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# Catalytic properties of the immobilized *Aspergillus tamarii* xylanase

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## Abstract

Xylanase from *Aspergillus tamarii* was covalently immobilized on Duolite A147 pretreated with the bifunctional agent glutaraldehyde. The bound enzyme retained 54.2% of the original specific activity exhibited by the free enzyme (120 U/mg protein). Compared to the free enzyme, the immobilized enzyme exhibited lower optimum pH, higher optimum reaction temperature, lower energy of activation, higher $K_m$ (Michaelis constant), lower $V_{max}$ (maximal reaction rate). The half-life for the free enzyme was 186.0, 93.0, and 50.0 min for 40, 50, and 60 °C, respectively, whereas the immobilized form at the same temperatures had half-life of 320, 136, and 65 min. The deactivation rate constant at 60 °C for the immobilized enzyme is about $6.0 \times 10^{-3}$, which is lower than that of the free enzyme ($7.77 \times 10^{-3}$ min). The energy of thermal deactivation was 15.22 and 20.72 kcal/mol, respectively for the free and immobilized enzyme, confirming stabilization by immobilization. An external mass transfer resistance was identified with the immobilization carrier (Duolite A147). The effect of some metal ions on the activity of the free and immobilized xylanase has been investigated. The immobilized enzyme retained about 73.0% of the initial catalytic activity even after being used 8 cycles.

**Key words:** *Aspergillus tamarii* – xylanase – immobilization – properties

## Introduction

Hemicellulose is one of the most abundant organic substances, accounting for 5–50% of the dry weight of plant materials. Xylan is the most common component of hemicellulose (Puls and Poutanen 1989). Extensive degradation of xylans to monosaccharides is achieved by the cooperative action of multi-enzymes including: endo β-1,4-xylanase (β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8), usually called xylanase and β-xylosidase (1,4-xylan xylohydrolase, EC 3.2.1.37) (Gilbert and Hazlewood 1993).

Xylanases are mainly responsible for the hydrolysis of xylan and xylo-oligosaccharides (Gilbert and Hazlewood 1993). Xylanases have attracted considerable research interest because of their potential industrial applications, including hydrolysis of lignocellulose to biofuel fermentable sugars, bread making, clarification of beer and juices (Royer and Nakas 1989). Xylanases are also believed to be essential in improving the nutritive quality of animal feed (Gilbert and Hazlewood 1993). In addition, specific xylanases can be used to facilitate pulp bleaching in order to reduce the amount of chlorine required to achieve a target pulp brightness (Gerber *et al.* 1999; Senior *et al.* 1992).

Immobilization of the enzymes on a water-soluble carrier becomes a subject of interest for industrial applications. The advantages of this technique include the possibility of enzyme reuse, enhanced thermal stability, ease of catalyst separation from the reaction mixture and ready application for automated continuous processes. The activities of the immobilized xylanases so far reported are low when compared to those reported for other immobilized enzymes ( Roy *et al.* 1984; Abdel-Naby 1993; Tyagi and Gupta 1995).

In a previous work, an active cellulase-free xylanase was produced by *Aspergillus tamarii* (Gouda 2000). In the present work, we describe the preparation of immobilized *Aspergillus tamarii* xylanase by covalent binding to Duolite A147. The changes of the characteristic features of the enzyme by the effect of immobilization

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have been studied. The catalytic properties and stability of the immobilized enzyme have been compared to those of the free enzyme.

## Materials and methods

*Microorganism. Aspergillus tamarii* was a local isolate identified by the "Centraal Bureau voor Schimmel Cultures, Baarn/Holland" (Gouda 2000). It was subcultured on malt extract agar and maintained at 4 °C.

*Carrier for enzyme immobilization.* Duolite A 147, a strongly basic ion exchange crosslinked polystyrene functionalized with quaternary ammonium groups, was from Fluka, Switzerland.

