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# Covalent immobilization of xylanase on the surface of alginate-glutaraldehyde beads decreases the 'catalytic efficiency' but provides 'low temperature stabilization' effect

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## Abstract

The manuscript aims to compare some of the kinetic properties of covalently immobilized xylanase on the surface of glutaraldehyde activated alginate beads with free xylanase. The immobilized enzyme displayed higher $V_{max}$, $K_m$ and kcat values but lower kcat/$K_m$ value in comparison to its free counterpart. Overall, the 'catalytic efficiency' of xylanase was found to decline with immobilization. The isokinetic temperature ($T_{isokin}$) of free and immobilized xylanase was recorded at 86.5ºC with *ln kd* value of about -4.0. The method of immobilization provided 'low temperature stabilization' effect to xylanase which makes it more thermostable at temperatures lower than the $T_{isokin}$. The ability of immobilized xylanase to work better than free enzyme could be explained by increase in $E_d$ and enthalpy along with a decrease in negative entropy after immobilization.

**Keywords:** Covalent immobilization, xylanase, glutaraldehyde-alginate beads, thermodynamics, isokinetic temperature, catalytic efficiency

## Introduction

Xylan, a polymer consisting primarily of β-1,4-linked xylose residues, is the main constituent of plant hemicellulose. Several enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase are involved in the hydrolysis of xylan polymers (Shallom and Shoham 2003). Among them, the most important one is β-1,4-xylanase (EC 3.2.1.8), which cleaves internal glycosidic bonds at random or specific positions of the xylan backbone and thus hydrolyzes xylan into xylooligosaccharides and xylose (Pal and Khanum 2011d). In recent years, xylanases have received tremendous attention due to their application potential in many industries such as pulp and paper, animal feed, baking, brewing, and biofuel, etc (Pal and Khanum 2010b).

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Filamentous fungus *A. niger* is known for its capacity to produce xylanases with different capacities that may provide the fungus with the maximum potential to degrade xylans from different sources (Pal and Khanum 2010a; Pal and Khanum 2011c). Immobilization of enzymes and enzyme systems, as well as their thermostabilization is of great practical and theoretical importance. Enzyme immobilization provides considerable advantages with the possibility of continuous processing, reuse of the enzyme and reduction of auto-digestion (Pal and Khanum 2011a). Microbial enzymes that play a crucial role in many industrial processes are usually immobilized on a food grade support like alginic acid. The immobilization procedure on alginate beads is not only inexpensive but also very easy to carry out and provides extremely mild conditions which provide considerable potential for industrial applications. The alginate matrix has been extensively used to immobilize many enzymes by entrapping (Arya and Srivastava 2006; Goel et al. 2006). But, for an enzyme like xylanase where the substrate is a large molecule, the entrapment method does not suit because of the poor accessibility of substrate to enzyme entrapped within the beads. The yield and efficiency of the immobilized xylanase is further reduced by the slow outward diffusion of hydrolytic products. To overcome these problems, a method of covalent immobilization of xylanase was standardized by us wherein the enzyme was attached to the surface of alginate beads through glutaraldehyde (Pal and Khanum 2011a). Once an enzyme has been immobilized, it is bound to have different kinetic constants in comparison to free enzyme. The present communication, in continuation, aims to compare some of the kinetic parameters of immobilized and free xylanase. The manuscript also focuses on how the process of covalent immobilization has provided 'low temperature stabilization' effect to xylanase.

## Materials and Methods

### *Microorganism and xylanase preparation*

The xylanase producing microorganism was isolated from a soil sample collected near fruit and vegetable debris in Mysore, India, and identified as *Aspergillus niger*, based on morphology, at Indian Type Culture Collection (ITCC), New Delhi, India. The isolate, named as *Aspergillus niger* DFR-5, was grown on Potato Dextrose Agar (PDA) slants at 30ºC for 5 days and subsequently stored at 4ºC. The inoculum was prepared by suspending the spores from

PDA slants by adding sterile 0.1% tween-80 to give a final count of ~1x$10^6$ spores/ml.

