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ORIGINAL ARTICLE

Isolation and characterization of a protease-producing novel haloalkaliphilic bacterium *Halobiforma* sp. strain BNMIITR from Sambhar lake in Rajasthan, India

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Abstract A novel haloalkaliphilic bacterium designated as strain BNMIITR was isolated from a soil sample collected from Sambhar lake, Rajasthan, in northern India. Colonies of the isolated strain were dark orange and comprised Gram-negative bacilli; there was a slight pleomorphism towards the stationary phase of growth. Experiments revealed that the isolate can grow in the range of 2–5 M NaCl, pH 6–11 and 18–55 °C, with optimum growth observed at 3 M NaCl, pH 8–8.5 and 45 °C. No growth was observed in culture medium without NaCl. The isolate showed no requirement for magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) for growth. Major cellular fatty acids were $C_{14:0}$, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso and $C_{20:2}$ w6, 9c. The result of 16S rRNA gene sequence analysis showed 98 % sequence similarity with *Halobiforma lacisalsi* and *Hbf. haloterrestris*. *Halobiforma* sp. strain BNMIITR showed resistance towards several antibiotics and produced an extracellular alkaline protease. The crude enzyme was found to be active in broad range of alkaline pH and temperature (30–80 °C).

Keywords *Halobiforma* · Protease · Haloalkaliphilic · Pleomorphism · Cellular fatty acids

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Introduction

Hypersaline environments on the Earth occur either naturally as permanent saline lakes and ephemeral salt pans or artificially as solar salterns. Hypersaline water bodies can be classified into two major categories, thalassohaline and athalassohaline. The salt composition of thalassohaline water bodies is similar to that of seawater with sodium and chloride ions being dominant. In contrast, athalassohaline water bodies, formed by evaporation and the crystallization of salt, are dominated by potassium or magnesium ions. Hypersaline ecosystems show a considerably rich diversity of microbes and are biologically very productive. These microorganisms (halophilic and extremely halotolerant) are present in all three domains of life: Archaea, Bacteria and Eukaryota (Oren 2002). Several culture-dependent and metagenomic approaches have been used to characterize the microbial communities in hypersaline environments.

The haloarchaea are considered to be a monophyletic group represented by order Halobacteriales and family *Halobacteriaceae* in which distantly related species show 83.2 % 16S rRNA gene sequence similarity (Grant et al. 2001). The first members of the family *Halobacteriaceae* were isolated more than a 100 years ago. The name *Halobacteriaceae* was first used by Gibbons (1974) in the 8th edition of Bergey's Manual of Determinative Bacteriology to refer to three species only: *Halobacterium salinarium*, *H. halobium* and *Halococcus morrhuae*. In terms of cell morphology, members of this family are rod shaped, coccoid pleomorphic or even flat and square to rectangular shaped. Only two alkaliphilic genera have been reported in the family (*Natronobacterium* and *Natronomonas*). At the present time family *Halobacteriaceae* is represented by 36 genera and 129 species, with the complete genome sequence known for at least 12 species (Oren 2012). Metagenomic studies have

shown that there are many species yet to be isolated and characterized (Bodaker et al. 2010; Narasingarao et al. 2012).

These organisms are chemo-organotrophic and need at least 1.5 M NaCl for growth, while some organisms show optimum growth at 3.5–4.5 M NaCl (Grant et al. 2001). All members of family *Halobacteriaceae* have orange–red-pigmentation except for some members of the genus *Natrialba* (Grant et al. 2001; Hezayen et al. 2001).

Halobacterial taxonomy prior to 1970 was mainly based on standard biochemical tests and morphology (Gibbons 1974). The current polypsasic taxonomy is based largely on 16S rDNA sequences and chemotaxonomic criteria, particularly polar lipid composition. Minimal standards for the description of a species includes cell morphology, pigmentation, optimum NaCl and $MgCl_2$ concentrations for growth, the range of salt concentrations enabling growth, temperature and pH ranges for growth, ability to grow on single carbon sources, catalase and oxidase tests, hydrolysis of starch, casein and Tween 80 and sensitivity to different antibiotics and polar lipids (Oren et al. 1997)

The genus *Halobiforma* was proposed by Hezayen et al. (2002), and it contains three species: *Halobiforma haloterrestris*, *Hbf. nitratireducens* (formerly *Natronobacterium nitratireducens*, Xin et al. 2001) and *Hbf lacisalsi* (Xu et al. 2005). The members of this genus are motile, Gram-negative rods or coccoid in shape (Hezayen et al. 2002).

Extreme halophiles are a rich source of a wide variety of biotechnological products, such as bacteriorhodopsins, biopolymers, biosurfactants, exopolysaccharides, polyhydroxyalkonates, flavouring agents, antitumour drugs and enzymes (Margesin and Schinner 2001). Among all extremozymes, halophilic proteases are the most widely exploited enzymes in industrial applications. Halophilic proteases have been isolated and characterized from several bacterial species, including *Bacillus* sp. (Setyorini et al. 2006; Shivanand and Jayaraman 2009), *Halobacillus* sp. (Namwong et al. 2006; Karbalaei-Heidari et al. 2009) and *Virgibacillus* sp. (Sinsuwan et al. 2010).

