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Production, Partial Purification and Biochemical Characterization of Thermostable Xylanase from *Bacillus brevis*

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ABSTRACT

Additional to industrial use of xylanase in kraft pulp production, thermostable xylanase has wider application in fishery, piggery, cattle food and human food. In this research, 1-4-β-D-endoxylanase was isolated from liquid state cultures of *Bacillus brevis* containing wheat straw as carbon source. Xylanase was purified to apparent homogeneity by gel filtration and ion exchange chromatography. The enzyme was optimally active at 55 °C and pH 7.0. The molecular weight of xylanase (determined through SDS-PAGE) was 23 kDa. The partially purified xylanase was found active in gel when birchwood xylan was used as substrate in zymogram analysis. The enzyme showed good thermal stability with half life of 3 h at 55 °C and 2 h at 70 °C, respectively.

Key words- xylanase, thermostable, *Bacillus brevis*, hemicellulose, β-1, 4- linked-D-xylopyranosyl residues, arabinofuranosyl residues,

INTRODUCTION

Xylan is the major component of the hemicellulose fraction in higher plant cell walls. This has a backbone of β-1, 4- linked-D-xylopyranosyl residues and, based on their origin, in soft wood their degree of polymerization varies from 70-130, while in hard wood xylan, it ranges from 150 to 200[1]. Some of the xylopyranosyl are acetylated at C-2 or C-3; α-1, 2-linked to glucuronic or 4-O—methylglucuronic acid groups; α-1, 3-linked to arabinofuranosyl residues[2]. Endo-1,4 β-xylanases (EC 3.2.1.8) are responsible to cleave internal glycosidic bonds and converts xylan into small oligomers[3]. Xylanase can be classified into family F/10 and family G/11 glycosyl hydrolases[4-5].

In recent years many microorganism including bacteria, fungi and yeast have been studied for xylanase production and characterization[6-9]. In the present scene promising application as a biobleach to reduce the demand of harsh chemical bleaching agents but for that the enzyme should be active at alkaline pH and high temperature. To meet the industrial requirements especially for kraft biobleaching the bacterial xylanases are preferred as compare to fungal xylanase because mostly fungal xylanase has celulase activity, which may adversely decrease the quality of paper[10-11].

Additional to industrial use of xylanase in kraft pulp production, thermostable xylanase has wider application in fishery, piggery, cattle food and human food[12]. The present study describes partial purification and characterization of a termostable xylanase from *Bacillus brevis* grown on wheat straw as substrate. Figure 1 shows production of xylanase using different substrates. To our knowledge, this is the first report describing the production of xylanase by *Bacillusbevis* using agrowaste for xylanase production and the enzyme was stable at higher temperature.

Methodology

The bacterial strain of *Bacillus brevis* (ATCC 8246^T) was grown at 37 °C for 72 h on rotatory shaking (100 rpm) in 1-L Erlenmeyer flasks containing 250 ml of medium. The composition of the medium (w/v) was as follows: 2% wheat straw, 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2 SO_4$, and 0.06% yeast extract. The contents of each flask were filtered through filter paper and used for enzyme activity measurement and purification process.

All purification steps were carried out at 4 °C unless otherwise specified. Aliquots (20 ml) of the concentrated culture filtrate were fractionated by ion exchange on DEAE-Sepharose (3.0 x 9.0 cm) column, pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Fractions (10-30) of 2.0 ml representing the pre-gradient xylanase peak were collected at a flow rate of 30 ml/h, pooled concentrated by freeze-drying and loaded on to a Sepharose G50 column (2.5 x 82 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Fractions were collected and those corresponding to the peak of xylanase activity were pooled and analyzed for xylanase activity.

