

Diversity of hydrolytic enzymes in Haloalkaliphilic archaea isolated from Lonar Lake



Microbiology

KEYWORDS : Archaea, Haloalkaliphiles, Extracellular enzymes, Lonar Lake

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ABSTRACT

Production of five hydrolytic enzymes was qualitatively studied in Haloalkaliphilic archaea isolated from Lonar Lake. All isolates were extreme haloalkaliphiles and were not able to grow under 15% salt and grew optimally at 20% with an optimum pH of 9.5. Phenotypic characteristics and 16S r-RNA sequence analysis showed the strains to be members of family Halobacteriaceae, belonging to genera Natrinema, Natrionalba and Natronobacterium. All the three isolates were able to produce xylanase. Natrinema sp. produces amylase, caseinase, and cellulase where caseinase showed maximum activity at 23% salt and at 60°C at pH 9.5. Natrionalba chahannaensis produces amylase, caseinase, gelatinase and cellulase. Natronobacterium innermongoliae produces thermostable amylase with optimum temperature of 55°C and pH 9.0 and caseinase which showed maximum activity at 45°C and with 14% salt. The present investigation reports isolation and studies on the enzymatic diversity of extremely Haloalkaliphilic Archaea for biotechnological applications.

INTRODUCTION

Extremely haloalkaliphilic archaea, a group of organisms with twin extremities of pH and salinity for growth, belong to 'archaea', a group of organisms recognized as the 'third domain of life' after the prokaryotes and eukaryotes. Evolutionarily, the domain archaea has learnt to thrive in extreme conditions including extremities of pH, temperature, pressure, salt, etc. and contribute significantly to planetary biomass in these environments. In this context the group's enzymes are of tremendous significance. Major interests have so far focused on the enzymes of thermophiles that can function at higher temperatures. The enzymes from halophilic and haloalkaliphilic organisms that can function in extreme pH and salinity have been less explored, but are now generating interest from this point of view (Horikoshi, 2008 and Kakhki et al, 2011). With the advancement in molecular tools, it would be possible to get an insight into the biocatalytic mechanisms for greater applications (Santos et al, 2009).

The world famous Lonar Lake in Buldhana district of central Maharashtra is a prime candidate for such haloalkaliphilic archaea as it is known to be highly alkaline with high salinity. The present investigation reports isolation and studies on the enzymatic diversity of three extremely haloalkaliphilic Archaea *Natrinema*, *Natrionalba* and *Natronobacterium* from this lake environment for potential biotechnological applications.

MATERIALS AND METHODS

Site description

Lonar Lake (Latitude 19°58', Longitude 76°36') is a unique ecosystem formed by meteorite impact in basaltic rock. It is situated in village Lonar of Buldhana district in Maharashtra State, India. It is an almost circular depression with diameter of 1875 meter and depth of 135 m (Fig1). Previously considered to be around 52,000 years old, a recent study by Jourdan et al, (2011) has on the basis of ⁴⁰Ar/³⁹Ar step heating experiments put it at 570 ± 47 ka.



Sample collection

Soil and water samples, 11 in number, were collected around the periphery of the Lonar Lake. Soil including the salt crust from the littoral zone was collected from the top 15 cm into sterile polythene bags. Water samples were collected following standard protocol. Samples were transported at ambient temperature to the laboratory and processed immediately or in case of delay kept at 4°C.

Enrichment and isolation

Aliquots of the soil and water samples were enriched by inoculation into specific Haloalkaliphilic (SH) medium of composition 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; Na₂CO₃, 18.5. The pH was self adjusted (8.5 as measured on a Toshniwal digital pH meter). NaCl and Na₂CO₃ were separately autoclaved at 121 °C /15' and incubated at 40 °C under aerobic conditions for 21 days. Enriched samples were then streaked on the same medium to obtain isolates and incubated at 40 °C up to 21 days. Pure isolates were obtained by successive cultivation on solid SH medium. Strains were stored under refrigeration. Pure cultures were then screened for extracellular hydrolytic enzymes like amylase, xylanase, cellulase and protease.

Identification of the isolates

Morphological, physiological, and biochemical characteristics of the isolates were studied in SH medium. Colony characters, Gram staining, motility, utilization of various carbohydrates and acid production were determined as described by Ventosa et al 1982.

To confirm the identity of the isolates, 16S rRNA gene sequencing was done in Microbial Culture Collection, National Centre for Cell Science Pune. Phylogenetic relationship of the isolates was determined by comparing the sequencing data with closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information, via BLAST search. Phylogenetic trees were constructed using the software package MEGA 4.0 (Tamura et al 2007).

Screening of strains for extracellular hydrolytic activities

Determination of extracellular amylase activity

The presence of amylolytic activity on plates was determined qualitatively by following the method described by Amoozegar et al. (2003), using starch agar medium containing 20 % (w/v) salt. After incubation at 40 °C for 21 days, the plates were flooded with Lugols iodine solution; a clear zone around the growth indicating the hydrolysis of starch.

