

CHARACTERIZATION AND CATALYTIC PROPERTY OF XYLAN DEGRADING ENZYME FROM MICROBIAL SOURCE

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ABSTRACT

Xylan degrading enzyme xylanase was extracted, purified and characterized from *Bacillus megaterium* SV1. The enzyme was purified to homogeneity by ammonium sulphate precipitation, gel permeation and ion exchange chromatographic techniques. During the series of steps, 28.5fold purification was obtained with 72.7U/mg specific activity of xylanase and the corresponding molecular weight of the enzyme was identified as 24kDa. The optimum conditions for maximal enzyme activity were identified at pH-8.0, temperature 40°C and with 5% of sodium chloride. It is observed that the reactants like calcium chloride, Dithiothreitol, β -mercaptoethanol was found to enhance the activity of enzyme and Mercuric chloride strongly inhibits the enzyme activity. Degradation of Birch wood Xylan was investigated to determine the kinetic parameters K_m and V_{max} values and was found to be 6.1mg/ml and 280 μ mol/min/mg respectively. It is also verified that the enzyme Xylanase extracted from *Bacillus* SV1 was identified as alkalophilic and halotolerant.

Keywords: Purification, Characterization, xylanase, *Bacillus megaterium* SV1.

INTRODUCTION

Hemicelluloses is a matrix of polysaccharides[1-3] contains major component as xylan and considered to be the second most abundant resource which can be renewable. Xylan is a type of heteropolymer, backbone of β -1,4 linked D-xylose, arabinose, glucuronic acid, mannose as branches. It is ubiquitous cellulose in plant cell walls and contains predominantly β -D-xylose units linked as in cellulose. Xylans are particularly important for the re-usage of vegetable wastes, as it contains 30% of the plant cell wall in hardwoods and crops. During the bioconversion, xylan will be converted to xylose (or) xylo-oligosaccharides. The core chain will be hydrolyzed by the action of xylanase and release xylo-oligosaccharides with different sizes [4,5].

Hydrolysis of xylan results in endo β -1,4-xylanase, β -xylosidase, α -L-arabinofuranosidase, α -glucuronidase and acetyl-xylan esterase.[6,7] The endo β -1,4-xylanase is dominating in depolymerization of xylan and gained much attention from the scientists, in the field of pulp and paper industries. [8,9]

The enzyme xylanase represents, one of the major group of enzymes used in industries with high marker demands and finds application in Kraft pulp pre-bleaching, agricultural wastes bioconversion, coffee and oils from the plant extraction, nutritional property improvement in agricultural silage, plant fiber degumming, etc.[10-11] Microorganisms include bacteria, fungi, yeast and actinomycetes have been reported to produce xylan degrading enzymes.[12,13] *Bacillus* is found to be the major producer of industrially important enzymes[14]. There are different ranges of xylanase from *Bacillus* such as acidic [15] (or) alkaline.[16,17] In the present research paper deals with the purification and characterization of alkalophilic moderate halotolerant xylanase producing *Bacillus* SV1.

MATERIALS AND METHODS

All the chemicals and reagents used were of analytical grade. Commercial Birchwood xylan, Sepharose and Sephadex were purchased from Sigma (St. Louis, USA). All the experiments were performed independently in triplicate and the results given were the mean of three values obtained.

Microorganism and culture conditions: Alkalophilic, moderate halotolerant xylanase producing strain *B. megaterium* strain SV1 was isolated and characterized previously (data not published). The

isolate was identified by biochemical and molecular analysis and the sequence was submitted in Gene bank and the Accession number given was JN015193. Birchwood contains xylan 5g/L, yeast extract 5g/L, peptone 5g/L, KH₂PO₄ 1g/L, MgSO₄·7H₂O 0.1g/L (pH-8.0).