In the study reported here, we isolated and characterized a novel haloalkaliphilic bacterium designated as strain BNMIITR based on 16S rRNA gene sequencing, lipid and cellular fatty acid analysis and physiological and biochemical characteristics. The isolate showed 98 % similarity with *Halobiforma lacisalsi* and *Hbf. haloterrestris*, but the physiological and biochemical data were not identical with any of the strains reported to date. The isolate was evaluated for the production of extracellular hydrolytic enzymes, and a crude alkaline protease was characterized. The aim of the study was to determine the taxonomic position of bacterium designated as strain BNMIITR and further evaluate the strain for the production of extracellular hydrolytic enzymes.

Materials and methods

Source and isolation

Strain BNMIITR was isolated from a soil sample collected from Sambhar lake, located at Sambhar, Rajasthan (North India). The soil sample was initially incubated at 45 °C on a rotatory shaker (180 rpm) in a complex halophilic medium (NaCl, 250 g/L; potassium chloride (KCl), 2 g/L; $MgSO_4 \cdot 7H_2O$, 20 g/L; tri-sodium-citrate, 3 g/L; yeast extract, 10 g/L; casamino acid, 7.5 g/L; agar, 20 g/L; $MnCl_2 \cdot 4H_2O$, 0.36 mg/L; $FeSO_4 \cdot 6H_2O$, 1.6 mg/L; Asker and Ohta 1999). The pH of the medium was adjusted to 9.0 with 1 M potassium hydroxide (KOH). After 8 days of incubation the isolate was further subcultured in the same liquid medium under the same experimental conditions. It was subsequently purified by serial dilutions and repeated streaking on agar plates having same composition as the liquid medium.

Morphological characterization

The cellular morphology of the isolate was studied by bright field microscopy (objective lens was 100× with oil immersion). Gram staining was performed according to Dussault (1955).

Physiological characterization

Salt requirement To ascertain the halophilic nature of the isolate, strain BNMIITR was grown in complex halophilic medium with salt concentrations ranging from 2 to 5 M for NaCl and from 1 to 4 M for KCl, at 45 °C on a rotatory shaker (180 rpm). The final pH was adjusted to 8.5 with 1 M KOH. Growth was measured in terms of optical density (OD) at 600 nm in UV–Vis spectrophotometer.

pH and temperature profiles The temperature range for growth was determined by incubating agar plates containing complex halophilic medium at temperatures ranging between 16 and 60 °C. The optimum pH for growth was determined by incubating agar plates at pH range 5–12.

Requirement of magnesium ion and carbon and nitrogen sources The requirement of $MgSO_4 \cdot 7H_2O$ for growth was tested by growing the strain BNMIITR in liquid medium supplemented with $MgSO_4 \cdot 7H_2O$ at concentrations ranging from 0 to 30 g/L.

Casamino acids, peptone, tryptone and yeast extract were tested as the sole nitrogen and carbon sources. These experiments were performed in liquid growth medium, and the concentration of casamino acids, peptone, tryptone and yeast extract, respectively, was kept at 10 g/L. While using casamino acids as the sole nitrogen and carbon source,

trisodium citrate and yeast extract were excluded from the growth medium, and growth was checked at regular intervals. When checking the suitability of peptone and tryptone as the sole carbon and nitrogen source, casamino acids, trisodium citrate and yeast extract were excluded from the growth medium. Similarly, for checking yeast extract as the sole carbon and nitrogen source, casamino acid and trisodium citrate were excluded from the growth medium. Growth in all media was checked at regular intervals.

Lipid and cellular fatty acid analysis Total lipid analysis was performed using thin layer chromatography (TLC) where the lipid types were analyzed after separation using TLC plates coated with silica gel. The developing solvent used was hexane/diethyl ether/formic acid (80:20:1, v/v/v). Lipids were visualized by acid charring: the developed plate was sprayed with 5 % concentrated sulphuric acid in 95 % ethanol followed by heating at 180 °C (Buckner et al. 1999). Total lipid extraction and polar lipid analysis on the silica gel TLC plate was performed as described by Elevi et al. (2004). After extraction, lipids were dissolved in a minimum volume of chloroform and applied onto TLC plate; the plate was then eluted with chloroform/methanol/acetic acid/water (85:22.5:10:4, v/v/v/v). Lipid spots were detected by spraying the plate with 0.5 % α-naphthol in 50 % methanol, followed by spraying with 5 % H_2SO_4 in ethanol and heating at 150 °C. Cellular fatty acid analysis was done by Royal Life Sciences Pvt. Ltd. (West Marredpally, India).

Sensitivity to antimicrobial agents The antibiotic sensitivity test was performed by streaking the log phase culture on solid complex halophilic medium containing 3 M NaCl and applying disks (octa-disks; HiMedia Laboratories Ltd., Mumbai, India) impregnated with the selected antimicrobial agents. In our study, we tested ampicillin (10 μg), penicillin-G (2 U), gentamicin (10 μg), tetracycline (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), erythromycin (15 μg), oxacillin (1 μg), vancomycin (30 μg), co-trimoxazole (10 μg), cloxacillin (5 μg), cefradine (30 μg), lincomycin (10 μg) and cefuroxime (30 μg). The culture was incubated at 45 °C for 7–10 days. Sensitivity was measured based on the presence of a growth inhibition zone.

Pigment profile Pigment was extracted from cell pellet with methanol/acetone (1:1 v/v) and the extract was analyzed by scanning the absorbance in the wavelength region of 300–800 nm using Varian UV–Vis spectrophotometer against the same solvent as blank (Oren 2002).