Determination of extracellular cellulase and xylanase activity

CMCase and xylanase activity of the isolates were screened in a SH agar medium supplemented with 1% CMC and 1% xylan. After incubation at 40 °C for 21 days, the plates were flooded with 0.1 % Congo Red solution; a clear zone around the growth with red background indicating cellulase and xylanase activity (Zhou and Li, 2004; Wejse and Ingvorsen, 2003).

Determination of extracellular protease (Gelatinase and caseinase) activity

Proteolytic activity of the cultures was screened in solid SH medium supplemented with 1% gelatin and casein. Clear zones around the growth after incubation at 40 °C for 21 days for caseinase and after the addition of Frazier’s reagent for gelatinase were taken as evidence of proteolytic activity.

Production of enzymes

After cultivation of isolates at 40 °C for 21 days in SH broth, cell-free supernatants after centrifuging at 10,000 rpm for 20 min at 4°C were collected and used as crude enzyme source.

Determination of optimum pH, temperature and salt concentration

Optimum pH, temperature and salt concentration for all the enzymes were determined by measuring the enzyme activity at various pH, temperature and salt concentrations using standard protocols (Jayaraman , 2000).

RESULTS AND DISCUSSION

Haloalkaliphilic Archaea were obtained from the soil and water from all around the periphery of Lonar Lake. These organisms were found to be grow optimally at 20% salt concentration but unable to grow in salt below 15% proving that they were true halophiles. Their optimum pH was in the range 8-10 with no growth below 7.0. Most isolates showed the typical orange red pigmentation as well and all stained Gram negative. From 16S r-RNA sequencing all were confirmed to be members of family Halobacteriaceae.

Upon qualitative analysis for extracellular hydrolase activity, three isolates *Natrinema sp.*, *Natrialba chahannaensis* and *Natronobacterium innermongoliae* showed great potential (Table 1). All the isolates produced amylase, caseinase and xylanase while gelatinase was produced only by *Natrialba chahannaensis* and *Natronobacterium innermongoliae* did not produce cellulase.

The various enzymes produced by these organisms were also characterized for their kinetic properties and the results are presented in Table 2.

It is obvious from the above table that all the enzymes are alkaline and Halostable with their optimum pH around 8.5-9.0 in range between 6.0 and 12.0 and salt tolerance up to 24%. Four enzymes, amylase and caseinase of *Natrinema sp.* caseinase of *Natrialba chahannaensis* and amylase of *Natronobacterium innermongoliae* shows an optimum salt requirement of 20% and above

Thermal stability of these enzymes is also very good and all of them functions optimally around the 55°C. The two proteases – caseinase and gelatinase of *Natrialba chahannaensis* are seen to be stable even at 70°C.

These extreme kinetic optima of the enzymes make them suitable for several biotechnological uses especially in detergents and several food fermentations such as fish, sauce etc.

Thus the possibility to have a wide variety of extreme haloalkaliphilic archaea producing extracellular hydrolytic enzymes will be of valuable importance for biotechnological applications.

Table 1

Isolate	Enzymatic activity				
	Amylase	Caseinase	Gelatinase	Cellulase	Xylanase
<i>Natrinema sp.</i>	+	+	-	+	+
<i>Natrialba chahannaensis</i>	+	+	+	+	+
<i>Natronobacterium innermongoliae</i>	+	+	-	-	+

Table 2. The effect of pH, temperature and salt concentration on the activity of enzymes produced by *Natrinema sp. SSBJUP-1*, *Natrialba chahannaensis SSBJUP-3* and *Natronobacterium innermongoliae SSBJUP-4*.

Organism and enzyme	pH		Temp.		Salt conc.	
	Range	Opt.	Range	Opt.	Range	Opt.
<i>Natrinema sp.</i>						
Amylase	6.0-9.5	8.0	40-60	55	14-22	20
Caseinase	8.0-10	9.5	45-65	60	16-24	23
Gelatinase	-	-	-	-	-	-
Cellulase	7-11	9.0	28-55	45	12-18	14
Xylanase	6.0-9.5	8.5	40-65	50	12-22	16
<i>Natrialba chahannaensis</i>						
Amylase	7.0-10	8.5	28-60	50	10-20	16
Caseinase	8.0-10	8.5	28-70	50	16-24	20
Gelatinase	8.0-10	9.5	28-70	55	12-20	16
Cellulase	7.0-10	8.5	40-60	55	12-20	14
Xylanase	7.0-9.5	8.5	28-55	45	12-20	18
<i>Natronobacterium innermongoliae</i>						
Amylase	6-10	9.0	37-60	55	15-20	20
Caseinase	7.0-10	8.5	28-60	45	12-20	14
Gelatinase	-	-	-	-	-	-
Cellulase	-	-	-	-	-	-
Xylanase	7.0-10	8.5	40-60	45	14-20	15

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