Isolation, characterization and exploring biotechnological potential of halophilic archaea from salterns of western India

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Abstract

Thirteen halophilic archaea were isolated from Kandla and Bhayander salt pans. These isolates were grouped into three different genera *Halobacterium*, *Haloferax* and *Haloarcula* based on morphological and biochemical characterization, polar lipid analysis, Amplified 16S rDNA restriction analysis (ARDRA) and 16S rDNA sequence analysis. Biochemical characterization suggested the ability of isolates to produce protease, amylase and poly-hydroxybutyrate (PHB) indicating their biotechnological potential. The isolates were further screened for the amount of extracellular protease produced. *Halobacterium* sp. SP1(1) showed significant protease production compared to other isolates. Protease producing ability of the isolate was influenced by several factors such as NaCl concentration, type of protein source, metal ions and surfactants, and presence of amino acid supplements in the production medium. Soybean flour, FeCl₃ and dicotylsulfo succinate were found to increase protease production by 2.36, 1.54 and 1.26 folds, respectively compared to production in basal medium. Effect of organic solvents used in paints (n-decane, n-undecane and n-dodecane) was also investigated on protease production by the isolate. Protease production by *Halobacterium* sp. SP1(1) was enhanced by 1.2 folds in presence of n-decane compared to control. Furthermore, the ability of isolate to hydrolyse fish protein was investigated using three different edible fishes (Pomfret, Flat fish and Seer fish) as sole protein source. Pomfret was found to be a good protein source for protease production by the isolate. These results revealed that *Halobacterium* sp. SP1(1) may have potential for paint-based antifouling coating preparations and fish sauce preparation by virtue of its extracellular protease.

Keywords ARDRA · Fish protein · *Halobacterium* · Halophilic archaea · Organic solvents · Protease · Salt pans

Introduction

Life exists over the whole range of salt concentrations encountered in natural habitats from freshwater environments to hypersaline lakes. Halophiles can be found in each of the three domains of life: Archaea, Bacteria and Eucarya (Ma et al. 2010). The aerobic halophilic archaeabacteria are the halophiles par excellence and are the main component of microbial biomass in hypersaline environments. These organisms resist the denaturing effects of salt mostly by accumulating KCl equal to the external concentration of

NaCl. However other strategy like accumulation of compatible solutes has been recently reported (Goh et al. 2011). Haloarchaeal proteins possess highly negative surface charge due to presence of acid amino acids which compensate for the extreme ionic conditions (Reed et al. 2013). Several halophilic archaea have been isolated from various habitats such as salt mines in Turkey, solar salterns of Goa in India, solar salterns and estuaries polluted with crude oil (Yildiz et al. 2012; Mani et al. 2012; Raghavan and Furtado 2000). Adaptation to such high salt containing environments has evolved unique properties in these microorganisms with considerable biotechnological potential. Halophiles produce a range of unique and stable biomolecules of practical application including. (1) Hydrolytic enzymes like proteases, gelatinases, DNAases, lipases, xylanases and amylases that find potential application in salt based and detergent industries (Oren 2010). (2) Membrane proteins like bacteriorhodopsin that is recognized commercially for use in artificial retina, holograms, photoelectric devices, optical computing etc.

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(Hampp and Oesterhelt 2008). (3) Biodegradable polymers and exopolysaccharides: Polyhydroxyalkanoates (PHAs), a biodegradable polymer produced by many haloarchaea can be used as alternative to non-degradable plastics (Don et al. 2006). In addition, exopolysaccharides of microbial origin also find application in high valued products like cosmetics, medicine, agriculture and food (Suresh et al. 2007) (4) Gas vesicles: unique properties of gas vesicles produced by some halophilic archaea has been explored for their use as drug delivery vehicle. Besides biomolecules by them, haloarcheal isolates themselves have potential application in bioremediation and food fermentation process (Martínez-Espinosa et al. 2007; Akolkar et al. 2010).

Most strains of halophilic archaea are proteolytic in nature and proteases produced by these organisms are capable of functioning under high salt conditions that lead to precipitation or denaturation of other non-halophilic proteins. Therefore, protease producing strains of halophilic archaea can be exploited for commercial salt-based applications such as fish sauce and antifouling coating preparations (Akolkar et al. 2008, 2010).

About 10,000 salt-production sites exist in India. Major saline habitats studied in the country are Sambhar salt lake (Rajasthan); coastal regions of Gujarat, Tamil Nadu, Maharashtra, Andhra Pradesh, Orissa and West Bengal. Major salt-producing sites in Gujarat are coastal regions of Kutch and Saurashtra at Kandla, Jamnagar, Maliya, Mithapur, Porbandar and Bhavnagar (www.nutritionfoundationofindia.org; www.indiaonline.com). However, the information related to the nature of haloarchaeal communities present at these sites is limited (Dave and Desai 2006). As a result of natural and man-made global changes, hypersaline environments are on increase. Moreover, hypersaline environments can easily be created by the concentration of sea water in arid environments. These facts, together with the occurrence of novel and stable biomolecules in halophilic archaea, suggest that these microorganisms will prove even more valuable in the future. Characterization of such extremely halophilic

archaea from newer habitats is, therefore, necessary to explore their biotechnological potential.

The present investigation deals with chemical characterization of brine samples from different salt pans, isolation, characterization of the isolates from these samples as well as screening of isolates for extracellular protease production and application.

Materials and methods

Habitat

Brine samples were collected from salt pans located in Kandla (23°00'48.94"N, 70°12'48.45"E, Gujarat) and Bhayandar (19°17'28.27"N, 72°50'55.32"E, Mumbai, Maharashtra) regions on the west coast of India. Figure 1a shows the location of sampling area. Figure 1b, c shows the salt pans of Kandla and Bhayandar, respectively from which brine samples collected.

Analysis of brine

The brine samples were analyzed for pH and concentrations of Na^+ , K^+ , Ca^{2+} , Cl^- , CO_3^{2-} , HCO_3^- and SO_4^{2-} ions at Gujarat State Fertilizers and Chemicals Ltd, Vadodara, India.

Growth medium and culture conditions

The medium used for isolation and cultivation of the organisms contained (g/l): NaCl, 250; KCl, 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20; tri-Na-citrate, 3; yeast extract, 10; agar, 20. The pH of medium was 7.2. Yeast extract and NaCl concentrations were altered depending on the study. The cultures were incubated at 37 °C for 48–72 h.

