



ISOLATION, PURIFICATION AND CHARACTERIZATION OF XYLANASE, PRODUCED BY A NEW SOIL BORN *BACILLUS SP.*

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ABSTRACT

Research on Xylanase enzyme has markedly increased due to its potential applications in several industries include pulping and bleaching processes, where it is using cellulose free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment. The present study was aimed at isolation and characterization of xylan degrading strain of *Bacillus sp* from soil for production of xylanase. Five isolates were obtained from soil samples of different areas in the Andhrahalli area of Bangalore, Karnataka and studied for detection of xylanase activity. One of the strains was identified as *Bacillus sp* and it was having close relation with *Bacillus cereus*. On the basis of the nucleotide sequence of the 16S rRNA gene, which produces xylanase extracellularly. In this work we had purified xylanase enzyme after treating ammonium sulphate and then that precipitation was used for purification. Purification was done by using DEAE-sepharadex and Hydroxyapatite column chromatography. The SDS-PAGE gave a single band at 32 kDa. For the purified enzyme, optimum temperature was found 30°C and pH was 6.0. The xylanase hydrolyzed oat spelt xylan, birch wood xylan and beech wood xylan efficiently but showed no activity towards cellulose, CM-cellulose. Thus it was a true and neutral xylanase. Reporting on isolation of xylanase from *Bacillus sp* is rare.

KEYWORDS: Xylan, Xylanases, Neutral xylanase, *Bacillus sp*.



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INTRODUCTION

Biodegradation of xylan, a component of the plant cell wall, is a complex process that requires the combined action of several enzymes, among which xylanase (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) which cleaves internal linkages on the β -1,4-xylose backbone, play a key role[1]. It has been shown that many kinds of bacteria and fungi hydrolyze β -1,4 xylan by the use of xylanolytic enzymes, such as β -1,4 xylanases, β -xylosidases, and esterases [2, 3]. β -1,4 xylanases are the key enzymes that hydrolyze the backbone structure of β -1,4 xylans to initiate degradation of the complex polysaccharides by microorganisms. A number of β -1,4 xylanases have been purified from fungi and bacteria, and the genes encoding β -1,4 xylanases have been cloned and characterized. Several microorganisms produce multiple xylanases, implying a strategy for effective hydrolysis of β -1,4 xylan. Each of the enzymes may have a specialized function in the degradation of the complex polysaccharides and those specialized functions of individual xylanases may be useful for applications in human consumption, animal feed, and the paper industry [4, 5]. Recently the interest in xylanases has markedly increased due to the potential applications in pulping and bleaching processes using cellulase free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment [6,7,8].

Among the 5 strains isolated from the different part of soil in Andhrahalli, Bangalore, Karnataka. One was characterized according to Bergey's manual of systematic bacteriology [7,8,9] and was again characterized on the basis of the nucleotide sequence of the 16S rRNA gene and the strain was found as *Bacillus* sp. There were many reports of xylanases from *Bacillus* sp. but report on xylanase from *Bacillus* sp was rare. This study reports the isolation, purification and characterization of xylanase from *Bacillus* sp obtained from soil.

MATERIALS AND METHODS

Bacillus sp strain was isolated from different areas in the Andhrahalli area of Bangalore,

Karnataka and was maintained in medium I as follows: 0.5% oat xylan, 0.2% yeast extract, 0.25% NaCl, 1.5% KH_2PO_4 , 3% NaH_2PO_4 0.5% NH_4Cl and 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0.

Isolation and screening of the bacterium

Sample from soil in sterilized water was poured and spread onto agar plate A. These plates were incubated at 37°C for 2 days. The colonies on the plates were transferred onto agar plate B, which were again incubated at 37°C for 2 days. Colonies producing clear zone in the plate were selected.

Characterization of the bacterium

The morphological properties and taxonomic characteristics of the bacteria was studied by the methods in Bergey's manual of systematic bacteriology [7,9,11, 22]. The isolated bacteria was an aerobic, gram-positive, spore forming, rod-shaped organism.

