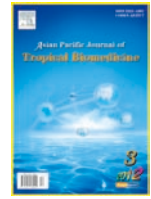




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# Production, purification and characterisation of alkali stable xylanase from *Cellulosimicrobium* sp. MTCC 10645

Rajashri D. Kamble<sup>1\*</sup>, Anandrao R. Jadhav<sup>2</sup><sup>1</sup> Department of Biotechnology Engineering, Tatyasaheb Kore Institute of Engineering and Technology, Warananagar Pin 416113. Tal. Panhala, Dist. Kolhapur, Maharashtra, India<sup>2</sup> Department of Microbiology, Smt. K.R.P. Kanya Mahavidyalaya, Islampur, Pin 415414 Tal: Walwa Dist: Sangli, Maharashtra, India

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## ABSTRACT

**Objective:** The aim of this experimental study was production, purification and characterization of alkali stable xylanase from locally isolated *Cellulosimicrobium* sp. MTCC 10645, which is an important industrial enzyme used in the pulp and paper industry. **Methods:** The enzyme was produced in Erlenmeyer flasks containing fresh basal salt medium supplemented with 1% oat spelt xylan. The enzyme was extracted and isolated using ammonium sulphate precipitation and dialysis. It was further purified using DEAE cellulose chromatography and purity was checked by SDS–PAGE. Effect of temperature and pH on activity and stability of enzyme was studied. The enzyme was also studied for its substrate specificity and kinetic parameters. **Results:** The isolate was identified on the basis of cultural, morphological, physiological and biochemical properties as well as 16S rRNA sequencing. Among the carbon sources tested, birchwood xylan found prominent for increased level of xylanase i. e. 96.33 U/ml. The enzyme was purified by DEAE cellulose chromatography at NaCl concentration of 0.25 M and had a molecular mass of 78.0 kDa. Xylanase was purified sixteen fold with a specific activity of 246.6 U/mg. Xylanase activity was maximum at 50°C. The enzyme was thermostable retaining 8% of the original activity after incubation at 60°C of 4 h. The enzyme was active over a pH range of 6.0–11.0, although its activity was optimal at pH 7.0. About 48.52% of the enzyme activity was retained after 4 h at pH 11.0. The enzyme was active on oat spelt and birchwood xylans but not on avicel, CMC, cellobiose, starch or p–nitrophenyl xylopyranoside. The xylanase had Km and Vmax values of 4.76 mg/ml and 232.5  $\mu$  mol/min/mg, respectively when birchwood xylan used as substrate. **Conclusions:** The xylanase showed a unique pattern of xylan hydrolysis releasing a large amount of intermediate products (xylotriose and xylobiose) with small quantity of xylose. Some of these characteristics make this enzyme potentially effective in xylan biodegradation and pulp bleaching.

## 1. Introduction

Xylan is one of the major components of hemicelluloses in plant cell walls, and is the second most abundant polysaccharide after cellulose [4]. In nature, complete hydrolysis of xylan requires the synergistic action of different xylanolytic enzymes, including endoxylanase,  $\beta$ –xylosidase, and accessory enzymes, such as  $\alpha$ –arabinofuranosidase, acetyl esterase and  $\alpha$ –glucuronidase. Among them, endo– $\beta$ –1,4–xylanase (EC 3.2.1.8) is very important to catalyze the hydrolysis of long–chain xylan into short xylooligosaccharides [4, 6]. In recent years, many kinds of xylanases have been isolated from various

microorganisms including bacteria, actinomyces, fungi and yeasts [1, 5, 9, 14]. Xylanase has been widely investigated and can potentially be adopted for hydrolysate production from agro–industrial wastes, improvement of the nutritional properties of agricultural silage, processing of food to increase animal feed digestibility and clarification of fruit juices [22]. Currently, the most important use of xylanase is in the prebleaching of kraft pulp to minimise the use of harsh chemicals in the subsequent stages of treatment [13]. To meet the specific industry's needs, an ideal xylanase should equip with specific properties, such as good pH and thermal stability, high specific activity. The present study describes production, purification and characterization of alkali stable xylanase from *Cellulosimicrobium* sp. MTCC 10645 isolated from soil samples collected at Kolhapur (M.S.), India. This enzyme shows superior pH stability from pH 6.0 to 11.0.