Standard haloarchaeal strains, *Halobacterium salinarum* (TIFR), *Haloarcula vallismortis* (MTCC 1600),

Fig. 1 a Map location of sampling area. b Kandla salt pan. c Bhayandar salt pan

Halorubrum saccharovorum (MTCC 1627) and *Haloferax mediterranei* (MTCC 1613) were cultivated in same growth medium under similar conditions.

Isolation of halophilic archaea

Brine samples (approximately 15 ml) were filtered through 0.45 µm membrane filters. The filters were subsequently placed on the above mentioned solid medium and incubated at 37 °C for 7–10 days. Different colonies that developed on the filters were then transferred to 10 ml of liquid medium and grown at 37 °C under shaking conditions (200 rpm) for 5–7 days. The isolated colonies were obtained by transferring the culture to solid medium using four sector streaking method. The well separated colonies were transferred to solid medium to obtain pure culture. Thirteen different isolates were obtained from different salterns.

Morphology and salt requirement of isolates

Cell shape and motility were examined on freshly prepared wet mounts by light microscopy of exponentially growing liquid culture. Gram nature of the isolates was determined by gram staining performed after fixing and simultaneously desalting the samples with 2% acetic acid for 5 min (Elevi et al. 2004).

Growth medium with NaCl concentrations ranging from 5, 10, 15, 20, 25, 30 and 35% were used to determine the salt requirement of isolates. Growth was monitored at intervals of 24 h. Cell lysis at low salt concentrations was confirmed by microscopic examination of cells and by reduction in turbidity of culture.

Sensitivity to antimicrobial agents

Antibiotic sensitivity test was performed by spreading the culture suspension on solid medium and applying disks (HIMEDIA octa-disks) impregnated with the antimicrobial agents-ampicillin, 10 µg; penicillin, 1 unit; gentamycin, 10 µg; kanamycin, 30 µg; tetracycline, 25 µg; ciprofloxacin, 10 µg; amikamycin, 10 µg; trimethoprin, 1.25 µg, sulfamethoxazole, 25 µg; bacitracin, 10 units; and novobiocin, 5 µg. The cultures were incubated at 37 °C for 5–7 days. The sensitivity was reported based on the presence of zone of growth inhibition.

Biochemical tests

The biochemical tests were performed as described by Elevi et al. (2004). Anaerobic growth in presence of arginine and nitrate was tested. Reduction of nitrate to nitrite was checked by adding 0.5% α-naphthylamine and 0.8% sulphanilic acid, both prepared in 5 N acetic acid. The presence of catalase

was determined by formation of bubbles on adding 3% (v/v) H₂O₂ solution to colonies on solid medium. Oxidase was detected by appearance of pink or violet colour within 30 s after streaking a colony on oxidase disk (HIMEDIA). Casein, starch and tween 80 hydrolysis was checked on agar plates containing casein, starch and tween 80 as substrates, respectively. Gelatin hydrolysis was tested in the solid medium containing 1% gelatine and a zone of clearance around the colonies was observed after flooding the colonies with 1% amidoblack which stains the unhydrolyzed gelatin. Acid production from glucose, mannose, galactose, fructose, maltose, sucrose, lactose, starch, glycerol, mannitol and sorbitol was tested in unbuffered growth medium supplemented with 1% of the above mentioned carbohydrates and sugar alcohols. The yeast extract concentration in this case was reduced to 0.5 and 0.001% (w/v) phenol red was added as pH indicator. Presence of polyhydroxyalkanoate (PHA) was determined by fluorescent microscopic examination using 1% aqueous solution of Nile blue for staining the PHA which appeared as bright orange coloured granules within the cells. Desalting with 5% acetic acid was done before staining.

Polar lipid analysis

Total polar lipid extraction and glycolipid analysis on silica gel G plates was followed as per the method described by Elevi et al. (2004).

Twenty milliliter of cultures were centrifuged at 10,000 × g for 15 min and the pellets were suspended in 1 ml water (for lysis of cell membrane). The lipids were extracted with 3.75 ml chloroform-methanol (1:2, v/v) for 4 h. The extract was collected and the pellet was re-extracted with 4.75 ml methanol-chloroform-water (2:1:0.8, v/v). Chloroform and water (2.5 ml each) were added to the combined supernatants to achieve phase separation and after centrifugation (4000 × g for 10 min) the chloroform phase was collected and dried. Lipids were then dissolved in 0.5 ml volume of chloroform.

The extracted lipids were applied to silica gel G plates. The plates were eluted with chloroform-methanol-acetic acid-water (85:22.5:10:4, v/v). For detection of glycolipids, plates were sprayed with 0.5% α-naphthol in 50% methanol, air dried, and then sprayed with 5% H₂SO₄ in ethanol followed by heating at 150 °C. Glycolipids appear as blue-purple spots. All other lipids are visualized by further heating as yellow-brown spots. Lipids extracted from standard strains of *Halobacterium salinarum*, Tata Institute of Fundamental Research (TIFR) Mumbai, India and *Haloferax mediterranei*, Microbial Type Culture Collection (MTCC) Chandigarh, India were used as reference for comparing the polar lipid pattern of isolates.

16S rDNA sequence analysis and ARDRA (Amplified 16S rDNA restriction analysis)

The genomic DNA from the isolates was extracted by lysing log phase culture in purified water followed by ethanol precipitation as described by Dyll-Smith (2008). The 16S rRNA gene of isolates was amplified using archaeal primers. The forward primer, F27: 5'ATTCCGGTTGATCCTGCCGAAG3' (positions 6–27), was designed in the laboratory according to Gupta et al. (1983) and reverse primer, primer, R15215'AGG AGGTGATCCAGCCGCAG3' (positions 1540–1521), was referred from Xu et al. (2005). Sequencing was done using an automated DNA sequencer (Bangalore Genei). The sequences were analyzed by NCBI BLAST search and were submitted to NCBI GeneBank database.

