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Production and characterization of biosurfactant from *Halomonas* sp. BRI3

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In this report, the halotolerant bacterium *Halomonas* sp. BRI3 was studied for its biosurfactant (BS) producing ability. Effect of carbon source, nitrogen source, temperature, inoculum concentration and incubation on the production of BS was studied and maximum production (14 g/L) was obtained in modified medium containing 1% glucose, yeast extract and ammonium chloride (0.25% each) with 5% inoculum concentration at 30°C after 48 h, which was 2.8 fold higher as compared to original medium (5 g/L). Highest emulsification index (72%) of crude BS was obtained with kerosene followed by *n*-hexane > crude oil > *n*-heptane > soybean oil > hexadecane > mustard oil > olive oil > sesame oil. Our hydrocarbon degradation experiments using crude oil and soybean oil revealed 40% decrease in crude oil and 60% decrease in soybean oil concentration after 50 days in presence of glucose, whereas, it was 20% and 50% in the absence of glucose, respectively. Based on TLC and FTIR analysis, the BS is chemically a glycolipo protein, demonstrated an significant antimicrobial and antiadhesive activity. We observed significant stability of BS over wide range of temperature (40 to 120°C) and pH (5.0 to 11.0), suggesting its potential for application in food, pharmaceutical and cosmetics industries.

Keywords: Antiadhesive, Biosurfactant, Emulsification, Glycolipoprotein, Halotolerant, Rhamnolipids

Biosurfactant (BS) is attracting attention due to advantages over chemical surfactants due to low toxicity and biodegradability[1]. Rhamnolipids released by *Pseudsomonas aeruginosa*[2], sophorolipids from *Candida* species[3], surfactin and iturin produced by *Bacillus subtilis* strains[4] *etc*. are some of the previous examples of microbial biosurfactants. Earlier, we have reported BS form *Oceanobacillus* sp. BRI10 isolated from Antarctic water sample[5], which showed stability at high temperature and pH and also antimicrobial activity. On the contrary, few species producing BS have been reported in the genus *Halomonas*. Microorganisms belonging to this genus are found in marine habitat and are halophilic or halotolerant. BS producing psychrotrophic *Halomonas* sp. has been reported by Pepi *et al*. (2005)[6]. The authors have documented isolation, characterization and chemical analysis of the emulsifier. We have isolated *Halomonas* sp. BRI3 from oceanic water sample. The present work deals with production and characterization of BS from BRI3.

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Suppl. data available on respective page of NOPR

Materials and Methods

Chemicals

All the media components and chemicals were purchased from Hi-Media and Merck (Mumbai, India) and were of Analytical Reagent (AR) grade.

Organism

Halomonas sp. BRI3 was isolated from oceanic water sample near Antarctic region (latitude S56°32'03.6", longitude E63°05'57.9") using marine salt medium (MSM) at 15°C. It was characterized morphologically, physiologically, biochemically[7] and also tested for (i) hemolytic activity[8], (ii) oil displacement test[9], and (iii) drop-collapse test[10]. The isolate was identified by 16S rRNA gene sequencing by the procedure as described previously. The sequence contigs were generated using Chromas Pro and then analyzed using online databases *viz*. NCBI-BLAST to find the closest match of the contiguous sequence. Phylogenetic analysis was carried out using MEGA software package, version 5.2.

BS production

Halomonas sp. BRI3 was initially examined for its BS producing ability by using β-hemolysis test and oil displacement test[5]. BRI3 was cultivated in BS

producing medium (BPM) {composition (g/L): KH_2PO_4 (2.7), K_2HPO_4 (13.1), NaCl (5.0), KNO_3 (1.0), $MgSO_4$ (1.0), NH_4Cl (1.0), yeast extract (0.5), pH 7.0} and incubated at 30°C for 48 h at 120 rpm[11]. This was used as inoculum (0.5 McFarland standard) at 5% (*v/v*) level for further experiments. All the experiments were performed in 250 mL flasks containing 50 mL medium by varying one parameter at a time and keeping other parameters constant. BS production was measured by calculating an emulsification index (E24) as described below after each experiment. To examine effect of carbon source, glucose, sucrose, fructose, *n*-hexadecane and soybean oil at the concentration of 1% (*w/v*) were added individually to the media and BPM was used as control. BPM with best carbon source was used to check effect of nitrogen source. For this, BPM was used without KNO_3, NH_4Cl and Yeast extract (YE). This was labeled as BPM-N. In this medium, (i) KNO_3, (ii) NH_4Cl, (iii) YE, (iv) KNO_3 + YE (1:1), (v) NH_4Cl + YE (1:1), (vi) KNO_3 + NH_4Cl (1:1), were added individually at 0.12% concentration. The concentration of carbon and nitrogen sources was individually varied to select the one yielding maximum BS. This medium was henceforth labeled as modified BPM (MBPM). Following media modification, effect of temperature (10 to 40°C), inoculum concentration (1 to 6%) and fermentation period (24 to 96 h) on BS production was examined.

