Int. J. Environ. Sci. Technol. (2014) 11:1379–1386
DOI 10.1007/s13762-014-0548-5

ORIGINAL PAPER

# Bioemulsification activity assessment of an indigenous strain of halotolerant *Planococcus* and partial characterization of produced biosurfactants

**G. Ebrahimipour · F. Gilavand · M. Karkhane · A. A. Kavyanifard · M. Teymouri · A. Marzban**

Received: 20 April 2013 / Revised: 22 January 2014 / Accepted: 12 March 2014 / Published online: 16 April 2014


**Abstract** Halotolerant bacteria are regarded as effective oil-scavengers in the polluted saltern and seawater. In this regard, a halotolerant *Planococcus* was isolated from oil-contaminated area of Dezful north springs, Iran, due to its capacity in biosurfactant (BS) production. To facilitate hydrocarbons degradation, in the current study, the efficiency of BS production as function of growth rate of the halotolerant *Planococcus* was investigated in the vicinity of heavy crude oil by emulsification index (E24). Subsequently, the BS characterization was made by thin-layer chromatography (TLC), gas chromatography (GC) and infrared spectra analysis, and the stability was determined by E24 value measurement over a certain pH (5–9), temperature (20–100 °C) and salt concentration (0–10 % w/v) ranges. The BS production was found to be growth-associated. Detection of a unique band on TLC and GC chromatogram showed the extensive refining capacity of the BS purification, using the medium supernatant under acetone alkaline precipitation followed by oil dissolution from the sediment by carbon tetrachloride. Accordingly, it was clarified that the BS ultimately accumulated outside the cells. The glycolipid quality of the BS was further determined by the routine chemical characterization on TLC and by IR spectra analysis. Moreover, there was no protein detected by lowery total protein assay. Finally, the optimal temperature, pH and NaCl concentration to reach highest E24 values (85.7, 77.0, and 79.0 %) were found at respective 40 °C, pH = 9 and 0 % w/v. Our results revealed the practically potential of strain Dezful Isolate for BS large-scale production as environmentally friendly oil-eliminating agents.

**Keywords** *Planococcus* · Biosurfactant · Halotolerant · Biodegradation

G. Ebrahimipour · F. Gilavand · A. A. Kavyanifard
Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran

M. Karkhane
Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

M. Teymouri · A. Marzban (✉)
Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
e-mail: marzban86@gmail.com

## Introduction

The role of petroleum degrading microorganisms as the candidate for oil decontaminations from polluted sites is recently highlighted among researchers who concern about environmental preservation (Tang et al. 2010). There are wide variety of microorganisms, including bacteria, yeasts and filamentous fungi, which are capable of consuming petroleum and their related compounds as their energy and carbon source (Kim et al. 1999; Kristanti et al. 2011; Mohsenzadeh et al. 2012; Sood and Lal 2009; Vyas and Dave 2011). Either every single of or any given consortium of these bioscavengers is found at a given locations. Some are distributed in vast areas of the globe, in terrestrial and aquatic environments, fresh and seawater, as well as almost every climate, while others are restricted to some specific locations whose productive life depends on seasonality (Jain et al. 2010; Menezes Bento et al. 2005; Singh et al. 2012). Biosurfactants are literally surfactants from biological sources. These compounds are produced by various microorganisms to considerably improve the bioavailability of water-immiscible hydrocarbons as carbon and energy

sources for them, whereby enhance their consumption rate (Bento et al. 2005; Erdogan and Karaca 2011; Pirollo et al. 2008). They have the ability to accommodate themselves between liquid phases with different degrees of polarity such as water–oil due to their amphiphilic nature, thereby increasing the oil–water interfaces (Singh et al. 2007; Van Hamme et al. 2003). In addition, they help hydrocarbon dissolution through surface intension reduction and/or emulsification (Suthar et al. 2008). In other word, BS play a large part in biodegradation process, which are regarded as helpful in eliminating hydrocarbon wastes and in averting their disastrous environment consequences (Van Hamme et al. 2003).