The production and purification studies of xylanase from *Aspergillus niger* DFR-5 has been reported by us earlier (Pal and Khanum 2010b; Pal and Khanum 2011d). Briefly, the extracellular xylanase was purified up to absolute homogeneity using $(NH_4)_2SO_4$ (30-65%) fractionation, size exclusion and ion-exchange chromatography. The preparation yielded a single peak in RP-HPLC confirming its purity. The molecular mass of xylanase as revealed by gel filtration and SDS-PAGE was ~32 kDa confirming its monomeric nature (Pal and Khanum 2011d). The absolutely pure enzyme preparation was covalently immobilized on the surface of glutaraldehyde-alginate beads. The immobilization process was optimized with a limited number of experiments designed using RSM and the process yield was more than 90% (Pal and Khanum 2011a).

*Enzyme assay and protein quantification*

Xylanase activity was assayed by the method of Khanna and Gauri (1993). The solution of oat spelt xylan and the enzyme preparation at appropriate dilution was incubated at 37°C for 30 min and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (1959), with xylose as the standard. The absorbance was recorded at 540 nm. One unit of xylanase is defined as the amount of enzyme required to release 1 µmol of reducing sugar as xylose equivalent/min under the assay conditions. All the experiments were done in triplicate and the results are expressed as mean±SD. The amount of protein in various samples was determined by Lowry method (1951) using bovine serum albumin as the standard.

*Determination of apparent kinetic parameters ($V_{max}$, $K_m$, kcat, kcat/$K_m$)*

To determine the maximum velocity ($V_{max}$) and Michael–Menten constant ($K_m$) of the free and immobilized xylanases, initial reaction rates were measured by using xylan substrate at different concentrations (2.5-25.0 mg/ml). The $K_m$ and $V_{max}$ values were calculated by means of the Lineweaver and Burk (1934).

$$\frac{1}{v} = \left(\frac{1}{V\max}\right) + \left(\frac{Km}{V\max}\right)\frac{1}{[S]}$$

Turn over number (kcat) was calculated by dividing $V_{max}$ by molar concentration of enzyme. Time required for one molecule of substrate to convert into product was calculating by dividing 1 by kcat value.

The 'catalytic efficiency' was calculated by dividing kcat by $K_m$.

*Thermostability, thermodynamic parameters and isokinetic temperature ($T_{isokin}$)*

First order thermal deactivation rate constants ($k_d$) of free and covalently immobilized enzymes were deduced in the temperature range 45-70 ºC by incubating enzymes for 3 h. In all the cases, the activity measured at 0 min was taken as 100%.

The activation energy ($E_d$) for denaturation was determined by a plot of ln $k_d$ versus reciprocal of the absolute temperature (ºK) using the equation-

$$slope = \frac{-E_d}{R}$$

The $T_{isokin}$ was calculated from a cross of Arrhenius plot of free and covalently immobilized xylanase.

## Results and Discussion

In our previous study, a method was developed to covalently immobilize xylanase, purified from *A. niger* DFR-5, on the surface of glutaraldehyde activated alginate beads (Pal and Khanum 2011a). The developed method can overcome the problem of reduced permeability of xylan, a high molecular weight substrate, to its enzyme which is conventionally entrapped within the alginate beads. In continuation, the present work compares some of the kinetic properties of covalently immobilized xylanase with free xylanase.

*Determination of apparent kinetic parameters*

Change in kinetic parameters upon immobilization is a critical point to evaluate the efficacy and success of an immobilization protocol. Both the free and covalently immobilized xylanases showed increased activity with increasing concentration of substrate (2.5-25.0 mg/ml) (Fig. 1). The apparent kinetic parameters were calculated using Lineweaver-Burk plot (Fig. 2) and an increase in apparent $K_m$ (as described in the text, $K_m$ is an approximate measure of substrate affinity for the enzyme: it is numerically equal to the concentration of [S] at V = 0.5 $V_{max}$) value of immobilized xylanase (14.9 mg/ml) as compared to that of free enzyme (9.0 mg/ml) was recorded. The regression equations for the Lineweaver-Burk slopes of free and immobilized xylanases were y = 0.0013x + 0.0001 ($R^2$ = 0.8579) and y = 0.0019x + 0.0001 ($R^2$ = 0.9236), respectively. The increased value of $K_m$ indicates that the insoluble xylanase has an apparently lower affinity for its substrate than that of soluble one.

