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PURIFICATION AND CHARACTERIZATION OF AN ENDOXYLANASE FROM THE CULTURE BROTH OF *Bacillus cereus* BSA1

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An extracellular xylanase from the fermented broth of *Bacillus cereus* BSA1 was purified and characterized. The enzyme was purified to 3.43 fold through ammonium sulphate precipitation, DEAE-cellulose chromatography and followed by gel filtration through Sephadex G-100 column. The molecular mass of the purified xylanse was about 33 kDa. The enzyme was an endoxylanase as it initially degraded xylan to xylooligomers. The purified enzyme showed optimum activity at 55°C and at pH 7.0 and remained reasonably stable in a wide range of pH (5.0–8.0) and temperature (40–65°C). The K_m and V_{max} values were found to be 8.2 mg/ml and 181.8 μmol/(min mg), respectively. The enzyme had no apparent requirement of cofactors, and its activity was strongly inhibited by Cu^{++}, Hg^{++}. It was also a salt tolerant enzyme and stable upto 2.5 M of NaCl and retained its 85% activity at 3.0 M. For stability and substrate binding, the enzyme needed hydrophobic interaction that revealed when most surfactants inhihited xylanase activity. Since the enzyme was active over wide range of pH, temperature and remained active in higher salt concentration, it could find potential uses in biobleaching process in paper industries.

Xylan, the major component of hemicellulose, is present in plant cell wall as reserve carbon [1]. It is composed of a linear backbone of 1,4-β-linked-D-xylopyranosyl units that often has side chains of arabinosyl and methylglucuronyl substituents [2]. This backbone can be broken into small oligomers by the action of microbial xylanase (endo 1-4-β-D xylan-xylanohydrolase; EC 3.2.1.8). This microbial enzyme has tremendous industrial applications and along with cellulase and pectinase it occupies about 20% of global enzyme market [3]. The most important field for application of xylanase is in the process of biobleaching and biopulping. In these processes, xylanase hydrolyzes xylan and facilitates release of lignin from paper pulp and thereby reduces the use of bleaching agents like chlorinated substances [4, 5]. The application of xylanase in paper and pulp industries is not only cost effective but also it reduces about 40% pollution [2, 4]. Xylanase can also be applied in the saccharification of xylan from agro-wastes and agro-foods, and intensifies the preparation of bio-fuels, which is the potential use of this enzyme in modern biotechnology [6, 7]. The demand of pure xylanase is increasing day by day for production of rayon, cellophane, and several chemicals, such as cellulose esters like acetates, nitrates, propionates and butyrates, cellulose ethers like carboxymethyl cellulose, methyl and ethyl cellulose etc. [8].

A wide variety of microorganisms mainly bacteria and fungi are known to produce xylan degrading enzymes and among them members of Bacillaceae are more efficient for rapid production of the enzymes [9]. Previously we have isolated a potent xylanase producing bacteria *Bacillus cereus* BSA1 [10] from the garbage dump soil of Midnapur, West Bengal, India. The organism was cultured in liquid media and the secreted enzyme was purified and some of its important properties were investigated and reported in this article.

MATERIALS AND METHODS

Source of xylanase. Previously isolated and identified a bacterial strain, *Bacillus cereus* BSA1 [10] has been used in this study for enzyme production. Production of xylanase was made in 250 ml Erlenmeyer flasks containing 50 ml of liquid media that composed of (%): xylan – 0.5; NaH_2PO_4 – 0.05; NH_4NO_3 – 0.1; $MgSO_4$ – 0.05 and NaCl – 0.5. In this enriched culture media 1% (v/v) freshly prepared inoculum was added and fermented in a rotatory shaker (120 rpm) at 35°C for 72 h. The cell-free supernatant after centrifugation (5000 g for 10 min) was used as the source of crude enzyme.

