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Characterizing and improving the thermostability of purified xylanase from *Aspergillus niger* DFR-5 grown on solid-state-medium

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Abstract

The thermostability of absolutely purified xylanase from *Aspergillus niger* DFR-5 was improved using polyols. Supplementation of sorbitol at 2M concentration was found to increase the half-life and D-value of xylanase at elevated temperatures (45-70ºC). Thermodynamic parameters associated with the process were analyzed which revealed that stability at higher temperatures was due to the increased enthalpy (ΔH^o) and free energy (ΔG^o) change of enzyme denaturation in the presence of sorbitol. The negative values of ΔS^o (-150.093 J mol^{-1} K^{-1} at 70ºC) clearly indicated that enzyme underwent a significant process of aggregation during denaturation. The enzyme required divalent cations for maximum activity and inhibited by chelator. The diminution of activity by various thiol-binding agents and enhancement by reducing agents like β-ME confirmed the essentiality of cysteine for catalysis. The enzyme had a half-life and D-value of 277 and 921 days when stored at 4 ºC.

Keywords: Xylanase, thermodynamics, thermostability, half-life

Introduction

Hemicellulose is one of the most abundant polysaccharides in nature present in agricultural biomass waste and cannot be easily converted to simple monomeric sugars due to its recalcitrant nature (deMeneres et al. 2010). Xylan, the major component of hemicellulose, is the second most abundant polysaccharide after cellulose and mainly consists of β-1,4-linked xylopyranosyl residues which is further substituted, depending on plant sources to a varying degree with glucuronopyranosyl, 4-0-methyl-D-glucopyranosyl, α-L-arabinofuranosyl, acetyl, as well as linked to feruloyl and coumaryl components of lignin (Shallom and Shoham, 2003). Xylanases are groups of enzymes that depolymerize xylan molecules into xylose units which are used by microbial populations as a primary carbon source. The enzymatic hydrolysis of xylan is advantageous for the recovery of monomeric sugars to be used as raw materials in a wide number of biotechnological processes (Nath and Rao, 2001). Microbial xylanases represent one of the largest groups of industrial enzymes and have attracted a great deal of attention during the past few decades. Their potential biotechnological applications in various industries include the food, feed, fuel, textile, detergents, paper and pulp industries and in waste treatment (Dhiman et al. 2008, Pal and Khanum, 2010a).

In recent years, interest in thermostable enzymes has increased dramatically as resistance to thermal inactivation has become a desirable property of the enzymes used in many industrial applications. Thermostable enzymes are generally defined as those with an optimum temperature above that of the maximum growth of an organism or with exceptional stability above 50ºC over an extended period of time (Singh et al. 2000). One of the ways to identify enzymes, which are thermally stable, is to exploit natural sources such as thermophilic organisms. They are known to produce enzymes having higher thermostability than those derived from their mesophilic counterparts (George et al. 2001). The stability of enzymes can also be increased by chemical modification, cross-linking, immobilization, treatment with additives and protein engineering (Gupta et al. 1991, Gouda et al. 2003). Addition of small compounds to protein solution and changing its microenvironment provides a simple but practical means of increasing the stability of enzyme.

Our laboratory is actively involved in the studies on industrially important microbial proteins (Pal and Ramana, 2009 and 2010, Pal and Khanum, 2010a, b). The present communication, in continuation, describes some of the characteristics of absolutely pure xylanase from *Aspergillus niger* DFR-5 with an emphasis to increase its thermostability so that application can be carried out at higher temperatures.

Materials and methods

Microorganism

The xylanase producing microorganism was isolated from a soil sample collected near fruit and vegetable debris in Mysore, India,

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and identified as *Aspergillus niger*, based on morphology, at Indian Type Culture Collection (ITCC), New Delhi, India. The isolate, named *Aspergillus niger* DFR-5, was grown on Potato Dextrose Agar (PDA) slants at 30°C for 5 days and subsequently stored at 4°C. Inoculum was prepared by suspending the spores from PDA slants by adding sterile 0.1% tween-80 to give a final count of ~1x10^6 spores/ml.