In the case of hydrocarbon-related contaminations, due to very low water-soluble compounds present in oil, natural biodegradation of hydrocarbons in the polluted soil, fresh water and seawater is extremely slow. However, two biologically strategy became famous in recent decades: biostimulation (the addition of some materials which can help grow bioscavengers population) and bioaugmentation (addition of bioscavengers to the polluted sites). Both can be applied to eliminate hydrocarbons from nature more efficiently and rapidly (Alisi et al. 2009; Llado et al. 2012; Silva-Castro et al. 2012; Tahhan et al. 2011; Wang et al. 2012).

The aim of the current study is to find a correlation between the biomass and BS production in a halotolerant *Planococcus* Strain Dezful Isolate (DI) isolated during autumn of 2011 from Dezful north springs, Iran, and chemically characterize the BS nature. In addition, the BS stability at different temperatures, pH conditions and salt concentrations is determined by emulsification index.

## Materials and methods

### Microorganism inoculum preparation

The halotolerant *Planococcus* strain DI was screened from spring water in north Dezful, Iran, and transferred to nutrient rich broth medium as inoculum culture. To isolate the bacterium, 1 ml of water sample was diluted by autoclaved distilled water of onefold to fivefold dilutions. Then, 0.1 ml of each dilution was spread on nutrient agar medium by a sterile glass rod. The plates were overnight incubated at 30 °C. After isolating colonies, halotolerant *Planococcus* DI was characterized as biosurfactants producing bacterium and selected for further studies in more details.

### Medium culture for biosurfactant production

1 ml of *n*-hexadecane was transferred to a 250 ml Erlenmeyer flask containing 100 ml of Mineral salt medium (MSM) with the following composition (mg/100 ml): $NH_4Cl$, 29.4; $Fe_2(SO_4)_3$, 0.1; $MgSO_4$, 2.0; KCl, 0.5, $CaCl_2$, 0.1; and trace elements. After autoclaving, the mixture was inoculated with 1 ml of bacterial suspension (with equivalent turbidity to 0.5 McFarland units), supplemented with 1 ml of autoclaved $Na_2HPO_4$ (2.94 mg/100 ml), and placed on a rotatory shaker incubator (140 rpm) at 30 °C.

### Biomass determination

At 12-h intervals, the resultant precipitates of the centrifuged culture (1 ml) lysed by 0.3M NaOH (0.5 ml). The mixture containing cell debris then underwent a centrifugation, and the supernatant absorbance was recorded at 623 nm by Lowry method using bovine serum albumin as standard (Lowry et al. 1951).

### Biosurfactants isolation

Briefly, 200 ml of chilled acetone was added to 100 ml of the supernatants (pH adjusted to 8.5–9) and left for 10 h at 4 °C. After centrifugation, the acetone was evaporated and removed from the supernatant at 65 °C. To separate oil, chloroform (100 ml) was mixed with and allowed to be divided into two phases. The upper milky phase was dried by hot air oven at 50 °C to be weighted later. The lower oily phase was also blended with distilled water and left at 76 °C to eliminate the chloroform solvent and reserved in refrigerator.

### Emulsifying activity assay

Every 12 h, 2 ml of the culture medium containing 1 % crude oil was centrifuged; the supernatant was blended with crude oil (2 ml), mixed by vortex for about 1 min, and after maintaining at room temperature for 24 h, emulsification activity was calculated with the measurement of emulsified layer (Viramontes-Ramos et al. 2010). Determination of emulsification activity was done using the following formula:

$$\text{Emulsification activity (E24)} = \frac{\text{Emulsified layer height}}{\text{Mixture's total height}} \times 100$$

## Chemical characterization of biosurfactant

### TLC analysis

The dried sediment achieved through the BS isolation protocol was dissolved in distilled water. A small amount of the solution was spotted on a silica gel 60 plates (Merck)

