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Antibacterial Activity of Biosurfactant Produced from *Haloferax chudinovii* HB1RANIA

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ABSTRACT

Multidrug resistant organisms (MDRO) raised as a big issue world-wide, and research are in progress to get a new alternative to the old drugs. Bioactivities of secondary metabolites from halophilic archaeon considered the least studied among extremophiles especially antibacterial activity. Biosurfactants (BS) were presented as the secondary metabolites promising bioactive molecule substitutes for several previous antibiotic and chemical ones.Haloferax is a halophilic archaeon thatcan grow, thrive in salinity ranges (0.5-25%), and produce some secondary metabolites to inhabit aharsh environment.In this work, we screened the ability of Haloferax chudinovii HB1RANIA isolated from the salt work saltern of Mulund to produce BS with antibacterial activity properties. BS production was determined in a modified mineral salt medium (MSM) supplemented with 5% waste engine oil, 5 % NaCl, and 1% glucose. BS derived from strain HB1RANIA emulsified 32.14% of soybean oil, has reduced surface tension of pure water to 57.24mN/m and showed good oil displacement and hemolysis (10mm) activities. Antibacterial activity of crude BS onthe growth of clinical pathogenic bacteria has carried out on 96 wells microtiter plateand showngrowth inhibition % on Proteus mirabilis (44.1%), Staphylococcus aureus (45.98%), Enterococcus sp (44.13%), and Escherichia coli (4.784%). The study highlighted the significance ofBSderived from strain HB1RANIAto be considered as an alternative for some inefficient antibacterial drugs.

Keywords: *biosurfactants; antibacterial; Haloferax, archaea*
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INTRODUCTION

Living microorganisms (bacteria, yeasts, and fungus) produce amphiphilic chemicals known as biosurfactants,to lower the surface and interfacial tension of immiscible phases [1],[2], [3]. It is preferred over synthetic surfactants due to less toxicity, lower critical micellar concentration, higher foaming capacity, more active at extreme temperatures, pH, and salinity, safe, and biodegradable[4], [5].Different industry fields including, food, agriculture, and pharmaceuticals, were widely usedBS[6],[7]. BS demonstrated a variety of bioactive characteristics, including antioxidant, anti-inflammatory, antibacterial, anti-adhesive, and anti-biofilm activities[17], [18].Antibiotic-resistant organisms are currently becoming more prevalent, especially in bacteria classified as MDRO[8].Since there is a daily and global need for novel antibiotics to combat MDRO, BS were promoted as bioactive molecules with antibacterial activity [9], [10].

The main classification criteria for BS are their molecular weight, chemical structure, microbial origin, and extracellular or cell wall attaching [11]. Glycolipids and lipopeptides areLow molecular weight BS, which are efficient in surface and interfacial tension reduction,andpolymeric compounds, such as proteins, polysaccharides, or mixed forms of lipoproteins or lipopolysaccharides are high molecular weight BS, which may adhere to a variety of surfaces and function as bioemulsifiers, are divided based on molecular weight[3], [12], [13]. Both aquatic ecosystem and terrestrial ecosystem, as well as ecosystems with severe pH, temperature, or salinity, are inhabited by microorganisms that produceBS[14].

The majority of microorganisms that produceBS are bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Rhodococcus* sp., *Acinetobacter* sp., *Enterobacter* sp., *Halomonas* sp., and *Arthrobacter* sp. are some of the bacteria that have been studied the most in scientific investigations [15], [16]. Additionally, several probiotic bacteria, including some species of *Lactobacillus*, *Lactococcus lactis*, *Streptococcus thermophilus*, and *Propioni bacterium freudenreichii*, canproduce BS[16]. BS from lactic acid bacteria demonstrated dose-dependent antibacterial and antibiofilm effects against methicillin-resistant and sensitive staphylococcal isolates, with changes in cell surface integrity acting as evidence of cell death[19].Sophorolipids have antimicrobial properties between the exponential and stationary phases of

growth, and rhamnolipids exhibit decreased growth during the exponential phase, suggesting that they may alter cell division[20],[21].At salt concentrations above 150–200 (w/v), halophilic archaea are the predominate microorganisms andone of the biggest groupings in the domain Archaea is the class Halobacteria [22], [23].This study aimed to screen the ability of halophilic *archaeonHaloferax chudinovii* HB1RANIA to produce biosurfactant and determine the antibacterial properties of crude biosurfactant against a group of pathogenic Gram-positive as well as Gram-negative bacteria.

