column (Merck, Germany) according to the manufacturer's instructions.

Enzyme Immobilized in Mesoporous Silica SBA-15

Immobilization was performed as described previously with minor modifications [16]. Briefly, 1.5 ml of tannase (10 mg/ml) with 0.1 g SBA-15 was incubated at 16°C for 1 h on a rotator. The loaded SBA-15 (SBA-15-tannase) was recovered by centrifugation at 6,000 ×*g* for 10 min. The SBA-15-tannase was washed with 20 mM potassium phosphate buffer (pH 6.4) until the protein was no longer detected in the washing solution and then stored at 4°C until further use.

One gram of SBA-15-tannase was added to 4 ml of 2.5% (v/v) glutaraldehyde, and the mixture was gently stirred for 1 h at 25°C. The immobilized enzyme was recovered by centrifugation at 6,000 ×*g* for 10 min, washed with 20 mM potassium phosphate buffer (pH 6.4) and then stored at 4°C until further use.

Enzyme Immobilized in Chitosan

Tannase was immobilized on chitosan as described by Kim *et al*. [15]. Chitosan was solubilized in 30% acetic acid to a final concentration of 2% (w/v). The solution was filtered through Whatman GF/A filters and added dropwise through a capillary into a gently stirred coagulation liquid (1 M sodium hydroxide and 26% (v/v) ethanol). The obtained particles were centrifuged and washed with distilled water until neutrality. Immobilization on chitosan particles was carried out by adding 1 g (wet weight) of particles to a mixture of 3.5 ml of 20 mM potassium phosphate buffer (pH 6.4) and 1.5 ml of tannase (10 mg/ml). The mixture was gently shaken at 100 rpm for 2 h, and after a further 16 h at 25°C in the presence of 2.5% (v/v) glutaraldehyde, the mixture was centrifuged at 12,000 ×*g* for 2 min. The immobilized enzyme was washed with 20 mM potassium phosphate buffer (pH 6.4) until protein was no longer detected in the washing solution.

Enzyme Immobilized on Calcium Alginate

Tannase was immobilized on calcium alginate as described by Srivastava and Kar [27]. A total of 1.5 ml of the purified enzyme was mixed with 1.5 ml of 6% sodium alginate solution until homogeneous. Then, the mixture was added with constant agitation to a 2% $CaCl_2$ solution as droplets, using a syringe. The beads were kept in 2% $CaCl_2$ at 4°C for 1 h and then washed with 20 mM potassium phosphate buffer (pH 6.4) until protein was no longer detected in the washing solution. Aliquots of washing buffer and supernatant were collected to establish encapsulation efficiency.

One gram of beads was added to 4 ml of 2.5% (v/v) glutaraldehyde, and the mixture was gently stirred for 1 h at 25°C. Then, the beads were washed with 20 mM potassium phosphate buffer (pH 6.4) and stored at 4°C until further use.

Enzyme Immobilized on Amberlite IRC 50

Immobilization was performed according to Figueira *et al*. [8]. Briefly, 1.0 g of Amberlite particles was washed with 5 ml of distilled water. The resulting suspension was centrifuged at 4,000 ×*g* for 10 min at room temperature. The precipitate was washed with 5 ml of acetate buffer (100 mM, pH 4.5), and the supernatant was discarded. A total of 2.5 ml of a polyethyleneimine solution (100 g/l) was added to the precipitate, and the suspension was incubated at room temperature with stirring for 2 h. The mixture was centrifuged and the precipitate was washed with distilled water twice. The precipitate was incubated for 16 h with stirring at room temperature with 5 ml of a 10% (v/v) glutaraldehyde solution and centrifuged at 4,000 ×*g* for 10 min. The precipitate was washed with 5 ml of 20 mM potassium phosphate buffer (pH 6.4). The suspension was filtered through qualitative filter paper, and 1.5 ml of an enzymatic solution in 20 mM potassium phosphate buffer (pH 6.4) was added to the activated Amberlite. The suspension was incubated at 30°C with stirring (200 rpm) for 2 h and then was centrifuged, and the precipitate was washed twice with 20 mM potassium phosphate buffer (pH 6.4). The enzyme linked to the support was maintained at 4°C until use. Aliquots of washing buffers and supernatants were collected to establish ion-exchange adsorption efficiency.

