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CHARACTERIZATION OF XYLANASE AND CELLULASE FROM EXTREMELY HALOALKALIPHILIC ARCHAEON *NATRINEMA SP.* SSBJUP-1 ISOLATED FROM LONAR LAKE.

J. U. PATIL*[1] AND S. S. BAJEKAL [2]

[1] Department of Microbiology, Yashwantrao Chavan College of Science, Karad, Vidyanagar- 415124, Karad, Maharashtra (India)
[2] Department of Microbiology, Yashwantrao Chavan College of Science, Karad, Vidyanagar- 415124, Karad, Maharashtra (India)

ABSTRACT

The extremely halophilic archaeon *Natrinema sp.* strain SSBJUP-1, which produces extracellular cellulase and xylanase, was isolated from the Lonar Lake situated in Buldhana, Maharashtra. The enzymes were optimally active at pH 9–10 and temperature 40–60 ^{0}C and they were most stable up to pH 11 and 16 % of NaCl concentration. The xylanase showed maximum activity at 50^{0}C and cellulase at 40^{0}C. Xylanase kept excellent activity over a broad range of salt concentration from 12 to 22% with highest activity at 16 % NaCl while Cellulase showed maximum activity at 14% of salt. Unlike xylanase that was stimulated by Cu^{2+}, $Mg^{2+}$and Ca^{2+}, cellulase activity was reduced when treated with $Hg^{2+}$and Cu^{2+} and stimulated by $Mg^{2+}$and Ca^{2+}. This investigation showed that *Natrinema sp.* strain SSBJUP-1 is a potential source of xylanase and cellulase and an interesting candidate for application in biotechnological processes.

KEYWORDS*:* Archaea, Haloalkaliphiles, Xylanase, Cellulase, Natrinema sp.

J. U. PATIL
Department of Microbiology, Yashwantrao Chavan College of Science, Karad, Vidyanagar- 415124, Karad, Maharashtra (India)

***Corresponding author**

INTRODUCTION

Natrinema is a member of the extremely Haloalkaliphilic archaea group that belongs to family *Halobacteriaceae.* On the basis of 16S rRNA gene sequences, salt tolerance, chemotaxonomic and physiological characteristics, the genus *Natrinema* was created in 1998 to accommodate *Natrinema pellirubrum* (formerly *Halobacterium salinarum* NCIMB 786) and *Natrinema pallidum* (formerly *Halobacterium halobium* NCIMB 777)[1]. The extreme halophilic archaea require at least 1.5 M NaCl for growth. However, most strains grow best at 3.5–4.5 M NaCl[2]. They have been isolated from different habitats including alkaline and salt lakes, marine salterns, the Dead Sea and saline soils[3,4,5]. Halophilic archaea, in contrast to halophilic bacteria which maintain a cytoplasm with low concentration of salt by production of compatible solute, use a high salt in strategy in order to survive osmotic challenges associated with life in hypersaline environments[6]. Thus, they have enzymes which are active at up to 5 M or higher concentration of NaCl or 4M KCl [7]. These enzymes have catalytic function in the condition of low water activity, a situation common in the presence of organic solvents[8].

Cellulases are enzymes which hydrolyze the β-1,4-glycosidic linkage of cellulose and have traditionally been divided into endoglucanases (E.C.3.2.1.4) and cellobiohydrolases (E.C.3.2.1.91). Recently, the potential of cellulases was revealed in various industries, such as food, textiles and laundry, pulp and paper and agricultural industries as well as research and development [9]. Xylanases are also glycosidases, catalysing the endo hydrolysis of 1, 4 β D- xylosidic linkage in xylan. They are a widespread group of enzymes, involved in the production of xylose, a primary carbon source in cell metabolism and in plant infection by plant pathogens and are produced by organisms including bacteria, algae, fungi, protozoa and arthropods[10]. Xylanases constitute the major commercial proportion of hemicellulases but represent only a small percentage of the total enzyme sales. The sales figure is expected to rise as these enzymes have attracted attention due to their potential for use in several applications such as in brewing to increase wort filterability, in coffee extraction[11], in detergent, in the production of pharmacologically active polysaccharides as antimicrobial agents[12], as antioxidants and in the production of surfactants[13].

