Thermal Stability and Thermodynamics of Xylanase from *Melanocarpus albomyces* in Presence of Polyols and Salts

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An extracellular xylanase from the thermophilic fungus *Melanocarpus albomyces* IIS 68 was evaluated for its activity and stability in the presence of polyols and salts at 60 °C, and found to be an effective protecting agent for thermal deactivation of enzyme. Response surface methodology was employed to study the synergistic effects of glycerol and NaCl (thermo-stabilizers) for xylanase stability. The addition of these thermo-stabilizers resulted in more than a 10-fold increment in enzyme half-life. Activation energy (E_a) and thermodynamic parameters such as ΔH, ΔG, and ΔS were calculated for the thermal inactivation of free and immobilized xylanase. The immobilized enzyme underwent substantially less conformational changes because of its enhanced stability and increased compactness, providing better thermo-stability at elevated temperatures. These findings suggest that the combined effect of glycerol and sodium chloride serves as a potential stabilizer for extracellular thermophilic xylanase, which finds commercial application in many industries, especially in the pulp and paper industry.

Keywords: Polyols; Response surface methodology; Thermo-stabilizers; Thermodynamic; Xylanase

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INTRODUCTION

Xylanases [EC.3.2.1.8] are responsible for hydrolysis of xylan and the subsequent release of xylo-oligosaccharides in the form of xylose and xylobiose. The hydrolysed products in the form of soluble sugars have extensive biotechnological relevance in many fermentation processes and the functional food industry (Li *et al*. 2012).The filamentous fungus *Melanocarpus albomyces* IIS 68 produces extracellular xylanase that degrades xylan, a complex hemicellulose, into xylo-oligosaccharides by cleaving the xylose unit from non-reducing ends. The thermophilic fungus produces a high titre of active xylanases, which play a significant role in many industrial processes (Biswas *et al*. 2010).

The knowledge of xylanase opens up a wide range of biotechnological applications in multiple industries. The thermo-stable xylanase from *M. albomyces* IIS 68 can play an important role in the bakery industry by increasing shelf life and improving the quality of baked products (Bajaj and Manhas 2012). Xylanases, which are cellulose-negative and active at alkaline pH and elevated temperatures, are favoured for pulp and paper processes (Saleem *et al*. 2009). The promising application of xylanase in the paper industry is driven by its usage as a bio-bleaching agent, which reduces or eliminates the use of chlorine and eventually helps in reducing environmental pollution (Li *et al*. 2010). The use of xylanase

as an animal feed supplement along with multi-enzyme cocktails improves the digestibility of poultry feed (Nagar *et al*. 2012). Xylanase displays exciting potential in fruit-juice processing by enhancing sugar extraction and clarification before commercialization (Dhiman *et al*. 2011). Addition of xylanase to brewery mash significantly reduces the filtration rate and viscosity, making it a suitable candidate for application in the brewing industry (Qiu *et al*. 2010).

Immobilized enzymes are advantageous over free enzymes, as they can be recycled, reused, and save production costs at the industrial level by providing a broad spectrum of applications under different conditions (Pal and Khanum 2011b). The stabilizing effect of additives has been attributed to their ability to counteract the forces that lead to the denaturation of enzymes and loss of activity (Hall *et al*. 2008).

The present work deals with the stabilization of xylanase from *M. albomyces* IIS 68 for enhanced thermo-stability at 60 °C, in the presence of polyols and salts. The concentrations of polyols and salts were optimized using central composite design of response surface methodology (RSM) for evaluating the main and interactive effects of process variables. The stability of xylanase was demonstrated with decrease activation energy, higher half-life, and lower enthalpy, Gibb's free energy, and entropy. There has been no report on the influence of polyols and salts on the thermo-stability and thermodynamics for xylanase from *M. albomyces* IIS 68. Hence, this work could open up a promising debate and further research and development work concerning for combined utility of polyols and salts for industrially viable enzymes.

EXPERIMENTAL

Microorganism and Culture Maintenance

M. albomyces IIS 68 was obtained from the Indian Institute of Science, Bangalore, India. It is a thermophilic fungus that grows best at 45 °C. The culture was maintained on Emerson's YPSS agar (Cooney and Emerson 1964) at pH 7.5 (adjusted with 50% H_3PO_4). The Roux bottle slants were stored at 4 °C and sub-cultured every month.

Bioreactor Production of Xylanase

Xylanase was produced in a laboratory-scale 14-L stirred tank bioreactor (Cheamp-Fermenter, Switzerland) with a working volume of 10 L. The composition of optimized medium used in the bioreactor for the production of xylanase contained (g/L): wheat straw extract (WSE), 0.8; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.5; urea, 1.5; peptone, 1.0; and yeast extract, 1.0.The bioreactor was inoculated with a 36-h-old actively grown culture of *M. albomyces* IIS 68 at a 20% (v/v) inoculum. The fermentation was carried out at 45 °C for 42 h, with aeration and agitation rates of 0.3 vvm and 350 rpm, respectively. Samples were collected periodically after 12 h of fermentation with an interval of 6 h, centrifuged at 10,000×*g* for 10 min, and the supernatant was used to monitor xylanase activity (Gupta *et al*. 2013).

Xylanase Assay

For xylanase activity, 1% oat-spelt xylan was used as a substrate in sodium-phosphate buffer (0.05 M) with a pH of 6.0 (Saraswat and Bisaria 1997) by incubating the reaction mixture at 70 °C (Bailey *et al*. 1992) for 5 min. The release of reducing sugar was measured using the di-nitro salicylic acid method (Miller 1959) with xylose as a standard.

