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A Highly Thermostable Alkaline Cellulase-Free Xylanase from Thermoalkalophilic *Bacillus* sp. JB 99 Suitable for Paper and Pulp Industry: Purification and Characterization

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Abstract A highly thermostable alkaline xylanase was purified to homogeneity from culture supernatant of *Bacillus* sp. JB 99 using DEAE-Sepharose and Sephadex G-100 gel filtration with 25.7-fold increase in activity and 43.5% recovery. The molecular weight of the purified xylanase was found to be 20 kDA by SDS-PAGE and zymogram analysis. The enzyme was optimally active at 70 °C, pH 8.0 and stable over pH range of 6.0–10.0.The relative activity at 9.0 and 10.0 were 90% and 85% of that of pH 8.0, respectively. The enzyme showed high thermal stability at 60 °C with 95% of its activity after 5 h. The K_m and V_{max} of enzyme for oat spelt xylan were 4.8 mg/ml and 218.6 μM min^{-1} mg^{-1}, respectively. Analysis of N-terminal amino acid sequence revealed that the xylanase belongs to glycosyl hydrolase family 11 from thermoalkalophilic *Bacillus* sp. with basic p*I*. Substrate specificity showed a high activity on xylan-containing substrate and cellulase-free nature. The hydrolyzed product pattern of oat spelt xylan on thin-layer chromatography suggested xylanase as an endoxylanase. Due to these properties, xylanase from *Bacillus* sp. JB 99 was found to be highly compatible for paper and pulp industry.

Keywords *Bacillus* sp. JB 99 · Thermoalkalophilic · Xylanase · Purification · Glycosyl hydrolases

Introduction

Xylan is the major constituent of hemicellulose, a complex polysaccharide that is the most abundant biological carbon source in nature. Xylanase (E.C. 3.2.1.8) produced by microorganisms have attracted a great deal of attention during the past few decades because of their potential biotechnological applications in various industries including the food, feed, fuel, textile and paper and pulp industries, and in waste treatment [1]. Global

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markets for industrial enzymes growing from €510 billion in 2001 to €760 million in 2010, among which currently xylanase accounts for approximately 25–28% of total enzyme sales [16]. One of the most important large-scale biotechnological applications of recent years is the use of xylanases as bleaching agents in the pulp and paper industry. Treatment with xylanases facilitates the chemical extraction of lignin from pulp and leads to a significant reduction in the use of hazardous chemicals required for bleaching. For biobleaching applications, the candidate xylanase should be thermostable, alkali tolerant, and stable on kraft pulp, and its various properties, such as low molecular weight, net ionic properties, and specific action pattern must suit the process requirements. Moreover, to avoid damage to cellulose pulp, enzyme preparations should be free from cellulase activity as cellulases prove detrimental to yield and strength properties of pulp [11]. We have previously reported the production of thermostable alkaline xylanase from *Bacillus* sp. JB 99 on solid substrates [18] with large industrial importance due to its stability in alkali and high temperature. Many of the alkaliphilic microorganisms studied so far have been found to produce xylanases with pH optima in the near neutral region but very few xylanase having alkaline pH optima with high thermostability and low molecular weight. One the most alkaliphilic xylanases reported to date is XylB from *Bacillus* sp. AR-009, which has a pH optimum of pH 9–10 with 60 °C and *Bacillus* sp. TAR-1 having pH optimum between 6.0 and 8.0 with 70 °C [3, 16]. These low molecular weight enzymes are most active on long chain xylo-oligosaccharides, and indeed it has been found that they have larger substrate binding cleft [16]. In the present investigation, we have reported purification, characterization of low molecular weight xylanase from *Bacillus* sp. JB 99 which is highly significant compared to xylanases from already reported thermoalkalophilic *Bacillus* sp. In addition to purification, N-terminal sequence of xylanase was also determined to understand the mechanism involved in thermal and alkaline stability and evolutionary significance with other mesophilic xylanases.

Materials and Methods

Bacterial Strain

Bacillus sp. JB 99, used in this present investigation, was isolated in our laboratory from sugarcane molasses. The culture maintenance procedure was described earlier [4, 18].

