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Original Article

تولید و تعیین ویژگی های بیوسورفکتانت سویه بومی *میکروباکتریوم* تحمل کننده شوری جداشده از خاک های دریاچه نمک قم

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چکیده

سابقه و هدف: سورفکتانت ها با منشا زیستی، ترکیبات آلی تولیدی توسط میکروارگانیسم ها از جمله کپک ها، مخمرها و باکتری ها هستند که با قرار گرفتن در بین سطوح، باعث کاهش کشش سطحی و بین سحطی می شوند. در این تحقیق به جداسازی سویه های *اکتینوباکتریا* تولید کننده بیوسورفکتانت از خاک دریاچه نمک قم پرداخته شد.

مواد و روش ها: ۱۱۰ سویه *اکتینوباکتریا* از خاک جداسازی و برای تولید بیوسورفکتانت مورد سنجش قرار گرفتند. آزمون های رایج تولید بیوسورفکتانت (همولیز گلبول قرمز، آزمون پخش نفت، سنجش کشش سطح و غیره) صورت گرفت و در نهایت آنالیز *16S rRNA* روی جدایه برتر مولد بیوسورفکتانت انجام شد. آزمون های کروماتوگرافی لایه نازک، طیف سنجی مادون قرمز و آنالیز ساختاری روی بیوسورفکتانت صورت گرفت. بهینه سازی تولید بیوسورفکتانت در حضور منابع کربن و نیتروژن مختلف و عوامل دما، pH و دور همزن انجام شد.

نتایج: از بین ۱۱۰ سویه *اکتینوباکتریا*، ۱۵ سویه قادر به تحمل نمک تا ۱۰٪ بودند. با توجه به آزمون های سنجش تولید بیوسورفکتانت، ۸ سویه قادر به تولید بیوسورفکتانت بودند که از این میان سویه شماره ۹ به عنوان بهترین سویه انتخاب شد و با آنالیز *16S rRNA* در جنس *میکروباکتریوم* قرار گرفت. آنالیز های ساختاری گلیکولیپیدی بودن بیوسورفکتانت را مشخص نمودند. ساکارز و عصاره مخمر به عنوان بهترین منع کربن و نیتروژن و دمای ