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Immobilization of *Aspergillus oryzae* tannase and properties of the immobilized enzyme

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M.A. ABDEL-NABY, A.A. SHERIF, A.B. EL-TANASH AND A.T. MANKARIOS. 1999. Tannase enzyme from *Aspergillus oryzae* was immobilized on various carriers by different methods. The immobilized enzyme on chitosan with a bifunctional agent (glutaraldehyde) had the highest activity. The catalytic properties and stability of the immobilized tannase were compared with the corresponding free enzyme. The bound enzyme retained 20·3% of the original specific activity exhibited by the free enzyme. The optimum pH of the immobilized enzyme was shifted to a more acidic range compared with the free enzyme. The optimum temperature of the reaction was determined to be 40 °C for the free enzyme and 55 °C for the immobilized form. The stability at low pH, as well as thermal stability, were significantly improved by the immobilization process. The immobilized enzyme exhibited mass transfer limitation as reflected by a higher apparent K_m value and a lower energy of activation. The immobilized enzyme retained about 85% of the initial catalytic activity, even after being used 17 times.

INTRODUCTION

Tannins are high molecular weight polyphenolic compounds that exist in a variety of plant species. Tannase (tannin acyl hydrolase, EC 3.1.1.20) is the enzyme responsible for the decomposition of hydrolysable tannins, especially gallotannins, to glucose and gallic acid (Lbuchi *et al.* 1972).

Applications of tannase are concentrated in the leather-processing, food and pharmaceutical industries (Giovanelli 1989; Majumdar and Moudgal 1994).

For industrial application, the immobilized form of enzyme offers several advantages, including repeated use of the enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors. However, there are few reports on immobilized tannases (Weetal and Dater 1974; Katwa *et al.* 1981; Weetal 1985).

This work describes the immobilization of *Aspergillus oryzae* tannase. The properties of the immobilized enzyme were compared with those of the free enzyme.

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MATERIALS AND METHODS

Carriers for enzyme immobilization

Chitin, colloidal chitin, chitosan and Dowex 50 W were from Sigma, DEAE-Sephadex A-25 was from Pharmacia and acrylamide and Na-alginate were from BDH. All other chemicals were of analytical grade.

Preparation of tannase enzyme

Tannase enzyme was prepared from the cell-free extract of *A. oryzae* according to El-Tanash (1997). The specific activity of this crude enzyme was 72·2 U mg^{-1} protein. One unit of enzyme activity (U) is defined as the amount of the enzyme which releases one micromole of gallic acid from tannic acid per minute under the assay conditions.

Determination of tannase activity

Tannase activity was determined by measuring the amount of gallic acid released from tannic acid (Sigma). Unless otherwise specified, the assay mixture consisted of 0·5 ml tannic acid (2%, w/v) in citrate-phosphate buffer (0·1 mol l^{-1},

Lactobacillus vectors with replicons derived from small cryptic *Lactobacillus* plasmids and segregational instability of the introduced vectors. *Applied and Environmental Microbiology* **57**, 1822–1828.

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pH 5·5) and 0·5 ml enzyme solution or weighed amount of the immobilized enzyme. The reaction was incubated at 40 °C for 30 min (Nishira and Mugibayashi 1958). The released gallic acid was determined as reported by Nishira and Mugibayashi (1959). The results were confirmed by the method reported by Deschamps *et al.* (1983) for the estimation of tannase activity. One unit of enzyme activity (U) is defined as the amount of the enzyme which releases one micromole of gallic acid per minute under the assay conditions.

Immobilization methods

Physical adsorption. Aminoalkylsilane-alumina (AS-alumina) was prepared by the method of Weetal (1972). The carrier (AS-alumina or colloidal chitin, 1 g) was incubated with the enzyme solution (500 U *A. oryzae* tannase) dissolved in 1 ml 0·1 mol l^{-1} acetate buffer (pH 5·5) at 4 °C overnight. The unbound enzyme was removed from the carrier by washing with 0·1 mol l^{-1} acetate buffer (pH 5·5) until no activity or soluble protein was detected (Ohtakara and Mitsutomi 1987).