*Enzyme production.* The production medium used was that of Biswas *et al.* (1990) and had the following composition (g $l^{-1}$): $NaNO_3$, 9; KCl, 0.52; $KH_2PO_4$, 1.52; $COCl_2 \cdot 6H_2O$, 0.008; $MgSO_4 \cdot 7H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; casein hydrolysate, 1; oat xylan, 6. Mycelia were cultivated in 250 ml Erlenmeyer flasks, each containing 50 ml of the sterile medium. The culture was incubated at 30 °C for 7 days on a rotary shaker. At the end of the incubation period, the mycelia were removed by centrifugation (4500 xg) for 10 min at 4 °C. Proteins in the filtrate were precipitated by 70% ammonium sulfate. The pellet was removed by centrifugation and dissolved in 0.01-M citrate-phosphate buffer, pH 6.5 and dialysed in a Serva bag against the same buffer. This partially purified enzyme (specific activity 120 U/mg protein) was used for the preparation of the immobilized enzyme.

*Determination of xylanase activity.* Xylanase activity was determined by measuring the reducing sugar released from birchwood xylan (Sigma) according to the method of Miller (1959). Unless specified otherwise, the assay mixture consisted of 0.5 ml xylan (2%, w/v) in citrate-phosphate buffer (0.2 M, pH 6.5) and 0.5 ml of enzyme solution or weighed amount of the immobilized enzyme. The reaction was incubated at 30 °C for 10 min. One unit of enzyme activity (U) is defined as the amount of the enzyme which releases one μmole of reducing sugars (as xylose) per min under the assay conditions.

*Enzyme immobilization.* One g of Duolite A147 was shaken with 50 ml of 2.5% (v/v) glutaraldehyde (GA). The Duolite A147 was collected by filtration (using a sintered G1 glass funnel) and washed with distilled water to remove the excess GA. The wet Duolite A147 was mixed with 5.0 ml 0.01 M citrate-phosphate buffer (pH 6.5) containing a known amount of *Aspergillus tamarii* xylanase. The mixture was incubated for 12 h at 4.0 °C. The unbound enzyme was removed from the carrier by washing with distilled water until no activity or protein was detected in the washings.

*Properties of the free and immobilized xylanase*

*Effect of pH.* The effect of pH on the free and immobilized xylanase was studied using citrate-phosphate buffer (0.1 M pH 4.0–7.0), and phosphate buffer (0.1 M, pH 7.0–8.0).

*pH stability.* The free enzyme as well as the immobilized one were incubated in citrate-phosphate buffer (0.1 M, pH 4.5–8.0) at 20 °C. After 60 min, the enzyme samples were cooled at 4 °C and dialyzed 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 M) at the optimum pH value (pH 6.5 for the free enzyme and pH 6.0 for the immobilized one) at the designated temperature (40–60 °C) for 30–180 min. The residual activity was assayed under the standard conditions.

*Protein estimation.* Protein was estimated using the method of Lowery *et al.* (1951). The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the originally added protein.

*Operational stability of the immobilized enzyme.* One g of Duolite A147 immobilized xylanase (wet) containing about 500 U of *A. tamarii* xylanase was incubated with

**Table 1.** Maximal loading of *Aspergillus tamarii* xylanase on Duolite A147.

| Added enzyme | | Unbound enzyme | | Immobilized enzyme | | Specific activity (U/mg protein) | Immobilization yield = **I/(A–B)%** |
|---|---|---|---|---|---|---|---|
| Protein (mg/g carrier) | Activity (U/g carrier) **(A)** | Protein (mg/g carrier) | Activity (U/g carrier) **(B)** | Protein (mg/g carrier) | Activity (U/g carrier) **(I)** | | |
| 14.16 | 1700 | 9.95 | 1252.0 | 4.21 | 277.0 | 65.79 | 61.83 |
| 21.25 | 2550 | 15.32 | 1890.0 | 6.72 | 387.0 | 65.26 | 58.15 |
| 28.33 | 3400 | 21.61 | 2498.0 | 9.32 | 436.6 | 65.06 | 48.40 |
| 35.41 | 4250 | 26.09 | 3094.5 | 9.32 | 468.0 | 50.21 | 40.50 |
| 42.50 | 5100 | 31.30 | 3632.0 | 11.20 | 496.2 | 44.30 | 33.80 |