Xylanase preparation

The production and extraction studies of crude enzyme from *Aspergillus niger* DFR-5 has been reported by us earlier (Pal and Khanum, 2010a). The highest xylanase activity [2596 IU/g dry substrate (gds)] was achieved in medium that contained wheat bran (WB) and soybean cake (SBC) at a ratio of 70:30, moistened to 70% with MSS-2 mineral salt solution, and incubated for 6 days at 40°C. Water (10 ml/gds) at 37°C was used for enzyme recovery from moldy WB-SBC medium at 200 rpm for 60 min and 4465±52 IU/gds of xylanase was recovered. 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, 2011). The absolutely pure enzyme preparation was used in all the experiments.

Enzyme assay

Xylanase activity was assayed by the method of Khanna and Gauri (1993). The solution of xylan and the enzyme extract 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 released xylose was measured spectrophotometrically 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.

Effect of polyols on xylanase thermostability and thermodynamic analysis

To improve the thermostability of xylanase at higher temperature, initially the effect of three polyols (1M) additives namely ethylene glycol (2C), glycerol (3C) and sorbitol (6C) was studied at 60ºC. Later, the effect of different concentrations of sorbitol (0.5-2.5M) at 60ºC and finally 2M sorbitol in the range 45-70 ºC was studied.

The thermal stability of xylanase in the presence of polyols was investigated for varying periods of time in a temperature controlled water bath. The enzyme solution along with polyols was placed in a pre-warmed tube at the specified temperature, and aliquots were withdrawn at 30 min time intervals, ice-cooled and residual activity assayed. The stability of the enzyme was expressed as percent residual activity (%RA). The incubation was carried out in sealed vials to prevent change of volume of the sample and hence, the enzyme concentration due to evaporation. The data obtained from the thermal stability profile were used to analyze thermodynamic parameters related to the xylanase activity and compared with that of native enzyme. The experimental points were plotted according to the equation given below-

$$\ln \frac{A}{A_0} = k_d.t$$

Where A_0 is the initial activity, A is the residual activity after heat treatment, k_d is thermal inactivation rate constant (min^{-1}) and t is the exposure time (min).

The half-life of the xylanase ($t_{1/2}$, min^{-1}) was determined from the relationship-

$$t_{1/2} = \frac{\ln 2}{k_d}$$

The D-values (decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity) was calculated from the following relationship-

$$D - value = \frac{\ln 10}{k_d}$$

The z-value (temperature rise necessary to reduce D-value by one logarithmic cycle) was calculated from the slop of graph between log D versus T (ºC) using equation-

$$Slope = \frac{-1}{z}$$

The activation energy (E_d) for xylanase denaturation was determined by an Arrhenius plot of log denaturation rate constants (ln k_d) versus reciprocal of the absolute temperature (K) using the equation-

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

The change in enthalpy (ΔH^o, kJ mol^{-1}), free energy (ΔG^o, kJ mol^{-1}) and entropy (ΔS^o, J mol^{-1} K^{-1}) for thermal denaturation of xylanase were determined using the following equation-

$$\Delta H^\circ = E_d - RT\,,$$
$$\Delta G^\circ = -RT \ln \frac{k_d.h}{k_B.T}$$
$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T}$$

Where E_d is the activation energy for denaturation, T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol^{-1} K^{-1}), h is the Planck constant (11.04 X 10^{-36} J min), k_B is the Boltzman constant (1.38 X 10^{-23} J K^{-1}) and k_d is the deactivation rate constant (min^{-1}).

Effect of metal ions and chelator

To determine the requirement of metal ions for enzymatic activity, effect of various metal ions and chelator (EDTA) on xylanase activity was determined by incorporating them in the standard assay mixture with the final concentrations ranging from 1-20 mM. To avoid the formation of insoluble phosphates, 50 mM succinic acid buffer was used instead of the phosphate buffer. The activity was expressed as relative activity compared to control.