Enzyme Preparations

Bacillus sp. JB 99 was grown in the following medium consisting of (g/l) oat spelt xylan, 1.0; yeast extract, 2.5; peptone, 5.0; $NaNO_3$, 5.0; K_2HPO_4, 5.0; NaCl, 10.0; $MgSO_4$. $7H_2O$, 0.4; $CaCl_2.2H_2O$, 0.2; pH 10.0 was adjusted with sodium carbonate, which was autoclaved separately and added to the above medium just before inoculation. The 18-h-old culture inoculated flasks were incubated for 48 h at 50 °C with agitation of 220 rpm.

Enzyme Activity and Protein Assay

Xylanase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method as described earlier [5] at 70 °C for 10 min. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of xylose per minute per milliliter under the standard assay conditions. The cellulase activity was also measured by the DNS method.

The oat spelt xylan was replaced with 1% (*w*/*v*) carboxymethyl cellulose as the substrate, while other assay conditions were kept similar to those described earlier. Protein concentrations were determined by Bradford method using bovine serum albumin as the standard.

Purification of Xylanase

All purification steps were carried out at 4 °C. For purification purpose, the culture filtrate was centrifuged at 10,000×*g* for 10 min (Sorvall, RC5B Centrifuge), and the clear supernatant exhibiting xylanase activity was precipitated to 80% saturation of ammonium sulfate. The resulting precipitate was collected by centrifugation at 12,000×*g* for 15 min. The precipitate was dissolved in a minimal volume of 20 mM Tris–HCl buffer pH 8.0 and dialyzed for 18 h against three changes of same buffer.

DEAE-Sepharose Chromatography The resultant dialyzed enzyme fraction was applied to a DEAE-Sepharose column (1.5×9 cm) previously equilibrated with 20 mM Tris–HCl buffer pH 8.0. The column was washed with same buffer and then eluted with a linear gradient of 0 to 0.5 M NaCl in same buffer. The eluted fractions with xylanase activity were pooled and concentrated in U tube concentrator (3 kDa MWCO, Merck.).

Sephadex G-100 Chromatography The concentrated enzyme fraction was loaded on to a Sephadex G-100 column (1 cm×50 cm) previously equilibrated with 20 mM Tris–HCl pH 8.0. The enzyme was eluted by using same buffer at a flow rate of 20 ml/h. The purified xylanase was pooled, concentrated, and used for the further studies.

SDS-PAGE, Zymography Analysis, and N-Terminal Amino Acid Sequencing

The molecular weight and homogeneity of the purified xylanase were analyzed by SDS-PAGE electrophoresis. The SDS-PAGE (12%) was performed as described [7]. Proteins were visualized by staining with Coomassie brilliant blue R-250. Zymogram analysis was performed as described [12]. The polyacrylamide(12%) gel with separated proteins was washed four times for 1 h at 4 °C in a 50 mM Tris–HCl buffer pH 8.0 containing 2.5% Triton X-100 to remove SDS and renature protein in the gel; washed gel was layered on a 1% agarose gel plate containing 0.2% oat spelt xylan in 50 mM Tris–HCl buffer pH 8.0. After incubation at 45 °C for 30 min, the polyacrylamide gel was removed, and agarose plate was flooded with 0.5% (*w*/*v*) congo red dye for 30 min and washed with 1 M NaCl to visualize zones of clearance corresponding to xylanase activity. The N-terminal amino acid sequencing of PVDF membrane blotted protein was performed by Automated Edman degradation method using an Applied Biosystem model 494 Procise sequencer (Indian Institute of technology, Mumbai, India).

Determination of Temperature and pH Optima and Stability

The optimum temperature for xylanase activity was determined by carrying out assay with the purified xylanase at selected temperatures ranging from 30 to 90 °C. The temperature stability of the enzyme was also studied by incubating the enzyme at different temperatures from 30 to 90 °C for 5 h at pH 8.0. The residual activity was determined under standard assay conditions. The enzyme activities were also tested for pH optima ranging from 5.0 to

12.0. In order to determine the enzyme pH stability, the purified xylanase was incubated in different buffers at 4 °C for 24 h, and then the residual activities were determined under standard assay condition.