Many of these enzymes used in industries today appear to be of mesophilic or neutrophilic origin. Yet enzymes from extremophilic sources may be of tremendous utility in many biotechnological processes. Despite advances in understanding the diversity and systematics of haloarchaea, studying their hydrolytic enzymes such as amylases, xylanases, cellulase and protease and their characterization has received less attention. However, some special haloarchaeal enzymes are characterized in detail[14]. Characterization of cellulase and xylanase from such extremely halophilic archaea for their high alkalinity and high salinity, therefore, assumes significance from the biotechnological point of view. The aim of this study is thus to describe the characters of these two hydrolytic enzymes from the extremely Haloalkaliphilic archaeon *Natrinema sp.* isolated from this environment.

MATERIALS AND METHODS

Bacterial isolation, media and culture conditions

Soil samples were obtained from the Lonar Lake situated in Buldhana District (Lat.19^{0}58', long. 76^{0}34') of Maharashtra, India. Aliquots of the soil were enriched in Specific Haloalkaliphilic (SH) medium[15] containing (g/l): casamino acid, 7.5; yeast extract, 10; Tri-sodium citrate, 3; MgSO4.7H2O, 1; KCl, 2; FeSO4.7H2O, 0.05; NaCl, 200; Na_2CO_3, 18.5. The pH was self adjusted (8.5 as measured on a Toshcon digital pH meter). The sterilization of NaCl and Na_2CO_3 was carried out separately at 121^{0}C 15 min. Incubation was carried out at 40^{0}C under aerobic conditions for 15 days. Enriched samples were then streaked on the

same medium for isolation and incubated at 40°C for up to 20 days. Pure isolates were obtained by successive cultivation on solid SH medium. Pure cultures were screened for extracellular hydrolytic activity. Storage of pure cultures was on the slopes of the same medium at 4^0C.

Identification of the isolate

Morphological, physiological and biochemical characteristics of the isolate were studied in SH medium. Colony characters, Gram staining, motility, utilization of various carbohydrates and acid production were determined as described by Ventosa [16]. To confirm the identity of the isolate, 16S rRNA gene sequencing was done in Microbial Culture Collection, National Centre for Cell Science Pune.

Detection of hydrolytic enzyme activity

This was accomplished by streaking the pure culture on sterile SH agar supplemented with cellulose and xylan in Petri dishes, incubation at 40^0C for up to 21 days. Production of the respective enzymes was detected by conventional methods of flooding cellulose and xylan agar with 0.1 % Congo red and observing for appearance of clear zones around the growth.

Production, Partial Purification and assay of the enzymes

This was performed in SH medium supplemented with 1% CMC and 0.5% xylan. After cultivation of the isolate at 40^0C for 10 days, cell-free supernatants were collected by centrifuging for 20 min at 10,000 rpm at 4 ^{0}C. Cell-free supernatants were collected by centrifugation at 10000xg for 20' at 4^oC and the enzymes precipitated with ammonium sulphate, stirring for 30' and refrigeration at 4^oC overnight. The precipitates were collected through centrifugation at 5000xg for 15' at 4^oC, dissolved in 50ml 50mM Tris-HCl buffer (pH 8.5) and dialysed against the same buffer at 4^oC overnight. Xylanase and Cellulase activity was measured using the 3, 5-dinitrosalicylic acid (DNS) method. Here 2.5 mL of substrate solution (0.5% xylan and 1% carboxy methyl cellulose (CMC)) mixed in 2.5 mL of buffer, 1mL of cofactor (1% NaCl) and 0.5 mL of dialyzed supernatant, and the mixture was incubated at 37 °C for 15 min. The reaction was stopped by adding 0.5 ml inactivator (2N NaOH) and DNS reagent followed by heating in boiling water bath for 15 min. The amount of reducing sugar released was quantified by measuring the absorption at 540 nm in colorimeter and using Glucose/Xylose as the standard. One unit of enzyme activity was defined as the amount of enzyme releasing one microgram of reducing sugar per minute from soluble xylan and CMC under the assay conditions[17].

