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ORIGINAL ARTICLE

Identification and characterization of a halocin-producing haloarchaeon isolated from Pachpadra salt lake

R. Kaur and S.K. Tiwari

Department of Genetics, Maharshi Dayanand University, Rohtak, Haryana, India

Significance and Impact of the Study: The present study reports a new halocin-producing haloarchaeon. The halocin was stable at higher temperature, salt concentrations and inhibited-related strains of haloarchaea. These findings suggested its application in the safety of salted foods and leather industries to prevent the growth of unwanted haloarchaea.

Keywords
antimicrobial activity, cytocidal, halocin, *Haloferax larsenii* HA4, host-range.

Correspondence
Santosh Kumar Tiwari, Department of Genetics, Maharshi Dayanand University, Rohtak 124001, Haryana, India.
E-mail: santoshgenetics@gmail.com

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Abstract

Haloarchaea are known to produce antimicrobial proteins, halocins which are generally stable at extreme conditions suggesting their potential biotechnological applications. Here, we report a halocin-producing haloarchaeon isolated from salt lake and identified as *Haloferax larsenii* HA4 using partial 16S rDNA sequence and biochemical properties. Whole-cell methanolysate showed ether-linked lipids, which is a characteristic feature of haloarchaea. Strain HA4 was able to grow at pH 6·0–10·0 and 15–30% NaCl. The growth response was normal but antimicrobial activity was detected only during the log-phase. Crude halocin HA4 was active in the pH range of pH 2·0–10·0 with stability up to 100°C. Cell-free supernatant (CFS) was also stable in different organic solvents and detergents tested. However, halocin activity was reduced after treatment with proteinase K suggesting the proteinaceous nature of the active compound. Concentrated CFS showed the presence of several proteins from 6·5–66 kDa but bioassay suggested ~14 kDa protein as halocin. Crude halocin preparation showed cytocidal activity against indicator strain, *H. larsenii* HA10 and inhibited the growth of other related strains such as *H. larsenii* HA3, HA8, HA9 and HA10.

Introduction

Halophilic micro-organisms are found in all three domains of life: *Archaea*, *Bacteria* and *Eucarya* (Oren 2002). However, it has been reported that haloarchaea are most dominant in the extreme hypersaline environments (Hou and Cui 2018). They are phylogenetically separated from eukaryotes and bacteria due to their characteristic features such as replication, transcription and translation machineries. The ether-linkage in lipids of haloarchaea is similar to eukaryotes and cell membrane to few bacteria (DasSarma and DasSarma 2012). All members of this domain contain diether glycerolipids instead of fatty acids ester-linked glycerolipids found in bacteria (Litchfield 2011). They are able to grow at 1–5 mol l^{-1} NaCl and generally found in hypersaline environments such as high salinity lakes and solar salterns (Enache *et al.* 2008; Kumar *et al.* 2016). This is due to adaptation to live at extreme ion concentrations by accumulating potassium chloride in cytoplasm equal to the external NaCl concentration (Amoozegar *et al.* 2017). Consequently, the majority of haloarchaeal proteins execute optimum functions *in vitro* and *in vivo* at 4–5 mol l^{-1} NaCl (Oren 2013; Singh and Singh 2017).

In biological ecosystem, micro-organisms compete for space and nutrients. Therefore, produce proteinaceous antagonists that inhibit the growth of closely related strains (O'Connor and Shand 2002; Mazguene *et al.* 2017). Although, the production of antagonistic substances has been reported from domain Bacteria and Eucarya as bacteriocins and eukaryiocins respectively research in the field of halocins from haloarchaea is still

limited (Kumar *et al.* 2016; Mazguene *et al.* 2017). Haloarchaea produce bacteriocin-like antimicrobial peptides/proteins known as halocins. The production of halocin is dependent on growth conditions of producer strains such as pH, NaCl concentrations and temperature (Pasic *et al.* 2008; Karthikeyan *et al.* 2013). Since halocins are least explored in comparison to bacteriocins of bacteria, characterization of new halocin is of immense importance for developing alternate antibiotics and other possible applications in food sector and therapeutics. In this manuscript, a new isolate of haloarchaea from Pachpadra Salt Lake, Rajasthan, India has been identified as *Haloferax larsenii* HA4. The growth and halocin production was optimized under various conditions and crude halocin was characterized for stability, sensitivity and antimicrobial activity against target strains.

