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Production, Purification and Characterization of Xylanase Using Alkalo-Thermophilic *Bacillus halodurans* KR-1

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Abstract: Xylanase (EC. 3.2.1.8) has been isolated from an alkalo-thermophilic bacteria, *Bacillus halodurans strain KR-1* isolated from the soil near river bed at Indore. The bacteria secreted xylanase in the growth medium in the presence of xylan. The production of the enzyme was induced in the presence of glucose, mannose, lactose and maltose whereas presence of starch, cellulose and sucrose retarded in enzyme production. The presence of casein, peptone, sodium nitrate and potassium nitrate as nitrogen source in the growth medium resulted in more xylanase production, whereas presence of ammonium sulfate, ammonium nitrate and yeast extract resulted in lesser enzyme production. The enzyme has been partially purified using sodium sulfate fractionation, DEAE-cellulose and Sephadex G-200 chromatographies. The molecular weight of the enzyme has been found to be 45±02 kDa as determined by Sephadex G-200 chromatography as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme protein is monomeric exhibiting maximum activity at pH 9.0. The optimum temperature for exhibiting maximum activity has been found to be 40°C. The metal ions viz. Mg^{2+} and Fe^{2+} when present in the enzyme assay medium stimulated the xylanase activity, whereas Hg^{2+}, Co^{2+} and Mn^{2+} strongly inhibited the enzyme activity. The Km value for birchwood xylan was calculated to be 12.0 g/l.

Key word: Xylanase · Alkalo-thermophilic · *Bacillus halodurans* · Purification · Km

INTRODUCTION

Xylanase (Endo-1,4-β -D xylan, xylano hydrolase) (EC 3. 2.1.8) acts onβ-1,4 xylan and cleaves β-1,4 glycosidic linkage randomly [1]. The products are xylose, xylobiose and xylo-oligosaccharides. These products are useful feedstock for food and fine chemicals [2]. The enzyme belongs to the glycoside hydrolase family and randomly cleavesβ-1, 4 backbone of the complex plant cell wall polysaccharide xylan, the major constituent of hemicellulose. Xylan is the second most abundant biopolymer after cellulose and major hemicellulosic polysaccharide found in the plant cell wall [3]. The enzyme is of industrial importance and is used in paper manufacturing to degrade xylan, to bleach paper pulp increasing its brightness, improving the digestibility of animal feed and for clarification of fruit juices [4]. Use of xylanase avoids the use of chemical processes that are very expensive and cause pollution [5, 6].

Xylan degrading enzymes occur ubiquitously in wide diversity of sources viz. plants, animals and microbes, however not in mammals. Xylanase producing microbes include aerobic and anaerobic mesophiles and thermophiles. Multiple xylanases are reported in numerous microbes [7]. As mentioned above, xylanase is important for paper industry where kraft pulping is carried out under strong alkaline conditions and even after multiple washings; alkali continues to leach from the fibre. Therefore, activity under alkaline conditions is an important characteristic for an enzyme to function in enzymatic pre-bleaching. Enzymes active at higher temperatures and alkaline pH have great potential and they can be introduced at different stages of the bleaching process without requiring change in pH and temperature [8].

Besides, xylanase is used in variety of applications viz. clarification of wine, starch separation and production of functional food ingredients, improving the quality of

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bakery products and in animal feed biotechnology [9]. Xylanase showing transglycosylation activities can also be used for tailoring drugs and in the preparation of neoglycoproteins [10].

In the present study, an alkalo-thermophilic bacteria capable of growing under alkaline conditions and producing alkalophilic xylanase has been isolated from the soil near the river bed at Indore where mostly washermen wash clothes using highly alkaline detergents. The produced enzyme has been partially purified and characterized.

MATERIALS AND METHODS

Screening of the Bacteria: Semi-dried soil near the river bed at Indore, where mostly washermen wash clothes using highly alkaline detergents, was used as a source of the bacteria. Using autoclaved water, 10^2 to 10^7 times dilutions were made from a suspension of the soil. Serially diluted soil sample was plated on Luria Broth (LB) nutrient agar plates and incubated at 55°C for 24 h. The LB medium was comprised of bacto-tryptone, 10gm; yeast extracts 5gm; sodium chloride, 10gm; agar, 15 gm per litre and adjusted to pH 10 using 0.1N sodium hydroxide. Few single colonies with distinct morphology were isolated and transferred to petri plates having Emerson medium (peptone, 0.5%; K_2HPO_4, 0.1%; $MgSO_4.7H_2O$, 0.02%; pH adjusted to 9.0 with 1% Na_2CO_3), pH 9.0 and incubated at 55°C for 24 h. The medium was supplemented with 1% xylan in order to isolate xylan utilizing bacteria.

