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Comparison of the Kinetic Properties of Crude and Purified Xylanase from *Bacillus pumilus* with Commercial Xylanase from *Aspergillus niger*

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Xylanases showing better stability at alkaline pH and higher temperatures have potential applications in several industrial processes. The objective of this study is to compare the kinetic properties and stability of crude and purified xylanase from *Bacillus pumilus* with commercial xylanase from *Aspergillus niger*. *Bacillus pumilus* which can grow and produce xylanase above 40^{o}C and pH 9.0 was selected for this study. Crude, purified and commercial xylanases showed zero order kinetics for 10 minutes and highest activity was obtained at 60^{o}C and pH 9.0. The optimum pH for crude, purified and commercial xylanases were 9.0, 9.0 and 7.0 respectively at 60^{o}C and the Michaelis constant by Lineweaver-Burk Plot for xylan were 3.1, 2.3 and 0.03gL^{-1} under the respective optimized conditions. The half-life of the crude and purified xylanase was highest at pH 9.0 and 50^{o}C while that for the commercial enzyme was at pH 7.0 and at 60^{o}C.

Key words- Xylanase, Kinetic properties, Michaelis constant, Vmax, Stability, Half life

Introduction

Xylan is the second most abundant renewable polysaccharide in nature [1]. Xylan is present in appreciable amounts in pulp and in agricultural residues. Xylanases are used to convert the xylan to xylose in the paper-pulp industry [2], to treat the agricultural wastes and recently to improve the bread quality [3]. The *B. pumilus* strains reported so far, have produced xylanases showing optimal activities at pH 8.0 & 65^{o}C [4], pH 6.5 & 40^{o}C [5], pH 9.0 & 60^{o}C [6] and pH 6.5 & 50^{o}C [7]. In this report the kinetic properties of the crude and purified xylanase produced by *Bacillus pumilus,* (which was isolated and identified in our Laboratory) was compared with one of the commercially available xylanase from *Aspergillus niger*. If this enzyme is similar or superior to other enzymes reported, it could be recommended for the industries.

Materials and Methods

Materials

Commercial xylanase from *Aspergillus niger* was purchased from Sigma Chemical Company, USA.

Microorganism

The *Bacillus pumilus* which was isolated, identified and characterized in the Biochemistry laboratory, Faculty of Medicine was used [8].

Media

The activation medium contained (gL^{-1}) xylan 20.0 and nutrient broth 25.0. Fermentation medium contained (gL^{-1}) xylan, 20.0; peptone, 20.0; yeast extract, 2.5; $CaCl_2.2H_2O$, 0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4,

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2.5; KH_2PO_4, 1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0.

Production of crude xylanase and measurement of xylanase activity

Fermentation medium, inoculated with 16h old *Bacillus pumilus* (20%, v/v) was incubated at 40°C in at 100rpm for 42h. At the end of incubation at 42h the spent medium was centrifuged and the supernatant was used as a xylanase source. Commercial xylanase showing the optimum activity of 120mgmL^{-1} at pH 7.0 and 60°C was used. Xylanase purified in this laboratory was used [9].

Analytical methods

Xylanase activity was measured by standard methods [10]. One unit of xylanase activity is defined as the amount of enzyme that produces one µmol of reducing sugar in one minute at pH 9.0 and 60°C with 20 gL^{-1} xylan.

Kinetic studies

Activities of crude, purified and commercial xylanases were determined as a function of time with soluble xylan (20gL^{-1}, pH 9.0) at 60°C. The effect of temperature on xylanase activity was determined by incubating the appropriately diluted enzyme (0.01M Tris buffer at pH 9.0) with 20gL^{-1} soluble xylan at pH 9.0. The enzyme and substrate samples at pH 9.0 were preincubated at the respective temperatures considered for the studies for 5 minutes and then mixed to determine the activities. The effect of pH on xylanase activity was measured with 20gL^{-1} soluble xylan. The effect of substrate concentration on the activities of the three different xylanase preparations was studied by incubating with different concentrations (0.25 to 40gL^{-1}) of xylanin 0.01M Tris buffer and xylose produced was measured [10]. The Michaelis constant was calculated for all three preparations using Lineweaver Burk plot.