Results and discussion

Haloarchaeon was identified as *H. larsenii* HA4

Strain HA4 cells were stained red indicating Gram-negative and cell morphology was found to be rod-shaped (Fig. 1a). The colonies were able to produce effervescence after addition of H_2O_2, suggesting catalase-positive nature (Fig. 1b). It was found to be resistant to kanamycin, tetracycline, chloramphenicol, gentamicin, and sensitive to novobiocin, erythromycin and taurocholic acid. Approximately 700 bp of 16S rDNA was amplified showing 99% homology with *H. larsenii* JCM 13917 using an online BLAST (NCBI) as shown in Fig. 1c,d. The lipid profile of strain HA4 showed glycerol diether moeities (GDEM) with characteristic Rf (0·20) which was absent in *Lactobacillus plantarum* NRRL B-4496 and *Escherichia coli* ATCC 25922. The bacterial lipid profile showed ester-linked lipid, fatty acid methyl esters (FAME) with Rf value around 0·96 which was absent in strain HA4 (Fig. 2a). The FTIR spectrum of ether moiety showed strong absorption at long-chain groups (2800–3000 cm^{-1}), ether C-O-C groups (1065 cm^{-1}) and no absorption at range of 1730–1750 cm^{-1} related with ester linkage (Fig. 2b). Catalase-positive, sensitive to novobiocin, taurocholic acid and ether linkage in lipids are the characteristic feature of halophilic archaea (Enache *et al.* 2008; Kumar *et al.* 2016) demonstrated by strain HA4.

Halocin activity was detected only in log-phase

The growth response of strain HA4 showed all the phases and stationary phase was recorded up to 120 h. There was increase in pH from pH 7·2–8·8 during microbial growth. The zone of growth inhibition after 24 h was found to be 22 mm and remained stable up to 36 h. Thereafter, there was decrease in the activity and reduced to nil after 60 h (Fig. S1). The loss of activity during stationary phase could be due to proteolytic degradation of active compound(s) (Kumar *et al.* 2016). Production of many halocins has also been reported in early stationary phase of growth (O'Connor and Shand 2002; Pasic *et al.* 2008). However, maximum halocin activity at the end of the exponential growth phase of *Halobacterium salinarum* ETD5 and *H. salinarum* ETD8 was reported by Ghanmi *et al.* (2016).