Screening for Xylanase Activity: The colonies so obtained were screened for xylanase activity using Congo red dye method [11]. A replica plate was prepared and incubated at 55°C for 24 h. Thereafter, one of the replica plates was flooded with 0.5% Congo red dye and subsequently with 1 M sodium chloride. A clear zone around the colony was considered as indication of xylanase activity. The corresponding colonies from another replica plate were inoculated individually in medium broth, allowed to grow overnight at 55°C and 200 rpm in an orbital shaker. The xylanase activity was analyzed in the medium.

Morphology of the Bacteria: Gram staining was done using kit from Hi-media. After staining, slides were observed in a phase contrast microscope. For further studies, the culture was sent to Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh.

Production of Xylanase: The pre-culture was grown as above and 2% inoculum was used for 100 ml medium in a 250 ml Erlenmeyer flask. The culture was grown by incubation in an orbital shaker using 200 rpm at 50°C for 72 h. Thereafter, the broth was centrifuged at 10,000 x *g* for 10 min at 0 to 4°C. The resulting supernatant was analyzed for xylanase activity.

Enzyme Assay: Xylanase enzyme was assayed by measuring the release of the reducing sugars from birchwood xylan following the dinitrosalicylic acid (DNS) method [12]. A 0.9 ml sample of 1% birchwood xylan dissolved in 50 mM glycine- NaOH buffer, pH 9.0 was pre incubated at 50°C for 5 min. To this, 0.1 ml enzyme (supernatant of the broth) was added and incubated at 50°C for 15 min. The reaction was stopped by adding 1.5 ml of DNS solution and the tubes were put in a boiling water bath for 15 min. A control was also run simultaneously where enzyme was added after the addition of DNS. A blank was also prepared where no enzyme was added and against the blank, zero was set in the colorimeter. The D-xylose was used as standard during the colorimetric estimation. One unit of the xylanase activity was taken as the amount of the enzyme required to release one micromole of the reducing power equivalent to one micromole of xylose per min.

Under the conditions of the enzyme assay. Specific activity was taken as units per mg protein.

Protien Determination: Protein was estimated according to the procedure of Lowry *et al.* [13] using bovine serum albumin as a standard protein.

Enzyme Purification: Unless otherwise stated, the entire purification procedure was carried out at 0 to 4°C in a cold room. After centrifugation of the broth at 10,000 x *g* for 15 min in a Sorvall RC 5B super speed centrifuge, the supernatant was used as a source of the enzyme. To the supernatant, powdered sodium sulfate was slowly added to get 0 to 30% saturation and was incubated on a magnetic stirrer in an icebox with continuous stirring. After 2h incubation, it was centrifuged at 15,000 x g for 20 min and the supernatant having most of the activity was brought to 70% saturation with powdered sodium sulfate. After storage for 3 h, the suspension was centrifuged at 15,000 x g for 20 min. The pellet was dissolved in 0.05M sodium phosphate buffer, pH 9.0 (Buffer A), centrifuged and the clear supernatant was desalted using Sephadex G-25 column (2.5 x 30 cm) chromatography. The desalted fraction was loaded on to

a DEAE cellulose column (2.5 x 30 cm) previously equilibrated with Buffer A. The column was washed with 2-3 bed volumes of buffer A and the enzyme was eluted from the column using a linear sodium chloride gradient (0 to 1M). Fractions of 2ml were collected at a flow rate of 0.5ml/min. Fractions having xylanase activity constituting a single peak were pooled and concentrated by precipitation with sodium sulfate (0 to 80% saturation). The concentrated sample was loaded on to a Sephadex G-200 column (1.5 x 55 cm) equilibrated with buffer A and subsequently chromatographed. The active fractions constituting a single peak were pooled.

Molecular Weight Determination: Molecular weight was determined according to the procedure of Whitaker [14]. The partially purified enzyme was applied on to a Sephadex G-200 column (1.5 x 55 cm), pre-equilibrated with buffer A. The flow rate was 10 ml/ h. Bovine serum albumin (MW 66,000), tryptosinogen (MW 23,000) and lysozyme (MW 14,400) was used as standard proteins (all from Sigma, USA). Subunit molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10 % gel at pH 8.8 as described by Laemmli [15]. Standard Molecular markers having molecular weights of 97, 66, 45, 29, 21 and 14KDa (all from Helini Biomolecules, India) were used as standard proteins. To denature the protein, solutions were incubated with 1% SDS and 0.02% 2-mercaptoethanol at 100°C for 5 min.

