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Thermal stability of two xylanases from *Macrotermes subhyalinus* little soldier: kinetic and thermodynamic analysis

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Abstract

The knowledge on thermal inactivation kinetics of enzymes is necessary to allow their adequate utilization as natural biopreservatives in the food industry and technology applications. In this work, the kinetics of thermal inactivation was studied for the previously purified and characterized xylanases Xyl1 and Xyl2 from *Macrotermes subhyalinus* little soldier. Samples of xylanases were treated at different time-temperature combinations in the range of 5-60 min at 50-65°C and the kinetic and thermodynamic parameters for xylanases were calculated. The inactivation kinetic follows a first-order reaction with k-values between 0.0192 ± 0.0002 to 0.0405 ± 0.0003 and 0.0119 ± 0.0005 to 0.0418 ± 0.0004 min^{-1} for Xyl1 and Xyl2, respectively. Activation energy (*Ea*) and *Z*-values were estimated to 48.08 ± 1.84 kJ mol^{-1} and 43.47 ± 3.02°C for Xyl1, 75.52 ± 3.52 $kJ.mol^{-1}$ and 27.77 ± 1.87°C for Xyl2. The catalytic reactions of both xylanases are endothermic due to positive enthalpy. The high value obtained for the variation in enthalpy of activation indicates that a high amount of energy is required to initiate denaturation, probably due to the molecular conformation of xylanases. All results suggest that both xylanases are relatively resistant to long heat treatments up to 50°C.

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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 (de Meneres *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-o-methyl-D-glucopyranosyl, α-L-arabinofuranosyl, acetyl, as well as linked to feruloyl and coumaryl components of lignin (Shallom and Shoham, 2003 ; Ninawe *et al.,* 2008). 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 functional food industry (Li *et al.,* 2012). The knowledge of xylanase opens up a wide range of biotechnological applications in multiple industries. Thus, the thermostable xylanase can play an important role in the bakery industry by increasing shelf life and improving the quality of baked products (Bajaj and Manhas, 2012). The use of xylanase as an animal feed supplement along with multi-enzyme cocktails improves 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). Xylanolyitc enzymes have also opened new possibilities for the bioconversion of agricultural wastes in to easy fermentable sugars (Romdhane *et al.,* 2010). Although xylanases potentially offer a number of advantages over conventional chemical reagents, their application at an industrial scale remains limited. Consequently, there is on-going search for more potent strains of xylanase producers, especially those that can produce thermostable enzymes with greater yields (Viikari *et al.,* 2007).

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 50°C over an extended period of time (Singh *et al.,* 2000). Since high thermostability is required for industrial applications of enzymes (Sriyapai *et al.,* 2011), therefore the stabilization of enzymes remains an important concern especially during thermal processing. The loss of enzyme activity through-out the elevated temperature ranges is related to changes of enzyme conformation (Cui *et al.,* 2008; Fu *et al.,*2010).In fact, in order for a xylanase to achieve actual industrial application, it should ideally fulfill a number of specific requirements that are highly desired in the marketplace. However, very little information is available on kinetics of xylanases from *Macrotermes subhyalinus* little soldier. Therefore, the aims of this work were to evaluate the stability, then to determinate kinetic and thermodynamic parameters of xylanases Xyl1and Xyl2.

Material and methods

Enzymes

Xylanases (Xyl1 and Xyl2) used in this study were previously purified from little soldier of *Macrotermes subhyalinus* (Fagbohoun *et al.,* 2012; Fagbohoun, 2013). Theses enzymes were homogeneous on polyacrylamide-gel electrophoresis without sodium dodecyl sulphate (SDS).

Xylanases assays

Under the standard test conditions, xylanase activity was assayed spectrophotometrically by measuring the release of reducing sugars from Birchwood xylan. The reaction mixture (0.38 ml) contained 0.2 ml of 0.5 % xylan (w/v) dissolved in 20 mM acetate buffer (pH 5.0) and 0.1 ml enzyme solution. After 30 min of incubation at 45°C, the reaction was stopped by adding 0.3 ml of dinitrosalicylic acid solution and heating for 5 min in boiling water bath. The absorbance was measured at 540 nm after cooling on ice for 5 min.

One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg of protein).

Protein determination

Protein was determined according to Lowry method (Lowry *et al.,* 1951) using bovine serum albumin as standard.