\*Corresponding author: Department of Biotechnology Engineering, Tatyasaheb Kore Institute of Engineering and Technology, Warananagar Pin 416113. Tal. Panhala, Dist. Kolhapur, Maharashtra, India;

Tel: 02328–224012, 224013; Fax: 02328–223507;

E–mail: [rajashri.kamble@gmail.com](mailto:rajashri.kamble@gmail.com)

## 2. Materials and methods

### 2.1. Strain isolation

*Cellulosimicrobium* sp. MTCC 10645 was isolated from soil samples containing decaying agro-residues collected at Kolhapur (M.S.), India. Serially diluted soil sample was inoculated for enrichment in oat spelt xylan medium of composition (g/L) oat spelt xylan 7.0, Yeast extract 1.0, NaCl 5.0, K<sub>2</sub>HPO<sub>4</sub> 1.0, MgSO<sub>4</sub> 0.2, CaCl<sub>2</sub> 0.1 by adjusting pH 7.0 for 24 h. To detect xylanolytic activity, xylan agar supplemented with dye labelled substrate, xylan-cibacron brilliant 3BA was used. Enriched soil samples were again diluted in sterile distilled water and spread inoculated on xylan-red agar. The agar plates were incubated at 40°C. An isolate yielding a clear hydrolysis zone on agar plate was used for subsequent studies. The isolate was investigated to determine morphological, physiological and biochemical characteristics following standard procedures [19]; It has been deposited in International Depository, Institute of Microbial Technology (IMTECH), Chandigarh, India.

### 2.2. Phylogenic analysis for strain identification

The partial 16S rRNA sequences were retrieved on NCBI server using BLAST tool. Top 15 similar sequences were downloaded in FASTA format from NCBI server. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using ClustalW2 program. A phylogenetic tree obtained was analyzed for closely related organism. The evolutionary history was inferred using the Neighbor-Joining method [17].

### 2.3. Effect of carbon source on xylanase production

The effect of various carbon sources on xylanase production was evaluated in triplicate by culturing isolate in basal salt medium at 40°C. Approximately 0.5% (w/v) of birch wood xylan, xylose, galactose, lactose, glucose, sucrose, carboxymethylcellulose and arabinose (all from Sigma) were separately added to the medium. Combination of above carbon sources with xylan (0.5% xylan and 0.25% other carbon source) was also studied. After 48 h of growth in shake flasks, the various cultures were centrifuged at 4°C and 10,000 g for 20 min. The xylanase activity of the supernatant was estimated as described later.

### 2.4. Xylanase assay

Xylanase activity was measured according to Bailey et al. (1992) [2]. A reaction mixture containing 900 µl of 1% solubilised birchwood xylan solution and 100 µl of enzyme solution was added in a test tube. 1.5 ml DNS reagent was added and incubated at 50°C for 5 min in water bath. The absorbance was measured at 540 nm. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of reducing sugars equivalent to xylose per minute under the assay conditions described.

### 2.5. Protein assay

Total soluble protein was measured according to Lowry et al. (1951) [12]. 1.0 ml of properly diluted enzyme was taken in a test tube and 5 ml alkaline copper reagent was added to it. Mixed well and allowed to stand for 10 min. 0.5 ml Folin's phenol reagent was mixed with above mixture and incubated in dark at room temperature for 30 min. The absorbance was measured at 660 nm. A standard graph was prepared using 0–1000 µg bovine serum albumin.

### 2.6. Enzyme production

1 ml (O.D. 1.0) of the suspension of MTCC 10645 was transferred to Erlenmeyer flasks containing 50 ml of fresh basal salt medium supplemented with oat spelt xylan with 1% (w/v). The flasks were shaken at 150 rpm for 48 h at 40°C. The culture supernatant was obtained following centrifugation at 5000 g for 10 min at 4°C. Ammonium sulfate was added to the supernatant to 80% saturation. The mixture was left overnight and then the precipitate was recovered by centrifugation at 10,000 g for 20 min at 4°C. The precipitate was dissolved in 50 mM phosphate buffer (pH 7.0) and dialysed against the same buffer for 48 h. Dialysis was performed using tubing cellulose membrane with a molecular weight cutoff of 13.0 kDa (Sigma). This dialysed fraction was the crude enzyme extract used for further purification.

### 2.7. Purification of xylanase

All purification steps were performed at 4°C except where otherwise noted. The crude enzyme extract was purified using DEAE-cellulose column chromatography. The column was packed with activated DEAE-cellulose equilibrated with 50 mM phosphate buffer (pH 7.0). The height of column was 20 cm with the 2.5 cm diameter. The protein was eluted with the 0.0 to 0.5 M NaCl gradient. The 50 fractions were collected having 5 ml volume of each fraction with the flow rate of 1 ml/min.

### 2.8. Molecular weight determination by SDS-PAGE

Purity of the fractions, showing xylanase activity, was checked by SDS-PAGE by the method of Laemmli [10]. The bands were visualized by coomassie brilliant blue R-250. The molecular weight standard contained the following protein markers: phosphorylase B (103.0 kDa), bovine serum albumin (81.0 kDa), ovalbumin (47.0 kDa), carbonic anhydrase (35.0 kDa) and soybean trypsin inhibitor (27.0 kDa).