Optimum pH: The enzyme assay was carried out in the range varying pH 5.0 to 12.0 using citrate buffer for pH 5 to 6, phosphate buffer for pH 7 to 8 and glycine-NaOH buffer for pH 9 to 12 in order to find out the optimum pH at which the enzyme shows maximum activity.

Optimum Temperature: The enzyme assay was carried out in the temperature range of 20 to 70°C to find out the optimum temperature at which the enzyme shows maximum activity.

Hydrolysis Studies: To a 50 ml sample of 1% birchwood xylan in 50mM glycine-NaOH buffer, pH 9.0, 1 ml of xylanase enzyme was added and incubated at 40°C. Samples of 5ml were removed at intervals of 1, 3, 6 and 12 h. In these samples, un-used polysaccharides were precipitated out using isopropanol and removed by centrifugation at 10,000 *g* for 10 min at 0-4°C using Sorvall RC 5B super speed centrifuge. The supernatants having hydrolyzed products were concentrated by using rotatary vaccum evaporator. The concentrated samples were chromatographed on TLC plates (TLC Aluminium sheets 20 x 20 cm Silica gel 60 F254, Merck, Germany) using n- butanol-pyridine- water (6:3:1 v/v) as solvent. After chromatography, the separated products were detected by spraying a mixture of ethanol and H_2SO_4 (1:1) followed by heating at 120°C for 15 min. D-xylose and D-arabinose were used as standard sugars.

RESULTS AND DISCUSSION

Screening of the Bacteria: A xylanase producing bacteria was isolated from the soil near river bed. Its xylanase producing activity was screened by the presence of clear zone around the colony after staining with congo red dye (Fig. 1). The clear zones were enhanced on treating the plate with 1 M sodium chloride.

Morphological studies revealed it to be an oval shaped, gram positive, endospore forming bacteria. The spores were centrally placed. This infers the bacteria to be a *Bacillus sp.* The Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh also identified it as *Bacillus halodurans* and registered it as *Bacilllus halodurans*, MTCC No. 9534. The morphological characteristics of the strain are shown in Table 1.

Effect of Physiological Factors on the Bacterial Growth: The *Bacillus halodurans* was grown at various temperatures ranging from 4°C to 42°C. It showed good growth in the range of 25 to 42°C (Table 2). Effect of pH on the growth was checked in the pH range of 6.0 to 10.0 and was found to have good growth at all the tested pH. The effect of sodium chloride on the growth of the bacteria was also observed in the range of 2 to 8% sodium chloride and was found to have similar growth in the presence of various concentrations of sodium chloride (Table 2).

Biochemical Characteristics of the *Bacillus Halodurans*: Various tests viz. indole test, methyl red test, Voges Poskauer test, hydrogen sulfide production, gas production, casein hydrolysis, Mac Conkey test, citrate test were found negative with the bacteria (Table 3). The bacteria also showed negative test for esculin and urea hydrolysis whereas it showed positive test for nitrate reduction and Tween 20 hydrolysis. It also showed positive catalase and oxidase tests. The bacteria showed negative test for acid production from maltose and mannitol whereas it showed positive test for acid production from dextrose and xylose (Table 3).

Fig. 1: Screening of xylanase activity by using 0.5% Congo red dye method by *Bacillushalodurans* strain KR1

Table 1: Morphological characteristics of *Bacillus halodurans* strain KR1

Colony Morphology	KR-1
Configuration	Round
Elevation	Raised
Margin	Entire
Surface	Smooth
Pigment	Cream
Opacity	Opaque
Gram's Reaction	Positive
Cell Shape	Rods
Size (micro meter)	2-6
Spores	
Endospores	Positive
Position	Central
Sporangia	Non-Bulged
Shape	Oval
Motality	Position

Table 2: Effect of Physiological factors on the growth of *Bacillus halodrauns* strain KR-1

Growth temperature	
4°C	+
10°C	+
25°C	+
30°C	+
37°C	+
42°C	+
Growth at pH	
pH 6	+
pH 7	+
pH 8	+
pH 9	+
pH 10	+
Growth on NaCl	
2 %	+
4 %	+
6 %	+
8 %	+

Here + : Positive , - : Negative

Table 3: Various Biochemical tests on *Bacillus halodrans* strain KR-1

Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate utilization	-
H2S production	-
Gas production	-
Casein hydrolysis	-
Esculin hydrolysis	-
Gelatin hydrolysis	(-)
Strach hydrolysis	-
Urea hydolysis	-
Nitrate reduction	+
Arginine dihydrolase	-
Tween 20 hydrolysis	(+)
Tween 40 hydrolysis	-
Tween 80 hydrolysis	-
Catalase test	+
Oxidase test	+
Acid production from	
Dextrose	+
Maltose	-
Mannitol	-
Xylose	(+)
Mannose	-

+:Positive;-:Negative;(+):Weak Positive; (-): Weak Negative

Production of Xylanase: Xylanase enzyme was produced by using growth medium. Xylanase production was monitored up to 120 h. Maximum xylanase production was observed after 72 h. Thereafter, production of xylanase was almost stationary up to 120 h (Fig. 2).