Effect of temperature and pH on the activity of the enzymes

To determine the temperature optimum for the xylanase and cellulase the assay was carried out at various temperatures from 0 to 100 ^{0}C. The effect of pH on xylanase and cellulase activity was studied by incubating the reaction mixture at different pH values ranging from 3.0 to 11.0, in the following buffer systems: 0.1 M sodium acetate (pH 3.0–5.6); 0.1 M sodium phosphate (pH 6.0–7.0); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M carbonate bicarbonate buffer (pH 9.0–11.0).

Effect of NaCl concentration, metal ions and substrate concentrations on the activity of the enzymes

Xylanase and cellulase activity was assayed at optimum temperature and pH with different NaCl concentrations (0–26%) in the reaction mixture. To determine the effect of metal ions on xylanase and cellulase activity, the assay was carried out in metal ions Ca^{2+}, Mg^{2+} Cu^{2+}, Na^{2+}, Hg^{2+}, and Zn^{2+}. The effect of substrate concentrations was studied by incubating the reaction mixture with different substrate (soluble xylan and cellulose) concentrations.

RESULTS

Identification of Haloalkaliphilic isolate

Cells of the isolate are Gram variable cocci and non motile. Colonies on SH plate are reddish pink, circular, regular, convex and moist. In KOH method[18] the young culture showed Gram

negative nature while the older culture was Gram positive. The isolate grew well in 20% salt concentration. Optimal bacterial growth was observed at pH 9, 37–42^{0}C, and 20% NaCl. The isolate was positive for catalase, amylase, caseinase, cellulase and xylanase activities while lipase, gelatinase and oxidase activities were negative. Acid was produced from glucose, galactose, maltose and arabinose. The phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the isolate (GenBank accession number JX478270.1) was a member of the genus *Natrinema*. The two enzymes, xylanase and cellulase were produced by cultivation of the organism in respective liquid media and the yield of each found to be 26 U/ml and 35 U/ml respectively.

Effect of temperature on xylanase and cellulase activity

As shown in Fig. 1 and 2 both xylanase and cellulase showed similar responses to temperature with both showing maximum activity at 40^{0}C with fair activity at lower temperatures and up to 55^{0}C, rapidly losing it at high temperatures.

Figure 1 & 2
Effect of temperature on xylanase and cellulase activity

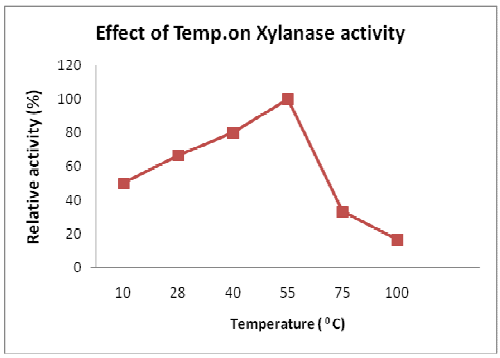


Figure 1

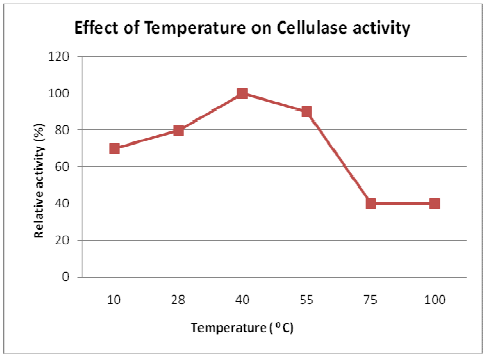


Figure 2

Effect of pH on xylanase and cellulase activity

Both xylanase and cellulase showed increasing activity from pH 4.0 upwards to reach their peaks in the alkaline range, with xylanase showing maximum activity at pH 8.0 and cellulase, at pH 9.0. The activity of both the enzymes declines rapidly immediately thereafter (Fig. 3 and 4).

