Purification Of Xylanase From *Bacillus Pumilus* And It's Characterization

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ABSTRACT

This study is aimed at purification and characterization of xylanase produced by thermophilic and alkalophilic *Bacillus pumilus* isolated from corncob decaying soil. . Xylanase with thermostable and alkalo tolerant properties are needed for the application in paper pulp industry as the bio-bleaching process. Culture supernatant (120.6Umg^{-1}) of *B. pumilus* the xylanase was purified by ammonium sulphate precipitation and Sephadex G 75 gel filtration. With different concentrations of $(NH_4)_2SO_4$, maximum amount of xylanase was precipitated at 50% of $(NH_4)_2SO_4$ saturation. This $(NH_4)_2SO_4$ precipitated sample was dialysed against distilled water for 24h and the sample (824.72 Umg^{-1}) was loaded to Sephadex G 75 column and eluted with 0.5M Tris buffer at the flow rate of 0.5mL/min. Eluted fractions which showed highest xylanase activity were pooled together (2250.13 Umg^{-1}), separated by Sodium Dodecyl Sulphate polyacrylamide (SDS) gel electrophoresis. Purified xylanase showed 2250.13 Umg^{-1} specific activity and the purification fold was18.6. The specific activity of the initial crude xylanase was 120.62 Umg^{-1} with a recovery yield of 34 %. The enzyme appeared as a single band on SDS-PAGE gel with the molecular mass of approximately 25kDa. Accurate molecular mass was determined as 25.42kDa by electrospray mass spectrometry (ES-MS). Purified xylanase showed zero order kinetics for 4 min and gave highest xylanase activity [193.7 (±0.26) UmL^{-1}] at 60°C and pH 8.4. Purified enzyme showed high specific activity against xylan and showed no activity with carboxymethyl cellulose, starch and Avicel. Two step purification method was able to provide this purified xylanase with no amylase and cellulase activities. Due to the purity and activity at alkaline pH and at 60°C this enzyme can be used for bio-bleaching of paper pulp.

Key words: Gel filtration, precipitation, purification, xylanase and xylan

1. INTRODUCTION

Xylanase with thermostable and alkalo tolerant properties are needed for the application in paper pulp industry as the bio-bleaching process is carried out at both high temperature and basic pH. Consequently several thermostable alkaline xylanases have been isolated and characterized from various microorganism such *Bacillus sp.* 45 M1 [1] and *Bacillus stearothermophilus* [2]. Purified xylanases are important for both analytical research and in the study of the structure and function of enzymes. A wide variety of combinations of several different steps such as precipitation, ultra filtration, ion-exchange chromatography, gel filtration chromatography, hydrophobic interaction chromatography and affinity chromatography have been used for the recovery and purification of enzymes [3]. In this paper we report the purification of xylanase produced by *Bacillus pumilus* and its characterization.

2. MATERIALS AND METHOD

Materials

Birchwood xylan (Roth, Germany), oat spelt xylan, Larchwood xylan, starch, carboxymethyl cellulose, Avicel, Acrylamide; Bis-stock solution 37.5:1, 40% and $(NH_4)_2SO_4$ (Sigma, USA), Sephadex G 75 (Pharmacia, Uppsala, Sweden) were used.

Microbial strain

Xylanase producing bacterial strain *Bacillus pumilus* isolated from corncob decaying soil was used for enzyme production [4].

Culture Media and culture conditions

The Xylan Nutrient Agar plates and slants containing (gL^{-1}) nutrient agar, 28.0 and Birchwood xylan, 20.0 at pH 8.5 was used for the storage of the isolates and incubated at 40°C for 24 h.

The activation medium contained (gL^{-1}) xylan, 20.0 and nutrient broth, 25.0 at pH 8.5. The bacterial colonies grown on the slant were transferred to 100mL conical flask

containing 10 mL of activation medium (1 loop/10mL) and incubated in a reciprocal shaker water bath (120 rpm) at 45°C and at pH 8.5, for 18 h.

Fermentation medium contained (gL^{-1}) xylan, 20.0; peptone, 2.0; yeast extract, 2.5; $CaCl_2.2H_2O$, 0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4, 2.5; KH_2PO_4, 1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0 at 8.5 pH.