Biochemical characterization

Carbon substrate usage by the isolate was determined using BIOLOG Phenotype GEN III plates, (MicroStation™ System/MicroLog; Biolog Inc., Hayward, CA). Each 96-well microtiter plate has 94 wells, with each well containing a single substrate, as well as one negative and one positive control well. Substrate usage was evaluated colourimetrically along with spectrophotomeric cellular growth measurement. The BIOLOG experiment was carried out using 15 % salt in universal inoculating fluid IF-A and IF-B (suitable for halophilic bacteria like *Virgibacillus*).

Genotypic analysis

For genotypic characterization, genomic DNA of strain BNMMIITR was isolated using the DNA extraction kit of HiMedia laboratories Ltd. The gene encoding 16S rRNA was amplified by PCR using the forward primer 5′-ATTCCGGT TGATCCTGCCGG-3′ and reverse primer 5′-TGGAGGTG ATCCAGCCGCAG-3′ (Xu et al. 2005). The PCR amplification programme consisted of six cycles of 45 s at 95 °C (denaturation), 45 s at 58 °C (annealing) and 2 min at 72 °C (polymerization), followed by 25 cycles of 45 s at 95 °C (denaturation), 45 s at 55 °C (annealing) and 2 min at 72 °C (polymerization), with a final extention at 72 °C for 10 min.

The 16S rRNA gene sequence was further determined by Eurofins Genomics India Pvt Ltd. (Bangalore, India). The 16S rRNA gene sequence was aligned and compared with available sequences in GenBank (National Centre for Biotechnology Information (NCBI)) using the basic local alignment search tool (BLAST). Multiple sequence alignment was done using clustal W tool and the phylogenetic tree was constructed by maximum likelihood (PhyML). The sequence (1,369 bp) was submitted to the NCBI GenBank under accession number KJ475544.

Screening of isolate for extracellular hydrolytic activities

In order to detect the production of extracellular hydrolases, we performed different enzymatic agar plate assays according to Onishi et al. (1983) for DNase, Samad et al. (1989) for lipase, Soares et al. (1999) for pectinase, Amoozegar et al. (2003) for amylase, Amoozegar et al. (2008) for protease and Kasana et al. (2008) for cellulase. Here we only report data related to protease activity.

Protease production by strain BNMIITR To measure protease production, we inoculated 1 mL of culture grown for 96 h into 100 mL of production medium (optimized in the laboratory: NaCl, 180 g/L; KCl, 2 g/L; $MgSO_4 \cdot 7H_2O$, 20 g/L; xylose, 10 g/L; $MnCl_2 \cdot 4H_2O$, 0.36 mg/L; $FeSO_4 \cdot 4H_2O$, 1.6 mg/L; 1 % casein protein) for culture at 45 °C on a rotatory shaker (180 rpm). The final pH of the production medium was set to 10 by 1 M KOH. Samples were withdrawn aseptically at different time intervals for determination of protease activity.

The cell-free supernatant was used as the crude enzyme source.

Protease activity assay The culture was centrifuged at 10,000 *g* for 1 min (4 °C) and the culture supernatant used as a source of protease. The proteolytic activity of the crude enzyme, with casein as the substrate, was determined by the modified method of Kunitz (1947). Briefly, 0.2 mL of crude enzyme was added to 0.8 mL of substrate solution (50 mM Tris buffer, pH 9.0, concentration of casein was 1 % in the final reaction mixture) and the mixture incubated at 55 °C for 30 min. The reaction was stopped by adding 1.0 mL of 10 % trichloroacetic acid (TCA) and kept at room temperature. After 15 min, the supernatant was separated by centrifugation (12,000 *g*, 15 min) and absorbance was recorded spectrophotometrically at 280 nm with tyrosine as the standard. The blank was prepared by adding 1.0 mL of TCA prior to adding the enzyme. One unit of protease activity was defined as the amount of enzyme required to produce 1 μg of tyrosine per minute.

Effect of pH on protease activity The effect of pH on the activity of the crude enzyme was studied by incubating the reaction mixture at pH values ranging from 7 to 12. Buffers used for the enzyme preparations were 50 mM phosphate (pH 7.0), 50 mM Tris–buffer (pH 8.0–9.0) and 50 mM glycine–NaOH (pH 10.0–12.0).

Effect of temperature on activity of enzyme The effect of temperature on the activity of crude protease was examined by incubating the reaction mixture within the temperature range 30–80 °C. The activity was determined as mentioned before.

Effect of additives and surfactants on protease activity The effects of phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and urea on crude protease activity were determined by including these substances in the reaction mixture at a concentration of 10 and 50 mM, respectively. The effects of the surfactants Triton X-100, Tween 80, Tween 40, sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) on enzyme activity were studied at a concentration of 0.5 and 1 %. The activities were measured and presented as relative activity. The activity of the enzyme, incubated without any additive or surfactant was taken as 100 % (control).

