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Screening and identification of biosurfactant producing marine bacteria from the Caspian Sea

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ABSTRACT

Marine microorganisms have unique physiological properties and novel metabolites such as biosurfactant to live in extreme habitats (e.g. oil polluted environment). The aim of this study was to characterize some biosurfactant-producing bacteria collected from sediment and seawater samples from the Caspian Sea. These bacteria were isolated using enrichment method in ONR7a medium with crude oil as sole carbon source. Five screening tests were used for selection of the bacteria including hemolysis in blood agar, oil spreading, drop collapse, emulsification activity and bacterial adhesion to hydrocarbon test (BATH). The bacteria isolated were identified using molecular methods. Eighteen biosurfactant-producing bacteria (from 44 different colonies isolated) were selected and 9 isolates were screened as predominant biosurfactant producer belonging to *Pseudomonas stutzeri* (Strain S1-4-2), *P. balearica* (Strain S1-4-1), *Pseudomonas* sp. (Strain S2-1), *P. stutzeri* (Strain R3-2), *P. chloritidismutans* (Strain K4-1), *Achromobacter xylosoxidans* (Strain K4-3), *Acinetobacter radioresistens* (Strain S1-2), *Acinetobacter calcoaceticus* (Strain K4-2) and *Citrobacter freundii* (Strain R3-1). The largest clear zone diameters for oil spreading method observed for *P. balearica* strain S1-4-1 (14 mm). Also, this strain has the best emulsification activity (100 %); surface hydrophobicity (BATH: 77 %) and reduction of surface tension (34.5 mN m^{-1}). Thus, this strain selected as the best isolated strains. The results of this study confirmed that there is sufficient diversity of biosurfactant-producing bacteria in the Caspian Sea and the environmental problem can be resolved by application of these bacteria in petrochemical waste water.

Key words: Biodegradation, Biosurfactant, Caspian Sea, Marine environment.

INTRODUCTION

Surfactants are surface-active compounds that capable to reduce surface and interfacial tension of liquids, solids and gases thereby allowing them to mix or disperse readily as emulsions in water or other liquids. Biosurfactants are a group of valuable microbial natural products with unique biochemical properties. From a biotechnology prospective, production of biosurfactants are important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries (Cappello *et al*. 2012a). These molecules chemically belong to various categories such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Cappello *et al*. 2012a; 2012c). Excellent detergency, emulsification, dispersing traits, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) are important characteristics of biosurfactants which make them suitable to replace with chemical surfactants. The largest

possible market for biosurfactant is the oil industry, both for petroleum production and for incorporation into oil formulations.

The marine environment represents the major component of the Earth's biosphere. It covers a majority (70%) part of earth's surface and makes 90% of the volume of its crust. Potent biosurfactant-producing microorganisms from marine environment demands reduced utilization of synthetic surfactants and probably favors the increased use of easily biodegradable and environmentally-benign biosurfactant molecules. Marine microorganisms have unique metabolic and physiological properties to thrive in extreme habitats and produce novel metabolites which are not often present in the microbes of terrestrial origin (Fenical 1993). Therefore, this rich marine habitat provides a magnificent opportunity to discover new compounds such as antibiotics, enzymes, vitamins, drugs, biosurfactant (BS), bioemulsifier (BE) and other valuable compounds of commercial importance (Jensen and Fenical 1994; Austin 1989; Romanenko *et al*. 2001; Lang and Wagner 1993; Hassanshahian *et al*. 2014a, 2014b).

Large quantities of crude oil, hydrocarbons, petroleum oil products and halogenated compounds discharge into the marine ecosystem through accidental spillage. To treat, emulsify or simply overcome such spills, on the one hand, many petroleum-based synthetic chemical surfactants often get used. Such synthetic compounds, however often have detrimental ecological effects. The use of biosurfactants and bioemulsifiers therefore may represent a better alternative to overcome the toxicity of synthetic compounds. The environmental roles of the biosurfactants produced by marine microorganisms have been reported earlier (Poremba *et al*. 1991; Schulz *et al*. 1991; Abraham *et al*. 1998).