Thermal inactivation

Thermal inactivation of each xylanase was investigated at various constant temperatures from 50 to 65°C after exposure to each temperature for a period of 5 to 60 min. The enzyme was heated in sealed tubes, which was incubated in 100 mM sodium acetate buffer (pH 5.0) in a thermostatically controlled water bath. Tubes were withdrawn at each time intervals and immediately immersed in an ice bath, in order to stop heat inactivation. The residual enzymatic activity, determined in both cases at 37°C under the standard test conditions, was expressed as percentage activity of zero-time control of the untreated enzyme.

Kinetic data analysis

First-order kinetic has been reported to describe thermal inactivation of xylanases (Guiavarc'h *et al.,* 2002).The integral effect of inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Eq. 1:

$$ln\ (At/Ao) = -kt \quad (1)$$

where, At is the residual enzyme activity at time t (min), Ao is the initial enzyme activity, k (min^{-1}) is the inactivation rate constant at a given condition. k-values were obtained from the regression line of $ln\ (At/Ao)$ versus time as slope.

D-value is defined as the time needed, at a constant temperature, to reduce the initial enzyme activity (Ao) by 90 %. For first-order reactions, the D-value is directly related to the rate constant k (Eq. 2)(Stumbo, 1973; Espachs-Barroso *et al.,* 2006):

$$D = 2.303/k \quad (2)$$

The Z-value (°C) is the temperature increase needed to induce a 10-fold reduction in D-value(Stumbo, 1973)and follows the Eq. 3:

$$log(D1/D2) = (T2 - T1)/Z \quad (3)$$

where, $T1$ and $T2$ are the lower and higher temperatures in °C or K, $D1$ and $D2$ are D-values at the lower and higher temperatures in min, respectively.

The Z-values were determined from the linear regression of log (D) and temperature (T).

Thermodynamic analysis

The Arrhenius equation is usually utilized to describe the temperature effect on the inactivation rate constants and the dependence is given by (Eq. 4 or 5):

$$k = Aexp(-Ea/RT) \quad (4)$$

$$or\ lnk = lnA - (Ea/RT) \quad (5)$$

where, k is the reaction rate constant value, A the Arrhenius constant, Ea ($kJ.mol^{-1}$) the activation energy, R (8.31 $J.mol^{-1}K^{-1}$) the universal gas constant and T (K) the absolute temperature.

When lnk is plotted versus the reciprocal of the absolute temperature, a linear relationship should be observed in the temperature range studied. The slope of the line obtained permitted to calculate the Ea and the ordinate intercept corresponds to lnA (Dogan *et al.,* 2002). The changes in enthalpy ($\Delta H^{\#}$, $kJ.mol^{-1}$), entropy ($\Delta S^{\#}$, $J.mol^{-1}.K^{-1}$) and Gibbs free energy ($\Delta G^{\#}$, $kJ.mol^{-1}$) for the thermal denaturation of xylanase were determined using following equations (Eq. 6; 7; 8) (Gummadi, 2003).

$$\Delta H^{\#} = Ea - RT \quad (6)$$

$$\Delta S^{\#} = R\ (lnA - lnKB / h - lnT) \quad (7)$$

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \quad (8)$$

where, KB (1.38 x 10^{-23} $J.K^{-1}$) is the Boltzmann's constant, h the Planck's constant (6.626 x 10^{-34} J.s) and T the absolute temperature.

Statistical analyses

Statistical analyses were carried out in triplicate. The results were processed by the software STATISTICA 7 (Statsoft Inc, Tulsa-USA, Headquarters).

Thus, results were expressed as means ± standard deviation. The statistical differences among the means of data were calculated using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Differences at $P < 0.05$ were considered significant.

Results and discussion

Thermal inactivation kinetics of xylanases

The purified enzyme was found to be relatively stable at 50 °C. The effect of heat treatment over a range of temperature from 50 to 65°C on both xylanases Xyl1 and Xyl2 was evaluated by determining the residual percentage activity (Table 1).

Table 1. Effect of treatment temperature and time on the inactivation of xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus*.