Partial Purification of Xylanase

Ammonium Sulphate precipitation

To the cell free supernatant ammonium sulphate was added and made up to 80% saturation. The precipitate was centrifuged at 10,000 rpm for about 10min at 4°C. The collected precipitate was dissolved in 50mM phosphate buffer (pH-7.5) and tested for their total protein content and enzyme activity. The precipitate was dialyzed against the same buffer and the dialyzed material was lyophilized and suspended in 1ml of 50mM phosphate buffer (pH-7.5). The suspension was applied to Sephadex G-100 (2X50cm) which is previously equilibrated and the samples were eluted with the same buffer. The flow rate maintained was 8.0ml/hr. The different fractions (1ml) were collected and all the collected fractions were tested for protein content at 280nm for enzyme activity using spectrophotometer. Single positive peak was obtained which was pooled together, lyophilized and used for further steps. All the assays were done in triplicate and the Standard Deviation values were found to be less than 0.05%.

Ion exchange chromatography: The lyophilized enzyme from gel filtration chromatography was loaded to Q-Sepharose (2.0X50cm), pre-equilibrated with 50mM phosphate buffer (pH-7.5) at a flow rate of 3 ml/min with NaCl salt gradient (0 to 0.5M) in the buffer. The active fractions were pooled together and concentrated.

Native PAGE

Electrophoresis was carried at 10% acrylamide. For identification of molecular mass bovine serum albumin (66kDa), egg albumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), trypsinogen (24kDa) and α -lactalbumin (14.2kDa) was used. The gel was stained with Coomassie Brilliant Blue R-250.

Characterization of Enzyme

Effect of pH and temperature

Ammonium sulphate precipitated enzyme was used for the characterization of enzyme. The partially purified enzyme (40U) was pre-incubated with different pH buffers (6.0 to 10.0), different temperatures (30, 35, 40, 45, 50, 55 and 60°C). The residual activity was measured under standard assay conditions.

Effect of NaCl, metal salts, surfactants

Effect of sodium chloride was determined by incubating the enzyme (40 U) at different sodium chloride concentration (0, 1, 3, 5 and 7%). Enzyme (40U) was incubated with metal ions includes $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, FeSO_4 , CoCl_2 , $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, HgCl_2 at 2mM concentration. Surfactants like SDS, Triton X-100, Tween 20 and 80, β -mercaptoethanol and dithiothreitol at 2mM concentration was mixed with the enzyme. The residual activity was measured after incubation, enzyme without any additives was taken as 100%.

Kinetic analysis

For the determination of efficiency (k_m) and catalytic properties (V_{max}) of enzyme, the enzyme was incubated with different concentration of birch wood xylan (0.5 to 20 mg/ml). Effect of enzyme (0.2 to 3.0 mg/ml) on constant substrate concentration (2.0 mg/ml) was determined at pH 8.0, 40°C.

Substrate Specificity

The xylanase activity was analyzed in different substrates carboxy methyl cellulose (CMC), and starch.¹⁸ Partially purified enzyme (40 U) was incubated with substrates 1% at pH 8.0 at 40°C

Color removal from synthetic kraft effluent

Synthetic kraft effluent was prepared by suspending the saw dust in distilled water containing bleaching powder (4mg/ml) and sodium hydroxide (0.1 M), pH adjusted to 10.0 to 11.0 and autoclaved, the extract was then filtered in fine muslin cloth.¹⁹ The prepared kraft pulp at different concentration (0.2, 0.4, 0.6, 0.8 and 1%), measured for absorbance at 465nm, was treated with xylanase (40U) and incubated at pH-8.0, 40°C for about 24hrs. After incubation, absorbance value was measured at 465nm to check for color reduction.

RESULTS AND DISCUSSION

Purification of Enzyme

The cell free culture supernatant was precipitated by the addition of ammonium sulphate and made up to 80% saturation (Table 1). Specific activity obtained was 31.87 U/mg with 2.3 fold purification fold. Then the precipitate was purified in gel permeation chromatography (Fig. 1) and resulted in 56 U/mg of specific activity. The enzyme was further purified in ion exchange chromatography (Fig. 2) yielded 5.3 fold purification with 72U/mg of specific activity.