The Caspian Sea is the largest enclosed inland body of water on Earth by area, variously classed as the world's largest lake or a full-fledged sea. It is an endorheic basin (it has no outflows) located between Europe and Asia. It is bounded to the northeast by Kazakhstan, to the northwest by Russia, to the west by Azerbaijan, to the south by Iran, and to the southeast by Turkmenistan.

The aims of this study was to realize the diversity of biosurfactant-producing bacteria in the Caspian Sea and especially at Iran shoreline. Isolation and characterization of these important bacteria is also another purpose of this research.

MATERIALS AND METHODS

Sampling

Marine samples (sediments and seawater) were collected from five stations in the Caspian Sea at Iran shorelines including Anzali Port (AP), Rasht City (RC), Lahijan Shoreline (LS), Mahshahr Port (MP) and Ramsar Shoreline (RS), 26°15, N; 54°15, E. These stations are located beside petrochemical plants and oil refinery. Sediment samples were taken from 1-12 cm below the surface using a sterile knife. Seawater samples were collected from a depth of 15 cm in sterile 100 ml bottles and transported on ice to the laboratory (Microbiology lab at Shahid Bahonar University of Kerman) for microbiological analysis.

Isolation and enrichment of biosurfactant producing bacteria

ONR7a medium was used for isolation of biosurfactant-producing bacteria. ONR7a is contained (per liter of distilled water) 22.79 g NaCl, 11.18 g $MgCl_2$ $6H_2O$, 3.98 g Na_2SO_4, 1.46 g $CaC1_2$ $2H_2O$, 1.3 g TAPSO {3-[N tris (hydroxymethyl) methylamino]-2-hydroxypropanesulfonic acid}, 0.72 g KCl, 0.27 g NH_4Cl, 89 mg Na_2HPO_4 $7H_2O$, 83 mg NaBr, 31 mg $NaHCO_3$, 27 mg H_3BO_3, 24 mg $SrCl_2$ $6H_2O$, 2.6 mg NaF, and 2 mg $FeCl_2$ $4H_20$. For solid media, Bacto Agar (Difco) (15 g L^{-1}) was added to the solution (Dyksterhous *et al.* 1995).

ONR7a medium was supplemented with 1 % crude oil (v/v) as a carbon source and energy. Portion of sediment (10g) or condensed seawater (10 ml) were added to Erlenmeyer flasks containing 100 ml of medium and the flasks were incubated for 10 days at 30°C on rotary shaker (180×g, INFORS AG). Then 5 ml

aliquots were transferred into fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out and phenotipically different colonies purified on ONR7a agar medium.

The procedure was repeated and the isolates with high growth on crude oil, were stored in stock media with glycerol at -20 °C for further characterization (Chaillan *et al*. 2004; Tebyanian *et al*. 2013).

Screening of biosurfactant-producing bacteria

Five different Methods were used for screening and selection of the best biosurfactant producing bacteria.

Hemolytic test

Hemolytic activity test was carried out as described by Carrillo *et al.* (1996).

Isolated strains were screened on blood agar plates containing 5% (v/v) blood and incubated at 30 °C for 24–48 h. Hemolytic activity was detected as the presence of a clear zone around a colony.

Drop collapse method

The drop-collapse technique was performed in the polystyrene lid of a 96-microwell plate (Biolog, Harward, CA, USA) as described by Jain *et al*. (1991) and Bodour and Miller-Maier (1998). In this protocol 100 µl culture supernatant was added to wells of a 96-well microtiter plate lid, and then 5 µl of crude oil was added to the surface of the culture supernatant. Biosurfactant-producing cultures gave flat drops.

Aliquots from a culture of each strain were analyzed on two separate microtiter plates.

Oil spreading method

Oil spread technique was carried out according to Morikova *et al*. (2000) and Youssef *et al*. (2004). In this method 50 ml distilled water was added to Petri dishes followed by addition of 100 µl crude oil to the surface of the water. Then, 10 µl of the culture filtrates was put on the crude oil surface.

The diameter of the clear zone on the oil surface was measured.

Liquid surface tension

The surface tension (ST) of the culture supernatants was measured by a digital surface tensiometer (DCAT, DataPhysics Instruments GmbH, Filderstadt, Germany) working on the principles of Wilhelmy plate method (Fernandes *et al*. 2007). The validity of the surface tension readings was checked with pure water (70.78 ± 0.02 Mille Newton's per Meter (mN m^{-1}) before each reading. All surface tension readings were taken in triplicate.