Kinetic Parameters for Purified Xylanase

The kinetic parameters (Michaelis–Menton constants) K_m and maximal velocity V_{max} of xylanase were determined from Line weaver–Burk plot under optimal assay condition (70 °C, pH 8.0 for 10 min) for oat spelt xylan concentration ranging from 1.0 to 10.0 mg/ml.

Substrate Specificity of Xylanase

The hydrolytic activity against 1% birch wood xylan, oat spelt xylan, beech wood xylan, pectin, avicel, and carboxymethyl cellulose (CMC) in 50 mM Tris–HCl buffer (pH 8.0) were determined to evaluate the substrate specificity of xylanase. The assay as described above was performed in triplicate.

Analysis of the Oat Spelt Xylan Hydrolysis Products

The products from hydrolyzed oat spelt xylan by purified xylanase were analyzed by thin-layer chromatography (TLC). The hydrolysis was carried out with 15 U of purified xylanase and 5 mg/ml oat spelt xylan in 50 mM Tris–HCl buffer (pH 8.0) at 70 °C for different intervals. Aliquots were removed periodically and inactivated by boiling, and 10 µl of each aliquot was spotted on to the TLC plate (Silica gel G, F_{254} Merck). The end products were separated by the solvent system chloroform/acetic acid/H_2O (60:70:10, *v*/*v*); xylo-oligosaccharides were visualized by spraying 0.2% (*w*/*v*) orcinol in sulfuric acid/methanol (10:90, *v*/*v*) solution on the plate followed heating at 120 °C for 10 min. Xylose and xylobiose were used as standards.

Results

Purification of Thermostable Alkaline Xylanase

The results for purification of xylanase from a culture supernatant of *Bacillus* sp. JB 99 are shown in Table 1. The culture supernatant was precipitated with 80% saturation by ammonium sulfate, and resultant precipitate was dissolved in 20 mM Tris–HCl pH 8.0, dialyzed against three exchange of same buffer. This step resulted in 87.4% recovery with 1.8-fold purification. In further purification step, xylanase activity was found in unbound eluted fractions from DEAE-Sepharose column. The unbound fractions with xylanase activity were concentrated using U tube concentrator resulting in 69.7% recovery, which was further analyzed by SDS-PAGE (Fig. 1). After gel filtration, only single peak with maximum xylanase activity was eluted at the flow rate of 20 ml/h, pooled, and concentrated to 25.7-fold purification with 43.5% of recovery.

SDS-PAGE, Zymogram, and N-Terminal Sequence Analysis

The purity and homogeneity of purified xylanase were analyzed after gel filtration by SDS-PAGE, which showed single purified band of low molecular weight of 20 kDa when

Table 1 Summary of purification of xylanase from *Bacillus* sp. JB 99.

Purification steps	Total (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Recovery (%)
Culture supernatant	87,924	1,420	61.9	1.0	100
Ammonium sulfate (dialyzed)	76,844	698	110.0	1.8	87.4
DEAE-Sepharose	61,282	93.4	656.1	10.6	69.7
Sephadex G-100	38,246	24.0	1,593.6	25.7	43.5

compared with standard protein molecular markers. After electrophoresis, zymogram analysis of purified xylanase on 0.2% oat spelt xylan plate revealed a clear zone of hydrolysis band (Fig. 1). The partial N-terminal sequence of xylanase from *Bacillus* sp. JB 99 was determined to be NH_2-Asn-Thr-Tyr-Trp-Gln-Tyr-Trp-Asp-Gly, which showed homology to glycosyl hydrolase family 11 xylanase from *Bacillus halodurans* C-125, *Bacillus firmus*, *Thermobacillus xylanilyticus*, and also other genus like *Geobacillus* sp., *Closridium cellulolyticum*, and *Cellulosilyticum ruminicola* (Fig. 2).

