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Full Length Research Paper

Purification and biochemical characterization of a xylanase purified from a crude enzyme extract for the determination of active site residues

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This paper describes the purification of a thermostable xylanase (Xyn) from a commercially available xylanase stock (Buzyme 2511 from Buckman Laboratories International, South Africa). The purified enzyme showed a Mr. of 66 kDa, and presented a dimeric form with two similar molecular weight chains of 33 kDa. The optimum pH and temperature were found to be 6.0 and 50°C, respectively. The enzyme was active over a broad pH (5 to 10) and temperature (50 to 90°C) range. The enzyme showed 90% residual activity at 70°C for more than 35 min. The substrate progress curve showed that the enzyme substrate reaction attains V_{max} within 30 min. The K_m value determined by Lineweaver-Burk Plot was 1 mg/ml by using birchwood xylan as a substrate. The inhibition of enzyme activity by chemical modifiers like *o*-phthaldehyde, trinitrobenzenesulphonic acid, diethyl pyrocarbonate and woodword's reagent K suggest the importance of Cys, His, Lys and Glu residues for the catalytic activity of the enzyme.

Key words: Xylanase, buzyme 2511, enzyme thermostability, steady state kinetics.

INTRODUCTION

The endo-1, 4-β-xylanase (EC 3.2.1.8) is the key enzyme (Wong et al., 1988) that depolymerizes xylan, which is the main component of hemicellulose and consists of 1, 4-glycosidically linked β-D-xylose. Xylanases are used in paper manufacturing, waste treatment, deinking, fuel and chemical production (Biely, 1985). The major interest of thermostable xylanases in pulp bleaching is that it helps in reducing the kappa number and increases the brightness of the pulp. Hence, the requirement of chlorine as a bleaching agent could be substantially reduced and this could lead to the development of environmentally friendly technologies (Coughlan and Hazelwood, 1993). There are some reports of xylanases that are active and stable at both alkaline pH and elevated temperatures (Nakamura et al., 1993; George et al., 2001a). Most of the commercial xylanases showing activity in the alkaline range are only active at 50°C (Viikari et al., 1994).

Investigations involving chemical modification of an enzyme can potentially yield insights into structure - function relationships. In comparison to the extensive studies on the biotechnological applications of xylanases, inadequate information is available concerning the mechanism of action of xylanases. The mechanism of action of xylanases is similar to the double displacement mechanism of lysozyme (Vernon and Banks, 1963). The role of carboxyl groups (Chauthaiwale and Rao, 1994; Ko et al., 1992) in catalysis and aromatic residues such as tryptophan (Nath and Rao, 1998) and tyrosine (Bray and Clarke, 1995) in substrate binding of xylanases are well documented. Crystal structure of family 10 xylanases has shown the presence of a His residue in the active site (White et al., 1994; Harris et al., 1996). Charnock et al. (1997) suggested the possible role of a Lys residue in positioning the substrate in the active site of a xylanase from *Pseudomonas fluorescence*.

The role of a Trp residue in the active site of the xylanase from the extremophilic *Bacillus* sp. was

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illustrated by Nath and Rao (1998). George et al. (2001b) reported the involvement of a Lys residue in the active site of a xylanase from Thermomonospora sp. Chemical modifiers such as OPTA (*o*-phthaldehyde), TNBS (trinitrobenzenesulphonic acid), DEP (diethyl pyrocarbonate) and WRK (woodword's reagent K) have been used to study the mechanism of enzymatic catalysis and determining the active site residues of many enzymes (George et al., 2001a; George et al., 2001b; Nath and Rao, 2001; Rawat and Rao, 1997; Anish and Rao,2007). In this paper, we describe the purification and biochemical properties of a thermostable xylanase (Xyn) and also identify active site amino acid residues by using chemical modifiers and fluorescent labeling. Buzyme 2511 is an impure commercial enzyme and needed to be characterized and purified to homogeneity which could help in molecular cloning of this enzyme. Further the data will help in understanding the mechanism of action of substrate hydrolysis by this xylanase.

MATERIALS AND METHODS

Materials

Commercially available xylanase stock (Buzyme 2511) was purchased from Buckman Laboratories International, South Africa. Ammonium sulphate, DNS (dinitrosalicylic acid), BSA (bovine serum albumin), sephadexG-100, CBB R-250 (coomassie brilliant blue R-250), β-mercaptoethanol, OPTA (*o*-phthalaldehyde), L-cysteine, TNBS (2, 4, 6-trinitrobenzene sulfoic acid), DEP (diethyl pyrocarbonate, WRK (Woodward's reagent K, N-ethyl-5-phenylisoxazolium-3-sulfonic) were purchased from Sigma-Aldrich, USA. Centricon$^+$20 30kDa cut off columns were from Millipore Corporation USA, Birchwood xylan was from Roth chemicals, Germany and precast 12% SDS-PAGE gels were from Bio-Rad Laboratories. All other chemicals were of analytical grade.

Enzyme assays

Xyn activity assays were carried out by incubating the enzyme with 0.5 ml of xylan (1%) in a final volume of 1 ml and incubating the mixture at 50°C, pH 7 for 30 min. The reducing sugar released was determined by the DNS method (Baily et al., 1992). One unit of Xyn activity was defined as the amount of enzyme that released 1 µmol of xylose equivalent per minute from xylan, under assay conditions. Protein concentration was determined according to the method of Bradford (1976).

Production and purification of Xyn

A xylanase from commercially available xylanase stock (Buzyme 2511) was purified to homogeneity. All purification steps were carried out at 4°C unless otherwise stated. The broth was subjected to fractional ammonium sulphate precipitation (40 to 60%). The precipitate was dissolved in a minimum amount of 50 mM sodium phosphate buffer, pH 7 and dialyzed against 1 L of the same buffer with several changes for 24 h. The dialyzed fraction was applied to a Sephadex G-100 column (150×2 cm), previously equilibrated with 50 mM sodium phosphate buffer at pH 7. Elution was carried out by using the same buffer at a flow rate of 1 ml/min a nd 4-ml fractions were collected during a period of 640 min.

The fractions having maximum specific activity were pooled and concentrated. The purity of the enzyme was checked by SDS-PAGE (Laemmli, 1970) followed by staining with CBB R-250. To further purify and concentrate the samples, active fractions were run through Centricon$^+$20, 30 kDa cut off columns. The supernatant was then assayed to check for activity to ensure the presence of active protein. Centricon$^+$20, 30 kDa cut off columns were used according to the information provided in the manufacturers manual.

Properties of purified Xyn

To determine the optimum temperature and pH of the enzyme, the activity of Xyn (1 µM) was determined at different temperature (20, 37, 50, 60, 70, 80 and 90°C) and pH (4 to 10) values. To determine the temperature stability, the enzyme (1 µM) was incubated at different temperatures (50, 70, 80 and 90°C) for 35 min at pH 6. The aliquots were withdrawn at different time intervals and the residual enzymatic activity was then measured. To determine its pH stability, the enzyme (1 µM) was incubated in buffer of desired pH (5 to 10) for 2 h at 50°C. Aliquots were withdrawn at different time intervals and the residual enzymatic activity was measured at 50°C and pH 7 for 30 min using xylan as a substrate (0.5 ml of 1%) in a final reaction mixture volume of 1 ml.

Determination of molecular weight of Xyn

The molecular weight of the enzyme was determined by gel filtration chromatography using Sephadex G-100 previously equilibrated with 50 mM phosphate buffer pH 7. The column was calibrated using the following marker proteins: rabbit muscle phosphorylase B (97,000), bovine serum albumin (66,000 Da), ovalbumin (44,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100) and lysozyme (14,400 Da) obtained from Amersham, USA. The void volume (V_o) of the column was determined by the elution volume (V_e) of blue dextran. The presences of subunits in the protein were determined using a 12% SDS-PAGE.

