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Research Article

ISOLATION, PURIFICATION AND CHARACTERIZATION OF ALKALI AND THERMO STABLE XYLANASE FROM *BACILLUS* SP. KS09

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ABSTRACT

Nine bacterial strains were isolated using xylan rich media. The bacterial strain KS09 was selected on the basis of qualitative and quantitative test. It was identified as *Bacillus* sp. via physiological, morphological and biochemical characterization. The xylanase was purified to homogeneity from crude extract of *Bacillus* sp. KS09 using ammonium sulphate fractioning and CM-Sephadex C-50. The final purification fold was 10.20 with a recovery of 36.18%. The enzyme was optimally active at 50°C, pH 7.0 and stable over a broad pH range of 6.0-11.0. The residual activity at 6.0-11.0 pH was 100% even upto 3 h of incubation. The enzyme showed 75, 70 and 60% thermal stability at 50, 55 and 60°C, respectively after 1 h of incubation. The kinetic parameters (K_m 22.59 mM; V_{max} 76.93 IU/mL) were estimated using Lineweaver-Burk plot for purified xylanase. The xylanase activity was inhibited by all the metal ions applied. The characteristic studies revealed that xylanase including its cellulase free nature, broad pH stability and temperature stability are particularly suited its industrial applications.

Keywords: Xylanase, purification, isolation, ammonium sulfate fractionation, Congo red dye.

INTRODUCTION

Xylan constitute up to 35% of the total dry weight of higher plant and is built from homopolymeric backbone chain of 1, 4-linked β-D-xylopyranose units, including short chains O-acetyl, α-L-arabinofuranosyl and D-glucuronyl or O-methyl-D-glucuronyl residues[1]. The degradation of lignocellulosic materials by hydrolyzing xylan is an important step for nature. Chemical hydrolysis of xylan by the industries is accompanied with the formulation of toxic components which is hazardous to environment[2]. The use of site specific microbial enzymes for xylan hydrolysis makes the process eco-friendly. Xylanases (EC 3.2.1.8), catalyze the hydrolysis of 1, 4-β-D-xylosidic linkages in xylan. They are characterized as exo- and endo-xylanases and release xylo-oligosaccharides and xylose. Initially the main hydrolysis products are β-D-xylopyranosyl oligomer, but at later stage, small molecules such as mono-, di- and trisaccharides of β-D-xylopyranosyl may be produced[3].

Xylanases are produced by diverse species of micro-organisms and have been studied mostly from bacteria, fungi and actinomycetes [4, 5]. Among bacterial xylanases, members of the genus *Bacillus* have been studied extensively. Xylanases are required for pre-bleaching in

pulp and paper industry, and this is the biggest potential application of xylanolytic enzymes[6]. Cellulase-free xylanases active at high temperature and pH are gaining importance in pulp and paper industry as they reduce the need for toxic chlorinated compounds making the bleaching process environment-friendly[5]. In contrast to paper and pulp industries xylanases have also been used for clarifying fruit juices/wines, improving the nutritional value of animal feed, bread making and for the extraction of coffee[7].

Generally, it have been accepted that suitability of an enzyme for an industry application depends upon the enzyme's homogeneity, thermal stability and other kinetic properties. The present study elaborates the isolation of *Bacillus* sp. KS09 strain, production, purification and characterization of the purified xylanase.