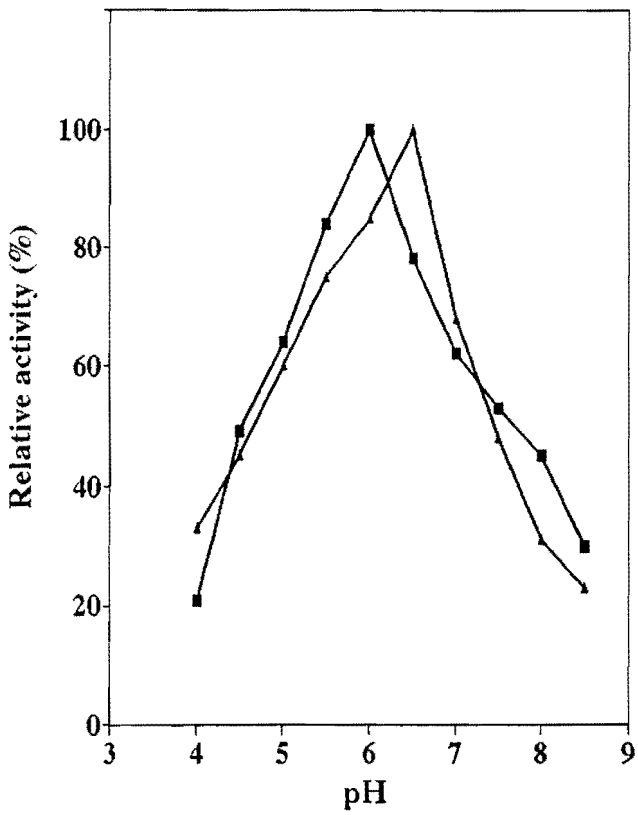


**Fig. 1.** Effect of pH on the activity of the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

100 ml of 1% (w/v) birchwood xylan in citrate-phosphate buffer (0.1 M, pH 6.0) at 55 °C for 10 min. At the end of the reaction, the immobilized enzyme was collected by centrifugation at 5000 rpm for 15 min, washed with distilled water, and resuspended in 100 ml of freshly prepared substrate to start a new run. The supernatant was assayed for reducing sugars.

*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

Xylanase enzyme from *Aspergillus tamarii* was immobilized by covalent coupling on Duolite A147. The specific activity of the immobilized xylanase (65.06 U/mg protein) was 54.2% of the original specific activity (120 U/mg protein) exhibited by the free enzyme. This drop in specific activity could be attributed to diffusional limitation of the substrate and product flux. The rate of mass transfer of the substrate and products to and from the immobilization matrix presents problems not found with free enzymes. For the substrate to be acted upon by an immobilized enzyme it must diffuse into the matrix and the product must diffuse out. These diffusional processes often result in lower concentration of the substrate and a higher concentration of product at the enzyme active site than in solution (Siso *et al.* 1990). These diffusional problems become more significant with macromolecular substrates, like xylan. Some of the results presented here can be interpreted as representing mass transfer effects, such as the apparent lower values of the energy of activation for the immobilized form as compared to the free enzyme (4.99 versus 5.75 kcal/mol, *see below*). This drop in activation energy compared to the free enzyme has been interpreted as an indication of diffusional limitation (Allenza *et al.* 1986; Kitano *et al.* 1982; Kusano *et al.* 1989).

Variation of xylanase concentration attached to the carrier altered the apparent specific activity of the immobilized enzyme. Increasing the bound protein higher than 6.71 mg/g carrier led to gradual decrease of the specific activity of the immobilized enzyme (Table 1). Hyndman *et al.* (1992) reported that excessive packing of the enzyme would lead to the development of steric effect around the enzyme active sites. This steric effect is commonly reflected by a decrease of the retained activity of the immobilized enzymes. Similar results were previously reported for other immobilized enzymes (Wang and Chio 1998; Abdel-Naby 1999).