Assay of Tannase Activity

Tannase activity was determined by detection of the gallic acid released from propyl gallate according to the method described previously [11]. The enzyme was incubated with 200 μl of 30 mM propyl gallate in 100 mM phosphate buffer at 30°C for 5 min. Then, 200 μl of the methanolic rhodanine solution (0.667% (w/v) rhodanine in 100% methanol) was added to the mixture. After incubation at 30°C for another 5 min, 200 μl of 500 mM KOH was added and the mixture was further incubated for 5 min at 30°C and diluted to 4 ml. The absorbance was measured at 520 nm using a spectrophotometer. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid in 1 min under the specified conditions.

Effects of pH and Temperature on Free and Immobilized Tannases

The optimum pH for free and immobilized tannases was determined in 100 mM sodium acetate buffer (pH 4.0–6.0), 100 mM phosphate buffer (pH 5.5–8.0), and 100 mM Tris-HCl buffer

(pH 7.5–9.0). The pH stability was determined by incubation of the enzyme at pH 4.0 to 9.0 at 30°C for 4 h. The optimum temperature was determined by examining from 25°C to 70°C. The thermostability of the enzyme activity was investigated by incubating the enzyme at 25°C to 65°C for 4 h and then the residual activity was measured as described above.

Effects of Various Chemical Substances on Free and Immobilized Tannases

The effects of Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, Co^{2+}, Hg^{2+}, Cr^{2+}, EDTA, and urea on tannase activity were investigated. The enzyme was incubated for 15 min at 30°C with the different metal ions at a final concentration of 1 mM. Then, the residual activity was measured using the standard conditions.

Storage Stability of Free and Immobilized Tan410

The activities of the free and immobilized tannases after storage in 100 mM phosphate buffer (pH 6.5) at 4°C were measured in a batch operation mode.

Operational Stability of the Immobilized Tan410

Immobilized Tan410 (0.3 g) was incubated with 2 ml of 15 mM propyl gallate in 100 mM phosphate buffer at 45°C for 20 min. At the end of the reaction, the immobilized enzyme was collected by filtration, washed with distilled water, and then resuspended in 2 ml of freshly prepared substrate. The supernatant fluid was assayed for tannase activity under the standard conditions.

Tannase Enzymatic Treatment of Green Tea Infusion

Preparation of green tea infusion. Green tea leaves were coarsely ground in a mortar, and 2 g of the pulverized product was mixed with 100 ml of boiling water in a 250 ml beaker. The beaker was then immediately placed in a water bath at 85°C for 30 min. The tea infusions were then filtered through Whatman No.1 filter paper and the filtrates were collected for enzyme treatment.

Immobilized tannase enzymatic treatment. A total of 500 ml of the tea infusion was treated with 0.5 g immobilized tannase beads at 45°C for 4 h. After incubation, the reaction was stopped by the addition of 3 ml of a 1.0 mg/ml bovine serum albumin solution prepared in a 170 mM sodium chloride solution in 200 mM acetate buffer (pH 5.0). The solution was then filtered and centrifuged at 10,000 ×*g* for 10 min at 4°C. The precipitate was dissolved in 1.5 ml of SDS-triethanolamine (1% (w/v) SDS solution containing 5% (v/v) of triethanolamine) and 0.5 ml of $FeCl_3$ (10 mM $FeCl_3$ in 0.01 N hydrochloric acid) and incubated for 15 min for stabilization of the color. The absorbance was measured at 530 nm.