MATERIALS AND METHODS

Chemicals

All the chemicals and media constituents used in the present investigation were of AR grade and of high purity. Birch-wood xylan, Oat spelt xylan, xylose, 3,5- dinitrosalicylic acid, N,N'-methylene bisacrylamide, tris (hydroxymethyl) aminomethane, N,N,N',N'-tetramethyl ethylenediamine (TEMED), bovine serum albumin (BSA), glycine, Ammonium per sulfate (APS) were purchased from Sigma Chemicals Co. Ltd., St. Louis, MO, USA. Agar-agar, peptone, yeast extract, beef extract, sodium hydroxide, sodium sulfite, phenol, sodium thiosulfate, sodium potassium tartarate, K_2HPO_4, KH_2PO_4, $CuSO_4$, $NaCO_3$, $MgSO_4$, NaH_2PO_4, Na_2HPO_4 and Congo red were procured from Hi-Media Laboratories India. Methanol, glacial acetic acid, silver nitrate, hydrochloric acid, glycerol, phenol reagent (Folin-ciocalteau's reagent) was purchased from Merck Laboratories, India. CM-Sephadex C-50 was purchased from Pharmacia, Sweden. Wheat bran was collected locally.

Isolation of microorganisms

The xylanase producing microbial strains were isolated by screening the soil samples collected from district Yamunanagar (Haryana), India. One gram of sample was suspended in 10 mL sterile distilled water and serial dilutions were made. The 50 µL of the diluted sample (10^{-6}) was spread on nutrient agar plates containing (g/L): peptone, 5.0; beef extract, 3.0; agar 20.0 and pH 7.0 followed by incubation at 37°C for 24 h. The selected purified colonies were transferred and grown on xylan agar medium plates containing (g/L): peptone, 5.0; yeast extract, 2.0; $MgSO_4$, 0.5; NaCl, 0.5; $CaCl_2$, 0.15; agar, 20.0; birch wood xylan, 20.0 and pH 7. Xylanolytic colonies were qualitatively recognized by the method of Teather and Wood[8]. The grown colonies were flooded with 1% aqueous Congo red dye for 1h followed by destaining with 1M NaCl. The xylanase producing *Bacillus* sp. KS09 strain was identified on the basis of their physiological, morphological and biochemical characteristics in our laboratory using *Bergey's* Manual of Systematic Bacteriology guidelines. The strain was stored and maintained on nutrient agar slants at 4°C. The various type of media used in the study were sterilized at 1.05 kg/cm^2 for 20 min before utilized in the process.

Seed culture preparation

The single colony of *Bacillus* sp. KS09 was grown in a test tube, which contained 5 ml of nutrient broth (in g/L: peptone, 5.0; beef extract, 3.0 and pH 7.0) followed by incubation at 37°C for overnight. Then, a loop full (~0.01 ml, Himedia SS-4) of the culture from the tube is transferred to 50 ml autoclaved nutrient broth and incubated at 37°C for 18 h under shaking at 200 rpm.

Xylanase production and extraction

Erlenmeyer flasks (250 mL) containing 50 mL of modified Horikoshi medium (in g/L: peptone, 5.0; yeast extract, 6; KNO_3; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1; wheat bran, 10.0) were autoclaved at 1.05 kg/cm^2 for 20 min and cooled. The flasks were then inoculated with 2% (v/v) of 18 h old culture and incubated at 37°C for 48 h under shaking at 200 rpm. Xylanase was harvested by centrifugation (Remi C-24 BL centrifuge) at 10,000 x g for 20 min at 4°C. The cell free supernatant was used as crude enzyme.

Enzyme assay & Protein determination

Xylanase and cellulase (Carboxymethyl cellulase and filter paper activity) activities were assayed by measuring the amount of reducing sugars liberated using 3,5-Dinitrosalicylic acid (DNSA)[9]. One unit of xylanase or cellulase activity was defined as the amount of enzyme catalyzing the release of 1µmol/min of reducing sugar equivalent to xylose or glucose under the specified assay conditions.

The protein concentration in chromatographic elutes was determined by measuring the absorbance at 280 nm. Total

soluble protein content at the end of each purification step was determined by the standard Lowry 's method using bovine serum albumin as standard.