The restriction analysis of amplified 16S rDNA of isolates was performed by digesting the PCR products with 5 units of restriction enzymes (tetra cutters) *Hae*III, *Hha*I and *Hinf*I. The reaction mixtures were incubated at 37 °C for 16 h (overnight). The digested 16S rDNA restriction fragments thus obtained were separated on 2% agarose gel (10 cm) prepared in 1 × TAE in an electric field strength of 7 V/cm. The patterns of bands of isolates were compared visually with those of reference strains *Halobacterium salinarum* (TIFR), *Haloarcula vallismortis* (MTCC 1600), *Halorubrum saccharovororum* (MTCC 1627) and *Haloferax mediterranei* (MTCC 1613). The band patterns obtained by ARDRA were compared and clustered using NTSYSpc 2.0 (Numerical Taxonomy SYStem) Exeter Software program (Rohlf 2009). A dendrogram was constructed based on the cluster analysis.

Screening of isolates for extracellular protease production

The isolates were screened for production of extracellular protease. Cultures were spotted on 1% skimmed milk-agar plates containing 25% NaCl and incubated at 37 °C for 5–7 days. Presence of zone of clearance surrounding the culture spot was taken as a measure of protease production. The isolates showing significant zone of clearance were further analyzed for the amount of protease produced. The protease production medium was similar to the growth medium except that the yeast extract (10 g/l) was replaced by same quantity of soybean meal. The isolates were grown in the production medium at 37 °C for 96 h under shaking conditions (200 rpm) and the enzyme activity was estimated at interval of 24 h.

Enzyme activity

The culture was centrifuged at 10,000×g for 20 min (4 °C) and the culture supernatant was used as a source of protease. The caseinolytic activity was estimated at 37 °C using 1%

casein as a substrate prepared in 50 mM Tris–HCl buffer (pH 7.2) containing 2 M NaCl. The concentration of NaCl was set at 2 M in the assay system as casein is known to lose its original conformation at higher NaCl concentrations (Capiralla et al. 2002). One milliliter of casein buffer solution was pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 1 ml of enzyme. After incubation for 10 min at 37 °C, the reaction was terminated by adding 3 ml of 5% (w/v) trichloroacetic acid (TCA). For blank tubes TCA was added before enzyme. The content was centrifuged and the absorbance of supernatant was measured at 280 nm. One unit of enzyme activity was defined as 1 µg of tyrosine released per minute.

Effect of NaCl on protease production

The selected isolate was grown in soybean meal-based production medium in presence of varying concentrations of NaCl at 37 °C under shaking conditions (200 rpm) for 96 h. Protease activity was estimated in the culture supernatant at intervals of 24 h.

Identification of nutrients influencing protease production

Influence of carbon and nitrogen source on protease production was determined by replacing yeast extract (10 g/l) in the growth medium with sugars like glucose and starch; nitrogen sources like (NH₄)₂SO₄, NH₄Cl, urea and NaNO₃; and complex protein sources like peptone, fish peptone, beef extract, gelatin, skimmed milk, soybean meal, chick pea flour, pigeon pea flour and soybean flour, in similar concentration. Effect of metal ions, surfactants and amino acids was determined by one-factor-at a time approach. The components used in the medium were (1) metal ions (1 mM): MnSO₄, ZnSO₄, CaCl₂ and FeCl₃; (2) surfactants (0.2 g/l): tergitol, hexamine hydrate, span 80, dicotylsulfosuccinate, triton X-100 tween 20, tween 80, SDS, CTAB and sodium taurocholate; and (3) amino acids (10 mM): L-alanine, L-valine and sodium glutamate.

Effect of organic solvents on protease production

Influence of organic solvents on protease production was determined by incubating the culture in soybean flour-based production medium containing organic solvents: n-dodecane (log P_{ow} = 7.0), n-undecane (log P_{ow} = 6.5) and n-decane (log P_{ow} = 5.5) in 2:1 ratio at 37 °C for 96 h under shaking conditions (200 rpm). The culture was centrifuged at 10,000×g for 20 min and the supernatant was used as a source of enzyme for production studies.

Protease production in fish containing medium

The ability of isolates to produce protease in medium containing fish powder as a sole source of carbon and nitrogen was investigated. The protease production medium was similar to growth medium except that yeast extract (10 g/l) was replaced by fish powder. The culture was grown in fish based production medium for 120 h at 37 °C under shaking conditions (200 rpm). The enzyme production was estimated in the culture supernatant at intervals of 24 h. Three different edible fishes, white Pomfret (*Pomus argenteus*), Flat fish (*Cynoglossus arel*) and Seer fish (*Scomberomorus guttatus*) in dry form were purchased from the local market, ground into powder and used for protease production.

Results

Chemical analysis of brine samples

The compositions of brine samples analyzed were similar to those described for thalassohaline environments, the sodium and chloride ions being dominant. The concentration of chloride ions exceeded that of sodium ions and the pH were around 6.0 for all the salterns (Table 1). The brine samples were colourless to pink in colour.

Isolation of halophilic archaea

All the colonies obtained on membrane filters showed different shades of pink, orange and red colours indicating the presence of halophilic archaea. Total 13 halophilic archaea were isolated, out of which 3 were from Kandla salt pans I and II each, 2 from Kandla salt pan III, 1 each from Kandla salt pans IV and V, 1 from Bhayandar salt pan I and 2 from Bhayandar salt pan II (Table 2).

Morphological and biochemical characterization of isolates

All the isolates exhibited similar morphology being rod shaped, motile and negative for Gram stain. The isolates showed growth in presence of varying NaCl concentrations. The minimum requirement of salt varied among the isolates from 10 to 20% (w/v) of NaCl. In general the isolates exhibited optimum growth at 20 to 25% (w/v) NaCl with significant growth at 35% (w/v) NaCl (saturating concentration) (Data not shown). All isolates were insensitive to the antibiotics that target peptidoglycan layer (ampicillin, penicillin, gentamycin and amikamycin) and sensitive to bacitracin and novobiocin. Sensitivity towards tetracycline, chloramphenicol, streptomycin and ciprofloxacin was variable among the isolates whereas the isolates were resistant to trimethoprim and sulfamethoxazole.

