Int.J.Curr.Microbiol.App.Sci (2016) *5*(11): 51-64

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 11 (2016) pp. 51-64**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.511.006

Extracellular Hydrolases producing Haloarchaea from Marine Salterns at Okhamadhi, Gujarat, India

Bhavini N. Rathod[1], Harshil H. Bhatt[2] and Vivek N. Upasani[1]*

[1]Department of Microbiology, M.G. Science Institute, Ahmedabad- 380009, India
[2]Kadi Sarva Vishwavidyalay Gandhinagar-23, India
**Corresponding author*

Keywords

Extremozymes, haloarchaea, hydrolases, *Halobacterium*, *Haloferax*, *Halopetinus*, Okhamadhi

Article Info

Accepted:
04 October 2016
Available Online:
10 November 2016

A B S T R A C T

Haloarchaea thrive in hypersaline environments such as marine salterns, saline soils, soda lakes, salted foods, etc. The lysis of marine phyto- and zoo-planktons such as algae, diatoms, shrimps, purple and green bacteria, fish, etc. releases biopolymers namely cellulose, starch, chitin, proteins, lipids, etc. in the saline ecosystems. The chemorganotrophic haloarchaea therefore, need to produce hydrolytic enzymes to utilize these substrates. However, the raw solar salt used for preservation can cause spoilage of foods due to the growth of halobacteria leading to economic loss. We report here the isolation and identification of extracellular hydrolases (substrates casein, gelatin, starch, and Tweens: 20, 60, 40, 80) producing haloarchaea isolated from the salt and brine samples collected from marine salterns at Okhamadhi, Gujarat, India. Morphological, physiological, detection of diether lipids, carotenoids, antibiotic sensitivity and molecular (16S rRNA) sequencing was carriedout. Majority of the isolates showed their potential to hydrolyze at least one substrate, while one strain BVM005 belonging to the genus *Haloferax* showed multiple hydrolytic activities against four substrates (casein, gelatin, starch and Tween 80).The 16S rRNA sequences of these strains were deposited in the GenBank, accession numbers: KP636732-33, 34, 35, 36, and 37. This is the first paper concerning the identification of hydrolases producing haloarchaea based on 16S rRNA sequencing from Okhamadhi, Gujarat.

Introduction

Microbes are ubiquitous in a wide range of extreme conditions (salinity, pH, temperature, pressure, light intensity, oxygen and nutrient limitations). Hypersaline environments can be classified as: Thalassohaline (originate by solar evaporation of seawater which contains sodium, magnesium, potassium, chloride and sulphate as the major components), and Athalassohaline (originate by solar evaporation of inland surface water) (Ventosa, 2006). The haloarchaea belonging to the family *Halobacteriaceae* are distinguished by their requirement for high concentration of salts (at least 1.5M NaCl), neutral or high pH, and insensitivity to antibiotics affecting cell-wall (peptidoglycan) synthesis, presence of diphytanyl glycerol ether core-lipids, red C_{50} carotenoids (bacterioruberins), etc. (Upasani

et al., 1994; Amoozegar *et al*., 2013). The growing demand for extremozymes has been satisfied either by improving the properties of the enzyme by site-directed mutagenesis or discovery of novel enzymes with desired characteristic from extremophiles. Genomic and structural analyses of halophilic enzymes has established that they are negatively charged due to an excess of acidic over basic residues, and altered hydrophobicity, which enhances solubility and promote function at conditions of low water activity (Oren, 2002; Siglioccolo *et al*., 2011; DasSarma and DasSarma, 2015).

Among the various enzymes of halophilic origin, amylase, proteases and esterases/lipases have potential applications in detergents, food, pharma industry, paper manufacturing, waste management and other allied industries (Schreck and Grunden, 2014). Recent application of halophilic proteases also includes their use in antifouling coating preparations provided the enzymes are organic solvent-tolerant. Lipases have been used widely in biofuel production, biotransformations, textile processing, waste treatment, environmental bioremediation, detergent additives, tea processing, cosmetics, leather processing, etc., (Boutaiba, 2006; Chakraborty *et al*., 2009; Muller-Santos *et al*., 2009; Ozcan *et al*., 2012). The advantage of marine algae-based biofuel systems have triggered interest in lipases from halophilic / halotolerant micro-organisms. There has been an interesting report on the industrial application of a recombinant lipase (LipBL) from *Marinobacter lipolyticus* SM19 expressed in *E. coli* to produce both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil (Perez *et al*., 2011). The aim of this study was to isolate and screen haloarchaea from Okhamadhi site, a marine solar saltern used for salt production in the coastal region of Gujarat by Tata Chemicals Limited (TCL), Mithapur viz. for industrially important enzymes. To the best of the author's knowledge, this is the first paper concerning the screening and characterization of hydrolyases producing haloarchaea from this site in India.

Methods

Sampling site and sample collection

Samples: water/brine and salt were collected in April 2014 (temperature 35^0C $\pm$ 2^0C) from the salt pans alongside the railway track at Okhamadhi salt works near Dwarka situated at 22.4^0 N and 69^0 E on the west coast Gujarat state. The samples were taken in sterile containers, preserved at 4^0C and processed within 48 hours.

Enrichment and isolation of haloarchaea

Haloarchaea were enriched by inoculating the samples (2.0ml water or 1.0g salt in 100ml medium in 250ml Erlenmeyer flasks) in different sterile media as follows: (I) Mullakhanbhai and Larsen (M-L) medium (Mullakhanbhai and Larsen, 1975); (II) Larsen medium (Larsen, 1981); and (III) Oren medium (Oren, 1983). The pH was adjusted to 7.0-7.5 before autoclaving. The flasks were incubated at 37^0C, 180 rpm for 7-10 days. The isolates were obtained by streaking on the respective medium, incubation at 37^0C, 7-10 days; and preserved on slants at 4-10^oC, in a refrigerator. For further studies the isolates BVM001, BVM003 and BVM004 were grown on M-L medium; BVM002 was grown on Oren medium; and BVM005 and BVM006 were grown on Larsen medium. The media contained 12.5 or 25 (% w/v) NaCl as the major salt for growth of extremely halophilic archaea.