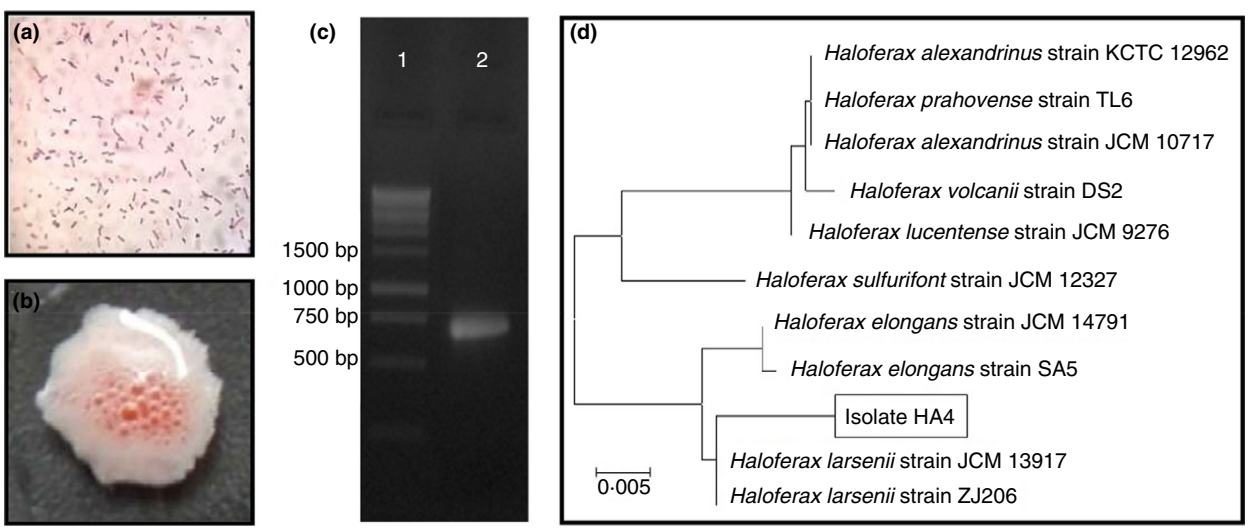

Figure 1 Identification of strain HA4 (a) Cell morphology, (b) catalase test, (c) 16S rDNA (lane 1: ladder, lane 2: PCR product), (d) phylogenetic analysis of strain HA4. [Colour figure can be viewed at wileyonlinelibrary.com]

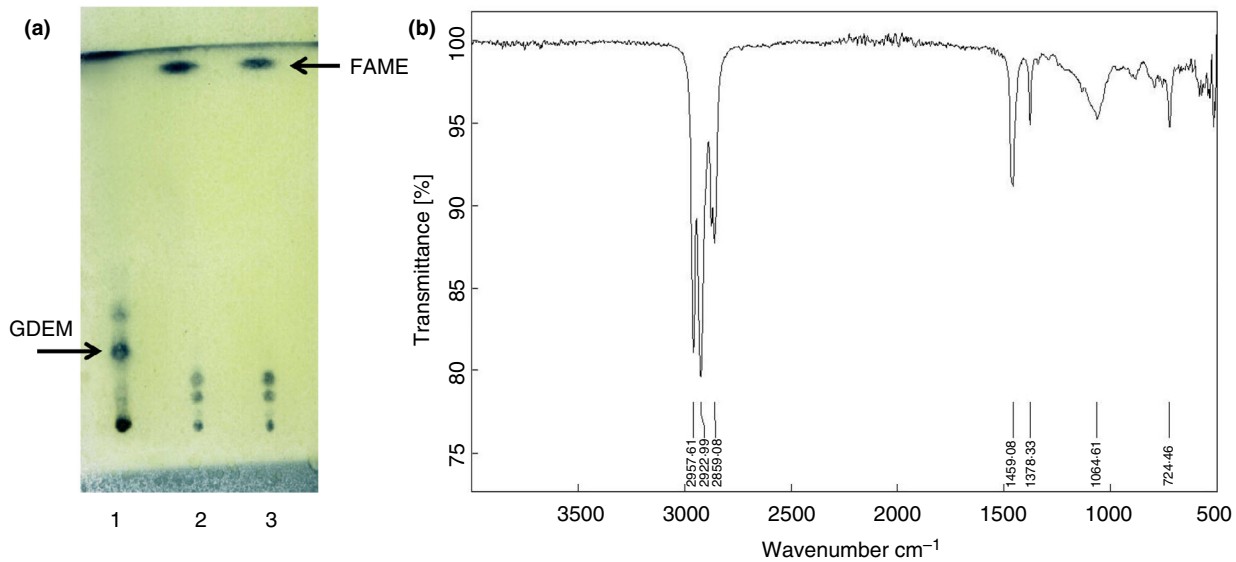

Figure 2 (a) Thin layer chromatography of whole-organism methanolysate of strain HA4 (lane 1) showing the presence of glycerol diether moieties (GDEM) characteristic feature of haloarchaea, whereas same was absent in bacteria, *Lactobacillus plantarum* NRRL B-4496 (lane 2) and *Escherichia coli* ATCC 25922 (lane 3), (b) Fourier Transform Infrared (FTIR) spectrum of purified ether moiety of strain HA4. [Colour figure can be viewed at wileyonlinelibrary.com]