to be separated by the solvent system of distilled water: acetic acid: methanol: ethyl acetate: chloroform as 25:20:15:10:5 (in volume). The same thin-layer chromatography (TLC) examination was also carried out for chloroform and acetone-free solution as control. The BS chemically was identified on silica gel by the following treatments: total lipids were exposed of the chromatogram plate at 100 °C for about 15 min following spraying $H_2SO_4$-brownish black spots (Hamilton and Hamilton 1992); carbohydrates-initially spraying of Alpha-naphthol/ethanol reagent on the plate and subsequently were identified to extra pure $H_2SO_4$ spraying purple spots (Dubois et al. 1956) and functional amine groups were exposed of the plate at 110 °C for 10 min following processing by ninhydrin/glacial acetic acid/*n*-butanol blue spots (Hamilton and Hamilton 1992). Protein content was examined by dissolving 0.2 g of the dried BS in 2 ml of distilled water according to the Lowry method (Lowry et al. 1951). Moreover, the acidified culture supernatant was qualified as rhamnose equivalent by Orcinol assay (Vyas and Dave 2011).

### GC analysis of biosurfactant

One microliter of the dried BS/methanol solution (0.01 g/2 ml) was injected into splitter. Gas chromatography was performed using Agilent 7890A instrument with thermal conductivity detector (column: HP-5 30 m × 0.25 mm; stationary phase; DB-5 (silica gel: phenyl, 95:5 ratio); carrier gas: different ratios of $He:O_2:H_2$ with 3 ml/min flow; initial and final temperature: 40 and 200 °C with temperature ramp of 10 °C/min; isothermal at 200 °C for 5 min) (Jain et al. 2010).

### Infrared spectra (IR)

After BS extraction with chloroform followed by drying by $Na_2SO_4$, the IR spectra of the BS was recorded on Bruker IFS113vFTIR-spectrometer, in 4,000–400 $cm^{-1}$ spectral region at 2 $cm^{-1}$ resolution, 100 scans for each spectrum, using a 0.23-mm KBr liquid cell.

### Biosurfactant stability assay

To demonstrate BS stability under different conditions, emulsification index (E24) of its partially purified solution (1 g/l) was examined in a given range of pH (5–9), temperature (20–100 °C) and NaCl concentration (0–10 % w/v). Briefly to characterize thermal stability, an equal amount of BS solution and sterile crude oil was blended together and get exposed in the temperature range for 15 min and allowed to cool to room temperature; to evaluate pH effect, pH was adjusted to various values by adding HCl or NaOH to solution at room temperature; and the assessment of salinity effect was performed by adding different concentration of NaCl solution to BS dry powder followed by mixing with crude oil and then recording the emulsifying activity after 30 min.

## Results and discussion

### Determination of effectiveness of isolating methods

In this study after precipitating bacterial cells by centrifugation (10,000×*g* for 10 min), different methods were used to extract the BS from supernatant. They include precipitation by lowering pH of medium supernatant, using chilled acetone, or ammonium sulfate and extraction by different organic solvents (Anandaraj and Thivakaran 2010; Deka and Das 2009). However, it was revealed that the most efficient way to extract BS was achieved by applying a modified method, i.e., first alkaline precipitation of culture medium supernatant in acetone and then oil dissolution of the resulted sediment in tetrachloride carbon (Bryant 1990). Regarding the high efficacy of our isolation method, preliminary emulsification activity and TLC tests on acetone-free supernatant and the subsequent lower chloroform-free phase determined lack of BS in these solutions, while emulsification activity was increased in milky phase (the data not presented).

The medium supernatant was able to completely disperse crude oil, implying the extracellular location of BS (Deka and Das 2009). Since more than 60 % of total production expenditures are attributed to recovery of product in biotechnological processes, the excretion of BS would decrease more remarkably industrial costs compared to those entrapped in cytosol or periplasmic environments.

### Bacterial growth, biosurfactant production and activity relationship

As evident from Fig. 1, the time course study of BS production by the isolate exhibits a logarithmic rise in E24 for up to 100 h after inoculation to the medium containing crude oil as carbon and energy source, which subsequently reaches a stationary phase. Regarding the biomass concentration, BS yield and its emulsifying power in the course of incubation, there are remarkable similarities