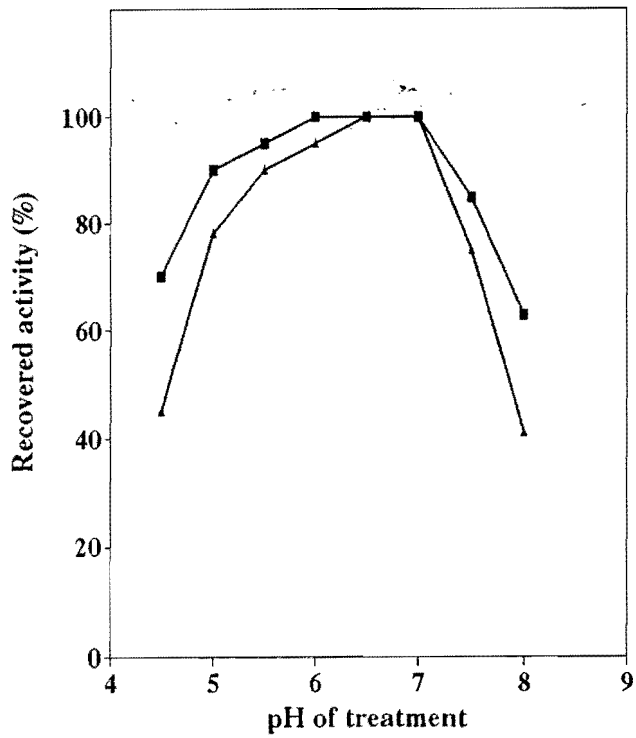


**Fig. 2.** pH stability of the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

Figure 1 shows the activity of the free and immobilized *Aspergillus tamarii* xylanase at different pH values. The optimum pH of the immobilized enzyme was slightly shifted to acidic range (pH 6.0) from pH 6.5 which was the optimum for the free enzyme. The acidic shift in the optimum pH for catalytic activity as an effect of immobilization on the cationic matrix (as the present case) was in agreement with the general observation that the positively charged supports displace pH-activity curves towards lower pH values (Krajewska *et al.* 1990).

The pH stability of the free and immobilized *Aspergillus tamarii* xylanase was compared in the pH range between 4.5 and 8.0 at 20°C during 60 min incubation periods (Fig.2). The pH stability of the immobilized xylanase was also slightly shifted to a more acidic range (pH 5.0–7.0) than that of the free enzyme (6.0–7.0). These results reveal the same trend as in the shift of the optimum pH. The stabilization in the acidic medium originates from the cationic character of the support used (Duolite A147). Similar observations of the stabilizing effect in acidic media by using carriers of cationic character have been reported for another immobilized xylanase (Abdel-Naby 1993).

The temperature dependence of the activity of soluble and immobilized *Aspergillus tamarii* xylanase was studied in 0.1 M citrate-phosphate buffer at the optimum