Steady-state kinetic studies using birchwood xylan

Substrate hydrolysis was measured by incubating the enzyme (1 µM) with substrate (1 mg/ml). At different time intervals, 100 µl aliquots were withdrawn and the presence of product was measured as described above. To obtain Lineweaver-Burk plot at different pH values, Xyn (1 µM) was incubated at pH values of 3 to 10 and assayed with increasing concentrations of birchwood xylan (0.5 to 10 mg/ml) at 50°C for 30 min. The reciprocals of substrate hydrolysis (1/v) at each pH value were plotted against the reciprocals of the substrate concentrations. The corresponding V_{max} and K_m values were obtained for each pH value. A graph of log V_{max}/K_m against pH was plotted to obtain the p*K*a values. The approximate p*K*a values of ionizing groups were determined by fitting straight lines to plots of the log V_{max}/K_m against pH (Kulkarni et al., 2008).

Modification of Xyn with OPTA

Modification was carried out by incubating 1 µM of Xyn in 50 mM sodium phosphate buffer, pH 7 with varying concentrations of OPTA (0, 100 , 200 and 400 mM), at 25±1 °C. Fresh OPTA solution was prepared in methanol for each experiment. At different time intervals, aliquots were withdrawn from the reaction mixture and the residual activity was measured on termination of the reaction by

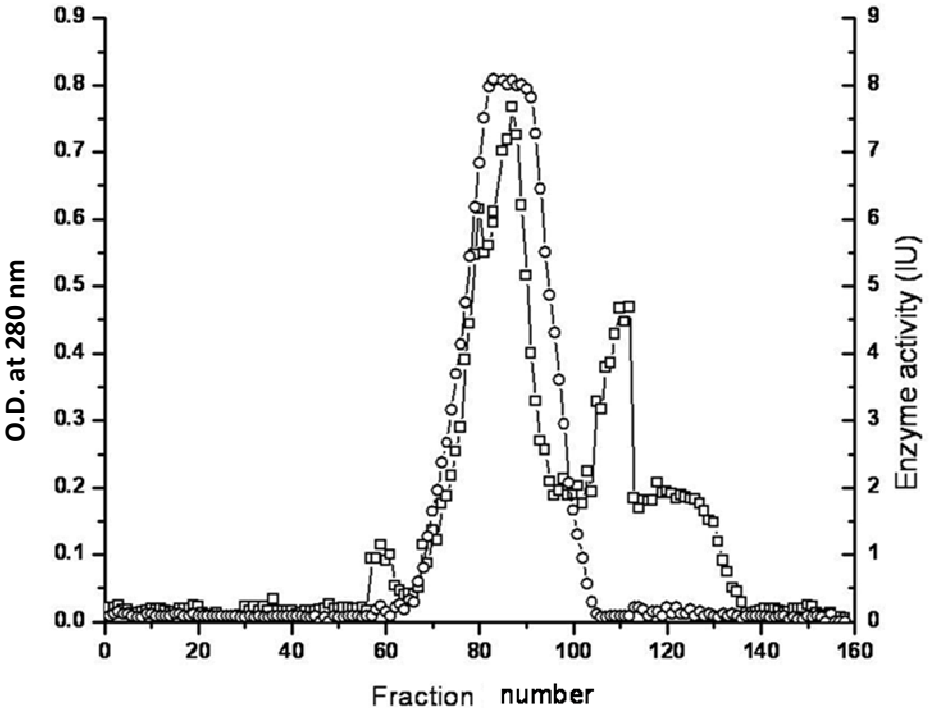

Figure 1. The Sephadex G-100 gel filtration column profile showing total protein at 280 nm (□) and xylan hydrolyzing activity (○) in the collected fractions.

adding 5 µl of 10 mM Cys. Methanol had no effect on the activity of the enzyme and was always present in concentrations of less than 2 % (v/v). The formation of Xyn-isoindole derivative was followed spectrofluorometrically by monitoring the increase in fluorescence at 410 nm with the excitation wavelength fixed at 338 nm. The modified enzyme was passed through a Biogel P-2 column to separate the isoindole derivative from the free OPTA and Cys in the reaction mixture. The isoindole content of the modified enzyme was calculated spectrophotometrically at 338 nm using a molar absorption coefficient of 7.66 $mM^{-1}cm^{-1}$ (Simons Jr. and Johnson, 1978).

Modification of Xyn with TNBS

Xyn (2 µM) was incubated with varying concentrations of TNBS (0, 100, 200 and 300 mM) in the presence of 0.25 ml 4% sodium bicarbonate at 37°C in a reaction volume of 0.5 ml (George et al., 2001b). Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adjusting the pH to 4.5. The degree of inactivation in presence of 1% xylan was also determined. Control tubes with only enzyme, TNBS (0, 100, 200 and 300 mM) and TNBS/substrate were incubated under identical conditions.

Modification with DEP

Xyn (2 µM) in 2 ml of 50 mM sodium phosphate buffer at pH 7 was incubated with varying concentrations of DEP (0, 100, 200 and 300 mM) at 25±1°C. Freshly prepared DEP in absolute ethanol was used. Samples were removed periodically at different time intervals and the reaction was arrested by the addition of 50 ml of 10 mM imidazole buffer, pH 7.5. The residual activity of the diluted enzyme derivative was determined and expressed as a percentage of the control. The DEP concentration was determined spectrophotometrically at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of $3000M^{-1}cm^{-1}$ (Melchior and Fahrney, 1970). Decarbethoxylation of the DEP modified enzyme was carried out according to Miles, 1977.

The DEP modified enzyme samples were incubated with 50 mM hydroxylamine, pH 7, at 25±1°C for 4 h and the enzyme activity was determined as described above.

Modification with WRK

Xyn (2 µM) was incubated with increasing concentrations of WRK (0, 10, 20, 30, 40 and 50 mM) in the presence of phosphate buffer (50 mM) pH 7 at 37°C for 0 to 60 min. The aliquots were withdrawn at specific time intervals and the residual activity of the diluted enzyme derivative was determined. The Xyn-WRK adduct was separated from the free enzyme by gel filtration on Sephadex G-25.

RESULTS

Purification of Xyn

The Xyn from Buzyme 2511 was purified to homogeneity by a 15.18 fold purification factor and a specific activity of 3267 IU/mg of total protein. Sephadex G-100 gel filtration column fractions 55 to 130 showed the presence of protein while fractions 70 to 99 showed Xyn hydrolyzing activity (Figure 1). The purity of the enzyme was

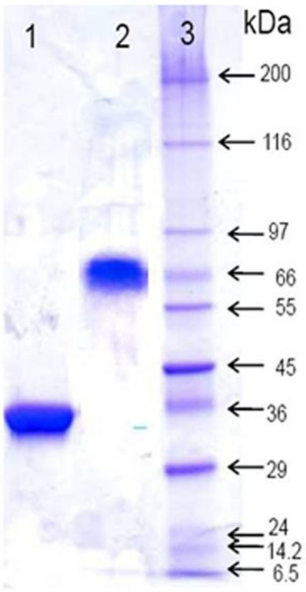

Figure 2. SDS - PAGE of Xyn. Lane (1), purified Xyn by sephadex G-100 column treated with DTT for 3 min in boiling water bath, (2) enzyme without DTT treatment and (3) molecular weight markers.

analyzed by SDS-PAGE. The difference in molecular weight of the purified Xyn as determined by by SDS–PAGE (33 kDa) (Figure 2) and gel filtration (66 kDa) (Figure 3) suggests the presence of two subunits in this protein. In the presence of DTT and boiling conditions, the disulphide bonds present in the enzyme are broken down and SDS-PAGE shows a band of 33kDa proving the presence of two equal subunits (Figure 2).