### 2.9. Effect of temperature and pH on activity and stability

The optimal assay temperature of the purified xylanase was determined by assaying standard reaction mixture as described earlier at the indicated temperatures. To evaluate thermal stability, the enzyme solution was incubated at temperatures of 30–80°C for up to 4 h. The residual enzyme activity was recorded at 1 h intervals during 4 h period. The effect of pH on enzyme activity was determined by incubating xylanase at pH ranging from 6.0 to 12.0. The

various buffers used were 50 mM sodium phosphate (pH 6.0, 7.0), 50 mM Tris HCl (pH 8.0, 9.0), 50 mM carbonate bicarbonate buffer (pH 10.0) and 50 mM glycine–NaOH buffer (pH 11.0, 12.0). To evaluate the stability of the enzyme at each pH, the purified enzyme was incubated into the respective buffer over a pH range of 6.0–12.0 for up to 4 h at optimum temperature. The relative enzyme activity was determined at 1 h interval during the 4 h period of incubation. The enzyme activity was determined as described earlier.

### 2.10. Substrate specificity

Substrate specificity of the xylanase was studied by using 1% (w/v) xylan, cellobiose, starch, carboxy methyl cellulose (CMC), p–nitrophenyl xylopyranoside and avicel as substrates.

### 2.11. Kinetic parameters

Initial reaction rates using birchwood xylan as substrate were determined at substrate concentrations of 0.5–10 mg/ml in 50 mM phosphate buffer (pH 7.0) at 40°C. The kinetic constants  $K_m$  and  $V_{max}$  were estimated using the linear regression method of Lineweaver and Burk [11].

### 2.12. Release of hydrolysis products

To 50 ml of birchwood xylan suspension (1% of birchwood xylan in 50 mM Phosphate buffer pH 7.0), 40  $\mu$ g of xylanase enzyme was added and incubated at 40°C. Samples (5 ml) were removed at 5 min, 10 min, 30 min, 60 min and 2 h, 4 h, 8 h and 12 h. The unused polysaccharide from the sample was precipitated using isopropanol. The precipitate was removed by centrifugation and the supernatant containing the hydrolyzed products was collected. The supernatant was concentrated using rotary vacuum evaporator and made to a known volume. Hydrolysis products were detected by thin layer chromatography (TLC) [21]. TLC (TLC plates, 0.25 mm layers of silica gel F 254, Merck, India) was performed using the mixture of acetone: n–butanol: H<sub>2</sub>O (8:1:1 v/v) as a solvent system. Compounds were detected by spraying with ethanol–sulphuric acid mixture followed by heating at 150°C for 5 min.

## 3. Results

### 3.1. Isolate identification

The present isolate was screened from a soil sample, and showed the clear zone on agar plates supplemented with dye labelled substrate, xylan–cibacron brilliant 3BA, indicating that it secretes considerable amount of xylanase. Table 1 presents cultural, morphological, physiological and biochemical properties of the isolate. Identification of a portion of the 16S rRNA gene resulted in a 1408 bp sequence (accession number FR729925.1). A BLAST search of the 16S rRNA gene against the GenBank database revealed that the strain was 98% similar to *Cellulosimicrobium cellulans*

(accession number AJ784811.1). Hence, the isolate was considered to be a strain of the genus *Cellulosimicrobium* as shown in Figure 1. We propose this isolate be designated *Cellulosimicrobium* sp. The strain was submitted at microbial depository and assigned Microbial Type Culture collection (MTCC) no. 10645 by Institute of Microbial Technology (IMTECH), Chandigarh, India.

Table 1 Morphological, physiological and biochemical properties of *Cellulosimicrobium* sp. MTCC 10645

Figure 1 Phylogenetic tree of isolate *Cellulosimicrobium* sp. (MTCC 10645)