Emulsification activity (E24%)

E24% of BS was estimated by adding 2 mL of kerosene to the same amount of cell free culture broth (CFCB). The mixture was vortexed for 2 min at high speed and allowed to stand for 24 h. The E24 index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm)[12].

$$E24 = \text{Height of emulsion formed}/\text{Height of solution} \times 100 \quad \ldots (1)$$

Emulsification index of BS present in CFCB was examined against different hydrocarbons *viz*. hexadecane, *n*-hexane, *n*-heptane, kerosene, crude oil, sesame oil, soybean oil, mustard oil and olive oil.

Stability of BS

Effect of temperature on stability of BS was examined by incubating 2 mL of CFCB at different temperatures ranging from 40 to 180°C for 30 min, cooled to room temperature. Subsequently, E24 was calculated[13]. In order to determine effect of pH, CFCB was subjected to different pH ranging from 2.0-12.0 at room temperature and then emulsification index was measured.

Extraction of BS

Halomonas sp. BRI3 was cultivated under conditions yielding maximum amount of BS. The CFCB was acidified to pH 2.0 with concentrated HCl and BS was extracted twice using equal volumes of chloroform: methanol (2:1) solution in a separatory funnel. The bottom layer was extracted and solvent was removed by Vacuum Rotary Evaporator (Kemi Science, Germany) at temperature below 40°C[5]. The dried extract (BS) was used for further studies.

Characterization and application studies of BS

Chemical analysis

Preliminary characterization of BS was carried out by using thin layer chromatography (TLC). A portion of crude BS was separated on silica plates (Si60F254, 0.25 mm, Merck) using chloroform: methanol: water (65:25:4) as a developing solvent system. Detection reagents used were iodine vapors for lipid, ninhydrin reagent for amino acids and alkaline permanganate (1% $KMnO_4$ and 2% Na_2CO_3) solution for sugars. Furthermore, proteins, carbohydrates and lipid content were estimated by Lowry's method[14], phenol–sulphuric acid method[15] and sulfo-phospho-vanillin test[16], respectively.

Fourier transforms infrared spectroscopy

The extracted BS was subjected to Fourier transform infrared spectroscopy (FTIR) analysis using Shimadzu FTIR system 8400 spectrometer in 4000-400 cm^{-1} wavenumber region with spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively[5] (Suppl. Fig. 1).

Hydrocarbon degradation

Hydrocarbon degradation experiments were carried out using crude oil (motor lubricating oil) and

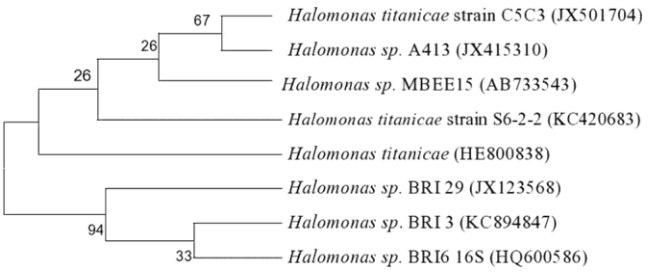

Fig. 1 — Phylogenetic analysis based on 16S rRNA gene sequences of the isolate BRI3 and related *Halomonas* species. GenBank accession numbers are listed with species names

soybean oil individually in 250 mL Erlenmeyer flasks containing 50 mL of MBPM and 2% oil. 5% inoculum was used for all the experiments.

The experiments were conducted in six different sets as follows: Set 1. MBPM (without glucose) + soybean oil + inoculums, Set 2. MBPM (without glucose) + crude oil + inoculums, Set 3. MBPM (with glucose) + soybean oil + inoculums, Set 4. MBPM (with glucose) + crude oil + inoculums, Set 5. MBPM + soybean oil (control) and Set 6. MBPM + crude oil (control).