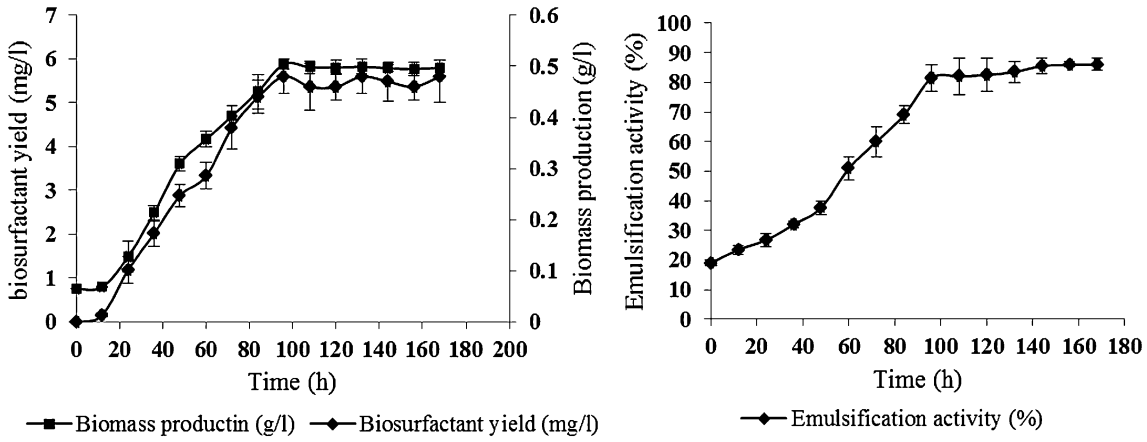


**Fig. 1** Time course of growth and BS production by halotolerant *Planococcus* strain DI (*right*), and its emulsification activity (*left*) exhibited a similar pattern with highest values for all three variables after 96 h

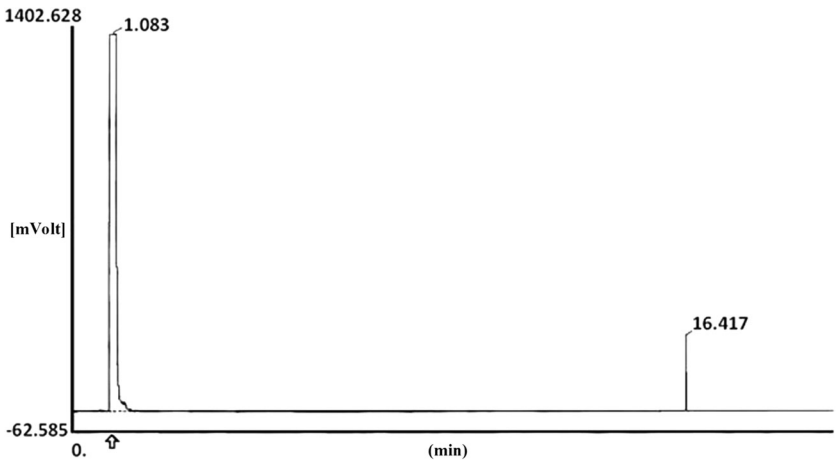


**Fig. 2** The GC chromatogram for BS of halotolerant *Planococcus* strain DI when hexadecane was used as the sole carbon source. The presence of a single sharp pick at 16.417 infers the outstanding BS purification. The first bigger pick at 1.083 is correlated with solvent

between their respective patterns. Accordingly, it presumed that the production and the emulsifying activity of the BS are associated to the bacterial growth. In the case of a growth-associated BS production, there is a parallel relationship between the substrate consumption, growth and BS production (Abbasi et al. 2012). On the other hand, accumulation of anionic compounds, e.g., BS in a medium, hinders bacterial growth rate and its BS productivity due to pH reduction in medium, which in turn leads to a less appropriate environment for bacterial reproduction and consequently BS synthesis (Anandaraj and Thivakaran 2010). Based on our results, the emulsification activity of the derived BS was determined to remain constantly highest (77 %) at the outset of log phase, using the emulsified layer height of oil in distilled water as control. This indicates the higher capacity of the strain DI for oil recovery than those of other bacteria (Vaz et al. 2012).