The xylanase activity was reported in terms of international units (IU), where 1 IU of xylanase activity was defined as 1 µmol of xylose produced in 1 min by 1 mL of undiluted enzyme.

Estimation of Reaction Rate Constant for Deactivation (k_d) and Half-life ($t_{1/2}$)

For thermal deactivation studies, appropriate thermo-stabilizers (polyols and salts) were selected for their stabilizing effect in response to enzyme half-life (min) at 60 °C, *i.e.*, the temperature of operating conditions in the pulp and paper industry. The thermo-stabilizers were added as protective agents to reduce the loss of enzyme activity during the process of thermo-stabilization. The compounds used as polyols include polyethylene glycol, carboxymethyl-cellulose (CMC), sorbitol, xylitol, and glycerol, whereas chloride salts of calcium, potassium, lithium, and sodium, as well as sodium salts of carbonate, sulphate, and nitrate, were employed as salts. To maintain the homogeneity, a 50-mL blend was prepared by the addition of suitable polyols and salts with culture supernatant, which was uniformly distributed in test tubes and incubated together in shaking water baths at 50 rpm and 60 °C for 1 h. The incubated test tubes were removed at regular intervals, cooled immediately in ice-cold water to stop the reaction, and used for estimation of the residual xylanase activity. The initial activity was considered to be 100%, and the residual xylanase activity was calculated relative to the initial activity.

To study the deactivation kinetics of xylanase with polyols and salts, $\ln(E/E_0)$ was calculated as the log fraction of final (E) and initial (E_0) enzyme activities plotted against time (min), where the slope gave the value of the deactivation constant (k_d) and the half-life (min) of xylanase was calculated using this slope value. The half-life of an enzyme can be defined as the time required by the enzyme to lose half of its initial activity, given by:

$$t_{1/2} = \ln 2 / k_d \quad (1)$$

Combined Effect of Glycerol and Sodium Chloride on Xylanase Stability at 60 °C

To determine the optimum level of selected variables (glycerol and sodium chloride) and to monitor their combined effect on the thermo-stability of xylanase, a central composite design (CCD) was applied to design the next set of experiments. The combined effect of glycerol and sodium chloride was evaluated using response surface methodology (RSM) to verify the correlations between these variables. The two independent variables studied were glycerol (5 to 10%) and sodium chloride (5 to 10%). "Design Expert" statistical software (Version 5.0, Stat-Ease Inc., USA) was used to generate a set of experiments, with five replicates at centre points and a single run for each of the other combinations, *i.e.*, a total of 13 experiments were designed and carried out to analyse response surface graphs and predict the optimum values of variables employed to obtain the maximum thermo-stability (half-life) of xylanase.

Determination of Thermodynamic Parameters

The activation energy (E_a) of xylanase with optimized levels of stabilizers (glycerol 7.5% and NaCl 7.5%) and without thermo-stabilizers was calculated by plotting the natural logarithm of the deactivation constant (k_d) obtained at different temperatures *versus* the inverse of the absolute temperature ($1/T$) (Arrhenius equation); other thermodynamic parameters, such as ΔH (enthalpy), ΔG (Gibb's free energy), and ΔS (entropy), were calculated using the following equations (Eyring 1935),

$$\Delta H = \Delta E_a - RT \quad (2)$$

where R (gas constant) = 8.314 $Jmol^{-1}K^{-1}$ and T is absolute temperature in Kelvins:

$$\Delta G = -RT \ln\{(k_d * h)/(k_b * T)\} \quad (3)$$

In Eq. 3, h is Plank's constant equal to 6.626×10^{-34} Js, and k_b is Boltzmann's constant equal to 1.381×10^{-23} J K^{-1}

$$\Delta S = (\Delta H - \Delta G)/T \quad (4)$$

Characterization of Xylanase

Effect of pH and temperature on xylanase activity and stability

The optimum pH for xylanase activity was determined by suitably diluting the culture supernatant in 0.05 M buffer at pH values ranging from 5.0 to 9.0. The buffers used were phosphate-citrate buffer (pH 5.0, 5.5, 6.0, and 6.5), sodium-phosphate buffer (pH 6.0, 6.5, and 7.0) and Tris-HCl buffer (pH 7.5, 8.0, 8.5, and 9.0). This was followed by assaying xylanase activity under standard assay conditions. The pH stability of xylanase was carried out by incubating an equal volume of culture supernatant with buffers of different pH for 24 h at room temperature (28 ± 2 °C). Thereafter, residual xylanase activity was estimated under standard assay conditions.

To check optimum temperature for xylanase activity, the reaction mixture was incubated at temperatures ranging from 40 to 80 °C in 0.05 M sodium-phosphate buffer at pH 6.0 for 5 min. The temperature stability was determined by pre-incubating the aliquots of culture supernatant for 1 h at temperatures ranging from 45 to 70 °C, followed by estimation of residual xylanase activity under standard assay conditions.

Effect of metal ions and chemical agents

The enzyme was incubated with metal ions (Ca^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} Ag^{+}, and Hg^{2+}), a chemical agent (β-mercaptoethanol), a chelating agent (EDTA), and a modifier (N-bromosuccinimide) at a final concentration of 1 mM, as well as the non-ionic detergent polysorbate 80 (Tween® 80) and sodium dodecyl sulphate at 0.1%, w/v for 1 h at room temperature (28 ± 2 °C). The residual xylanase activity was measured under standard assay conditions and expressed as percent residual activity obtained in the absence of ions.

Substrate specificity

Substrate specificity of the culture supernatant was determined using birch wood xylan, larch wood xylan, oat-spelt xylan, CMC, filter paper, and Avicel at 1% w/v. The xylanase activity was measured under standard assay conditions.