Purification of enzyme

The crude xylanase was brought to 0-80% ammonium sulphate (AMS) saturation with constant stirring and kept for sedimentation in a refrigerator and centrifuged (Remi C-24 BL centrifuge) at 10,000 x g for 30 min. The pellet was dissolved in sodium phosphate buffer (0.05 M, pH 6.0) and dialyzed in dialysis bags (12 kDa cut off; Sigma- Aldrich) against the same buffer for overnight. The dialyzed enzyme was loaded onto CM-Sephadex C-50 (30 x 3 cm) column that had been pre-equilibrated with 0.05M phosphate buffer, pH 6.0. The column was run with 0.05M phosphate buffer (pH 6.0) and fractions of 5 mL each were collected at a flow rate of 4 mL/min. The protein bound to CM-Sephadex C-50 column was eluted using a continuous salt gradient (0-1M NaCl). The fractions were monitored for their proteins content by measuring the absorbance at 280 nm on a double beam UV-Visible spectrophotometer (Systronics, Model-2202). The buffer was run through the column until the A_{280} of the eluent was zero against 0.05M phosphate buffer (pH 6.0) as the blank. The active fractions were pooled and xylanase activity and protein content were measured. The homogeneity of the purified xylanase was checked by native polyacrylamide gel electrophoresis using mini size vertical gel electrophoresis system (Laby Instruments Model E-2,). Electrophoresis was carried out using 10% acrylamide gel and protein bands were stained via silver staining.

Effect of temperature

To determine the temperature optimum xylanase activity was measured at different temperatures (30°C to 65°C) under standard assay conditions.

The temperature stability of xylanase was determined by pre -incubating an aliquot of enzyme at 30°C to 65°C for 1 h and the residual activity (%) was calculated.

Effect of pH

The effect of pH on xylanase activity was studied by using the substrate prepared in buffer (0.05 M) of different pH ranging from 4.0 to 11.0 viz, citrate buffer (pH 4-6); phosphate buffer (pH 6-8) and Tris-HCl buffer (pH 8-11), under standard assay conditions. The pH stability of the enzyme was determined by pre -incubating an aliquot of enzyme with buffers of different pH for 24 h at 37°C followed by measurement of xylanase activity using sodium phosphate buffer (0.05 M, pH 7) after varying time interval for 5 h. The residual activity (%) at each pH was calculated.

Kinetic parameters

The activity of purified xylanase was assayed with different concentration of birch wood xylan (0.5 to 20 mg/mL) at 55°C. Lineweaver – Burk plot was drawn to determine the values of K_m and V_{max} of the enzyme.

Effect of additives

The purified enzyme was incubated with different metal salts (10 mM) and additives (1 %) viz. NaCl, KCl, $MnCl_2$, $BaCl_2$, $HgCl_2$, $CaCl_2$, EDTA and SDS for 15 min at room temperature and the residual activities were measured under standard assay conditions.

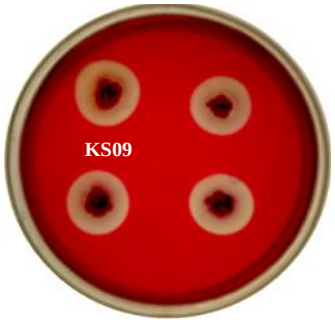
RESULTS AND DISCUSSION

Bacillus sp. KS09, thermo-alkalophilic xylanase producing microorganism was isolated and screened both qualitatively as well as quantitatively. Under liquid fermentation state it produced 232.64 ± 0.095 IU/mL of xylanase and the cellulase activities (CMCase and FPase) were undetectable, indicating xylanase was cellulase free. Xylanase after ammonium sulphate fractionation was subjected to ion-exchange chromatography through CM-Sephadex C-50 column and was purified to homogeneity with 10.20 fold increase in activity. Other research groups reported purification of xylanase by employing a combination of two or more different methods including salt fractionation, ion-exchange, gel filtration and hydrophobic interaction chromatography[10, 11]. All the experiments were carried out in triplicates and average result of each experiment is presented.