Emulsification activity and Bacterial Adhesion To hydrocarbon (BATH test)

The emulsification activity (E_{24}) was determined by adding hexadecane to the same volume of cell-free culture broth, mixed with a vortex for 2 min and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Emtiazi *et al*. 2009). The test of bacterial adhesion to hydrocarbon was carried out according to Pruthi *et al* (1997).

Molecular Identification of the isolates

Analysis of 16S rRNA gene sequences was performed for the taxonomic characterization of isolated strains. Total DNA extraction of bacterial strains was performed with the CTAB method (Winnepenninckx *et al*. 1993). The bacterial 16S rRNA loci were amplified using the forward domain specific bacteria primer, Bac27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer Uni_1492R (5′-TACGYTACCTTGTTACGACTT-3′).

The amplification reaction was performed in the total volume of 25 µl consisting of 2 mM $MgCl_2$ (1 µl), 10X PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 µl), 2 mM each dNTP (2 µl), 0.15 mM each primer (1 µl), 1U (0.5 µl) Taq DNA polymerase (Qiagen, Hilden, Germany) and 2 µl template DNA (50 pm). The distilled water was added for the remaining of reaction (15 µl). Amplification was performed in a thermal cycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept, 94 °C for

5 min, 94°C for 1 min, 54°C for 1 min, 72°C for 1.5 min, 35 cycles; then 72°C for 10 min and finally storage at 4°C (Troussellier *et al*. 2005). The 16S rRNA amplified was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Similarity rank from the Ribosomal Database Project (RDP) (Maidak *et al*. 1997) and FASTA Nucleotide Database. Query was used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of sequences were performed as described protocols (Yakimov *et al*. 2006; Cappello *et al*. 2012).

RESULTS

Isolation and screening of biosurfactant-producing bacteria from marine samples collected from the Caspian Sea

Forty four morphologically-distinct microbial colonies were isolated from collected seawater and sediment samples. These isolates belong to these geographic zones: 12 isolates were obtained from the Anzali Port (AP) samples, 6 from the Rasht City (RC), 9 from the Lahijan Shoreline (LS), 10 from the Mahshahr Port (MP) and 7 from the Ramsar Shoreline (RS). 90% of the bacterial isolates (36 out of 44) were characterized as gram-negative bacteria. These isolates were assayed for biosurfactant producing using three screening tests. However the responses of these isolates to screening methods were different. Drop collapse method has the most positive response, whereas hemolysis on blood agar has the lowest one. However, the positive and negative responses of oil spreading method were the same as each other.

Biosurfactant production by isolated strains

Eighteen isolated strains out of 44 isolates, exhibiting high growth rate on crude oil, were selected for further studies.

Table 1 illustrates the results of screening tests for isolates in the present study. As shown in this Table, the best isolates, displaying sufficient response to all screening tests, were found to be strains S1-4-1, S2-1, K4-2, R3-2, K4-1, and K1-3. However, some isolated strains such as K1-2, S2-2, and R3-4 exhibited positive response to one or two screening tests (Table 1).

Cell surface hydrophobicity, emulsification index and decreasing surface tension by isolated strains

Emulsification activity, bacterial adhesion to hydrocarbon (BATH) and reduction of surface tension were examined for each strain, separately. Data obtained from these tests were shown in Table 2. Strains S1-4-1, S1-2, K4-2, K4-3, R3-1 and R3-2 had the highest values of emulsification activity and cell surface hydrophobicity.

These strains also produced more biosurfactant and dramatically decreased surface tension than other strains. The results indicated that there are a direct relationship between cell surface hydrophobicity and biosurfactant production with emulsification activity (Table 2).

Molecular identification and phylogenetic analysis of isolated strains

Molecular identification of the isolates were performed for S1-4-1, S2-1, R3-2, S1-4-2, K4-1, S1-2, K4-2, R3-1 and K4-3 strains by amplification and sequencing the 16S rRNA gene sequencing along with comparing them to the database of known 16S rRNA sequences. The molecular identification of these strains showed that these strains belong to: *Pseudomonas stutzeri* (Strain S1-4-2), *P. balearica* (Strain S1-4-1), *Pseudomonas* sp. (Strain S2-1), *P. stutzeri* (Strain R3-2), *P. chloritidismutans* (Strain K4-1), *Achromobacter xylosoxidans* (Strain K4-3), *Acinetoba cterradioresistens* (Strain S1-2), *Acinetobacter calcoaceticus* (Strain K4-2) and *Citrobacter freundii* (Strain R3-1).