Effect of Different Carbon Sources on Xylanase Production: Different carbon sources viz. cellulose, sucrose, starch, lactose, mannose, maltose and glucose were used for xylanase production. Emerson medium

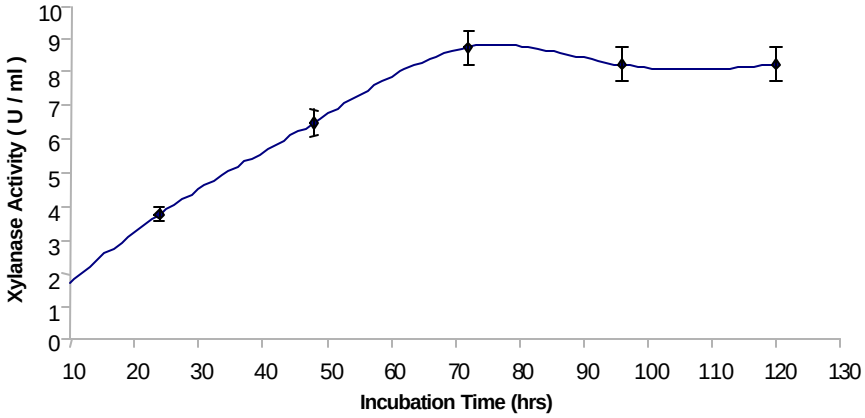
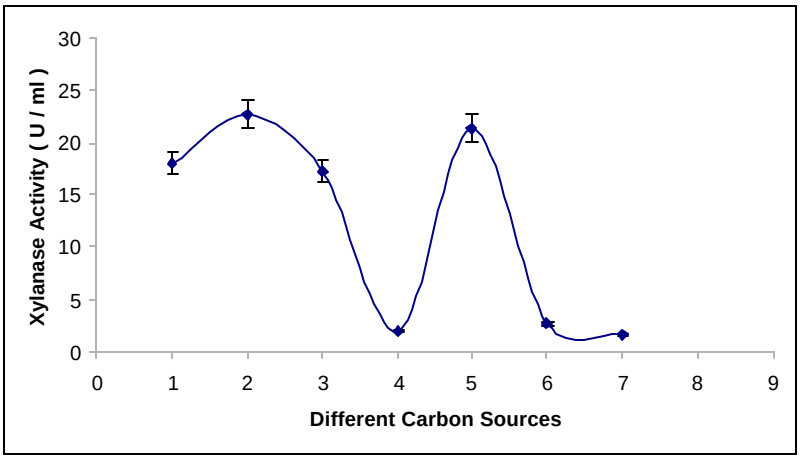
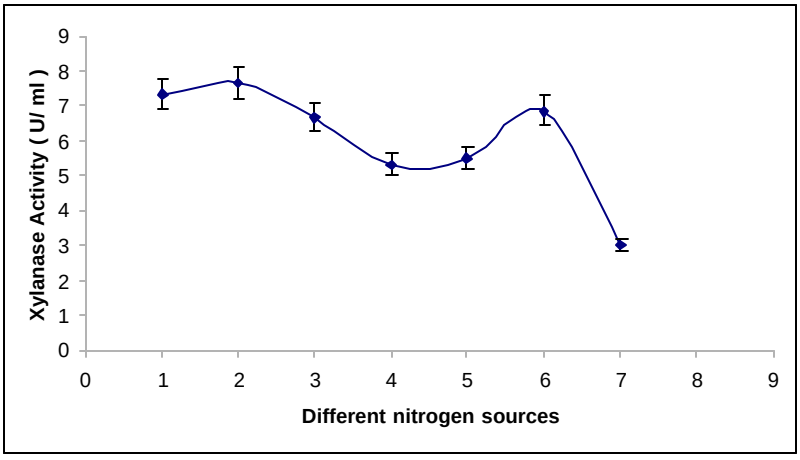


Fig. 2: Growth profile of *Bacillus halodrans* strain KR-1 MTCC 9534 when grown on birchwood xylan



1- Glucose , 2 - Maltose , 3 - Mannose , 4 - Sucrose , 5 - Lactose , 6 - Starch , 7 - Cellulose