Growth of strain HA4 at different pH, temperature, salt concentrations and carbohydrates

Strain HA4 was not able to grow at or below pH 4·0 and however, grew at pH 6·0–10·0 with optimum growth at pH 7·2. Thereafter, decline in growth was recorded at pH 8·0 (OD_{600} 0·7) and 10·0 (OD_{600} 0·6). It was able to demonstrate antimicrobial activity between pH 6·0 and 10·0 with optimum activity at pH 7·2 (24 mm) (Fig. S2a). Generally, haloarchaea grow optimally at pH 7·0–7·5 as suggested by Pasic *et al.* (2008). In contrast, haloarchaeon Sech7a, was reported to grow optimally at pH 8·0 and highest halocin activity was recorded at neutral pH (Pasic *et al.* 2008). Strain HA4 was not able to grow below 5% NaCl and started growing at 10% NaCl but grew optimally at 15% NaCl (OD_{600} 1·2). Thereafter, growth was declined at higher concentrations of NaCl. However, there was 50% reduction in growth at 30% NaCl in comparison with 15% NaCl but showed 23 mm zone of growth inhibition. At 15–30% NaCl, the antimicrobial activity (~24 mm) was almost similar while decline in the growth was observed (Fig. S2b). The optimal growth and production of halocin by *H. salinarum* ETD8 was also reported at 15% NaCl, whereas *H. salinarum* ETD5 and *Haloterrigena thermotolerans* SS1R12 were reported to grow optimally at 20% NaCl (Ghanmi *et al.* 2016). Strain HA4 grew optimally at 42°C with OD_{600} 1·47 and demonstrated 23 mm zone of growth inhibition (Fig. S2c). Similarly, optimum halocin activity by *Natrinema* sp. was also recorded at 42°C, whereas optimum growth and activity of haloarchaeon Sech7a was reported at 45°C (Pasic *et al.* 2008; Karthikeyan *et al.* 2013). Strain HA4 utilized fructose, glucose, lactose, maltose, mannitol, sucrose and xylose with lesser extent. It grew up to OD_{600} ~0·4–0·6 but was not able to utilise arabinose and mannose (OD_{600} ~0·1–0·2). In positive control (AS-169 medium), the growth was OD_{600} ~1·2 but in negative control (AS-169 medium without casein and yeast extract), growth (OD_{600} ~0·1) was recorded. Antimicrobial activity was recorded only in medium containing sucrose and xylose (~10 mm) but significant activity (23 mm) recorded in positive control (Fig. S2d).

Effect of different pH, temperature, organic solvents and proteases on the stability of halocin

Crude halocin HA4 retained 100% antimicrobial activity (20 mm) after treatment at 60, 80 and 100°C, whereas activity (16 mm) was reduced by 20% after autoclaving at 121°C and 15 psi. The decrease in activity of cell-free supernatant (CFS) of *H. salinarum* ETD5 and *H. salinarum* ETD8 was reported after heating at 80°C for 15 min (Ghanmi *et al.* 2016). The CFS of *Haloferax volcanii* and *Haloarcula japonica* were reported to be thermo-stable at 100°C for 30 min and further heating resulted in complete loss of activity (Salgaonkar *et al.* 2012). Crude halocin HA4 was found to be active at pH 2·0–10·0 and showed 20 mm zone of growth inhibition.