Isolates were differentiated based on biochemical properties as shown in Table 3. Out of the thirteen haloarchaeal isolates only three isolates, SP1(1), SP1(2b) and SP3(2) grew anaerobically on arginine which indicated their closest similarity to members of genus *Halobacterium*. Majority of the isolates exhibited gelatin and casein hydrolysis, which means that the isolates could be source of extracellular protease. Ability to hydrolyze starch by SP2(1), SP2(2) and SP4

Table 2 Isolates obtained from various salt pans

No.	Source	Isolates
1	Kandla (Gujarat, India)	Salt pan I SP1(1), SP1(2a), SP1(2b)
2		Salt pan II SP2(1), SP2(2), SP2(3)
3		Salt pan III SP3(1), SP3(2)
4		Salt pan IV SP4
5		Salt pan V SOP
6	Bhayandar (Mumbai, Maharashtra, India)	Salt pan I BS1
7		Salt pan II BS2a, BS2b

Table 1 Chemical composition of brine samples

Ionic composition	Kandla salt pans					Bhayandar salt pans	
	I	II	III	IV	V	I	II
Na ⁺ (g/l)	70.5	68.5	90.0	83.0	100.2	53.5	58.0
K ⁺ (g/l)	4.2	4.1	7.0	8.3	7.2	9.8	9.8
Mg ²⁺ (g/l)	7.477	6.912	16.032	19.477	11.927	40.824	40.776
Ca ²⁺ (g/l)	2.07	2.4	1.4	2.28	1.92	1.16	1.16
CO ₃ ²⁻ (g/l)	3.6	0.00	0.00	0.00	0.00	0.00	0.00
HCO ₃ ⁻ (g/l)	7.93	1.464	1.342	2.074	1.22	0.366	0.488
Cl ⁻ (g/l)	134.9	186.73	184.6	188.15	191.7	177.5	177.5
SO ₄ ²⁻ (g/l)	0.00	0.00	4.821	0.76	0.975	37.541	46.652
Total soluble salts	230.7	270.11	305.2	304.052	314.943	320.691	334.38
pH	6.16	6.17	6.19	6.17	6.31	5.92	6.0

Table 3 Biochemical characteristics of isolates

Characteristics		Isolates											
	SP1(1)	SP1(2a)	SP1(2b)	SP2(1)	SP2(2)	SP2(3)	SP3(1)	SP3(2)	SP4	SOP	BS1	BS2a	BS2b
Cell Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	M	M	M	M	M	M	M	M	M	M	M	M	M
Pigmentation	Blood red	Orange	Blood red	Orange	Pink	Pink	Orange	Blood red	Orange	Orange	Pink	Pink	Pink
Gram Nature	-	-	-	-	-	-	-	-	-	-	-	-	-
Minimum NaCl (% w/v) required to prevent lysis	20	15	20	20	15	10	15	10	15	15	15	15	10
Optimum NaCl (% w/v) required for growth	20-25	20	20-25	25	20-25	25	20	25	25-30	20	15-25	20-25	15-25
Range of NaCl (% w/v) that supports growth	20-35	15-35	30-35	20-35	20-35	15-35	10-35	15-35	10-35	15-35	15-35	15-35	10-35
Anaerobic growth on arginine	+	-	+	-	-	-	-	+	-	-	-	-	-
Anaerobic growth on nitrate	-	-	-	+	+	-	+	-	+	+	+	-	+
Catalase and oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	+	+	-	-	-	+	-	-	-	-
Gelatin hydrolysis	+	+	+	-	-	+	+	+	-	-	-	-	-
Caseine hydrolysis	+	+	+	-	-	+	+	+	-	-	-	-	-
Tween 80 hydrolysis	+	+	+	+	+	+	+	+	+	-	+	+	-
PHA	-	+	-	+	+	+	+	-	+	-	-	-	-
Acid from carbohydrates													
Glucose	-	+	-	+	+	+	+	-	+	+	+	+	+
Mannose	-	-	-	-	-	-	-	-	-	+	+	-	-
Galactose	-	-	-	+	+	-	-	-	+	-	-	-	-
Fructose	-	+	-	+	+	+	+	-	+	+	+	+	+
D-Xylose	-	-	-	+	+	-	-	-	+	+	+	+	+
Maltose	-	+	-	+	+	-	-	-	+	+	+	+	+
Sucrose	-	-	-	+	+	-	-	-	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	+	+	-	-	-	+	-	-	-	-
Glycerol	-	+	-	+	+	+	+	-	+	+	+	+	+
Mannitol	-	-	-	+	+	-	-	-	+	-	-	-	-
Sorbitol	-	-	-	+	+	-	-	-	+	-	-	-	-

M motile, - negative, + positive

indicated production of enzyme amylase by these organisms. All the isolates, except SOP and BS2b, hydrolyzed tween 80 which revealed the presence of esterase activity in isolates. Among the thirteen isolates six were found to possess polyhydroxyalkanoate (PHA) granules suggesting their possible use in bioplastic production. Utilization of carbohydrates was found to be variable among the isolates. Isolates SP2(1), SP2(2) and SP4 showed identical pattern of acid production from different sugars whereas, the isolates SP1(1), SP1(2b) and SP3(2) did not utilize any of the sugars indicating their similarity with *Halobacterium* species as these are not known to utilize sugars. Rest of the isolates showed a variable pattern of acid production in presence of carbohydrates.

Polar lipid analysis

Membrane glycolipids and phosphatidylglycerosulfate (PGS), the taxonomic markers of halophilic Archaea, were analysed by thin layer chromatographic technique. Glycolipids appeared as blue-purple or dark pinkish spots and PGS was observed as yellow–brown spot. The isolates were grouped according to their glycolipid signature and presence or absence of PGS. Figure 2a, b represent the polar lipid pattern of all isolates and reference strains. Isolates SP1(1), SP1(2b) and SP3(2) had a glycolipid signature similar to that of reference *Halobacterium salinarum* with presence of PGS. The glycolipid composition of isolates Sp1(2a), SP2(3), SP3(1), SOP, BS1, BS2a and BS2b resembled

glycolipid pattern of reference *Haloferax mediterranei* and showed absence of PGS while, isolates SP2(1), SP2(2) and SP4 showed presence of PGS and possessed same type of glycolipids but differed from the two reference strains used in the study and hence, might belong to a different genus. However, according to literature the polar lipid pattern of these isolates was similar to that possessed by members of genus *Haloarcula* (Kushwaha et al. 1982). These results revealed the occurrence of members belonging to three different genera, *Halobacterium*, *Haloferax* and *Haloarcula* in salt pans of Kandla and Bhayander.