Determination of K_m and V_{max}

To define the Michaelis-Menton constant (K_m) and maximal velocity (V_{max}), a Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934) for xylanase activity *versus* substrate concentration was employed. Oat-spelt xylan was used as a substrate in

the range of 2.5 to 20 mg/mL in sodium-phosphate buffer (0.05 M), pH 6.0 at 70 °C. Xylanase activity was measured per standard assay protocols.

RESULTS AND DISCUSSION

Effect of Polyols on Thermo-Stability of Xylanase

The effect of different polyols was investigated in terms of their thermo-stabilization efficiency for thermophilic xylanase from *M. albomyces* IIS 68 at 60 °C. After heat treatment, residual xylanase activity was measured at different time intervals over 1 h and plotted against time for calculating the deactivation constant (k_d), followed by xylanase half-life ($t_{1/2}$). The half-life quantifies the shielding effect and the extent of enzyme stabilization by thermo-stabilizers, which can be defined as the ratio of enzyme half-life with and without thermo-stabilizers. Higher half-life in the presence of thermo-stabilizers corresponds to high enzyme stability. Figure 1 displays the deviation in stabilizing effects of various polyols at altered concentrations.

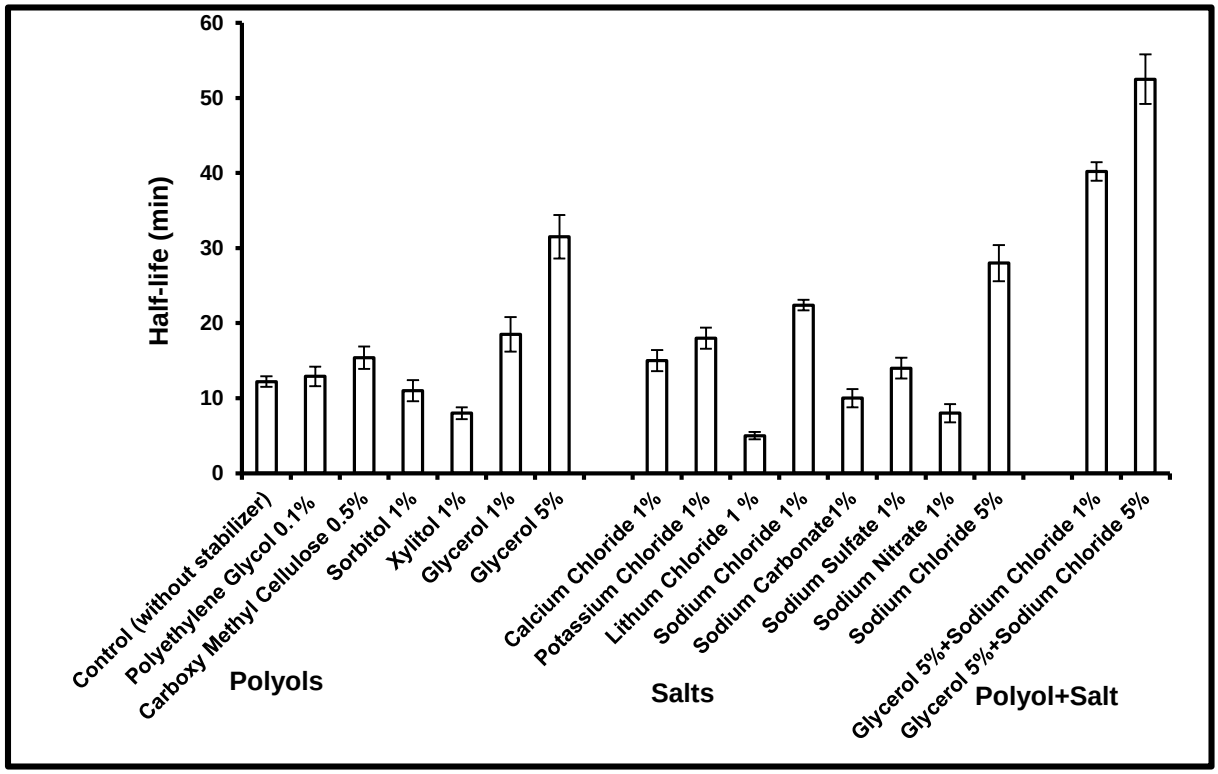

Fig. 1. Thermo-stabilizing effect of polyols, salts, and their combinations on xylanase at 60 °C as a function of half-life (min)

For polyethylene glycol and CMC, no significant effect was observed, whereas among the polyols ranging from C3 to C6 (glycerol, xylitol, and sorbitol), glycerol showed an excellent stabilizing effect by enhancing xylanase half-life by 1.5- and 2.6-fold at concentrations of 1 and 5% (w/v), respectively. This could be attributed to an overall increment in hydroxyl groups moving from C3 to C6 with the gradual decrease in deactivation constant, indicating the interaction of the enzyme with hydroxyl groups as a

vital parameter for safeguarding and maintenance of active structure of the enzyme. Elsewhere, the use of a polyol (glycerol) for glucose dehydrogenase from a *Haloferax mediterranei* halophilic enzyme enhanced the thermo-stability of the enzyme at 60 °C (Obon *et al*. 1996). A similar improvement in the thermo-stabilization in the presence of glycerol was observed for lipase from *Rhizomucor miehei* (Noel and Combes 2003).