MATERIALS AND METHODS

All chemicals and media were purchased from Himedia Laboratories, India, andclinical pathogenic bacterial cultures were collected from Shankarao Chavan government medical college, Nanded, MS, India.

Sample collection and Bacterial strain isolation

Strain HB1RANIA was isolated fromthesalt sample which was collected in a clean polythene bag from Jamasp salt work saltern, Mulund, Mumbai. Fiveg of salt sample was added into 50ml of brain heart infusion broth (BHIB) media, incubated at 37℃, 120rpm for 14 days. Ten-fold serial dilutions till 10^{-3}were prepared from inoculated BHIB and 100µl was transferred and spread on nutrient agar plates with 5 % NaCl concentration.

Bacterial strain identification

On the NA agar plate (5% NaCl), HB1RANIA's morphological properties were examined. In a PE 9700 thermal cycler, the 16S rRNA gene was amplified by PCR using universal oligonucleotide primers hybridizing at positions 8–27 and 1488–1511 in respect to the *E. coli* 16S rRNA numbering (Perkin Elmer, USA). Purified PCR products were sequenced and analyzed using kit from Applied Biosystems, Inc. California. The produced sequences were examined for closed homology using the BLASTn algorithm and related sequences for the isolates were retrieved from the NCBI database and aligned using the CLUSTAL X2 multiple sequence alignment program. The Neighbor Joining Method analysis was then used to determine the phylogenetic evolutionary history[24].MEGA 4.0 was used to carry out the phylogenetic analyses and to create the phylogenetic trees [25].

Biosurfactant production

Strain HB1RANIAwas cultivated on 25ml modified minimal salt media (MSM) with the following composition (g/l): $NaNO_3$ 0.25; $MgSO_4$ 0.04; NaCl 50; Kcl 0.1; $Cacl_2$ 0.001; NaH_2PO_4 4.4; andglucose1% after autoclaving 1ml sterile trace mineral;and 5% waste engine oil were added and PH 6.5. The inoculated flasks were incubated at 37℃, 120rpm for 14 days. The culture was separated from the broth by centrifuging the broth at 10,000 rpm, 4℃ for 10 min. the supernatant was used for biosurfactant production screening.

Biosurfactant production screening

Hemolysis test

A 5% blood-supplemented blood agar base with an overnight-grown active HB1RANIA strain was streaked on it, and it was then incubated at 37°C for 24-48 hours. The incubated plates were examined for positive hemolytic activity in a clean zone surrounding the streaked area.

Oil displacement

A Petri dish containing 40 ml of distilled water (DW) was placed on top of an aliquot of 1000 µl of waste engine oil. 10 µl of the crude biosurfactant, which had been prepared through centrifugation, was then dumped directly onto the center of the oil surface. A positive control and a blank were sodium dodecyl sulphate (SDS)1% and MSMwithout inoculum, respectively. The diameter of the oil displace zone on the oil surface was observed and measured As a sign of the existence of biosurfactants [26].

Emulsification index (E24 index)

1 ml crude biosurfactant which was obtained by centrifugation was mixed with 1 ml of soybeans oil in test tubes and mixed vigorously for 2 min. Tubes are allowed to stand for 24h at 37℃. SDS 1% and MSM without inoculum were used as a positive control and blank respectively. The E24 index was calculated using the following equation:

$$\text{E24 index} = \frac{Emulsion\ hight}{Total\ liquid\ hight} * 100$$

Surface tension reduction measurement

Traub's stalagnometer was used to measure surface tension using the drop weight method. To measure the surface tension (σ) of water (30℃) in presence of crude BS and absence of crude BS. The weight of the counted drops ofwater in absence of crude BS (mH2O) and in presence of crude BS (m)was determinedin triplicate. 1% SDS and MSM without inoculum were used as a positive control and blank respectively. Surface tension was calculated as the following formula:

$$\sigma = \sigma H2O \times m \div mH2O$$

where, σ – surface tension, σH2O – water surface tension (71.2 mN/m at 30°C), m –the mass of water in presence of BS, and mH2O – the mass of water in absence of BS[27].