Results

Tannase Production and Recovery

The optimum incubation time for maximal production of tannase was determined by drawing samples after a 2 h interval from the culture flasks. Tan410 was the most active after incubating at 25°C for 12 h. As the recombinant tannase was expressed in a pET-28a vector under the control of the *T7 lac* promoter with an N-terminal 6×His tag, the recombinant enzyme was purified on a Ni-NTA His-Bind resin column. Highly purified recombinant enzyme was obtained. After the purification, 125 mg of the recombinant tannase with a specific activity of 28.8 U/mg was obtained per 1 L of culture.

Enzyme Immobilization

From the supports that were screened, the best result (Table 1) was obtained with calcium alginate encapsulation (62%, immobilization yield). Amberlite IRC 50 proved the least efficient support (5%, immobilization yield). Other immobilized supports or methods were no less than 48% efficient. Thus, the remaining research was carried out using enzyme immobilized in calcium alginate.

Effects of pH and Temperature on the Activity of the Free and Immobilized Enzymes

The optimum activity of the free and immobilized enzymes was measured over a pH range of 4.0–9.0 and a temperature range of 20–60°C with propyl gallate as a substrate. The immobilized enzyme reached maximal activity at pH 7.0 whereas the free enzyme had its highest activity at pH 6.4 (Fig. 1). The optimum temperature was shifted from 30°C

Table 1. Immobilization of tannase.

Carrier	Enzyme added *A* (U)	Unbound enzyme *B* (U)	Immobilized enzyme *I* (U)	Immobilization yield (*I*/(*A*-*B*) %)	Specific activity of immobilized enzyme (U/mg)
SBA-15	3,600	380	1,610	50.0	14.4
SBA-15 + glutaraldehyde	3,600	380	1,571	48.8	14.0
Chitosan	3,600	640	1,480	50.0	14.4
Calcium alginate	3,600	438	1,960	62.0	17.9
Calcium alginate + glutaraldehyde	3,600	438	1,770	56.0	16.1
Amberlite IRC 50	3,600	3,009	29.55	5.0	1.4

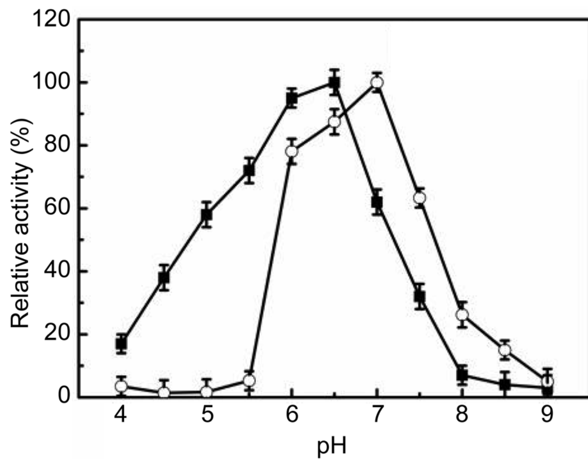

Fig. 1. Effect of pH on the activity of free (■) and immobilized (○) Tan410.

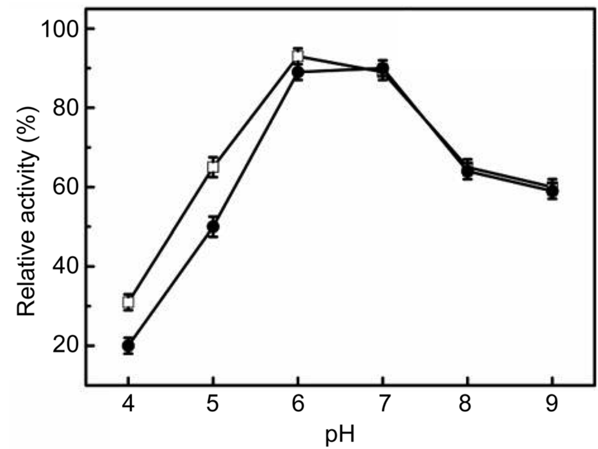

Fig. 3. Effect of pH on the stability of free (●) and immobilized (□) Tan410.

for free enzymes to 45°C for immobilized enzymes (Fig. 2). The profile of pH stability showed no obvious difference (Fig. 3) in the biocatalytic activity of the free and immobilized enzymes under the experimental conditions. For the thermostability, the immobilized enzyme retained about 75% of its initial activity after incubation at 50°C for 4 h, whereas the free enzyme only retained 50% of its initial activity when exposed to the same condition (Fig. 4).