Isolation and identification of microbial strain

A total of nine strains were isolated which were Gram positive, moderate thermophilic up to 55 °C. The strains were alkalophilic also being capable of growing at pH 11.0. Among the nine bacterial strains KS03 produced lowest zone diameter of 1.8cm and KS09 produced highest zone of 3.0 cm on xylan agar plates (table 1). Several workers have reported the similar isolation medium used for the screening of xylan degrading microorganisms[12, 13]. The zone of hydrolysis produced by *Bacillus* sp. KS09 is shown in figure 1.

Figure 1 Zone of substrate hydrolysis by *Bacillus* sp. KS09 xylanase producing isolate



Production of enzyme

All the nine bacterial isolates were quantitatively screened for the production of xylanase. The isolate KS09 showed maximum activity (232.64 IU/mL) of xylanase using wheat bran as substrate (Table 1) under liquid fermentation state. The unoptimized xylanase production in this study was higher than optimized production by other strains of *Bacillus* [5,10, 14, 15].

Table1 Qualitative and quantitative analysis of the bacterial isolates for xylanase

Isolate No.	Diameter of zone of hydrolysis (cm)	Xylanase Activity (IU/mL)
KS01	1.9	17.08 ± 0.075
KS02	2.0	37.63 ± 0.025
KS03	1.8	6.65 ± 0.050
KS04	2.1	52.70 ± 0.252
KS05	2.0	40.24 ± 0.072
KS06	2.2	71.92 ± 0.096
KS07	2.0	39.23 ± 0.052
KS08	2.5	172.03 ± 0.100
KS09	3.0	232.64 ± 0.095

Purification of xylanase

Xylanase purification was performed by AMS precipitation (0-80% saturation) followed by ion-exchange chromatography through CM-Sephadex C-50 (Table 2). The elution profile of xylanase through CM-Sephadex C-50 cation exchange column is shown in Figure 2. The xylanase activity was detected in fraction no. 60-70 and the specific activity of the pooled fractions reached 1248.76 IU/mg with no cellulase activity observed (Fig. 2). The enzyme was purified to 10.20 fold with a recovery of 36.18% (Table 2). The purity of the enzyme was checked by running Native-polyacrylaminde gel electrophoresis (Native-PAGE). A single band was observed by silver staining, which indicated that the enzyme was purified to homogeneity (Figure 3). The purification fold of the purified enzyme from *Bacillus* sp. KS09 following two step protocol was higher than reported from *B. amyloliquefaciens* to 7.3 fold using Diethylaminoethyl-Sephadex A50, $(NH_4)_2SO_4$ fractionation and hydrophobic interaction chromatography on butyl Sepharose 4B column (Breccia et al. 1998) and *B. circulans* Teri-42 to 2-fold by Q-Sepharose and Sephadex G-50 column chromatography (Qureshy et al. 2002). Further, the recovery was better than that obtained from *Arthrobacter* sp. with 14 % yield using $(NH_4)_2SO_4$ fractionation, Sephadex 200, Diethylaminoethyl-Sepharose FF, CM-Sepharose FF chromatography (Khandeparker and Bhosle 2006).

Table 2 Purification of xylanase from *Bacillus* sp. KS09. The enzyme was produced in submerged fermentation using wheat bran and the crude extract obtained was subjected to purification.

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Recovery (%)	Purification fold
Crude extract	34896.00	285.16	122.37	100.00	1
Ammonium sulfate	15938.00	43.00	370.65	45.67	3.03
CM-Sephadex C-50	12625.00	10.11	1248.76	36.18	10.20

Temperature optima and stability

Xylanase activity at different temperatures showed that the

activity increased up to 50°C and then declined progressively (Figure 4). Thus the optimum temperature of the purified xylanase was 50°C. The enzyme was found to be thermotolerant and showed 75, 70 and 60% thermal stability at 50, 55 and 60°C, respectively after 1 h of incubation (Figure 4). Significant enzyme stability at higher temperatures would be important for its industrial application. Purified xylanases exhibiting almost similar temperature optima have been reported by other researchers from several *Bacillus* sp.[16, 17]. However, some xylanase showed higher temperature optima [10, 11].