Fig. 3: Effect of different carbon sources on the production of xylanase



1 - KNO_3 , 2- $NaNO_3$, 3 - $(NH_4)_2NO_3$, 4 - $(NH_4)_2SO_4$, 5 – Casien, 6- Peptone , 7 - Yeast Extract

Fig. 4: Effect of different nitrogen sources on the production of xylanase.

with xylan containing different carbon sources at the concentration of 0.5% were used for enzyme production. Maximum xylanase activity was found with maltose with almost same activity with lactose, mannose and glucose. In the presence of sucrose, starch and cellulose, there was only about 10% xylanase activity. The data indicated that reducing sugars acted as inducers and non-reducing carbohydrates were unable to induce xylanase production (Fig. 3). Bakri *et al.* [16] showed induction of xylanase in the presence of xylan and cellulose where as glucose and sucrose was not found to induce xylanase production in *Cochliobolus sativus*. Khandeparkar and Bhosle [1] showed maximum production of xylanase on birchwood xylan, oat spelt xylan, xylose, arabinose and glucose and least production on cellobiose, glactose and sucrose by *Arthrobacter sp.* Anuradha *et al.* [17] reported production of xylanase having maximum xylanase activity of 52 U/ml on oat spelt xylan followed by starch and sucrose, 25 U/ml and 18U/ml, respectively and least activity on xylose and Arabinose 8U/ml and 13 U/ml, respectively. Altaf *et al.* [18] reported xylanase production by *Pleurotus erungii* and *Flamulina velutipes* using different carbon sources and their different concentrations. They reported highest production of xylanase in the presence of 0.2% xylan as well as xylose. They showed that type and size of carbon sources are important for xylanase production. Knob and Carmona, [19] reported maximum xylanase activity of 7.82 ± 0.25 U/ ml by *Penicillium sclerotiorum* using oat spelt xylan.

Effect of Different Nitrogen Sources on Xylanase Production: Different nitrogen sources viz. yeast extract, peptone, casein, ammonium sulfate, ammonium nitrate, sodium nitrate, potassium nitrate at a concentration of 0.5% in Emerson medium were used for xylanase production. Sodium nitrate and potassium nitrate were found to be better nitrogen sources for xylanase production. Ammonium nitrate, ammonium sulfate and casein were also found to be almost equally effective nitrogen sources. However, surprising yeast extract was found to be a poor nitrogen source for xylanase production (Fig 4). Contrarily, in *Cochliobolus sativus*, *Arthrobacter sp.* and *Paecilomyces themophila* J18, yeast extract has been shown to be a good source of nitrogen for production of xylanase [1, 16, 20] . In *Cochliobolus sativus*, sodium nitrate, potassium nitrate and peptone have been shown to be good nitrogen sources for production of xylanase. Whereas ammonium sulfate, ammonium nitrate, ammonium chloride and urea have been shown to be poor nitrogen sources for xylanase production [16]. Khandeparkar and Bhosle [1] also showed ammonium chloride and peptone to be better nitrogen sources for xylanase production in *Arthrobacter sp.*

Effects of Metals Ions on Xylanase Production: Different metal ions viz. Mg^{2+}, Fe^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} were used in the growth medium to check the amount of xylanase production. There was more than double xylanase production in the presence of 10 mM Mg^{2+} and Fe^{2+} whereas inhibition of xylanase production was observed in the presence of Hg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} (Table 4). In *Arthrobacter sp.* Khandeparkar and Bhosle [1] reported xylanase production to be 175, 161, 193, 212, 154% in the presence of Co^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+} and Mg^{2+} respectively. However, there was 73% inhibition in the presence of Hg^{2+}. In *Bacillus sp.*, xylanase production has been shown to be 108, 106, 104, 101 and 105% in the presence of $CaCl_2$, $CuSO_4$, $MgCl_2$, $MgSO_4$ and $ZnSO_4$ respectively, whereas presence of $FeCl_3$, $FeSO_4$ and $HgCl_2$ in the growth medium showed more than 90% inhibition of the xylanase production [21].

Enzyme Purification: The summary of purification of xylanase from *Bacillus halodurans* KR-1 is given in Table 5. The DEAE cellulose chromatography of the 30 to 70% sodium sulfate fraction revealed only one peak of xylanase activity that was eluted at 0.2 M NaCl. The concentration of NaCl in the eluent was calculated by using the formula given by Morris and Morris [22]. After DEAE cellulose chromatography, the enzyme was chromatographed on Sephadex G-200 column which also yielded a single peak indicating absence of multiple forms of the enzyme. The Sephadex G-200 chromatography resulted in a 23.64 fold purification of xylanase with 20% recovery from the initial extract. The specific activity of the enzyme was found to be 14.61 U/mg protein.