Effect of Salts on Thermo-Stability of Xylanase

Various salts were studied relative to their thermo-stabilization competence for thermophilic xylanase from *M. albomyces* IIS 68 at 60 °C following a similar protocol as for polyols; half-life of xylanase was calculated to observe stabilizing effect with each salt. As shown in Fig. 1, LiCl had a marginal effect, while $CaCl_2$ and KCl had a positive effect on xylanase stability. Among the sodium salts, sodium carbonate, sodium sulphate, and sodium nitrate were found to have borderline effects. Sodium chloride gave maximum xylanase half-life in comparison to all other sodium salts, which in turn suggests that chloride ions (Cl^-) gave more encouraging effect on xylanase half-life than sodium ions (Na^+), as Na^+ ions were already present in other sodium salts; only the introduction of chloride ions from NaCl led to an increased xylanase half-life. The effect of salts depends on their nature, concentration, and the type of enzyme used, as chloride ions increased half-life of xylanase by 1.8 and 2.3-fold at concentrations of 1 and 5% (w/v), respectively, as observed in Fig. 1. Hence, NaCl was found to be a potential enzyme stabilizer, and it could be concluded that chloride ions have an encouraging effect on xylanase stability. These results were comparable with observations made for lipase from *Rhizomucor miehei* (Noel and Combes 2003).

Combined Effect of Glycerol and NaCl on Thermo-Stability of Xylanase

Among all the polyols and salts studied, glycerol and NaCl displayed enhanced xylanase half-life, probably due to reduced surface tension by glycerol and salting-in/out property of NaCl, which stabilizes the native structure of protein. They were therefore chosen to investigate their possible interactive effect on xylanase stability (Obon *et al*. 1996; Noel and Combes 2003). Figure 1 reveals excellent stabilizing effects of various combinations of glycerol and NaCl, which appeared to be more or less compatible with each other. This synergistic effect enhanced xylanase half-life by more than 4.2-fold compared to their individual effects. The maximum half-life of 52 min, which was observed with the combination of glycerol and NaCl (5%), was much higher than that obtained with the addition of any individual thermo-stabilizer. These results indicate that the combination of glycerol and NaCl expressively affected xylanase half-life at 60 °C. As the concentrations of these thermo-stabilizers and their balance is an important factor for improving the half-life, the statistical approach of central composite design was employed to qualify and quantify the interaction between these variables.

Response Surface Modelling for Thermo-Stabilization

To determine optimal levels of glycerol (5 to 10%, w/v) and NaCl (5 to 10%, w/v) for maximum half-life, a total of 13 experiments were performed, as shown in Table 1. By applying multiple regression analysis on experimental data, the following second order polynomial equation was found to explain half-life:

$$\text{Half-life (min)} = 125.18+13.88*A-10.79*B-37.53*A^2-32.38*B^2+4.20*A*B \quad (5)$$

where A and B are glycerol and NaCl, respectively. From the analysis of variance (ANOVA) for this quadratic model, it is clear that the linear and square terms of glycerol and NaCl had a significant effect on xylanase half-life (on the basis of their *t* and p-values, where $p \leq 0.0001$ was considered to be significant), as shown in Table 2.

Table 1. Results of CCD Showing Observed and Predicted Response

Run	A:Glycerol % (w/v)	B:NaCl % (w/v)	Half-life Observed (min)	Half-life Predicted (min)
1	7.5	7.5	126.1	125.1
2	7.5	7.5	121.7	125.1
3	5.0	5.0	54.2	56.4
4	10.0	10	61.6	62.5
5	7.5	7.5	128.6	125.1
6	7.5	7.5	125.8	125.1
7	5.0	10.0	30.2	26.4
8	3.96	7.5	28.7	30.5
9	7.5	3.96	81.5	75.7
10	10.0	5.0	68.8	75.7
11	11.04	7.5	74.7	69.8
12	7.5	11.04	42.5	45.1
13	7.5	7.5	123.7	125.1

Table 2. ANOVA for Determination of Half-life by CCD

Factors	Coefficient estimate	Error	*t* value	Pr> |*t*|
Intercept	125.18	2.17		
A-Glycerol	13.88	1.71	8.11	<0.0001
B- NaCl	-10.79	1.71	-6.3	0.0004
A^2	-37.53	1.84	-20.44	<0.0001
B^2	-32.53	1.84	-17.63	<0.0001
AB	4.2	2.42	1.73	0.1264

The statistical significance of the model equation was evaluated by Fisher's *F*-test for analysis of variance (ANOVA), as shown in Table 3. The Fisher's *F*-test ratio of 150.82 suggests that the model was significant, with low probability value for a regression model [(prob> F) < 0.0001], which confirmed that the model was appropriate for describing optimization of thermo-stabilizers with respect to the stability of xylanase from *M. albomyces* IIS 68. The coefficient of determination (R^2) was calculated to be 0.9908, which indicates that the model could explain up to 99% of the variability. The adjusted determination coefficient (adjusted $R^2 = 0.9842$) and predicted determination coefficient ($R^2 = 0.9431$) strongly confirmed the significance of model. The model also determined to have a statistically insignificant lack of fit ($p = 0.0486$), which further supported the adequacy of second-order polynomial model. "Adequate Precision" measures signal to noise ratio, and a ratio greater than 4 is desirable. The ratio of 30.02 indicated a significant adequate signal to noise ratio; therefore, the model could be considered appropriate for explaining xylanase half-life.