16S rDNA sequence analysis and ARDRA

16S rRNA gene from all isolates was amplified by colony PCR method using archaeal primers. Amplicon of size 1500 bp was obtained from all the isolates and the reference strain of *Halobacterium salinarum*. Phylogenetic grouping of isolates was done by restriction analysis of amplified 16S rDNA. Thirteen haloarchaeal isolates were subjected to ARDRA using restriction enzymes *Hae*III, *Hha*I and *Hinf*I. The phylogenetic tree constructed based on banding pattern of digested 16S rDNA using NTSYSpc 2.0 software (Fig. 3) revealed close relatedness of isolates SP1(1), SP1(2b) and SP3(2) to *Halobacterium salinarum* with similarity coefficient of 1.00 (100% similarity). Isolates SP1(2a), SP2(3) and SP3(1) were clustered with *Haloferax mediterranei* whereas SP2(2) and SP4 clustered to *Haloarcula vallismortis* as they

Fig. 2 **a** Thin layer chromatography of polar lipids from haloarchaeal isolates. Lanes 1 and 8 represent reference *Halobacterium salinarum* and *Haloferax mediterranei*; lanes 2–7 represent isolates SP1(1), SP1(2b), SP3(2), SP2(1), SP2(2) and SP4, respectively. Blue-purple/pink spots represent glycolipids and yellow–brown spot represent PGS. Spots of PGS are seen in lane 1–7. **b** Thin layer chromatography

of polar lipids from haloarchaeal isolates. Lanes 1 and 9 represent reference *Halobacterium salinarum* and *Haloferax mediterranei*; lanes 2–8 represent isolates SP1(2a), SP2(3), SP3(1), SOP, BS1, BS2a and BS2b, respectively. Blue-purple/pink spots represent glycolipids and yellow–brown spot represent PGS. Faint spot of PGS is seen in lane 1

Fig. 3 Dendrogram based on ARDRA showing diversity among 13 haloarchaeal isolates and four reference strains

showed similarity coefficient of 1.00 with the respective reference strains. Another group which was closely clustered to *Haloferax mediterranei* consisted of SP2(1), SOP, BS1, BS2a and BS2b which showed 91% similarity (similarity coefficient 0.912) with the reference strain.

The amplified 16S rDNA obtained using colony PCR method were subjected to sequencing at Bangalore Genei Pvt Ltd, India. The sequence alignment of isolates also revealed the existence of three different haloarchaeal genera: *Halobacterium*, *Haloarcula* and *Haloferax*. Isolates SP1(1), SP1(2b) and SP3(2) showed close relatedness to *Halobacterium salinarum*; SP2(1), SP2(2), and SP4 to *Haloarcula hispanica*; SP1(2a), SP2(3) and SP3(1) to *Haloferax mediterranei*/*Haloferax lucentense*; SOP and BS1 to *Haloferax volcanii*; BS2a to *Haloferax alexandrinus*; and BS2b to *Haloferax volcanii*/*Haloferax alexandrinus*. These results were in agreement with the results of biochemical tests, polar lipid analysis and ARDRA. The sequences were submitted to NCBI Gene Bank database. The extent of similarity in terms of percentage and the accession numbers for sequences as given by NCBI are shown in Table 4.

Table 4 16S rDNA sequence analysis of isolates

Isolate	Best match using NCBI BLAST search	NCBI accession number
SP1(1)	<i>Halobacterium salinarum</i> (99%)	DQ465014
SP1(2a)	<i>Haloferax lucentense</i> (97%) <i>Haloferax mediterranei</i> (97%)	DQ465020
SP1(2b)	<i>Halobacterium salinarum</i> (98%)	DQ465019
SP2(1)	<i>Haloarcula hispanica</i> (98%)	DQ899739
SP2(2)	<i>Haloarcula hispanica</i> (99%)	DQ899743
SP2(3)	<i>Haloferax mediterranei</i> (97%)	DQ899740
SP3(1)	<i>Haloferax mediterranei</i> (98%) <i>Haloferax lucentense</i> (98%)	DQ899744
SP3(2)	<i>Halobacterium salinarum</i> (98%)	DQ983320
SP4	<i>Haloarcula hispanica</i> (98%)	DQ899745
SOP	<i>Haloferax volcanii</i> (99%)	DQ465024
BS1	<i>Haloferax volcanii</i> (99%)	DQ465021
BS2a	<i>Haloferax alexandrinus</i> (100%)	DQ465022
BS2b	<i>Haloferax volcanii</i> (99%) <i>Haloferax alexandrinus</i> (99%)	DQ465023

Screening of isolates for extracellular protease production

Most halophilic archaea are known to secrete proteases into the external environment that can have unique properties for application to biotechnology. The isolates were therefore, screened for protease production based on the zone of clearance surrounding the growth on skimmed milk agar plates

at 37 °C (data not shown). The zone of clearance appears due to the digestion of protein present in skimmed milk by the action of extracellular protease produced by the isolates. *Halobacterium* sp. SP1(1), SP1(2b) and SP3(2) showed significant zone of clearance. Isolates SP1(2a), SP2(3) and SP3(1) related to *Haloferax mediterranei* (as revealed by 16S rDNA sequence analysis) showed comparatively very less zone of clearance whereas, rest of the isolates belonging

to genus *Haloferax* and *Haloarcula* did not show clearance on skimmed milk plates.

These results were indicative of *Halobacterium* species as potential protease producers. Hence, the three *Halobacterium* sp. SP1(1), SP1(2b) and SP3(2) were selected for further quantification of protease produced. Protease production by these organisms in soybean meal-based production medium was studied as a function of time (Fig. 4). Maximum protease production (18.16 ± 1.55 U/ml) was observed from *Halobacterium* sp. SP1(1) at 72 h. Protease production by *Halobacterium* sp. SP1(2b) (9.99 ± 0.74 U/ml) and SP3(2) (12.76 ± 0.09 U/ml) was comparatively less and was also achieved at 72 h of growth. These results were suggestive of *Halobacterium* sp. SP1(1) as potential isolate for production of extracellular protease. All the further studies were carried out using *Halobacterium* sp. SP1(1).