Table 1: Summary of purification steps of xylanase from *Bacillus SV1*

Purification	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Purification fold	Yield
Crude extract	56	4.1	13.65	1	100
NH_4Cl precipitation	51	1.6	31.8	2.33	91
GPC	28	0.5	56	4.1	50
Ion Exchange chromatography	16	0.22	72.2	5.32	28.5

*GPC-Gel permeation Chromatography

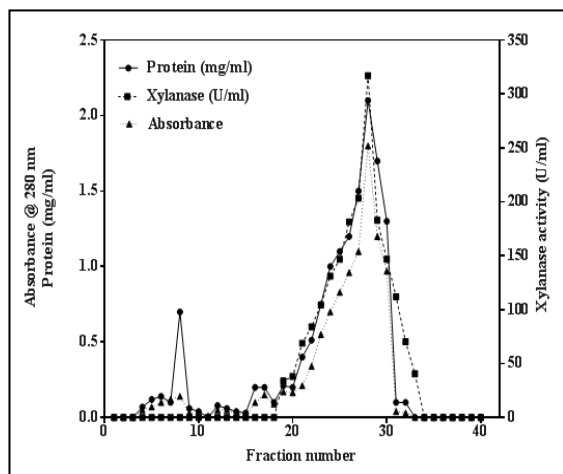


Fig. 1: Fraction elution profile of xylanase from *Bacillus SV1* in Gel permeation chromatography

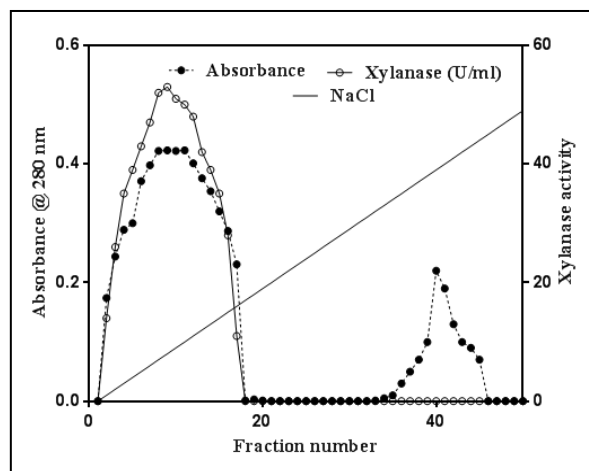


Fig. 2: Fraction elution profile of xylanase from *Bacillus SV1* in Ion Exchange Chromatography

Molecular weight determination

The partially purified enzyme obtained from ion exchange chromatography was applied to SDS-PAGE for molecular weight determination. The molecular mass for the xylanase enzyme was 24 kDa. Single band was obtained showing the purified enzyme was a monomer consisting of single polypeptide. This result is in accordance with the previous reports *B. circulans* AB16[20], *Bacillus* sp. strain TAR-1[21], *Bacillus licheniformis* A99[22], *Arthrobacter*[23] and *Paecilomyces themophila*[24]. In contrast, larger molecular weight 55kDa has been reported[25].

Effect of pH and Temperature

Influence of pH on enzyme activity was checked for xylanase from *B.megaterium* SV1 by incubating the enzyme at different pH buffers (6.0 to 10.0). Maximum activity was obtained at pH 8.0 (Fig. 3 & 4) was taken as 100%. This result is in accordance with a study showing optimum activity at 8.0 for *Bacillus arseniciselenatis*[26]. At pH 10.0 and 6.0, 81 and 75% of activity was retained. Thus the enzyme possess broad range of pH for its activity and could better used in potential application. Most of reported xylanase showed optimum pH in acidic or slight alkaline condition [27,28]. Effect of temperature on enzyme activity was studied at different temperatures (30, 35, 40, 45, 50, 55 and 60°C). Highest activity was observed at 40°C was taken as 100%. This is in accordance with a study[29,30] showing optimum activity at 40°C. In contrast, a report[31] showed maximum activity at 80°C.