Results and discussion

Morphological and physiological characterization of the isolate

Colonies growing on complex halophilic agar medium were circular, convex, translucent, smooth and dark orange (Fig. 1a). The isolate was initially grown on complex

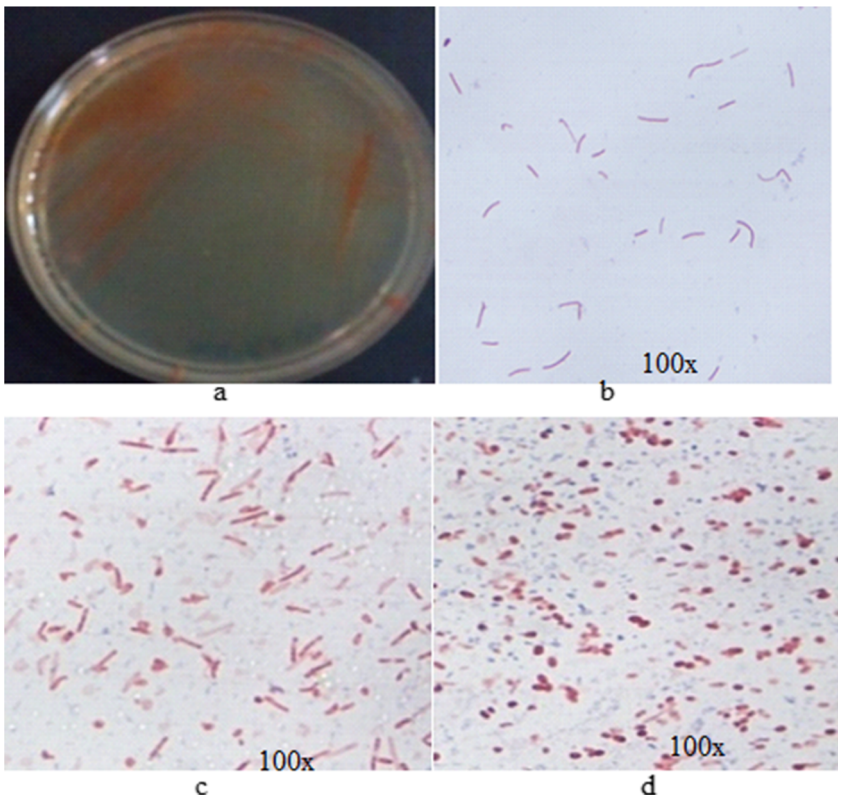

Fig. 1 Morphology of isolate BNMIITR. **a** Colonies growing on agar plates, **b** phase-contrast micrograph showing long Gram-negative rods (early stages of growth), **c** phase-contrast micrograph showing Gram-negative rods and coccoid cells (early log phase), **d** phase-contrast micrograph showing Gram-negative coccoid cells (stationary phase). Magnification100×, with oil immersion

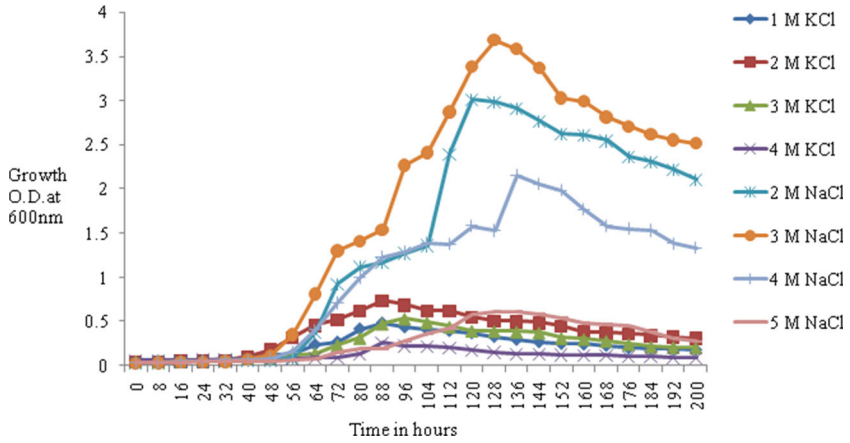

Fig. 2 Growth curve of isolate *Halobiforma* sp. strain BNMIITR in the presence of 1–4 M KCl and 2–5 M NaCl

halophilic agar medium containing 0–5 M NaCl where we observed that it was not able to grow when the NaCl concentration was below 1.8 M. During its early stages of growth in the liquid medium, examination under the microscope revealed that the cells were rod shaped and Gram negative (magnification 100×, with oil immersion; Fig. 1b), but at the later stages of growth the cells acquired a coccoid and pleomorphic shape (Fig. 1c, d).

The growth profile of the isolate in the presence of NaCl (2–5 M) and KCl (1–4 M) was studied separately in liquid medium. The growth profile (Fig. 2) in NaCl medium showed that optimum growth occurred at 3 M NaCl, with poor growth at 5 M NaCl. Growth in KCl medium was 5-fold less than that in NaCl medium (Fig. 2). These results indicate that this isolate is a borderline extreme halophilic bacterium which requires 3 M NaCl for optimal growth (Margesin and Schinner 2001).

The isolate was grown on agar plates with different pH (5–12). Growth was observed at pH 6–10 but faster growth was obtained at pH 8.0–8.5. Similarly it was observed that the isolate could grow in a broad temperature range (18–55 °C) but that faster growth occurred at 45 °C. Cells of the isolate lysed in water when the NaCl concentration was below 2 M.

The isolate was tested for its ability to use different substrates for growth. Strain BNMIITR was found to be able to use casamino acids, peptone, tryptone and yeast extract as the sole carbon and nitrogen source, but maximum and fast growth was observed when peptone (7.5 g/L) and yeast extract (10 g/L) were used in combination. Sugars were found to be a better source of carbon than trisodium citrate as the former supported faster and better growth when used in the complex medium. Maximum growth was observed when D-xylose was used as the carbon source. The isolate did not require $MgSO_4 \cdot 7H_2O$ for growth.