**Table 1.** Morphological, physiological and biochemical properties of *Cellulosimicrobium* sp. MTCC 10645

Tests	Characteristics	Tests	Characteristics
Colony morphology		Growth on NaCl (%)	
Configuration	Circular	2.0	+
Margin	Entire	4.0	+
Elevation	Raised	6.0	–
Surface	Moist	10.0	–
Colony color	Lemon–yellow	12.0	–
Opacity	Opaque	Biochemical tests	
Gram's reaction	Positive	Methyl red	–
Cell shape	Short rods	Voges Proskauer	–
Size ( $\mu$ m)	0.5–1.0	Casein hydrolysis	+
spores	–	Citrate	–
Motility	Motile	Indole	–
Anaerobic growth	Facultative	Gelatin hydrolysis	+
Morphological tests		Starch hydrolysis	+
Growth at temperatures		Esculin hydrolysis	+
4°C	+	Catalase test	+
15°C	+	Oxidase	–
25°C	+	Growth on MCA	–
30°C	+	Tween–40	–
37°C	+	Tween–60	–
40°C	++	Tween–80	–
55°C	–	Urease	–
Growth at pH		Arginine	–
5.0	–	Nitrate	+
6.0	+		
7.0	++		
8.0	+		
9.0	+		
10.0	+		
11.0	+		
12.0	–		

+: Positive; –: Negative

### 3.2. Effect of carbon source on enzyme production

Effect of various carbon sources on xylanase production is shown in Figure 2. Increased level of xylanase 96.33 U/ml was observed when *Cellulosimicrobium* sp. MTCC 10645 grown in medium supplemented with birchwood xylan (0.5% w/v).

Figure 2 Effect of different carbon sources on the production of xylanase by *Cellulosimicrobium* sp. MTCC 10645

**Table 2.**

Summary of Purification of xylanase enzyme isolated from *Cellulosimicrobium* sp. MTCC 10645

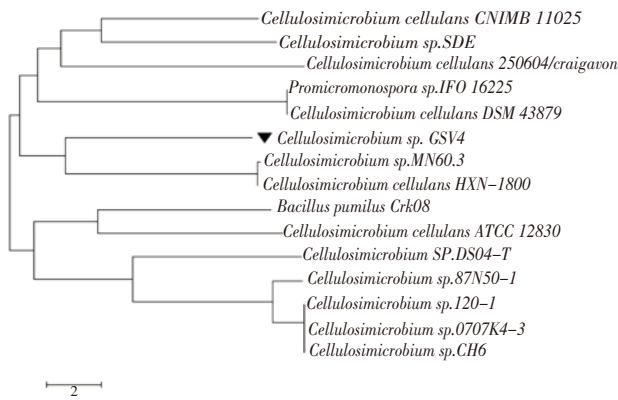
Purification step	Total Activity (U)	Protein (mg)	Sp. act (U/mg)	Purification fold	Yield (%)
Culture supernatant	182.0	12.0	15.16	1.0	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation (80%)	94.70	2.3	41.17	2.71	52.03
DEAE Cellulose	4.933	0.02	246.6	16.2	2.71

**Table 3.**

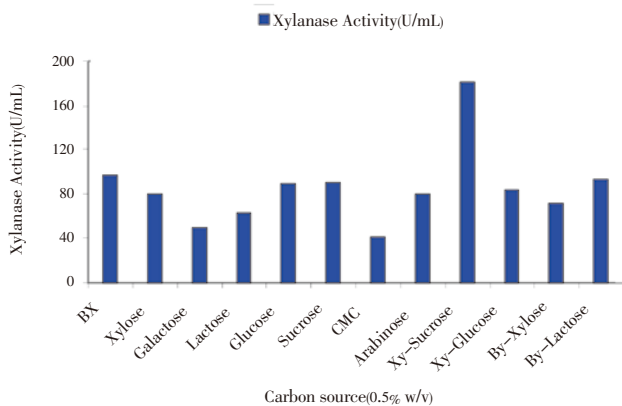
Substrate specificity of purified xylanase obtained from *Cellulosimicrobium* sp. MTCC 10645

Substrates	Xylanase activity (U/mg protein)
Oat spelt xylan	215.3 ± 0.40
Birchwood xylan	247.5 ± 0.35
Cellobiose	0.0 ± 0.0
Starch	0.0 ± 0.0
Carboxy methyl cellulose (CMC)	0.0 ± 0.0
p-Nitrophenyl xylopyranoside	0.0 ± 0.0
Avicel	0.0 ± 0.0