Ionic binding. Anion exchanger (DEAE-Sephadex A-25, 1 g) equilibrated with citrate buffer (0·1 mol l^{-1}, pH 6·0), or cation exchanger (Dowex 50 W, 1 g) equilibrated with tris-HCl buffer (0·1 mol l^{-1}, pH 8·0), was incubated with the enzyme solution (500 U *A. oryzae* tannase) dissolved in the same buffer for 12 h at 4 °C. The unbound enzyme was removed by washing with 0·01 mol l^{-1} NaCl until no activity or soluble protein was detected (Kusano *et al.* 1989).

Covalent binding. Chitosan (1 g) was dissolved in 100 ml 0·1 mol l^{-1} HCl containing 2·5% (v/v) glutaraldehyde (GA) for 2 h at 30 °C. The solubilized chitosan was precipitated by the addition of 1 ml 1·0 mol l^{-1} NaOH. The precipitate was separated by filtration (using a sintered glass funnel) and washed with distilled water to remove the excess GA. The wet chitosan was mixed with 5·0 ml of the enzyme solution (500 U *A. orzyae* tannase) and stirred for 1 h at 30 °C. The unbound enzyme was removed by washing with distilled water until no protein or activity was detected (Ohtakara and Mitsutomi 1987). Chitin (1 g) was shaken with 10 ml 2·5% (v/v) GA. Chitin was then collected by filtration (using a sintered glass funnel) and washed with distilled water to remove the excess GA. The wet chitin was mixed with 5·0 ml of the enzyme solution (500 U *A. oryzae* tannase) for 2 h at 30 °C. The unbound enzyme was removed by washing with distilled water as described above (Ohtakara and Mitsutomi 1987).

Entrapment.

(i) In polyacrylamide: The entrapment of the enzyme was carried out in 7% (w/v) acrylamide with a 3·0% (w/w, of the total monomer content) crosslinker (*N,N* methylene-bis-acrylamide) concentration, as described by Roy *et al.* (1984). The added enzyme activity for entrapment was 100 U (*A. oryzae* tannase) 10 ml^{-1} gel. The gel was washed with saline, cut into 2 × 2 × 2 mm fragments and kept in tris-HCl buffer (10 mmol l^{-1}, pH 7·5) at 4·0 °C for 72 h to remove the unbound enzyme.

(ii) In Ca-alginate: 10 ml 5% (w/v) Na-alginate were mixed with 100 U of *A. oryzae* tannase. The entrapment was carried out by dropping the mixture into 100 mmol l^{-1} $CaCl_2$ solution. The resulting beads (1·0–1·5 mm diameter) were collected and washed with distilled water to remove the unbound enzyme (Abdel-Naby 1993).

Properties of the free and immobilized tannase

Effect of pH. The effect of pH on the free and immobilized tannase was studied using citrate buffer (0·1 mol l^{-1}, pH 3·5–5·5), citrate-phosphate buffer (0·1 mol l^{-1} pH 6–7·5) and phosphate buffer (0·1 mol l^{-1}, pH 7·0–8·0).

pH stability. The free and immobilized enzyme were incubated in universal buffer (0·1 mol l^{-1}) of various pH values (3·0–10) at 35 °C. After 30 min, the enzyme samples were cooled at 4 °C and dialysed against distilled water. The residual enzyme activity was assayed under the standard conditions.

Thermal stability. The enzyme samples were incubated with citrate-phosphate buffer (0·1 mol l^{-1}) at the optimum pH value (5·5 for the free enzyme and 4·5 for the immobilized enzyme) at a designated temperature (50–70 °C) for 5–60 min. The residual activity was assayed under the standard conditions.

Protein estimation

The protein was estimated using the method developed by Bradford (1976). The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the protein originally added.

Operational stability of the immobilized tannase

Chitosan-immobilized tannase (1 g, wet) containing about 19·31 U *A. oryzae* tannase was incubated with 20 ml 2% (w/v) tannic acid in citrate-phosphate buffer (0·1 mol l^{-1}, pH 4·5) at 55 °C for 60 min. At the end of the reaction, the immobilized enzyme was collected by centrifugation at

5000 rev min^{-1} 2500 *g* for 15 min, washed with distilled water, and resuspended in 20 ml 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 represented the mean values within ± 5·0% of the individual values.

RESULTS AND DISCUSSION

Tannase enzyme from *A. oryzae* was immobilized on various carriers by different methods of immobilization including: physical adsorption on AS-alumina and colloidal chitin; ionic binding onto Dowex 50 W and DEAE-Sephadex A-25; covalent binding on chitosan and chitin; and entrapment on polyacrylamide and Ca-alginate. The immobilized tannase activity was evaluated (Table 1). The immobilized enzyme prepared by covalent binding to chitosan had the highest immobilized activity (107 U g^{-1} carrier) and the highest immobilization yield (26·6%). Thus, chitosan was used as a carrier through this study.