Flasks were incubated at 30°C for 50 days with shaking at 120 rpm. The residual hydrocarbon in the CFCB was extracted thrice with *n*-hexane in 1:2 (*v/v*) ratios in pre weighed beaker and evaporated using vacuum rotary evaporator (Kemi Science, Germany) at 40°C to a constant mass[17]. The percentage of oil degradation was calculated as follows:

$$\text{Hydrocarbon degradation (\%)} = (\text{HDC} - \text{HDT})/\text{HDC} \times 100 \quad \ldots (2)$$

where, HDC is the hydrocarbon degradation control sample, and HDT is the hydrocarbon degradation test[18].

Antibacterial activity

The antibacterial activity of the crude BS was determined by the micro dilution method using 96-well flat-bottomed tissue culture plates as described by Gudina *et al*. (2010)[19]. For this,125 µL of sterile double strength medium [nutrient broth for *E.coli* (NCIM 2065), *Staphylococcus aureus* (NCIM 2079), *Proteus mirabilis*, *Klebesillia pneuomoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa* (NCIM 2200) and de Man, Rogosa, Sharpe medium for *Enterococcus fecalis* (all the cultures except NCIM strains were clinical isolates)] was placed into the first column of the 96-well micro plate. 125 µL of sterile single strength growth medium was added to the remaining wells and 125 µL of BS solution in phosphate buffered saline (PBS, 100 mg/mL) was added to the first column of the micro plate which was mixed with the medium. This resulted in BS concentration of 50 mg/mL. Further, 125 µL was transferred to the subsequent wells, discarding 125 µL of the mixture in the tenth column, so that the final volume for each well was 125 µL. This procedure resulted in two-fold serial dilution of the BS in the first 10 columns (50-0.097 mg/mL). Columns 11 and 12 did not contain BS and served as negative and growth controls, respectively. All wells (except for the 11th column) were inoculated with 2.5 µL of a test culture grown overnight in respective media at 37°C diluted to a final OD 1.0 at 600 nm. Micro plates were covered and incubated for 48 h at 37°C. Following this, the optical density was determined at 600 nm for each well. The growth percentages at different BS concentration for each microorganism were calculated as:

$$\text{\% Growth} = (OD_c/OD_0) \times 100 \quad \ldots (3)$$

OD_c = the optical density of the well with a BS concentration c

OD_0 = is the optical density of the control well (without BS).

Anti-adhesion assay

The anti-adhesive activity of the crude BS was examined against different microbial strains (as mentioned in section 2.9 except *E. coli*). The experiment was carried out according to the procedure described by Luna *et al*. (2011)[20]. The wells of a sterile 96-well flat-bottomed tissue culture plate were filled with 100 µL of a crude BS solution in PBS. Different BS concentrations used were in the range of 10 to 0.012 mg/mL. The plate was incubated for 18 h at 4°C and subsequently washed twice with PBS. Control wells exclusively contained the PBS buffer. An aliquot of 100 µL of each washed test bacterial suspension (OD 1 at 600 nm in PBS) was added to the wells individually and incubated in the wells for 4 h at 4°C. Unattached microorganisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 µL of 99% methanol per well, after 15 min, the plates were emptied and left to dry. Each well in the plates was stained by using 200 µL of 2% crystal violet for 5 min. Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air dried. The dye bound to the adherent microorganisms was re-solubilized with 200 µL of 33% (*v/v*) glacial acetic acid per well and the optical density readings of each well were measured at 595 nm. The % inhibition for bacterial adhesion at different BS concentrations for each microorganism was calculated as:

$$\text{\% Inhibition of microbial adhesion} = (1 - (OD_c/OD_0)) \times 100 \quad \ldots (4)$$

OD_c= represents the optical density of the well with a BS concentration c

OD_0 is the optical density of the control well.

Statistical analysis

The experiments were performed in triplicates and standard deviation was calculated. One-way ANOVA was applied to determine significant value ($P< 0.05$).