Table 3. ANOVA for Response Surface Quadratic Model

Source	Sum of squares	Mean square	DF	*F*-Value	P>F
Model	17686.43	3537.29	5	150.82	<0.0001
Residual	164.18	23.45	7		
Lack of fit	136.95	45.65	3	6.71	0.0486
Pure error	27.23	6.81	4		
Total	17850.61		16		
Root MSE	4.84	CV	5.89	Adq. Precision	30.02
Adjusted R^2	0.9842	Predicted R^2	0.9431		

To understand the effect of variables employed for thermo-stabilization of xylanase, the predicted model was further assessed using RSM. The response surface curves plotted in as three-dimensional plots provide a means to visualize the interactions between the variables and to estimate optimum level of each variable for maximum response. The model was used to plot for glycerol and NaCl concentrations against half-life (min) as a function of response in 3D surface plot and contour plot, as shown in Figs. 2a and 2b. The most favourable concentrations obtained were glycerol 7.5% and NaCl 7.5% (w/v) which enhanced xylanase half-life up to 125 min, which was close to the predicted value of 124.2 min. The combined effect of selected variables corresponded to 10.2-fold increment in half-life of xylanase compared to the case without thermo-stabilizer at 60 °C. Similarly, xylanase from *Bacillus pumilus* SV-205 was optimized using RSM (CCD), resulting in an 83.65% improvement in enzyme immobilization (Nagar *et al*. 2012).

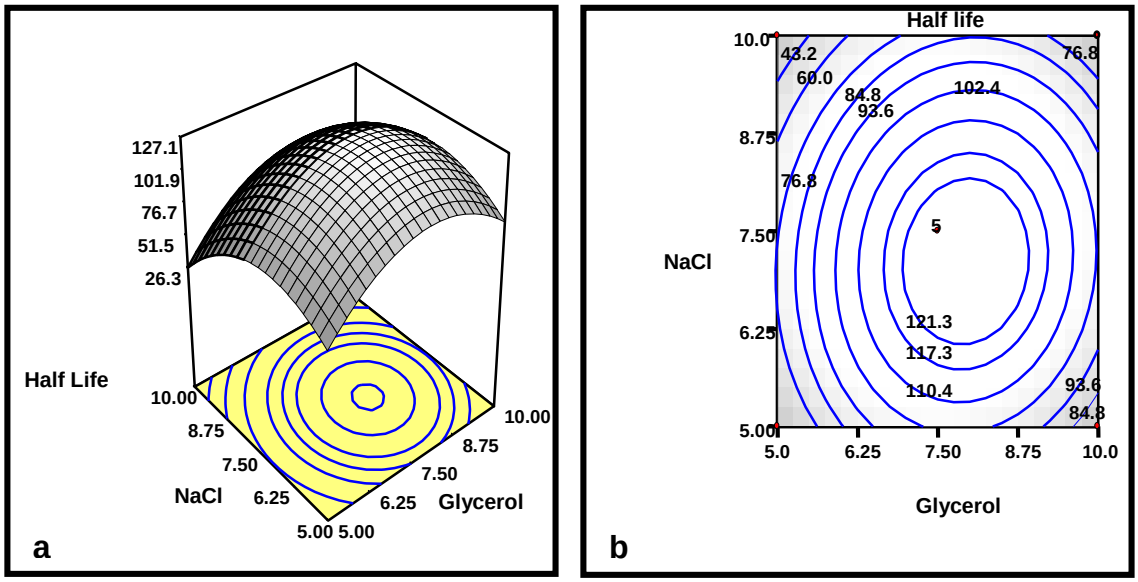

Fig. 2. Response surface plots of CCD showing interactive effects on xylanase at 60 °C; (a) three-dimensional plot for glycerol and NaCl as a function of half-life (min) and (b) contour plot for glycerol and NaCl as a function of half-life (min)

Thermodynamic Parameters for Free and Immobilized Xylanase

The activation energies (E_a) for free and immobilized xylanase were calculated using an Arrhenius plot, as shown in Fig. 3. The use of thermo-stabilizers enhanced covalent bonds by lowering E_a of product formation from 150.1 to 78.8 kJ mol^{-1}, resulting in higher catalytic efficiency. The requirement of lower E_a for product formation may be

considered as potential indicator for thermo-stability during thermal inactivation of enzymes at high temperatures (Adams and Kelly 1998). The low E_a value indicated the thermo-stabilizers contributed to a more stable metabolic network for the enzyme than was present without stabilizers (Bokhari *et al*. 2010).

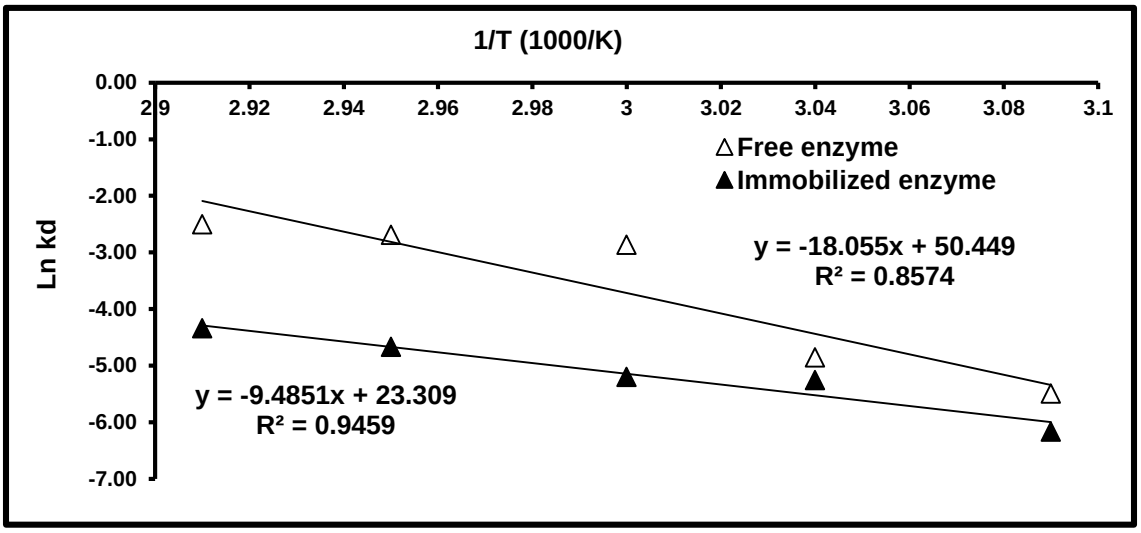

Fig. 3. Arrhenius plot for calculating activation energy (E_a) of free and immobilized xylanase