Effect of Temperature and pH on Enzyme Activity

The effect of temperature on the enzyme activity and stability was studied (Fig. 3). The purified xylanase showed activities over broad range of temperature 40–90 °C. Under standard assay condition, pH 8.0, the enzyme exhibited maximum activity at 70 °C. The stability of enzyme was tested by incubating at 30–90 °C for 5 h. The enzyme retains 95% and 82% of its activity at 60 and 70 °C, respectively, which is highly stable compared to other reported *Bacillus* sp. The effect of pH was also studied over broad range of pH 5.0–11.0 (Fig. 4). The enzyme was most active in the pH range of 8.0–10.0, but exhibited maximum activity at pH 8.0. With respect to stability, the enzyme was found to be highly stable between pH 7.0 and 10.0. Even after 24 h of incubation, enzyme retains 86% and 90% of its original activity at pH 8.0 and 9.0, respectively.

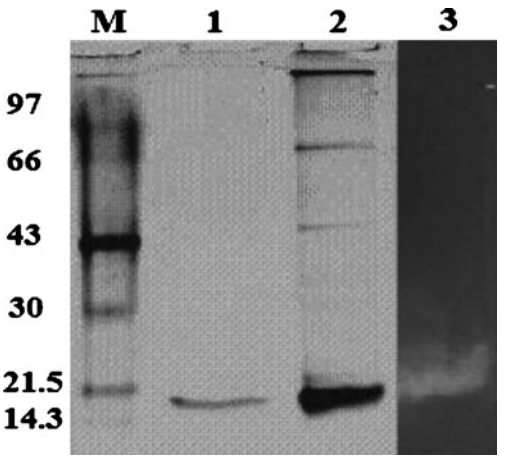

Fig. 1 SDS-PAGE of purified xylanase from *Bacillus* sp. JB 99; steps of purification were analyzed on 12% polyacrylamide gel. Purified xylanase after gel filtration (*lane 1*) and after ion exchange (*lane 2*) molecular weight markers (*lane M*—phosphorylase, 97 kDa; albumin, 66 kDa; oval albumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; alpha lacto albumin, 14.4 kDa)

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Cellulosilyticum ruminicola      ATDYWQNWTDGGGYVNAVNGQGGNYSV
Geobacillus sp. Y412MC10         ATDYWQNWTDGGGTVNAVNGSGGNYSV
Clostridium cellulolyticum       ATDYWQNWTDGGGTVNVTNGSGGNYSV
Paenibacillus sp. DG-22          ATDYWQYWTDGGGTVNATNGSGGNYSV
Bacillus sp. JB99                -NTYWQYWTDG----------------
Thermobacillus xylanilyticus     -NTYWQYWTDGIGYVNATNGQGGNYSV
Bacillus firmus                  -NTYWQYWTDGGGTVNATNGPGGNYSV
Bacillus halodurans C-125        -NTYWQYWTDGGGTVNATNGPGGNYSV
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Fig. 2 Multiple alignment of N-terminal sequence of xylanase from *Bacillus* sp. JB 99 with other glycosyl hydrolase family 11 endoxylanases. Accession numbers for the indicated sequences are as follows *Cellulosilyticum ruminicola* (ACZ98621); *Geobacillus* sp. Y412MC10 (YP_003243577); *Clostridium cellulolyticum* (YP_002506194); *Paenibacillus* sp. DG-22 (ABI9699); *Thermobacillus xylanilyticus* (CAJ87325); *Bacillus firmus* (AAQ83579); *Bacillus halodurans* C-125 (NP_241765)

Substrate Specificity of Xylanase and Kinetic Parameters

The hydrolytic activity of xylanase on different commercial xylan substrates was studied. This xylanase exhibited different specificity and activity towards xylan substrates, which was observed by incubating 15 U of enzyme with 1% of birch wood xylan, beech wood xylan, oat spelt xylan, pectin, avicel, and CMC. The xylanase exhibited maximum activity towards birch wood xylan compared to other substrates and no activity for CMC, pectin, and avicel (Table 2). These results indicate that the xylanase is cellulase free and it is a true xylanase. The kinetic parameters (K_m and V_{max}) were determined using Line weaver–Burk plot at 70 °C (pH 8.0) with different concentrations of birch wood xylan ranging from 1.0 to 10.0 mg/ml. The K_m and V_{max} of purified xylanase were 4.8 mg/ml and 218.6 μmol mg^{-1} min^{-1}, respectively. This indicates that specificity towards birch wood xylan substrate is high (K_m value is low) when compared with the earlier reported.