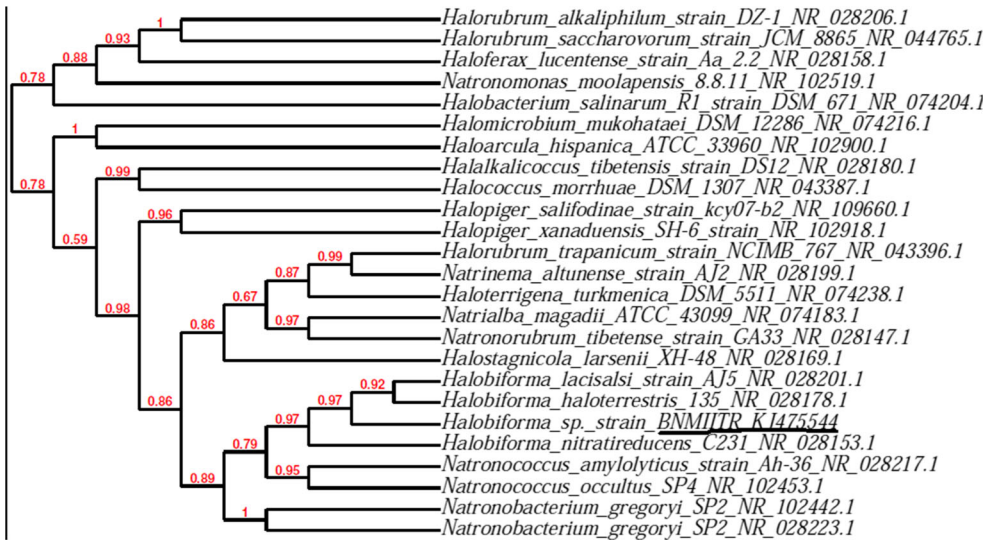

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequence data showing the phylogenetic positions of isolate *Halobiforma* sp. strain BNMIITR and of a number of related taxa. Tree was constructed by maximum likelihood estimation (PhyML). The *number at each branch point* represents the bootstrap support

Table 1 Comparision of the isolate *Halobiforma* sp. strain BNMIITR with other members of the genus *Halobiforma*

Characteristics	*Hbf. haloterrestris*	*Hbf. lacisalsi*	*Hbf. nitratireducens*	*Hbf.* sp. strain BNMIITR
NaCl range (M)	>2.2	>1.7	2.5–5.2	2–5
NaCl optimum	3.4 M	2.5–4.3 M	3.5 M	3 M
pH range	6–9.2	6.5–9.0	8–10.5	6–10
pH optimum	7.5	7.5	8.9	8–8.5
Temperature range	Up to 58 °C	24–57 °C	26–44 °C	20–55 °C
Temperature optimum	42 °C	42–45 °C	36–41 °C	45 °C
Mg required for growth (mM)	0	0.2	0.4	0
Catalase	+	+	+	+
Oxidase	+	+	+	−
Hydrolysis of casein	+	−	−	+
Fructose	−	+	−	+
Mannitol	NR	−	+	+
Maltose	+	W	−	+
Sucrose	W	−	−	W
Glycerol	W	+	−	W
Glucose	W	+	W	W
Gelatin	+	+	−	−
Tween 40	+	+	NR	−
Penicillin G	R	NR	R	R
Chloramphenicol	R	NR	R	R
Tetracycline	R	W	NR	R
Fusidic acid	S	NR	NR	R
Nalidixic acid	R	NR	NR	R
Erythromycin	R	NR	S	R
Vancomycin	NR	S	NR	R
Ampicillin	NR	S	R	R
Carotenoid	494, 528, 470	NR	NR	469, 493, 527
Minor pigments	370, 390	NR	NR	350, 355, 360, 368, 388
Total lipids	4	NR	NR	6
Glycolipids	Sulphated triglycosyl diether and a triglycosyl diether were produced as sole glycolipids	NR	NO	2
Major fatty acids	NR	NR	NR	$C_{14:0}$,$C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso, $C_{20:2}$ w6,9c

+, Positive; −, Negative; W, weak; NR, not reported; NO, not present

[a] *Halobiforma* (*Hbf.*) *haloterrestris* according to Hezayen et al. 2002; *Hbf. lacisalsi* according to Xu et al. 2005; *Hbf. nitratireducens* according to Xin et al. 2001; *Halobiforma* sp. strain BNMIITR (this study)

The strain BNMIITR was found to have resistance against many antibiotics, including ampicillin, penicillin-G, gentamicin, tetracycline, cephalothin, chloramphenicol, clindamycin, erythromycin, oxacillin and vancomycin. However, it was sensitive to co-trimoxazole, cloxacillin, cefuroxime, lincomycin and cefradine [Electronic Supplementary Material (ESM) Fig. 1].

Pigment extract in methanol/acetone (1:1, v/v) showed an absorption maximum at 469, 493 and 527 nm, with minor peaks at 350, 355, 360, 368 and 388 nm (ESM Fig. 2). The nature of the orange pigment of the isolate has yet to be characterized, but its absorption spectrum indicates the presence of carotenoid (Cuadros Orellanaa et al. 2006). The presence of multiple pigments cannot be ruled out in the isolate. It is interesting to note that the isolate does not require $MgSO_4 \cdot 7H_2O$ for growth, but pigment production was markedly reduced in the absence of $MgSO_4 \cdot 7H_2O$ in the liquid medium. We observed that a minimum 5 g/L of $MgSO_4 \cdot 7H_2O$

was required for prominent pigment production. Similarly D-xylose was found to be a good carbon source for growth and pigment production.