Table 4 summarizes thermodynamics parameters for free and immobilized xylanase. The half-life of xylanase was monitored at temperatures ranging from 50 to 70 °C. With an increase in temperature, k_d increased and $t_{1/2}$ decreased. Higher k_d led to a slowing down of enzyme destabilization at elevated temperatures. Immobilization of xylanase using glycerol and NaCl was demonstrated to be long-lasting for all temperatures, resulting 10-fold increment in xylanase half-life (min) at 60 °C. The covalent attachment of thermo-stabilizers with enzyme allowed enhanced thermo-stability at higher temperatures (Cobos and Estrada 2003). The enthalpy of denaturation (ΔH) for free and immobilized xylanase at 60 °C was found to be 147.3 and 76.09 kJ mol^{-1}, respectively. The enthalpy for immobilized xylanase was lower than the enthalpy of free xylanase, which clearly indicates that the enzyme underwent considerably less conformational changes during the process of denaturation at higher temperature (Bhunia *et al*. 2013). The value of ΔH after immobilization remained almost constant at for all temperatures.

Table 4. Thermodynamic Parameters for Free and Immobilized Xylanase

Parameters		Temperature (°C)				
		50	55	60	65	70
k_d (min^{-1})	F	0.0041	0.0078	0.0568	0.068	0.082
	IE	0.0021	0.0052	0.0055	0.0094	0.013
Half-life (min)	F	169	88.8	12.2	10.1	8.4
	IE	330	133.2	126	73.7	53.3
ΔH (kJ/mol)	F	147.4	147.3	147.3	147.2	147.2
	IE	76.17	76.13	76.09	76.04	76
ΔG (kJ/mol)	F	94.08	93.82	89.8	90.68	91.5
	IE	95.8	94.8	96	96.1	96.6
ΔS (kJ/mol)	F	165	163	172	167	162.7
	IE	-60.77	-56.92	-59.78	-59.34	-60.05

F: Free enzyme, IE: Immobilized enzyme

The Gibbs free energy (ΔG) for thermal unfolding at 60 °C was determined to be 89.8 and 96.0 kJ mol^{-1} for free and immobilized xylanase, respectively. The value of ΔG increased slightly with increase in temperature for immobilized xylanase, whereas it decreased for free xylanase; the difference between them was marginal. This indicates immobilization did not affect thermal unfolding at higher temperatures; rather it facilitate thermal stability at higher temperatures (Nagar *et al*. 2012). The values of ΔG for immobilized xylanase at 65 and 70 °C was close to that at 60 °C, indicating high resistance of immobilized xylanase towards thermal unfolding at higher temperatures, providing enhanced stability and resistance for immobilized enzyme at elevated temperatures. Entropy of inactivation (ΔS) is accompanied by splitting of different bonds and linkages and in turn increased randomness or entropy of deactivation, resulting in a disorganized system. The positive entropy for free xylanase (172 kJ mol^{-1}) revealed that the enzyme was less stable in its native state compared to its immobilized state (-59.78 kJ mol^{-1}) at 60 °C. The negative value for change in entropy (ΔS) signified better sustainability at higher temperatures, as highly stable systems always have low energies. The lower values of ΔS for immobilized enzyme suggests an increased compactness and high resistance for thermal inactivation (Pal and Khanum 2011a). This change improved the consistency of immobilized enzyme by stabilizing different forces and provided better thermo-stability to the enzyme at elevated temperatures (Anema and McKenna 1996).

The native form of enzymes could be made more thermo-stable by incorporating non-covalent bonds, salt-bridges, and hydrophobic-interactions, or by decreasing the entropy (ΔS) of unfolding (Rashid and Siddiqui 1998). The thermo-stabilization of enzymes is usually accompanied by negative ΔS and a decreased ΔH with increasing temperature (Pal and Khanum 2012). The favourable and larger reaction enthalpy (ΔH) was counterbalanced by negative entropy (ΔS) (Garidel *et al*. 2009). The value of ΔG, which measures the spontaneity of inactivation of the process, was higher than the value of ΔH for immobilized enzyme; this was due to negative entropy (ΔS) during the process of inactivation (Tanaka and Hoshino 2002).

Characterization of Xylanase

Effect of pH on xylanase activity and stability

The pH affects enzymatic activity due to presence of charges on substrate binding sites and/or of enzyme molecules. Xylanase from *M. albomyces* IIS 68 was investigated over a wide range of pH values from 5.0 to 9.0 using oat-spelt xylan as a substrate. As shown in Fig. 4a, xylanase retained more than 60% of its activity in the pH range of 5.0 to 7.5 with maximum at 6.0, which is within the optimal pH range from other thermophilic xylanases, such as *Thermobifida halotolerans* YIM 90462^{T} (Zhang *et al*. 2012).