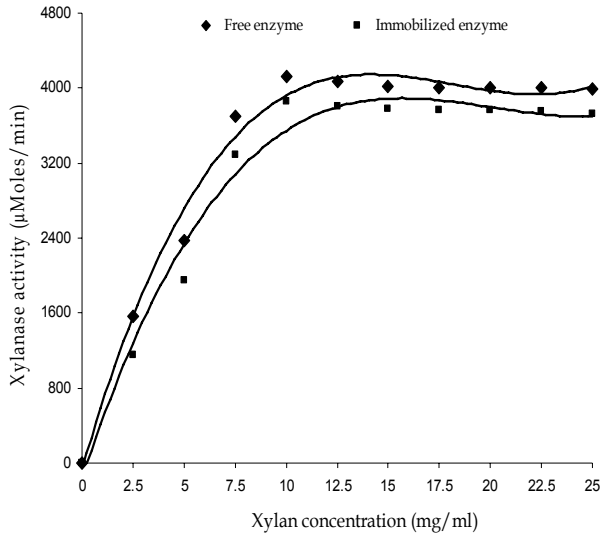



Figure 1. Effect of substrate concentration on xylanase (free and covalently immobilized) activity

The apparent low affinity for substrate may be explained by the steric hindrance of the active site caused by support or by the loss of sufficient enzyme flexibility necessary for substrate binding. Usually, most of the immobilized enzymes show increase in $K_m$ values especially when assayed with high molecular weight substrates like xylan (Abdel-Naby 1993; Gouda and Abdel-Naby 2002; Dalal et al. 2007).

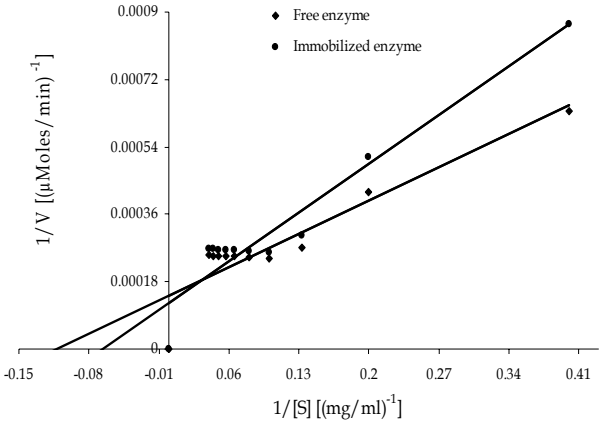
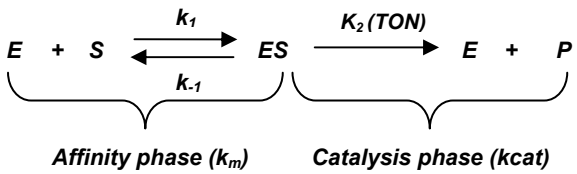



Figure 2. Lineweaver-Burk plot for the estimation of kinetic parameters

The free enzyme had an apparent $V_{max}$ of 7092 µmoles/min which was found to be marginally increased in case of immobilized xylanase (8000 µmoles/min) indicating a slight improvement in the catalytic efficiency of covalently immobilized enzyme. The apparent $K_m$ value of covalently immobilized xylanase was 65.5% higher than that of free xylanase, while the difference in apparent $V_{max}$ was small (only 12.8%). The increase in apparent $V_{max}$ upon immobilization is an unusual phenomenon but has been reported by Ai et al (2005) also, in case of xylanase immobilized on Eudragit S-100.