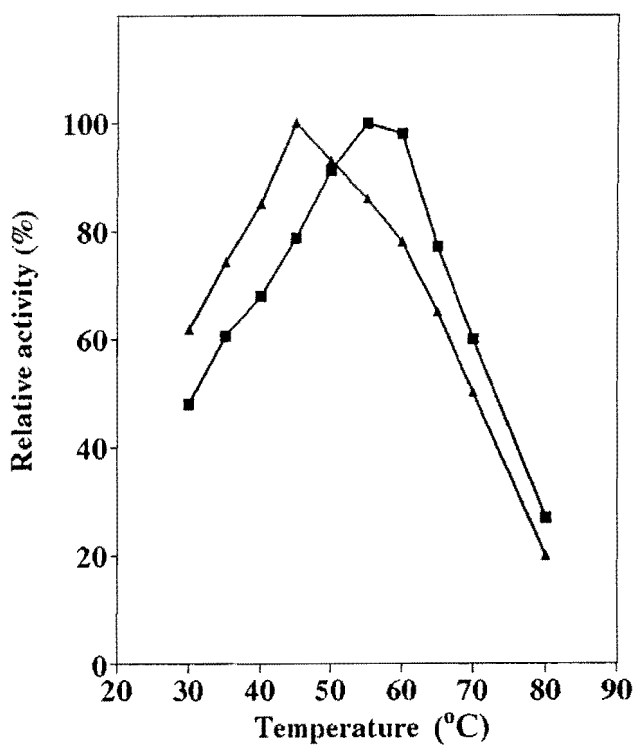


**Fig. 3.** Effect of temperature on the activity of the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

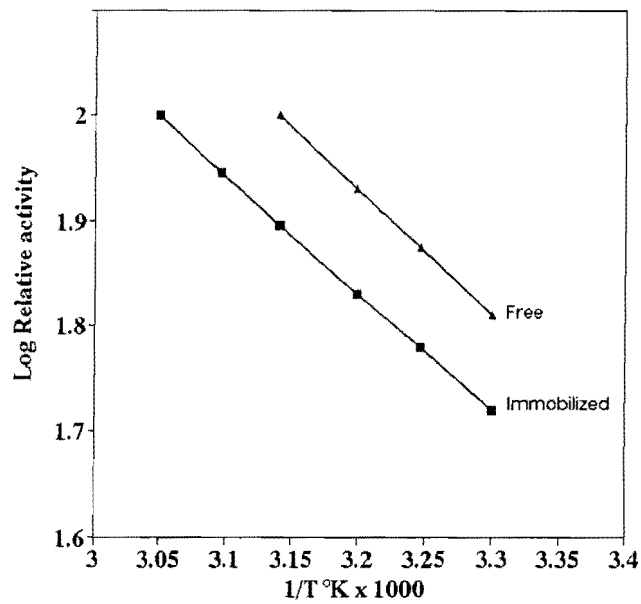


**Fig. 4.** Arrhenious plots, from data in Fig. 3.

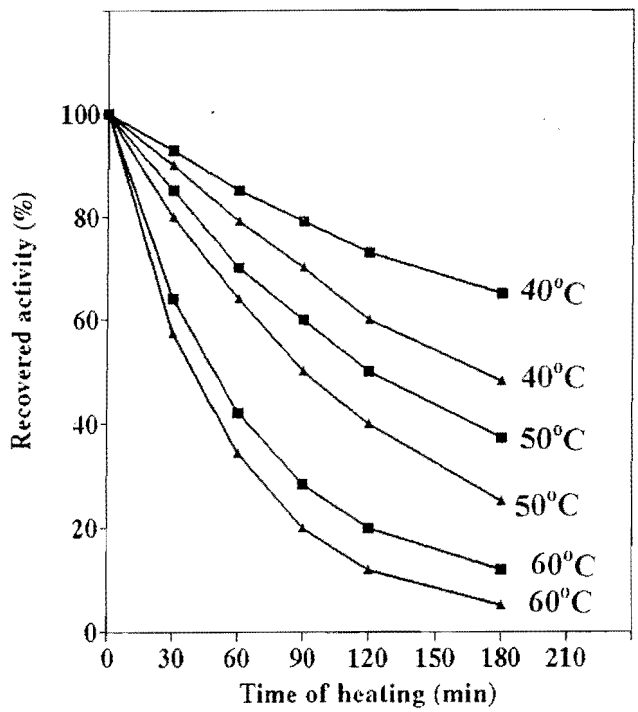


**Fig. 5.** Heat inactivation of the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

pH for the catalytic activity (pH 6.5 for the free enzyme and pH 6.0 for the immobilized enzyme). The free enzyme had an optimum temperature of about 45°C, whereas that of the immobilized enzyme was shifted to 55°C (Fig. 3). The increase of the optimum temperature probably is a consequence of enhanced thermal stability.