Analysis of 16S rRNA gene

The partial sequence of 16S rRNA was done by using a pair of the universal primer EU10F and UN1513R with 30 cycles of the following thermal program: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 120 sec. The amplified DNA fragments were cloned with a TOPO TA cloning kit for sequencing (Invitrogen). The nucleotide sequence was determined with a Big Dye terminator Cycle sequencing Kit (Applied Biosystems) using universal rRNA-specific primers, M13 forward and reverse primer on an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Xylanase purification

Bacillus sp was aerobically grown at 37°C. for 48 hrs in a liquid medium as described above. The cells were separated by centrifugation at 12,000 \times g for 10 min and used as crude enzyme. The purification of the xylanase was done at 4°C. The crude xylanase was precipitated with ammonium sulfate at a concentration corresponding to 80% saturation. The resultant precipitate was collected by centrifugation at 15,000 \times g for 20 min, dissolved in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer

and applied to the DEAE-sepharose column. The elution was done from 0-0.5 M NaCl. The xylanase active fraction was eluted at 0.25 M NaCl gradient. The active fractions from DEAE-sepharose column were combined, mixed with the same volume of 3 M ammonium sulfate and put onto Phenyl 5 PW column, which was previously equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 1.5 M ammonium sulfate. Adsorbed proteins were eluted with a descending linear gradient of ammonium sulfate. Xylanase activities were eluted at 0.2 M ammonium sulfate. The active fraction from phenyl 5 PW was dialyzed against 5 mM sodium phosphate buffer of pH 7.0. The dialyzed enzyme solution was put onto a hydroxyapatite column previously equilibrated with the same buffer. The absorbed protein was eluted with a linear gradient of 5-100 mM sodium phosphate buffer (pH 7.0). The active fraction was eluted at 50 mM sodium phosphate buffer. Purity of xylanase in the fractions from column chromatography was analyzed by SDS-PAGE.

Protein measurement

Protein concentration was measured by the method of Lowary *et al.* [8,13,17]. Bovine serum albumin was used as a standard.

Effect of temperature and pH on xylanase activity

The reaction mixture containing 0.9 ml of oat spelt xylan solution and 0.1 ml of the purified enzyme preparations was incubated at 30-80°C. The effect of pH on xylanase activity was

studied in the following buffers; 50 mM acetate buffer of pH 4.0, 50 mM acetate buffer of pH 5.0, 50 mM sodium phosphate buffer of pH 6.0, 50 mM sodium phosphate buffer of pH 7.0, 50 mM Tris buffer of pH 8.0 and 50 mM glycine buffer of pH 9.0.

Xylanase activity assay

Xylanase activity was assayed using 1% solution of oat spelt xylan as the substrate as described by Baily *et al* [9,17,18,20] and the amount of reducing sugars released was determined by the dinitrosalicylic acid method [10,15,19,22]. One unit of enzyme activity was defined as 1 mM xylose equivalent produced per minute under the given conditions.

RESULTS AND DISCUSSION

Isolation and screening of the bacterium

For the preliminary experiment of this study, bacteria samples were collected from soil. Xylanolytic clear and transparent zone (on the xylan agar plate) producing bacterial strains were collected and incubated at 37°C for 48 h. After collection of bacterial strains by pure culture technique, the colonies were screened by the staining method, microscopic examination, and oxidase and catalase test. Finally we tried to verify one of the strains by sequencing the 16S rRNA gene described in the materials and methods section. As a result, the 16S rRNA gene of the strain revealed 94% identity with *Bacillus cereus* (Table 1).

Table 1
The Nucleotid sequences of the partial 16SrRNA gene of the isolate identified as *Bacillus*. Sp 16S rRNA Gene Sequence

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AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG
AATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGCACGGGTGAGTAACACGTGGGTAAC
CTGCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACC
GCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTTCGCATTA
GCTAGTTGGTGAAGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAAC
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Purification of xylanase

Results of xylanase purification from *Bacillus sp* are summarized in table 2. The purification of xylanase was done by four steps; ammonium-

sulphate precipitation, DEAE-sepharose, Phenyl-5PW and Hydroxyapatite column chromatography as described above. The xylanase was purified 16.3 fold, with a final

yield of 13.4 %. The specific activity of the purified enzyme was 19.8 mM/min/mg. Analysis of the purified enzyme by SDS-PAGE revealed a single band with a molecular mass of 31 kDa as determined by SDS-PAGE (Fig. 1). Recently, the cloning and characterization of xylanase A from the strain *Bacillus sp.* BP-7

was done and reported by Gallardo *et al.* [11]. They found the low molecular weight xylanases of family 11 and it was 27 kDa. Comparatively the xylanase from *Bacillus sp.* is little higher molecular weight than the xylanase from *Bacillus sp.* BP-7.