The specific activity of the immobilized tannase (14·65 U mg^{-1} protein) was 20·3% of the original specific activity (72·2 U mg^{-1} protein) exhibited by the free enzyme. This drop in specific activity may be due to diffusional limitation (i.e. resistance of the substrate to diffuse into the immobilization matrix and resistance of the products to diffuse out), as reflected by the apparent lower energy of activation for the immobilized enzyme (5·77 *vs* 6·75 kcal mol^{-1}, see below). This apparent lower energy of activation for the immobilized enzyme has been reported to be an indication of diffusional limitation (Kitano *et al.* 1982). On the other hand, Gottschalk and Jaenicke (1991) reported that the immobilization of the enzyme by covalent binding (as in the present case) would lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in catalytic activity. The decrease in specific activity after enzyme immobilization has been previously reported (Siso *et al.* 1990; Gottschalk and Jaenicke 1991).

The optimum pH of the immobilized enzyme was shifted to acidic range (pH 4·5) from pH 5·5, which was the optimum for the free enzyme (Fig. 1). The acidic shift in the optimum pH for catalytic activity as an effect of immobilization on the cationic matrix (as in the present case) was in agreement with the general observation that the positively-charged supports displace pH-activity curves of the enzymes attached to them towards lower pH values (Krajewska *et al.* 1990).

The pH stability of the free and immobilized *A. oryzae* tannase was compared in the pH range 3·0–10·0 at 25 °C during 30 min incubation periods (Fig. 2). The pH stability of the immobilized tannase was also shifted to a more acidic

Table 1 Immobilization of *Aspergillus oryzae* tannase

Carrier	Added enzyme (A)	Unbound enzyme (B)	Immobilized enzyme (I)	Specific activity of the immobilized enzyme	Immobilization yield = I/(A − B) %
Physical adsorption	(U g^{-1} carrier)	(U g^{-1} carrier)	(U g^{-1} carrier)	(U mg^{-1} protein)	
AS – alumina	500	340	31·5	10·80	19·64
Colloidal chitin	500	235	59·5	12·31	22·45
Ionic binding	(U g^{-1} carrier)	(U g^{-1} carrier)	(U g^{-1} carrier)	(U mg^{-1} protein)	
Dowex 50 W	500	168·5	23·5	8·20	7·09
DEAE-Sephadex	500	97·4	48·0	10·50	11·83
Covalent binding	(U g^{-1} carrier)	(U g^{-1} carrier)	(U g^{-1} carrier)	(U mg^{-1} protein)	
Chitin	500	109·0	87·4	11·23	22·25
Chitosan	500	98·4	107·0	14·65	26·60
Entrapment	(U 10 ml^{-1} gel)	(U 10 ml^{-1} gel)	(U 10 ml^{-1} gel)	(U mg^{-1} protein)	
Polyacrylamide*	100	27·3	13·3	10·77	18·29
Ca-alginate†	100	67·9	4·7	8·67	14·64

* Final gel concentration, 7%.
† Final gel concentration, 5%.

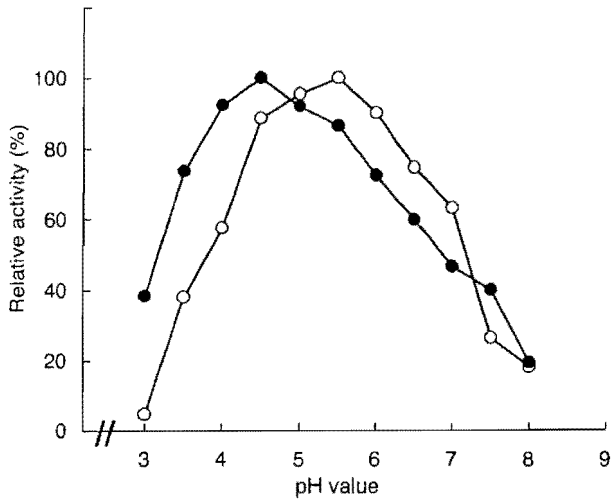

Fig. 1 Effect of pH on the activity of free and chitosan-immobilized tannase. (○), Free enzyme; (●), immobilized enzyme