Purification and determination of molecular weight of xylanase. The culture supernatant was brought to 80% saturation of ammonium sulphate and after overnight preservation at 4°C, the precipitated enzyme was separated by centrifugation (10000 g for 30 min). Precipitate was dissolved in phosphate buffer (0.02 M, pH 7.0) and the enzyme solution was dialyzed against the same buffer for 48 h. The concentrated enzyme was loaded onto a DEAE cellulose column (3 × 20 cm) and active enzyme was eluted with a linear gradient of NaCl (0.25–1.5 M). Finally the partially purified enzyme was passed through Sephadex G-100 column (2 × 30 cm) and eluted at a flow rate of 0.2 ml/min with 0.02 M phosphate buffer (pH 7.0). The fractions

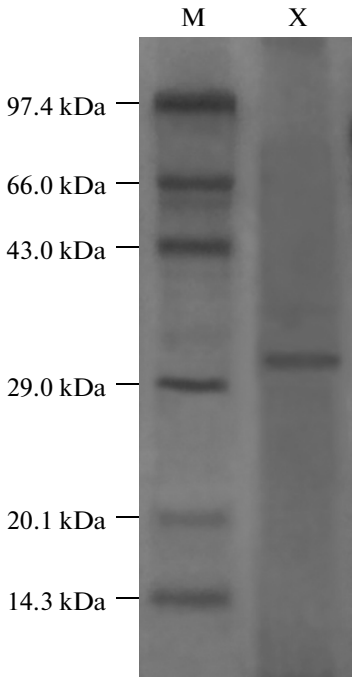

Fig. 1. SDS-PAGE analysis of purified xylanase from *B. cereus* BSA1: M – molecular weight markers; X – purified xylanase.

with enzyme activity were collected, concentrated by lyophilization and used for further experiments.

To analyze the purity and to determine the molecular weight of the enzyme, the active fraction from Sephadex G-100 column was loaded onto sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) following the method of Laemmli [11]. The molecular weight of the bacterial xylanase was determined by comparing its mobility with the medium range marker proteins (14.3–97.4 kDa).

Xylanase assay. Xylanase activity was measured by estimating released reducing sugar from the birch wood xylan (Fluka, USA) with 3,5-dinitrosalicylic acid [12]. The reaction mixture containing 0.4 ml phosphate buffer (0.2 M, pH 7.0), 0.4 ml of 1% (w/v) xylan solution and 0.2 ml enzyme solution. The enzymatic reaction was carried out at 55°C for 30 min and then stopped by the addition of 1ml of DNS (3%, w/v). The solution was then boiled in a water bath for colour development and the absorbency was measured at 540 nm (Systronics spectrophotometer 105, India) against the enzyme blank. Xylanase activity was determined by using a calibration curve of D-xylose (Sigma, USA).

One unit of xylanase activity (IU) was defined as the amount of enzyme required to produce 1μmol of reducing sugars as xylose by hydrolyzing xylan per min under the above assay conditions.

Effect of temperature on enzyme activity and stability. To determine the optimum temperature for enzymatic activity, the catalytic reactions were carried out at different temperatures ranging from 30 to 65°C in a controlled water bath.

Thermostability of the enzyme was studied by preincubating it (without substrate) at different temperature (40–65°C) and then residual activity was measured at standard assay conditions.

Effect of pH on enzyme activity and stability. The enzymatic reactions were carried out at different pH (4.0–10.0) by varying the type of reactant buffers like sodium acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0) and carbonate – bicarbonate buffer (pH 9.0–10.0). Stability of the enzyme at different pH was studied by incubating the enzyme (without substrate) at different pH ranging from 4.0 to 10.0 for 24 h at 4°C and then activity was measured at optimum conditions.

Kinetic parameter. The K_m and V_{max} of the purified xylanase were determined by using different concentrations of birch wood xylan as substrate following the Lineweaver-Burk plot [13].

Effect of metal ions and additives on xylanase activity. Different metal ions and surfactants (1–10 mM) were added to the enzyme substrate reaction and studied their influences on the rate of catalysis.

Some of the common enzyme inhibitors like sodium azide, urea, sodium arsenate, iodoacetate, cysteine were also tested (1–10 mM) to study their effect on xylanase activity.

Salt tolerance. Salt stability of the enzyme was studied by incubating the enzyme with different concentration of NaCl (0.5–5.0 M) for over night at 4°C and then activity was measured at standard conditions.

Time course of xylan hydrolysis by purified xylanase. The periodic hydrolytic products of xylan by the action of purified xylanase were examined under the standard assay conditions. The hydrolytic products were examined in paper chromatography by using a solvent system of n-butanol: acetic acid: water (4 : 1 : 5).