Figure 2 Purification profile of xylanase by ion-exchange chromatography through CM-Sephadex C-50 column. The bound proteins were eluted using a linear gradient of 0-1.0 M NaCl at a flow rate of 4 mL/min. Xylanase activity (*hollow circle*), absorbance at 280nm (*black circle*), NaCl (*dotted line*).

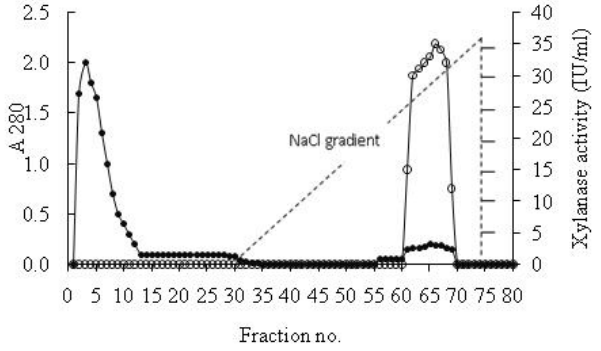


Figure 3 Native polyacrylaminde gel electrophoresis (Native- PAGE) analysis of the purified xylanase from *Bacillus* sp. KS09. Lane 1: crude xylanase; Lane 2: Ammonium sulphates fractionate and Lane 3: purified xylanase.

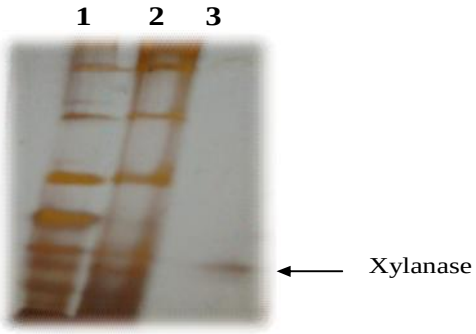
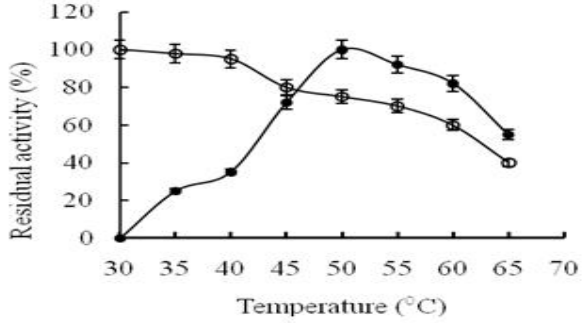


Figure 4 The effect of temperature (30-65°C) on xylanase from *Bacillus* sp. KS09: Temperature optima (*black circle*) and thermo stability (*hollow circle*). The residual xylanase activity (%) was calculated

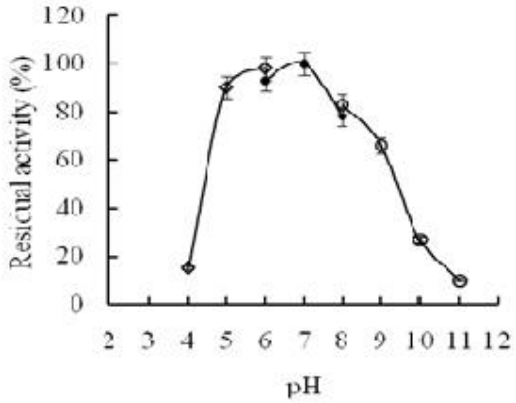


pH optima and stability

The profile of xylanase activity as a function of pH is shown in Figure 5 and displayed optimum activity at pH 7.0.