Screening for extracellular hydrolases

All the tests were performed by spot inoculation of 3-4 days old culture on the respective medium. The plates were incubated at 37^0C and the results were noted periodically from 4th to 7th day. The diameter of zone of clearance or halos was measured. The isolates were screened thrice for the hydrolase activities.

Amylase activity

In order to test for amylase activity, starch (2.0 g %) was added to the medium as previously described by (Amoozegar *et al*., 2003). After incubation the plates were flooded with I_2-KI solution (0.1% I_2 – 0.2% KI). The presence of a clear zone around the colony indicated starch hydrolysis.

Protease activity

The isolates were screened for proteolytic activity by using medium supplemented with skimmed milk (10.0 % v/v) or casein (1.0 % w/v) or gelatin (1.0 % w/v). Casein hydrolysis (caseinase activity) was detected based on the presence of a clear zone around the growth on milk and casein containing medium. The gelatinase activity was observed as a clear zone around the growth after adding Frazier's reagent for 5 mins.

Lipase activity

Screening for lipase (lipolytic enzyme) was done by two different methods as follows:

Method described by Gutierrez and Gonzalez (Gutierrez, 1972)

This was done by spot inoculation of the active culture suspension on the respective solidified medium containing 1.0 ml per liter either Tween 20 or 40 or 60 or 80. After incubation the presence of lipolytic activity was demonstrated by the formation of conspicuous halos. The diameter of the colony and zone of opaqueness (halos) around it was measured. Relative enzyme activity (REA) was calculated using the formula (Kanlayakrit and Boonpan, 2007), from three experimental readings.

REA = Diameter of the opaque zone / Diameter of colony

Method using Rhodamine B Agar plates (RBP) (Bhatnagar *et al*., 2005)

The six halophilic archaeal isolates were screened for lipolytic activity as mentioned above for all the Tweens (20, 40, 60 and 80) in respective medium to which 0.001% Rhodamine B was added, and incubated at 37^0C for 7 days. Lipase production was monitored from 3rd to 7 th day of incubation by using UV-transilluminator (350nm) to observe orange halos around the colonies.

Morphology and biochemical characterization

Gram staining was performed by fixing the smear with 2.0 (%v/v) glacial acetic acid (Dussault, 1955). Motility was checked by hanging drop method. Routine biochemical tests were also performed as per standard microbiological methods .

Studies on growth requirements

The growth response of the isolates to various factors namely NaCl (%w/v) 0.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0 and 30); pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5); temperature (refrigeration 5-10, 25, 37, and 45^0C) was studied in the respective medium (50ml broth in 250ml flask) and incubation on an environmental shaker, 100rpm. Growth was

monitored at A_{620} using a Systronics spectrophotometer 104 at different time intervals (24, 48, 72, 96 upto 7 days).

Absorption spectra of pigments

Biomass was obtained by centrifugation at 10,000 rpm (10^0C) after 5-7 days of growth in liquid medium. Carotenoid pigments were extracted in methanol: acetone (1:1 v/v), and absorption spectra was taken in a UV-Vis double beam spectrophotometer (Gochunauer *et al*., 1972).

Detection of archaeal / diether lipids

Diether core-lipids were extracted in hexane from whole-organism methanolysate and analysed by thin layer chromatographic (TLC) method using silica gel HF_{254}. The solvent system used was petroleum ether (B.P. 60-800°C) / acetone (95:5 v/v) and lipids were detected by using 10% dodecaphosphoric acid in ethanol or iodine vapours (Ross *et al.*, 1981; Upasani and Desai, 1990).

Antibiotic sensitivity testing

Sensitivity to chemotherapeutic agents was tested using the discs obtained from Pathoteq Biological Laboratories (Bio-Disc-12), Gujarat, India. The plates were incubated at 37^0C for 5 days and the diameters of inhibition zones were measured in mm. The agents tested were ampillicillin (20µg), cotrimoxazole (25µg), ceftizoxime (30µg), ofloxacin (5µg), gentamicin (10µg), ikacin (30µg), gatiftoxacin (10µg), piperacillin (100µg), chloramphenicol (30µg), roxythromycin (15µg), lincomycin (2µg), cloxacillin (1µg), amikacin(30µg), bacitracin (10units), erythromycin (10µg), novobiocin (30µg), tetracycline (30µg), cefo-taxime (30µg), ciprofloxacin (5µg), levoflox-acin (5µg), linezolid (30µg), cephalexin (30µg).

Phylogenetic analysis

Molecular identification of the six strains was performed by amplification of 16S rRNA by PCR method, with the following forward and reverse primers: 5'-TCCGGTTGATCCTGCCG (position8–24, *Escherichia coli* numbering) and 5'–GGAGGTGATCCAGCCG (position1540 – 1525) (Enache *et al*., 2008) at Chromous Biotech Bangalore, India. The identification of phylogenetic neighbors was initially carried out by the BLASTN program against the database containing type strains with validly published EzTaxon-eDatabase developed (Kim *et al*., 2012). Multiple sequence alignment was done with Clustal W1.7 program. Phylogenetic tree (neighbor-joining analysis) and datamatrix were performed by MEGA6 software (Tamura *et al*, 2013; http://www.megasoftware.net).The 16S rRNA gene sequences of these isolates (BVM001-BVM006) were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the Accession numbers: KP636732-33, 34, 35, 36, and 37. The amplicon sizes (bp) of the products were 775, 746, 778, 800, 764 and 741, respectively.