Biochemical, genotypic and lipid analysis

Carbon substrate utilization by the isolate was determined using the BIOLOG system. We inoculated the isolate in inoculating fluid IF-A (default protocol) and IF- B separately. The results are summarized in ESM Table 2, and they clearly indicate that the isolate was unable to use amino acids as a carbon source, with the exception of *D*-serine, and showed borderline reaction for L-aspartic acid and L-histidine. The isolate was unable to utilize organic acids as a carbon source, except for D-galacturonic acid, glucuronic acid, acetoacetic acid and acetic acid, with a borderline reaction for α-ketoglutaric acid, propionic acid, α-ketobutyric acid and D-saccharic acid. The isolate was able to use D-mannitol, fructose-6-phosphate and glucuronamide, with a borderline reaction for D-raffinose, α-D-glucose, D-sorbitol, α-D-lactose, D-mannose, D-arabitol, D-trehalose, D-galactose, D-fucose, D-glucose-6-phosphate, stachyose, inocine, sodium lactate and sucrose. Similarly, it showed positive reaction for maltose, D-fructose, dextrin, L-rhamnose and a borderline reaction for pectin, D-mannose, D-galactose, D- and L-fucose, D-turanose and *N*-acetylgalactosamine. Sabet et al. (2009), after analyzing 35 halophilic cultures isolated from solar salterns, concluded that the bacterial isolates utilized more carbon substrates than archaeal isolates.

Strain BNMIITR was isolated from a soil sample collected near Sambhar lake, Rajasthan India. On the basis of its pigmentation, NaCl-dependent growth, antibiotic susceptibility, physiological, biochemical characterization and 16S rRNA gene sequence, we identified strain BNMIITR as a member of the family *Halobacteriaceae*. The complete 16S rRNA gene sequence showed 98 % similarity with *Halobiforma lacisalsi* and *Hbf. haloterrestris* and 96 % with *Hbf. nitratireducens* (Fig. 3). Thus, the strain is phylogenetically related to genus *Halobiforma*. Genus *Halobiforma* has been reported from the soda lakes of China and Egypt. *Hbf. haloterrestris* has been isolated from hypersaline soil close to Aswan, Egypt (Hezayen et al. 2002), *Hbf. lacisalsi* has been isolated from a water sample of Ayakekum salt lake in China (Xu et al. 2005) and *Hbf. nitratireducens* has been isolated from a soda lake in China (Xin et al. 2001). We report here for the first time the isolation and characterization of genus *Halobiforma* from Sambhar lake (India). Sambhar lake is the largest inland salt lake in India and is characterized by a predominance of NaCl, sodium carbonate, sodium bicarbonate and sodium sulphate and lacked divalent cations (calcium and magnesium), pH values of 9.5–9.7 and temperature 37–45 °C (Upasani and Desai 1990). Based on the observation of lower growth in the presence of KCl and no requirement for $MgSO_4 \cdot 7H_2O$ for growth, it can be concluded that the strain was better adapted to the environmental conditions under which it was isolated (NaCl, pH and temperature). $MgSO_4 \cdot 7H_2O$ was required only to maintain pigmentation. Most of the archaeal strains isolated from Sambhar lake cannot utilize Tween 40 (Upasani and Desai 1990). Carotenoid pigment isolated from archaea show absorption maxima at 526, 494, 388 nm, with a shoulder at 470–475 nm (Cuadros Orellanaa et al. 2006). In addition to the carotenoids, strain BNMIITR also had some minor pigments (absorption maximum at 350, 355, 360 nm) which have not been previously reported in any of the species of genus *Halobiforma*. These pigments possibly developed by lateral gene transfer, as shown in *Salinibacter* (Sharma et al. 2007).

All three representative species of genus *Halobiforma* are catalase- and oxidase-positive, but we found strain BNMIITR to be catalase-positive and oxidase-negative.

The temperature profile of strain BNMIITR is similar to that of other members of the genus. The optimum pH for *Hbf. haloterrestris* and *Hbf. lacisalsi* is 7.5, but strain BNMIITR had a pH optimum at 8.5, which is similar to that of *Hbf. nitratireducens* (8.7) to which it showed 96 % similarity in 16S rRNA gene sequence. All three representative members of *Halobiforma* genus can hydrolyze Tween 40, whereas strain BNMIITR cannot hydrolyze it. A detailed comparison of strain BNMIITR with other members of the genus *Halobiforma* is given in Table 1.