Stability studies

Effect of pH on the stability of the enzyme preparations at different pH values ranging from 8.0 to 10.0 was studied at 60°C and the residual activity was determined with 20 gL^{-1} xylan under optimized conditions.

Stability of the xylanase preparations was measured at the temperatures ranging from 50 to 70°C, at the optimum pH values of the crude purified and commercial enzymes.

Results

Kinetic properties

The influence of incubation time on the production of xylose from the reaction of xylanase enzyme with xylan (20gL^{-1}) was studied for 4h at pH 7.0 and at 60°C. All three-

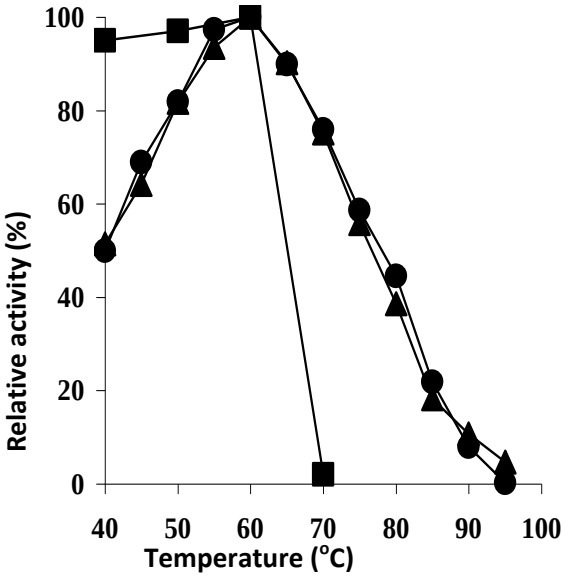

Figure 1: Effect of temperature on the activity of (●), crude; (▲), purified and (■), commercial xylanase with commercial xylan (20gL^{-1}) -0.01M Tris buffer at pH 9.0.

enzyme preparations showed a linear relationship between the time and product formation up to 10 minutes. Hence, it was decided to fix the reaction time for 10min. All three enzymes preparations showed maximum activity at 60°C (Figure 1). Hence 60°C was chosen as the optimum temperature for the assay of all three xylanase preparations.

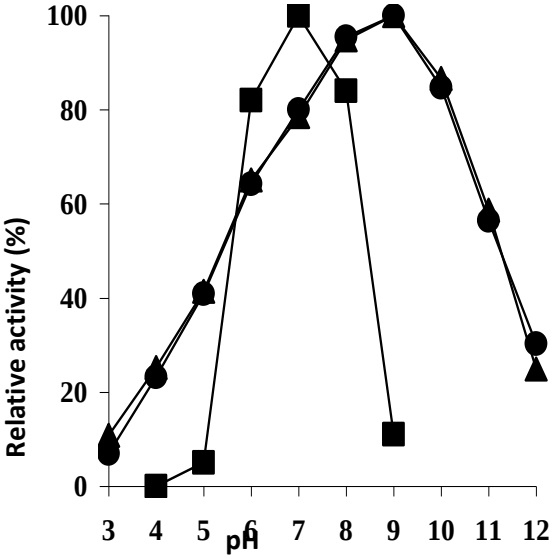

Figure 2: Effect of pH on (●), crude; (▲), purified and (■), commercial xylanase activities at 60°C with xylan (20gL^{-1}).

Citrate-phosphate (pH 3.0, 4.0, 5.0 and 6.0), sodium phosphate (pH 7.0), Tris (pH 8.0), Glycine-NaOH buffer (pH 9.0) and carbonate-bicarbonate (pH 10.0, 11.0 and 12.0) buffers were used. The pH value of the reaction mixtures were measured and presented in the figure.