Identification of amino acids essential for catalysis

To identify the amino acids essential for enzymatic activity, xylanase activity was assayed in the presence of various thiol-binding agents and tryptophan oxidizing agent. The activity was expressed as relative activity compared to control.

Effect of reducing agents

The effect of various reducing agents on xylanase activity was determined by incorporating them in the standard assay mixture

with the final concentrations ranging from 1-20 mM. The activity was expressed as relative activity compared to control.

Storage stability of xylanase

To determine the storage stability of enzyme, the purified xylanase was stored at 4, 15 and 37ºC for three months. Activity measurements were carried out, taking samples every day, to determine the storage stability and results were expressed as inactivation rate constants (k_d), half-lives ($t_{1/2}$) and D-values.

Results and Discussion

Effect of polyols on the thermostability of xylanase

Thermostability of purified native xylanase was studied and it was found stable up to 40ºC. With further increase in temperature, stability decreased and showed a $t_{1/2}$ of 92.4 min at 70ºC. To improve the thermostability of xylanase at higher temperature, the effect of polyols (1M) additives such as ethylene glycol (2C), glycerol (3C) and sorbitol (6C) was studied initially at 60ºC. The stability of xylanase improved with addition of all the polyols additives (Fig. 1) but the thermostabilizing effect was proportional to their molecular size, which can further be correlated with the number of hydroxyl groups per polyol molecule. Incubation of enzyme at 60ºC in the presence of 1M sorbitol resulted in 59% retention of original activity after 180 min which is higher as compared to activity retention of 41% of control. An increase in the concentration of sorbitol further improved the retention of activity with maximum at 2M concentration.

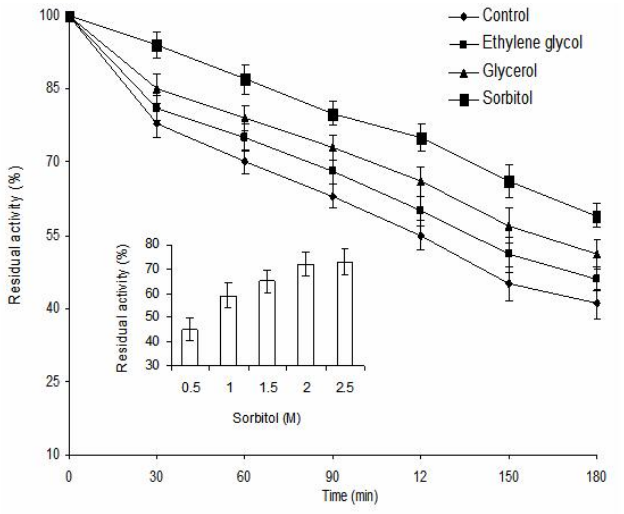

Figure 1. Thermal stability of xylanase at 60ºC in the presence of 1M polyols. Inset is the effect of different concentrations of sorbitol on thermostability at 60ºC.

These compounds have been found to show similar effect on xylanases isolated from *Thermomonospora* sp. (George et al. 2001) and *Arthrobacter* sp. MTCC 5214 (Khandeparkar and Bhosle, 2006). Polyols have the capability to form hydrogen bonds that play key role in supporting and stabilizing the native conformation of protein. The stabilizing effect of additives is not an absolute effect valid for all enzymes, but it depends on the nature of the enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with the additive (George et al. 2001). However, the improvement of xylanase stability in the presence of polyhydric alcohols such as sorbitol suggests their importance in improving the xylanase thermostability.