Results and Discussion

Sampling site and sample collection

One of the largest integrated marine chemicals complexes in the South East Asia is located at TCL, Mithapur, on the west coast of Gujarat State, India. Salt (pink to red) and water (brine) samples having salt concentration ranging from 20.0 – 25.0 % w/v (NaCl), pH 7.5 + 1.0, orange to red colour, were used for enrichment and isolation of haloarchaea (Figure 1).

Enrichment and isolation of haloarchaea

Three serial transfers were carried out to enrich extremely halophilic archaea, which

was indicated by orange to red colored growth in the medium. Eleven isolates with different morphological and cultural characteristics (pigmented and non-pigmented) were isolated. All the isolates were moderate to extremely halophilic, gram negative, aerobic, motile (except BVM006) and chemoorganotrophs. Six haloarchaeal strains that showed pigmentation varying from orange to red color were selected for further studies (Table 1).

Growth conditions for haloarchaeal strains

The salt requirement for optimal growth was 12.5 and 25.0 (%w/v) NaCl, for the isolates: BVM001, BVM002, BVM003, BVM004; and BVM005, BVM006, respectively. The salt range of the four isolates was 10.0 to 20.0 (%w/v), whereas two isolates (BVM005, BVM006) grew in the range 15.0 to saturation. Thus, moderately as well as extremely halophilic archaea were isolated and studied further. All the isolates grew optimally at pH 7.0 - 7.5 and temperature 35-37^{0}C.

Screening for extracellular hydrolases

It was interesting to observe that all the isolates tested showed hydrolytic activity for at least one substrate. Some of the isolates produced multiple hydrolases (Figure 2). The summarized results are given in Table 2.

Screening for amylase activity

The four strains that demonstrated amylase activity were *Haloferax larsenii* (BVM001, BVM003 and BVM005), and *Haloferax elongans* (BVM004) as shown in Table 2. It was interesting to know that *Halobacterium noricense* (BVM002) and *Halopenitus persicus* (BVM006) lacked this hydrolytic enzyme activities. BVM003 and BVM005 showed maximum amylase activity as compared to the other two isolates.

Screening for lipase activity

Out of the six isolates tested five isolates (except BVM006) produced opaque halos on Tween containing medium (Figure 2) indicating lipase production as per the method (Gutierrez and Gonzalez, 1972).The haloarchaeal isolates BVM004 and 005 hydrolyzed all Tweens and showed maximum REA (Table 3). Similar results for lipase activity were observed on Rhodamine B agar plates (fluorescent halos around the colony under UV irradiation) Figure 3.

Screening for protease activity

Most of the halobacteria are known to produce proteolytic enzymes. Caseinase activity was maximum in *Haloferax larsenii* BVM005, which also showed excellent gelatinase activity. Gelatinase activity was found in all other isolates, except BVM006.

Absorption spectra of pigments

The absorption spectra of the pigments extracted in acetone: methanol (1:1) showed maxima at 526, 494, and 388. With a shoulder at 470-475 nm, this indicates the presence of bacterioruberins (C_{50} isoprenoid pigments).

Detection of archaeal / diether lipids

Whole-organism methanolysate prepared from the isolates showed the presence of glycerol-diether lipids as a single spot by TLC analysis (Rf 2.0), confirming the archaeal identity.

Antibiotic susceptibility

All the isolates were found to be susceptible to bacitracin, rifampicin, and novobiocin.

They were insensitive / resistant to ampicillin, norfloxacin, tetracycline, azithromycine, neomycin, chloramphenicol, penicillin G, and vancomycin. Strains were also resistant to known eubacterial inhibitors, while they were sensitive to the inhibitors which are effective on haloarchaea, i.e. novobiocin and bacitracin.

Phylogenetic tree analysis

The Phylogenetic tree showing evolutionary relationship of BVM isolates was constructed based on 16S rRNA sequences (range of partial sequences 740 to 800bp), as shown in Figure 4. Based on 99.0% or more relatedness the isolates were assigned to the haloarchaeal genera *Haloferax*, *Halobacterium* and *Halopenitus* (Table 4). The alignment results showed that four strains had high similarity to the genus *Haloferax* (different species), while one was related to *Halopenitus* species.

The members of the family *Halobacteriaceae* displays extreme polymorphism, ranging from rods to pleomorphic rods, cocci, disc-shaped, square and triangular forms. The interest in extremozymes from haloarchaea resulted from identifying them as the cause of spoilage of salted food (meat and fish). The halophilic hydrolases in many cases are also thermostable, and show activity over a broad pH range (Mellado and Ventosa, 2003; Ozcan *et al*., 2009; Rohban *et al*., 2009; Enache and Kamekura, 2010; Setati, 2010; Yue *et al*., 2014). A method for simultaneous detection for protease and Tween hydrolysis (lipase production) by haloarchaea was described by Gonzalez and Gutierrez (Gonzalez and Gutierrez, 1970). They reported lipase activity in 35 out of 56 *Halobacterium* strains tested. Twenty-three (23) *Halobacterium* isolates had both proteolytic and lipolytic activities. Extra-cellular esterase and lipase activities were reported in 18 out of 118 halophilic archaeal strains, using Rhodamine agar plates (Ozcan *et al*., 2009).