Results and Discussion

Organism

The isolate BRI3 was identified using 16S rRNA (1232 bp) gene sequencing. The sequence was deposited in EMBL + GenBank under the accession number KC89487. The analysis indicated that BRI3 is a member of the genus *Halomonas* which showed 98% similarity with *Halomonas* sp. BRI6 (HQ600586) and *Halomonas* sp. BRI 29 (JX123568) as shown in (Fig. 1). *Halomonas* sp. BRI3 a Gram-negative rod grew well at 15°C and tolerated temperature up to 40°C. It cannot grow at 45°C or above, indicating its psychrotolerant nature. It showed good growth in alkaline conditions (pH 9.0 -11.0). Growth temperature range of 5-30°C and pH in the alkaline range had been reported earlier for Antarctic microorganisms[21]. Our isolate BRI3 could grow at 20% salt concentration confirming its halo tolerant character. Poli *et al*. (2007) have reported *Halomonas alkali antartica* sp. nov. growing optimally at 10% NaCl[22]. The moderately halophilic character (NaCl tolerance 12.5%) of Antarctic microorganisms has been also described by Bozal *et al*. (2003)[23]. Biochemical characterization of the isolate BRI3 indicated its ability to utilize glucose, sucrose, maltose and lactose. Also it showed positive results for nitrate reduction, methyl red, oxidase, citrate and catalase tests.

Halomonas sp. BRI3 was examined for its ability to produce BS. Maximum displacement of 6.02 cm^2 was observed in oil displacement test representing significant surface activity. It also showed β-hemolysis when tested on blood agar plates.

BS Production

Halomonas sp. BRI3 was cultivated in BPM and E24 was found to be 49%. The yield of extracted BS was 5 g/L. Maximum production (E24 = 64%) was observed in the media containing glucose**.** High concentrations of BS are normally seen under nitrogen limiting conditions. We observed combination of ammonium chloride and yeast extract (1:1) as the best suitable nitrogen source for BRI3. It resulted in percentage increase of E24 to 66.4%. Further, effect of change in glucose concentrations (0.2-1.4%) was examined on BS production and maximum emulsification activity of 67% was recorded at 1% glucose. High concentration of sugars like 2% sucrose and 4% glucose were previously reported to maximize BS production from *Streptomyces* sp. B3[24] and *Bacillus subtilis* SPB1[8], respectively. In comparison, higher yield of BS at 1% glucose concentration with our isolate BRI3 appears beneficial. Following this, concentration of ammonium chloride + yeast extract (1:1) was varied in the range of 0.10 to 0.35% in the media containing 1% glucose. Highest emulsification activity of 69% was noted at 0.25% concentration of ammonium chloride + yeast extract. Thus we obtained 13% increase in BS (in terms of E24) as compared to BPM. Maximum emulsification activity of 70% was obtained at temperature 30°C using MBPM. Plethora of papers on BS from marine microorganisms report 30°C as optimum temperature for BS production[20]. Most favorable percentage of inoculum for BS production is 5% of the fermentation medium. It resulted in highest emulsification activity of 71%. Increase in the yield of BS was also reported in the genus *Bacillus*[4,11]. Gutiérrez *et al.* (2007) had published their studies on BS from *Halomonas* sp. TG39 and TG67[25]. They obtained yield of 131.0 mg/L and 28·0 mg/L, respectively. Higher yields of BS (more than 9 g/L) were reported by Praveesh *et al*. (2011) and George and Javachandran, 2009[26,27]. It is a well-known fact that adequate density of the inoculum is determinant for high BS production as it affects biomass and yield of final product[28,29]. BRI3 was cultivated in MBPM at 30°C for different time intervals to evaluate the effect of incubation period. Maximum BS production (E24 72%) was obtained at the end of 48 h, with the yield of 14 g/L (Table 1). MBPM (g/L) containing 1% glucose (10g/L), 0.25% ammonium chloride + yeast extract (1:1) inoculated with 5% inoculum at 30°C for 48 h, yields 14 g/L with highest emulsification index of 72%. It resulted in 46% increase in E24 as compared to BPM.