The phylogeny trees of these isolated strains were shown in the Figs. 1-2. All sequences of the seven bacteria were submitted to Genetic sequence database at the National Center for Biotechnical Information (NCBI). Gene Bank ID of these strains in NCBI is: LN854578 to LN854586.

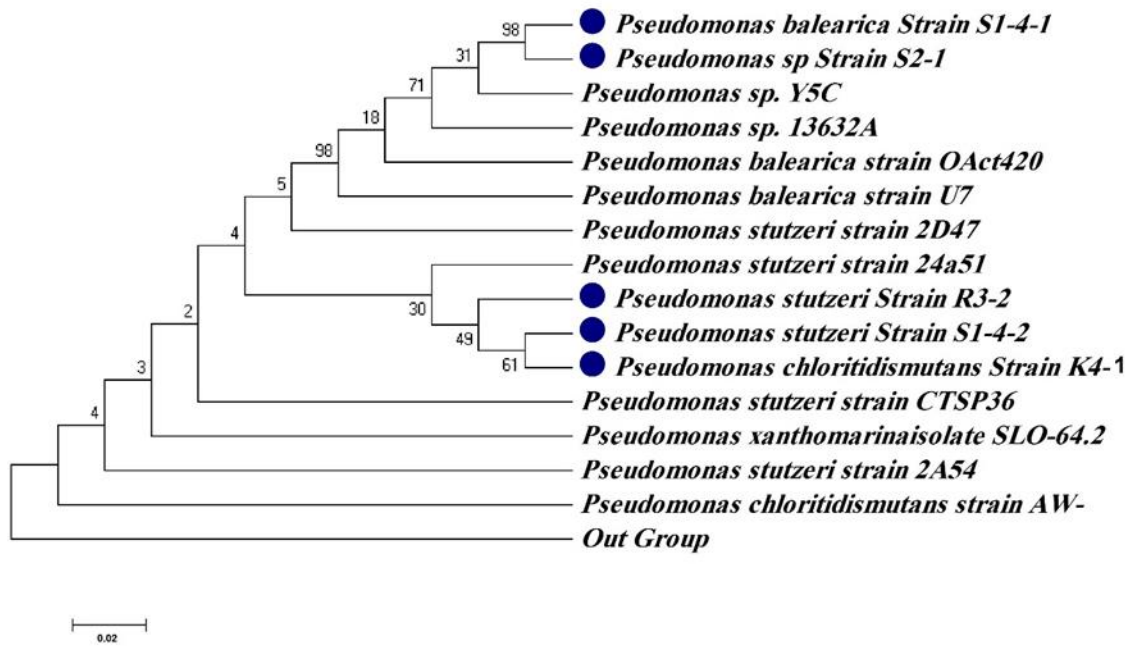

Fig. 1. Phylogenetic tree of 16S rRNA sequences of the isolated strains obtained from the Caspian Sea. The tree was constructed using sequences of comparable region of the 16S rRNA gene sequences available in public databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.02 % sequence divergence. Sequenced data shows the location of isolated strains.

Table 1. Biosurfactant production assay for isolated strains.

Isolate	Hemolytic test	Drop Collapse (shape of drop in comparison to blank)	Oil Spreading (area of oil displaced in mm)
S1-4-2	-	+++	10 mm
S1-4-1	+	+++	14 mm
S1-3	+	++	8 mm
S1-2	+	+++	8 mm
S2-1	+	+++	13 mm
S1-1	_	_	10 mm
K4-2	+	+++	10 mm
K4-3	+	+++	10 mm
R3-1	+	+++	8.5 mm
R3-2	+	+++	10 mm
K4-4	-	+	6 mm
K4-1	+	++	11 mm
R4-3	_	++	7 mm
K1-3	+	++	9 mm
S2-2	+	-	7 mm
R3-4	-	+	6 mm
K1-2	-	-	5 mm
K3-2	+	+	6 mm

Abbreviations:

+, Positive response; _, Negative response (For hemolytic test).