RESULTS AND DISCUSSION

Purified microbial enzymes have attracted attention of biotechnological industries due to high selectivity and efficiency. The demand and applications of purified xylanase in the paper, food and chemical industries are increasing day by day.

In the present study xylanase from the culture broth of *B. cereus* BSA1 was purified by conventional methods like ammonium sulphate precipitation, DEAE-cellulose column chromatography and finally by gel filtration through Sephadex G-100 column. The efficiency of the purification processes was summarized in Table 1. The procedure followed in this study had made 3.43-fold purification with 22.15% yield. Kiddinamoorthy et al. [7] also reported 27% recovery of xylanase from *Bacillus* sp. GRE7 by using DEAE-cellulose column chromatography followed by Sephadex G-75 gel filtration.

Table 1. Purification steps of xylanase from *B. cereus* BSA1

Purification steps	Xylanase activity, IU	Protein, mg	Specific activity, IU/mg	Purification, fold	Yield, %
Culture broth	5200	186	27.45	1	100
$(NH_4)_2SO_4$ precipitation	2236	65	34.4	1.23	43
DEAE-cellulose column	1612	27	59.7	2.13	31
Sephadex G-100	1152	12	96	3.43	22.15

Homogenity of the purified xylanase was tested by PAGE and subsequently gel staining by coomassie brilliant blue revealed only one band (Fig. 1). The apparent molecular mass of the xylanase was estimated to 33 kDa. Most of the xylanases from *Bacillus* sp. were reported to have the molecular weight within the range of 15 to 49 kDa [5].

The optimum temperature of xylanase from *B. cereus* BSA1 was found to be 55°C (Fig. 2a) and the enzyme was completely stable at 40°C, only looses 25% activity when incubated at 55°C for 1 h (Fig. 2b). Biobleaching with xylanase at high temperature improves pulp quality (degree of delignification) and brightness (bleaching effectiveness) [14]. Current research in the area of xylanase has been focused on developing new xylanse preparations that are able to work at high temperature (approximately 60°C) [15–17]. In this respect xylanase from *B. cereus* BSA1 can be qualified as a suitable enzyme in prebleaching process.

Xylanase from *B. cereus* BSA1 showed maximum activity at pH 7.0 (Fig. 2c). It was also stable at pH 7.0 and loosed only 18 and 28% activity at pH 5.0 and 8.0 respectively (Fig. 2d). This pH probably helped proper ionization of amino acid residues in the active site of enzyme to make strong bonding with the substrate [18]. This wide range of pH stability of the enzyme has placed it in high rank in relation to other xylanase from actinomycetes and bacteria [19–23].

The purified xylanase showed K_m and V_{max} of 8.2 mg/ml and 181.8 μmol/(mg min) respectively (Fig. 3). These kinetic parameters were found to be comparable to other reported to xylanase produced by *Bacillus* (Table 2).

Enzyme generally requires a specific activator for expressing their absolute catalytic power. Cofactor mediated activation is important in industry in order to achieve total catalytic efficiency. From this experiment it was found that calcium, magnesium and po-