Okhamadhi is one of the sites for production of marine solar salt by TCL. All the strains isolated from this habitat belongs to the haloarchaea as they require high salt (NaCl) concentration for growth (minimum 10.0 % w/v, and some could grow upto saturation), possessed diether lipids, typical absorption spectra for carotenoids, sensitivity to bacitracin and novobiocin. The six halophilic archaeal strains studied were identified as *Haloferax larsenii* (BVM001, BVM003 and BVM005), *Halobacterium noricense* (BVM002), and *Haloferax elongans* (BVM004) and *Halopenitus persicus* (BVM006). Multiple hydrolytic activities [amylase, protease (caseinase and/or gelatinase) and lipase (one or more tween hydrolysis)] was detected in five strains (>80.0). The three *Hf. larsenii* strains differed in their salt requirement and hydrolase activity. *Hf. larsenii* was first reported from the solar salterns at Zhe-Jiang Province, China and showed amylase, gelatinase and esterase (Tween 80) activities. *Haloferax mediterraneii* demonstrated caseinase activity along with these (Xu *et al*., 2007). Our study also showed that *Haloferax larsenii* (BVM 005) had better multiple hydrolytic activities as compared to the *Haloferax* species (so far reported). *Haloferax elongans* was isolated from the microbial mats collected from Hamelin Pool, Shark Bay, Western Australia (Allen *et al*., 2008). It had hydrolyases profile similar to that of *Haloferax mediterraneii* and our strain BVM004. Among the extremely halophilic archaea (*Haloferax prahovense*, *Halobacterium sp.*, and *Halorubrum sp.*) isolated from the coastal areas of western Maharashtra, India; hydrolases (protease) was detected in

Haloferax prahovense, while the strains belonging to the remaining two genera did not possess amylase, cellulase and gelatinase activities (Pathak and Rajurkar, 2014). Among the nine unidentified haloarcheal strains isolated from Balta Alba salt lake in Romania, none had multiple hydrolases, few of them showed either amylase or esterase activity (Rao *et al*., 2009; Neagu *et al*., 2014). Screening of moderately halophilic bacteria from various saline habitats in India for producing extra-cellular hydrolases (amylases, lipases and proteases) including sea water from Somnath and Veraval in Gujarat has been reported. However, out of the 108 isolates, 21 were identified belonging to various genera; however none belonged to the haloarchaea group (Kumar *et al*., 2012).

Common halophilic amylases are cyclomaltodextrinases (EC 3.2.1.54) and used to hydrolyze starch and have been used in commercial detergents (Ross *et al*., 1981; Moreno *et al*., 2013; Delgado-Garcia *et al*., 2012). Amylases from halophilic bacteria including *Halomona smeridiana*, *Halobacillus* spp., *Halothermothrix orenii*, and *Chromohalobacter* sp. has been reported by (Amoozegar *et al*., 2003; Tan *et al*., 2008). Amylase from *Haloarcula* sp. S-1 was tolerant to organic solvents (Fukishima *et al*., 2005). In a recent paper (Yan and Wu, 2016) have used phylogenetic and statistical methods for analyzing evolutionary relationship between 3118 α-amylases and 280 β-amylases from all the three domains of life to provide information for cloning and expression of amylase gene in different organisms.

Extremely halophilic archaea (identified based on morphological, biochemical and physiological properties, without 16S rRNA sequencing) possessing amylase, caseinase, gelatinase and lipase activities belonging to the genera *Haloferax* and *Halobacterium* have been reported earlier from the marine salterns behind TCL, Mithapur. However, these isolates varied in their hydrolases profile, but all could hydrolyse gelatine, and one or more tweens (Upasani VN, 1988; Upasani *et al.*, 1996). Lipolytic activity has been reported in 35 out of 54 haloarchaeal strains isolated from a salt lake in the Algerian Sahara (Boutaiba *et al*., 2006). Similar studies have been reported on the isolation of these archaea belonging to the genera *Haloferax* and *Haloarcula* from the salt lakes in Prahova, Romania (Enache *et al*., 2008; Chamacho *et al*., 2009). Based on 16S rRNA sequence analysis and growth requirements a novel genus, namely *Halopenitus* was described for a pale pink-pigmented haloarchaeal isolate - strain DC30 (T) obtained from the Aran-Bidgol salt lake, Iran, to include two new species namely *Haloplenitus persicus* and *Halopenitus malekzadehii* (Amoozegar *et al*., 2013). We report here for the first time the isolation and characterization of *Hp. persicus* BVM006 from Okhamadhi salterns, India. However, it was interesting to note that it didn't possess any hydrolases activity. The lipase producing strain BVM002 (hydrolyzed Tween 40) included in this study was identified as *Halobacterium noricense*, which was isolated from a bore core of an alpine Permian salt mine in Austria (Gruber *et al*., 2004). Screening results showed the thirty five (35) haloarchaea among the cultures in an Algerian culture collection had lipase activity (Boutaiba *et al*., 2006). Organic-solvent tolerant, thermostable, alkali-stable lipase from *Haloarcula* strain G41, has been isolated from the saline soil of Yuncheng Salt Lake, China, with a molecular mass of 45kDa, and maximum activity at 70 ^{0}C, pH 8.0, and 15 % NaCl has been reported. This study also revealed the use of lipases from haloarchaea for biodiesel production (Li and Yu, 2014). Proteases are widely applied for

laundry additives, pharmaceuticals, waste management and food processing (Setati, 2010). The preparation of fish sauce requires 6–18 months and proteolytic activities of the halobacteria (Stefansson, 1993). It is hypothesized that augmenting the fish sauce preparation with the extracellular protease-producing *Halobacterium* spp. should accelerate the fish soy sauce fermentation process. Most of halophilic proteases are also alkali tolerant or alkaliphilic that is desirable for laundry industry.