Determining the pH stability profile for any enzyme is a vital step before its industrial application. The pH stability test determined that the xylanase from *M. albomyces* IIS 68 was highly stable within a wide pH range, as shown in Fig. 4b. Xylanase retained more than 88% activity over the extensive pH range of 5.0 to 9.0 for 24 h of incubation at room temperature. In short, xylanase exhibited good stability under alkaline conditions, permitting direct enzymatic treatment of alkaline pulp by avoiding pH re-modification (Mishra and Thakur 2011). Xylanase from *Aspergillus niger* DFR-5 was found to be stable in the narrow pH range of 4.0 to 6.5 (Pal and Khanum 2011), while that of *Enterobacter* sp. MTCC 5112 maintained 100% of its activity between pH 7.0 and 9.0 (Khandeparkar and Bhosle 2006).

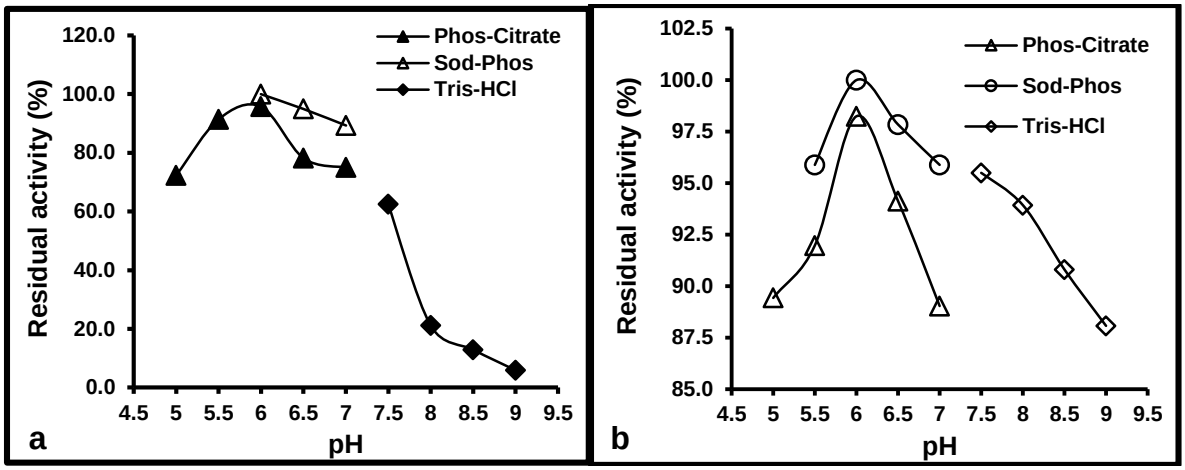

Fig. 4. Optimal (a) pH and (b) stability of xylanase from *M. albomyces* IIS 68

Effect of temperature on xylanase activity and stability

The optimum temperature for xylan hydrolysis was determined to be 70 °C, as shown in Fig. 5. Further increasing the temperature resulted in a gradual reduction in xylanase activity, down to 67.3% and 34.8% at 75 and 80 °C, respectively. In a study carried out with *Kluyvera* sp. OM3, the optimum temperature for maximum xylanase activity was reported to be 70 °C (Xin and He 2013).

Figure 5 shows that the pre-incubated xylanase from *M. albomyces* IIS 68 retained 100% of its original activity for 1 h at 45 °C, while it was significantly affected above 45 °C; further increasing the temperature adversely affects xylanase residual activity.

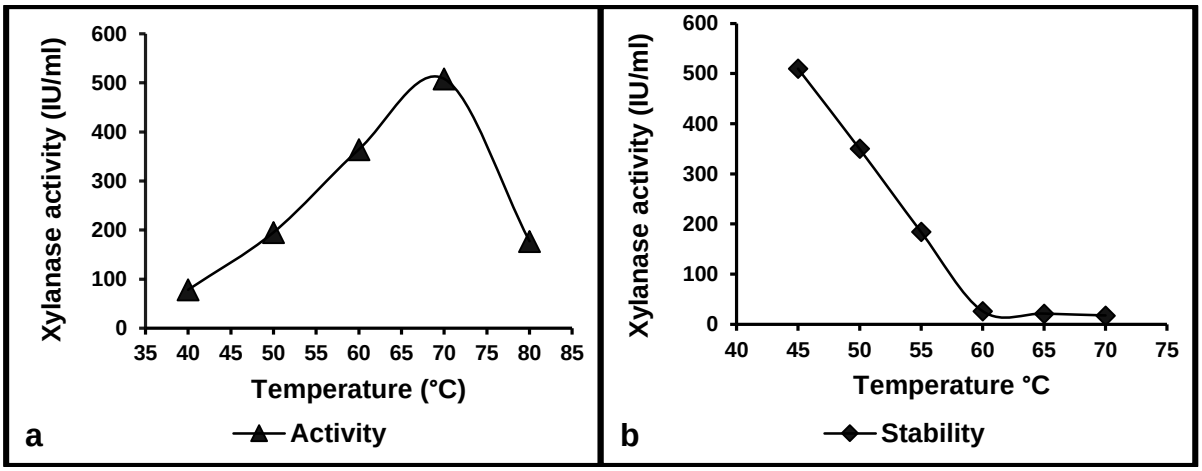

Fig. 5. Effect of (a) temperature and (b) stability on xylanase activity from *M. albomyces* IIS 68