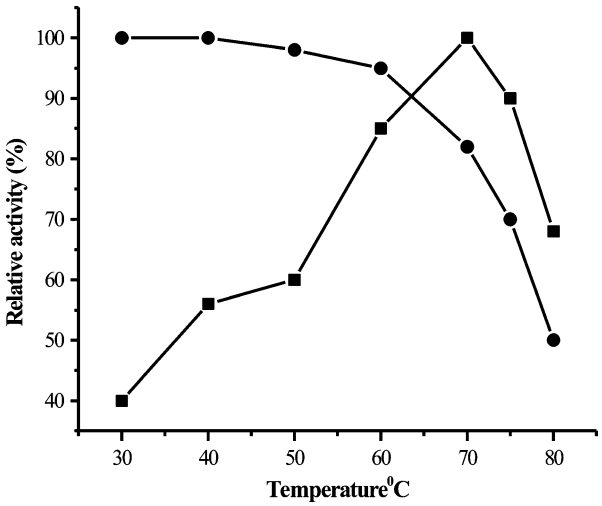

Fig. 3 Effect of temperature on xylanase activity (*squares*) and stability (*circles*); assay was carried at various temperatures for 10 min using 1% oat spelt xylan as substrate in 50 mM Tris–HCl buffer pH 8.0

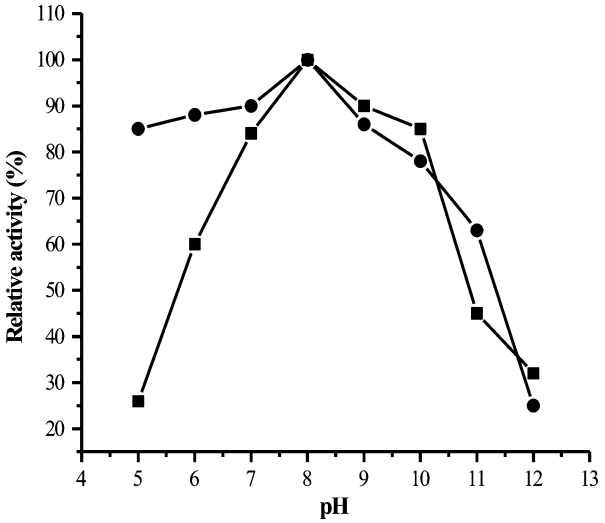

Fig. 4 Effect of pH on xylanase activity (*squares*) and stability (*circles*); the effect was measured at 70 °C for 10 min using 1% oat spelt xylan as substrates in the following buffer 50 mM systems; sodium acetate (pH 5.0); phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 8.0– 9.0), and glycine–NaOH buffer (pH 10.0–12.0)

TLC Analysis of Enzymatic Hydrolysis Product of Oat Spelt Xylan

The mode of action of purified xylanase was analyzed by TLC of hydrolyzed product of oat spelt xylan. At different time intervals, the enzyme liberated different level of xylo-oligosaccharides mainly xylobiose. After 6 h of incubation, the level of xylobiose was high along with xylotriose, xylotetrose, and xylopentose, indicating xylanase from *Bacillus* sp. JB 99 as end- acting xylanase (Fig. 5).

Discussion

Vast studies are carried in search of highly alkaline and thermostable xylanase from microbial sources especially bacterial due its advantages over fungal xylanase. However, there are few reports on low molecular weight cellulase-free xylanase from *Bacillus* sp. having pH optima ≥9.0 except XylB from *Bacillus* sp. AR-009, which has a pH optimum of 9.0 at 60 °C [14, 16]. In this investigation, we have purified highly active xylanase from

Table 2 Relative activity of xylanase on different commercial substrates.