Vahino and Nakane [23] purified xylanase from a *Bacillus species* and showed specific activity of the purified enzyme to be 5.33 U/mg protein. Blanco *et al.* [24]

Table 4: Effect of metal ions on xylanase production by *Bacillus halodrans strain* KR-1

Metal ions	% Residual enzyme activity
None	100
$MgSO_4.7\,H_2O$	200
$CuSO_4$	0
$FeSO_4.\,7\,H_2O$	261.1
$CoCl_2.\,5H_2O$	83.33
$HgCl_2$	0
$MnSO_4.\,H_2O$	0
$ZnSO_4.\,7\,H_2O$	66.66

Table 5: Purification profile of xylanase from *Bacillus halodrans* strain KR-1

Sample	Total Volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Purification yield
Crude extract	700	1165.5	1883.70	0.618	1.00	100.00
Sodium sulfate fraction (30-70%)	40	732.8	333.96	2.1942	13.06	62.87
Sephadex G-25	40	719.28	332.12	2.164	12.88	61.71
DEAE Cellulose	10	299.7	28.29	10.59	17.13	25.71
Sephadex G 200	8	233.76	16.56	14.61	23.64	20.05

purified xylanase from a *Bacillus sp.* and reported its specific activity to be 40.2 U/mg proteins. Nakamura *et al.* [25] reported specific activity of purified xylanase from a *Bacillus* species to be 310 U/mg proteins. However, Amare [21] reported presence of two multiple forms of xylanase in a *Bacillus* species having specific activity of 524.2 and 367.9 U/mg protein, respectively. From the data, it is evident that there is wide variation in the specific activity of xylanase from different *Bacillus* species. In the present case, specific activity is comparatively low. However, our preparation is not homogeneously pure. The preparation is nearly 70 % pure as checked using polyacrylamide gel electrophoresis.

Molecular Weight : The molecular weight of xylanase has been found to be 45±02 kDa as determined by using Sephadex G-200 chromatography according to the procedure of Whitaker 14]. Bovine serum albumin, trypsinogen and lysozyme were used as standard markers (Fig. 5). On SDS polyacrylamide gel electrophoresis, the major band was calculated to have a molecular weight of 45±02 kDa indicating monomeric nature of the enzyme (Fig. 5). Wamalwa *et al.* [26] reported molecular weight of xylanase from *Bacillus halodurans* to be to be 22 kDa. Nakamura *et al.* [25] reported molecular weight of the enzyme from *Bacillus* species to be 36 kDa as determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Blanco *et al.* [24] reported the molecular weight 32kDa of the enzyme from *Bacillus* species using SDS-PAGE. Vahino and Nakane [23] reported the molecular weight of the enzyme from *Bacillus species* to be 56kDa. From these reports, it is clear that there is wide variation in the molecular weight of xylanase from different *Bacillus* species. Molecular weight of xylanase from *Aeromonas caviae* ME -1 and *Alcaligenes sp. XY*-234 has been reported to be 46kDa[27, 28]. Lin *et al.* [29] reported molecular weight of xylanase from *Thermomyces lanuginosus*-SSBP to be 23.6kDa [29].

Optimum pH: Xylanase activity has been found to be maximum at pH 9.0. (Fig 6). Earlier, Wamalwa *et al.* [26] reported optimum pH 7 for xylanase from a strain of *Bacillus halodurans*. However, Nakamura *et al.* [25] reported optimum pH 9 for xylanase from a *Bacillus species* strain 41M-1. Blanco *et al.* [24] reported optimum pH 5.5 of xylanase from a *Bacillus species* strain BP-23. Vahino and Nakane [23] showed optimum pH 4 for the enzyme from a *Bacillus species.* Kubata *et al.* [27] reported optimum pH 6.8 for xylanase from *Aeromonas caviae* ME -1. An optimum pH 7.5 has been reported for xylanase from *Alcaligenes sp. XY-234* [28]. Lin *et al.* [29] reported optimum pH 6.5 for xylanase from *Thermomyces lanuginosus*-SSBP. Knob and Carmona [19] reported optimum pH 4.5 for xylanase from *Penicillium sclerotiorum.*