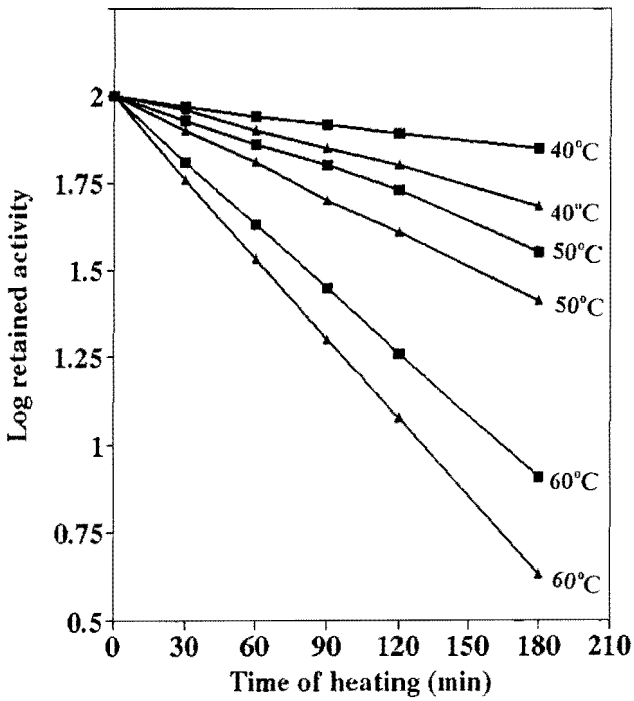


**Fig. 6.** Log of activity retained as a function of time for the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

Arrhenius plots of the temperature data of both the free and immobilized enzyme were linear (Fig. 4). The calculated value of the activation energy for the free enzyme was 5.75 kcal/mol. However, the calculated value for the immobilized enzyme was 4.99 kcal/mol. The lower value of the activation energy of the immobilized enzyme compared to the free enzyme may be attributed to mass transfer limitations. (Allenza *et al.* 1986; Kitano *et al.* 1982).

The rates of heat inactivation of soluble and immobilized xylanase were investigated in the temperature range between 40 and 60 °C (Fig. 5). As shown in Fig. 6, the thermal inactivation process of the immobilized enzyme corresponds well with the theoretical curves of a simple first-order reaction. Probably because, the steric position of the enzyme molecules immobilized in the active form is relatively similar to each other (Zanin and DeMoraes 1998). In general, the immobilization process of the enzyme on Duolite A147 protects the enzyme against heat inactivation. For example, the calculated half-life values (Table 2) show that the heat inactivation of the free enzyme at 40, 50, and 60 °C are 1.72, 1.46 and 1.3 times faster than those of the immobilized enzyme. In addition, the deactivation rate constant at 60 °C for the immobilized xylanase is $6 \times 10^{-3}$ min$^{-1}$, which is lower than that of the free enzyme ($7.77 \times 10^{-3}$ min$^{-1}$). Based on the Arrhenius equation, the energy of thermal deactivation was 15.22 and 20.72 kcal/mol, respectively, for the free and immobilized enzyme, confirming stabilization by immobilization (Table 2).

Linweaver-Burk plots of the free and immobilized *Aspergillus tamarii* xylanase gave $K_m$ values (Michaelis constant) of 3.57 mg/ml and 5.26 mg/ml, respectively, with birchwood xylan (Fig. 7). The $V_{max}$ (maximum reaction rate) of the free and immobilized xylanase were 192.3 and 137.0 U/mg protein, respectively. The increase of $K_m$ value and decrease of the $V_{max}$ after xylanase immobilization were previously reported (Roy *et al.* 1984; Abdel-Naby 1993; Tyagi and Gupta 1995). This increase of the $K_m$ value after the immobilization may be partially due to mass transfer resistance of the substrate into the immobilization medium Duolite A147. This mass transfer resistance drastically appears with macromolecule substrate like xylan.