Xylanase activity was routinely determined by mixing 50 µl of enzyme solution with 100 µl of birchwood xylan (1% w/v) in 50 mM sodium phosphate buffer, pH 7.0 at 55 °C for 5 min. Dinitrosalicylic acid reagent method [13] was used to measure the reducing sugars released by the xylanase activity. The xylanase was measured in IU, µmol of reducing sugars formed per ml in unit time by the enzyme solution. The determination of optimum temperature of xylanase was carried out in the temperature range of 37-95 °C in 50 mM phosphate buffer, pH 7.0 as shown in figure 2. The optimum pH shown in figure 3 was determined by measuring the activity at 55 °C. 50mM sodium acetate (pH 3.0-6.0); 50 mM sodium phosphate (pH 6.5-8.0) and Tris buffer (pH 8.5-9.5) were used for different pH ranges. The temperature stability of xylanase was determined by pre-incubating the enzyme at 55 °C and 60 °C and removing aliquots at intervals to measure the xylanase activity as described earlier. Protein concentration was measured by [14] using BSA as the standard. Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE, 12%) was carried out as prescribed [15]. After electrophoresis, the protein bands were silver stained, following standard protocol. Zymogram was developed by Congo reg solution (1%) for 30 min at 37 °C, 1M NaCl was used for distaining while 0.5% acetic acid was used as fixative[16].

RESULTS

Xylanase from *B. brevis* was purified by a combination of ammonium sulphate precipitation, gel filtration and ion exchange chromatography techniques. The summary of purification data presented in Table 1clearly shows that finally specific activity of 4380 was achieved. The activity yield of the purified enzyme was 2.4% of the original activity[17]. Earlier reported purification yields in the range of 1-30% for xylanase. Due to the synergistic affect of xylan–degrading enzymes the yield and fold purification values were underestimated[1,18]. The purified xylanase showed single band of 23 kDa on SDS-PAGE (Figure 4).

Out of wheat bran, rice bran, wheat straw and rice straw used as substrate for xynanase production only wheat straw showed maximum enzyme production (Fig. 2). Enzyme activity is affected by change in pH values. Extremely high

Table 1: Summary data on purification of xylanase from Bacillus brevis

	Total protein (µg)	Total activity (IU)	Specific activity (units/mg)	Fold purification	Yield (%)
Crude	840	380	452	1	100
30-60% Ammonium precipitation	980	250	2551	5.6	65
Sephadex G 50	8.4	32	3809	8.4	8.4
DEAE Sepharose	2.1	9.2	4380	9.6	2.4