Effects of Different Chemical Substances on Free and Immobilized Enzyme

The effects of different chemical substances on free and immobilized enzyme activity are shown in Fig. 5, where Mg^{2+}, Ca^{2+}, and urea significantly activated both the free (residual activity was 120%, 126%, and 106%, respectively) and immobilized enzymes (residual activity was 110%, 115%, and 113%, respectively). Co^{2+}, Zn^{2+}, and EDTA only enhanced the immobilized enzyme activity (residual activity was 105%, 108%, and 106%, respectively). Hg^{2+} only slightly affected the activity of the immobilized enzyme (residual activity was 93.7%), whereas it completely inhibited the free enzyme activity.

Storage Stability of the Free and Immobilized Enzymes

The storage stability curves for both the free and immobilized enzymes are shown in Fig. 6. The activity of the free enzyme was quickly lost upon storage and decreased to zero by 16 days. For the immobilized enzyme, there was a slight decrease in the activity during the same

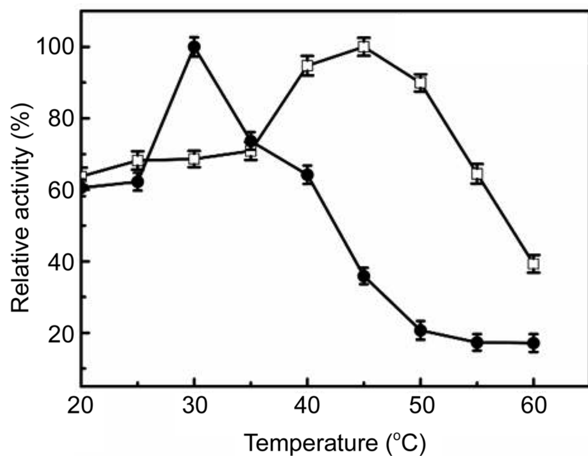

Fig. 2. Effect of temperature on the activity of free (●) and immobilized (□) Tan410.

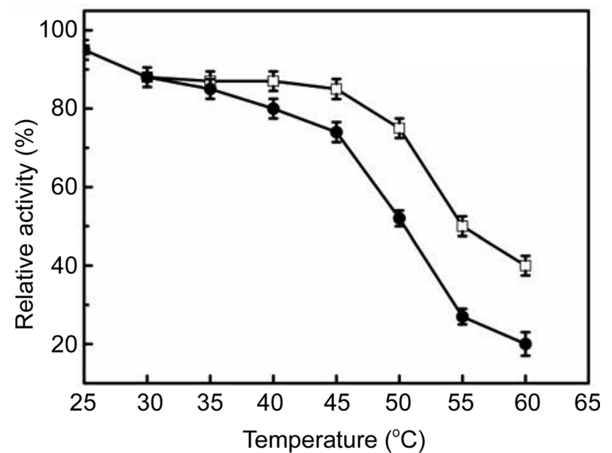

Fig. 4. Effect of temperature on the stability of free (●) and immobilized (□) Tan410.