Characterization of Xyn

The temperature optimum of the purified Xyn was 50°C (Figure 4a) and retained 52% relative activity at 37°C and 10% relative activity at 90°C. The enzyme was thermostable, retaining 95% activity at 50°C and 90% activity at 70°C for more than 35 min (Figure 4b). The Xyn was active in the range of pH 5 to 9, with optimum activity at pH 6.0 (Figure 5a). It was stable in a broad range of 5 to 10 with more than 60% residual activity after incubation at pH 5 for 2 h and about 20% residual activity after incubation at pH 10 for 2 h (Figure 5b).

Study state kinetics and pH dependence of the enzyme activity

The substrate progress curve analysis showed that V_{max} for the enzyme substrate reaction is attained within 30 min at pH 7 and at 50°C (Figure 6). The Lineweaver-Burk plot at pH 7 showed a K_m value of 1mg/ml and a V_{max} of 33 µM/min^{-1} (Figure 7a). The plot of initial velocity and substrate concentration was hyperbolic at each pH investigated and all Lineweaver-Burk plots were linear. The plots indicate the dependence of Xyn activity on the ionization of at least four groups. The ionizable group essential for the function of Xyn activity was determined through the effects of pH on V_{max}/K_m values and the plots of V_{max}/K_m against pH are shown in Figure 7b. The log V_{max}/K_m versus pH plot shows pK values of 4.1, 6.0, 8.3 and 10.4 for the enzyme substrate complex. The values obtained from the acidic limb of the curves are consistent with the participation of a carboxylate group of Glu and Asp residue in the Xyn. However, the basic limb of the rate profile may reflect the ionization of a Lys residue since Lys groups in proteins have a pK value of 10.54 (Raetz and Auld, 1972).

The pK values of 6.0 and 8.3 represents ionization of His and Cys residues. The slopes of the acidic and basic limb of the curves are less than unity, indicating that the simple model of the pH dependence of enzyme action does not describe adequately the Xyn enzyme system. The divergence from unit slopes may arise from electrostatic perturbation of ionization constants, multiple intermediates or conformational effects. This may indicate the interaction of ionizable groups on the enzyme suggesting a complex mechanistic pathway for the Xyn system (Wakim et al., 1969). The nature of the essential residues for the function of Xyn activity was resolved through specific chemical modification of the enzyme.

Modification of Xyn by OPTA: Role of Cys and Lys residue in the active site

Fluorescence excitation and emission spectroscopic data showed that an isoindole derivative was formed following the reaction between Xyn and OPTA. The modification of Xyn by OPTA resulted in concomitant increase in fluorescence at 410 nm (excitation wavelength, 338 nm), which is characteristic for the formation of an isoindole derivative (Figure 8a). Therefore, this indicates that the SH and NH_2 groups of Xyn involved in the reaction with OPTA are situated at the active site of Xyn. OPTA forms a fluorescent isoindole derivative by cross-linking the proximal thiol and amino groups of the protein. Upon complete inactivation, an average of one isoindole derivative per molecule of enzyme was found, based on the increased absorbance at 410 nm. As shown in Figure 8b the increase in the amount of isoindole derivative formed correlated well with the decrease in enzyme activity, suggesting that OPTA causes modification of the Xyn by the formation of a single isoindole derivative. OPTA has been used as a fluorescent probe and a modifier of Cys and Lys residues (Levi et al., 1963;

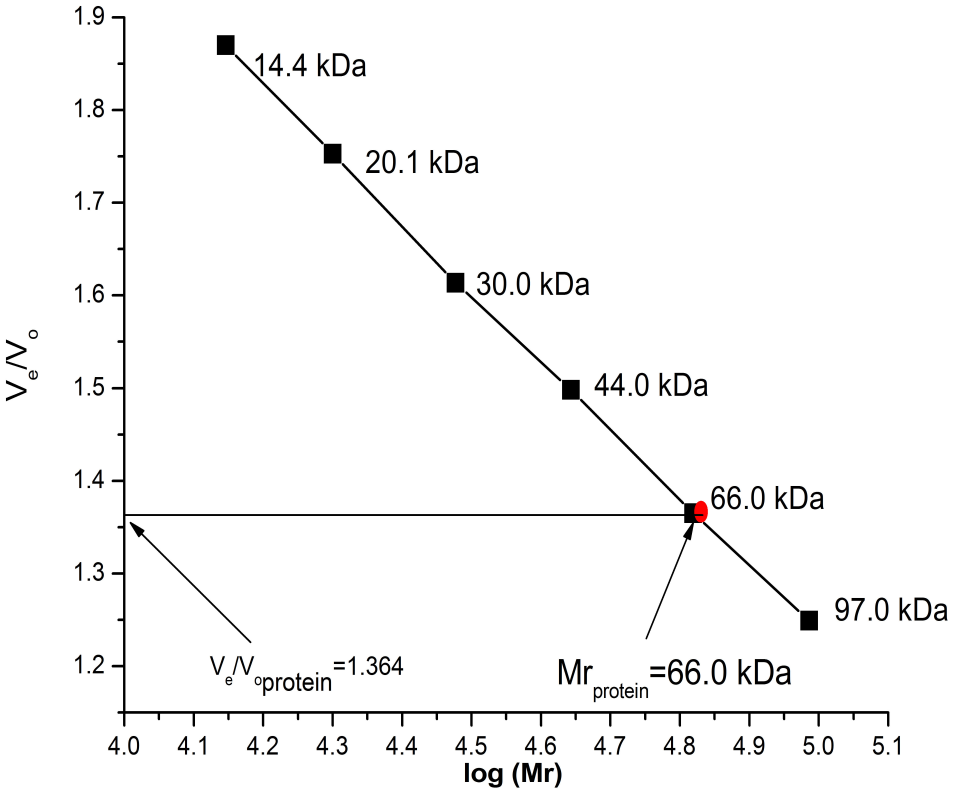

Figure 3. Gel filtration chromatography by Sephadex G 100: rabbit muscle phosphorylase B (Mr 97,000); bovine serum albumin (Mr 66,000); ovalbumin (Mr 44,000); carbonic anhydrase (Mr 30,000); soybean trypsin inhibitor (Mr 20,100); lysozyme (Mr 14,400) Red circle indicate the V_e/V_o and Mr of purified enzyme.

Simons and Johnson, 1978; Palczewski et al., 1983). The incubation of Xyn with increasing concentrations of OPTA (0 to 400 mM) resulted in a time dependent decrease in enzyme activity.

The natural logarithm of percent activity remaining was plotted against time to obtain linear pseudo first order plots (Figure 8b). When the pseudo first order rate constants obtained at each concentration were re-plotted against OPTA concentration, a linear relationship was observed. Analysis of the order of inactivation with respect to OPTA concentration using methods described by Levy et al. (1963) yielded a slope of 1.09, indicating that one molecule of OPTA binds to one molecule of enzyme at the active site (Figure 8b). Modification of Lys by TNBS and Cys residue by DTNB of the enzyme abolished the ability of the enzyme to form an isoindole derivative with OPTA supporting the above assumption.

Modification of Lys residue by TNBS: Role of Lys residue in the active site

Incubation of the Xyn (2 µM) with different concentrations of TNBS resulted in a time and concentration dependent loss of enzyme activity as shown in Figure 9. The reaction followed pseudo first order kinetics. The pseudo first order rate constants (k) were linearly related to the concentrations of the reagent, suggesting that no reversible complex was formed during the inactivation process. Furthermore a reaction order of 2.2 with respect to the modifier was determined from the slope of the double logarithmic plots (Figure 9), indicating that 2 mol of TNBS inactivated 1 mol of enzyme. Hence the TNBS induced inactivation of Xyn is a result of direct chemical modification of two essential Lys residues.