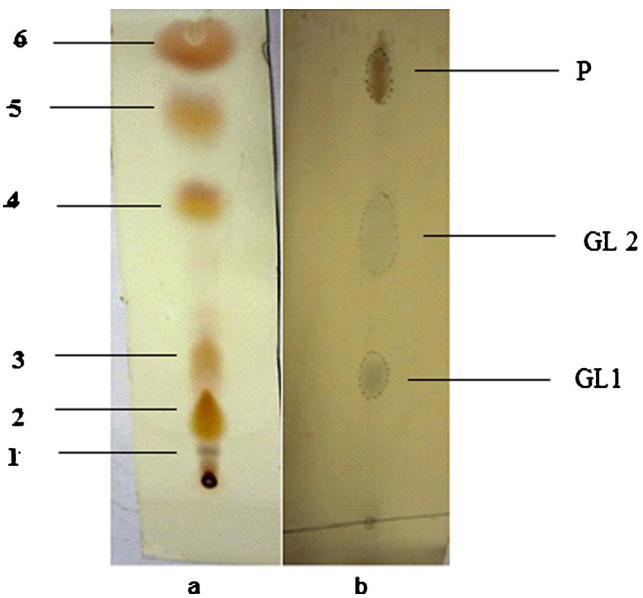

Fig. 4 Thin-layer chromatogram of: **a** total lipids (*1–6*), **b** polar lipids from *Halobiforma* sp. strain BNMIITR showing glycolipids (*GL1, GL2*) and phospholipid (*P*)

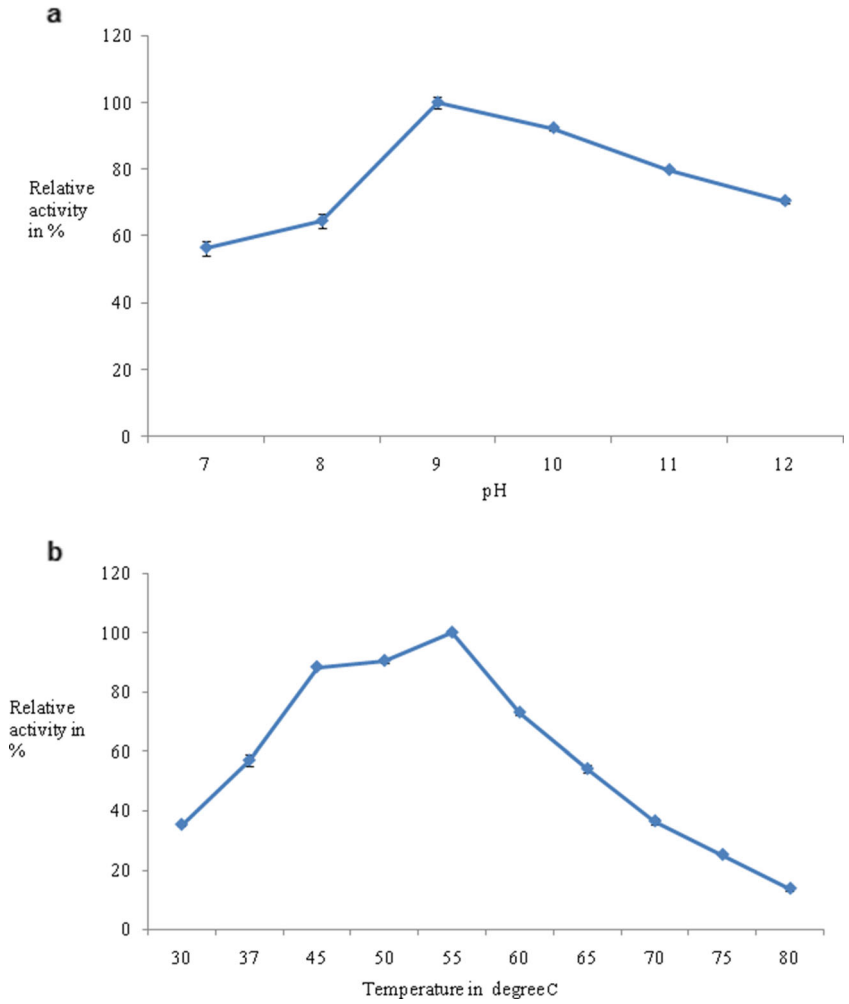

Fig. 5 Effects of pH (at 45 °C) (**a**) and temperature (at pH 9.0) (**b**) on caseinolytic activity of the crude enzyme extract from isolate *Halobiforma* sp. strain BNMIITR. Values are presented as the mean ± standard deviation (SD) (n=3)

Halobiforma lacisalsi is sensitive to tetracycline and vancomycin, but our results revealed that strain BNMIITR is resistant to both, *Hbf. lacisalsi* can use amino acids, such as glutamate and arginine, but these could not be utilized by