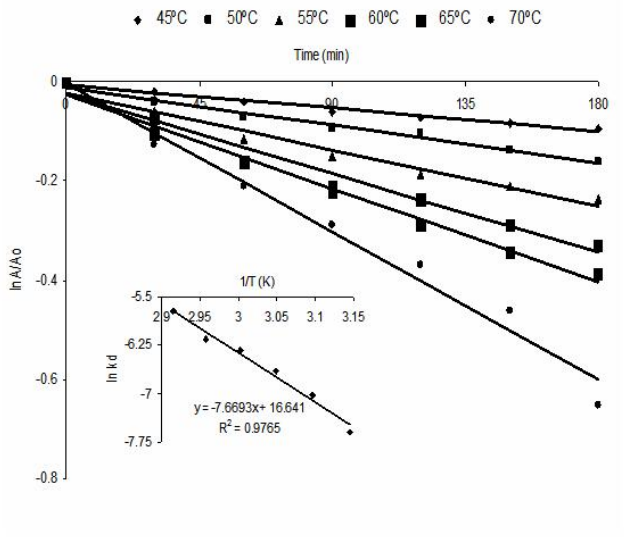

Figure 2. First order thermal deactivation of the sorbitol treated xylanase. Inset is the Arrhenius plot to calculate activation energy (E_d) for thermal denaturation.

The kinetics and thermodynamics of irreversible thermal denaturation of 2M sorbitol treated xylanase from *A. niger* DFR-5 was studied at 45-70ºC and compared with that of native enzyme. The plots of the residual activity of sorbitol treated xylanase versus incubation time were linear, with R^2 >97%, indicating that the inactivation could be expressed as first order kinetics (Fig. 2). Results of the thermoprotecting effect of 2M sorbitol are presented in Table 1. It was observed that the rate of enzyme deactivation (k_d) decreased in the presence of sorbitol indicating the thermostabilizing nature of sorbitol as a lower rate constant means

Table 1: Inactivation kinetic parameters of native and sorbitol treated xylanase towards thermal processes

Temp	k_d (min^{-1})		$t_{1/2}$ (min)		D-value (min)	
(ºC)	N	S	N	S	N	S
45	0.0015	0.0005	462	1386	1535	4606
50	0.0026	0.0009	267	770	886	2559
55	0.0036	0.0013	193	533	640	1772
60	0.0048	0.0018	144	385	480	1279
65	0.0063	0.0021	110	330	366	1097
70	0.0075	0.0033	92.4	210	307	698

z-value (ºC) = 36.6 (N) and 32.8 (S), k_d = Thermal inactivation rate constant; $t_{1/2}$ = Half-life; D-value = Decimal reduction time; N = Native xylanase; S = Sorbitol treated xylanase

the enzyme is more thermostable (Marangoni, 2003). Half-life ($t_{1/2}$) determinations are more accurate and reliable especially when computing the effect of additives on thermostability. Native xylanase showed a $t_{1/2}$ of 462 min at 45ºC. With increase in temperature, the $t_{1/2}$ decreases and showed a value of 92.4 min at 70ºC indicating that the enzyme is unstable at higher temperatures. On the other hand, sorbitol enhanced the thermostability of enzyme and displayed a $t_{1/2}$ of 210 min at 70ºC. The 2.27 fold increase in half-life clearly indicates that sorbitol treated xylanase was more stable at higher temperatures. Also, the time needed to reduce the enzyme activity by 90%, i.e. D-value, also increased in presence of sorbitol further proving its thermoprotecting effect on xylanase. All these results confirm that presence of sorbitol results into a thermostable enzyme.

The temperature increase required to decrease the D-value by one log cycle i.e. z-value, of native and sorbitol treated xylanase, calculated from the slope of graph between log D versus temperature, was 36.6 and 32.8ºC, respectively (Fig. 3). The high magnitudes of z-values mean more sensitivity to the duration of heat treatment and lower z-values mean more sensitivity to increase in temperature (Tayefi-Nasrabadi and Asadpour, 2008). Therefore, the lesser z-value of sorbitol treated xylanase (32.8ºC) as compared to 36.6ºC of native xylanase indicates that sorbitol makes the enzyme more sensitive to increase in temperature rather than its duration. Studies pertaining to this aspect of xylanase characterization are lacking to compare and discuss our findings.

