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Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp AR-135

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An xylanase producing alkaliphilic *Micrococcus* sp was isolated from an alkaline soda lake. Xylose and xylan induced enzyme production but no activity was detected when it was grown using other carbohydrate sources. The level of xylanase production was higher in the presence of xylose than in the presence of xylan. The enzyme was purified to homogeneity and its molecular weight was estimated to be 56 kD on SDS-PAGE. The optimum temperature and pH for xylanase activity were 55°C and 7.5–9.0, respectively. Sixty per cent of the maximum activity was displayed at pH 11. The enzyme was very stable in the pH range of 6.5–10 and up to a temperature of 40°C. Xylanase activity was inhibited by Cu^{2+} and Hg^{2+}.

Keywords: alkaliphile; alkaline xylanase; *Micrococcus* sp

Introduction

World-wide a large amount of hemicellulose is released annually in the form of agricultural, municipal, and industrial waste. Development of enzymatic processes for the saccharification of such waste has been considered very important both as a way of waste management and for the production of simple sugars which can be used as feedstocks in different industrial processes [1,11]. Because xylan is the major component of hemicellulose, attention is mainly focused on the study of xylanases, enzymes which are involved in the hydrolysis of xylan. Xylan has better solubility at alkaline pH. As a result xylanases active at alkaline pH have interesting potential application for the hydrolysis of xylan.

Another important area of application for alkaline xylanases is for the bleaching of kraft pulp [2,5,25,28,31,33]. It has been shown that xylanase-treated pulp requires less chlorine during subsequent bleaching operations. As a result the amount of chlorinated organic compounds released in the bleach effluent is substantially lowered thus minimizing the risk of environmental pollution [2,25]. Since the kraft process is carried out at alkaline pH, the use of alkaline xylanases will have many advantages.

The great majority of xylanolytic microorganisms known to date are neutralophilic strains producing xylanases having optimum activity in the neutral or acidic pH range. Although different xylanolytic alkaliphiles have been reported from different laboratories, most of them produce xylanases optimally active around neutrality [4,6,12,13,15,21–23,30]. Most alkaliphiles known so far have been isolated from neutral soil samples [9,10]. Isolation and screening of microorganisms from naturally occurring alkaline habitats is expected to provide new strains producing alkaline enzymes which may be important from a scientific and application point of view. In this paper we report the purification and characterization of an alkaline xylanase from an alkaliphilic bacterial strain isolated from an alkaline soda lake.

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Materials and methods

The organism

A sample collected from Lake Arenguadie, an alkaline soda lake in Ethiopia, was streaked onto nutrient agar plates containing 1% Na_2CO_3. Individual colonies were isolated, purified through repeated streaking and transferred to xylan-containing agar plates. Xylanase production was detected following the congo red staining method [29]. Xylanase positive isolates on solid media were further grown on xylan-containing liquid media and the level of extracellular xylanase activity was determined from the cell-free culture supernatant. Liquid medium used for enzyme production was composed of (g L^{-1}): xylan, 5; peptone, 5; NaCl, 5; K_2HPO_4, 1; $MgSO_4$, 0.2; $CaCl_2$, 0.1; and Na_2CO_3, 10. Sodium carbonate was sterilized separately and added to the rest of the medium after cooling. One hundred milliliters of the above medium in 500-ml baffled flasks were inoculated with a 24-h inoculum culture and incubated at 32°C with rotary shaking. Afer 48 h the culture was harvested and cells were separated by centrifugation at $5000 \times g$ for 15 min.

Xylanase purification

The cell-free culture supernatant was precipitated using solid ammonium sulfate to 70% saturation. After centrifugation the pellet was dissolved in 10 mM Tris-HCl buffer, pH 8, and dialyzed against three changes of the same buffer. The dialyzed enzyme was applied to DEAE-Sepharose column (2.5×12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl at a flow rate of 90 ml h^{-1}. Fractions containing xylanase activity were pooled, concentrated, and dialyzed against 10 mM Tris-HCl buffer, pH 8. The concentrated enzyme preparation was applied to

Sephadex G-75 column (1.5 × 112 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8 and eluted at a flow rate of 12 ml h^{-1}.

Enzyme essay

Xylanase activity was assayed at 40°C in 50 mM Tris-HCl buffer pH 8. The amount of reducing sugar released from xylan was determined following the dinitrosalicylic acid (DNS) method as described previously [8]. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per min.