Emulsification activity against different oils

Stability of an oil and water emulsion is commonly used as an indicator of surface activity[24]. Kerosene and *n*-hexane were the best components with E24 72% and 60%, respectively. Emulsification of different hydrocarbons by the BS present in CFCB was in the order of kerosene >*n*-hexane > crude oil >*n*-heptane > soybean oil > hexadecane > mustard oil >olive oil >sesame oil. The emulsion against each hydrocarbon was stable up to 48 h. Emulsification of nine different hydrocarbons by the BS reflects

Table 1 — Optimization of BS production

	BS producing medium (BPM)	Modified BPM (MBPM)
Media composition	KH_2PO_4 (2.7), K_2HPO_4 (13.1), NaCl (5.0), KNO_3 (1.0), $MgSO_4$ (1.0), NH_4Cl (1.0), yeast extract (0.5) with 5% inoculum at 30°C for 48 h at 120 rpm	1% glucose (10g/L), 0.25% ammonium chloride + yeast extract (1:1) inoculated with 5% inoculum at 30°C for 48 h at 120 rpm
Yield of BS	5 g/L	14 g/L
Emulsification activity	49%	72%

the possibility of its application against different hydrocarbon pollution. Earlier Gutiérrez *et al*., 2007[28] reported E24 of 60% for BS from *Halomonas* sp. TG39 with *n*-hexadecane. The authors observed effective emulsification of various food oils at neutral and acidic pH.

Stability of crude BS

The applicability of BS in several fields depends on its stability at different pH and temperatures. We observed E24 of 65.14% and 60.14% at 120°C and 180°C, respectively at the end of 30 min for BS from *Halomonas* sp. BRI3 (Fig. 2A). Accordingly, BS retained 90.4% and 83% emulsification activity, respectively (considering emulsification index of 72% as 100%). Similar experiment at various pH values indicated E24 of 50% even at pH 12 (Fig. 2B) indicating 69% residual activity at high pH. The ability of BS from BRI3 to withstand extreme conditions of temperature and pH imply its potential application in food, pharmaceutical and cosmetics industries. The effect of pH and temperature on emulsification activity of BSs has been previously reported from different microorganisms[29,30].

Characterization and application studies

Characterization of BS

Results of TLC experiments indicated that BS is a mixture of carbohydrate, lipid and amino acids. The total content of sugar, amino acid and lipids in extracted BS were found to be 4.15%, 1.05% and 94.8%, respectively. The FTIR analysis of the BS exhibited strong and broad band covering a wide range of 2800-3500 cm^{-1} (for the OH stretch). A prominent and stake shaped band was located near 1700 cm^{-1} (for the C=O ester bond). C−H stretching bands of CH_2 and CH_3 groups were observed in the region 2850–2960 cm^{-1}. CH_2 and CH_3 bends were confirmed at (1465 and 1377 cm^{-1}). Wave numbers

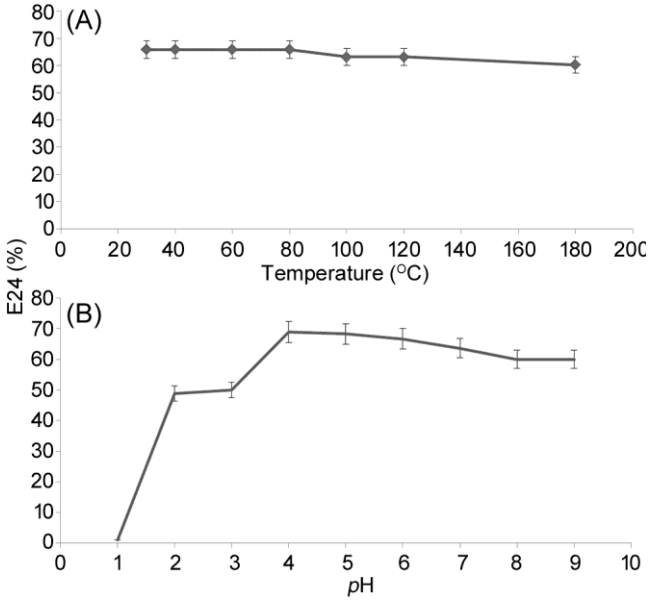

Fig. 2 —Stability of crude extracted BS from *Halomonas* sp. BRI3; (A) Effect of temperature (2 mL of CFCB was incubated at different temperatures ranging from 40 to 180°C for 30 min, cooled to room temperature and E24 was calculated); and (B) Effect of pH (CFCB was exposed to different pH ranging from 2.0-12.0 at room temperature and then emulsification index was measured)

3282 and 3358 cm^{-1} inferred the presence of N−H/C−H bonds of amino acids. This was confirmed with wave numbers 1531 and 1625 cm^{-1} indicating NH bend in amino acids. This preliminary analysis suggested the glycolipoprotein nature of the BS. Most of the literature on BS published so far, describe glycolipid or glycoprotein characteristics of biosurfactant[9,30]. Comparatively few biosurfactants are of glycolipoprotein nature *e.g*. BS produced by *Oceanobacillus* sp. BRI10[5].