Table.1 Morphological and growth characteristics of haloarchaeal isolates

Designation	Morphology	Colony pigmentation	Incubation (days)
BVM001	Short rods, pleomorphic	Orange	3-5
BVM002*	Short rods	Pink	8-10
BVM003	Short rods, pleomorphic	Orange	6
BVM004	Short rods	Orange	6
BVM005	Short rods, pleomorphic, nonmotile	Orange	5
BVM006	Pleomorphic rods	Red	8-9

*grew well in medium containing 12.5 & 16.5g% NaCl and $MgCl_2$, respectively.

Table.2 Extracellular hydrolases produced by each halophilic archaeal strains

Enzyme Activity	Isolates with strain designation					
	Haloferax larsenii			*Halobacterium noricense*	*Haloferax elongans*	*Halopenitus persicus*
	BVM001	**BVM003**	**BVM005**	**BVM002**	**BVM004**	**BVM006**
Amylase (starch agar)	++	+++++	+++++	--	++++	-
Caseinase (casein agar)	-	-	++++	++	++	-
Caseinase (milk agar)	-	-	++++	+	+	-
Gelatinase	++++	++++	+++++	+++	+++	-
Lipase						-
a. Tween 20	++	+++++	+++++	+	++++	-
b. Tween 40	+	+	+++++	++++	+++++	-
c. Tween 60	+++	+	++++	++	+++++	-
d. Tween 80	+	+++	+++++	++	++	-

*Identification based on 16S rRNA sequencing

Table.3 Lipase activity of the isolates in terms of Relative enzyme activity (REA) with different Tweens

	Haloarchaeal strains					
Tween	**BVM001**	**BVM002**	**BVM003**	**BVM004**	**BVM005**	**BVM006**
20	3.0±0.1	1.03±0.10	1.03±0.14	2.1±0.07	3.6±0.10	0.06±0.04
40	1.1±0.1	2.8±0.10	2.30±0.14	3.03±0.10	3.9±0.10	-
60	2.06±0.15	0.9±0.10	1.06±0.10	3.0±0.08	4.1±0.04	-
80	1.5.0±0.1	1.80±0.14	2.30±0.12	3.10±0.14	3.90±0.10	0.13±0.04

*REA values are calculated based on three independent experimental readings and S.D. calculation. ; (-) indicates negative results

Table.4 Identification of the BVM isolates based on 16S rDNA sequence analysis

Strain	**Closest matches**	**Accession**	**Accession**	**Completeness (%)**
BVM001	*Haloferax larsenii JCM 13917*	NR_113442	KP636732	99.0
BVM002	*Halobacterium noricense HmC1*	KM258002	KP636733	99.0
BVM003	*Haloferax larsenii ZJ204*	AY838279	KP636734	99.0
BVM004	*Haloferax elongans JCM 14791*	NR_112862	KP636735	99.62
BVM005	*Haloferax larsenii ZJ203*	DQ458847	KP636736	99.0
BVM006	*Halopenitus persicus IARI-MAAB3*	KJ875320	KP636737	99.0

[a]GenBank sequence accession numbers of most closely related sequences.
[b]GenBank sequence accession numbers of isolated strains.

Fig.1 Satellite image of Okhamadhi salt works, Gujarat (India) covering an area of approximately 8.11 km^2 showing red-pink colouration due to growth of extreme halophiles (source: Google maps)

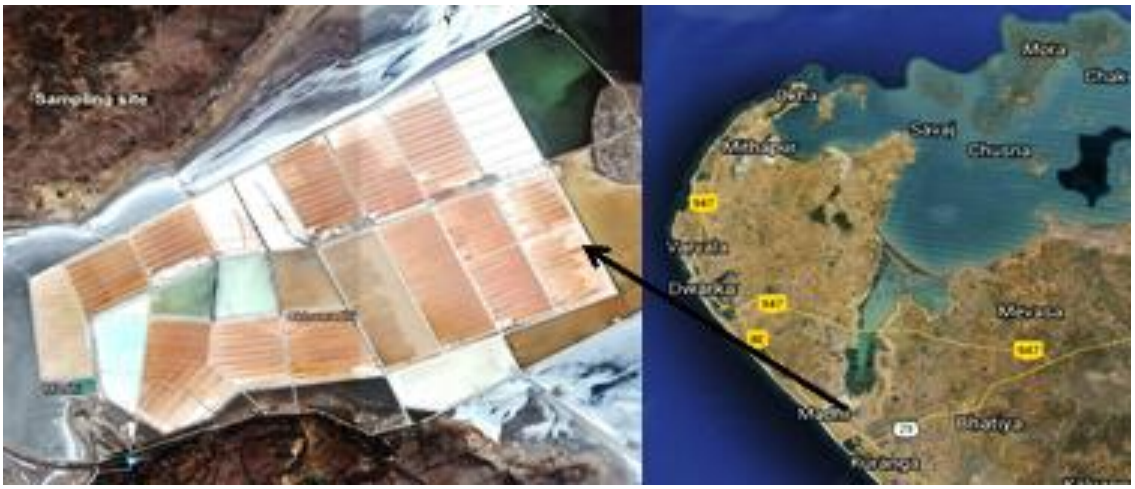
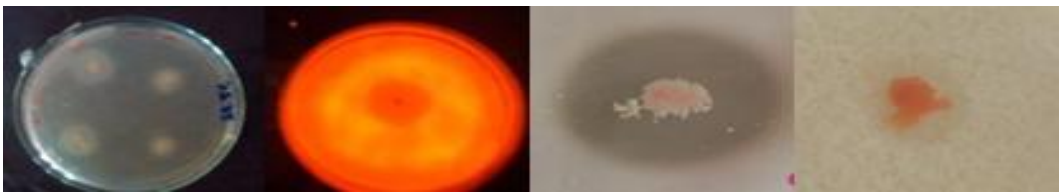