Note: Each value represents the mean ± standard error values

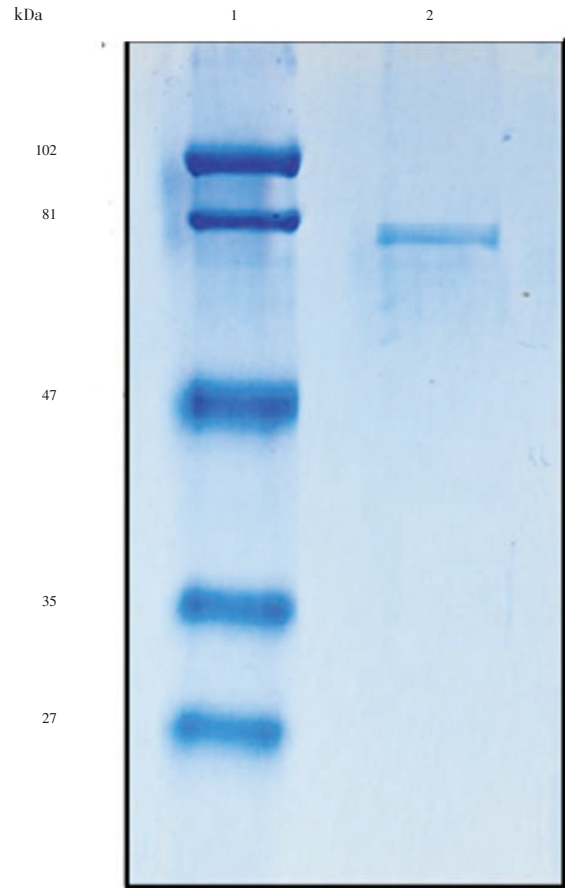
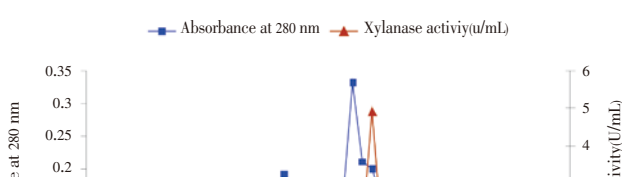


**Figure 1.** Phylogenetic tree of isolate *Cellulosimicrobium* sp. (MTCC 10645)



**Figure 2.** Effect of different carbon sources on the production of xylanase by *Cellulosimicrobium* sp. MTCC 10645. Birchwood xylan; CMC: Carboxy methyl cellulose; Xy: xylan

Sucrose was found to be the second best of the carbon sources used, producing 91.10 U/ml of xylanase. Xylan in combination with sucrose found stimulatory for increased production of xylanase.



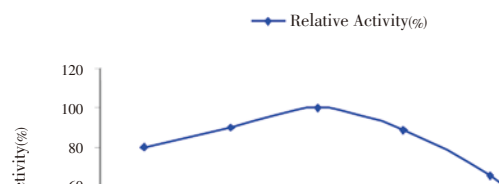
**Figure 4.** SDS-PAGE of the purified xylanase from *Cellulosimicrobium* sp. MTCC 10645. Proteins were separated on a 12.5 % SDS-polyacrylamide gel and stained with coomassie brilliant blue R-250: lane 1, Standard marker proteins; lane 2, the purified xylanase

**3.3. Enzyme purification**

Xylanase was produced by the culture grown on saline medium supplemented with 0.5% (w/v) birchwood xylan. The crude enzyme, obtained as a cell-free supernatant, was precipitated using ammonium sulfate to 80% saturation. The enzyme was eluted from DEAE cellulose column at a NaCl concentration of 0.25 M as shown in Figure 3. The fractions (no. 25–32) having maximum specific activity were concentrated. Xylanase was purified 16 fold with a specific activity of 246.6 U/mg. A summary of purification of xylanase enzyme isolated from *Cellulosimicrobium* sp. MTCC 10645 is given in Table 2.

Figure 3 Elution profile of xylanase from DEAE-cellulose column chromatography

Table 2 Summary of Purification of xylanase enzyme isolated from *Cellulosimicrobium* sp. MTCC 10645



3.4. Molecular weight determination

The purified xylanase obtained from *Cellulosimicrobium* sp. MTCC 10645 was subjected to SDS- PAGE as shown in Figure 4, which resulted into a single band. Compared with the standard protein markers, xylanase had a molecular weight of 78.0 kDa.

Figure 4 SDS-PAGE of the purified xylanase from *Cellulosimicrobium* sp. MTCC 10645

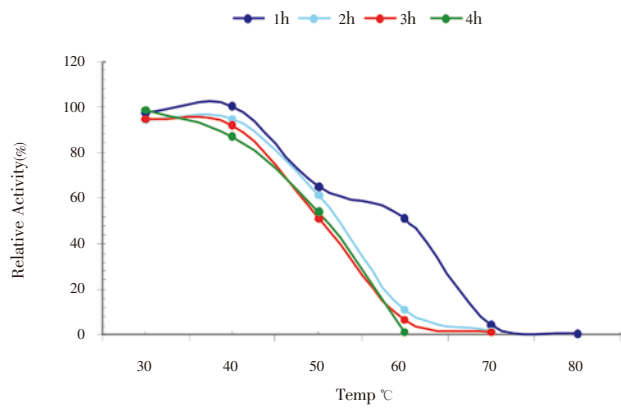


Figure 6. Effect of Temperature on stability of xylanase from *Cellulosimicrobium* sp. MTCC 10645

3.5. Effect of temperature and pH on xylanase activity and stability

Effect of temperature was studied on enzyme activity by varying the values from 30°C to 80°C. Figure 5 shows the effect of temperature on activity of xylanase from *Cellulosimicrobium* sp. MTCC 10645. From figure it was found that enzyme activity was 100% at 50°C. Xylanase activity was found to be gradually increased with increasing temperature and declined significantly at 80°C.