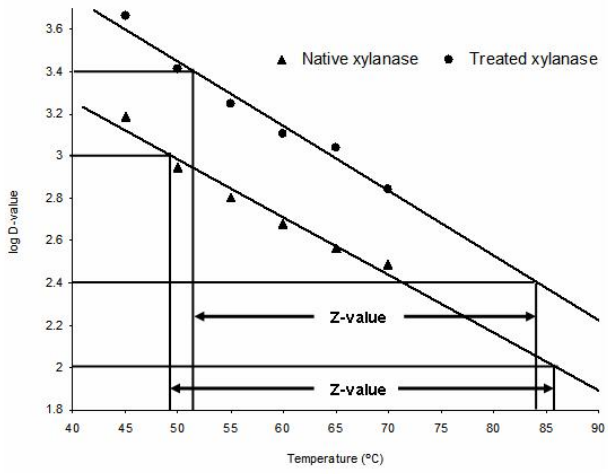

Figure 3. Temperature dependence of the decimal reduction of native and sorbitol treated xylanase to calculate z-values.

In order to determine the thermodynamic parameters for thermal stability, the energy of activation (E_d) for thermal denaturation was determined by applying the Arrhenius plot. The E_d for sorbitol treated xylanase was found 15.185 kcal/mol which was higher as compared to 13.59 kcal/mol of untreated enzyme indicating a very stable and compact xylanase that is highly resistant to heat denaturation. The higher value of E_d means more energy is required to denature the treated enzyme as postulated by Mainer et al. (1997) and Tayefi-Nasrabadi and Asadpour (2008). A significant increase in E_d in the presence of sorbitol also indicates that stabilization of xylanase was of conformational origin (Gouda et al. 2003).

The ΔHº of native xylanase was 54.414 kJ mol^{-1} at 45ºC while that of sorbitol treated form was 61.119 kJ mol^{-1} at 45ºC which clearly indicates that more energy is required for thermal denaturation of enzyme in the presence of sorbitol (Bhatti et al. 2005). The values decrease with rise in temperature, in both cases, revealing that lesser energy is required to denature enzyme at high temperatures but still more in treated xylanase (Table 2). The observed change in ΔHº also indicates that enzyme undergoes considerable change in conformation at higher temperatures even after treatment with sorbitol (Marin et al. 2003).

The Gibbs free energy (ΔGº) for thermal unfolding at 45ºC was 106.086 kJ mol^{-1} for native xylanase while that of sorbitol treated was 108.992 kJ mol^{-1}. With increase in temperature ΔGº values increased in both cases but more in treated enzyme. Moreover, a higher value of free energy of thermal denaturation at 70ºC (112.393 kJ mol^{-1}) indicates the resistance of treated enzyme towards thermal unfolding at higher temperatures which indirectly indicates its stability.

Table 2: Thermodynamic parameters for thermal inactivation of native and sorbitol treated xylanase.

Temp (ºC)	ΔHº (kJ mol^{-1})		ΔGº (kJ mol^{-1})		ΔSº (J $mol^{-1}K^{-1}$)	
	N	S	N	S	N	S
45	54.41	61.11	106.08	108.992	-162.492	-150.544
50	54.37	61.07	106.31	109.168	-160.825	-148.885
55	54.33	61.03	107.11	109.897	-160.939	-148.966
60	54.28	60.99	107.99	110.713	-161.285	-149.306
65	54.24	60.95	108.89	111.984	-161.680	-150.979
70	54.20	60.91	110.05	112.393	-162.816	-150.093

E_d = 15.185 kcal/mol (63.762 kJ/mol), ΔHº = Variations in enthalpy; ΔGº = Variations in free energy; ΔSº = Variations in entropy; N = Native xylanase; S = Sorbitol treated xylanase