Treatment time (min)	Residual activity (%) at each temperature (°C) of heat treatment							
	50		55		60		65	
	Xyl1	Xyl 2	Xyl1	Xyl 2	Xyl1	Xyl 2	Xyl1	Xyl 2
5	90.48 ± 3.51	92.68 ± 2.82	87.80 ± 3.84	91.39 ± 2.34	81.87 ± 3.18	81.87 ± 2.98	74.08 ± 1.48	74.30 ± 1.88
10	81.87 ± 1.35	90.48 ± 2.24	79.45 ±1.18	81.87 ±1.74	74.08 ± 1.78	72.61 ± 3.64	65.20 ± 1.31	65.05 ±2.84
15	75.37 ± 1.98	81.87 ± 1.56	72.61 ± 2.35	67.07 ± 1.38	60.04 ± 1.48	59.45 ± 1.54	54.88 ± 2.08	49.01 ± 1.78
20	65.70 ± 2.15	75.57 ± 1.38	65.70 ± 2.22	67.03 ± 2.75	51.17 ± 1.72	54.88 ± 1.42	44.93 ± 1.02	38.86 ± 1.14
25	61.36 ± 1.88	74.08 ± 1.82	56.05 ± 1.78	59.45 ± 1.85	44.57 ± 1.25	44.91 ± 2.15	36.05 ± 0.68	33.97 ± 1.96
30	55.43 ± 0.48	69.07 ± 1.46	52.86 ±1.92	52.20 ± 1.78	34.99 ± 1.52	39.08 ± 1.52	27.25 ± 1.25	27.25 ± 1.45
35	50.15 ± 1.42	65.05 ± 1.29	46.76 ± 1.12	46.34 ± 1.57	30.72 ±1.86	31.66 ± 1.36	24.65 ±1.28	21.65 ± 1.76
40	45.03 ± 1.45	61.98 ± 1.62	42.74 ± 1.56	40.65 ± 1.08	26.44 ± 2.84	27.25 ± 1.38	18.63 ± 1.15	18.26 ± 0.64
45	42.74 ± 1.08	60.65 ± 1.34	37.15 ± 1.45	36.78 ± 2.12	22.31 ± 0.82	21.98 ± 1.68	16.04 ± 1.52	14.95 ± 0.63
50	38.67 ± 0.64	54.88 ±1.82	34.64 ± 1.32	31.66 ± 1.94	20.18 ± 1.44	20.18 ± 0.34	13.53 ± 0.78	13.26 ± 1.24
55	35.34 ± 1.75	52.20 ± 1.22	29.52 ± 1.92	28.65 ±1.29	16.86 ± 1.84	16.52 ± 1.24	11.08 ± 0.56	10.12 ± 1.56
60	32.30 ± 1.56	49.65± 2.25	26.71 ± 2.51	25.92 ± 2.76	13.94 ± 0.94	13.53 ± 1.64	09.07± 0.84	09.07± 0.18

Here, values represent mean ± SD three independent determinations.

Thus, we note an enzyme activity peak usually referred to the optimum temperature and which varies for different enzymes(Trasar-Cepeda *et al.,* 2007).

The activity of both xylanases was decreased with increasing heating time (5-60 min) and temperature (50-65°C). Indeed, between 50 to 65°C, heat-denaturation of Xyl1 and Xyl2 occurred after incubation for 5 min (90.48 ± 3.51 to 74.08 ± 1.48 % and 92.68 ± 2.82 to 74.30 ± 1.88 %, respectively).

Therefore, the heat treatment at 50°C during 60 min caused a high inactivation of 32.30 ± 1.56 % for Xyl1 and a partial inactivation of 49.65 ± 2.25 % for Xyl2.

A partial inactivation of 52.86 ± 1.92 and 52.20 ± 1.78 % was also observed for Xyl1 and Xyl2, respectively, after heating at 55°C during 30 min. However, a strong inactivation of both enzyme activities was obtained after 60 min of heat treatment at 65°C. Moreover, xylanases from *Macrotermes subhyalinus* little soldier showed a temperature-dependent inactivation profile in the presence of the substrate used. At higher temperature, the enzyme most likely underwent denaturation and lost its activity. Stauffer (1989) states that denaturation is the heat induced spontaneous, irreversible breakdown of the secondary and tertiary structure of the enzyme protein such that the enzyme will no longer function and cannot re-activate.

Table 2. k-values and $T_{1/2}$ for thermal inactivation of xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus* at temperature range (50-65°C).