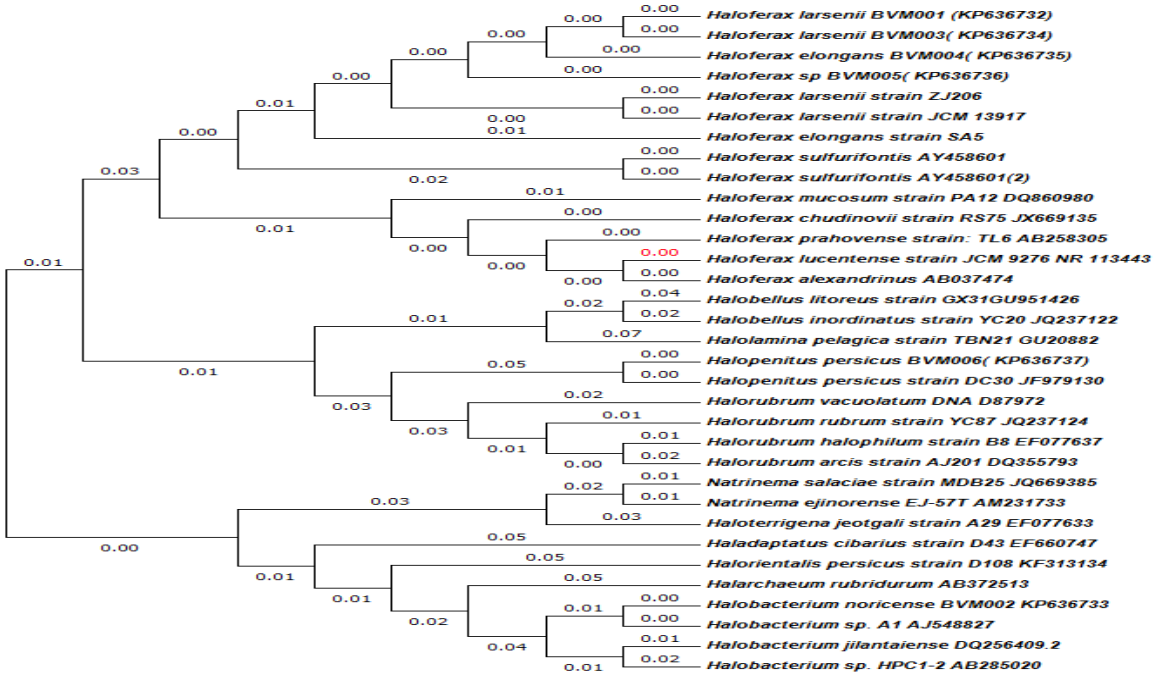
Fig.2 Amylase, gelatinase and caseinase activities demonstrated by the isolate BVM 005 (earlier BVM 008).

Fig.3 (a) and (b) Lipase activity demonstrated by isolate BVM005: halos (left without Rhodamine B), and (right containing Rhodamine B) in medium containing Tween 80; (c) and (d) Caseinase activity on casein agar and milk agar plate (s) demonstrated by BVM 003.



(a) **(b)** **(c)** **(d)**

Fig.4 Phylogenetic tree showing the position of the isolates. Phylogenetic trees depicting the interrelationships of 16S rRNA sequence of halophilic archaeal isolates (BVM001-006), with closely related halophilic isolates of their respective genera. The Accession numbers for the investigated strains are included in brackets.



A recent review on hydrolases from marine extremophiles and their biotechnological applications includes the use of esterases for producing building blocks for anti-AIDS drugs, degradation of organic pollutants in saline waste water, and offer a new solution for the treatment of oil field waste in saline environments (Schreck and Grunden, 2014; Dalmaso *et al.*, 2015). Two of our isolates namely *Haloferax larsenii* (BVM005), and *Haloferax elongans* (BVM004) could hydrolyze all substrate tested. Among these two, BVM005 was extremely halophilic, and had better amylolytic, lipolytic and proteolytic activity as compared to BVM004. Thus, the former strain is a superior candidate as sources of halophilic hydrolyases for biotechnological applications. In summary, our study has resulted in demonstrating the diversity of haloarchaea in the salt/brines from Okhamadhi, having extracellular hydrolyases activity for biotechnological applications.

In conclusion, the halophilic enzymes from haloarchaea have been explored for biotechnological applications due their stability in high salt concentration and efficiency in organic solvents. The six halophilic archaeal strains isolated were identified as *Haloferax larensii* (three), *Haloferax elongans* (one), *Halobacterium sp.* (one), and *Halopenitus persicus* (one). The *Haloferax* isolates BVM004 and BVM005 will be further studied as they possessed amylase, protease and lipase activities. To the best of the author's knowledge, this is the first paper concerning the phylogenetic analysis of extracellular enzymes producing haloarchaea from marine salterns at Okhamadhi (Gujarat, India).

Acknowledgments

The authors are grateful to the management at the institute and Ahmedabad Education Society (AES) for providing laboratory facilities. The facilities availed under the DBT (New Delhi) Star College Scheme is also gratefully acknowledged.

References

Allen, M.A., Goh, F., Leuko, S., Echigo, A, Mizuki, T., Usami, R., Kamekura, M., Neilan, B.A.and Burns, B.P. 2008. *Haloferax elongans* sp. nov.and *Haloferax mucosum* sp. nov., isolated from microbial mats from Hamelin Pool, Shark Bay, Australia. *Int. J. Syst. Evol. Microbiol.* 58(4):798-802.