There was no change in the k_{cat} values of native and modified enzyme, while the K_m values were 1 and 6 mg/ml respectively. The comparable k_{cat} values suggested that Lys residues were not present in the catalytic site, while the significant increase in the K_m indicated the presence of Lys at the substrate-binding site. The modification of the Lys residue present in the substrate binding site results in a decrease in the efficiency of binding of the substrate to the substrate binding site and results in an increase of K_m. The complete protection of the Xyn against inactivation by TNBS and OPTA, by the substrate, confirms the presence of Lys at the substrate-binding site.

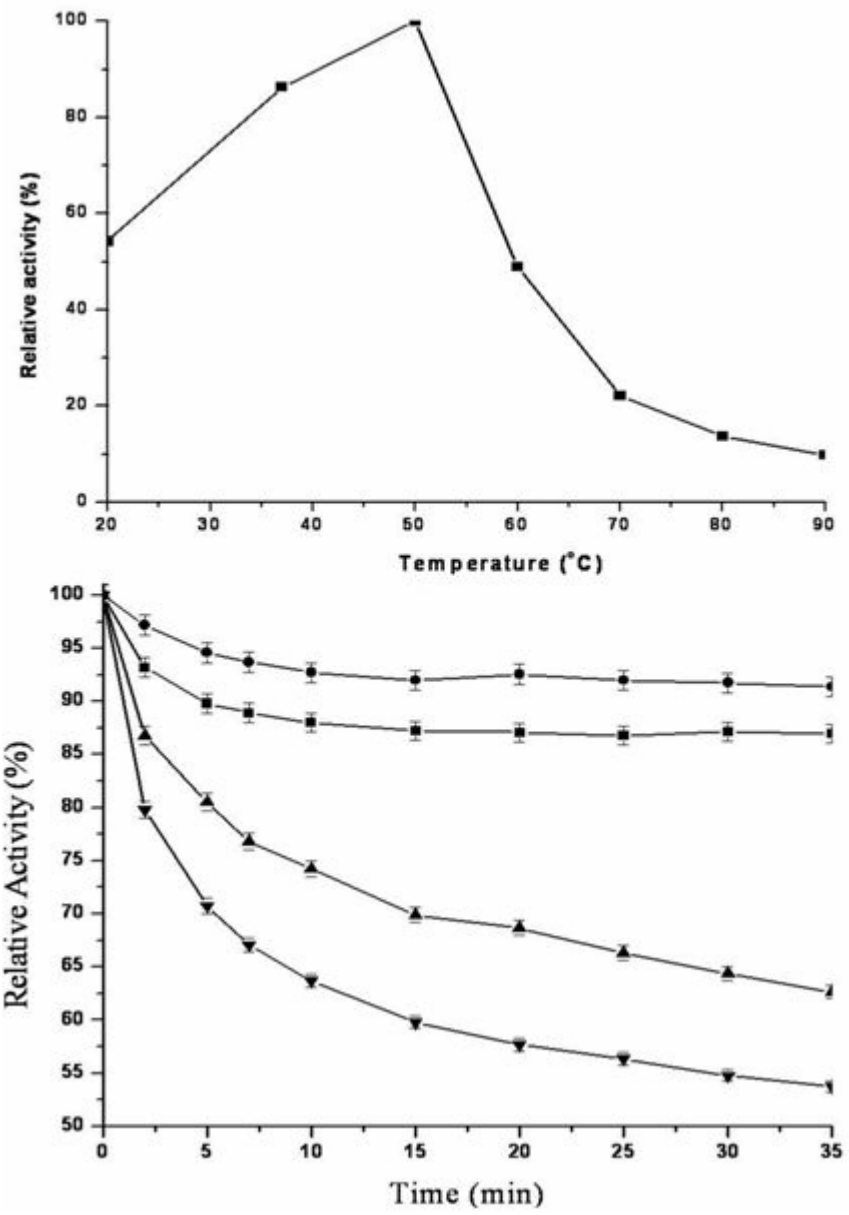

Figure 4. (A) Optimum temperature of Xyn. (B) Stability of Xyn at 50°C (●), 70°C (■), 80°C (▲) and 90°C (▼) for 35 min at pH 6.

Modification of Xyn with DEP: Role of His in the active site

Reaction of the Xyn with DEP resulted in progressive loss in activity. The inactivation was dependent on the concentration of DEP. The pseudo first order rate constants for different concentrations of DEP (0, 100, 200 and 300 mM) were calculated and the slope of the plots of log K against log (DEP) produced a value of 1.15 (Figure 10). These results showed that the loss in activity was due to the modification of a single His residue. DEP is specific for His at neutral pH, but it also reacts to a lesser extent with tyrosine and Lys (Miles, 1977). The modification of tyrosine residues by DEP was excluded, as there was no decrease in the absorbance of the modified protein at 278 nm. Moreover, there was no loss in activity on treating the purified enzyme with N-acetylimidazole, which is a tyrosine specific reagent. The recovery of total activity in the presence of hydroxylamine further proved the involvement of DEP in His modification and negated the modification of Lys by DEP.

The kinetic parameters for the Michaelis-Menten constant (K_m) and turnover number (k_{cat}) were obtained from the Lineweaver-Burk plots for native and DEP