In shaker flask experiments, media volume to shaker flask volume ratio was maintained as 1:10. All the experiments were carried out in triplicate.

Assay of xylanase activity

Assay mixture consisted of 0.25mL of diluted enzyme solution and 0.25mL of 20 gL^{-1} xylan in 0.05M Tris-HCl buffer (pH 8.4) was incubated at 60°C for 4 min and the increase in reducing sugars was determined by Dinitrosalicylic acid (DNS) method [5] with xylose as the standard.

One unit of xylanase activity is defined as the amount of enzyme that releases one µmol of reducing sugar equivalent to xylose per minute at 60°C and pH 8.4 with 20gL^{-1} xylan.

Enzyme Purification

Production and Extraction of xylanase

Fermentation medium was inoculated with the activated culture (20%, v/v) and incubated was at 45°C with shaking at 120rpm for 32h. Then cells were removed by centrifugation at 3500rpm for 15 min and the culture supernatant was used as enzyme source.

Ammonium Sulphate Precipitation

The crude enzyme solution with 10-70% of $(NH_4)_2SO_4$ saturation were prepared and centrifuged for 8000rpm at 4°C for 30 min. Xylanase activities and protein contents of the supernatants and the precipitates were analysed. The $(NH_4)_2SO_4$ saturation which gave the precipitate with highest specific activity was dialysed against distilled water and selected for further studied.

Gel filtration

Sephadex G 75 suspended in 0.05M Tris HCl buffer (pH 8.4) overnight, was packed in a column (1.6 cm × 45 cm) and equilibrated with the same buffer. The selected protein precipitate (1mL) obtained with $(NH_4)_2SO_4$ was applied to the column. The protein was eluted with the same buffer with a flow rate of 0.5 mL/min by collecting 1mL fractions. The fractions which showed xylanase activity were pooled and used for characterization.

Gel electrophoresis

Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed [6] with standard markers of molecular mass.

Electrospray - mass spectrometry

The protein separated by gel filtration was used to determine the molecular weight by Electrospray - Mass Spectrometry (ES-MS). Sample was re-suspended in 10% of acetonitrile and injected by direct infusion at 100µL/min. Mass spectrum was in the range from 600- 3000 m/z. Spectrum deconvolution was performed with the program Analyst QS soft ware.

Characterization Of The Purified Enzyme

Effect of Reaction time

Xylan solution (20gL^{-1}, 0.25mL, pH 8.4) was mixed with 0.25mL of diluted purified enzyme at 60°C and the amount of xylose production was monitored.

Effect of Temperature

The effect of temperature on the xylanase activity was determined by incubating the diluted and purified enzyme containing Tris-HCl buffer (pH 8.4) with soluble xylan (20gL^{-1}) at different temperatures, ranging from 40 to 65°C.

Effect of pH

The effect of pH on the xylanase activity was measured by incubating diluted purified enzyme in the appropriate buffers ranging from pH 7.0-9.0, 20gL^{-1} of xylan solutions at respective pH values and at optimum temperature. The buffers used were phosphate buffer (pH 7.0- 7.5) and Tris amino methane buffer (pH 8.0-9.0).

Substrate specificity

Activity of the xylanase was tested by using 2% (w/v) Birchwood xylan, oat splet xylan, Larchwood xylan, carboxymethyl cellulose (CMC), starch and Avicel respectively as substrates.

3. RESULTS AND DISCUSSION

Purification

Amount of protein decreased with the increase in the saturation percentage of ammonium sulphate while the xylanase activity increased up to 50% saturation of ammonium sulphate (Table 1). Highest xylanase activity (736.15UmL^{-1}) and highest amount of protein (0.37mg) were obtained with 50% of ammonium sulphate saturation

with the specific activity of 1989.59 $UmL^{-1}mg^{-1}$ and 40.4% yield. Hence the precipitate formed with 50% $(NH_4)_2SO_4$ saturation was selected and loaded. Fractions 13 to 23 had protein content. Among the fractions, fraction 21 had highest xylanase activity. The fractions from 19-23 were pooled and this pooled (05 fractions) sample showed 517.32 U enzyme activity with 0.23 mg protein content. Other fractions contained non xylanase protein. By this gel filtration purification, the specific activity of xylanase was increased from 824.72 to 2250.13 Umg^{-1} proteins, which was 18.6 fold higher than that of the initial crude xylanase and the yield was 34 % (Table 2).