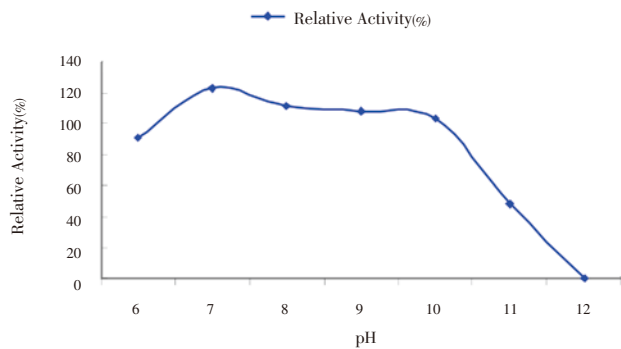


Figure 7. Effect of pH on activity of xylanase from *Cellulosimicrobium* sp. MTCC 10645

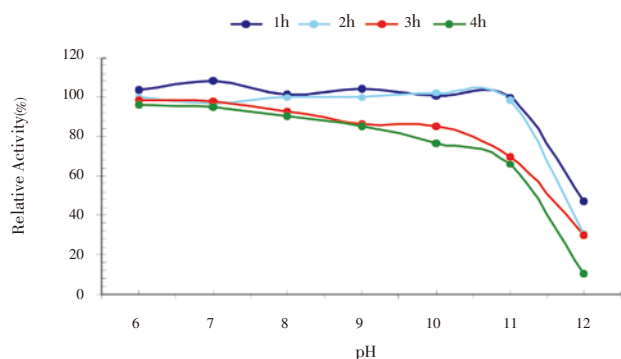


Figure 8. Effect of pH on stability of xylanase from *Cellulosimicrobium* sp. MTCC 10645

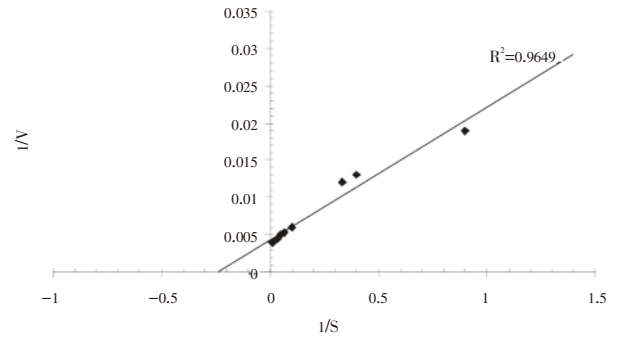


Figure 9. Double reciprocal plot for determining the Km and Vmax values of xylanase when acted on Birchwood xylan

Figure 5 Effect of Temperature on activity of xylanase from *Cellulosimicrobium* sp. MTCC 10645

Stability of the enzyme was the most important factor in studying characteristics. Xylanase obtained from *Cellulosimicrobium* sp. MTCC 10645 was more stable at 30°C and 40°C for 4 h of incubation and retained upto 86% of the activity. At 50°C temperature relative activity was comparatively constant upto 4 h of incubation. Above 50°C there was significant decline in xylanase stability as shown in Figure 6.

Figure 6 Effect of Temperature on stability of xylanase from *Cellulosimicrobium* sp. MTCC 10645

The effect of pH on activity and stability of xylanase from *Cellulosimicrobium* sp. MTCC 10645 is shown in Figures 7 and 8 respectively. The xylanase purified from *Cellulosimicrobium* sp. MTCC 10645 was active at a wide range of pH from 6.0 to 11.0. It showed highest activity at pH 7.0 and found more stable at pH 7.0 upto 4 h of incubation. About 48.52% of the enzyme activity was retained after 4 h at pH 11.0. The most desirable characteristic was its steady stability at alkaline pH.