Similar findings have also been reported that change in pH did not affect the antimicrobial activity of CFS of *H. salinarum* ETD5, *H. salinarum* ETD8 and *H. thermotolerans* SS1R12 and found to be stable in acidic and alkaline conditions (Ghanmi *et al.* 2016). Complete activity (20 mm) was retained after treatment with organic solvents, surfactants and detergents tested suggesting robust nature of the halocin HA4. The activity of halocin HalS8, HalR1 and HA1 was also unaffected on treating with organic solvents (O'Connor and Shand 2002; Kumar *et al.* 2016). The activity of crude halocin HA4 was stable after treatment with trypsin and papain but there was complete loss in activity after treatment with proteinase K (Table 1) suggesting proteinaceous nature of the antimicrobial compound present in CFS of *H. larsenii* HA4. Several halocins, for example, halocin HalS8, HalR1 and HA1 have also been reported to be sensitive to proteinase K (O'Connor and Shand 2002; Kumar *et al.* 2016).

Bioassay and molecular weight determination of halocin

Tricine SDS-PAGE of crude halocin showed multiple protein bands but the bioassay of gel showed zone of growth inhibition corresponding to ~14 kDa protein band confirming proteinaceous nature of antimicrobial compound present in CFS (Fig. 3a). There was significant drop in the viable cell count up to $\log_{10}$ ~4 and 6 in comparison to control ($\log_{10}$ ~12) after treatment with 0·5 and 1 ml of crude halocin respectively. There was gradual decrease in viable cell count of indicator strain and complete loss in the viability was recorded after 48 and 72 h in the set treated with 1 and 0·5 ml respectively (Fig. 3b) indicated cytocidal nature of halocin HA4. It was found to be active only against other haloarchaea strains such as *H. larsenii* HA3, HA8, HA9 and HA10 but did not inhibit the growth of bacterial and fungal strains tested (Table 1). The above properties of halocin HA4 such as thermo-pH stability, robust nature and inhibition of haloarchaea strains suggested its applications in preservation of salted foods and leather industry to prevent haloarchaea growth (Charlesworth and Burns 2015; Kumar *et al.* 2016).

Table 1 Biochemical properties of crude halocin HA4

S. no.	Treatments	Antimicrobial activity Zone of growth inhibition (mm)
	Control (Untreated)	20·6 ± 1·0*
1	Temperature	
	60°C (15 min)	20·5 ± 1·0
	80°C (15 min)	20·6 ± 0·7
	100°C	20·3 ± 0·5
	121°C (15 min, 15 psi)	16·8 ± 1·0
2	pH	
	2·0	19·3 ± 1·0
	4·0	19·3 ± 0·7
	6·0	20·3 ± 0·5
	7·0	20·6 ± 1·0
	8·0	20·5 ± 0·8
	10·0	20·3 ± 1·0
3	Organic compounds	
	Methanol	20·3 ± 1·0
	Ethanol	20·3 ± 0·5
	Acetone	19·5 ± 1·0
	Isopropanol	19·5 ± 0·5
	Tween-80	20·5 ± 1·5
	Acetonitrile	20·6 ± 0·8
	sodium dodecyl sulphate	21·5 ± 1·5
	Triton X-100	20·3 ± 0·7
	Urea	20·5 ± 0·5
4	Proteolytic enzymes	
	Trypsin	20·6 ± 1·0
	Papain	20·3 ± 0·5
	Proteinase K	0
5	Host-range	
	Micrococcus luteus MTCC106, *Enterobacter cloaceae* NRRL B-14298, *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-4525, *L. curvatus* subsp. *curvatus* NRRL B-4562, *L. plantarum* NRRL B-4496, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 29905, *Aspergillus fumigatus* ITCC 4517, *Candida* sp.	0
	H. larsenii HA10	22·8 ± 0·28
	H. larsenii HA3	17·0 ± 1·0
	H. larsenii HA8	20·0 ± 1·5
	H. larsenii HA9	17·0 ± 0·5

*Values are mean of three independent experiments and shown along with standard deviation (±SD).