## Biosurfactant purification and biochemical analysis

The preliminary assessment of the BS purification of the isolating method described in the "Materials and methods" section was carried out by TLC. The development of a unique fluorescent pink band on the gel under UV spectrum indicates its purification (the result is not shown). It was further confirmed by post-gas chromatogram exhibiting only one sharp pick at 16.417 min besides the one correlated to solvent (1.083 min) (Fig. 2). There are some reports taking advantage of TLC method to determine BS purity (Satpute et al. 2010). Moreover, gas chromatography has been considered as the more powerful method in the separation of different organic mixtures and in the determination of purity of a wide variety of compounds. Our results showed high BS purity when strain DI used *n*-hexadecane, receiving more attention for further studies.

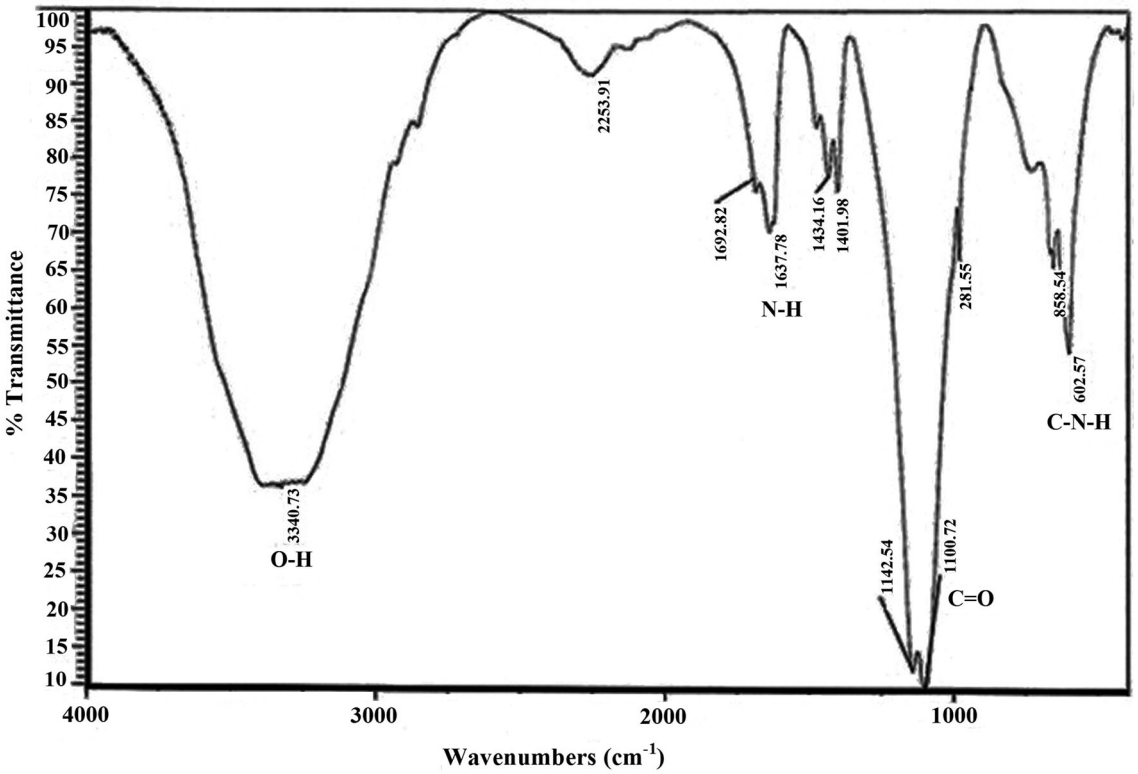


**Fig. 3** FTIR spectrum of the BS bears a remarkable resemblance to those of glycolipids

The result of the TLC plate exposure to $H_2SO_4$, ninhydrin and alpha-naphthol showed that our BS structure was composed of carbohydrate, fatty acid parts and functional amine groups when *n*-hexadecane was used as the only carbon source. However, there was no protein available in the BS using lowery total protein assay. The result of UV–Vis spectral analysis for the orcinol assay showed that the glycolipid part was probably associated with rhamnose. The same result was also achieved for BS derived from *Candida* sp. SY16 when Kim et al. (1999) applied these treatments on silica plate. The BS was further presumed to be glycolipid in nature by FTIR analysis, as the characteristic absorption bands corresponding to typically functional groups of glycolipids could be observed for the BS (Fig. 3). The absorption bands include 3,340 1/cm O–H stretching, the stretching bands of the amide and ester C=O groups at approximately 1,637 and 1,100 1/cm as well as N–H and C–N–H vibrations at around 1,637 and 600 1/cm, respectively. Based on the characteristics have already been evident from FTIR spectra of carbohydrates, the wide picks denote high hydroxyl and amine functional groups' frequencies in their structures (Kačuráková and Wilson 2001; Kuhn 1950). Overall, detection of carbohydrate and lipid compounds, the high repetition of O–H and C=O as well as amine functional groups present in the FTIR spectrum and protein absence in the BS samples infer that the BS produced by strain DI probably belongs to glycolipids category. This is in accordance with results of Engelhardt et al. (2001) which determined structurally BS class of oil-degrading *Planococcus alkanoclasticus* as glycolipid (Engelhardt et al. 2001). However, the current IR spectrum is needed to be further compared with other standard ones to find out more details.