An overall enzyme catalyzed reaction




It can be seen above that there are two distinct phases one after the other. First one is the 'affinity phase' which decides $K_m$ value (in general, a lower value of $K_m$ means tighter substrate binding) and the second is 'catalysis phase' which decides kcat (the kcat corresponds to the maximum number of substrate molecules converted to product per molecule of enzyme). It is worth mentioning that neither kcat nor $K_m$ are suitable parameters individually to describe and compare the catalytic functions of enzymes since $K_m$ neglects the catalysis step while kcat pre-supposes that the reaction starts with ES complex and completely neglects the impact of substrate binding. A more meaningful analysis would include both of them i.e. kcat and $K_m$, in the form of 'catalytic efficiency' (kcat/$K_m$). A comparison of kcat/$K_m$ for the same enzyme with different substrates, or for two enzymes with their different substrates, is widely used as a measure of enzyme effectiveness (Eisenthal et al. 2007). Therefore, the experimental initial rates were further used for determination of kcat and calculation of 'catalytic efficiency' (kcat/$K_m$).

The covalently immobilized xylanase showed kcat and kcat/$K_m$ values of 9.09 x $10^4$ $min^{-1}$ (1.51 x $10^3$ $sec^{-1}$) and 101.67 ml/mg.sec, respectively, compared to 8.06 x $10^4$ $min^{-1}$ (1.34 x $10^3$ $sec^{-1}$) and 148.88 ml/mg.sec for the free xylanase (Table 1). The analyzed data indicates that TON of covalently immobilized xylanase was 12.7% higher than that of free enzyme. From the study, it can also be concluded that one soluble enzyme molecule needs 0.74 milliseconds to convert one molecule of substrate into product while one molecule of covalently immobilized xylanase requires 0.66 milliseconds of time. The results indicate that considering kcat alone, covalently immobilized xylanase has better enzymatic activity.

**Table 1.** Apparent kinetic parameters of free and covalently immobilized xylanase

| Constants | Free xylanase | Covalently immobilized xylanase |
|---|---|---|
| $K_m$ (mg/ml) | 9.0 | 14.9 |
| $V_{max}$ (µmoles/min) | 7092 | 8000 |
| kcat ($min^{-1}$) | 8.06 x$10^4$ | 9.09 x $10^4$ |
| kcat ($sec^{-1}$) | 1.34 x $10^3$ | 1.51 x $10^3$ |
| kcat/$K_m$ (ml/mg.sec) | 148.88 | 101.67 |

It is also worthwhile to mention that the apparent $V_{max}$ and kcat values obtained for the covalently immobilized xylanase were comparable to those of the free enzyme with a variation below 15% while a larger variation (~32%) was observed with regard to the kcat/$K_m$ values. The 'specificity constant' ('catalytic efficiency') of the enzymes towards oat-spelt xylan, represented by the values of kcat/$K_m$, showed ~32% decrease for immobilized xylanase. This important decrease could be attributed entirely to the difference in $K_m$ value since $V_{max}$ of the enzyme is not much affected by immobilization.

In spite of having a higher kcat value, the covalently immobilized xylanase exhibits 31.7% lesser 'catalytic efficiency' (kcat/$K_m$) as compared to free xylanase which indicates clearly the inefficiency of kcat alone to draw the exact inference and necessity to calculate the 'catalytic efficiency' while comparing the two forms (soluble and insoluble) of enzymes. Overall, the results of study clearly indicate that immobilization process proceeds with a decline in 'catalytic efficiency' of the enzyme.

*Thermostability, thermodynamic parameters and isokinetic temperature ($T_{isokin}$)*

The thermodynamic parameters of free and covalently immobilized xylanases have already been reported by us (Pal and Khanum 2011a) but being reproduced in Table 2 to discuss the present findings. Fig. 3 shows the dependences of *ln kd* on the inverse temperature (ºK) for free and covalently immobilized xylanase. There is an intersection of the Arrhenius plots at a point corresponding to 86.5°C with *ln kd* value of nearly -4.0, under which the immobilized enzyme is more stable and above which the free enzyme is more stable. This temperature is designated as the temperature of compensation (Tc) or isokinetic temperature ($T_{isokin}$) and is defined by Barnes (1969) as related reactions in which the mechanism of thermal unfolding or nature of transition state is identical. No effect is observed at Tc while opposite effects are observed on either side of the isokinetic temperature which is normally found in the range –10 and 100°C for most of the proteins (Urabe et al. 1973; Rashid and Siddiqui 1998). In contrary to the present findings, a parallel Arrhenius plot of native CMCase and thermostable GAM15 has also been reported which imply a different transition state for the unfolding of enzymes (Siddiqui et al. 1997).