Temperature (°C)	Xyl 1		Xyl 2	
	k-values (min^{-1})	$T_{1/2}$ (min)	k-values (min^{-1})	$T_{1/2}$ (min)
50	0.0192 ± 0.0002^{a}	36.09 ± 1.08^{g}	0.0119 ± 0.0005^{b}	58.23 ± 1.82^{a}
55	0.0218 ± 0.0003^{c}	31.78 ± 1.84^{f}	0.0224 ± 0.0007^{e}	30.93 ± 0.78^{b}
60	0.0329 ± 0.0004^{d}	21.06 ± 1.08^{e}	0.0327 ± 0.0002^{h}	21.19 ± 1.65^{c}
65	0.0405± 0.0003^{b}	17.11 ± 0.05^{a}	0.0418 ± 0.0004^{d}	16.57 ± 0.07^{f}

The results of the heat inactivation studies suggest that these enzymes belong to the group of thermostable enzymes. Compared to Xyl2, results show that Xyl1 was the most thermostable because it retained about 60 and 55 % activity after 15 min at 60 and 65°C, respectively. Based on the semi-log plots linear of xylanase activities versus heat treatment time at temperature ranged from 50 to 65°C (Table 1), it can be concluded that thermal inactivation described a first-order reaction (Fig. 1).

(Driss *et al.,* 2014) and for purified xylanase from *Aspergillus niger* DFR-5 (Pal and Khanum, 2010).

The inactivation rate constant (*k*) value and half-life ($t_{1/2}$) of xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus* are presented in Table 2.

Table 3. D, Z and Ea-values for thermal inactivation of xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus* at temperature range (50-65°C).

Kinetic parameters	Xyl 1	Xyl 2
D values (min)		
D_{50}	119.32 ± 2.78^{j}	154.56 ± 1.96^{d}
D_{55}	105.64 ± 1.27^{h}	96.35 ± 3.75^{e}
D_{60}	70.00 ± 1.49^{k}	66.56 ± 1.54^{i}
D_{65}	56.86 ± 0.68^{b}	55.36 ± 1.82^{d}
Z value (°C)	43.47 ± 3.02^{b}	27.77 ±1.87^{a}
Ea ($kJ.mol^{-1}$)	48.08 ± 1.84^{a}	75.52 ± 3.52^{c}

Results showed clearly that the rate of *k*-value increased with the temperature, indicating the thermostabilizing nature of xylan, as a lower rate constant means the enzyme is more thermostable (Marangoni, 2002). Rate of Xyl1 and Xyl2 inactivation, after logarithmic transformation, decreased linearly with the inverse of temperature (Fig. 2).

This relationship is described by the equation: ln k = -5786 (1/T) + 13.90 (R^2= 0.96) and ln k = - 9088 (1/T) + 23.78 (R^2= 0.96) for Xyl1 and Xyl2, respectively, where T represents absolute temperature (°K).

(Arogba *et al.,* 1998). As shown in Table 2, $t_{1/2}$ determinations are more accurate and reliable on thermostability. With the increasing temperature, the $t_{1/2}$ decreased and showed values ranged between 36.09 ± 1.08 and 17.11 ± 0.05 min for Xyl1, and between 58.23 ± 1.82 and 16.57 ± 0.07 min for Xyl2.

This would indicate that the enzymes are unstable at higher temperature (Lappe, *et al.,* 2009). In order to establish the link between treatment and enzyme activity, the decimal reduction time (*D*-value) needed for 90% reduction of the initial enzyme activity was calculated. The corresponding *D*-values for Xyl1 and Xyl2 are given in Table 3.

D-values decreased with increasing temperature from 50 to 65°C, indicating a faster inactivation of xylanasesat higher temperatures. Similarly to the works of Bankeeree *et al.* (2014); Pal and Khanum, (2010) and Sant'Anna *et al.*(2011), thermal stability of

tropical isolate xylanase from *Aureobasidium pullulans*, purified xylanase from *Aspergillus niger* DFR-5and peptide P34 decreased respectively at higher temperature. *D*-values for Xyl1 and Xyl2 ranged from 119.32 ± 2.78to 56.86 ± 0.68min and 154.56 ± 1.96to 55.36± 1.82min, respectively.

In comparison, it should be noted that at 55°C and 60°C, the *D*-values for Xyl1 inactivation were higher than the corresponding values for Xyl2 inactivation. This is probably due to the relative higher thermal stability of Xyl1.

Table 4. Thermodynamic parameters xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus* under heat treatment between 50 and 65 °C (assuming a 1st-order kinetic model).