Antibacterial activity of biosurfactant

A modified method was followed to screen the antibacterial activity of biosurfactant was carried out using 96 wells microtiter plate by taking OD_{600nm}using microplate reader (Bio-Rad) of 0.5 MacFarland standard dilution growth of activated clinical pathogenic bacteria(*Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus* sp, and *Escherichia coli)* in Mueller Hinton broth(MH) after incubating with crude biosurfactant(A treatment) for 24h at 37°C and compared with growth on MH broth without biosurfactant(A control)[10], [28]. The antibacterial activity of biosurfactant was calculated using the following equation:

$$\%\text{Bacterial growth inhibition} = \frac{A\ (Control)\ -\ A\ (Treatmen)}{A\ (Control)} * 100$$

RESULTS AND DISCUSSION

Bacterial strain identification

Based on morphological characterization, the isolate HB1RANIA was a Gram-negative, rod-shaped and motile bacterium. Colonies' characteristics are polymorphic, cream, opaque, and irregular on the NA agar plate. It grows well over a wide range of salt concentrations (0.5 to 25%). Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the isolate HB1RANIA (accession number in GenBank: LC730651) falls within the branch encompassing the members of the genus *Haloferax* (Figure 1). Thus, it was named as *Haloferaxchudinovii* HB1RANIA.

Biosurfactant production screening

One of the most fundamental qualitative BS screening assays is the indirect measurement of BS production by taking into consideration the hemolytic potential of the target bacteria. On newly made blood agar, HB1RANIA demonstrated the mild or partial lysis (alpha) of erythrocytes with a diameter (of 10mm) (Table 1). while the oil displacement test is more sensitive to the presence or absence of surface-active compounds. The oil displacement ability of the crude biosurfactant fromHB1RANIA strainwas observed in comparison with the blank (MSM) and positive control (SDS1%)which showed oil displacement area capability witha diameter of 0.13 cm, no oil clearance,andan oil displacement of 72.35 cm, respectively (Table 1). A quick approach to assess the emulsification potential and potency of surface-active compounds is the E24 index. HB1RANIA strain showed a considerably higher E24 index when compared to the blank (MSM) (table 1). E24 index showed 32.14% of soybean oil in comparison with SDS1% which was shown as 64.29%[29].

Surface tension reduction measurement was lookedthe main test to screen the potentiality of biosurfactant production. Strain HB1RANIA can reduce the surface tension of DW at 30°Cfrom 71.2mN/m to 57.24mN/mand compared the result withSDS1% which was shown 49.12 mN/m (Table 1.).The same results were found by Barakat et al (2017) by two isolates *B. amyloliquefaciens* strain SH20 and *B. thuringiensis* strain SH24, which reduced surface tension to a value of 57.7 ± 2.885 mN/m [30].

Table 1. Emulsification index (E−24), oil displacement, and hemolytic capabilities of HB1RANIA.

	Hemolytic activity (mm)	Oil displacement (Cm)	EI_{24} (Cm)	Surface Tension at 30°C(mN/m)
HB1RANIA (BS)	10	0.13	32.14	57.24
Blank (MSM)	0.00	0.008	28.57	58.07
DW	0.00	0.00	0.00	71.2
SDS 1%	0.00	72.35	64.29	49.12

Antibacterial activity of biosurfactant

Table 2. displays the effects of the crude biosurfactant produced by HB1RANIA against clinical pathogenic bacteria (*Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus* sp, and *Escherichia coli).* The crude biosurfactant inhibited the growth of *Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus* sp, and *Escherichia coli* with 44.1%, 45.98%, 44.13%, and 4.784%, respectively. Biosurfactant demonstrated antibacterial activity by making the cell membrane more permeable, which increased protein release and caused leaking of the absorbing intracellular contents[21]. Many studies have reported using biosurfactants as antibacterial agents, one study showed an active inhibitory effect (> 10 mm) on all of the pathogens that were tested against selected Gram-negative and positive bacteria (*Salmonella typhimurium, Pseudomonas aeruginosa, E. coli, Micrococcus luteus, Staphylococcus aureus, and Bacillus cereus*), and other reported partially purified biosurfactant produced from halophilic

Halobacilluskarajiensis MB588 shown 94% inhibition towards *Klebsiella pneumoniae* ATCC 4617. *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and *Enterococcus faecalis* JH22 [26], [28] .