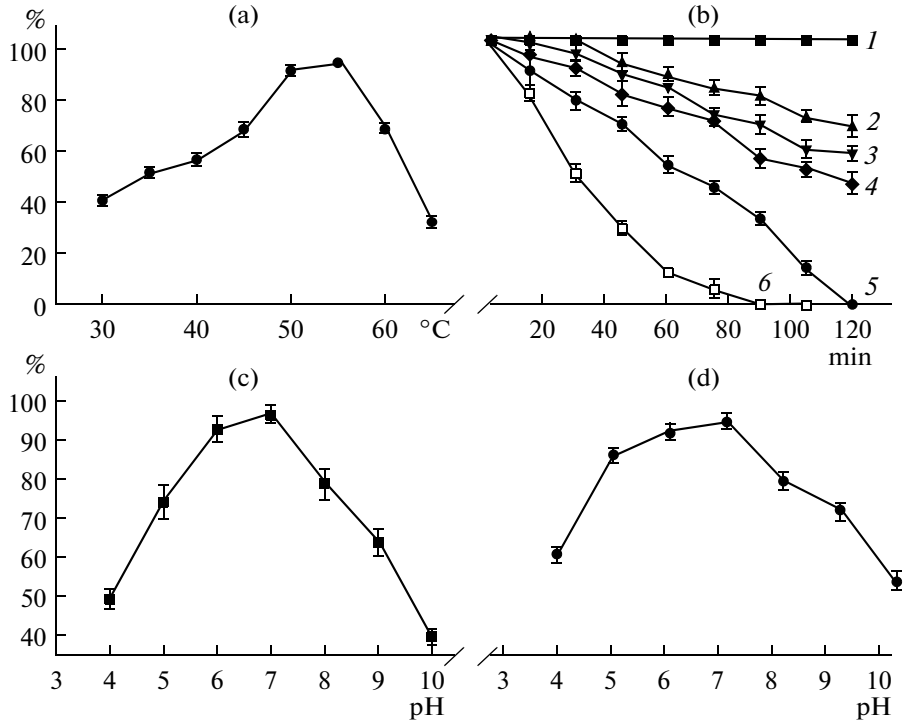

Fig. 2. Effect of temperature on activity (a), stability at 40 (*1*), 45 (*2*), 50 (*3*), 55 (*4*), 60 (*5*), 65°C (*6*) (b); and effect of pH on activity (c), stability (d) of purified xylanase from *B. cereus* BSA1.

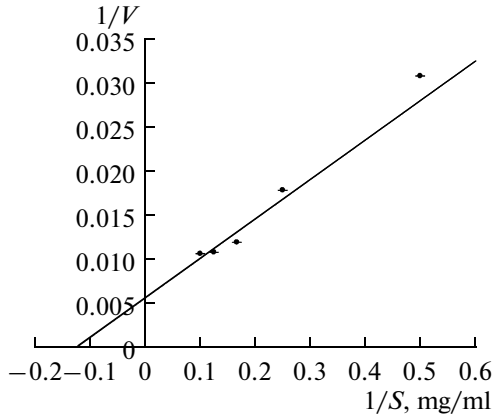

Fig. 3. Double reciprocal plot for determination of V_{max} and K_m of purified xylanase against birch wood xylan at 55°C.

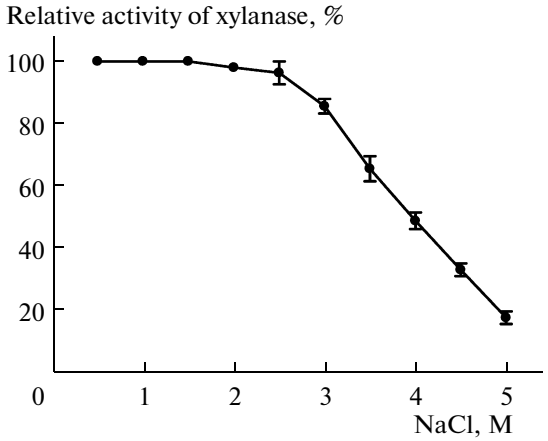

Fig. 4. Salt tolerance capability of xylanase from *B. cereus* BSA1.

tassium ions had small stimulatory effects, whereas zinc, manganese, ferrous, nickel and other heavy metals showed some inhibition on the activity of xylanase (Table 3). Sodium ion showed non-significant stimulation or inhibition. The catalytic inhibition by heavy metals may be due to their interaction with sulfhydril groups in active site of the enzyme [24]. The enzymatic activity was not changed by the addition of EDTA, a metal chelating agent (result not incorporated). This result indicates that the enzyme needs no such metal for its activation or it is devoid of any co-factor. Similar type of result had also been shown by Khasin et al. [25] in case of xylanase from *Bacillus stearothermophilus* T-6.