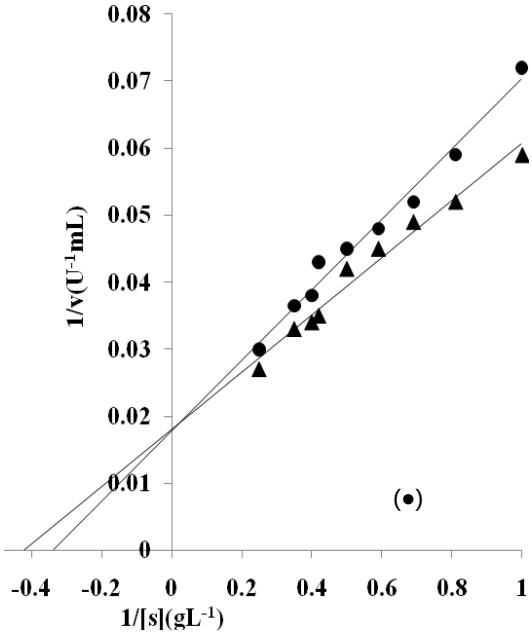

Figure 3a: Lineweaver-Burk plot of (●), crude; and (▲), purified xylanase activity at pH 9.0 and 60°C with different concentrations of xylan.

The influence of pH on crude, purified and commercial xylanase activities was studied (Figure 2). The optimum pH for the activities of crude, purified and commercial xylanases were pH 9.0, 9.0 and 7.0 respectively.

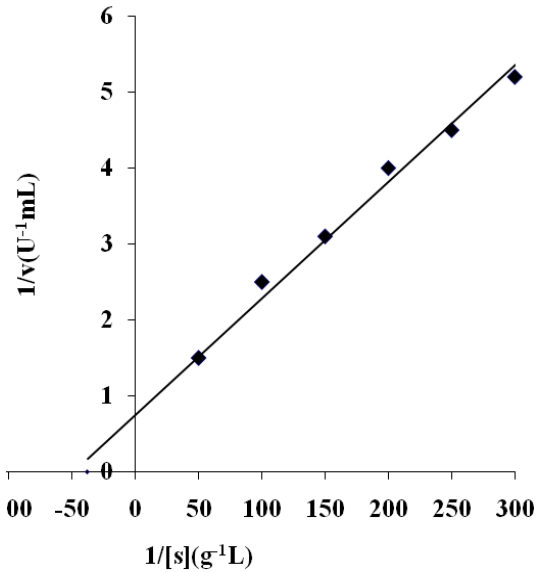

Figure 3b: Lineweaver-Burk plot of commercial xylanase activity atpH 7.0 and 60°C with different concentrations of xylan.

The Michaelis constants of crude and purified xylanases from *Bacillus pumilus* were 3.12 and 2.34gL^{-1} at pH 9.0 and 60°C (Figure 3a) while that of the commercial xylanase was 0.03gL^{-1} at pH 7.0 and 60°C(Figure 3b).

Stability of xylanase

At 60°C, crude xylanase retained 20 and 1.6% of its initial activity at 20 and 30 min respectively (Figure 4a). At 50°C the crude

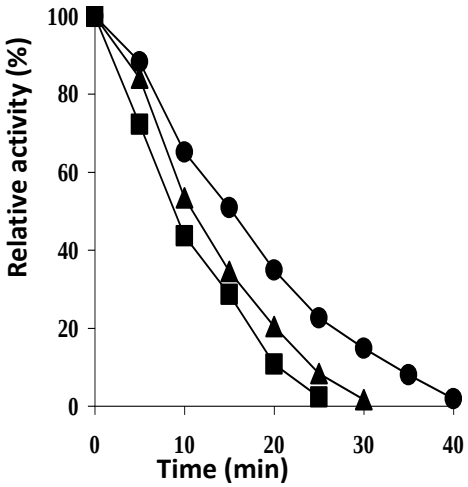

Figure 4a: Stability of crude xylanase at different temperatures of (●), 50; (▲), 60 and (■), 70 at pH 60°C with 20gL^{-1}xylan (0.01M Trisbuffer) at pH 9.0.

enzyme retained 14.7 and 1.89% of the initial activity at 30 and 40min respectively (Figure 4a). At 70°C, the crude enzyme retained 2.28