Substrates	Relative activity[a] (%)
Oat spelt xylan	100
Birch wood xylan	112
Beech wood xylan	98.6
Avicel	ND
Carboxy methyl cellulose	ND
Cellulose	ND
Pectin	ND

[a] Assay was carried out at pH 8.0 and 70 °C for 20 min with purified xylanase

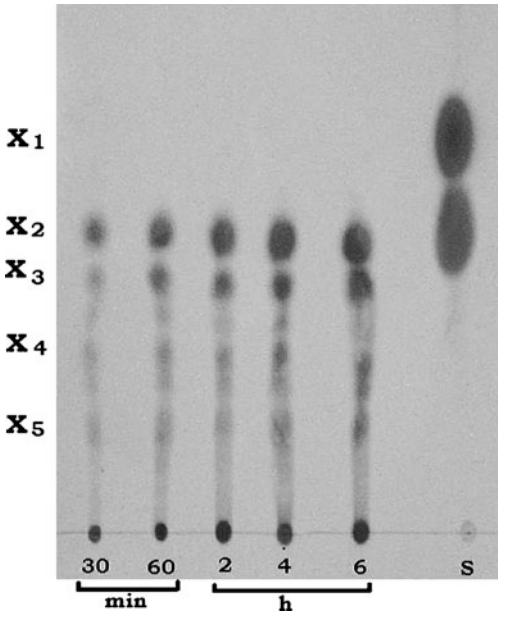

Fig. 5 Thin-layer chromatogram of hydrolyzed products of oat spelt xylan by xylanase from *Bacillus* sp. JB 99. The purified xylanase was incubated with 5 mg/ml of oat spelt xylan in 50 mM Tris–HCl buffer pH 8.0 at 70 °C for 6 h. The samples (10 μl) at different intervals are analyzed on TLC. The standards used were xylose (X_1), xylobiose (X_2), and indicated xylotriose (X_3), xylotetrose (X_4), and xylopentoses (X_5)

Bacillus sp. JB99 with pH optima 8.0 and thermostability of 70–80 °C. The high temperature and pH optima as well as cellulase free nature of such enzymes facilitate their usage for application in paper and pulp factory since these very features are prime requisite of the industrial pulping process [2]. The molecular weight determination by SDS-PAGE and zymography analysis reveals the presence of single purified 20-kDA protein, which is almost similar with that of xylanase from thermoalkalophilic *Bacillus* sp. K-1 [6], *Bacillus sp.*TAR-1[3], and *Geobacillus thermoleovorans* [13]. The low apparent molecular weight with basic p*I* of xylanase could also be advantageous for pulp treatment application; as such, enzymes would have greater access to the xylan component of the wood matrix [14, 17]. Also, determination of N-terminal sequence of xylanase reveals that the xylanase belongs to glycosyl hydrolase family 11, low molecular xylanase with basic p*I*; this sequence is highly homologous to xyn11A from *B. firmus* [10]. Based upon the substrate specificity and kinetic parameters determination, the xylanase possesses high specificity and activity towards different xylan substrates, and it exhibited no other activities except xylanase. The result from thin-layer chromatography analysis of hydrolysis product of oat spelt xylan indicates xylanase as endoxylanase. These findings are comparable with other *Bacillus* sp. in the literature already reported [1], which suggest that family 11 xylanase is considered as a true xylanase because of their higher substrate specificity. In summary, because of its occurrence in culture supernatant, broad pH stability, high thermal stability, and xylooligosaccharide production profile, the xylanase is an attractive candidate for paper and pulp industry and food industry for production of xylosaccharides. The potential use of these xylanase is its selective removal of only hemicellulose component with minimal damage to cellulose pulp, which is of tremendous importance in pulp bleaching to avoid drop in quality of paper pulp. The industry highly demands for high alkaline (pH 8.0–10.0) and thermostable xylanase (70–80 °C), which are employed in bleaching of paper pulp and conversion of agricultural residues into valuable by-products for bioethanol production [1, 8, 9, 15]. However, completed nucleotide sequence of xylanase encoding gene from

Bacillus sp. JB 99 (work in progress) would help in better understanding thermal and alkaline stability and also help us in understanding the evolutionary significance.

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