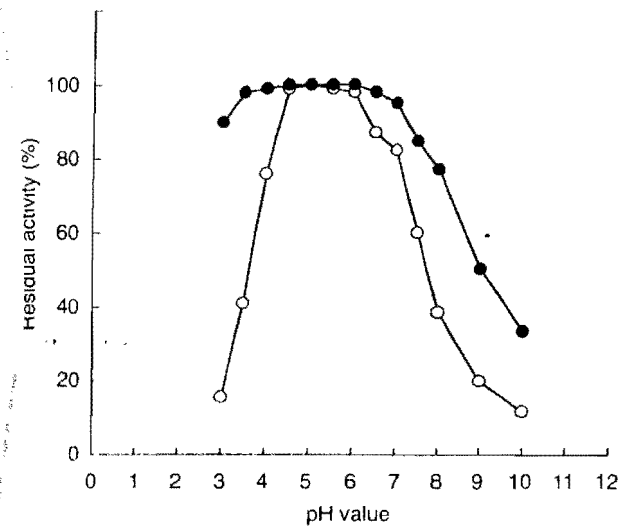

Fig. 2 pH stability of free and chitosan-immobilized tannase. (○), Free enzyme; (●), immobilized enzyme

range (pH 3·0–6·5) than that of the free enzyme (4·5–6·0). This effect may have been caused by the microenvironmental pH of the chitosan matrix. At high H^+ concentrations, the amino groups of chitosan would be protonated, thereby attracting hydroxyl ions which would maintain a higher microenvironmental pH than in the bulk solution, and thus stabilize the immobilized enzyme (Bissett and Sternberg 1978).

The temperature dependence of the activity of soluble and immobilized tannase was studied in 0·1 mol l^{-1} citrate-phosphate buffer at the optimum pH for the catalytic activity (5·5 for the free enzyme and 4·5 for the immobilized enzyme). The free enzyme had an optimum temperature of about 40 °C, whereas that of the immobilized enzyme was shifted to 55 °C (Fig. 3). The increase in the optimum temperature is probably a consequence of enhanced thermal stability. The activation energy (obtained from Arrhenius plots) for the immobilized enzyme was 5·77 kcal mol^{-1}, which is lower than that of the free enzyme (6·75 kcal mol^{-1}). Kitano *et al.* (1982) and Allenza *et al.* (1986) reported that the activation energy of the immobilized enzyme was lower than that of the free enzyme because the internal diffusion of the substrate into the carrier-enzyme system was the rate-limiting step.

The rates of heat inactivation of soluble and immobilized tannase were investigated in the temperature range 50 to 70 °C. As shown in Fig. 4, the thermal inactivation process of the immobilized enzyme corresponded well to the theoretical curves of a simple first-order reaction. This is probably because the steric position of the enzyme molecules immobilized in the active form is relatively similar (Ivony *et al.* 1983). In general, the immobilization process on chitosan protected the enzyme against heat inactivation. For example, the calculated half-life values (Table 2) show that heat inactivation of the free enzyme at 50, 60,and 70 °C are 1·33, 2·22 and 3·13 times faster, respectively, than those of the immobilized enzyme. The deactivation rate constant at 60 °C for immobilized tannase is $1{\cdot}73 \times 10^{-2}\,min^{-1}$, which is lower than that of the free enzyme ($3{\cdot}80 \times 10^{-2}\,min^{-1}$). Using the Arrhenius equation, the activation energy for thermal denaturation of the free enzyme is about 20·9 kcal mol^{-1}, which is 4·1 kcal mol^{-1} lower than that of the immobilized enzyme (Table 2).

Lineweaver-Burk plots of the free and immobilized *A. oryzae* tannase gave K_m (Michaelis constant) of 12·5 mg ml^{-1}