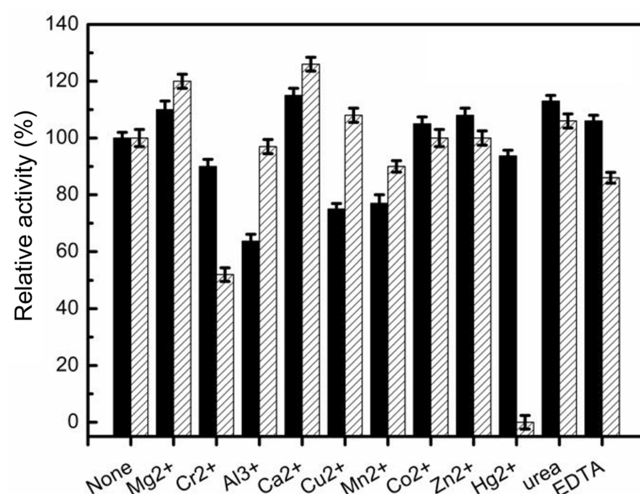

Fig. 5. Effects of different additives on free and immobilized Tan410 activities.

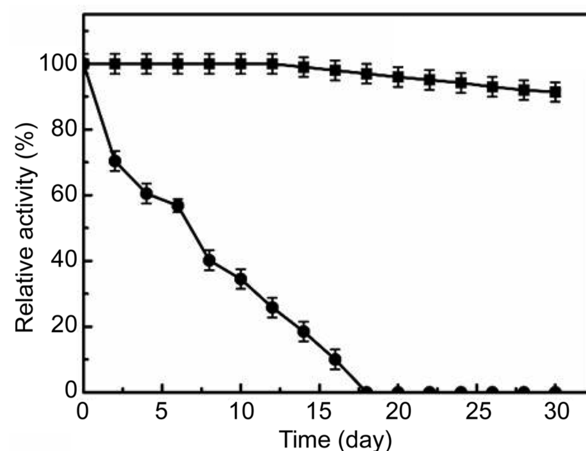

Fig. 6. Storage stability of free (●) and immobilized (■) Tan410.

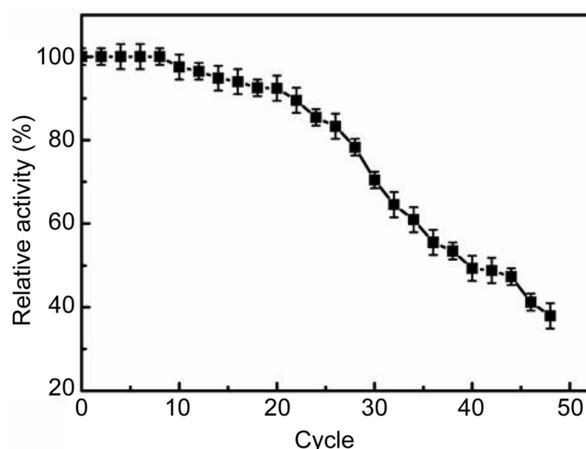

Fig. 7. Operating stability of immobilized Tan410.

storage period, but more than 90% of its initial activity was preserved after 30 days of storage.

Operational Stability of the Immobilized Enzyme

The operational stability curve for the immobilized enzyme is shown in Fig. 7. The immobilized enzyme retained its initial activity over 10 cycles and retained 80% of its initial activity after 26 cycles. After that, the activity of the immobilized enzyme was quickly lost, retaining about 40% of its initial activity after 46 cycles.

Application of Immobilized Tannase for Tannin Removal from Tea Infusion

The immobilized tannase was used to remove tea tannin from a fresh infusion and the results showed that 80% of the tea tannin was hydrolyzed after 4 h in the first run. The beads were used for six successive runs resulting in 56% hydrolysis of the tannins after six runs.

Discussion

Enzyme immobilization is a useful biotechnology suitable for enzyme recycling. There is a growing demand and application of tannase in different sectors, especially the tea, wine, dye, and pharmaceutical industries [19]. In this study, immobilization of tannase to Amberlite did not yield a good result as compared with other carriers. This observation was in agreement with earlier results from the Amberlite immobilization of β-glucosidase [8]. However, Sharma *et al*. [25] used Amberlite IR 1204 and Amberlite XAD-7 as supports to immobilize *Penicillium variable* tannase, resulting in immobilization yields of 69% and 25%, respectively. Glutaraldehyde was used for the immobilization and may have been responsible for the loss in enzyme activity; hence the relatively low yields were observed, which are consistent with the results reported by Chang and Juang [5] and Jiang *et al*. [13]. Sodium alginate was determined to be the best support to immobilize Tan410, with an immobilization yield of 62%, which was higher than that of immobilized *Aspergillus niger* tannase (36.8%) [30], but lower than that of the immobilized tannase (93.6%) reported by Srivastava and Kar [27].