The enzyme retained 66% and 27% residual activity at pH 9.0 and 10.0, respectively. This indicated that enzyme was alkalophilic. The purified xylanase was stable over a broad range of pH. The residual activity at 6.0-11.0 pH was 100% even upto 3 h of incubation (Figure 5). The xylanase from *B. stearothermophilus* has optimum pH at 6.5 but it was stable up to pH 10.0 [18]. Xylanase from alkalophilic *Bacillus* sp. strain 41M-1 showed a broad pH profile of 4-11 [19]. A considerable stability of *Bacillus* sp. KS09 xylanase at alkaline pH values makes it potentially effective for use in industry.

Figure 5 pH optima of the purified xylanase from *Bacillus* sp. KS09. The buffers used were sodium citrate pH 4.0-6.0 (*black diamond*), sodium phosphate pH 6.0-8.0 (*hollow circle*) and Tris- HCl pH 8.0-11.0 (*black triangle*). The residual activity was measured using standard assay

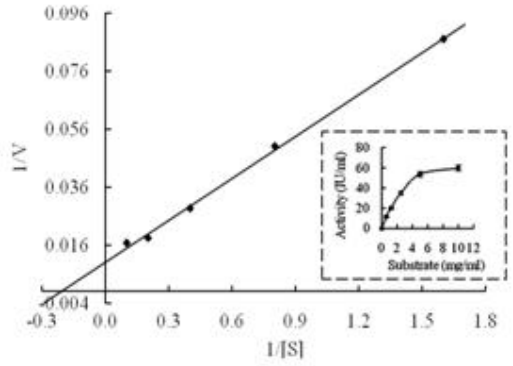


Determination of Km and Vmax

The activity of purified xylanase exhibited a rectangular hyperbolic response w. r. t. various concentrations of birch

wood xylan thus obeying Michaelis-Menten Kinetics (Figure 6). K_m and V_{max} values obtained from the Lineweaver-Burk plot were 22.59 mM (3.39 mg/mL) and 76.93 IU/mL, respectively (Figure 6). The *B. polymyxa*[20] and *S. cyaneus* SN32[21] exhibited K_m value of 17.7 and 11.1 mg/ml. Some xylanases were reported to have lower K_m ranging from 0.025 mg/mL to 1.7 mg/mL [10, 11, 15].

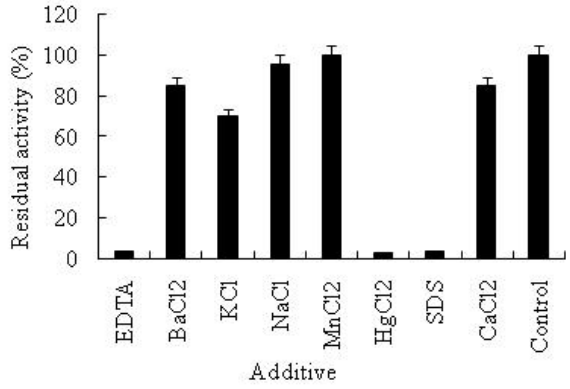
Figure 6 Lineweaver-Burk plot for the purified *Bacillus* sp. KS09 xylanase with a substrate saturation curve is shown in inset



Effect of various additives

Xylanase activity was assayed in the presence of several additives which included metal ions, SDS and EDTA. Some metal ions added inhibited xylanase activity and maximum inhibition was observed in the presence of $HgCl_2$ (Figure 7). Inhibition of enzyme activity by $HgCl_2$ [15, 22] and EDTA has been reported earlier also [13, 23].

Figure 8 Effect of various additives on purified *Bacillus* sp. KS09 xylanase after 15 min of pre-incubation



CONCLUSIONS

In this present study, xylanase producing organism was isolated from soil and identified as *Bacillus* sp. KS09 on the basis of physiological, morphological and biochemical characteristics. Xylanase was purified to homogeneity using two step methods with 10.2 purification fold, which was produced by submerged fermentation using a cheap agro waste residue as carbon source. This enzyme was found to be cellulase free and exhibits alkalophilic and thermophilic properties that suggest its potential role in industrial application.

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