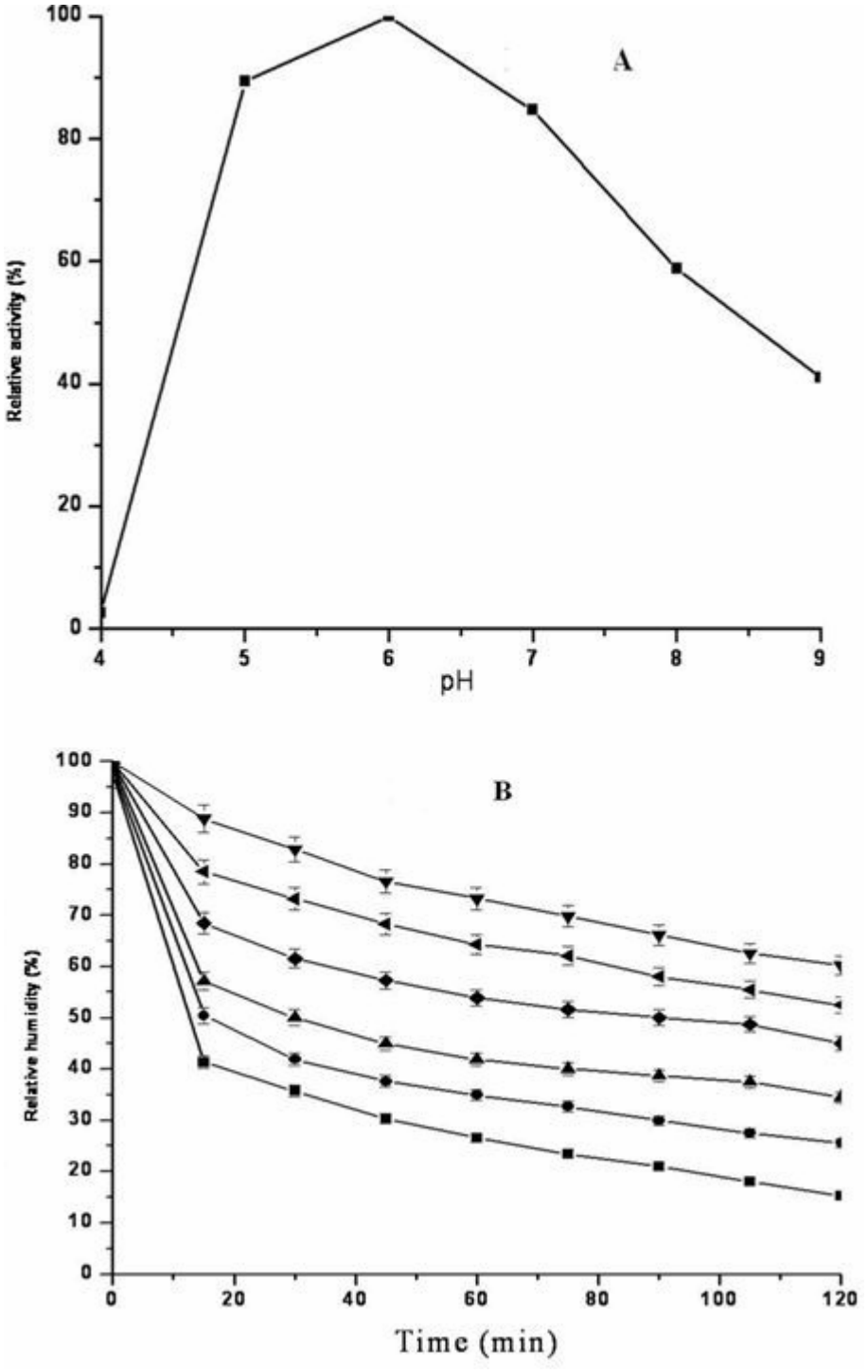

Figure 5. (A) Optimum pH of Xyn. (B) Stability of Xyn at pH 5 (◄), pH 6 (▼), pH 7 (♦), pH 8 (▲), pH 9 (●) and pH 10 (■) for 2 h and 10.4 for the active ionizable groups at the active site of the enzyme corresponding to Glu, His, Cys and Lys residues, respectively.

modified Xyn. There was no change in the K_m values of modified and native enzyme, while the k_{cat} values were 6530 and 23420 min^{-1} respectively. The similar K_m values and the significant decrease in the k_{cat} suggested that His was not present in the substrate binding site and was present at or near the catalytic site of the Xyn. Xylan did not provide any protection to the Xyn against inactivation by DEP, which may be because xylan binds only at the

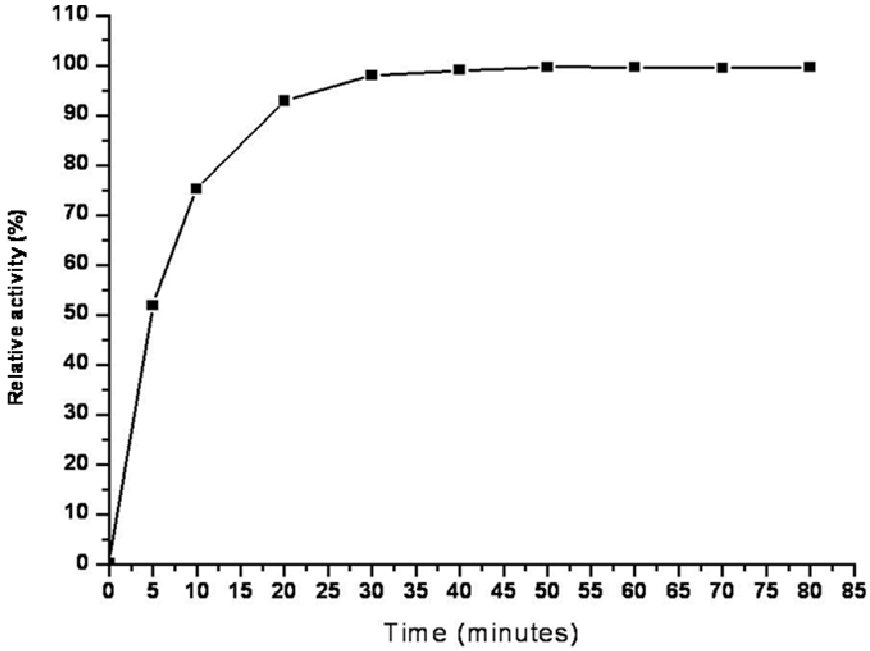

Figure 6. % relative activity of purified Xyn on substrate over the time.

substrate binding site, therefore making the catalytic site susceptible to attack by DEP. The His residue in the active site may be involved in a network of hydrogen bonds, which are responsible for maintaining the ionization state of the two catalytic residues responsible for the hydrolysis of the β-1,4 glycosidic bonds.

Inhibition of Xyn with WRK

WRK has been generally used as a chemical modifier of enzyme carboxyl groups. Incubation of the Xyn (2 µM) with different concentrations of WRK (0 to 50 mM) resulted in a time and concentration dependent loss of enzyme activity as shown in Figure 11. The reaction followed pseudo first order kinetics. The pseudo first order rate constants (*k*) were linearly related to the concentrations of the reagent, suggesting that no reversible complex was formed during the inactivation process. Furthermore a reaction order of 1.18 with respect to the modifier was determined from the slope of the double logarithmic plots (Figure 11), indicating that 1 mol of WRK inactivated 1 mol of enzyme. Hence, the WRK induced inactivation of Xyn is a result of direct chemical modification of essential Asp or Glu residue.

DISCUSSION

In this paper, the purification of Xyn from a commercially available xylanase stock, Buzyme 2511 was described. A plethora of xylanases have been reported which contain carboxyl and tryptophan residues in the active site and there are also a few reports of Lys and His. To the best of our knowledge there are few reports of the presence of Lys in the active site of a xylanase from *P. fluorescens* and Thermomonospora sp. (Harris et al., 1996; Charnock et al., 1997; Nath and Rao, 1998). Our studies suggest the presence of essential Lys, His and Asp or Glu residues in the active site of the studied Xyn by fluorescent chemo-affinity labeling and group specific modification. The xylanase purified in this study was more thermostable when compared to most of the other reported thermostable xylanases from wild bacterial strains. It was more stable than the xylanase isolated from Thermotoga sp. strain FjSS-B.1, which had a half life of 8 min at 100°C (Simpson et al., 1991). However, a recombinant xylanase whose gene has been isolated from *Thermotoga neopalitina* and expressed in *E. coli* had a half-life of 30 min at 100°C (Velikodvorskaya et al., 1997).

The xylanase isolated from *Thermus thermophilus* was also highly thermostable with 61% residual activity after incubation at 90°C for 48 h (Lyon et al., 2000). Many authors have reported several xylanases from bacterial and fungal sources which can be used in paper and pulp industry. *Bacillus* SSP-34 was reported to produce a xylanase with activity of 506 IU/ml (Subramaniyan, 2000). A *Bacillus* sp. strain NCL 87-6-10 produced 93 U/ml of xylanase in a zeolite induced medium (Balakrishnan et al., 2000). Streptomyces sp. QG-11-3 was also found to produce a xylanase with an activity of 96 U/ml (Beg et al.,