Figure 3 & 4
Effect of pH on xylanase and cellulase activity

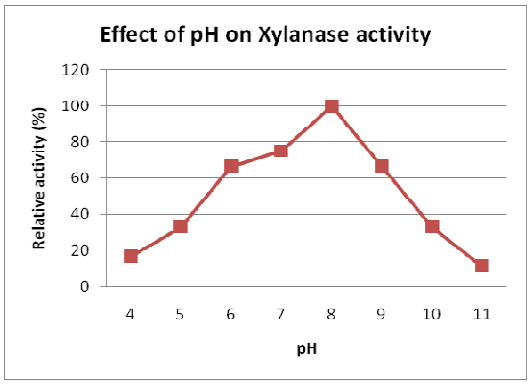


Figure 3

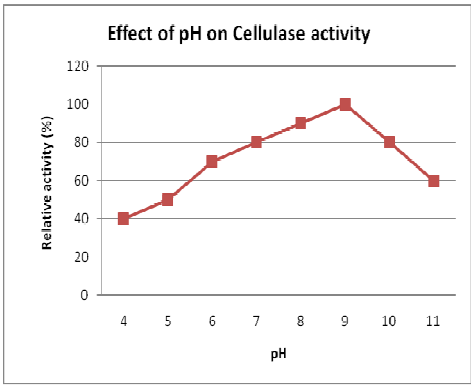


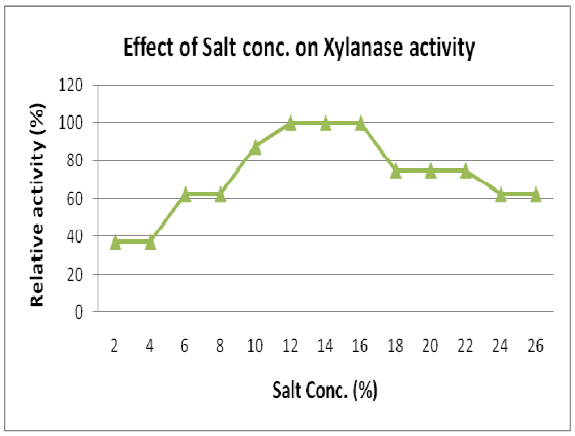
Figure 4

Effect of NaCl concentrations on xylanase and cellulase activity

The enzyme activity was also determined at different NaCl concentrations (0–26%) under optimum temperature and pH conditions. Xylanase kept excellent activity over a broad range of salt concentration from 12 to 22% and the highest activity was obtained at 16% NaCl. At higher salinities (22%), more than 80% of the activity was retained. The activity below 10% NaCl is significantly lower with around 70% lost, indicating the halophilic nature of the enzyme (Fig. 5). Cellulase showed maximum activity at 14% of salt conc. While above and below this, it shows steady activity over broad range (Fig. 6).