+, low flat; ++, middle flat, +++, completely flat (For drop collapse test).

Table 2. Measurement of Emulsification activity (E_{24} %), Cell surface hydrophobicity (BATH %) and decrease in surface tension in millinewtons per meter (mN m^{-1}) by strains in this study. All data that reported in this table is the mean of three values and cited value in the table is standard deviation.

Isolate	Emulsification activity (E24%)	Cell surface hydrophobicity (BATH %)	Surface Tension* (mN m^{-1})
S1-4-2	62	50	42.1
S1-4-1	100	77	34.5
S1-3	12	8	48.4
S1-2	66	40	44.6
S2-1	58	32	43.3
S1-1	33	6	49.6
K4-2	54	43	41.5
K4-3	100	56	40.3
R3-1	58	69	41.3
R3-2	75	47	39.5
K4-4	62	30	43.8
K4-1	58	54	44.8
R4-3	25	1.7	50.2
K1-3	8	3.7	49.8
S2-2	12	5	51.2
R3-4	13	8	49.7
K1-2	18	7	48.6
K3-2	14	6	51.6

*: surface tension of ONR medium without bacteria as blank is 53.6.

DISCUSSION

Biosurfactants have various physiological roles and provide ecological advantages to the producing strains (Ron & Rosenberg 2001). For instance, they improve the bioavailability of water insoluble substrates by emulsification, thus enhancing biodegradation of several environmental pollutants. Biosurfactant-producing microbes can be found in various ecosystems, although the environments that are impacted by hydrophobic contaminants such as refinery wastes and petroleum, are more yielding than undisturbed ones (Bodour & Miller-Maier 1998; Batista *et al.* 2006). In this study, the bacteria dominated in the enrichment media, were selected due to their ability for utilizing hydrophobic substrates, since hydrophobic pollutants existed in collected seawater and sediment samples, induced growth of these bacteria. Therefore, microorganisms in these samples are adapted to survive with hydrophobic compounds as carbon source. These cultures yielded 44 isolates that were also differentiated based on colony morphology on ONR7a-agar plates. Are there any biosurfactant producers among the isolated bacteria? To answer this question, we adopted a variety of screening assays as suggested by Walter *et al.* (2010). The isolated bacteria were searched for potential biosurfactant producers via three screening phases. To get a preliminary indication, these isolated bacteria were grown on ONR7a containing crude oil as a sole carbon source and inducer of biosurfactant production (initial screening phase). Emulsification of the crude oil in the growth medium indicated production of biosurfactant by the examined bacteria. The cultures which exhibited weak emulsification, showed scarce or no turbidity in the aqueous phase. In these cultures the cells might be adhering to the emulsified oil slicks (Bredholt *et al.* 1998). Emulsification of the crude oil in water is a prerequisite that paves the way for biodegradation of this environmental pollutant by many bacteria. It enhances the bioavailability of the oil and thus increases the biodegradation rate (Bredholt *et al.* 1998) Minf *et al.* (2011). However, visual inspection of the crude oil cultures for biosurfactant production is an indirect screening method, as the growth with hydrophobic compounds indicates the production of biosurfactants, but does not always correlate with this trait (Willumsen & Karlson 1997).