Optimum Temperature: In the present case, xylanase activity has been found to be maximum at 40°C (Fig. 7). Wamalwa *et al.* [26] also reported optimum temperature 40°C for xylanase from an strain of *Bacillus halodurans*. However, Nakamura *et al.* [25] reported optimum temperature 50°C for xylanase from *Bacillus species* strain 41M-1. Blanco *et al.* [24] also reported optimum temperature 50°C for the enzyme from a *Bacillus species* strain BP-23. However, Vahino and Nakane [23] showed optimum temperature 80 °C for xylanase from a *Bacillus* species. Kubata *et al.* [27] showed optimum temperature of xylanase from *Aeromonas caviae* ME -1 to be 30°C and 37°C [28]. Araki *et al.* [28] reported optimum temperature 40°C for xylanase from *Alcaligenes sp.*XY-234. On the other hand, an optimum temperature of 70-75°C has been reported for xylanase from *T hermomyces lanuginosus*-SSBP [29]. Knob and Carmona [19] reported optimum temperature 50°C for xylanase from *Penicillium sclerotiorum.* Therefore, it is clear that there is wide variation in the optimum temperature of xylanase from different *Bacillus species*.

Km Value: Xylan saturation curve has been found to be rectangular hyperbolic and a linear double reciprocal plot (Fig. 8) indicated that the enzyme obeyed classical Michaelis kinetics. The Km value of xylanase for birchwood xylan was calculated to be 12.0 g/l. Wamalwa *et al.* [26] reported Km value of xylanase from *Bacillus halodurans* for birchwood xylan to be 9.47mg/ml.

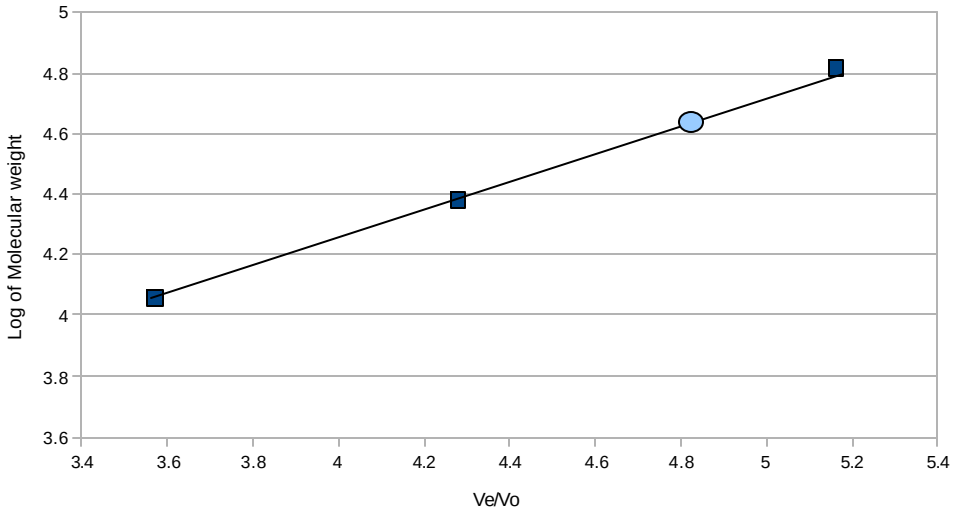


Fig. 5: Molecular weight determination of xylanase using Sephadex G-200 gel filtration chromatography (Whitaker method)

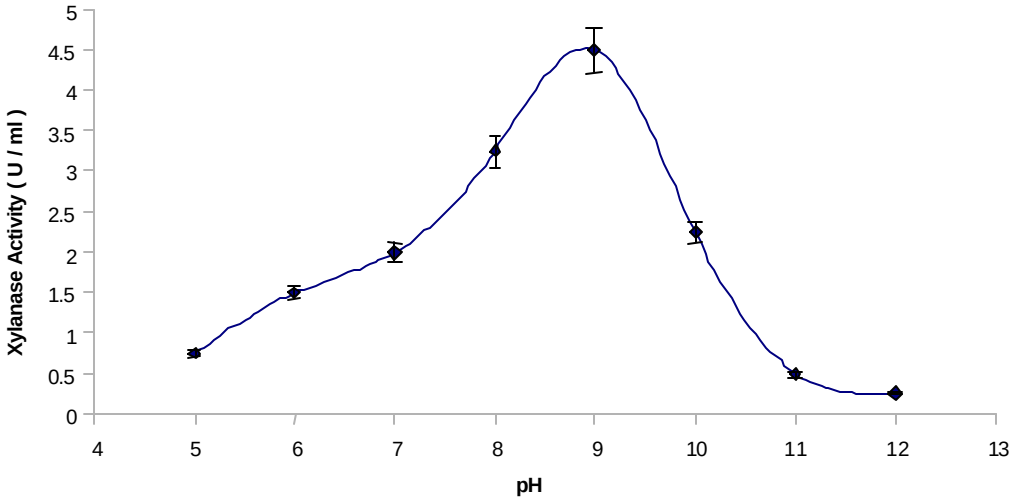


Fig. 6: Effect of pH on the activity of xylanase from *Bacillus halodrans* strain KR-1