Figure 7 Effect of pH on activity of xylanase from *Cellulosimicrobium* sp. MTCC 10645

Figure 8 Effect of pH on stability of xylanase from *Cellulosimicrobium* sp. MTCC 10645

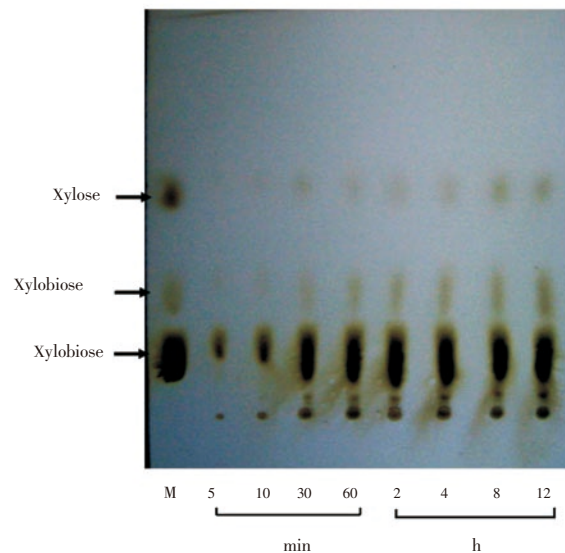


Figure 10. TLC analysis for hydrolysis products released from oat spelt xylan by xylanase from *Cellulosimicrobium* sp. MTCC 10645

### 3.6. Substrate specificity

The action of the purified xylanase towards various substrates was studied. The details of substrate specificity of purified xylanase obtained from *Cellulosimicrobium* sp. MTCC 10645 is summarized in Table 3. The enzyme was active on oat spelt and birchwood xyans but not on avicel, CMC, cellobiose, starch or p-nitrophenyl xylopyranoside. Purified xylanase was not active on avicel, CMC, cellobiose, starch and p-nitrophenyl xylopyranoside even when the enzyme concentration was 5 times greater than used in normal assay at an incubation period of 20 minutes rather than 5 minutes suggesting that enzyme is a true xylanase.

Table 3 Substrate specificity of purified xylanase obtained from *Cellulosimicrobium* sp. MTCC 10645

### 3.7. Kinetic parameters

The kinetic parameters  $K_m$  and  $V_{max}$  of the enzyme were determined from Lineweaver–Burk double reciprocal plots of xylanase activity at 40°C using various concentrations of birchwood xylan as substrate (Figure 9). The  $K_m$  values of xylanase were 4.76 mg/ml and  $V_{max}$  232.5  $\mu$  mol/min/mg respectively.

Figure 9 Double reciprocal plot for determining the  $K_m$  and  $V_{max}$  values of xylanase when acted on Birchwood xylan

### 3.8. Release of hydrolysis products

The hydrolysis patterns of soluble xylan by both enzymes were studied by thin layer chromatography. Figure 10 shows the TLC analysis for hydrolysis products released from oat spelt xylan by xylanase from *Cellulosimicrobium* sp. MTCC 10645. The enzyme showed an identical endo mode of action. Thus, as xylan degradation occurred, a large amount of intermediate products (xylotriose and xylobiose) was obtained, but a very small quantity of xylose appeared. Even for long (24 h) incubation periods, xylotriose was the most abundant product.

Figure 10 TLC analysis for hydrolysis products released from oat spelt xylan by xylanase from *Cellulosimicrobium* sp. MTCC 10645

## 4. Discussion

The present isolate was screened from a soil sample and identified *Cellulosimicrobium* sp. on the basis of cultural, morphological, physiological and biochemical properties as well as portion of the 16S rRNA gene. The strain was deposited and designated MTCC no. 10645 by Microbial type culture Collection, Chandigarh, India. The strain was screened for xylanase production on xylan red agar by observing zone of clearance. Effect of various carbon sources on xylanase production was studied for enhanced production of xylanase. Birchwood xylan (0.5% w/v) was found effective among all carbon sources. The behaviour