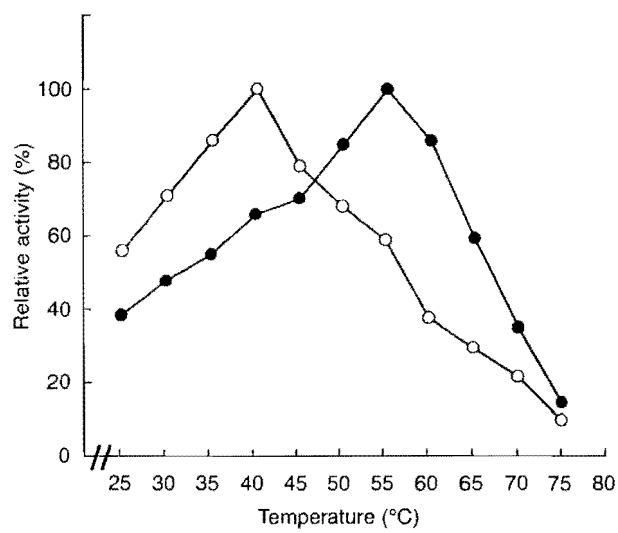

Fig. 3 Effect of temperature on the activity of free and chitosan-immobilized tannase. (○), Free enzyme; (●), immobilized enzyme

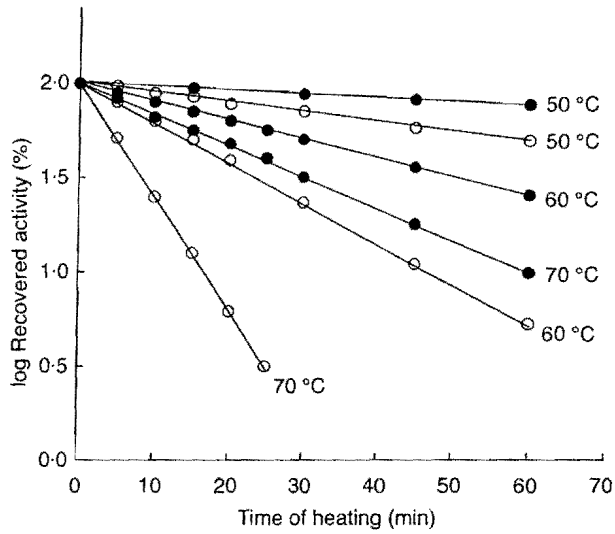

Fig. 4 Heat inactivation of free and chitosan-immobilized tannase. (○), Free enzyme; (●), immobilized enzyme

Table 2 Comparison of thermal stabilities of both free and chitosan-immobilized *Aspergillus oryzae* tannase

Property	Free enzyme	Immobilized enzyme
Half-life (min)		
50 °C	90	120
60 °C	18	40
70 °C	8	25
Deactivation constant rate (min^{-1})		
50 °C	$0{\cdot}77 \times 10^{-2}$	$0{\cdot}57 \times 10^{-2}$
60 °C	$3{\cdot}80 \times 10^{-2}$	$1{\cdot}73 \times 10^{-2}$
70 °C	$8{\cdot}60 \times 10^{-2}$	$2{\cdot}77 \times 10^{-2}$
Activation energy of denaturation (kcal mol^{-1})	20·9	25·0

and 20 mg ml^{-1}, respectively, with tannic acid. The V_{max} (the maximum reaction rate) of the free and immobilized tannase were 83 and 40 U mg^{-1} protein, respectively. This increase in K_m value after immobilization may be partially due to mass transfer resistance of the substrate into the immobilization medium (chitosan). Mass transfer resistance appears to be drastic in macromolecule substrates such as tannins. Also, it could explain why the substrate had low accessibility to the enzyme-active sites. On the other hand, multiple fixation of the enzyme by the effect of covalent binding (as in the present case) would also lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in catalytic activity (Gottschalk and Jaenicke 1991). The increase in K_m value and decrease in V_{max} after enzyme immobilization have also been reported by other investigators (Bissett and Sternberg 1978; Ohtakara and Mitsutomi 1987; Krajewska *et al.* 1990; Abdel-Naby 1993).

The activities of both free and immobilized *A. oryzae* tannase towards different tannin substrates are listed in Table 3. The immobilized enzyme, like the free enzyme, decomposes a variety of tannins. However, the rate of gallic acid release from tannin obtained from the leaves and bark of *Acacia nilotica* and *A. salinga*, relative to tannic acid, was lower with the immobilized enzyme. It was not clear whether this was due to a change in the affinity of the enzyme towards these substrates, or to the rate of mass transfer peculiar to the immobilized system.