## Study of biosurfactant stability

The partially purified BS (1 g/l) was used to study the stability of its emulsifying power under different conditions (Table 1). The optimum conditions for the highest E24 values were provided at 40 °C, pH 9 and zero salt concentration, giving maximum emulsification activity of about 85.5, 77.0 and 79.0 %, respectively. However, there was no remarkable difference found for E24 values between pH of 8 and 9 in terms of significance level of 0.05. Except for the salt concentration, this is somehow in consistent with Ilori et al. (2005) results, determining 40 °C, pH 7–8 and 5 % w/v NaCl concentration as the best environmental condition for emulsification activity of

**Table 1** Emulsification indices (EI, %) for the BS of halotolerant *Planococcus* strain DI were determined at different temperature, NaCl concentration and pH points

| Treatment | Temperature (°C) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| EI (%) | 70.7 ± 1.5 | 78 ± 3.0 | 85.7 ± 3.1 | 73.7 ± 3.5 | 51 ± 3.0 | 42.7 ± 3.1 | 31.7 ± 1.5 | 27.0 ± 1 | 22 ± 2 |
| Treatment | NaCl (g/l) | | | | | | | | |
| | 0 | 1 | 3 | 5 | 7 | 10 | | | |
| EI (%) | 79.0 ± 5.3 | 63.3 ± 3.5 | 57.3 ± 1.5 | 42.0 ± 3.0 | 32.3 ± 2.5 | 24.5 ± 0.5 | | | |
| Treatment | pH | | | | | | | | |
| | 5 | 6 | 7 | 8 | 9 | 10 | | | |
| EI (%) | 36.3 ± 4.0 | 46.3 ± 3.5 | 65.7 ± 3.1 | 74.7 ± 3.5 | 77.0 ± 5.0 | 60.3 ± 3.5 | | | |

Measurements were performed at ambient temperature for the salt concentration and pH assessment

glycolipid BS derived from *Aeromonas* sp. Unlike our previous anticipation, our strain DI has better emulsifying activity at zero salt concentration. This indicates their greater suitability to biodegrade oil contamination at oil extraction bases, which is being encompassed by areas containing underground fresh water. According to the present data, the emulsification index was gradually decreased around the optimal determined values toward the both extremes. Although our data did not show the expected thermostability for the BS, the EI remained in acceptable range from 20 to 60 °C, indicating its effectiveness at oil cleanup enhancement in all seasons and most climates. There are some reports about disruptive effects of salts on oil–water emulsions and on emulsifying activity of surfactants (Ferhat et al. 2011). This provides a clue as to why BS emulsifying ability gradually declines when salt concentration in medium increases. Since the DI strain was collected from spring water; they would be regarded as rather inherently superior oil-scavengers than marine microorganisms in terrestrial polluted areas, whose heavy reliance upon restricted salt concentration occurring in sea water confined their reproductive life, regarding the seasonality of the circumstances. The pH increase has a positive effect on emulsion stability, since the more alkaline solution provides the better conditions for prolonged stability of fatty acid surfactant micelles (Lakatos-Szabo and Lakatos 1999). These conditions are naturally occurred in our DI strain's habitats, that is, the most terrestrial areas and their associated ground waters located on moderate climates. Therefore, among all oil-degrading species, our DI strain is probably more efficient at oil bioremediation in these areas.