Table 2
Purification of xylanase from *Bacillus sp.*

Steps	Volume (ml)	Protein (mg)	Activity (mmol./min)	Recovery (%)	Specific activity (mmol/min/mg)	Purification (fold)
Culture	20	512	623	100	1.21	1
Ammonium	4	220	323	51.8	1.46	1.21
DEAE	6	53	127	20.3	2.39	1.98
Phenyl	5	13.5	97	15.5	7.18	5.93
Hydroxy-apatite	4	4.24	84	13.4	19.8	16.3

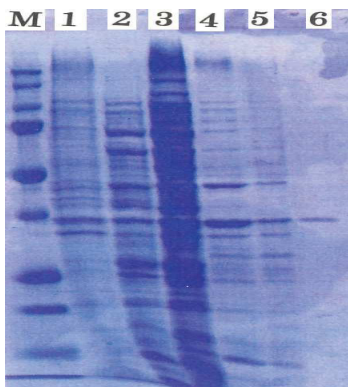


Figure 1

SDS-PAGE analysis of xylanase purification from *Bacillus sp.* Lane. M, protein standard marker (molecular mass from top: 200kDa, 150 kDa, 100 kDa, 75 kDa, 37 kDa and 25 kDa); 1, culture supernatant; 2, ammonium sulphate precipitant; 3, DE AE-sepharose; 4, Phenyl 5, PW and 6, Hydroxy-apatite. Lane 6 single band indicates purified enzyme.

Optimum temperature and pH of xylanase

The effect of temperature on activity of xylanase against oat spelt xylan was examined in the temperature range of 30-80°C. The xylanase showed maximum activity at 30°C (Fig. 2). The xylanase exhibited greatest activity in the pH range of 5-8, having

maximum value at 6.0 (Fig. 3). The optimum temperature of purified xylanase in this study is similar to the optimum temperature of alkaline xylanase from *Bacillus pumilus* [12,16,21]. The optimum pH is similar to high molecular weight xylanase from *Aeromonas caviae* W-61 reported by Roy *et al.* [13, 17,18].

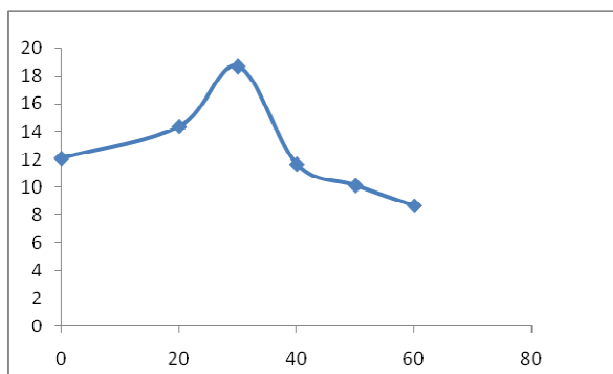


Figure 2
Effect of temperature on purified enzyme activity

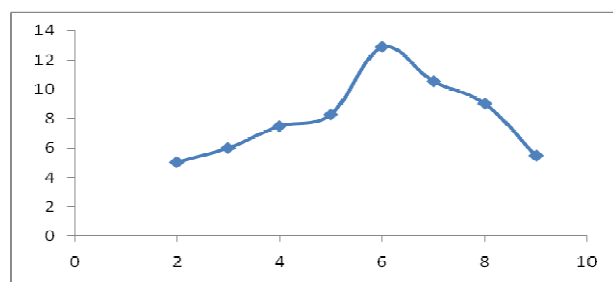


Figure 3
Effect of pH on purified enzyme activity

Substrate specificity of xylanase

The substrate specificity of purified xylanase was studied using various polysaccharides as substrates (Table 3). From the table, it was shown that the xylanase strongly hydrolyzed oat spelt xylan, birch wood xylan and beech wood xylan. However, the xylanase could not hydrolyze cellulose, CM-cellulose. These properties were found in the xylanase 1, 2 and 3 reported previously [14, 15] and other

xylanases from *Bacillus pumillus* [16,19,22] and *Bacillus subtilis* [17,18,20]. Thus it was a true xylanase. The isolation of xylanase from *Bacillus sp* is rare. Its maximum activity was found at 30°C and pH 6.0. So it may be considered as a neutral xylanase. It should be interesting to further study the molecular biology and structural features of this type of xylanase.

Table 3
Substrate specificity of xylanase from *Bacillus sp*

Substrate	Relative activity (%)
Oat spelt xylan	98
Birch wood xylan	100
Beech wood xylan	94
Cellulose	86
CM-cellulose	0
Avicell pH 101	0

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