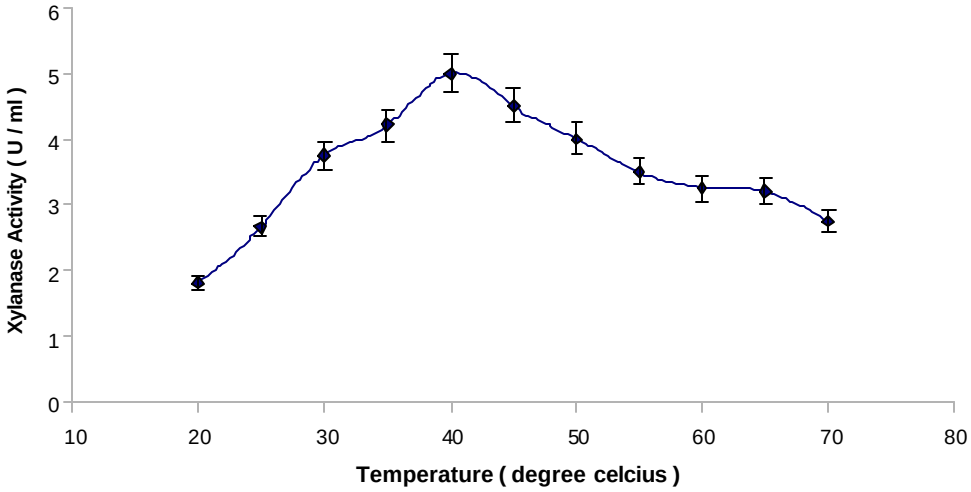


Fig. 7: Effect of temperature on the activity of xylanase from *Bacillus halodrans* strain KR-1

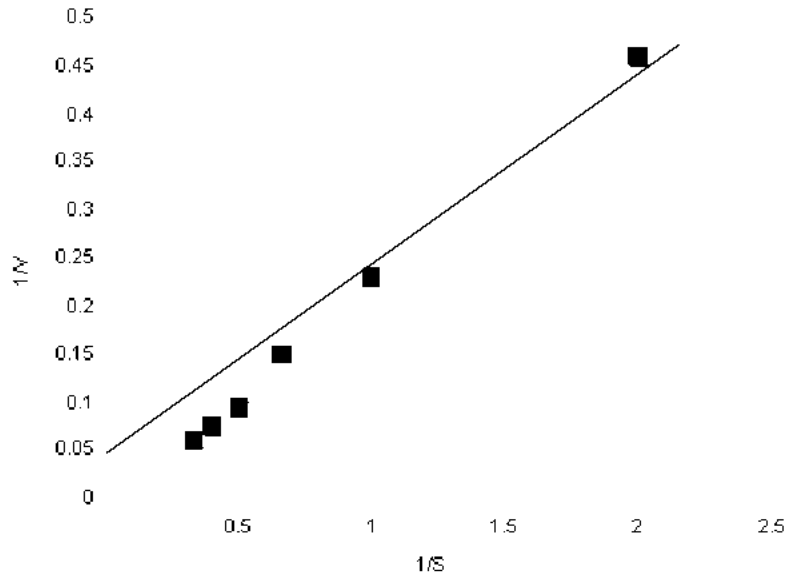


Fig. 8: Double reciprocal substrate saturation plot for xylanase from *Bacillus halodrans* strain KR-1

Vahino and Nakane [23] reported Km value of 1.68 mg xylose eq/ml for xylanase from a *Bacillus* sp. using largewood xylan. Kubata *et al*. [27] reported Km value of xylanase from *Aeromonas caviae* ME - 1 to be 2 mg/ml. The Km value of xylanase from *Alcaligenes sp*. XY-234 and from *Thermomyces lanuginosus*-SSBP has been reported to be 4 mg/ml and 3.26 mg/ml xylan, repectively [28, 29]. The Km value of the enzyme from *Thermomyces lanuginosus*-SSBP has been reported to be 3.26mg/ml xylan [29].

Hydrolysis Studies: Hydrolysis studies of xylan were carried out at 40°C by using xylanase. Hydrolysis products were analyzed after 1, 3, 6 and 12 h time intervals. Up to 6 h of hydrolysis, no significant amount of any intermediate product(s) could be observed. After 12 h incubation, intermediate products were found to be formed in the reaction; however no xylose formation could be observed. These results indicated that xylanase cleaves the xylan into xylo-oligosaccarides. The present enzyme may be exploited for industrial use in paper bleaching due to its alkalophilic properties.

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