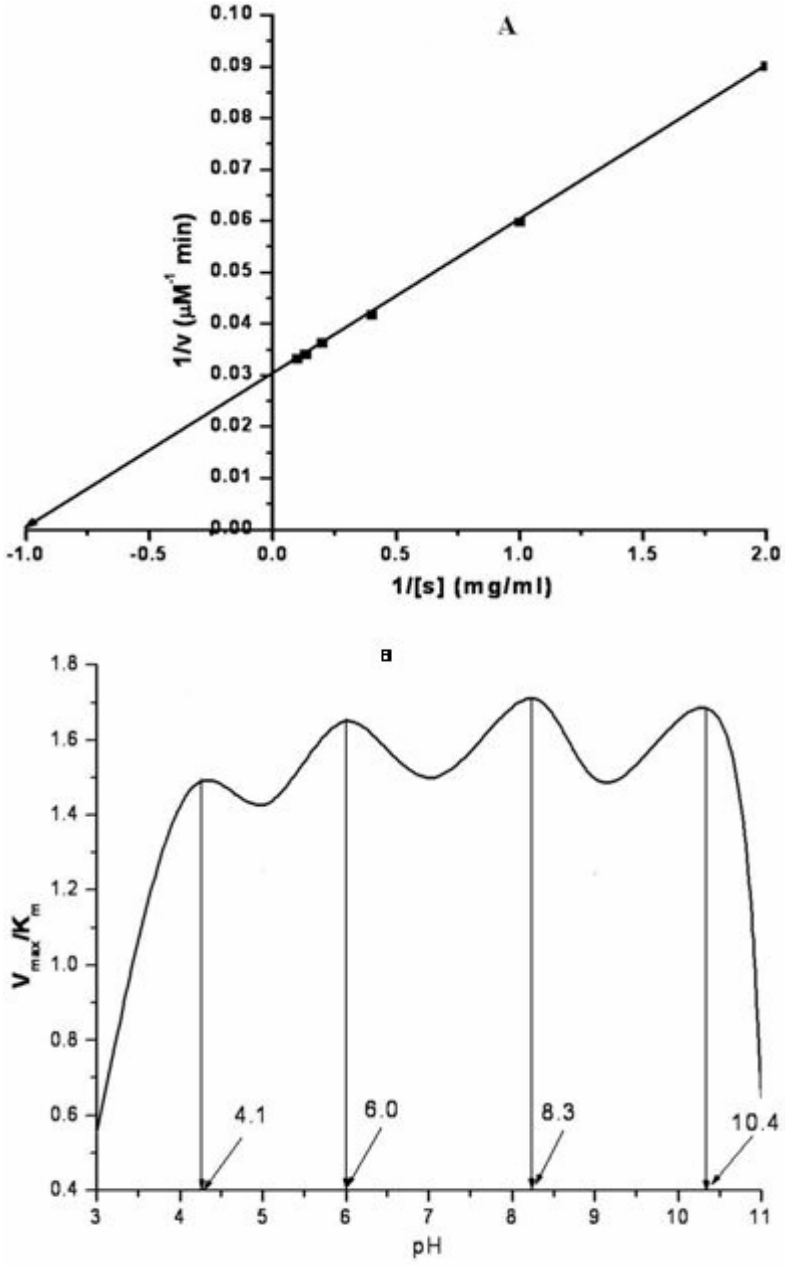

Figure 7. (A) Lineweaver-Burk plot at pH 7 to determine the K_m and V_{max} value of Xyn- (B) Identification of p*K*a values of ionizable side chains of active site amino acids. The secondary plot of the log V_{max}/K_m versus the pH provided p*K*a values of 4.1, 6.0, 8.3.

2000). *Rhodothermus marinus* was found to be producing thermostable xylanases of approximately 1.8 to 4.03 IU/ml (Dahlberg et al., 1993; Hreggvidsson et al., 1996). *Caldocellum saccharolyticum* possesses xylanases with optimum activities at pH values 5.5 to 6.0 and at a temperature of 70°C (Luthi et al., 1990). Xylanases from *Dictyoglomus* sp. showed optimum activities at pH 5.5 and 90°C, however, merits the significant pH stability at pH values 5.5 to 9.0. Gomes et al. (1992) reported xylanase activity (188.1 U/ml-optimum pH 5.2) from *Trichoderma viride*. Similar to *T. viride*, *T. reesei* was also known to produce higher xylanase activity – approximately 960 IU/ml (Bailey et al., 1993). Singh et al. (2000) reported a xylanase activity of 59,600 nkat/ml (approximately 3576 U/ml) from a *Thermomyces lanuginosus* strain. Column chromatographic techniques, mainly ion exchange and size exclusion are the generally utilized schemes for xylanase purification (Wong and Saddler,

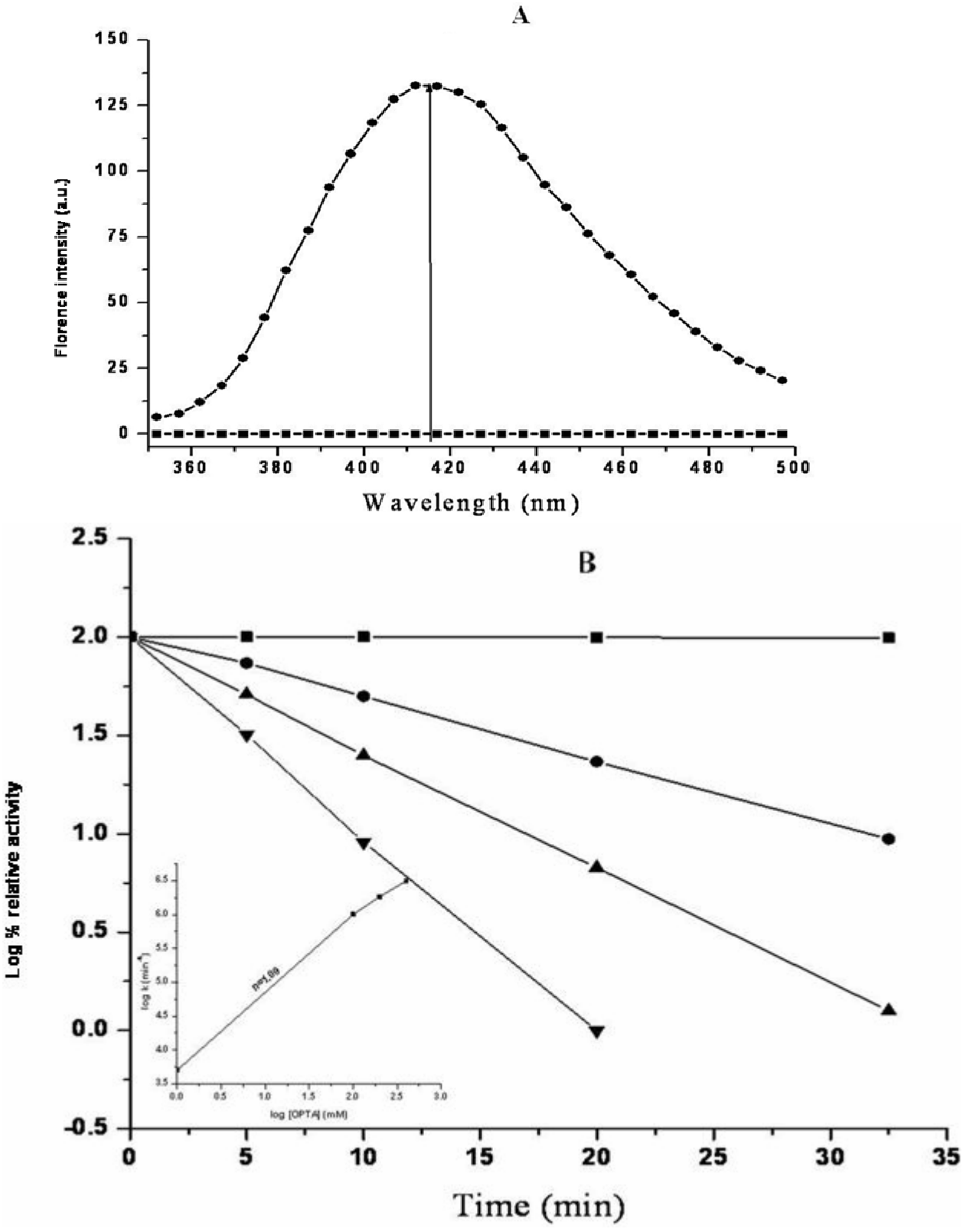

Figure 8. (A) Modification of Xyn with OPTA (fluorescence measured at 415 nm. excitation wavelength= 338 nm) for native Xyn (■) and for Xyn incubated with 400 mM OPTA (●) for 32 min. (B) Xyn activity after incubation with 0 (■),100 mM (●), 200 mM (▲) and 400 mM (▼) OPTA . Inset: Double logarithmic plots of pseudo first order rate constants as a function of OPTA concentration.