Materials and methods

Microbial strains, culture media and growth conditions

Haloferax larsenii HA4 and indicator strain, *H. larsenii* HA10 were previously isolated from Pachpadra Salt Lake, Rajasthan. For routine work, these isolates were grown and maintained in AS-169 medium (g l^{-1} casein 10, magnesium sulphate 20, tri-sodium citrate 3, potassium chloride 2, yeast extract 1, ferrous sulphate 0·023, sodium chloride 150, pH 7·2) (Kaur and Tiwari 2018) with initial inoculum ~10^6 CFU per ml at 42°C and 200 rev min^{-1} in an incubator shaker. Reference lactic acid bacteria were obtained from ARS Culture Collection (NRRL), USA. Pathogenic strains were obtained from Post Graduate Institute of Medical Sciences (PGIMS), Rohtak, Haryana. Other LAB and haloarchaea strains were available in our laboratory culture collection as mentioned in Table 1.

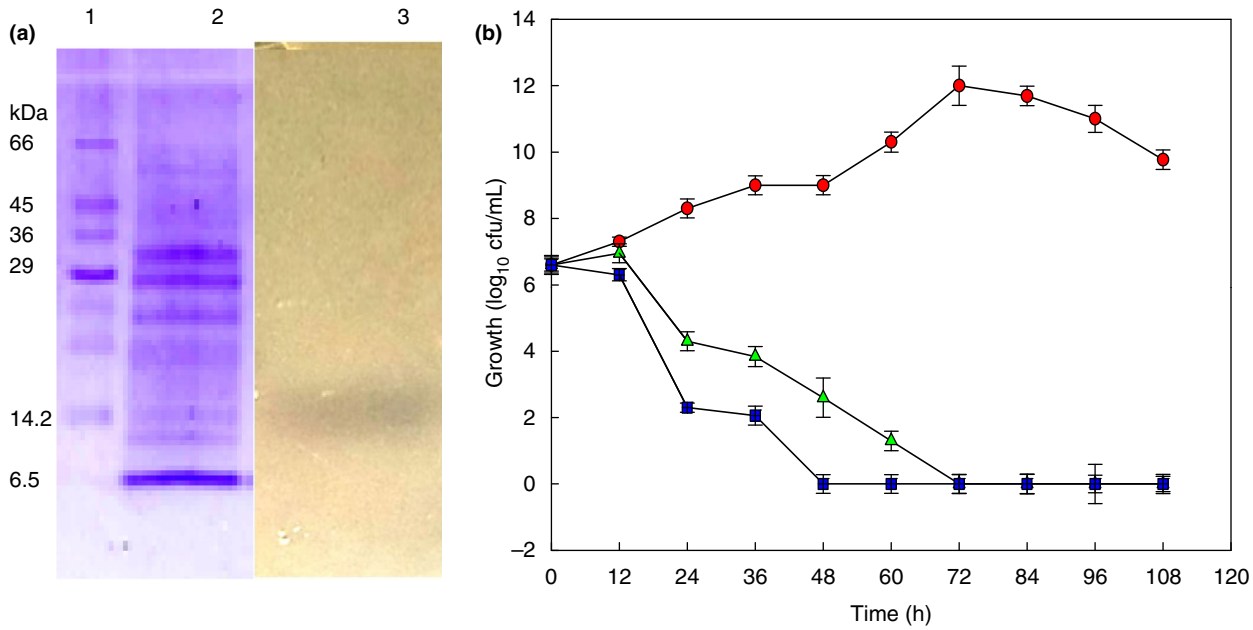

Figure 3 (a) Tricine SDS-PAGE (lane 1: molecular weight marker and lane 2: crude halocin) and bioassay of crude halocin (lane 3) showed zone of growth inhibition against indicator strain *Haloferax larsenii* HA10 corresponding to ~14 kDa (b) Effect of crude halocin HA4 on growth of indicator strain, *H. larsenii* HA10. (–●–) Control (Untreated); (–▲–) Treated (0·5 ml) and (–■–) Treated (1 ml). Y-axis: Growth ($\log_{10}$ cfu ml^{-1}). [Colour figure can be viewed at wileyonlinelibrary.com]

LAB strains were grown in MRS medium, pathogenic strains in Nutrient Broth, *E. coli* in Luria Bertani and fungi in Potato Dextrose Agar (PDA) medium at 37°C. The media components, enzymes and chemicals were procured from Sisco Research Laboratories (Mumbai, India), HiMedia (Mumbai, India) and Sigma-Aldrich (St. Louis, USA).