Table 1: Effect of $(NH_4)_2SO_4$ saturation on the precipitation of xylanase.

$(NH_4)_2SO_4$ (%)	Xylanase activity (U)	Protein (mg)	Specific activity (Umg^{-1})
0	376.9	0.8	471.12
10	198.03	0.563	351.74
20	214.68	0.22	997.03
30	294.00	0.18	1667.41
40	520.45	0.36	1443.40
50	736.15	0.37	1989.59
60	585.38	0.34	1650.29
70	562.44	0.34	1647.82

Table 2: Summary of xylanase purification from Bacillus pumilus.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Purification fold	Recovery (%)
Crude enzyme	1507.7	12.5	120.62	1	100
50% $(NH_4)_2SO_4$ Saturation	610.24	0.74	824.72	6.8	40.4
Gel filtration	517.53	0.23	2250.13	18.6	34

Pooled fractions from the gel filtration was analyzed by SDS-PAGE, and it gave a single band after separation and with a molecular mass of approximately 25kDa (Figure 1). Based on the ES-MS spectrum molecular weight of the purified xylanase was determined as 25.42kDa. Xylanase from *Paecilomyces thermophilia* also showed a single band on SDS-PAGE gel with the molecular mass of approximately 25.8kDa [7] and purified enzyme from *Bacillus sp.* K8 showed the mass of 24 kDa [8].

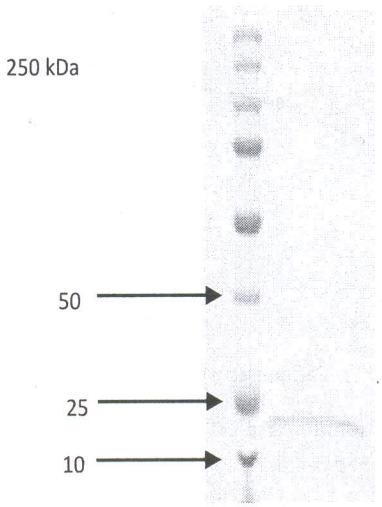

Fig 1: SDS–PAGE of the purified xylanase. Lane 1: purified protein, Lane M: protein molecular weight markers.

Characterization of the purified xylanase

Xylanase obtained from *B.pumilus* showed zero order kinetics for 4 min. Therefore the reaction time of the enzyme was fixed as 4 min (Figure 2) in the following experiments.

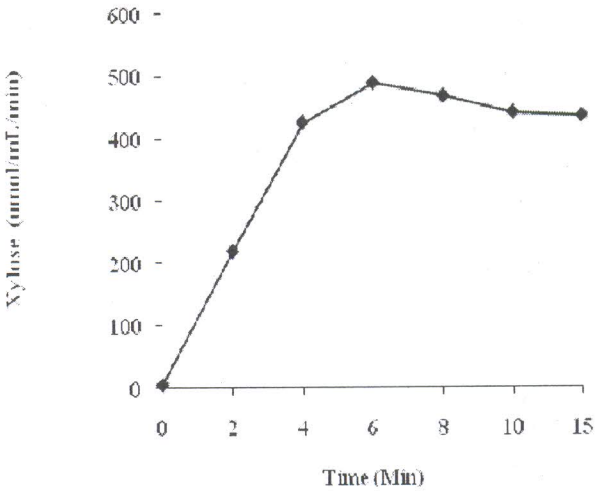

Fig 2: Activity of xylanase produced by *B. pumilus* with Birchwood xylan as a function of time at pH 8.4 and 60°C.

The activity of xylanase obtained from *B.pumilus* was assayed at different temperatures ranging from 40-65°C at pH 8.4. The optimum temperature was 60°C (Figure 3). The enzyme gave 67 and 75% of its maximum activity [214.0(±0.36) UmL^{-1}] at 40 and 60°C respectively. The partially purified xylanase obtained from *Streptomyces* sp was optimally active at 60°C [9]. Which that of *Paenibacillus sp* showed it height activity at 55°C [10].