Amoozegar, M. A., Makhdoumi-Kakhki, A., Mehrshad, M., Shahzadeh Fazeli, S. A., and Ventosa, A. 2013. *Halopenitus malekzadehii* sp.nov. an extremely halophilic archaeon isolated from a salt lake. *Int. J. Syst. Evol. Microbiol.*, 63: 3232-3236.

Amoozegar, M.A., Malekzadeh, F., and Malik, K.A. 2003. Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. *J. Microbiol. Methods*, 52(3): 353-359.

Bhatnagar, T., Boutaiba, S., Hacene, H., Cayol, J., Fardeau, M. L., Ollivier, B., and Baratti, J. C. 2005. Lipolytic activity from halobacteria: screening and hydrolase production. *FEMS Microbiol. Lett.*, 248(2):133-40.

Boutaiba, T., Bhatnagar, H., Hacene, H., Mitchell, D. A., and Baratti, J. C. 2006. Preliminary characterization of alipolytic activity from an extremely halophilic archaeon, *Natronococcus sp. J. Mol. Catal. B. Enzym.*, 41:21-26.

Chakraborty, S., Khopade, A., Kokare, C., Mahadik, K., and Chopade, B. 2009. Isolation and characterization of novel α-amylase from marine *Streptomyces* sp. D1. 58:17-23.

Chamacho, R. M., Mateos, J. C., González-Reynoso. O., Prado, L. A. and Cordova, J. 2009. Production and characterization of esterase and lipase from *Haloarcula marismortui*. *J. Ind. Microbiol. Biotechnol.,* 36(7):901-909.

Dalmaso, G.Z.L., Ferreira, D. and Vermelho, A.B. 2015. Marine extremophiles: a source of hydrolases for biotechnological applications. *Mar drugs*, 13(4):1925–1965.

DasSarma, S.,and DasSarma P. 2015. Halophiles and their enzymes: negativity put to good use. In *Environ. Microbiol. Extremophiles*, 25: 120-126.

Delgado-García, M., Valdivia-Urdiales, B., Aguilar-González, C. N., Contreras-Esquivel, J. C., and Rodríguez-Herrera, R. 2012. Halophilic hydrolases as a new tool for the biotechnological industries. *J. Sci. Food. Agric.*, 92(13): 2575-2580.

Dussault, H.P. 1955. An improved technique for staining halophilic bacteria. *J. Bacteriol.* 70(4):484-485.

Enache, M., and Kamekura, M. 2010. Hydrolytic enzymes of halophilic microorganisms and their economic values. *Rom. J. Biochem.* 47(1):47–59.

Enache, M., Itoh, T., Kamekura, M., Popescu, G. Dumitru, L. 2008. Halophilic archaea isolated from man-made young (200 years) salt lakes in Slănic, Prahova, Rom. *Open Life Sci*. 3(4):388-395.

Fukishima, T., Mizuki, T., Echigo, A., Inoue, A., and Usami, R. 2005. Organic solvent tolerance of halophilic α-amylase from a haloarchaeon, *Haloarcula* sp. strain S-1. *Extremophiles,* 9(1):85-89.

Gochnauer, M. B., Kushwaha, S. C., Kates, M., Kushner D. J. 1972. Nutritional control of pigment and isoprenoid compound formation in extremely halophilic bacteria. *Arch. Microbiol.*, 84(4):339-349.

Gonzalez, C., and Gutierrez, C. 1970. Presence of lipase among species of extremely halophilic bacteria. *Can. J. Microbiol.* 16(12):1165-1166.

Gruber, C., Legat, A., Pfaffenhuemer, M., Radax, C., Weidler, G., Busse, H. J., and Stan-Lotter, H. 2004. *Halobacterium noricense* sp. nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of *Halobacterium* sp. NRC-1 as a strain of *H. salinarum* and emended description of *H. salinarum*. *Extremophiles*, 8(6):431-439.

Gutiérrez, C., and González, C. 1972. Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. *Appl. Microbiol.*, 24(3):516-517.

Kanlayakrit, W., and Boonpan, A. 2007. Screening of halophilic lipase producing bacteria and characterization of enzyme for fish sauce quality improvement. *Kasetsart J. Nat. Sci.*,41:576-585.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J.H., Yi, H., Won, S. and Chun, J. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.*, 62(3):716-721.

Kumar, S., Karan, R., Kapoor, S., Singh, S. P., Khare, S. K. 2012. Screening and isolation of halophilic bacteria producing industrially important enzymes. *Braz. J. Microbiol.*, 43(4): 1595-603.

Larsen, H. 1981. The family *Halobacteriaceae* In: The

Prokaryotes: A handbook on habitats, isolation and identification of bacteria (Eds.) M. P. Starr, H. Stolp, H. G.Trüper, A. Balows and H. G. Schlegel. Berlin: Springer-Verlag. vol 1. Pp. 985–994.

Li, X., and Yu, H. Y. 2014. Characterization of an organic solvent-tolerant lipase from *Haloarcula sp.* G41 and its application for biodiesel production. *Fol. Microbiol.,* 59(6):55-463.

Mellado E. and Ventosa A. 2003. Biotechnological potential of moderately and extremely halophilic microorganisms In: Microorganisms for health care, food and enzyme production (Eds.) Barredo J. L. Res. Signpost; Kerala, India. Pp. 233–256.

Moreno ML, Dolores Pérez, María Teresa García, and Mellado E. 2013. Halophilic bacteria as a source of novel hydrolytic enzymes. *Life (Basel).*, 3(1): 38–51.

Mullakhanbhai, M. F., and Larse, H. 1975. *Halobacterium volcanii* spec.nov., Dead Sea halobacterium with a moderate salt requirement. *Arch. Microbiol.* 104(3):207-214.