Protein assay

Protein concentration was determined following the bicinchoninic acid (BCA) assay method [26] using bovine serum albumin as a standard.

Results and discussion

The organism

Strain AR-135 was isolated from an alkaline soda lake in Ethiopia. It grows in the pH range of 8–11 but does not grow at pH 7. The organism is aerobic, Gram-negative, cocci, non-motile, catalase positive, and negative for Vogus Proskaur reaction. It forms a distinct clearing zone on xylan-containing agar plates, but does not hydrolyze gelatin, casein, starch, Tween 80, and cellulose. No acid and gas formation was detected from glucose. Colonies were deep yellow in color on nutrient agar plates. On the basis of these properties the strain was identified as a species of the genus *Micrococcus* following Bergey's Manual of Systematic Bacteriology [27].

Although a diverse group of microorganisms are known to produce xylanases, relatively few are alkaliphiles. Among xylanase-producing alkaliphilic strains known so far the great majority belong to the genus *Bacillus*, most of them isolated from neutral soil samples [9,10]. Naturally occurring alkaline habitats, which up to now are not properly exploited, are potential sources of new microbial strains. In this regard strain AR-135, isolated from a naturally occurring alkaline habitat, is among the few exceptions of xylanolytic alkaliphiles that do not belong to the genus *Bacillus*.

Effect of different carbohydrate sources on enzyme production

Xylanase production by AR-135 was induced by xylan and xylose (Table 1). Compared to xylan, xylose was a better inducer of xylanase activity. No extracellular xylanase activity was detected when the organism was grown in the presence of other carbohydrate sources. However, the organism grew equally well in all the carbohydrate sources tested. In most xylanolytic microbial strains xylose is known to either suppress enzyme production or support a lower level of xylanase production than xylan [8,16,17,24,31]. In addition, in many strains some basal xylanase activity has been detected in the presence of other carbohydrate sources [8,16,17,32]. In this regard the regulatory mechanism(s) of xylanase production by AR-135 appears to be different from most other xylanolytic microorganisms.

Table 1 Effect of different carbon sources on xylanase production by *Micrococcus* sp AR-135. The carbon sources were added at a concentration of 1% and the culture was harvested after 48 h. Each value is the mean of three independent experiments

Carbon source	Enzyme production (U ml^{-1})
Birch wood xylan	2.7
Oat spelt xylan	2.5
Xylose	3.7
Arabinose	NA
Glucose	NA
Sucrose	NA
Starch	NA
Lactose	NA
None	NA

NA: No detectable activity.

Enzyme purification

AR-135 xylanase was purified after ammonium sulphate fractionation, DEAE-Sepharose ion exchange chromatography and Sephadex G-75 gel filtration chromatography. The result of the purification is summarized in Table 2. After the final purification step the enzyme was purified 228-fold with a specific activity of 112 U mg^{-1} and 50% recovery. The purified xylanase preparation was homogenous on SDS-PAGE (Figure 1) and its molecular weight was estimated to be 56 kD.

Effect of pH on activity and stability

The pH profile of the enzyme was determined using different buffers of varying pH values. The enzyme was active in a broad pH range with an optimum at pH 7.5–9.0 (Figure 2a). At pH 11, 60% of the maximum activity was displayed. The effect of pH on stability, determined using different buffers of varying pH values at 40°C, is shown in Figure 2b. The enzyme was stable in the pH range of 6.5–10. Most xylanases known to date are active at acidic or neutral pH [1,3,29]. Even xylanases from many alkaliphilic microbial strains are optimally active around neutrality [4,6,12,13,15,21–23,30]. To date few microbial strains producing xylanases with optimum activity above pH 8 have been reported [8,19,20]. Xylanases active in the alkaline pH range are important for different applications. For example, in the production of pulp using the kraft process a large amount of hemicellulose-containing waste is released in the effluent. Because the effluent has an alkaline pH the use of alkaline xylanases for the hydrolysis of such waste is expected to allow the development of a cost-effective saccharification process by reducing the amount of acid required for pH readjustment and thus lowering the cost of ion exchange media needed during downstream processing. Alkaline xylanases are also important for the bleaching of kraft pulp [2,5,25,32]. Since the kraft process of pulp production is carried out at alkaline pH, the use of alkaline xylanases would reduce the need for pH re-adjustment thus minimizing the overall cost of enzyme-based pulp bleaching.