Effect of NaCl on protease production

Since haloarchaea require high salt concentrations (3–5 M NaCl i.e., ~ 17–30% w/v) for growth and haloarchaeal proteases are salt dependent, effect of NaCl on protease production by *Halobacterium* sp. SP1(1) was studied. The maximum protease production at 25% (17.6 ± 1.2 U/ml), 30% (17.28 ± 0.95 U/ml) and 35% (17.81 ± 0.53 U/ml) NaCl was achieved at 72 h of growth (Fig. 5). Drastic reduction in protease production (3.07 ± 0.15 U/ml) was observed at 20% (w/v) NaCl, the minimum concentration required for growth of the organism.

Effect of carbon and nitrogen source on protease production

The initial protease production medium consisting of 1% soybean meal in place of yeast extract in the growth medium yielded 17.86 U/ml of enzyme. The effect of various carbon and nitrogen sources on protease production was monitored (Fig. 6). Maximum protease production (42.23 U/ml) was obtained at 96 h in medium containing 1% soybean flour which was 2.36 folds higher compared to the initial,

Fig. 4 Protease production (U/ml) by *Halobacterium* sp. SP1(1), SP1(2b) and SP3(2) as a function of time (h)

Fig. 5 Protease production (U/ml) by *Halobacterium* sp. SP1(1) at different NaCl concentrations as a function of time (h)

soybean meal-based, production medium. The other protein rich flours, chick pea and pigeon pea showed relatively very less protease production whereas; yeast extract and peptone failed to support the production. Growth in presence of gelatin was poor and no protease production was observed. The carbon sources like glucose and starch, and simple nitrogen sources like $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NaNO_3 and urea did not support growth. Further studies were conducted using soybean flour instead of soybean meal in the production medium.

Effect of metal ions, surfactants and amino acids on protease production

The level of protease production by *Halobacterium* sp. SP1(1) in presence of different metal ions has been given in Fig. 7. Significant increase (1.54 fold) in protease

Fig. 6 Effect of protein sources on growth and protease production (U/ml) by *Halobacterium* sp. SP1(1)

production was observed on addition of FeCl_3 (1 mM) to soybean flour-based production medium. Addition of other metal ions, Mg^{2+} , Mn^{2+} and Ca^{2+} did not affect protease production whereas, Zn^{2+} failed to support growth of the isolate. The effect of various surfactants on protease production was investigated (Fig. 8). Dicotylsulfo-succinate increased the protease production by 1.26 fold. SDS reduced the production by 2.3 fold whereas, addition of CTAB and Na-taurocholate to the production medium inhibited growth of the isolate. The surfactant such as tergitol, hexamine hydrate and span 80 caused a marginal decrease in enzyme production while, triton X-100, tween 20 and tween 80 did not affect the production. Members of genus *Halobacterium* have characteristic requirement of amino acids for growth, hence, amino acids L-alanine, L-valine and Na-glutamate were incorporated into the production medium to investigate their effects on protease production by *Halobacterium* sp. SP1(1). All the three amino acids inhibited the production to varied levels, with maximum inhibition (1.93 fold) caused by L-alanine (Fig. 9).

Fig. 8 Effect of surfactants on growth and protease production (U/ml) by *Halobacterium* sp. SP1(1)

Protease production in presence of organic solvents

Effect of organic solvents, n-decane ($\log P_{ow} = 5.5$), n-undecane ($\log P_{ow} = 6.5$) and n-dodecane ($\log P_{ow} = 7.0$), on protease production by *Halobacterium* sp. SP1(1) was studied (Fig. 10). All the three organic solvents tested increased the protease production in the order n-decane > n-undecane > n-dodecane > control. Maximum protease production achieved in presence of n-decane was 69.56 ± 2.57 U/ml which was 1.2 fold high compared to control without solvent (57.9 ± 1.92 U/ml).

Fig. 7 Effect of metal ions on growth and protease production (U/ml) by *Halobacterium* sp. SP1(1)

Protease production in presence of edible fishes

Initial screening for fish protein hydrolysis by the isolate was done using three different edible fishes found in Gujarat; Pomfret, Flat fish and Seer fish. Dry powdered form of these fishes was used in the growth medium as the sole source of protein for growth and protease production by *Halobacterium* sp. SP1(1). Maximum protease production (17.8 ± 0.17 U/ml) by *Halobacterium* sp. SP1(1) was with Pomfret powder followed by 10.86 ± 0.26 U/ml with flat fish (Fig. 11). Medium containing Seer fish powder showed least protease production (4.62 ± 0.06 U/ml).

Fig. 9 Effect of amino acid supplementation on growth and protease production (U/ml) by *Halobacterium* sp. SP1(1)

Fig. 10 Growth and protease (U/ml) production in presence of organic solvents by *Halobacterium* sp. SP1(1)

Fig. 11 Growth and protease production (U/ml) by *Halobacterium* sp. SP1(1) using edible fish powder as protein source

Discussion

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Hypersaline environments are those with salt concentrations above that of sea water (3.3–3.5% total dissolved salts) (Kerkar 2004). Based on their origin, they can be classified into thalassohaline and athalassohaline environments. Thalassohaline habitats are those with

salt composition similar to that of sea water: sodium and chloride being the dominating ions and the pH is near neutral or alkaline as these environments result from evaporation of sea water. Athalassohaline hypersaline environments are those in which the ionic composition differs greatly from that of sea water: the concentration of divalent cations (Mg^{2+} and Ca^{2+}) exceeds that of monovalent cations (Na^{+} and K^{+}) and the pH is relatively low (Kerkar 2004). The compositions of brine samples analyzed were similar to those described for thalassohaline environments, the sodium and chloride ions being dominant. The pH was around 6.0 for all the salterns. The near neutral thalassic nature of brines suggested their suitability for isolating halophilic microorganisms.

Thirteen halophilic archaea were isolated from Kandla and Bhayander salt pans. Colonies were selected based on their red pigmentation and colony size. Colonies appearing with different shades of red were indicative of haloarchaeal existence. Halophilic archaea appear red due to the presence of C50 carotenoids (bacterioruberins) (Grant and Larsen 1989). Rod shaped cell and gram negative nature of isolates confirmed their bacterial morphology. Extreme halophilicity of isolates was evidenced by the ability of isolates to grow significantly at saturating NaCl concentration (35%, w/v) and lack of growth below 10–20% (w/v) NaCl. Halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement of NaCl. Examples of well adapted and widely distributed extremely halophilic microorganisms include archaeal *Halobacterium* species, cyanobacteria such as *Aphanothece halophytica*, and the green alga *Dunaliella salina*. Cell envelop of non-cocoid forms of haloarchaea possess hexagonal pattern due to regular packing of glycoprotein subunits which are held together only in presence of salt.