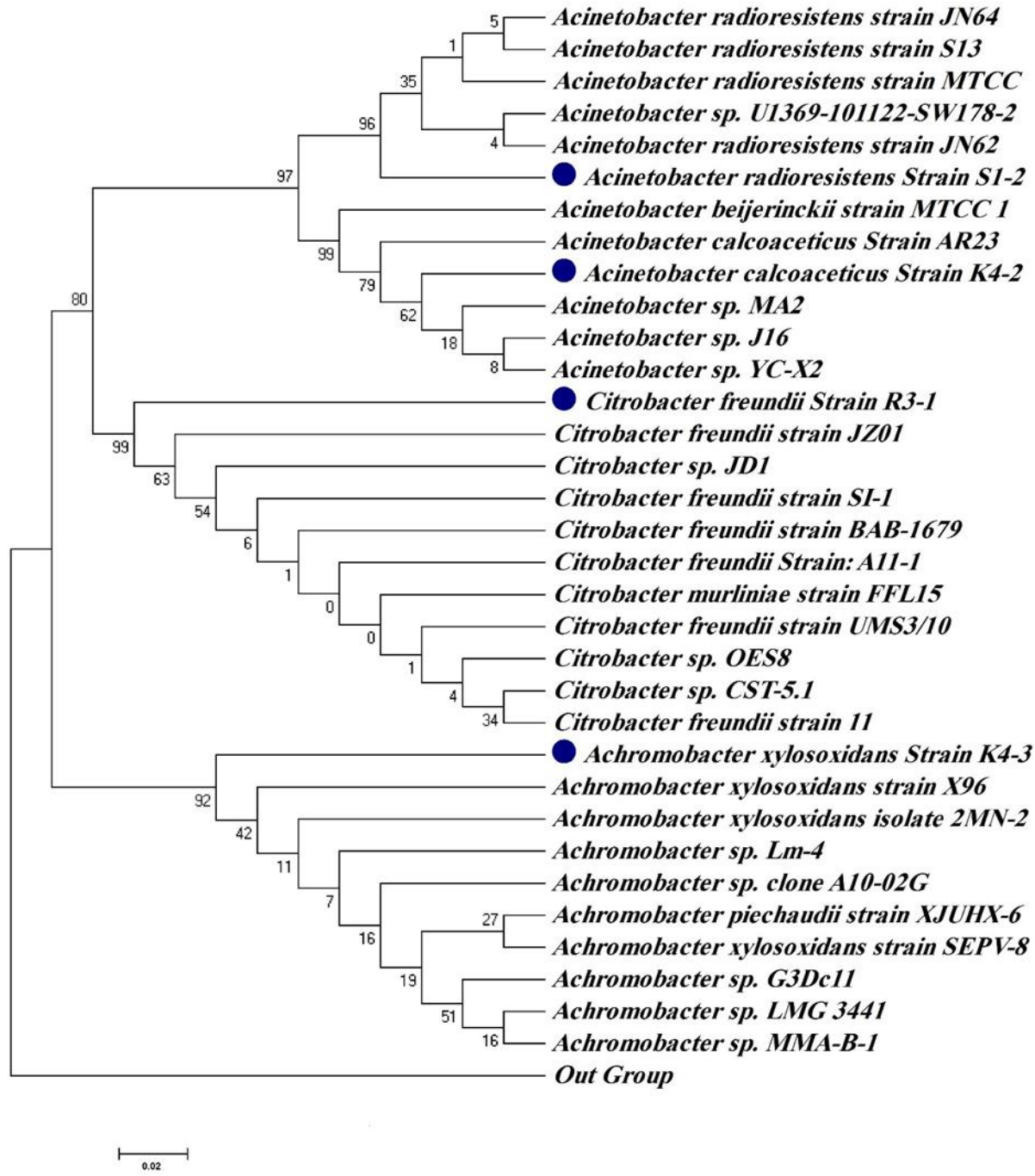

Fig. 2. Phylogenetic tree of 16S rRNA sequences of the isolated strains obtained from the Caspian Sea. The tree was constructed using sequences of comparable region of the 16S rRNA gene sequences available in public databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.02 % sequence divergence. Sequenced data shows the location of isolated strains.

Second screening phase to further investigation on biosurfactant production contain three tests: oil spreading, drop collapse and hemolytic activity assays. The clear zone diameters were larger on cultures of the following strains: S1-4-1, S2-1, K4-2, R3-2, K4-1, and K1-3. However, the largest diameters observed for strain S1-4-1, hence indicating the presence of higher

biosurfactant concentrations. This is in good agreement with the drop collapse and hemolytic activity results. Microbial production of the surface-active compounds on crude oil and other hydrophobic substrates has frequently been reported (Kumara *et al.* 2006). The results of this research confirmed that these bacteria can produce surface active agents, in accordance with the previous researches (Hassanshahian et al., 2014b; Batista *et al.* 2006; Cappello *et al.* 2012b).The data of the third screening phase (surface tension reduction and emulsification activity) confirmed unequivocally the production of biosurfactants by the mentioned strains. Taken together, the data of the screening assays in the three phases are coherent and support each other. Since all the screening assays were performed with cell-free culture supernatants, it can be concluded that the cells produce biosurfactant and secrete them to the medium. Similar results have been reported in many cases (Batista *et al.* 2006; Cappello *et al.* 2012b).