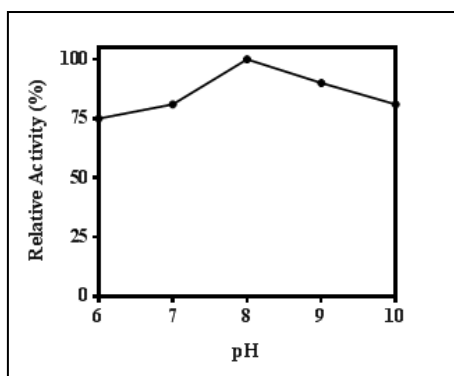


Fig. 3: Effect of pH on enzyme activity by Bacillus SV1 incubated at 40°C.

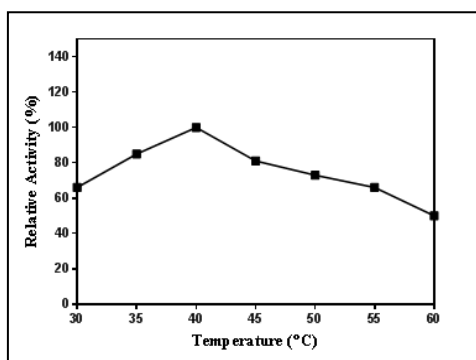


Fig. 4: Effect of temperature on enzyme activity by Bacillus SV1 incubated at pH-8.0

Effect of NaCl, Metal salts and surfactants

Effect of sodium chloride was tested in different concentration (0, 1, 3, 5 and 7%). Maximum activity was seen with 5% NaCl (Fig. 5) suggesting it as moderate halotolerant enzyme. Higher salt concentration decreased the enzyme activity.

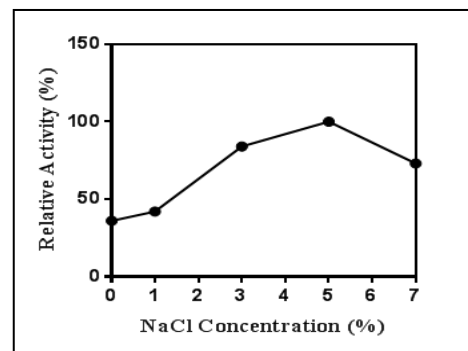


Fig. 5: Effect of NaCl concentration on enzyme activity by Bacillus SV1 incubated at pH 8.0, 40°C.

Metal salts includes CaCl_2 , MgSO_4 , FeSO_4 , CoCl_2 , MnSO_4 , ZnSO_4 , HgCl_2 at 2mM concentration and surfactants like SDS, Triton X-100, β -mercaptoethanol and dithiothreitol at 2mM was tested for the effect on enzyme activity. The activity of the control (without addition of additives) was taken as 100%. CaCl_2 , ZnSO_4 increased the enzyme activity (Table 2). This is in accordance with a previous report[32].

Table 2: Effect of additives on enzyme activity by Bacillus SV1 xylanase

Additives	Relative Activity (%)
Control	100
CaCl_2	107
MgSO_4	100
FeSO_4	101
CoCl_2	99
MnSO_4	100
ZnSO_4	110
HgCl_2	76
SDS	81
Triton X-100	94
DTT	101
β -mercaptoethanol	105

FeSO_4 , CoCl_2 , MnSO_4 and MgSO_4 had neutral effect on enzyme activity[33]. HgCl_2 inhibited the enzyme activity which is correlating with standard protocol[34], showing inhibiting effect of HgCl_2 . The reason for inactivation was the reaction of ions with sulphhydryl groups and cysteine residue in the active site of the enzyme[35]. β -mercaptoethanol and DTT had increased the enzyme activity. This is similar with a study reported highest activity for xylanase from *Bacillus* sps[36]. EDTA has neutral effect on enzyme activity it neither reduced nor increased the activity. SDS, Triton X-100 had reduced the xylanase activity.

KINETIC ANALYSIS

The K_m and V_{max} values were determined by incubating the enzyme at different concentration of birch wood xylan (0.5 to 20 mg/ml) and measured for enzyme activity. The K_m and V_{max} value was found as 6.1 mg/ml and 280 $\mu\text{mol}/\text{min}/\text{mg}$ respectively. The values are consistent with the previous reported range[37] for microbial xylanases (0.27-14 mg/mL^{-1}). The result is correlated with previous reports for *Bacillus arseniciselenatis* 5.26 mg/mL (K_m) and 277.7 $\mu\text{mol}/\text{min}/\text{mg}$ (V_{max}) value[26]; *Bacillus* sp. NTU-06, 3.45 mg/mL and 387.3 $\mu\text{mol}/\text{min}/\text{mg}$ [38] as shown in the fig. 6. Effect of enzyme concentration (0.2 to 3.0 mg/ml) was studied at constant substrate concentration (2.0 mg/ml). Up to 1.6 mg/ml of enzyme concentration, the activity was increased and then the activity becomes constant.