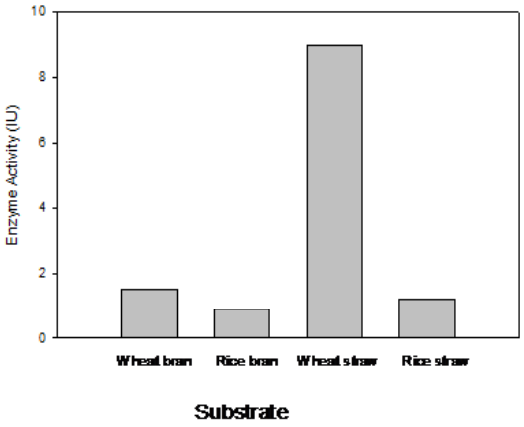


Fig. 1: Production of xylanase from *B. brevis* using different substrates

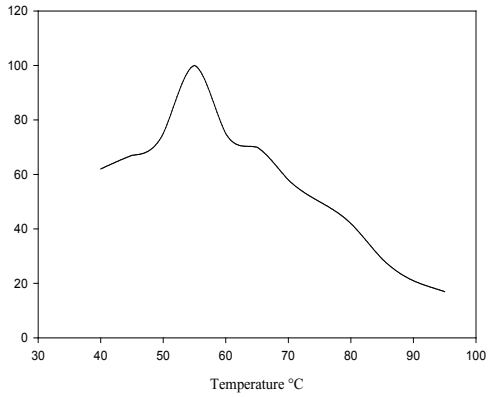


Fig. 2: Effect of Incubation temperature on xylanase activity

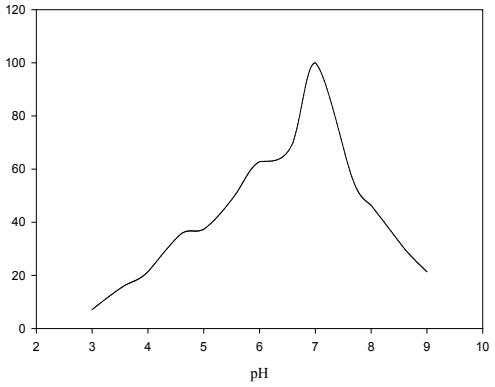
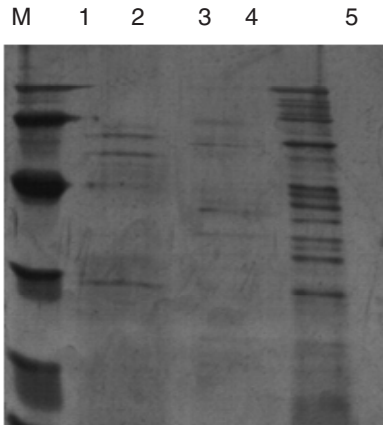


Fig. 3: pH optimization of xylanase activity



M. Marker, 1. Gel filtration, 2. Gel filtration fraction pool, 3. Crude, 4. Blank well to avoid sample mixing, and 5. Ion exchange

Fig. 4: Confirmation of purified xylanase on SDS-PAGE

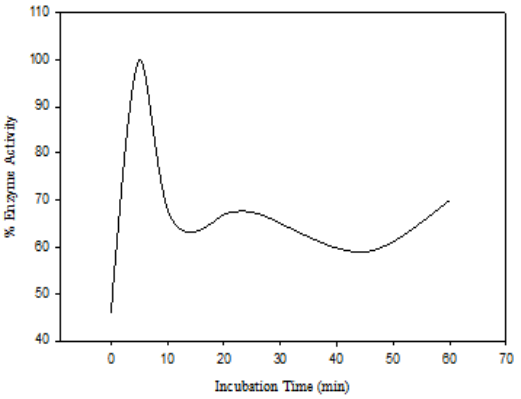


Fig. 5: Incubation time optimization

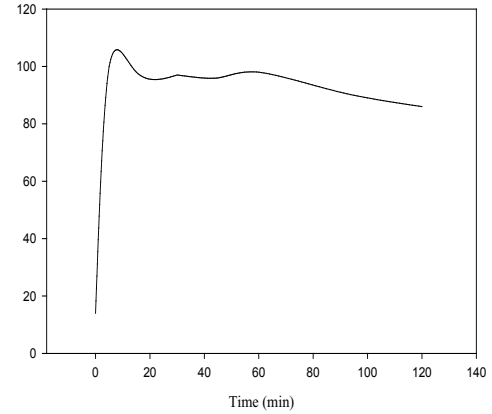


Fig. 6: Thermal stability of xylanase at 55°C

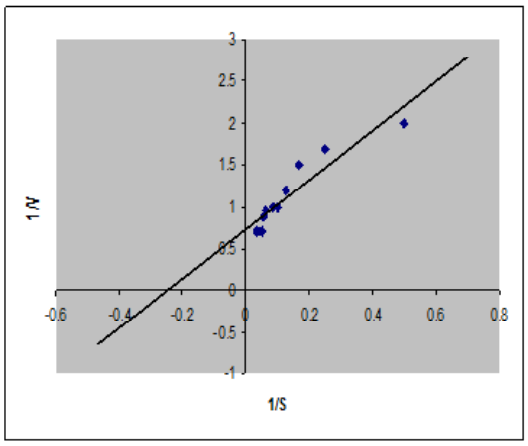


Fig. 7: Double reciprocal plot for xylanase enzyme

or low pH generally causes loss of enzyme activity for most enzymes. Therefore, the effect of pH on xylanase enzyme activity was studied by varying the pH of the buffer in the range of 3.0 to 9.0. *B. brevis* xylanase showed maximum activity of 14 IU at pH 7.0 (Fig. 3). Any change from this optimum pH resulted in decrease in enzyme activity. Good enzyme activity was maintained up to pH.8. 4 pH followed by sharp decrease.