Table 2. Antimicrobial activity of biosurfactant produced by *Haloferax chudinovii* HB1RANIA.

Clinical pathogenic bacteria	Bacterial inhibition %
Proteus mirabilis	44.1
Staphylococcus aureus	45.98
Enterococcus sp	44.13
Escherichia coli	4.784

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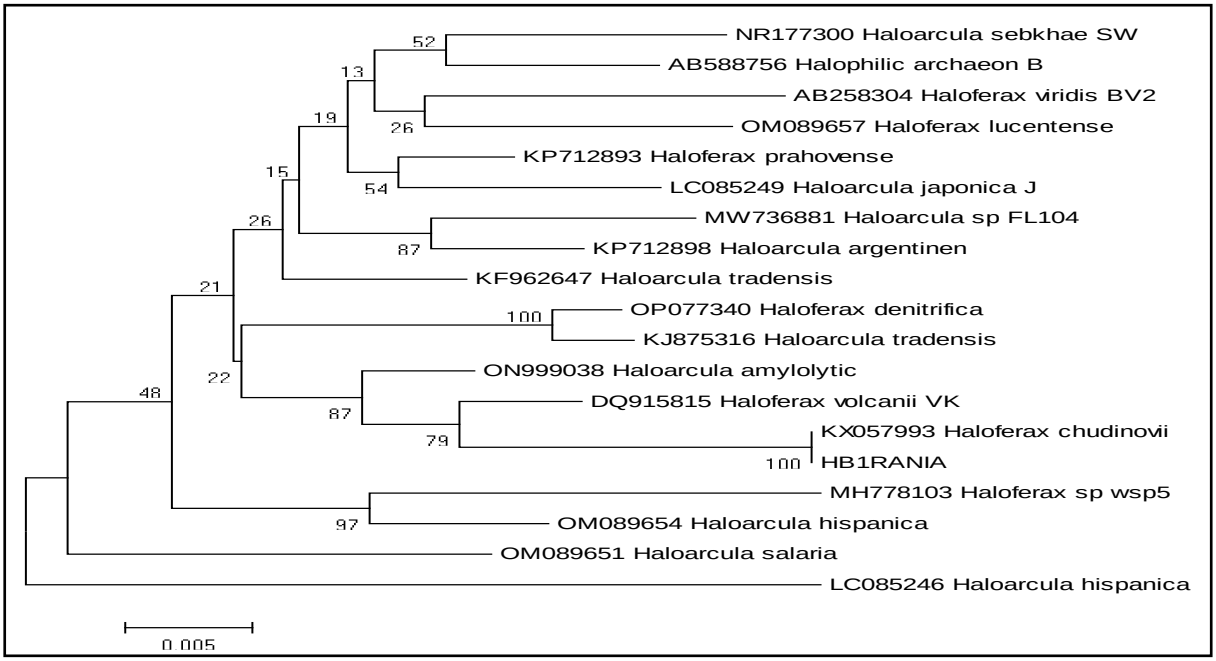

Figure 1. Phylogenetic tree based on 16S rRNA sequences illustrating the relations between the isolate HB1RANIA and other Haloferax species. After the strain identification, the accession numbers of the sequences utilized in this investigation are displayed in parentheses. Only values larger than 50% are displayed in the node numbers, which are percentage bootstrap values based on 1,000 replications. Per nucleotide location, there are 0.005 substitutions

CONCLUSION

Halophilic archaeon *Haloferax chudinovii* HB1RANIA has the potential to producebiosurfactant.Gram-positive and Gram-negative pathogenic bacteria were both sensitive to crudebiosurfactant from the HB1RANIA strain.Our work opens up the possibility for the futuristic use of this abundant biosurfactant as an antimicrobial agent in both food and pharmaceutical industries to combat the increasedthreat of MDRO by taking into account the complex mode of inhibitory action of biosurfactant.

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