## Conclusion

The results of the present study clearly proved that the isolated halotolerant *Planococcus* strain DI could produce extracellular biosurfactants and utilize crude oil or hexadecane as the sole carbon and energy source. The high purifying efficacy of the BS isolation method was revealed by TLC and GC analysis, suggesting the top quality of the BS product suitable for a variety of applications. Moreover, the strong association between the bacterial growth and BS production is regarded as good prospect for its convenient scale-up of yield process as just one-stage continuous culture system is necessitated. The glycolipid characteristics of BS became evident from IR analysis. Furthermore, the examination of the emulsifying index (EI) in various simulated environmental conditions revealed the BS considerable activity even in partially alkaline extremes, that is, the strain DI might well tolerates the changes in most land and fresh water throughout seasons, without considerably decline in the growth and the BS activity. The negative correlation between EI and NaCl concentration does not rule out the emulsifying capacity of the BS in salty environments. Overall, we concluded that the strain DI could be recommended as an outstanding candidate for biosurfactant production and for enhanced oil removal in contaminated environments such as seawater, saltern, polluted land and spring water.

**Acknowledgments** This work was carried out with cooperating biology students in Microbiology research laboratory. Herein, it is necessary that authors thank Mr. Javad Fakhari for providing of facilities and supplements to work this research.

## References


Abbasi H, Hamedi MM, Lotfabad TB, Zahiri HS, Sharafi H, Masoomi F, Moosavi-Movahedi AA, Ortiz A, Amanlou M, Noghabi KA (2012) Biosurfactant-producing bacterium, *Pseudomonas aeruginosa* MA01 isolated from spoiled apples: physicochemical and structural characteristics of isolated biosurfactant. J Biosci Bioeng 113:211–219

Alisi C, Musella R, Tasso F, Ubaldi C, Manzo S, Cremisini C, Sprocati AR (2009) Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Sci Total Environ 407:3024–3032

Anandaraj B, Thivakaran P (2010) Isolation and production of biosurfactant producing organism from oil spilled soil. J Biosci Technol 1:120–126

Bento FM, Camargo FAD, Okeke BC, Frankenberger WT (2005) Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol Res 160:249–255

Bryant FO (1990) Improved method for the isolation of biosurfactant glycolipids from *Rhodococcus* sp. Strain H13A. Appl Environ Microbiol 56:1494–1496

Deka M, Das K (2009) Effect of biosurfactant from two strains of Pseudomonas on germinating seedlings of *Cicer arietinum* L. and *Phaseolus mungo* Roxb. Afr J Biotechnol 8:6621–6626

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Engelhardt MA, Daly K, Swannell RP, Head IM (2001) Isolation and characterization of a novel hydrocarbon-degrading, Gram-positive bacterium, isolated from intertidal beach sediment, and description of *Planococcus alkanoclasticus* sp. nov. J Appl Microbiol 90:237–247

Erdogan EE, Karaca A (2011) Bioremediation of crude oil polluted soils. Asian J Biotechnol 3:206–213

Ferhat S, Mnif S, Badis A, Eddouaouda K, Alouaoui R, Boucherit A, Mhiri N, Moulai-Mostefa N, Sayadi S (2011) Screening and preliminary characterization of biosurfactants produced by *Ochrobactrum* sp. 1C and *Brevibacterium* sp. 7G isolated from hydrocarbon-contaminated soils. Int Biodeterior Biodegrad 65:1182–1188

Hamilton RJ, Hamilton S (1992) Lipid analysis, a practical approach. Oxford University Press Inc, New York, pp 83–96

Ilori MO, Amobi CJ, Odocha AC (2005) Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere 61:985–992

Jain PK, Gupta VK, Pathak H, Lowry M, Jaroli DP (2010) Characterization of 2T engine oil degrading indigenous bacteria, isolated from high altitude (Mussoorie), India. World J Microbiol Biotechnol 26:1419–1426

Kačuráková M, Wilson RH (2001) Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. Carbohydr Polym 44:291–303