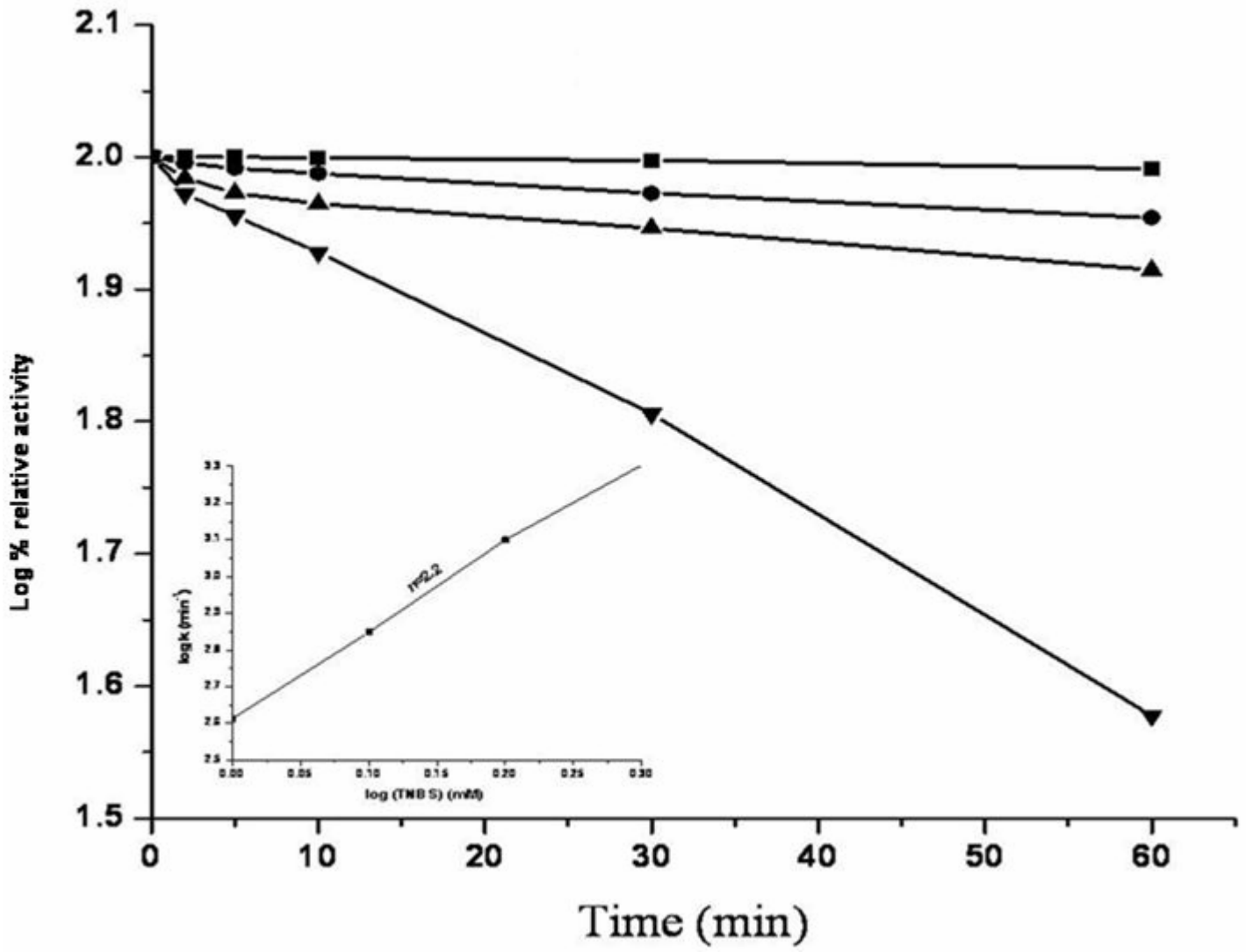

Figure 9. Modification of Xyn by TNBS: Xyn activity after incubation with 0 (■), 100 mM (●), 200 mM (▲) and 300 mM (▼) TNBS. Inset: Double logarithmic plots of pseudo first order rate constants as a function of TNBS concentration.

1992).

There are several reports regarding the purification of xylanases to electrophoretic homogeneity, however, the yield and purification fold varies in different cases. In all the cases the culture supernatants are initially concentrated using precipitation or ultrafiltration techniques. A moderately thermostable xylanase was purified from *Bacillus* sp. Strain SPS-0 using ion exchange, gel and affinity chromatographies (Bataillon et al., 2000). Thermostable xylanases from thermophilic organisms like Dictyoglomus and Thermotoga spp which grow at a temperature higher than 80°C could be easily purified by the inclusion of one additional step of heating (Bergquist et al., 2001). Takahashi et al. (2000) purified a low molecular weight xylanase (23 kDa) from *Bacillus* sp. strain TAR-1 using CM Toyopearl 650 M column. This xylanase with optimum activity at 70°C had broad pH profile. Kimura et al. (2000) purified *Penicillium* sp. (Beg et al., 2000) xylanase with molecular weight 25 kDa which was induced by xylan and repressed by glucose. A dimeric xylanase also reported from *Thermotoga thermarum* with a Mr of 266 kDa with subunits of 105 kDa. Thorough information about the xylanase can be obtained from a review by Subramaniyan and Prema (2002). The three dimensional structure of family 10 xylanases have revealed several highly conserved residues that are on the surface of the active site. Charnock et al. (1997), have suggested that Lys-47 plays an important role in positioning the substrate into the active site of xylanase A from *Pseudomonas fluorescens*.

The structural analysis of the active site of family 10 xylanases from *Cellulomonas fimi*, *P. fluorescens* and *Penicillium simplicissimum* have shown the presence of a hydrogen bonding network involving His residues in the