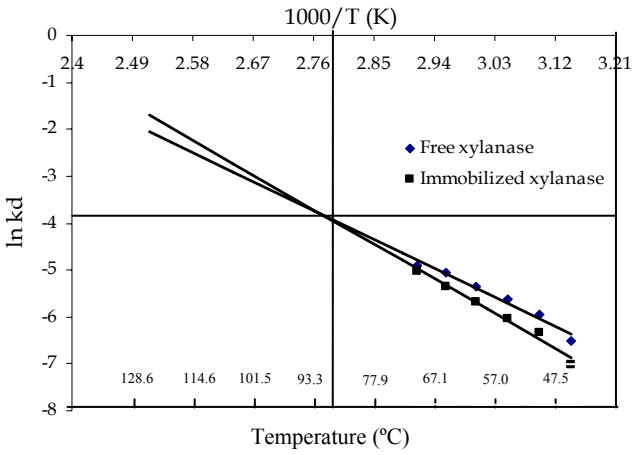



Figure 3. Arrhenius plot for determination of isokinetic temperature

**Table 2.** Thermodynamic parameters for thermal inactivation of free and covalently immobilized xylanase

| Temp (ºC) | ΔHº (kJ mol$^{-1}$) | | ΔGº (kJ mol$^{-1}$) | | ΔSº (Jmol$^{-1}$K$^{-1}$) | |
|---|---|---|---|---|---|---|
| | F | I | F | I | F | I |
| 45 | 54.414 | 65.724 | 106.086 | 107.437 | -162.492 | -131.173 |
| 50 | 54.373 | 65.683 | 106.319 | 107.307 | -160.825 | -128.867 |
| 55 | 54.331 | 65.641 | 107.119 | 108.225 | -160.939 | -129.829 |
| 60 | 54.289 | 65.599 | 107.997 | 108.952 | -161.285 | -130.189 |
| 65 | 54.248 | 65.558 | 108.896 | 109.720 | -161.680 | -130.657 |
| 70 | 54.206 | 65.516 | 110.052 | 110.416 | -162.816 | -130.904 |

$E_d$ (Activation energy for denaturation)=57.058 (F), 68.368 KJ/mol (I)
ΔHº = Variations in enthalpy; ΔGº = Variations in free energy;
ΔSº = Variations in entropy; F = Free and I = Immobilized xylanase

The thermostabilization effect of immobilization can be explained as follows- The $E_d$ of the covalently immobilized xylanase (68.368 KJ/mol) is about 20% greater than that of the free enzyme (57.058 KJ/mol) (Table 2), indicating the decreased inactivation of the immobilized one. The increase in $E_d$ leads to an increase in ΔHº since ΔHº = $E_d$-RT. The covalent immobilization also decreases the negative entropy (-ΔSº) of the irreversible thermoinactivation. A 20.9% increase in ΔHº and 19.6% decrease in -ΔSº produces a thermostabilization effect. The dependences of *ln kd* on 1/T for a native and immobilized xylanase intersect outside the temperature range studied. The immobilized enzyme is more thermostable than the free one at the temperatures lower than $T_{isokin}$. In other words, the thermostabilization effect of immobilization increases with a decrease in temperature and is known as 'low-temperature stabilization' effect.

## Conclusion

Results of the investigation showed that covalently immobilized xylanase on the surface of glutaraldehyde activated alginate beads display higher $V_{max,}$ $K_m$ and kcat values but lower kcat/$K_m$ value in comparison to its free counterpart. The 'catalytic efficiency' of xylanase was found to decline with immobilization which could be attributed mainly to increased $K_m$ value since change in $V_{max}$ was marginal. However, the decreased 'catalytic efficiency' can, to some extant, be considered compensated by 'low temperature stabilization' effect of immobilization process which makes the immobilized enzyme to work better at temperature lower than the isokinetic temperature. The ability of immobilized xylanase to work better than free enzyme could be explained by increase in $E_d$, and enthalpy along with decrease in negative entropy. Further work on the use of immobilized xylanase in different juice clarification is in progress.

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