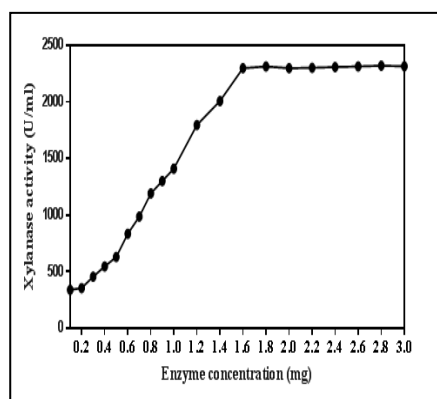


Fig. 6: Effect of enzyme concentration on constant substrate concentration (2 mg/ml) by *Bacillus* SV1 incubated at pH 8.0, 40°C.

Color removal from synthetic kraft effluent

The bio-bleaching efficiency of xylanase enzyme from *Bacillus* sps was tested. Increase in kraft effluent concentration, the color removal efficiency was also increased. Maximum reduction was seen in 1% kraft effluent (Table. 3).

Table 3: Color Removal efficiency of xylanase on synthetic kraft effluent

Kraft Concentration	Effluent	Absorbance at 465 nm (Color)	
		Initial	Final
0.20%		1.423	0.45
0.40%		1.616	0.538
0.60%		1.856	0.557
0.80%		2.67	0.622
1%		3.108	0.811

Substrate specificity

Specificity of enzyme on different substrates was determined. There was no activity on the tested substrates CMC and starch. This is in accordance with a report [18,36]. Degradability of native xylan is minimal; it is present as a formidable substrate, whereas alkaline extraction of the native form de-esterifies the substrate and is responsible for the removal of acetyl groups and breakdown of cross linkages, hence increasing the enzyme degradability of xylan. Recently report stated that, strain producing xylanase in solid state fermentation using agricultural residues (wheat bran, sugarcane, bagass and saw dust) as substrates without enrichment of the medium.

CONCLUSION

To conclude that, *Bacillus megaterium* SV1 strain produces xylanase. Characterization and purification xylanase enzyme has been done and the molecular weight is identified as 24kDa with a basic optimum pH and thermo stability. Thus, the enzyme from *Bacillus* SV1 was identified and concluded as alkalophilic and halotolerant xylanase producer. Amino acid sequence of the xylanase enzyme is under progress.

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REFERENCES

1. J. Perez, J. Munoz-Dorado, T. De-La-Rubia, J. Martinez. *Int. Microbiol.* 2002, 5, 53.
2. B. C. Saha. *Bioresour. Technol.* 2003, 90, 33.
3. M. Tuomela, M. Vikman, A. Hatakka, M. Itavaara. *Bioresour. Technol.* 2000, 72, 169.
4. R. A. Prade. *Biotechnol. Genet. Eng. Rev.* 1995, 13, 101.
5. E. T. Chivero, A. N. Mutukumira, R. Zvauya. *Food. Chem.* 2001. 72, 179.