The kinetics of the enzyme bound on the porous particle can be affected by external or internal diffusional

**Table 2.** Comparison of thermal stabilities of both free and immobilized *Aspergillus tamarii* xylanase on Duolite A147.

| Property | Free enzyme | Immobilized enzyme |
|---|---|---|
| Optimum Temperature (°C) | 45 °C | 55 °C |
| Activation energy (kcal/mol) | 5.75 | 4.99 |
| Half – life (min): | | |
| 40 °C | 186.0 | 320.0 |
| 50 °C | 93.0 | 136.0 |
| 60 °C | 50.0 | 65.0 |
| Deactivation constant rate (min$^{-1}$) | | |
| 40 °C | $1.66 \times 10^{-3}$ | $0.94 \times 10^{-3}$ |
| 50 °C | $3.33 \times 10^{-3}$ | $2.22 \times 10^{-3}$ |
| 60 °C | $7.77 \times 10^{-3}$ | $6.0 \times 10^{-3}$ |
| Activation energy of denaturation (kcal/mol) | 15.22 | 20.72 |

**Table 3.** Effect of some metal ions on the activity of free and Duolite A147 immobilized *Aspergillus tamarii* xylanase.

| Metal ion (10mM) | Residual activity (%) | |
|---|---|---|
| | Free enzyme | Immobilized enzyme |
| None* | 100.00 | 100.00 |
| $AgNO_3$ | 48.72 | 56.23 |
| NaCl | 100.13 | 105.35 |
| KCl | 100.25 | 100.34 |
| $CoCl_2$ | 110.47 | 113.29 |
| $CuSO_4$ | 47.39 | 65.93 |
| $MnSO_4$ | 82.31 | 90.12 |
| $ZnSO_4$ | 101.71 | 100.34 |
| $HgCl_2$ | 40.92 | 59.75 |

resistance, which respectively correspond to the transport of the substrate and products from the bulk solution to the outer surface of the enzymatic particle, and to the internal transport of these species inside the porous system of the particle (Siso *et al.* 1990). Eadie-Hofstee plots are useful for discerning between external (concave plots) and internal (sigmoidal plots) diffusional limitations. Linear plots are found in the absence of these effects, i.e for the enzyme in solution (Segel 1975).

In Fig. 8, Eadie-Hofstee plots for the immobilized xylanase showed concave plots. This means that, external diffusion limitation are found for *Aspergillus tamarii* xylanase bound to Duolite A147.

The effects of various metal ions on the activity of the immobilized *Aspergillus tamarii* xylanase as compared with that of the free enzyme are listed in Table 3. Cobalt ions slightly activate the free and immobilized enzyme. On the other hand, $Ag^{+}$, $Cu^{2+}$, 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. Similar results were reported for other immobilized enzymes (Krajewska 1991; Abdel-Naby 1999).

The operational stability of the immobilized *Aspergillus tamarii* xylanase was evaluated in repeated batch process. The immobilized enzyme is able to keep producing considerably good yield of reducing sugars (74.4–54.1 µmole xylose/ml/min) with high 73% of the

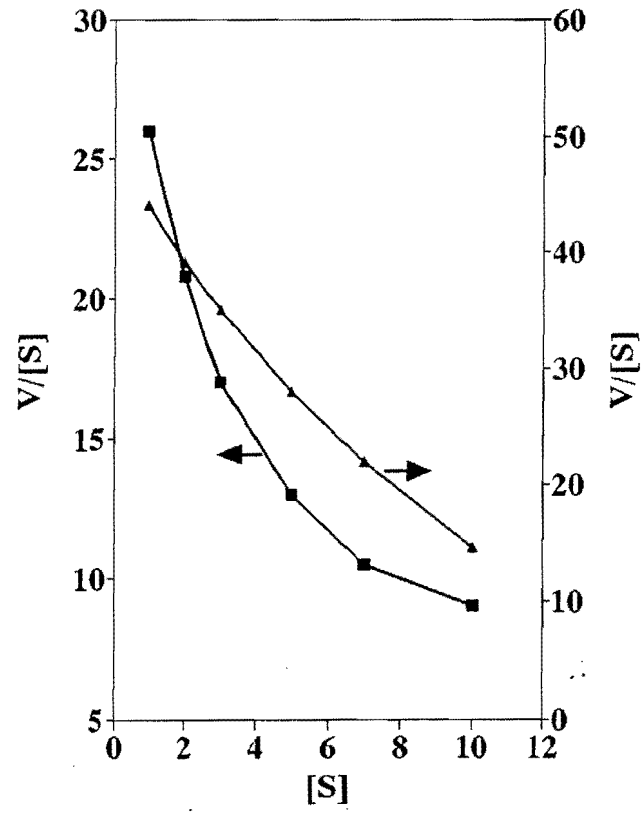


**Fig. 8.** Eadie-Hofstee plots for the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase
V = reaction rate in U/gram carrier, (S) = xylan concentration (mg/ml).