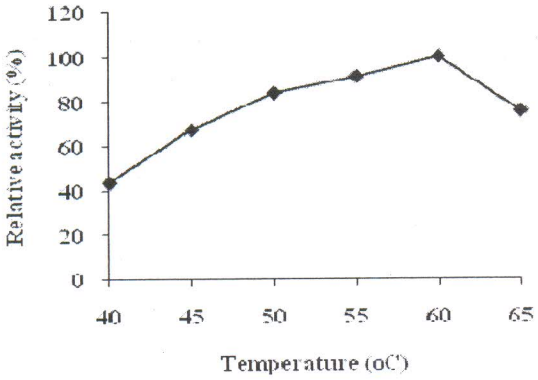

Fig 3: Effect of temperature on the activity of xylanase from *B. pumilus* at pH 8.4.

The optimum pH value of the enzyme was 8.4 at 60°C (Figure 4). Xylanase from this *B.pumilus* showed broad activity between the pH ranges of 7.5- 8.4. Xylanases with alkaline pH optimum are most preferred for industrial applications. Xylanase from *B.pumillus* showed 59% of its maximum activity [193.7(±0.26) UmL^{-1}] at pH 9.0. The isolate *Bacillus pumilus* produced xylanase demonstrating maximal activity at 35°C and at pH 7.0 [11]. The optimum pH for the activity of purified xylanase from *Bacillus amyloliquifaciens* and *Rhodothermos marinus* was 6.8-7.0 [12]. The optimum pH of the xylanase extracted from *Paenibacillus sp* was 7.0 when the activity was measured over a pH range from 4.0 to 9.0 [10].

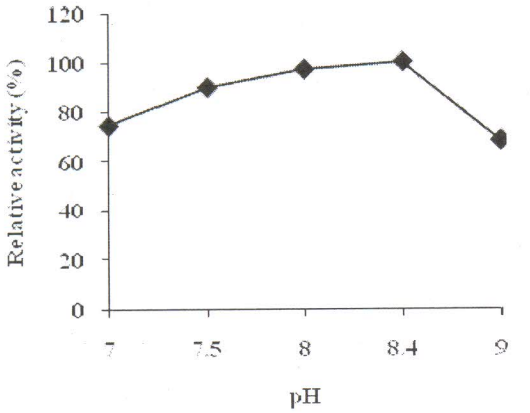

Fig 4: Effect of pH on the activity of xylanases from *B.pumilus* at 60 °C.

The enzyme showed a high specificity towards different xylans tested. The highest activity (125.8%) was observed with Larchwood xylan. The xylanase did not act towards CM-cellulose, Starch and Avicel (Table 3). Many xylanases show both xylanase and low cellulase activities [13] such as that from *P. variotibainier* and *T. lanuginosus* strains [14, 15]. The purified xylanase only hydrolyzed xylans and was free from all other enzyme activities examined. This substrate specificity may also be a desirable property for pulp bio-bleaching.

Table 3: Effect of different carbon sources (2% w/v) on the activity of purified enzyme at 60°C and at pH 8.4 (0.05M Tris buffer) when incubated for 4min.

Substrates	Actual activity (UmL^{-1})	Relative activity (%)
Birchwood xylan	215.66	100
Oat spelt xylan	161.25	74.7
Larchwood xylan	271.38	125.8
CMC cellulose	0	0
Starch	0	0
Avicel	0	0

4. CONCLUSION

Culture supernatant of *Bacillus pumilus* was precipitated with 50% of $(NH_4)_2SO_4$ saturation and separated in Sephdex G 75 column. The enzyme fractions eluted with Tris-HCl which showed highest xylanase activity were pooled together and used for characterization. The purified xylanase showed single band on SDS-PAGE with the molecular weight of 25kDa. This was confirmed by ES MS. The enzyme showed optimum activity at 60°C and pH 8.4 and substrate specificity only towards xylan.

5. ACKNOWLEDGEMENT

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