	Thermodynamic parameters					
Temperature (°C)	$\Delta H^{\neq}$ (kJ.mol^{-1})		$\Delta S^{\neq}$ (J.mol^{-1}K^{-1})		$\Delta G^{\neq}$ (kJ.mol^{-1})	
	Xyl1	Xyl 2	Xyl1	Xyl 2	Xyl1	Xyl 2
50	45.40 ± 0.02^{f}	72.83 ± 0.02^{d}	74.98 ± 0.01^{n}	57.95 ± 0.02^{a}	21.17 ± 0.02^{e}	54.11 ± 0.02^{d}
55	45.36 ± 0.01^{e}	72.79 ± 0.01^{c}	74.86 ± 0.02^{m}	57.82 ± 0.02^{b}	20.79 ± 0.01^{f}	53.82 ± 0.02^{e}
60	45.31 ± 0.02^{h}	72.75 ± 0.01^{e}	74.73 ± 0.02^{k}	57.69 ± 0.01^{c}	20.42 ± 0.02^{g}	53.53 ± 0.01^{b}
65	45.27 ± 0.01^{k}	72.71 ± 0.02^{b}	74.61 ± 0.01^{l}	57.57 ± 0.02^{d}	20.04 ± 0.01^{h}	53.24 ± 0.02^{c}
Mean	45.34 ± 0.01^{j}	72.77 ± 0.01^{c}	74.79 ± 0.03^{k}	57.76 ± 0.01^{b}	20.60 ± 0.01^{f}	53.67 ±0.02^{e}

The effect of temperature on *D*- and *Z*-values of xylanasesXyl1 and Xyl2 from little soldier of *Macrotermes subhyalinus* are shown in Table 3.

The temperature increase required to decrease the D-value by one log cycle i.e. Z-value, of both xylanases Xyl1 and Xyl2, calculated from the slope of graph between log D versus temperature, was 43.47 ± 3.02 and 27.77 ± 1.87°C, respectively (Figure 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 temperature (Tayefi-Nasrabadi and Asadpour, 2008). Therefore, the lesser Z-value of xylanase Xyl2 (27.77 ± 1.87°C) as compared to 43.47 ± 3.02°C of xylanase Xyl2 indicates that xylan makes the xylanase Xyl2 more sensitive to increase in temperature rather than its duration. Therefore, the *Z*-values for cooking and nutrients degradation (25-45°C) are generally greater than microbial inactivation (7-12°C)(Awuah *et al.,* 2007). In fact, differences between the *D*- and *Z*-values of enzyme and nutrients are exploited to optimize thermal processes and can be exploited also to maintain xylanases activity after treatment. This indicates that any change in temperature processing affects more intensely the stability of Xyl1 thanXyl2. In this study, *D*-, *Z*- and *k*-values indicate that both xylanases are heat stable and then can be used in high temperature short time (HTST) and low temperature long time (LTLT) industrial processes such as pasteurization, where values of 65°C for 3-5 min and 55°C for 30 min, respectively, are generally considered. In fact the *D*- and *Z*-values of xylanases are exploited to optimize thermal processes and to preserve enzyme activity after treatment.

The energy of activation (*Ea*) for thermal denaturation can be seen as the energy absorbed or released needed to the molecules be able to react (Van Boekel, 2008). In this study, the two enzymes presented in Table 3 had high activation energy (Ea) values and they were calculated to be 48.08 ± 1.84 and 75.52 ± 3.52 kJ.mol^{-1} for Xyl1 and Xyl2, respectively. It means that this enzymatic reaction doesn't occur easily, that is requires more energy to reach the transition state. In fact, as it is a biological catalyst, it is expected that severe conditions are not required for the reaction to occur, since biological reactions occur in mild pH (4.5 to 6) and temperature conditions (50 to 65°C) (Lopes *et al.,* 2013). However, the xylanase Xyl2 had higher activation energy (75.52 ± 3.52 kJ.mol^{-1}), than that of xylanase Xyl1 (48.08 ± 1.84kJ.mol^{-1}) requiring more energy to reach the transition state, that is it occurs less easily than that Xyl1. These values were lower than those of endoglucanase from *Humicola insolens* (108.69 kJ.mol^{-1}) and of beta-glucosidase from

Cardisoma armatum (172.98 kJ.mol^{-1}) (Riaz *et al.,* 2014; Ya, *et al.,* 2014). Both xylanases (Xyl1 and Xyl2) had high relative activation energy values, which could indicate an increased stability at higher temperatures and that the enzyme conformation was still stable at these temperatures (Leite *et al.,* 2007).