The unfolding of enzyme structure is accompanied with an increase in disorder or entropy of deactivation, but xylanase from *A. niger* DFR-5 has negative entropy (ΔSº) which revealed that native form is in more ordered state (Table 2). Sorbitol treated xylanase also exhibited a negative ΔSº but the magnitude was lesser in comparison to untreated enzyme. The negative values for change in entropy indicate that there are significant processes of aggregation, since had this not happened, the values would have been positive (Marin et al. 2003). However, the negative values for treated enzyme were lower in comparison to untreated enzyme indicating the lesser compaction of treated enzyme which further indicates resistance to inactivation. The change in ΔSº in the presence of sorbitol can be explained in terms of an enhancement of the order and integrity of the structure, thus favoring inter- and/or intramolecular stabilizing forces and subsequently increasing the stability of enzyme. A significant change in the ΔSº and E_d in the presence of sorbitol indicated that stabilization of xylanase was of conformation origin (Gouda et al. 2003).

It is also worth mentioning that ΔGº values, which are measures of the spontaneity of the inactivation processes, are higher than the ΔHº values in sorbitol treated as well as native xylanase. This is due to the negative entropic contribution during the inactivation process as stated by Tanaka and Hoshino (2002).

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrate, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate (Sarath Babu et al. 2004, Bhati et al. 2006). Thermal denaturation may occur in two steps as shown below-

Where, N is native enzyme, U is unfolded enzyme that could be reversibly refolded upon cooling and D is the denatured enzyme formed after prolonged exposure to heat and therefore cannot be recovered on cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with a concomitant increase in the enthalpy of deactivation (Srivastava et al. 2005). The opening up of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of inactivation (Vieille and Zeikus, 1996). But, the negative ΔSº observed for native as well sorbitol treated xylanase suggests that there is an aggregation process in which a few inter- and/or intramolecular bonds are formed, enzyme proceeds to the aggregated complex and the state of order of the system increases (Anema and McKenna, 1996).

Table 3: Effect of metal ions and chelator on xylanase activity

Additives	Enzyme activity (% of control)* Concentration (mM)				
	1	2	5	10	20
Li^{+1}	108±8.2	119±9.8	128±10.3	127±9.1	128±8.7
Na^{+1}	112±11.4	123±12.5	132±11.7	138±9.8	136±13.2
K^{+1}	114±9.2	129±11.6	138±14.2	134±12.	135±10.7
Mg^{+2}	134±13.2	145±14.2	146±11.6	140±12.	143±13.8
Mn^{+2}	112±8.9	125±9.4	149±13.4	179±14.	177±12.6
Ca^{+2}	120±8.9	134±11.6	143±12.4	154±14.	153±13.4
Cu^{+2}	94±8.7	83±9.5	69±8.2	60±5.9	52±4.7
Co^{+2}	102±8.6	106±10.4	104±9.8	105±8.4	104±10.8
Zn^{+2}	104±9.4	106±10.5	104±11.2	105±8.7	106±9.5
Fe^{+2}	112±8.9	119±7.9	128±12.5	130±11.	129±11.2
Hg^{+2}	68±5.9	44±4.2	26±2.5	12±1.1	0±0.0
EDTA	89±8.2	75±6.8	64±5.7	55±5.2	34±2.9

*Values are Mean±SD of three experiments

Effect of metal ions and chelator

The divalent metal ions such as Mn^{+2} and Ca^{+2} stimulated the enzyme activity by more than 50% while monovalent cations like Li^{+1}, Na^{+1} and K^{+1} had small stimulating effect on the activity at variable concentrations. Zn^{+2} and Co^{+2} did not show any discernable effect on enzyme activity and metal ions like Cu^{+2} and Hg^{+2} inhibited the enzyme activity substantially (Table 3). Inhibition of enzyme activity by $HgCl_2$ has been reported by Khanna and Gauri (1993), Kang et al. (1996), Bataillon et al. (2000) and Qureshy et al. (2002). The addition of EDTA inhibited the enzyme activity, suggesting that metal ions are needed for the enzymatic reaction.