One of the features by which archaea differ from eubacteria is the absence of peptidoglycan in cell wall. Insensitivity of the isolates to the antibiotics that target peptidoglycan layer suggested their relatedness to archaea. Most halophilic archaea are sensitive to novobiocin and bacitracin. Novobiocin is a DNA gyrase inhibitor and acts on the same target in archaea as in sensitive eubacteria. Novobiocin has also been reported to block carotenoid biosynthesis (Tomlinson et al. 1986). Bacitracin inhibits incorporation of high molecular weight saccharide into the cell wall glycoprotein of non-cocoidhalophilic archaea and may also inhibit lipid biosynthesis in these organisms (Basinger and Oliver 1979; Wieland et al. 1982). Sensitivity of all the isolates to novobiocin and bacitracin revealed their haloarchaeal nature.

Several biochemical tests were performed to differentiate between the isolates. Certain biochemical properties of microorganisms also relate to their potential in biotechnology. Among the neutrophilic halophilic archaea, *Halobacterium* is the only genus that exhibits fermentative growth

in presence of arginine (Grant and Larsen 1989). Growth of isolates SP1(1), SP1(2b) and SP3(2) on arginine under anaerobic conditions was indicative of their relatedness to *Halobacterium* species. Ability to reduce nitrate to nitrite under anaerobic conditions is characteristic of some halobacterial species mainly belonging to genus *Haloarcula* and *Haloferax* (Mancinelli and Hochstein 1986). Isolates SP2(1), SP2(2), SP3(1), SP4, SOP, BS1, BS2a and BS2b showing anaerobic growth on nitrate might therefore belong to one of the two genera. All the isolates were catalase and oxidase positive which was in agreement with that reported for members of family Halobacteriaceae in Bergy's Manual of Systematic Bacteriology (Grant and Larsen 1989). Gelatine and casein hydrolysis, starch hydrolysis and tween80 hydrolysis by the isolates suggested their potential for production of enzymes like proteases, amylases and esterases, respectively. Certain halobacteria produce intracellular granules of polyhydroxyalkanoate (PHA) as a storage polymer when they are grown under appropriate growth conditions (Lillo and Rodriguez-Valera 1990). PHA is used commercially as bioplastic and therefore, the organisms that can produce PHA may be exploited for commercial application. Six out of 13 isolates were found to possess PHA granules. Halophilic archaea are also characterized by their differences in utilization of carbohydrates. Members of genus *Halobacterium* have an absolute requirement of amino acids for growth and are not known to utilize sugars. Others may utilize sugars with acid production (Oren et al. 1997). The property of not utilizing any of the sugars by the isolates SP1(1), SP1(2b) and SP3(2) indicated their similarity with *Halobacterium* species. The pattern of acid production on different sugars was found to be similar for isolates SP2(1), SP2(2) and SP4 which was in agreement with other biochemical tests and may cluster in the same group. Rest of the isolates showed varied response for acid production in presence of sugars.

The polar lipid composition has proven quite useful in the taxonomy of extreme halophiles. A newly isolated extreme halophilic archaea can be assigned to a genus by the nature of glycolipids and presence of phosphatidylglycosulfate (PGS) (Oren et al. 1997). Based on the presence or absence of PGS and glycolipid signature the 13 isolates were grouped into 3 different genera: *Halobacterium*, *Haloarcula* and *Haloferax*. The genus *Halobacterium* is characterized by the presence of PGS; and glycolipids S-TGD (sulfated triglycosyldiether) and S-TeGD (sulfated tetraglycosyldiether) whereas the members of genus *Haloarcula* possess PGS and glycolipid TGD-2 (triglycosyldiether) as the major glycolipid. Members of genus *Haloferax* are characterized by the absence of PGS and presence of glycolipid S-DGD (sulfated diglycosyldiether) (Kamekura et al. 1992). The isolates were also clustered by Amplified rDNA (Ribosomal DNA) Restriction Analysis. The method was originally developed by Vaneechoutte et al. to characterize

Mycobacterium species (Vaneechoutte et al. 1993). ARDRA has been used effectively to study the microbial diversity of hypersaline environments such as Maras Salterns and Tuz Lake (Maturrano et al. 2006; Mutlu et al. 2008). A fair correlation between the results of polar lipid analysis and ARDRA was observed. The isolates were clustered to genus *Halobacterium*, *Haloarcula* and *Haloferax*. Treatment with all three enzymes *Hha* I, *Hae* III and *Hinf* I showed identical grouping of isolates obtained by comparing the band pattern among the isolates confirming their relatedness to the particular genus. A more precise classification of isolates was obtained by 16S rDNA sequence alignment. The Results again confirmed existence of genera *Halobacterium*, *Haloarcula* and *Haloferax* from salt pans of Kandla and Bhayander. Seven isolates showed identity to *Haloferax* species and three each to *Halobacterium* and *Haloarcula* species. *Haloferax* species have been found to produce halocines which are salts dependent peptide antibiotics active across the major divisions of archaea and may account for their abundance in salt pans (Haseltine et al. 2001; Platas et al. 2002).

Many strains of halophilic archaea are known to secrete proteases which enable to degrade proteins and peptides in the natural environments. These enzymes function in presence of high concentrations of salt (3–4 M NaCl) and permanently lose their conformation at low salt concentrations. Such unique adaptation often limits the application of haloarchaeal proteases. However, recent research advances have revealed application of halophilic proteases in fish sauce preparation, antifouling coating preparations and peptide synthesis in organic solvents (Kim and Dordick 1997; Yongsawatdigul et al. 2007). Owing to such applications the haloarchaeal isolates were screened for the presence of extracellular protease on skimmed milk agar plate. Out of 13 halophilic archaea *Halobacterium* sp. SP1(1) was found to be a good protease producer. Halophilic archaea have high salt requirement for growth as well as protease production. NaCl had a marked effect on production of protease by *Halobacterium* sp. SP1(1) with consistent production at and above 25% (w/v) NaCl while a drastic decrease at 20% (NaCl). This suggested a high salt requirement of *Halobacterium* sp. SP1(1) for extracellular protease production. Protease production at high salt concentration (35% NaCl) even after 120 h suggests the potential of these microorganisms in salt based applications.