Isolation of biosurfactant producing from marine environment was reported by other authors in the world. For example Coelho *et al.* (2003) reported production of biosurfactant by quinolone-degrading marine *Pseudomonas* sp. strain GU 104. *Alcanivorax* sp. produces a potent glucose–lipid surfactant (Abrahamet *et al.* 1998). In the present study, nine different genera of biosurfactant-producing bacteria were isolated from the Caspian Sea at Iranian shorelines.

Pruti *et al.* (1997) screened biosurfactant-producing bacteria by assaying the cell surface hydrophobicity, finding a direct relationship between this assay and biosurfactant production. Batista *et al.* (2006) isolated 17 biosurfactant producing-bacteria from oil-contaminated beaches in Brazil. They found that six strains can reduce surface tension below 40 mN m^{-1}, in agreement with our results.

CONCLUSION

Marine microorganisms producing biosurfactant, have sufficient diversity and ability in the Caspian Sea. In this study, 9 strains out of 18 biosurfactant-producing bacteria, can produce high biosurfactant and reduce surface tension. Also we found a direct relationship between cell surface hydrop- hobicity and biosurfactant production with emulsification activity. So that, the strain S1-4-1 exhibiting high biosurfactant production, also had efficient emulsification activity.

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غربالگری و شناسایی باکتری های تولید کننده بیوسورفکتانت از دریای خزر

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چکیده

میکروارگانیسم‌های دریایی دارای صفات فیزیولوژیک بی‌نظیر و تولید متابولیت‌های جدید از قبیل بیوسورفکتانت برای زنده ماندن در محیط‌های سخت از قبیل محیط‌های آلوده هستند. هدف از این تحقیق تعیین ویژگی تعدادی از باکتری‌های تولید کننده بیوسورفکتانت است که از نمونه‌های رسوبات و آب دریای خزر جداسازی شده‌اند. باکتری‌های تولیدکننده بیوسورفکتانت با استفاده از روش غنی‌سازی در محیط ONR7a با نفت خام به‌عنوان تنها منبع کربن جداسازی شدند. پنج روش غربالگری برای انتخاب باکتری‌های تولید کننده بیوسورفکتانت به‌کار رفت که عبارت‌اند از: ۱) همولیز در محیط بلاد آگار ۲) گسترش نفت ۳) ریزش قطره ۴) فعالیت امولسیونه کنندگی ۵) چسبندگی باکتری‌ها به هیدروکربن‌ها. باکتری‌های جداسازی شده به روش مولکولی شناسایی شدند. هیجده باکتری تولید کننده بیوسورفکتانت (از ۴۴ کلنی جداسازی شده) انتخاب شدند و ۹ جدایه به عنوان سویه‌های برتر تولید کننده بیوسورفکتانت انتخاب شدند که به جنس و گونه‌های زیر تعلق داشتند.

Pseudomonas balearica (strain S1-4-1), *P.* sp. (Strain S2-1), *P. stutzeri* (strain R3-2), *P. chloritidismutans* (strain K4-1), *Achromobacter xylosoxidans* (strain K4-3), *Acinetoba- cter radioresistens* (strain S1-2), *Acinetobacter calcoaceticus* (Strain K4-2) and *Citrob- acter freundii* (Strain R3-1).

بالاترین قطر هاله شفاف در روش گسترش نفت مربوط به سویه *P. balearica* strain S1-4-1 به میزان ۱۴ میلی‌متر بود. همچنین این سویه دارای بهترین فعالیت امولسیونه کنندگی (۱۰۰٪) بود. آبگریزی سطح سلولی این سویه ۷۷٪، و کاهش کشش سطحی در این سویه ۳۴ واحد بود. بنابراین، این سویه به‌عنوان بهترین سویه تولید کننده انتخاب شد. نتایج این تحقیق ثابت کرد که تنوع مناسبی از باکتری‌های تولید کننده بیوسورفکتانت در دریای خزر وجود دارد و مشکلات زیست محیطی می‌تواند به وسیله کاربرد این باکتری‌ها در پساب پتروشیمی‌ها حل شود.

*مولف مسئول