Kim HS, Yoon BD, Choung DH, Oh HM, Katsuragi T, Tani Y (1999) Characterization of a biosurfactant, mannosylerythritol lipid produced from *Candida* sp. SY16. Appl Microbiol Biotechnol 52:713–721

Kristanti AR, Hadibarata T, Toyama T, Tanaka Y, Mori K (2011) Bioremediation of crude oil by white rot fungi *Polyporus* sp. S133. J Microbiol Biotechnol 21:995–1000

Kuhn LP (1950) Infrared spectra of carbohydrates. Anal Chem 22:276–283

Lakatos-Szabo J, Lakatos I (1999) Effect of alkaline materials on interfacial rheological properties of oil-water systems. Colloid Polym Sci 277:41–47

Llado S, Solanas AM, de Lapuente J, Borras M, Vinas M (2012) A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil-contaminated soil. Sci Total Environ 435–436:262–269

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mohsenzadeh F, Chehregani Rad A, Akbari M (2012) Evaluation of oil removal efficiency and enzymatic activity in some fungal strains for bioremediation of petroleum-polluted soils. Iran J Environ Health Sci Eng 9:26

Pirollo MP, Mariano AP, Lovaglio RB, Costa SG, Walter V, Hausmann R, Contiero J (2008) Biosurfactant synthesis by *Pseudomonas aeruginosa* LBI isolated from a hydrocarbon-contaminated site. J Appl Microbiol 105:1484–1490

Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM, Chopade BA (2010) Methods for investigating biosurfactants and bioemulsifiers: a review. Crit Rev Biotechnol 30:127–144

Silva-Castro GA, SantaCruz-Calvo L, Uad I, Perucha C, Laguna J, Gonzalez-Lopez J, Calvo C (2012) Treatment of diesel-polluted clay soil employing combined biostimulation in microcosms. Int J Environ Sci Technol 9:535–542

Singh A, Van Hamme JD, Ward OP (2007) Surfactants in microbiology and biotechnology: part 2. Application aspects. Biotechnol Adv 25:99–121

Singh B, Bhattacharya A, Channashettar VA, Jeyaseelan CP, Gupta S, Sarma PM, Mandal AK, Lal B (2012) Biodegradation of oil spill by petroleum refineries using consortia of novel bacterial strains. Bull Environ Contam Toxicol 89:257–262

Sood N, Lal B (2009) Isolation of a novel yeast strain *Candida digboiensis* TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions. J Environ Manag 90:1728–1736

Suthar H, Hingurao K, Desai A, Nerurkar A (2008) Evaluation of bioemulsifier mediated microbial enhanced oil recovery using sand pack column. J Microbial Methods 75:225–230

Tahhan RA, Ammari TG, Goussous SJ, Al-Shdaifat HI (2011) Enhancing the biodegradation of total petroleum hydrocarbons in oily sludge by a modified bioaugmentation strategy. Int Biodeterior Biodegrad 65:130–134

Tang JC, Wang RG, Niu XW, Wang M, Chu HR, Zhou QX (2010) Characterisation of the rhizoremediation of petroleum-contaminated soil: effect of different influencing factors. Biogeosciences 7:3961–3969

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549

Vaz DA, Gudina EJ, Alameda EJ, Teixeira JA, Rodrigues LR (2012) Performance of a biosurfactant produced by a *Bacillus subtilis* strain isolated from crude oil samples as compared to commercial chemical surfactants. Colloid Surf B 89:167–174

Viramontes-Ramos S, Portillo-Ruiz MC, Ballinas-Casarrubias MD, Torres-Munoz JV, Rivera-Chavira BE, Nevarez-Moorillon GV

(2010) Selection of biosurfactan/bioemulsifier-producing bacteria from hydrocarbon-contaminated soil. Braz J Microbiol 41:668–675

Vyas TK, Dave BP (2011) Production of biosurfactant by *Nocardia otitidiscaviarum* and its role in biodegradation of crude oil. Int J Environ Sci Technol 8:425–432

Wang X, Wang QH, Wang SJ, Li FS, Guo GL (2012) Effect of biostimulation on community level physiological profiles of microorganisms in field-scale biopiles composed of aged oil sludge. Bioresour Technol 111:308–315