Figure 5 & 6

Effect of salt concentration on xylanase and cellulase activity



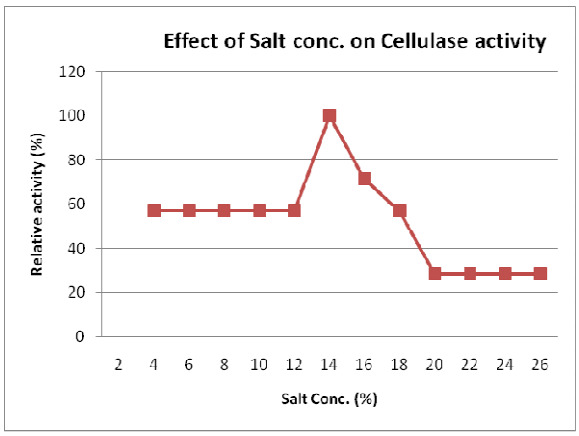


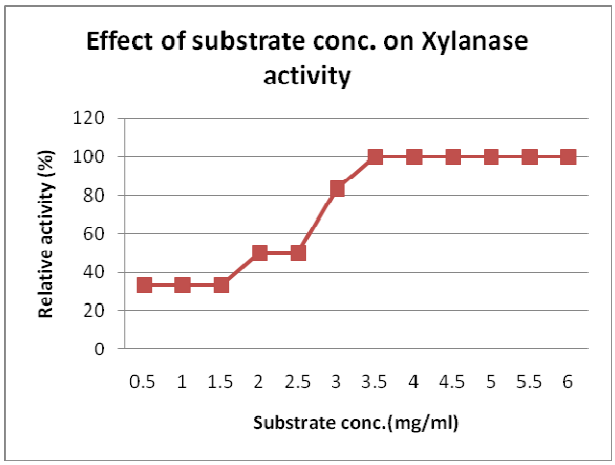
Figure 5 **Figure 6**

Effect of substrate concentration on xylanase and cellulase activity

The xylanase showed activity over a wide range of xylan concentration above 3 to 6 mg/ml. Initially xylanase showed a gradual increase in activity with the increase in substrate concentration up to 3.5 mg/ml beyond which it remains steady showing saturation of enzyme (Fig.7). The cellulase enzyme showed saturation with 8 mg/ml concentration of CMC (Fig. 8)

Figure 7 & 8

Effect of substrate concentration on xylanase and cellulase activity



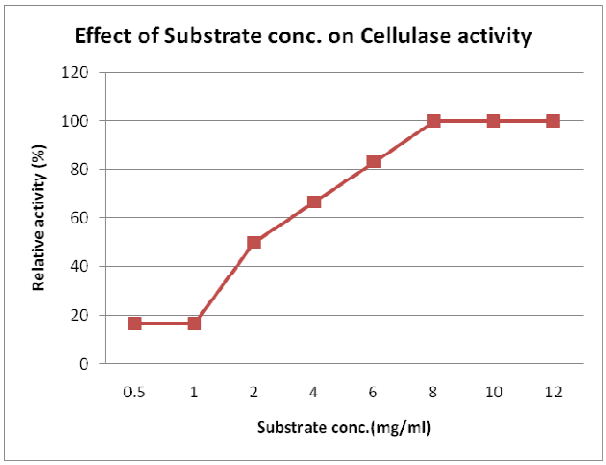


Figure 7 **Figure 8**

Effect of various metal ions on xylanase and cellulase activity

The effect of metal ions on xylanase activity showed that activity was markedly stimulated by metal Ca^{2+}, Cu^{2+} and Mg^{2+} in variable range of metal ion concentration compared with Na metal but was totally inhibited by Hg^{2+}. The Mg^{2+} and Na^{+} ions showed a stimulatory effect on cellulase activity while Ca^{2+} and Hg^{2+} were inhibitory. Complete inhibition of the xylanase by Hg^{2+} while stimulation by Ca^{2+} providing evidence that the xylanase was metalloenzyme and are Ca^{2+}dependent[22].

DISCUSSION

In recent years, there has been an increasing interest in studying the microbial flora of soda lakes since these naturally occurring alkaline hypersaline environments are the potential source of assorted microorganisms. Halophiles, alkaliphiles and haloalkaliphiles commonly found in such environments are useful for the development of new bioprocesses and novel microbial products of commercial interest[19]. The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter, Haloferax, Halobacterium, Halorhabdus, Marinococcus, Micrococcus, Natronococcus, Bacillus, Halobacillus* and *Halothermothrix.* However, many of these enzymes have not been investigated in detail or applied[20]. Moreover, these studies have not included the haloalkaliphilic archaea and this makes them very attractive for screening of novel enzymes with unusual properties. Makhdoumi et al[21], based on studies on several isolates obtained from a salt lake, have suggested that the *Natrinema* genus is a very good candidate for production hydrolytic enzymes. Among the extremophilic xylanases, the thermophiles, acidophiles and alkaliphiles have been extensively studied while halophilic xylanases have been much less investigated[22]. In this study, some extreme haloalkaliphilic archaea were isolated from Lonar Lake among which, the isolate identified as the genus *Natrinema sp.*SSBJUP-1 was found to be very promising for further studies as it happened to be the best producer of extracellular xylanase and cellulase. The properties of these two enzymes from this isolate indicate that they may prove interesting candidates for application in biotechnological processes, such as the treatment of foodstuff wastewater with starch, xylan, in textiles and laundry, pulp and paper etc. To our knowledge, xylanase and cellulase have not so far been reported from Haloalkaliphilic archaea. The present study indicates that *Natrinema sp.*SSBJUP-1 may be a potential source of xylanase and cellulase produced under high salinity and may be of commercial value.

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