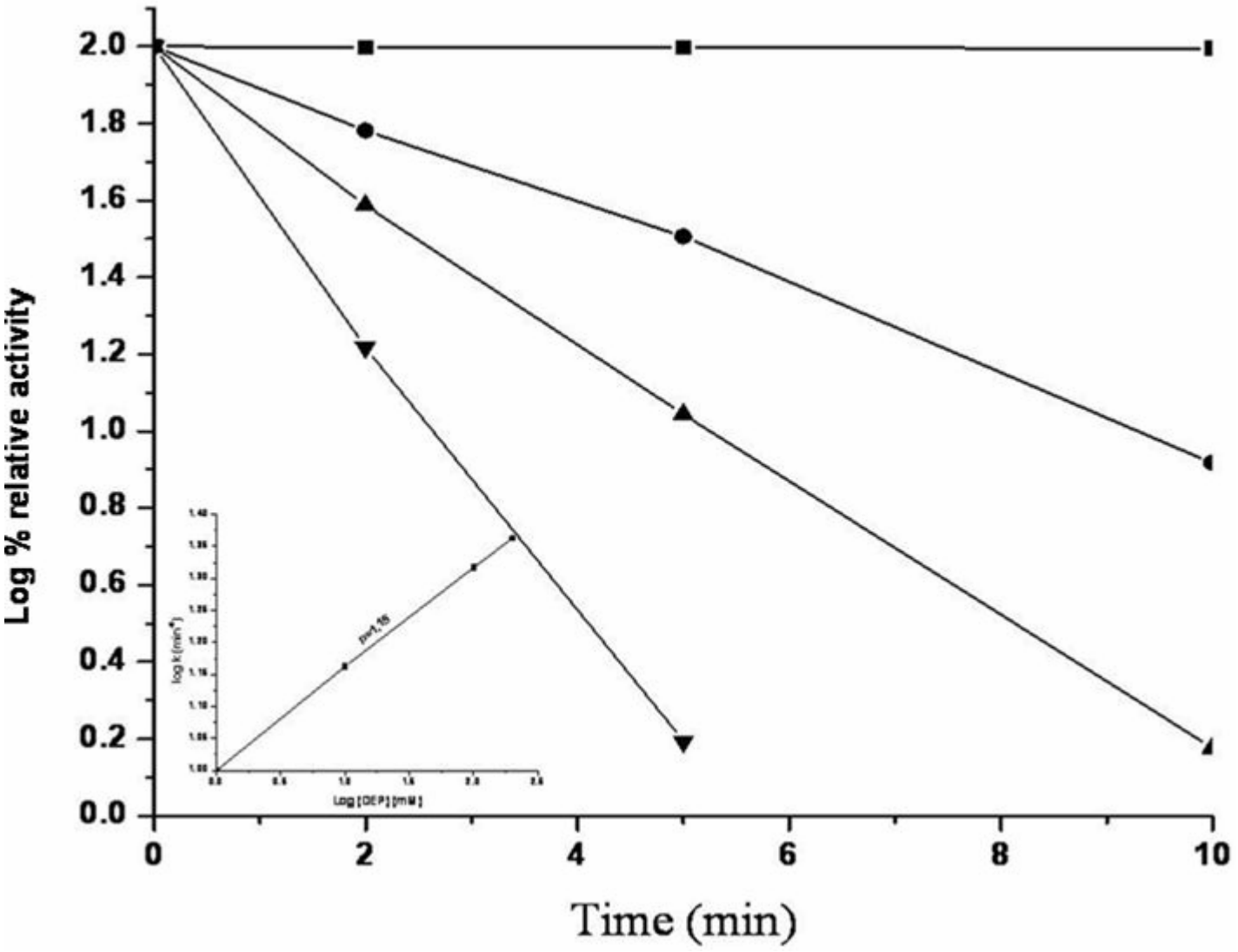

Figure 10. Modification of Xyn by DEP: Xyn activity after incubation with 0 (■), 100 mM (●), 200 mM (▲) and 300 mM (▼) DEP. Inset: Double logarithmic plots of pseudo first order rate constants as a function of DEP concentration.

active site (White et al., 1994; Harris et al., 1996; Schmidt et al., 1998). White et al. (1994) reported that the trios of residues Glu233-His205-Asp235 are highly conserved within family 10. Roberge et al. (1997), have undertaken the site directed mutagenesis for xylanase A derived from *Streptomyces lividans*. They have proved that His81 and His207 are very important in the hydrogen bonding network of the active site. Mutation of these residues modifies the interaction necessary to maintain the ionization state of the two catalytic Glu residues of xylanaseA. His residue present in the active site of the Xyn may play a role similar to that played by the conserved His residues in other family 10 xylanases. The insight into the active site of the enzyme will provide the information which will be helpful in enhancing its catalytic activity.

In conclusion, the enzyme showed its thermostable nature by exhibiting 90% residual activity at 70°C for more than 35 min. Cys, His, Lys and Glu residues were found to be important for the catalytic activity of the enzyme. After characterization, this enzyme can be used effectively in the pulp and paper industry. The N- and C-terminal sequencing of the enzyme are worth investigating and accordingly primers can be designed for the purpose of cloning and hence, higher production of the enzyme can be achieved.

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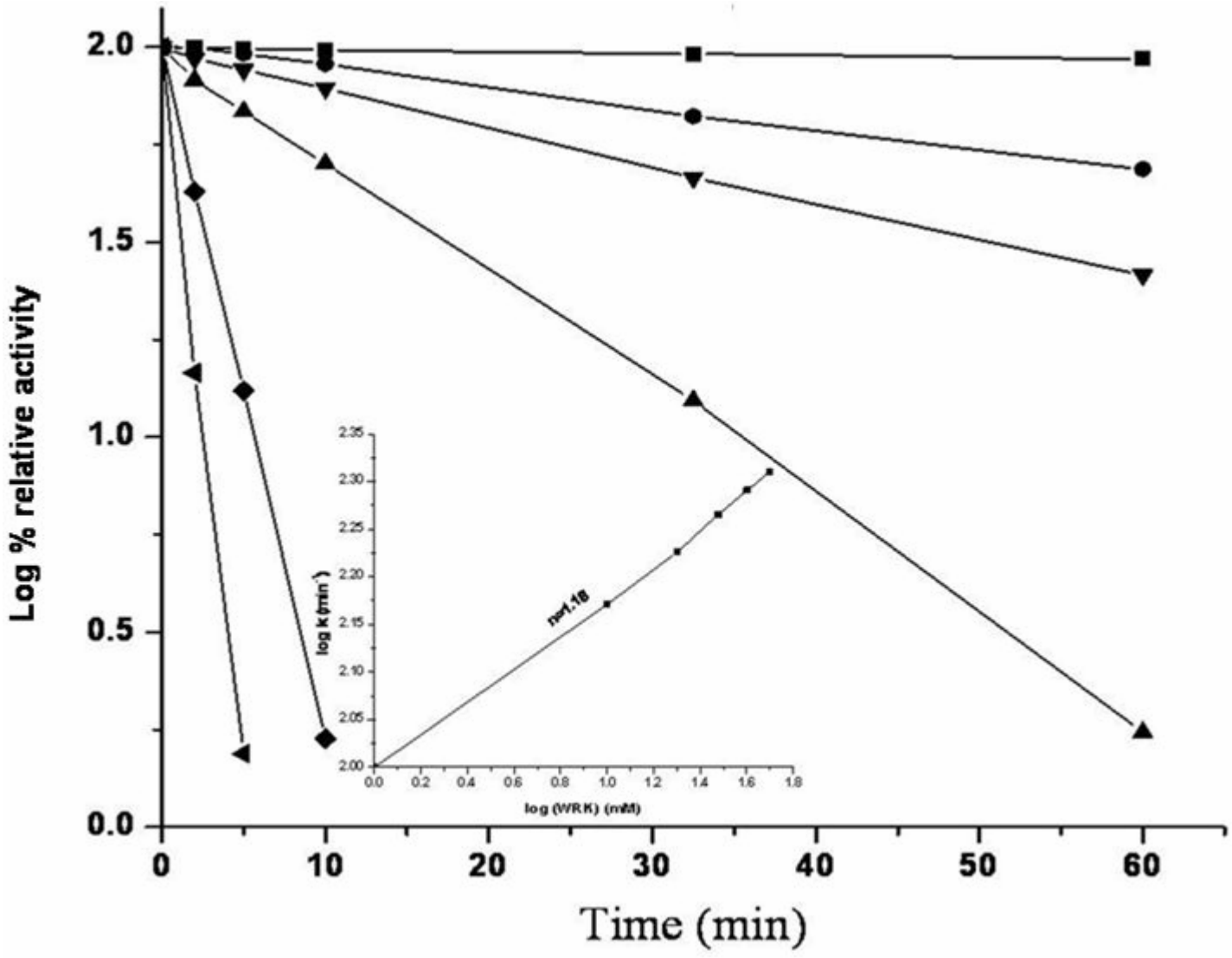

Figure 11. Modification of Xyn by WRK: Xyn activity after incubation with 0 (■), 10 mM (●), 20 mM (▼), 30 mM (▲), 40 mM (♦) and 50 mM (◄) WRK. Inset: Double logarithmic plots of pseudo first order rate constants as a function of WRK concentration.

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