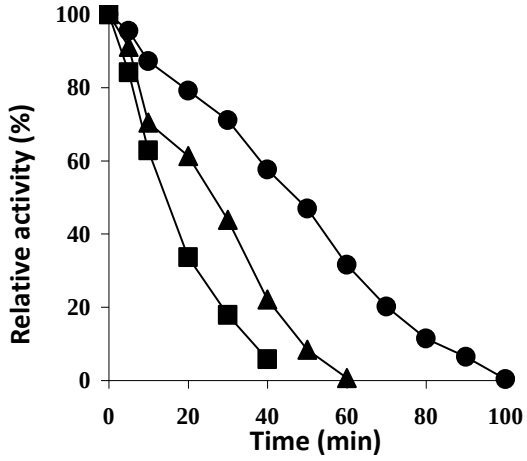

Figure 4b: Stability of purified xylanase at different temperatures of (●), 50; (▲), 60 and (■), 70 at pH 60°C with 20gL^{-1}xylan (0.01M Tris buffer) at pH 9.0

and 72.27% of the xylanase at 25 and 5min respectively. As the optimum temperature for crude and purified xylanase of *Bacillus pumilus* was 60°C, the pH stabilities of the enzymes were determined at this temperature (Figure 4a and 4b). At pH 8.0 the crude enzyme completely lost its activity at 30min. It retained 44.7% of the original activity at pH 10 and 60°C for 10min. At pH values 8.0 and 9.0, 31.2 and 34.5% of the activities were retained respectively at 15min (Figure 5a). At pH 8.0, purified xylanase lost all of its activity at 60min (Figure 5a). The purified enzyme retained 79.6

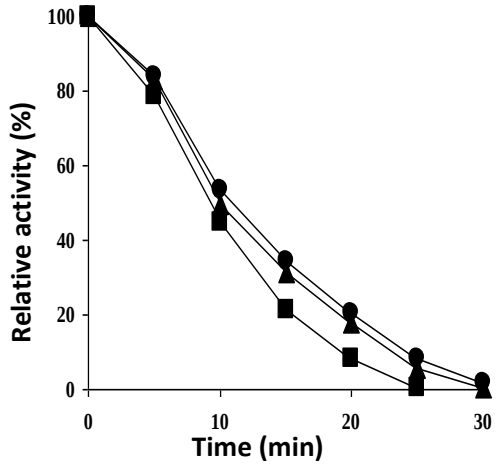

Figure 5a: Stability of crude xylanase at different pH values of (▲), 8; (●), 9 and (■), 10 at 60°C with 20gL^{-1}xylan.

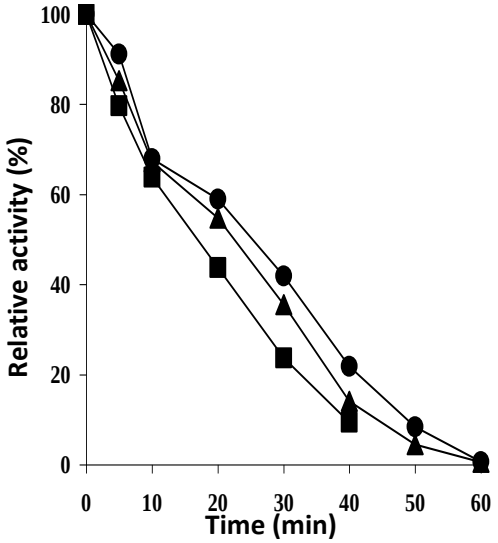

Figure 5b: Stability of purified xylanase at different pH values of (▲), 8; (●), 9 and (■), 10 at 60°C with 20gL^{-1}xylan.

and 9.4% of the enzyme activity at 5 and 40min respectively at pH 10.0 and 60°C.

At pH 8.0, 85 and 35.5% of the initial activities were retained at 5 and 30min respectively and 41.8% of the activity was retained at 30min and pH 9.0 (Figure 5b). These results indicated that the purification increased the stability of the enzyme and the enzyme was more stable at pH 9.0.

Discussion

Crude and purified xyalanse showed same period for zero order kinetics. Xylanase from *Bacillus pumilus* showed zero order kinetics similar to the commercial enzyme (that from *Aspergillus niger*). Hence the kinetic properties of *Bacillus pumilus* xylanse are comparable to that commercial xylanase.