6. Q. K. Beg, M. Kapoor, L. Mahajan, G. S. Hoondal. *Appl. Microbiol. Biotechnol.* 2001, 56, 326.
7. T. Collins, C. Gerday, G. Feller. *FEMS. Microbiol. Rev.* 2005, 29, 3.
8. S. Subramaniyan, M. Prema. *Microbiol. Lett.* 2000, 183, 1.
9. M. C. Srinivasan, M. V. Rele. *Curr. Sci.* 1999, 77, 137.
10. P. Biely, O. Markovic, D. Mislovikova. *Anal. Biochem.* 1985, 144, 147.
11. E. C. Carmona, M. B. Fialho, E. B. Buchgnani, G. D. Coelho, M. R. Brocheto-Braga, J. A. Jorge. *Proces. Biochem.* 2005. 40, 359.
12. C. Techapun, N. Poosaran, M. Watanabe, K. Sasaki. *Proces. Biochem.* 2003, 38, 1327.
13. A. K. Badhan, B. S. Chadha, K. Jatinder, H. S. Saini, M. K. Bhat. *Bioresour. Technol.* 2007, 98, 504.
14. N. Kulkarni, A. Shendye, M. Rao. *FEMS. Microbiol. Rev.* 1999, 23, 411.
15. P. Bajpai. *Adv. Appl. Microbiol.* 1998, 43, 141.
16. P. Morales, A. Madarro, A. Flors, J. M. Sendra, J. A. Perez-Gonzalez. *Enzym. Microb. Technol.* 1995, 17, 424.
17. J. D. Breccia, F. Sineriz, D. M. Baigori, G. R. Castro, R. Hatti-Kaul. *Enzym. Microb. Technol.* 1998, 22, 42.
18. G. Mamo, R. Hatti-Kaul, B. Mattiasson. *Enzym. Microb. Technol.* 2006, 39, 1492.
19. S. Kortekaas, R. E. Wijngaarde, J. W. Klomp, G. Lettinga, J. A. Field. *Wat. Res.* 1998, 32, 3362.
20. A. Dhillon, J. K. Gupta, S. Khanna. *Proces. Biochem.* 2000, 35, 210.
21. H. Takahashi, R. Nakai, S. Nakamura. *Biosci. Biotechnol. Biochem.* 2000, 64, 887.
22. B. Archana, T. Satyanarayana. *World. J. Microbiol. Biotechnol.* 2003, 19, 53.
23. R. Khandeparkar, N. B. Bhosle. *Bioresour. Technol.* 2007, 98, 897.
24. L. Li, H. Tian, Y. Cheng, Z. Jiang, S. Yang. *Enzym. Microb. Technol.* 2006, 38, 780.
25. K. B. Bastawade. *World. J. Microbiol. Biotechnol.* 1992, 8, 353.
26. R. D. Kamble, A. R. Jadhav. *Int. J. Microbiol.* 2012, 1.
27. B. K. Bajaj, N. P. Singh. *Appl. Biochem. Biotechnol.* 2010, 162, 1804.
28. A. Sanghi, N. Garg, V. K. Gupta, A. Mittal, R. C. Kuhad. *Braz. J. Microbiol.* 2010, 41, 467.
29. J. W. Lee, K. S. Gwak, S. I. Kim, M. Kim, D. H. Choi, I. G. Choi. *J. Microbiol. Biotechnol.* 2007, 17, 1811.
30. P. F. Franco, H. M. Ferreira, E. X. Filho. *Biotechnol. Appl. Biochem.* 2004, 40, 255.
31. J. W. Lee, J. Y. Park, M. Kwon, I. G. Choi. *J. Biosci. Bioeng.* 2009, 107, 33.
32. R. Khandeparkar, Preeti Verma, Deepti Deobagkar. *New. Biotechnol.* 2011, 28, 814.
33. B. C. Saha. *Proces. Biochem.* 2002, 37, 1279.
34. J. X. Heck, S. H. Flores, P. F. Hertz, M. A. Z. Ayub. *Proces. Biochem.* 2005, 40, 107.
35. E. T. Chivero, A. N. Mutukumira, R. Zvauya. *Food. Chem.* 2001, 72, 191.
36. M. Bataillon, A. P. Nunes Cardinali, N. Castillon, F. Duchiron. *Enzym. Microb. Technol.* 2000, 26, 187.
37. Q. K. Beg, M. Kapoor, L. Mahajan, G. S. Hoondal. *Appl. Microbiol. Biotechnol.* 2001, 56, 326.
38. C. Y. Wang, H. Chan, H. T. Lin, Y. T. Shyu. *Annal. Appl. Biol.* 2010, 156, 187.