Muller-Santos, M., de Souza, E. M., Pedrosa, Fde, O., Mitchell, D. A, Longhi, S., Carriere, F., Canaan, S., and Krieger, N. 2009. First evidence for the salt-dependent folding and activity of an esterase from the halophilic archaea *Haloarcula marismortui*. *Biochim. Biophys. Acta.*, 1791(8):719-729.

Neagu, S., Enache, M., and Cojoc., R. 2014. Extracellular hydrolytic activities of halophilic microorganisms isolated from Balta Albă salt lake. *Rom. Biotechnol. Lett.* 19(1):8951-8.

Oren, A. 1983. *Halobacterium sodomense* sp. nov., a Dead Sea halobacterium with an extremely high magnesium requirement. *Int. J. Syst. Bacteriol.* 22(2):381-386.

Oren, A. 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J. Ind. Microbiol. Biotechnol.* 28(1):56-63.

Ozcan, B., Ozyilmaz, G., Cihan, A., Cokmus, C., and Caliskan, M. 2012. Phylogenetic analysis and characterization of lipolytic activity of halophilic archaeal isolates. *Mikrobiologia.* 81(2):205-213.

Ozcan, B., Ozyilmaz, G., Cokmus, C., and Caliskan, M. 2009. Characterization of extra cellular esterase and lipase activities from five halophilic archaeal strains. *J. Ind. Microbiol. Biotechnol.* 36(1):105-110.

Pathak, A. P., and Rajurkar, A. A. 2014. Isolation and characterization of salt stable protease producing archaea from marine solar saltern of Mulund, Mumbai. *Ind J. Mar. Sci.* 43(3):412-417.

Pérez, D., Martín, S., Fernández-Lorente, G., Filice, M., Guisán, J. M.,Ventosa, A., Garcia, M. T. and Mellado, E. 2011. A novel halophilic lipase, LipBL, showing high efficiency in the production of eicosapentaenoic acid (EPA). *PLoS One.* 6(8): e23325.

Rao, L., Zhao, X., Pan, F., Li, Y., Xue, Y., Ma, Y., and Lu J. R. 2009. Solution behavior and activity of a halophilic esterase under high salt concentration. *PLoS One.* 4(9):e6980.

Rohban, R., Amoozegar, M. A., and Ventosa, A. 2009. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *J Ind. Microbiol. Biotechnol.,* 36(3):333-40.

Ross, H. N. M., Collins, M. D., Tindall, B. J., and Grant, W. D. 1981. A rapid procedure for the detection of

archaebacterial lipids in halophilic bacteria. *J. Gen. Microbiol.* 123:75-80.

Schreck, S. D., and Grunden, A., M. 2014. Biotechnological applications of halophilic lipases and thio-esterases. *Appl. Microbiol. Biotechnol.* 98(3):1011-21.

Setati M. E. 2010. Diversity and industrial potential of hydrolase-producing halophilic/halotolerant eubacteria. *Afr. J. Biotechnol.* 9(11):1555-1560.

Siglioccolo, A., Paiardini, A., Piscitelli, M., and Pascarella, S. 2011. Structural adaptation of extreme halophilic proteins through decrease of conserved hydrophobic contact surface. *BMC Struct. Biol.* 22:11-50.

Stefansson, G. 1993. Fish processing In: Enzymes in food processing (Eds.) Nagodawithana, T. and Reed, G. Academic Press Inc., New York.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30 (12):2725-2729.

Tan, T. C., Mijts B. N., Swaminathan, K., Patel, B. K., and Divne, C. 2008. Crystal structure of the polyextremophilic α-amylase AmyB from *Halothermothrix orenii*: Details of a productive enzyme-substrate complex and an N domain with a role in binding raw starch. *J. Mol. Biol.* 378(4):852–70.

Upasani V. N. 1988. Studies on halophilism. Ph. D. Thesis, Gujarat University, India.

Upasani, V. N., and Desai, S. G., Moldoveanu, N., and Kates. 1994. Lipids of extremely halophilic archaeobacteria from saline environments in India: a novel glycolipid in *Natronobacterium* strains. *Microbiol.* 140:1959-1966.

Upasani, V. N., Desai, S. G., Kates, M., and Mullakhanbhai, M. F. 1996. Isolation and characterization of halophilic archaeobacteria from marine salterns, Mithapur, India. *Biores. J.* 1(1):23-32.

Ventosa, A. 2006. Unusual micro-organisms from unusual habitats: hypersaline environments In: Prokaryotic diversity: mechanism and significance (Eds) Logan N. A., Lappin-Scott H. M., Oyston P. C. F. Cambridge University Press, Cambridge, Pp. 223–253.

Xu, Xw., Wu, Y.H., Wang, CS., Oren, A., Zhou, P.J., and Wu, M. 2007. *Haloferax larsenii* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int. J. Syst. Evol. Microbiol.*, 57(4):717-720.

Yan, S., and Wu, G. 2016. Analysis on evolutionary relationship of amylases from archaea, bacteria and eukaryota. W. *J. Microbiol. Biotechnol.*, 32: 24.

Yue, H., Ling, C., Yang, T., Chen, X., Chen, Y., Deng, H., Wu, Q., Chen, J., and Chen, G.Q. 2014. A seawater based open and continuous process for polyhydroxyalkanoates production by recombinant *Halomonas campaniensis* LS21 grown in mixed substrates. *Biotechnol. Biofuels*, 7(1): 108.

How to cite this article:

Bhavini N. Rathod, Harshil H. Bhatt and Vivek N. Upasani. 2016. Extracellular Hydrolases producing Haloarchaea from Marine Salterns at Okhamadhi, Gujarat, India. *Int.J.Curr.Microbiol.App.Sci.* 5(11): 51-64. doi: http://dx.doi.org/10.20546/ijcmas.2016.511.006