Optimum temperatures for the activities of all three enzyme preparations were 60°C. Hence this enzyme is well suited for the commercial uses in bio-bleaching and in other food industries [2]. The temperature optima of the xylanases from *Streptimyces* sp was 60°C [11]), *Rhodothermus marinus* was 80°C [12], *Paenibacilus* sp was 55°C [13] and *Melanocarpus albomyces* was 65°C [14]. Generally the rate of enzyme catalysed

reactions increase with the rise in temperature within the temperature range in which the enzyme is stable and retains its activity. The increase in activity results from the increased kinetic energy of the reacting molecules. Above 60^{o}C, due to the heat denaturation of the enzymes the activities of the enzyme preparations were decreased. The kinetic energy of the enzyme molecules increases with the raise in temperature. When the temperature exceeds the energy barrier for breaking the secondary bonds responsible in maintaining the native catalytically active state of the enzyme, the secondary and tertiary structures of the enzyme are lost, which subsequently leads to the loss of catalytic activity [15].

The optimum pH of the enzyme from *Bacillus pumilus* was 9.0. Industrially the alkaline xylanases have wide applications. The pH optima of xylanases vary with the organisms. The pH optima of purified xylanase from *Bacillus amyloliquifaciens* and *Rhodothermos marinus* was in the range of 6.8-7.0 [16], *Paenibacillus sp* was 7.0 [13], *Streptomyses* sp was 8.6 [11] and *Melanocarpous albomyces* was 6.6 [14]. The dependence of enzyme activity on pH is a consequence of the amphoteric properties of proteins [17]. Different ionizable groups with different pK_a values are present on the surface of the protein molecules and surface charge distribution on the enzyme molecules varies with the pH of the environment. These changes in charges may affect the enzyme activity either by changing the structure or by changing the charge on a residue functional in substrate binding or catalysis [14]. In addition, many enzyme reactions proceed through charged transition states. So these electrostatic effects have an important function in enzyme catalysis [18]. This means purification affects the kinetics of enzymes. However, the optimum pH of *Bacillus pumilus* xylanase was unaffected by the purification procedure.

Purification of *Bacillus pumilus* xylanase has increased the affinity between xylan and xylanase, probably by removing the impurities in the enzyme preparation. The Michaelis constants of the crude and purified xylanase preparations were 104 and 78times of that of commercial xylanase. Therefore the *Bacillus pumilus* xylanase has more potential than the commercial xylanse considered in this study and other studies (*Melanocarpus albomyces* xylanase has the K_m of 0.3mgL^{-1}; 14) to be used in the industries. However the xylanases from *Paenibacillus* sp and *Bacillus amyloliquifaciens* had the K_m of 9.1[13] and 4.5gL^{-1}[19], which could be used with very high substrate concentrations.

The results indicated that Xylanse from *Bacillus pumilus* was more stable at 50^{o}C than at 60 and 70^{o}C. The crude and purified enzymes showed better stability at 50^{o}C than the other temperatures tested. The paper industry and food industry need which are xylanases stable at or above 50^{o}C [11]. Xylanases from *Trichoderma launginosus* [20] and *Trichoderma launginosus* [21] was inactivated very easily at acidic, neutral and alkaline pH values and at 70^{o}C. Xylanase from *Bacillus* sp was stable for 2 hours at the temperatures ranging from 30-50^{o}C [22]. Thermal stability of two different xylanases obtained from *Melanocarpus albomyces* was differed. Purified *Bacillus pumilus* xylanase was more stable than the crude enzyme. Salt bridge numbers are higher in most thermophilic proteins. This fact can be explained since salt bridges and their networks rigidify protein structures. A high concentration of salt bridges, particularly networks stitches the structure of a protein, making it more resistant to local deformation or melting or unfolding at high temperatures [23]. The stability also depends on the organism producing the xylanase [5].

Half-lives at 60^{o}C of the crude xylanase from *Bacillus pumilus* at pH 8.0, 9.0 and 10.0 were 10.4, 11.6 and 8.2 respectively, while those of the purified xylanase were 18.82, 21.03 and 15.30 respectively. These results also have indicated that the purification has increased the stability of the enzyme and the enzyme was more stable at pH 9.0, which is well suited for the industrial applications.

Acknowledgements

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