Hydrocarbon degradation

We have come across various reports on crude oil pollution of water indicating the necessity of biological methods for its remediation[30,31]. Our results of emulsification index using crude oil (58.33%) and soybean oil (51.72%) prompted us to examine the ability of BRI3 for their degradation. Maximum degradation of 60% of soybean oil was noted on 50th day in presence of glucose. It was used by the isolate to a lesser extent (50%), in the absence of sugar. The isolate also had significant crude oil degradation ability as illustrated in (Fig. 3A). On 50th day, 40% crude oil was used up by BRI3 in presence of glucose while 20% of oil was degraded in absence of sugar. Increase in hydrocarbon degradation in the medium amended with glucose may be

attributed to increased BS production causing increase in hydrocarbon solubility making it more accessible for biodegradation[32]. These results indicate potential of *Halomonas* sp. BRI3 in bioremediation. Earlier *Halomonas* sp. C2SS100 isolated from petroleum reservoir was found to have an ability to use refined petroleum products like diesel, lubricating oil, *n*-hexadecane as the sole carbon source[33].

Anti-bacterial activity

The extracted crude BS was used to evaluate its antibacterial activity in the range of 6.25 mg/mL to

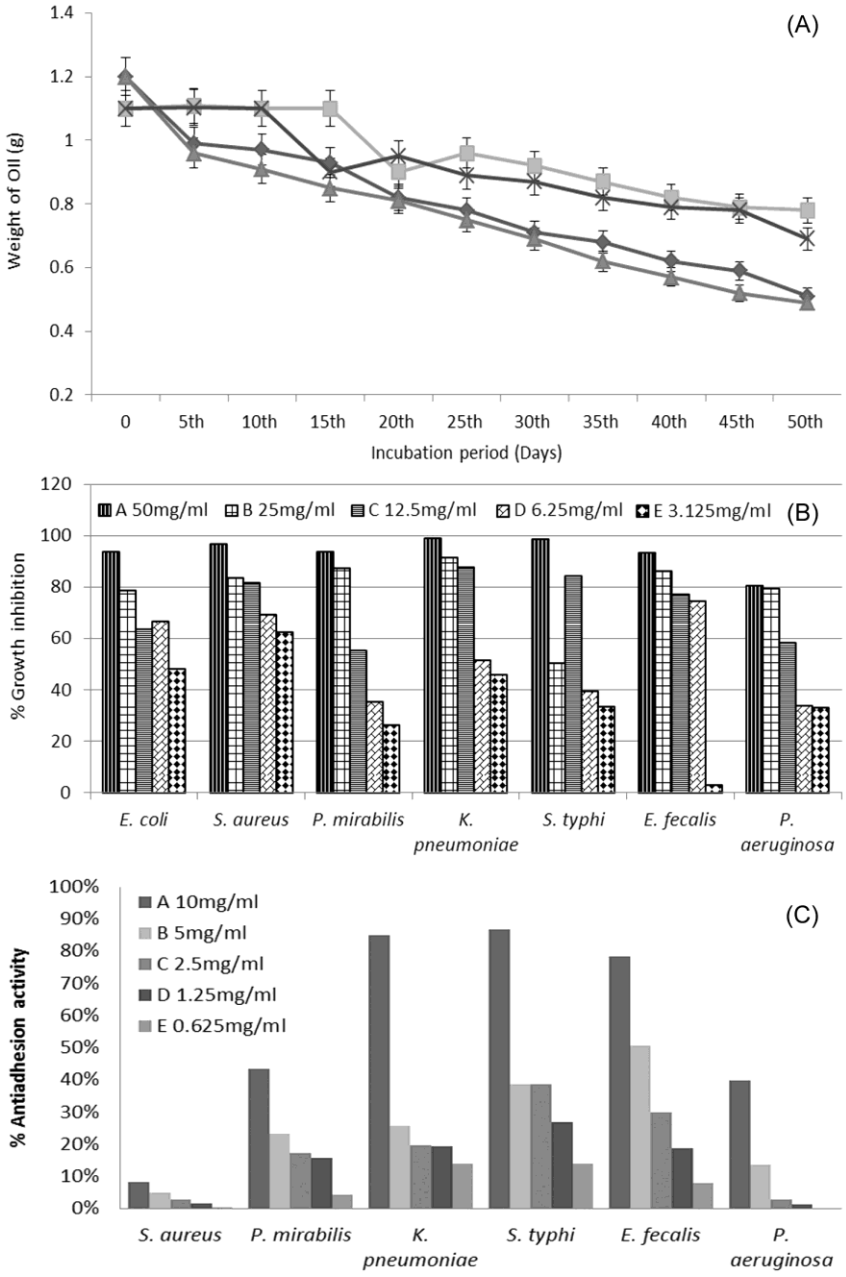

Fig. 3 — Characterization and application studies (A) Hydrocarbon degradation (Crude oil and soybean oil were used each at 2% concentration individually with 5% inoculum. Flasks were incubated at 30°C for 50 days with shaking at 120 rpm); ◆ BPM + soybean oil (1 mL) as a carbon source, ■ BPM + crude oil (1 mL) as a carbon source, ▲ BPM + soybean oil (1 mL) as carbon source + glucose as carbon source, ✕ BPM + crude oil (1 mL) as carbon source + glucose as carbon source (1%); (B) Antibacterial assay (Crude BS was tested in the concentration range of 6.25 mg/mL to 50 mg/mL against selected bacterial strains using micro dilution method. Micro plates were covered and incubated for 48 h at 37°C); and (C) Anti-adhesion assay (Crude BS was used in the range of 10 to 0.012 mg/mL against different bacterial strains)