Table 2 A summary of the purification of xylanase from alkaliphilic *Micrococcus* sp AR-135

	Total protein	Total activity	Specific activity	Purification (fold)	Recovery (%)
Culture filtrate	1511.4	738.7	0.49	1.0	100
Ammonium sulfate	324.7	554.9	1.7	34.9	75
Ion exchange	13.5	500.0	42.5	86.7	68
Gel filtration	3.3	368.0	111.7	228.0	50

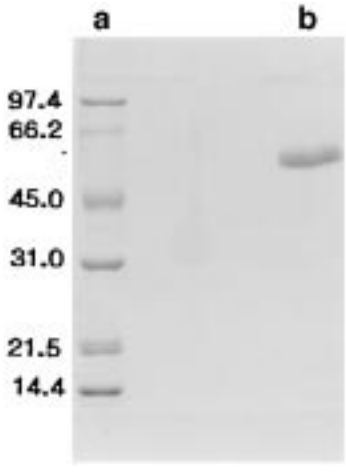

Figure 1 SDS-PAGE of purified AR-135 xylanase. (a) Molecular weight markers; (b) purified AR-135 xylanase.

Table 3 Effect of different metal ions on AR-135 xylanase activity

Metal ion (1 mM)	Relative activity (%)
None	100
NaCl	100
KCl	102
$CaCl_2$	106
$MgSO_4$	93
$BaCl_2$	90
$CoCl_2$	99
$ZnSO_4$	60
$FeCl_3$	43
$HgCl_2$	0
$AlCl_3$	87
$Pb(CH_3)COO^-$	66
$MnCl_2$	94
$CuSO_4$	1

Effect of temperature on activity and stability

The optimum temperature for xylanase activity was determined by varying the reaction temperature at pH 8. The enzyme was optimally active at 55°C (Figure 3a). Thermal stability of AR-135 xylanase was determined by heating the purified enzyme for 15 min at different temperatures in 50 mM Tris-HCl buffer, pH 8. The data presented in Figure 3b show that up to 40°C the enzyme was very stable followed by a rapid loss of activity above 45°C. The low thermal stability of the enzyme could be a disadvantage for some applications, such as in the pulp and paper industries where a xylanase having good activity and stability at elevated temperature and alkaline pH is preferred [33]. Cloning and sequencing of the gene for AR-135 xylanase may enable site-directed mutagenesis to be carried out, aimed at improving the thermal stability and other properties of the enzyme. Previously such an approach has been shown to give an appreciable increase in the thermal stability of xylanase A of *Streptomyces lividans* 1326 [18].

Effect of metal ions on activity

The effects of different metal ions on AR-135 xylanase was tested by assaying enzyme activity in the presence of different metal ions. Strong inhibition was observed in the presence of Hg^{2+} and Cu^{2+} while Fe^{3+} Pb^{+}, and Zn^{2+} resulted

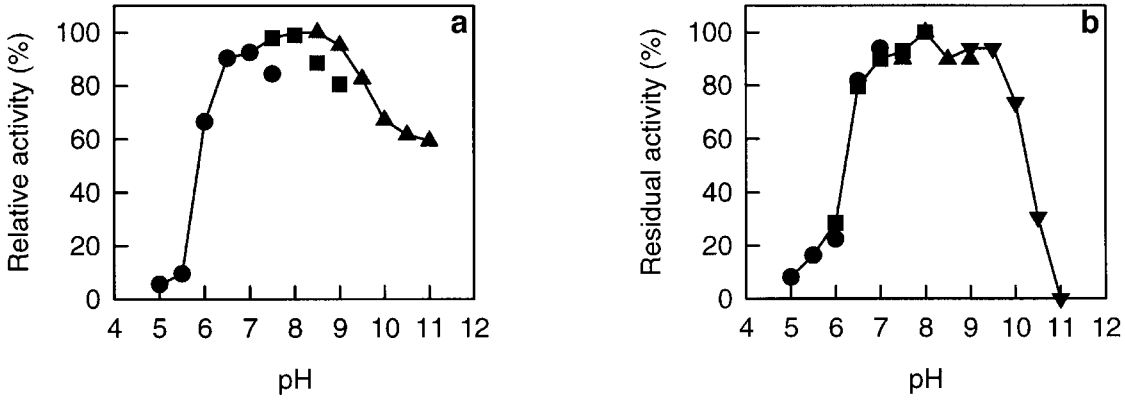

Figure 2 Effect of pH on activity (a) and stability (b) of AR-135 xylanase. The pH profile was determined in different buffers of varying pH values at 40°C. The buffers used were: citrate-phosphate (●), Tris-HCl (■), and glycine-NaOH (▲). The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 30 min at 40°C and the residual activity assayed following standard assay procedure. Buffers used were: citrate-phosphate (●), phosphate (■), Tris-HCl (▲), and glycine-NaOH (▼).