Table 4: Effect of thiol binding and tryptophan oxidizing agents on xylanase activity

Additives	Enzyme activity (% of control)* Concentration (mM)				
	1	2	5	10	20
Iodoacetamide	18±1.8	3±0.4	0±0.0	0±0.0	0±0.0
N-maleiamide	15±1.2	0±0.0	0±0.0	0±0.0	0±0.0
DTNB	13±0.9	0±0.0	0±0.0	0±0.0	0±0.0
PHMB	16±1.1	0±0.0	0±0.0	0±0.0	0±0.0
N-bromosuccinamide	98±8.9	95±7.8	91±6.6	87±7.5	85±8.2

DTNB = 5,5'-dithio-bis-(2-nitrobenzoic acid); PHMB = p-hydroxy-mercury benzoic acid, *Values are Mean±SD of three experiments

Identification of amino acid essential for catalysis

Tryptophan and cysteine have been shown to be involved in the active site of different xylanases (Marui et al. 1985, Keskar et al. 1989, Biswas et al. 1990). To test whether these residues are present in the active or binding site of xylanase of *A. niger* DFR-5, the effect of different modifying agents was studied (Table 4). The enzyme was completely inhibited by thiol-binding agents such as iodoacetamide, N-maleiamide, 5,5'-dithio-bis-(2-nitrobenzoic acid) and p-hydroxymercuribenzoic acid suggesting that there is an important cysteine residue in or close to the active site of the enzyme. The presence of cysteine at the active site of xylanase has been confirmed in earlier reports also (Bataillon et al. 2000, Kimura et al. 2000, Gupta et al. 2000, Saraswat and Bisaria, 2000, Ohta et al. 2001).

In contrast, only about 15% inhibition was detected after treatment with 20mM of N-bromosuccinimide (a potent oxidizing agent with specificity for tryptophan residue), indicating that tryptophan is not involved in catalysis. The slight inhibition is probably due to the reaction of this modifier with other residues (Khasin et al. 1993).

Table 5: Effect of reducing agents on xylanase activity

Reducing agents	Enzyme activity (% of control)* Concentration (mM)				
	1	2	5	10	20
Dithiothreitol	104±9.5	112±10.3	123±12.4	125±11.4	127±9.5
Glutathione	108±10.2	117±9.4	128±11.3	134±10.8	136±10.6
β-ME	118±8.5	134±12.5	146±11.8	157±13.4	155±12.1
L-Ascorbic Acid	112±8.9	121±10.3	137±10.8	135±11.7	134±12.3
Cysteine	115±10.8	129±10.1	136±12.4	135±11.6	130±12.6
Vanillin	106±8.4	117±10.2	123±11.5	124±9.8	120±10.5

*Values are Mean±SD of three experiments

Effect of reducing agent

The effect of a number of reducing agents, most widely used for the reduction of disulfide bonds, was tested on xylanase activity (Table 5). At a concentration of 10 mM in the reaction mixture, β-ME activated the enzyme activity maximally to an extant of 57%. The other reductive agents such as DTT, glutathione, cysteine, ascorbic acid and vanillin also displayed behaviors identical to that of β-ME but to a lesser extent. The stimulation of xylanase activity by reducing agents and inhibition by thiol-binding agents clearly indicates that there is a relationship between the reduced form of the cysteine residues and the activity of the xylanase (Khanna and Gauri, 1993).