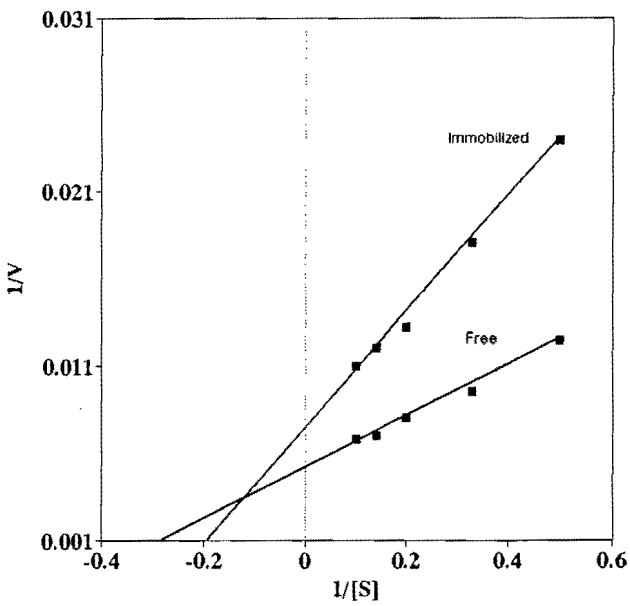


**Fig. 7.** Lineweaver-Burk plot for the determination of $K_m$ values for the free (▲) and Duolite A147 immobilized (■) *Aspergillus tamarii* xylanase.

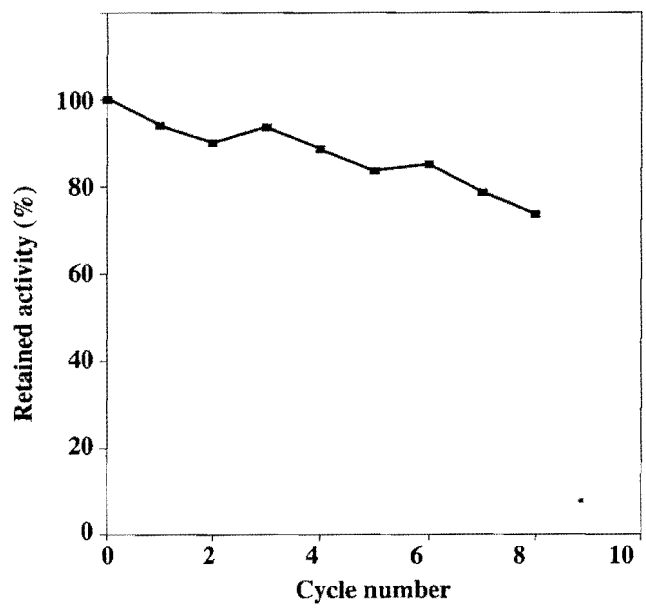


**Fig. 9.** Operational stability of Duolite A147 immobilized *Aspergillus tamarii* xylanase in repeated use. The reaction mixture is described in the text.

initial catalytic activity after 8 cycles (Fig. 9). The operational stability of the immobilized *Aspergillus tamarii* xylanase seems to be superior to that reported by Tagyi and Gupta (1995) for *Aspergillus niger* xylanase immobilized on magnetic latex beads (retained only 14% of the original activity after three cycles).

The overall performance of the immobilized *Aspergillus tamarii* xylanase with respect to catalytic activity, thermal and pH stabilities and reusability is promising compared with that of the free enzyme. Accordingly, it is suggested that *Aspergillus tamarii* xylanase immobilized on Duolite A147 by covalent binding is suitable for practical application in food, chemical and pharmaceutical industries.

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