The effects of various metal ions on the activity of the immobilized tannase compared with that of the native enzyme are listed in Table 4. Calcium ions showed no action on the free and immobilized enzymes. On the other hand, Mg^{-2}, Co^{2+}, Fe^{2+} and Hg^{2+} adversely affected the activity of both native and immobilized enzymes. However, it was observed that the inhibitory effect of these ions was less pronounced with the immobilized enzyme. This may be due to the protection of the immobilized enzyme by the carrier. This protection may result from at least two effects: (i) structure changes in the enzyme molecule introduced by the applied immobilization procedure and consequently, lower accessibility of inhibiting ions to the active site of the enzyme; and (ii) the chelating effect of chitosan which is known to be a very powerful chelating agent (Muzzareli 1973). Similar results have been reported for other immobilized enzymes (Kimura *et al.* 1989; Krajewska 1991).

Table 3 Relative activity of free and chitosan-immobilized *Aspergillus oryzae* tannase on various tannins

	Relative activity (%)	
Origin of tannin substrate	Free enzyme	Immobilized enzyme
Tannic acid	100	100
Commercial tannic acid	84·1	88·8
Commercial red tea	22·4	25·0
China green tea	69·1	60·1
Leaves of *Acacia nilotica*	39·0	9·5
Bark of *A. nilotica*	38·3	8·5
Bark of *A. salinga*	37·8	4·6
Bark of *A. ehrenbergina*	8·5	0·0

The activities with tannic acid (100%): free enzyme, 72·20 U mg^{-1} protein; immobilized enzyme, 14·65 U mg^{-1} protein.

Table 4 Effect of some metal ions on the activity of free and chitosan-immobilized *Aspergillus oryzae* tannase

	Residual activity (%)	
Metal ion (5 mmol l^{-1})	Free enzyme	Immobilized enzyme
None*	100	100
$CaCl_2$	100	100
$MgCl_2$	71·6	86
$CoCl_2$	50·7	76
$CuSO_4$	41·0	56
$FeSO_4$	38·7	72
$ZnCl_2$	44·6	71·7
$HgCl_2$	16·3	70·0

* The original activities (100%): free enzyme, 72·20 U mg^{-1} protein; immobilized enzyme, 14·65 U mg^{-1} protein.

The operational stability of the immobilized *A. oryzae* tannase was evaluated in repeated batch process. The results (Fig. 5) indicated that the catalytic activity of the immobilized enzyme was durable under repeated use. Thus, the immobilized enzyme was able to keep producing a good yield of hydrolysis products (0·78–0·66 mmol l^{-1} gallic acid), with as high as 85% of the initial catalytic activity after 17 runs.

The overall performance of the immobilized *A. oryzae* tannase catalytic activity, thermal and pH stabilities, and its possible re-use, are more promising than for the free enzyme. In addition, the immobilized activity of *A. oryzae* tannase (107 U g^{-1} carrier, 14·65 U mg^{-1} bound protein) reached by covalent binding on chitosan was higher than for treatment of tea cream for degradation of tannin content (Weetal and Dater 1974), improving the fermentation rate and stability of beer (Giovanelli 1989), and improving the protein digestibility (Carmen *et al.* 1992) and synthesis of gallic acid esters of *n*-propanol and amyl alcohol (Weetal 1985). Accordingly, it is suggested that *A. oryzae* tannase immobilized on chitosan by covalent binding is suitable for practical application.

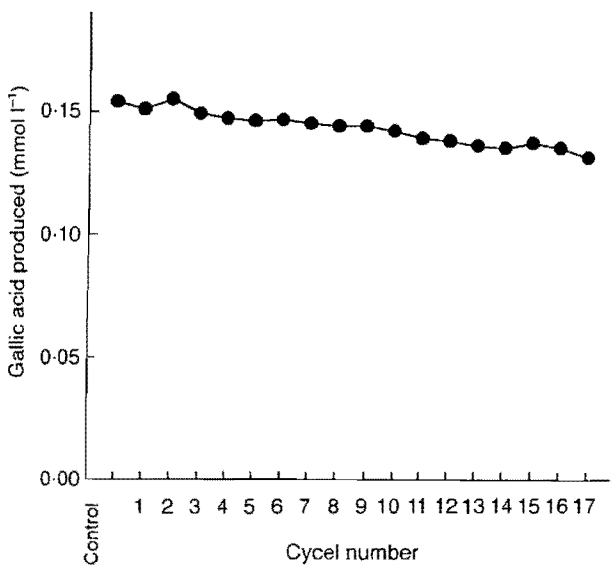

Fig. 5 Operational stability of chitosan-immobilized tannase after repeated use. The reaction mixture is described in the text

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