50 mg/mL against all the bacterial strains tested (Fig. 3B). Percent inhibition increased with an increase in BS concentration. Its minimum inhibitory concentration was found to be 50 mg/mL in case of *Klebsiella pneumoniae* and *Salmonella typhi* as total growth inhibition was observed in these microorganisms. For other microorganisms assayed, we observed high growth reductions (73.60% to 96.88%) at the same BS concentration. Remarkably, lower concentrations of 25 mg/mL and 12.5 mg/mL resulted in growth reduction for every microorganism tested. Even at 6.25 mg/mL, reduction in growth up to 50% was recorded in case of *Salmonella typhi* Previously, biomedical characterization of BS from *Halomonas* sp. BS4 revealed its antifungal and antibacterial characteristics[34]. Complete growth inhibition by BS was earlier reported by Gudina *et al*. (2010) in case of *Escherichia coli*, *Streptococcus agalactiae* and *Streptococcus pyogenes* at concentration of 25 mg/mL[19]. While Luna *et al*. (2011) reported highest percentage of inhibition for *Streptococcus oralis* J22, *Candida albicans* and *Staphylococcus epidermidis* at concentration of 10 mg/mL[20]. Considering this, our BS has moderate antibacterial activity which may be improved on further purification.

Anti-adhesion activity

The extracted crude BS was found to possess antiadhesive activity against all the microorganisms assayed (Fig. 3C). The highest anti-adhesive percentages were obtained for *Salmonella typhi* (86.8%), *Enterococcus fecalis* (78.48%) and *Klebsiella pneumoniae* (84.87%) at BS concentration of 10 mg/mL. The isolated crude BS from *Lactobacillus paracasei ssp paracasei* A20 showed highest antiadhesive activity of 72.0% against *Staphylococcus aureus* at 25 mg/mL[19]. The role of BS in microbial adhesion has been well documented in the literature [10,35-37]. The Adsorbing ability of BS on solid surfaces might prove as an effective strategy to reduce bacterial adhesion, thereby preventing colonization by pathogenic microorganisms indicating its potential in medicine and healthcare areas. In view of this, BS from BRI3 is very promising for further studies aiming to reduce microbial colonization on different surfaces.

Conclusion

Presumptive analysis suggested glycol lipoprotein nature of BS from *Halomonas* sp. BRI3. In modified production medium, crude BS yield is increased from 5 g/L to 14 g/L. It was stable under extreme conditions of pH and temperature. The organism was able to utilize soybean oil and crude oil with 60% and 40% degradation, respectively at the end of 50 days. Besides, present work revealed antimicrobial and antiadhesive characteristics of BS. Thus, our observations indicate BRI3 as a potential candidate for the production of BS.

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