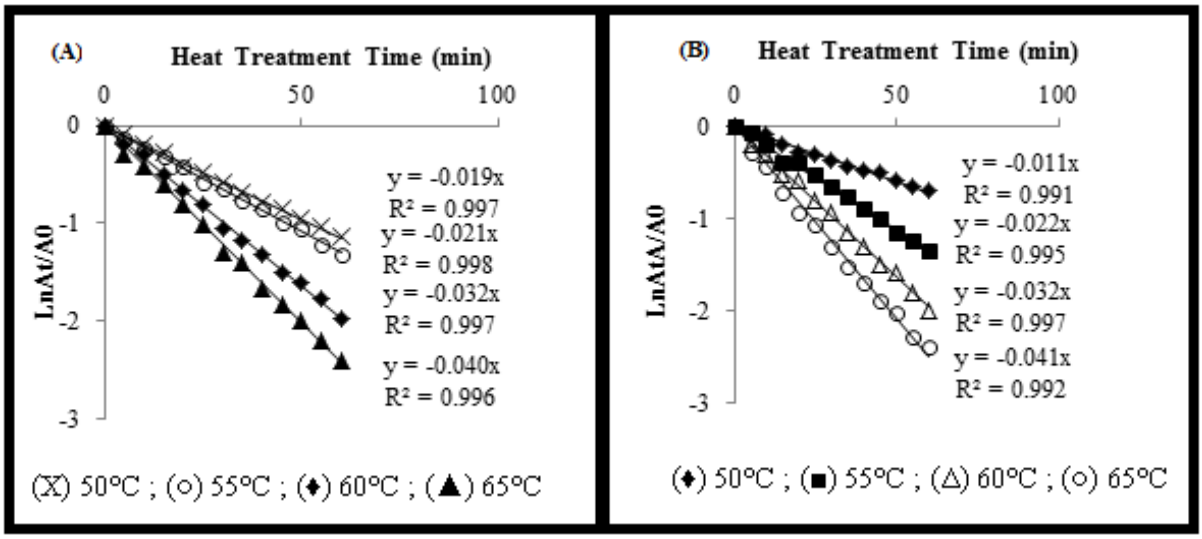

Fig. 1. Thermal inactivation of xylanases Xyl1 and Xyl2 at temperature range (50-65°C) from in sodium acetate buffer pH 5.0 in the temperature ranged from 50 to 65°C. A_o is the initial enzymatic activity and A_t the activity at each holding time. [A]: xylanase Xyl1, [B]: xylanase Xyl2.

Thermodynamic studies of xylanases

The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Srivastava *et al.,* 2005). Thus, thermostability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate, while thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of substrate (Georis *et al.,*2000; Sarath Babu *et al.,* 2004; Bhatti *et al.,* 2013).

In the study of the mechanism of thermal inactivation of proteins, valuable information can be obtained by identifying some inactivation parameters, such as enthalpy ($\Delta H^{\#}$), entropy ($\Delta S^{\#}$) and Gibbs free energy ($\Delta G^{\#}$). Thus, the determination of these thermodynamic parameters was carried out by measuring the xylanase activities at different temperatures (50-65°C). Table 4 shows these thermodynamics parameters for xylanasesXyl1 and Xyl2.

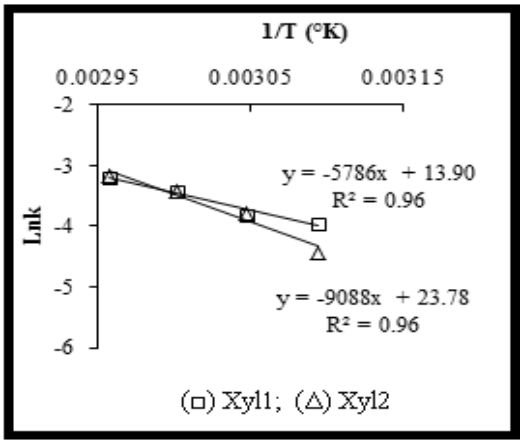

Fig. 2. Temperature dependence of inactivation rate constant for thermal inactivation xylanase Xyl1 and Xyl2 from of *Macrotermes subhyalinus* little soldier. 1/T represents the reciprocal of the absolute temperature. (□): xylanase Xyl1; (Δ): xylanase

Thus, the values of $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ were respectively 45.34 ± 0.01 kJ.mol^{-1}, 74.79 ± 0.03 J.mol^{-1}K^{-1} and 20.60 ± 0.01 kJ.mol^{-1} for Xyl1 and 72.77 ± 0.01 kJ.mol^{-1}, 57.76 ± 0.01 J.mol^{-1}K^{-1} and 53.67 ± 0.02 kJ.mol^{-1} for Xyl2. The enthalpy ($\Delta H^{\#}$) change in the system corresponds to the heat released or absorbed in the transformation (constant pressure).