Identification of strain HA4

Gram-staining, catalase test and sensitivity to different antibiotics and taurocholic acid were performed as described by Kumar *et al.* (2016). *Lactobacillus plantarum* NRRL B-4496 was used as negative control and *E. coli* ATCC 25922 as positive control. For lipid detection, freshly grown culture of strain HA4 was centrifuged. The cell pellet was lyophilized (Macflow, New Delhi, India) and 100 mg of it was mixed with methanol, toluene and concentrate H_2SO_4 (3 : 3 : 0·1) followed by incubation at 50°C for 18 h. After incubation, *n*-hexane (1·5 ml) was added in the mixture and vortexed. The upper layer containing lipids was collected in fresh tube and spotted on silica gel $60F_{254}$ aluminium-backed TLC plate (Merck, Mumbai, India) and run using mobile phase consisted of petroleum ether and diethyl ether (85 : 15). After 10 cm run, plate was air dried and sprayed with 10% dodecamolybdophosphoric acid prepared in absolute ethyl alcohol. Plate was heated at 150°C for 15 min and observed for blue spots. The Rf value was calculated using template. For further confirmation, the blue spot was scratched and resuspended in minimum volume of *n*-hexane and infrared absorbance spectrum was acquired using a Fourier Transform Infrared (FTIR) spectrophotometer (Bruker, Ettlingen, Germany) on diamond-attenuated total reflectance (ATR) accessory. Opus software was used for spectra acquisition.

The species level identification of strain HA4 was performed using 16S rDNA amplification and sequencing. Genomic DNA was isolated using HiPurA^{TM} Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia, Mumbai, India). Forward primer: 5′ ATTCCGGTTGATCCTGCCGG 3′ and reverse primer: 5′ AGCTACGGACGCTTTAGGC 3′ from conserved region of 16S rDNA sequence were used for polymerase chain reaction (Almeida-Dalmet *et al.* 2015). The PCR reaction was performed using 2 μmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs, 2 μmol l^{-1} of each primer, 0·1 U Taq polymerase, GoTaq buffer (1×), 50 ng of template DNA and sterile ddH_2O (final volume of 25 μl). Initial denaturation and cycle denaturation were consisting of 94°C for 2 min and 30 s respectively. The annealing temperature was 55°C for 1 min, extension and final extension at 72°C for 1 and 5 min respectively. The amplified PCR product was analysed using 1·2% agarose gel electrophoresis and

sequenced from University of Delhi South Campus, New Delhi. The sequence was analysed using online Basic Local Alignment Search Tool (BLAST) in NCBI. Top 10 sequences were used for phylogenetic analysis using MEGA 6.0. The 16S rDNA sequence was submitted to GenBank, NCBI with accession number KC608756.

Growth response, pH change and antimicrobial activity

Strain HA4 was inoculated (~10^6 CFU per ml) in AS-169 medium and grown at 42°C and 200 rev min^{-1}. The growth (OD_{600}), pH and antimicrobial activity in terms of zone of growth inhibition were monitored at 12 h intervals up to 120 h. The grown culture was centrifuged at 8000***g*** for 15 min at 4°C (Sigma, Osterodeam Harz, Germany). After centrifugation, CFS was collected and filtered through 0·2 μm syringe filter (Axiva, Sonipat, India) and considered as crude halocin. The antimicrobial activity in CFS was determined in terms of zone of growth inhibition using agar well diffusion assay (AWDA) as described previously (Kaur and Tiwari 2016).