Similarly, the optimum temperature for *B. brevis* xylanase enzyme activity was determined. For this, the xylanase was incubated for 5 minutes at a temperatures ranging from 37 °C - 95 °C. Very low activity was measured between 37 °C and 45 °C followed by maxima at 55 °C (Fig. 4). Almost 50% activity was lost between temperatures 55 °C and 85 °C, while only traces of enzyme activity was observed between 85 °C to 95 °C. Presence of high temperature tolerance in *B. brevis xylanase* can be a very promising character for using this xylanase in Kraft pulp bio-bleaching.

Time of incubation for *B. brevis* xylanase was optimized at optimum temperature, pH and substrate concentration as shown in figure 3. Xylanase activity was measured at 5 min interval; results summarized in figure 5 show a maximum xylanase activity after 5 min of incubation and no further increase in enzyme activity was observed even increasing the incubation time up to 1 h. This could be due the occupancy of most of the active sites of enzyme by substrate within 5 min of incubation and after that, not many sites are available for further binding of enzyme to the substrate.

The thermal stability of xylanase was studied at the optimam temperature i.e 55ºC. The enzyme was incubated at 55ºC and the enzyme activity was measured after every 5 min interval starting form 0 min up to 120 min. The results indicate that the enzyme, xylanase was stable for 2 h at 55 ºC with a loss of 5-10 % activity after 20 minutes (Figure 6). But From the Line-Weaver Burk (LB) plot (Figure 7), the values of V_{max} (1.33 IU/ml) K_m (5.75 mg/ml) were determined. By analyzing the results, it can be concluded that the xylanase from *B. brevis* has a higher affinity for the substrate xylan and can catalyze it more efficiently and faster than various other bacterial xylanases as it has a high value of V_{max}.

DISCUSSION

The first report on xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa. The purified enzyme of *Bacillus* sp. C-59-2 exhibited a broad pH optimum ranging from 6 to 8. An examination of xylanase multiplicity in *Bacillus* spp. suggested that these bacteria produce two types of xylanases: one is basic (pH 8.3-10.0) with low molecular weight (16-22 kDa) and the other is acidic (pH 3.6-4.5) with high molecular weight (43-50 kDa). Many of the xylanases produced by alkaliphilic organisms such as *Bacillus* sp.[19] and *Aeromonas* sp. 212 [20] with optimum growth at pH 10 showed remarkable stability at pH 9-10. The enzymes from *Bacillus* sp. TAR-1, C-125 [21-22], Bacillus sp. NTU-06 (Wang *et al.*, 2010) [23] *Bacillus arseniciselenatis* DSM 15340 [24] and alkaliphilic *Bacillus* sp. (NCL-86-6-10) [25] were optimally active at pH 9-10.

Xylanase production and characterization has been reported from many microbial strains but we did not find any report claiming xylanase production and characterization from *Bacillus brevis*. Probably this would be the first report on *Bacillus brevis* xylanase. Xylanase from fungal sources has a great attraction due to high level of production and thermal stability but generally they are produced in association with cellulases. In our study, we

found that the *Bacillus brevis* xylanase has pH and temperature optima of 7.0 and 55 °C, respectively. The interesting part of this study is that the enzyme was stable in a broad range of temperatures (45-90 °C). *Bacillus brevis* xylanase showed good thermal stability at 70 °C with half life of 2 h. In purification, the final specific activity of 4380 IU/mg was attained with 2.4 fold purification.

CONCLUSION

The xylanase enzyme from *Bacillus brevis* showed activity in neutral to alkaline pH and stability at higher temperature of >55 °C with high K_m (5.75 mg/ml) and V_{max} (1.33 IU/ml) values. Molecular weight, sequence analysis and catalytic properties of *B. brevis* xylanase suggest that it belongs to family G/11.

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