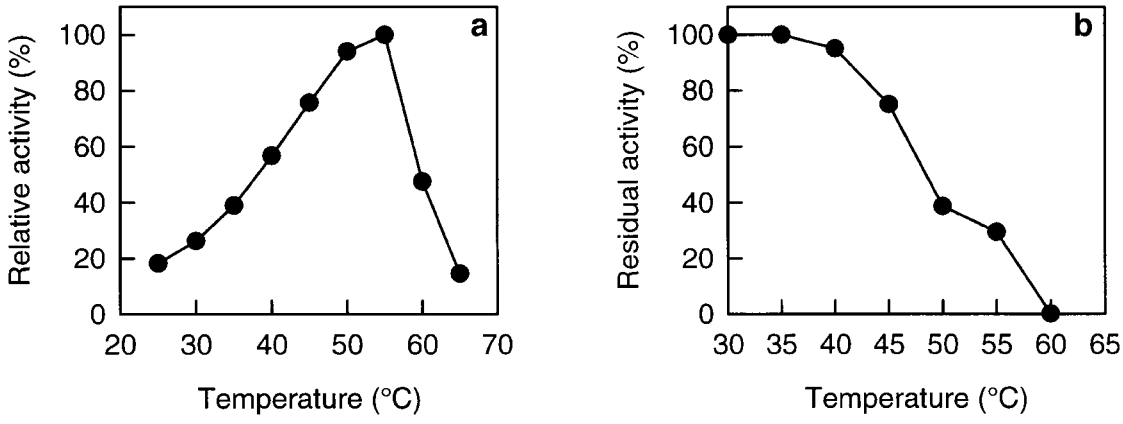

Figure 3 Effect of temperature on activity (a) and stability (b) of AR-135 xylanase. The temperature profile was determined by assaying enzyme activity at different temperature values using 50 mM Tris-HCl buffer, pH 8. The effect of temperature on stability was tested by incubating the enzyme at different temperature values for 15 min in 50 mM Tris-HCl buffer pH 8 and the residual activity was assayed at 40°C.

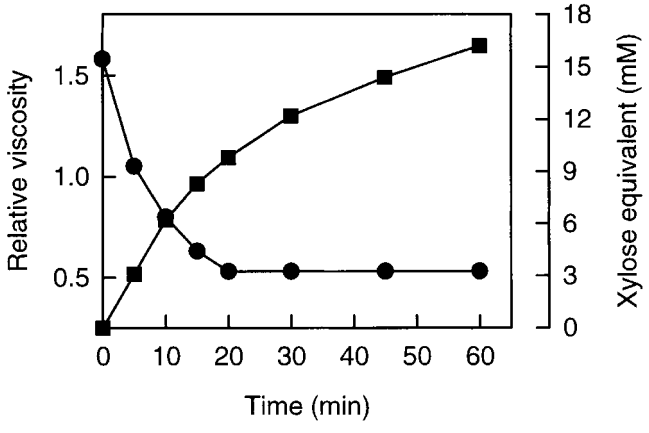

Figure 4 Viscosity reduction (●) and formation of reducing sugar (■) during degradation of xylan with AR-135 xylanase. The reaction was carried out using 0.5% xylan at 40°C.

in partial inhibition (Table 3). Resistance to inhibition by different metal ions differs among xylanases derived from different organisms and even isoenzymes of the same organism [7,14,19,30].

Mode of action

The mode of action of AR-135 xylanase was determined by measuring the rate of viscosity reduction of oat spelt xylan as described by Khasin *et al* [14]. The enzyme resulted in a rapid reduction in viscosity and corresponding rapid rise in reducing sugar concentration (Figure 4) indicating that it is an endoxylanase.

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