of the enzyme was similar to that of xylanases from other strains [6, 18, 22, 23]. Xylanase activities produced with the various carbon sources were in the order: xylan > sucrose > glucose > arabinose > xylose > lactose > galactose > CMC. Comparatively, lesser xylanase activity in the culture supernatant was obtained when the organism was grown on xylose and arabinose, the major constituents of xylan, than that produced by the culture grown on xylan. Garg et al. also observed no xylanase production by *Bacillus halodurans* MTCC 9512 when media supplemented with xylose, glucose and CMC [5]. The observations by Gupta and Kar stated that xylanase activity was not observed until the glucose was depleted from culture medium during the growth of *Streptomyces cyaneus* SN32 in medium containing both xylan and glucose [6]. These results are consistent with our observations. Xylanase was produced by growing culture in saline medium supplemented with 0.5% (w/v) birchwood xylan. The crude enzyme was precipitated using ammonium sulfate to 80% saturation. Xylanase was further purified by DEAE cellulose ion exchange column with 16 fold purity and specific activity of 246.6 U/mg. Menon et al. purified xylanase from *Bacillus pumilus* Strain GESF-1 obtaining 21.21 fold with a specific activity of about 112.42 U/mg protein [9]. Multistep purification procedures were also used for the purification of xylanase from *Aspergillus niger* [16]. In contrast, some workers have reported single step purification procedure like used in present work for *Bacillus stearothermophilus* T-6 [22]. The purified xylanase had a molecular weight of 78.0 kDa. Dhiman et al. reported that many xylanase belong to the category of low molecular weight (16–22.0 kDa) with basic pI or high molecular weight (43.0–50.0 kDa) with acidic pI [4]. Xylanase purified in the present work was suitable for second category of xylanases. Most of the xylanases are often found as monomeric protein with a molecular weight ranging from 11.0 kDa – 80.0 kDa [16]. Although, xylanases of molecular weight as high as 537.0 kDa [7], 350.0 kDa [8], 340.0 kDa [5], 212.0–253.0 kDa [14], 23–30 kDa [7] and as low as 5.5 kDa [7] have also been reported. The results from present study are in agreement with the result obtained by Knob et al. reported two different xylanases from *Penicillium sclerotiorum* of molecular weights 23.8 kDa and 30.8 kDa [7]. Enzyme activity was 100% at 50°C and found gradually increased with increasing temperature upto 80°C. Several xylanases were reported showing maximum activity at 50°C. Knob et al. reported two different xylanases purified from *Penicillium sclerotiorum* active at 50°C and 55°C [7]. Garg et al. characterized xylanase from *Bacillus halodurans* MTCC 9512 was most active at 55°C [5]. Xylanase was more stable at 30°C and 40°C for 4 h of incubation and retained upto 86% of the activity. At 50°C temperature relative activity was comparatively constant upto 4 h of incubation. The optimum temperature for xylanase activity in most of the bacteria studied ranges from 30°C to 60°C. Bacterial xylanases are more thermostable than fungal xylanases. Most of the thermostable xylanases are produced by mesophilic bacteria [15]. The xylanase purified from *Cellulosimicrobium* sp. MTCC 10645 showed highest activity at pH 7.0 and found more stable at pH 7.0 for 4 h of incubation. The most desirable characteristic was its steady stability at alkaline

pH showed 48.52% of the enzyme activity after 4 h at pH 11.0. Garg et al. purified alkali stable xylanase from *Bacillus halodurans* MTCC 9512 showed maximum activity at pH 9.5 [5]. Bai et al. isolated a thermophilic and alkaliphilic xylanase from *Alicyclobacillus* sp. A4 and reported that almost 100% activity shown by the xylanase at wide range of pH 5.5 to 9.0 [1]. Similar observation was reported by Ko et al., Ouyang et al. [8, 13]. Many of the alkaliphilic microorganisms studied have been found to produce xylanases with pH optima near neutral region but with relatively high activities being retained in alkaline conditions [1, 14, 16]. The enzyme was true xylanase acts on only oat spelt and birchwood xyans but not on avicel, CMC, cellobiose, starch or p-nitrophenyl xylopyranoside. Similarly, xylanase with absolute substrate specificity was purified from *Penicillium sclerotiorum* by Knob et al. [7]. Zhao et al. purified xylanase from *Paenibacillus* sp. 12–11 did not showed activity towards glycans such as starch, pachyman, avicel (microcrystalline cellulose) and carboxymethyl cellulose (CMC) [23]. The Km values of xylanase were 4.76 mg/ml and Vmax 232.5  $\mu$  mol/min/mg respectively. Wang et al. (2010) reported Km and Vmax values of xylanase isolated from *Bacillus* sp. NTU–06 were 3.45 mg/ml, and 387.3  $\mu$  mol/min/mg, respectively [51]. Menon et al. also reported kinetic properties of xylanase from *Bacillus pumilus* Strain GESF–1 such as Km and Vmax were 5.3 mg/mL and 0.42  $\mu$  mol/min/mL (6593.4  $\mu$  mol/min/mg protein) [9]. TLC analysis for hydrolysis products released from oat spelt xylan by xylanase showed large amount of intermediate products (xylotriose and xylobiose) and a small quantity of xylose. Analysis of hydrolytic products of xylan by the xylanase of white-rot fungi *Pleurotus* showed that xylan was degraded to various xylo-oligosaccharides without a significant accumulation of xylose [46]. Xylobiose and xylotriose were the main hydrolysis products when xylanase of *Bacillus* sp. NTU–06 reacted with oat spelt xylan and resulted oligosaccharides were then cleaved to form xylose by the  $\beta$ -xylosidase action [22].

### Conflict of interest statement

We declare that we have no conflict of interest.

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