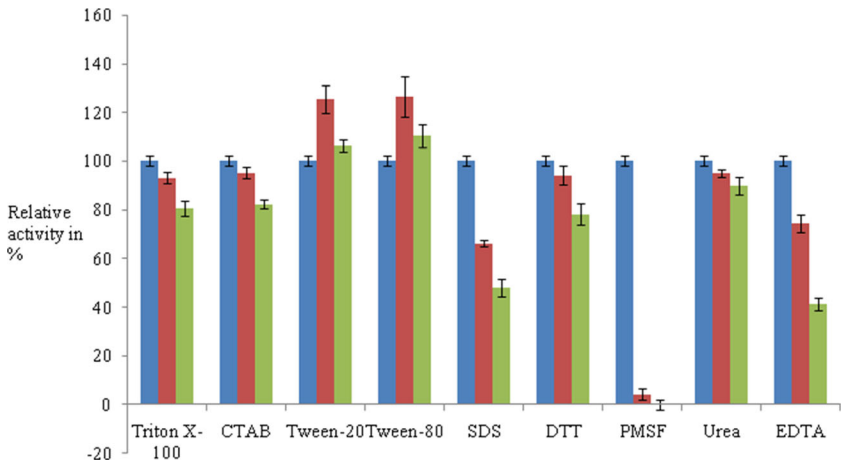

Fig. 6 Protease activity of crude enzyme in the presence of different enzyme inhibitors. For Triton X-100, cetyltrimethylammonium bromide (*CTAB*), Tween 20, Tween 80, sodium dodecyl sulphate (*SDS*): *leftmost column* (*blue*) control (100 % concentration), *middle column* (*red*) 0.5 %, *right column* (*green*) 1 % concentration. For dithiothreitol (*DTT*), phenylmethylsulphonyl fluoride (*PMSF*), urea, ethylenediaminetetraacetic acid (*EDTA*): *leftmost column* (*blue*) control (100 % concentration), *middle column* (*red*) 10 mM, *right column* 50 mM. Values presented are the mean ± SD (n=3)

strain BNMIITR and *Hbf. lacisalsi* cannot hydrolyze casein, but casein was hydrolyzed by strain BNMIITR in our experiments. Mannitol and sucrose cannot be utilized by *Hbf. lacisalsi*, whereas strain BNMIITR was observed to utilize both of these as a carbon source.

The thin-layer chromatogram of total lipids from *Halobiforma* sp. strain BNMIITR showed the presence of six different types of lipids, whereas Hezayen et al. (2002) reported the presence of four different types of lipids in *Hbf. haloterrestris*. The polar lipid profile showed the presence of two glycolipids and one phospholipid (Fig. 4). A sulphated triglycosyl diether and a triglycosyl diether were produced as the sole glycolipids in *Hbf. haloterrestris*; these are absent in the majority of neutrophilic and alkaliphilic members of the family *Halobacteriaceae* (Hezayen et al. 2002). No glycolipid was detected from *Hbf. nitratireducens* (Xin et al. 2001). Major cellular fatty acids, based on gas chromatography results (ESM Fig. 3), were $C_{14:0}$, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso and $C_{20:2}$ w6, 9c (ESM Table 1). Fatty acid synthetase has been reported to be sensitive to high salt concentrations and, consequently, fatty acids are not usually a part of archaeal membrane lipids. However, small amounts of fatty acids (C14, C16 and C18) have been detected in the membrane proteins of *Halobacterium salinarum* (Pugh and Kates 1994). Likewise, we found only trace amounts of fatty acids in *Halobiforma* sp. strain BNMIITR. The fatty acid composition of the members of this genus has not been reported previously. Our lipid analysis of strain *Halobiforma* sp. strain BNMIITR showed the presence of six lipids and three polar lipids (two glycolipids and one phospholipid), whereas only four different lipids were reported in *Hbf. haloterrestris* (Hezayen et al. 2002) and two polar lipids were reported from *Hbf. nitratireducens* (Xin et al. 2001). On the basis of all these physiological, biochemical, phylogenetic analyses, we concluded that strain BNMIITR should be treated as a novel bacterium and designated it as *Halobiforma* sp. strain BNMIITR. We also screened strain BNMIITR for the production of many extracellular hydrolytic enzymes and found that it produced an extracellular alkaline protease (ESM Fig. 4). Other enzymes were not detected in plate assays. We then characterized this crude enzyme in subsequent experiments.

Effect of pH and temperature on protease activity

The effect of pH on the protease activity of strain BNMIITR was studied at various pH values at 45 °C (Fig. 5a). The crude enzyme was highly active at pH range 7–12, with optimum activity at pH 9.0. The protease activity of BNMIITR was also examined at different temperatures (30–80 °C). The enzyme was active within a wide range of temperature, with an optimum at 55 °C (Fig. 5b). Protease from *Geomicrobium* sp. EMB2 was stable at pH range 6.0–12.0 and at temperatures up to 60 °C, with an optimum pH and temperature at 10.0 and 50 °C (Karan and Khare 2010). Protease from *Halobacillus karajensis* undergoes thermal activation above 30 °C, with maximum activity between 45 and 55 °C, followed by thermal deactivation above 60 °C (Hamid Reza et al. 2009).

The effect of inhibitors and surfactants on the protease

The effects of different enzyme inhibitors, such as group-specific reagents and chelating agents, on enzyme activity were examined. The enzyme was found to be completely inhibited by the serine protease inhibitor (PMSF 10 mM), indicating the possibility of a serine-type protease. The activity of the crude enzyme was also reduced by the metalloprotease inhibitors EDTA and SDS. However, the activity of the crude enzyme remained unaffected by urea. Minor reductions of activity were observed with Triton X-100, CTAB and dithiothreitol. Interestingly, enzyme activity increased in presence of Tween 20 and Tween 80 (Fig. 6).

Protease is one of the most important industrial enzymes, accounting for about 60 % of total enzyme sales. Proteases incorporated into detergent formulations must exhibit significant activity and stability at high pH and in the presence of detergent compounds, such as surfactants (Haddar et al. 2009). Most of the enzymes which have been recovered from thermophiles are unstable at alkaline pH, whereas enzymes from alkaliphilic bacteria are usually thermolabile (Banerjee et al 1999). Thus, there is a continuous demand for new proteases that are active at high temperatures and pH. The protease isolated in our study showed activity over a broad range of pH and temperatures and good activity in the presence of various surfactants, suggesting that it can be used in the detergent industry.

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