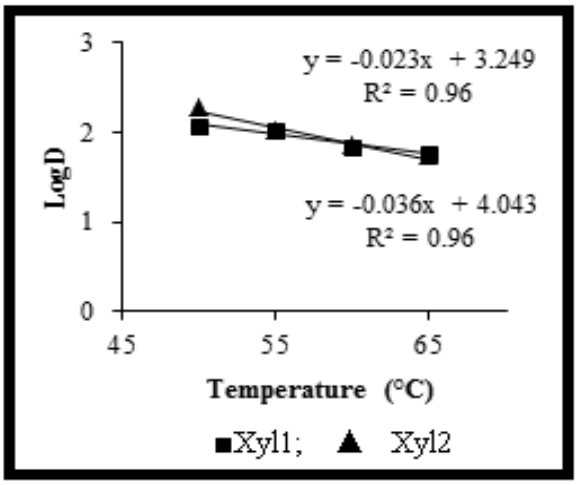

Fig. 3. Effect of temperature on D-values for inactivation of xylanases Xyl1 and Xyl2from *Macrotermes subhyalinus* little soldier ■ xylanase Xyl1; ▲ xylanase Xyl2.

Thus, the positive value of this parameter indicates that the catalytic reaction is endothermic that the heat of reaction is extracted from the surroundings in the transformation of the substrate into products under conditions of constant temperature and pressure (Yaws, 1999).Results also show that the Xyl2 enthalpy was higher than that of Xyl1.

The high enthalpy ($\Delta H^{\#}$) change in the system clearly indicates that more energy is required for thermal denaturation of enzyme (Bhatti *et al.,* 2005). The observed change in $\Delta H^{\#}$ also indicates that enzyme undergoes considerable change in conformation at higher temperatures even after treatment (Marín *et al.,* 2003).

The opening up of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation(Vieille and Zeikus, 1996). Thus, the both xylanases Xyl1 and Xyl2 have positive entropy ($\Delta S^{\#}$) which revealed that the native form of the enzyme is in less ordered state (Table 4). According to Anema and Mckenna (1996), the positive values of entropy ($\Delta S^{\#}$) for the hydrolysis reaction of xylan indicate that the reaction proceeds with less speed and is characterized by low regularity. Small changes in the values of $\Delta S^{\#}$ indicates a preferential destruction of weak bonds (hydrogen and electrostatic), resulting in a lower loss of catalytic activity.

The positive values for change in $\Delta S^{\#}$ also indicate that there are no significant processes of aggregation for both xylanases. Furthermore, the high values obtained for $\Delta S^{\#}$ variation probably reflect an increased disorder of the active site or the structure of each xylanase, which is the main driving force of heat denaturation (D'amico *et al.,* 2003). Generally, activation entropy has a dominant role in thermal inactivation of proteins in aqueous solutions (Bromberg *et al.,* 2008).

The Gibbs free energy change ($\Delta G^{\#}$) indicates the spontaneity of the reaction catalyzed under the conditions of temperature and pressure used. In this study, $\Delta G^{\#}$ values were positive, indicating that the processes were endergonic and not spontaneous.

Conclusion

From the characterization analyses of the enzymes, it can be concluded that the thermal inactivation of two xylanasesXyl1 and Xyl2 can be explained by the first-order model. Based on an isothermal experiment in the temperature range from 50 to 65°C and using Arrhenius equation, the *D*-, *Z*-, *k*-values, indicate that Xyl1 and Xyl2 are heat stable and then could be utilized in pasteurization conditions, maintaining part of their biological activity.

The high values obtained for activation energy (*Ea*) and change in enthalpy ($\Delta H^{\#}$) indicated that a high amount of energy was needed to initiate denaturation of these xylanases most likely due to its stable molecular conformation.

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