Growth and halocin production under different conditions

Strain HA4 was grown in AS-169 medium at pH 2·0–10·0, NaCl concentrations (0–30%) and temperatures (30–50°C) with initial cells ~10^6 CFU per ml and incubated at 42°C, 200 rev min^{-1} for 36 h. The effect of different carbohydrates on growth and halocin production was also observed. For this, filter-sterilized arabinose, fructose, glucose, mannose, lactose, maltose, mannitol, sucrose and xylose (1%) were added in different sets of AS-169 medium devoid of casein and yeast extract. Complete AS-169 medium and AS-169 medium devoid of casein and yeast extract were used as positive and negative control respectively. Growth (OD_{600}) and antimicrobial activity was recorded as mentioned earlier.

Stability of crude halocin

The crude halocin HA4 was treated at 60°C and 80°C in a water bath for 15 min, flame-heated up to boiling (100°C) and autoclaved (121°C, 15 psi for 15 min). To monitor the effect of different pH on the activity of crude halocin, it was adjusted to pH 2·0–10·0 with 1 mol l^{-1} NaOH/HCl and incubated at 37°C for 2 h. The crude halocin HA4 was mixed with methanol, ethanol, acetone, isopropanol, Tween-80, acetonitrile, sodium dodecyl sulphate (SDS), Triton X-100 and urea (1% v/v or w/v) and incubated at 37°C for 2 h. The CFS was also treated with proteinase K, trypsin and papain (1 mg ml^{-1}) for 2 h at 37°C followed by heating at 100°C for 5 min to terminate enzyme activity. Residual antimicrobial activity was measured using AWDA and compared with untreated control.

Tricine SDS-PAGE and bioassay

To know the molecular weight of antimicrobial compound in crude preparation, tricine SDS-PAGE and bioassay was performed as described previously by Kaur and Tiwari (2018). The CFS of isolate HA4 was concentrated using 3 kDa cut-off vivaspin (GE Healthcare, Danderyd, Sweden) and treated with loading dye (5×) at 37°C for 15 min. After incubation, sample was centrifuged at 8000***g*** for 5 min and loaded in duplicate along with molecular weight marker (Sigma, St. Louis, USA). One half of the gel was cut and used for staining while the other half was processed for bioassay. For bioassay, the other half of gel was washed with sterile distilled water with frequent changes. The gel was placed on AS-169 agar medium and overlaid with soft agar seeded with ~10^6 cells of freshly grown indicator strain. The plate was incubated at 42°C for 48 h and zone of growth inhibition was observed as described by Kaur and Tiwari (2018).

Cell viability and host-range

Crude halocin (0·5 and 1 ml) was added in AS-169 medium (final volume 20 ml) containing freshly grown cells (~10^6) of indicator strain, *H. larsenii* HA10 and incubated at specified growth conditions for 5 days. The control set was grown without halocin. Samples were withdrawn at regular interval of 12 h and viability of indicator strain was monitored in terms of CFU per ml. The antimicrobial activity of crude halocin was tested against bacteria, fungi and haloarchaea strains using AWDA as mention in Table 1.

Statistical analysis

The experiments were repeated three times and results are mentioned as mean of three experiments with standard deviation (mean ± SD). The level of statistical significance was estimated as $P \leq 0{\cdot}05$. SigmaPlot 11.0 software was used to plot graphs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Growth (●), pH change (▲) and antimicrobial activity (■) at different time intervals of growth response of strain HA4.

Figure S2 Growth (□) and antimicrobial activity (■) of strain HA4 at (a) different pH, (b) NaCl concentrations, (c) temperatures and in the presence of different carbohydrates (d) (C1-arbinose, C2-fructose, C3-glucose, C4-mannose, C5-lactose, C6-maltose, C7-mannitol, C8-sucrose, C9-xylose, without carbohydrate (WC) as negative control and AS-169 medium as positive control.