Common enzyme inhibitors prevented the catalytic efficiency of the purified xylanase at a considerable level (Table 4). The inhibition of xylanase activity was noted by addition of surfactants like SDS, tween 40, tween 60, tween 80 and tween 100 (Table 5). This indicated that hydrophobic interaction is essential for

Table 2. Comparative accounts of characters of xylanase of *B. cereus* BSA1 with some other bacterial xylanases

Characters	Xylanase from *B. cereus* BSA1	Xylanase from *Bacillus* sp. GRE7 [7]	Xylanase from *Arthrobacter* sp. MTCC 5214 [24]	Xylanase from *Acido-bacterium capsulatum* [30]	Xylanase from *Bacillus* sp. BP-7 [31]	Xylanase from *B. poly-myxa* CECT 153 [19]	Xylanase from *B. stearother mophilus* T-6 [25]	Xylanase from *Bacillus* sp. NCIM 59 [19]
Molecular weight, kDa	33	42	20	41	22–120	61	43	15.8, 35
Temperature for optimum activity, °C	55	70	100	65	55	50	75	50–60
pH for optimum activity	7	7	9	5	6	6.5	6.5	6
Temperature stability, °C	40	60–80	–	20–50	65	–	–	50
pH stability	5–8	5–11	7–8	3–8	8–9	–		7
K_m, mg/ml	8.2	2.23	0.9	3.5	–	17.1	1.63	1.58, 3.50
V_{max}, μmol/(mg min)	181.8	296.8	3571	403	–	112	288	0.017, 0.742
Requirement of cofactors	Not required	Mn^{++}, Co^{++}	–	–	–	–	Not required	
Inhibitory metal ion(s)	Cu^{++}, Hg^{++}	Cu^{++}, Fe^{++}	Hg^{++}	–	–	–	Zn^{++}, Cd^{++}, Hg^{++}	–
Salt tolerance	Upto 2.5 M of NaCl	–	–	–	–	–	–	–

Table 3. Effect of metal ions on activity of xylanase, from *B. cereus* BSA1

Salt	Relative activity of xylanase, %		
	1 mM	5 mM	10 mM
$BaCl_2$	100	98	95
Na_2MoO_4	93	98	100
KCl	94	102	106
$MnCl_2$	100	98	85
$ZnCl_2$	101	78	41
$CuSO_4$	48	35	22
$FeSO_4$	87	78	63
$Ni(NO_3)_2$	81	71	61
NaCl	100	102	102
$CaCl_2$	98	102	105
$HgCl_2$	42	35	15
$MgCl_2$	95	93	104
Control	100		

Table 4. Effect of some inhibitors on activity of xylanase, from *B. cereus* BSA1

Inhibitor	Relative activity of xylanase, %		
	1 mM	5 mM	10 mM
Sodium azide	81	70	45
Urea	60	75	73
Sodium arsenate	67	41	36
Iodoacetate	39	33	25
Cysteine	101	98	79
Control	100		

folding of protein's structure or enzyme-substrate interaction [26]. Another cause could be the alteration in substrates moiety by reaction with surfactants [27].

The enzyme did not loose its original activity even at 2.5 M NaCl solution and retained about 74 and 20% of activity at 3.0 and 5.0 M of salt respectively (Fig. 4). This salt-tolerance property of the enzyme will be very useful

Table 5. Effect of surfactants on activity of xylanase, from *B. cereus* BSA1

Surfactant	Relative activity of xylanase, %		
	1 mM	5 mM	10 mM
SDS	51	34	28
Tween 40	84	70	35
Tween 80	61	51	40
Tween 100	39	22	20
Triton 100	53	31	23
Control	100		

in pollution control mechanisms for the treatment of hemicellulosic effluent in hard water [28].

Paper chromatographic study of the periodic hydrolysis of xylan by the purified xylanase was revealed that the enzyme initially splitted xylan into xylo-oligomers, which were further hydrolyzed into xylose (data not incorporated). This indicated that the studied xylanase was an endoacting enzyme. Our finding is also consistent with the finding of Khandeparkar and Bhosle [24] and Kar et al. [29]

The characteristic features of the purified xylanase from *B. cereus* BSA1 has been compared with the xylanases of other bacteria and represented in Table 2. It was found that some characters of the studied xylanase are more or less similar to other. The uniqueness of the xylanse from *B. cereus* BSA1 is that it can able to tolerate 2.5 M of NaCl.

In conclusion, it can be said that the purified extracellular xylanase from *B. cereus* BSA1 was found to be a novel as it is salt tolerant, wide range of pH tolerant and moderately thermo-tolerant enzyme and, these properties would make this enzyme potentially very effective for industrial applications.

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