Table 6: Storage inactivation parameters of purified xylanase

Temp (ºC)	k_d (day^{-1})	R^2	$t_{1/2}$ (day)	D-value (day)
4	0.0025	0.8972	277	921
15	0.0065	0.9542	107	354
37	0.0341	0.9227	20	68

k_d = Storage inactivation rate constant; $t_{1/2}$ = Half-life; D-value = Decimal reduction time

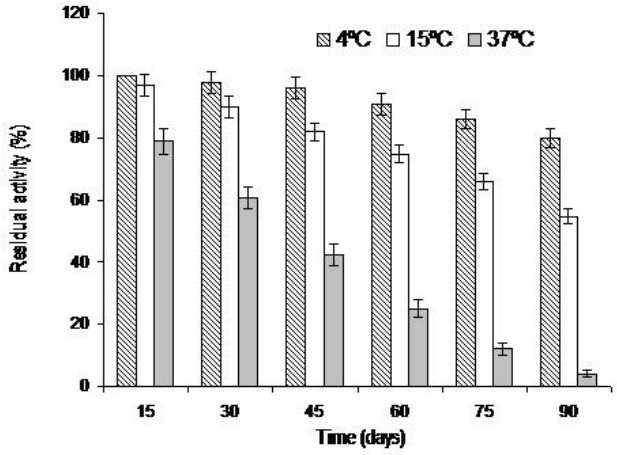

Figure 4a. Storage stability of purified xylanase

Storage stability of xylanase

The %RA of purified xylanase stored at three different temperatures is shown in Fig. 4a. The xylanase activity was absolutely intact when stored at 4ºC for 15 days while decreased slowly at 20ºC. The enzyme inactivation was faster when stored at 37ºC and negligible activity was detected after 3 months of storage at this temperature.

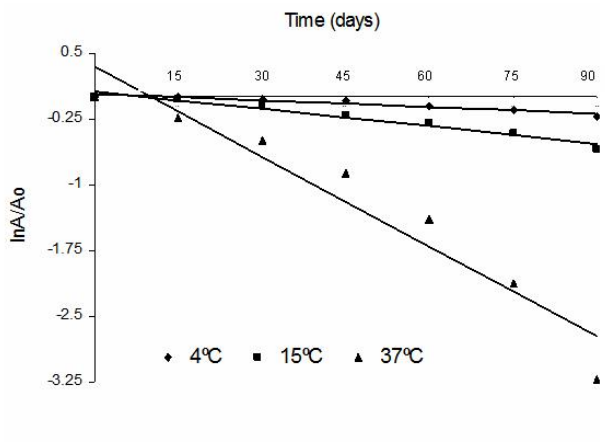

Figure 4b. First order storage deactivation of purified xylanase.

The plots of the residual activity of xylanase versus storage time at all temperatures were linear indicating that the storage deactivation could be expressed as first order kinetics (Fig. 4b). The deactivation rate constants (k_d), $t_{1/2}$ and D-values of xylanase stored at different temperatures are summarized in Table 6. The rate of enzyme deactivation was proportional to its storage temperature with maximum at 37ºC. It is clear that enzyme was most stable when stored at 4ºC with a maximum $t_{1/2}$ and D-value of 277 and 921 days, respectively. The half-life decreased with increasing storage temperature and showed a value of 107 and 20 days at 20 and 37ºC, respectively.

Conclusion

Some of the properties of xylanase purified from *Aspergillus niger* DFR-5 were studied. The enzyme stability at higher temperatures was improved by the supplementation of 2M sorbitol in the enzyme preparation. The effects were quantified in terms of $t_{1/2}$, D- and z-value of xylanase. Various thermodynamic parameters were also determined to study the mechanism of enhancement of thermostability. It was found that improved stability at higher temperatures was due to the increased enthalpy and free energy of deactivation in the presence of sorbitol. The negative entropy change at all temperatures indicated that xylanase proceeds toward compaction during denaturation. Increased activity in the presence of metal ions indicated that xylanase is a metal requiring enzyme which was further supported by its decreased activity in the presence of EDTA. Various thiol-binding agents abolished the enzyme activity confirming the necessity of cysteine for catalysis. Moreover, various reducing agents increased the enzyme activity to varying extant confirming that reduced form of cysteine is necessary for enzymatic activity. Half-life of xylanase stored at 4ºC was found maximum with a value of 277 days. Further work on immobilization and application of xylanase in juice clarification is in progress.

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