Effect of metal ions and chemical agents

The effects of various metal ions and chemical agents were studied to evaluate the extent of potential inhibitors or activators for xylanase activity. As shown in Table 5, xylanase activity increased in the presence of Ca^{2+} (125.1%), Ni^{2+} (115.2%), Fe^{2+} (109.7%), Mg^{2+} (108.3%), Mn^{2+} (104.5%), and Zn^{2+} (102.4%), which displayed that the enzyme catalysis involved metal ions and have stimulatory effects (Xin and He 2013). On other hand, Co^{2+} (96.7%) and Cu^{2+} (94.8%) exhibits slight inhibitory effects (Sayari *et al*. 2012). The strong suppression by Ag^{+} (8.2%) revealed the presence of cysteine residues in

close vicinity with the active site of xylanase, and its interaction with sulfhydryl group led to strong inhibition of xylanase activity; similar observations were also reported for other xylanases (Beg *et al*. 2001). The substantial effect of Hg^{2+} (2.5%) on xylanase activity reveal the important participation of tryptophan residues in the catalytic action of enzyme; as Hg^{2+} can oxidize the indole ring, it was believed to interact with aromatic ring present in tryptophan, contributing in decrease of xylanase activity (Liu *et al*. 2010; Zhang *et al*. 2007). The thiol group-bearing chemical agent β-mercaptoethanol (121.6%), the non-ionic detergent polysorbate 80 (115.2%), and the chelating agent EDTA (105.4%) all showed stimulatory effects on xylanase activity.

Table 5. Effect of Various Metal Ions and Chemical Reagents on Xylanase Activity

Agents	Residual activity (%)	Agents	Residual activity (%)
Control	100 ± 1.5	$CuSO_4$	94.8 ± 0.8
$CaCl_2$	125.1 ± 0.5	$AgNO_3$	8.2 ± 0.7
$NiCl_2$	115.2 ± 1.4	$HgCl_2$	2.5 ± 0.5
$FeSO_4$	109.7 ± 0.4	β-mercaptoethanol	121.6 ± 1.5
$MgSO_4$	108.3 ± 0.7	Tween-80	115.2 ± 1.1
$MnCl_2$	104.5 ± 0.9	EDTA	105.4 ± 0.4
$ZnSO_4$	102.4 ± 0.7	SDS	85.5 ± 1.2
$CoCl_2$	96.7 ± 1.2	N-bromosuccinimide	1.8 ± 0.6

Similar observations were reported for an alkaliphilic endo-β-1,4-xylanase from the microbial community EMSD5 (Lv *et al*. 2008).The xylanase activity was slightly inhibited by sodium dodecyl sulphate (SDS; 85.5%); however, a firm suppression was observed when a modifier like N-bromosuccinimde (1.8%) was used, indicating the role of tryptophan residues at the active site of xylanase, as reported for other xylanases (Kumar and Satyanarayana 2011). These results can be used to determine the ions, detergents, or modulators added or removed at appropriate concentrations were useful for industrial operations.

Substrate specificity

The substrate specificity of culture supernatant was assayed against different substrates. The highest activity was observed for oat-spelt xylan; only 68 and 35% xylanase activity was observed towards birch wood and larch wood xylan, respectively. However, no activity was noticed for cellulosic substrates such as CMC, filter paper, and Avicel, confirming the absence of cellulolytic activity. Thus, the cellulose-negative xylanase from *M. albomyces* IIS 68 has potential applications in pulp and paper industry for obtaining high-quality pulp. Similar observations were made for thermo-stable alkaline xylanase from alkaliphilic *Bacillus halodurans* S7 (Mamo *et al*. 2006).

Kinetic parameters of xylanase

The kinetic parameters for xylanase were determined using a Lineweaver-Burk double reciprocal plot with oat-spelt xylan as the substrate at pH 6.0 and 70 °C, as shown in Fig 6. The K_m and V_{max} for xylanase obtained from *M. albomyces* IIS 68 were 3.9 mg/mL and 27.4 µmol/min, respectively. Similar K_m and V_{max} values for xylanase from *Bacillus halodurans* S7 were calculated using oat spelt xylan at 70 °C (Mamo *et al*. 2006).

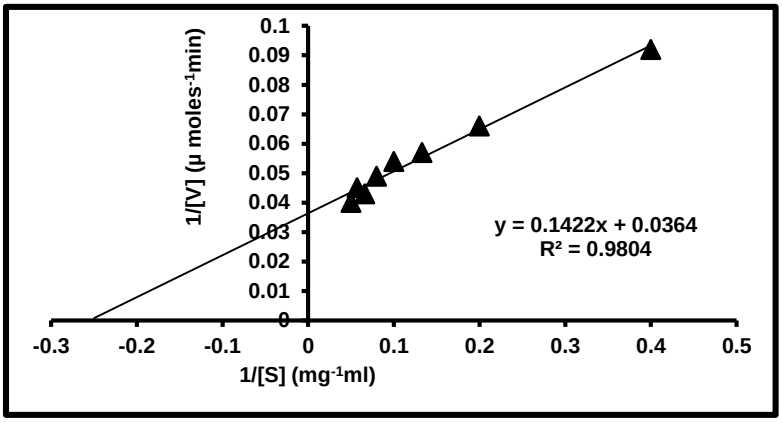

Fig. 6. Lineweaver–Burk double reciprocal plot for xylanase from *M. albomyces* IIS 68

CONCLUSIONS

1. The combined effect of glycerol and NaCl as a thermo-stabilizer extended xylanase stability, measured as the half-life, by more than 10-fold at elevated temperatures, leading to lower activation energy and higher catalytic efficiency.
2. The studied thermodynamic parameters revealed lower enthalpy (ΔH) and free energy (ΔG) after thermo-stabilization, leading to enhanced stability at higher temperatures. The negative value for the change in entropy (ΔS) for thermo-stabilizers indicated compactness during denaturation, resulting in improved tolerance at higher temperatures.
3. The wide pH range for activity and stability makes this enzyme suitable for enzymatic treatment of alkaline pulp in the paper industry.

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