The optimal temperature of the immobilized Tan410 was higher than that of the free form. The shift of the optimum temperature to higher values after immobilization has been reported previously [25, 28]. However, Enemuor and Odibo

[7] reported that the optimum temperature of immobilized tannase from *Aspergillus tamarii* was close to that of the free form. The immobilized Tan410 exhibited higher thermal stability than the free form, which agreed with the finding of Tanash *et al*. [28]. However, Enemuor and Odibo [7] found that the immobilized tannase showed lower thermostability than the free form. The optimum pH of immobilized Tan410 was shifted to a more alkaline range compared with the free enzyme. The shift of pH optima to more alkaline values has been reported for other immobilized tannases [30]. However, the optimum pH values of tannases from *Aspergillus aculeatus* and *A. niger* were shifted to more acidic values than the free forms [28]. Srivastava and Kar [27] also found that the optimum pH of tannase from *A. niger* was shifted to a more acidic value after immobilization. Variations in the biochemical properties of immobilized tannases could be attributed to the kind of carriers used and the immobilized methods employed [7].

The effects of chemicals on the free and immobilized Tan410 activities were also studied. Mg^{2+}, Ca^{2+}, and urea were significantly activating for both free and immobilized Tan410. However, after the addition of Mg^{2+}, Ca^{2+}, Al^{3+}, Cu^{2+}, and Mn^{2+}, immobilized Tan410 had lower activity than the free form. The explanation is that these metal ions increase the reaction of mass transfer resistance, resulting in the reduction of the immobilized Tan410 activity. Hg^{+} and Cr^{2+} only slightly affected immobilized Tan410, whereas they strongly inhibited the enzyme activity of the free form. This may be due to the structural changes in the enzyme molecule induced by the immobilization procedure, which made it difficult for the inhibiting ions to come close to the active site of the enzyme [28].

The operational and storage stability of the immobilized tannase are the most important factors that affect the release of gallic acid in tannin bioconversion and in other industrial applications such as in food, beverage, and juices where it is used to remove the undesirable effects of tannins. In this study, the immobilized Tan410 showed high operational and storage stabilities. There was no change in enzyme activity after 10 cycles of reuse, and only 10% of the immobilized Tan410 activity was lost after storage for 30 days, which was in accordance with the results for immobilized tannase from *A. niger* [27]. However, tannase from *A. niger* immobilized on concanavalin A-Sepharose had lower operational stability, retaining only 80% of its enzyme activity after six cycles of reuse [24]. Tannase from *A. aculeatus* immobilized on gelatin also had lower operational stability, where less than 40% of the enzyme activity remained after 12 cycles [28]. The immobilized Tan410 also had good operational stability when it was used to remove tannins from a green tea infusion, where 56% of tea tannins were hydrolyzed after six cycles. Srivastava and Kar [26] reported that tannase from *A. niger* ITCC6514.07 immobilized on sodium alginate can hydrolyze 68.8% of tannins in fresh aonla juice in the first run, but can only hydrolyze 37.7% and 24.4% of tannins after the second and third runs, respectively.

In conclusion, a tannase from a metagenomic library was immobilized by entrapment in calcium alginate beads. The immobilized Tan410 had a higher optimum temperature and more alkaline pH optimum than the free form. The immobilized tannase also revealed high operational and storage stabilities. All these properties support the investigation of this immobilized Tan410 as an interesting candidate for biological applications.

Acknowledgments

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