Halophilic archaea provide a valuable resource of proteases which have potential for application to biotechnology. The protease production by microorganisms is known to be influenced by various nutritional factors such as carbon and nitrogen source, metal ions, easily utilizable sugars such as glucose and rapidly metabolizable amino acids (Akolkar 2009). But, the effect of these nutritional factors on production of protease in halophilic archaea has been poorly

investigated. Highest protease production by *Halobacterium* sp. SP1(1) was observed in presence of protein rich soybean flour, whereas, simple sugars and nitrogen sources failed to support growth as well as protease production by the isolate. These results can be corroborated with the fact that the members of genus *Halobacterium* utilize amino acids, peptides and proteins as carbon and energy source while; carbohydrates are not utilized by these microorganisms. The observations are in contrast to those reported for haloalkaliphilic archaeon *Natrialba magadii* and the bacterium *Bacillus* sp. where yeast extract and gelatine increased protease production, respectively (D'Alessandro et al. 2007; Patel et al. 2005). Hence, protein rich flours can be used as cheaper alternative sources of carbon and nitrogen for enzyme production. Vidyasagar et al. has used skimmed milk for production haloalkaline proteases from *Halogeometricum* sp. TSS101 (Vidyasagar et al. 2006). *Halobacterium* sp. SP1(1) showed higher production of protease in soybean flour (prepared directly by grinding the soybean seeds) compared to soybean meal. This may be attributed to soybean meal preparation which includes heat processing leading to availability of easily utilizable peptides and amino acids. Availability of metal ions such as Fe^{3+} , Ca^{2+} , Mg^{2+} and Zn^{2+} has been shown to affect protease production in microorganism (Adinarayana et al. 2003). Supplementing the medium with FeCl_3 increased the production of protease (1.54 fold) by *Halobacterium* sp. SP1(1). This finding contradicted the earlier report on *Halogeometricum* sp. TSS101 in which Ca^{2+} at 200 mM concentration enhanced the protease production from 52 to 65 U/ml and Fe^{2+} was found to be inhibitory (Vidyasagar et al. 2006). Ca^{2+} at the same concentration inhibited the growth of *Halobacterium* sp. SP1(1). Iron regulated proteases have been reported in *Pseudomonas aeruginosa* and *Nisseria meningitidis* strain MC58 (Shigematsu et al. 2001; Grifantini et al. 2003). Surfactants are known to affect cell membrane permeability leading to increased secretion of extracellular enzymes. Protease production by *Halobacterium* sp. SP1(1) was increased in presence of dicotylsulfo succinate, whereas, triton X-100, tween 80 and tween 20 did not affect the production. These results differ from earlier reports on extracellular protease production by *Bacillus cereus* and *Rhizopus oryzae* which was enhanced in presence of triton X-100 and tween 80 (Banerjee and Bhat-tacharya 1992; Esakkiraj et al. 2009). Inhibition of protease production by all the three amino acids tested revealed the possibility of catabolic repression of genes for protease production by rapidly metabolized amino acids in the medium. The catabolite repression by amino acids is a well known phenomenon in the yeast *Saccharomyces cerevisiae* (Winderickx et al. 2003). *Bacillus intermedius* also exhibited nitrogen catabolite repression, in which easily metabolized nitrogen sources repressed the expression of genes encoding transporters and proteases (Balaban et al. 2004). However,

extracellular protease production by haloalkaliphilic *Bacillus* sp. was stimulated by amino acids (Patel et al. 2005).

Halophilic archaea are known to secrete proteases that are active and stable at high salt concentrations. A high salt concentration reduces water activity. Similar phenomenon of reduction of water activity is observed when proteins are suspended in organic solvents. Therefore haloarchaeal enzymes have advantage over the non-halophilic counterparts of being active in presence of these solvents. Extracellular protease production by *Halobacterium* sp. SP1(1) was studied in presence of solvents. No inhibitory effect on production was observed. Increase in protease production was observed in presence of all the tested solvents with maximum production in presence of n-decane (1.2 fold). The fact that increased protease production was observed with decreasing $\log P_{ow}$ values of solvents suggests the possible role of solvents in enhanced membrane permeability. Biofilms produced by the microorganisms often contain proteinaceous material as exo-polymeric substance and are the cause of serious corruptions occurring on the marine surfaces. Solvent tolerant halophilic proteases can be effective alternate to the conventional copper and tin based coatings as they can be directly mixed with paints.

Another application of halophilic proteases can be in fish sauce preparations. Fish sauce, which is known by various names according to the country of origin, is one of the most popular fermented fish products used in Asia. Salt contributes to the sensory and most importantly hygienic quality of the final fish sauce. Addition of salt in range 20–30% to protein rich substrates results in a controlled protein hydrolysis that prevents putrefaction and food poisonings such as botulism and yields meaty, savory, amino acid/peptide sauces that provide very important condiments particularly for those unable to afford much meat in their diets. It has been widely accepted that proteases from the fish viscera are responsible for fish tissue breakdown in fish sauces. Thongthai et al. (1990) however, have shown that the visceral enzymes were inactivated at higher NaCl concentrations and majority of protease activity found in liquid surrounding the fish was stable at 4 M NaCl, a unique characteristic of halobacterial proteases. This suggested the role of halobacterial proteases in the fermentation process. Hence, protease production by *Halobacterium* sp. SP1(1) in presence of three different edible fishes commonly found in Gujarat was investigated. Pomfret was found to be the best protein source for protease production by the isolate. These results revealed that *Halobacterium* sp. SP1(1) has the ability to grow and produce protease on edible fishes and therefore, may have potential in fish fermentation process.

In summary, halophilic archaea isolated from salt pans produce several molecules of biotechnological significance, extracellular protease being one of them. Salt dependent protease production by these organisms can be enhanced by

optimizing the production medium. Further, *Halobacterium* species by virtue of their extracellular protease may have application in antifouling coating preparations and fish sauce fermentation.

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Compliance with ethical standards

Conflict of interest Authors report no conflict of interest.

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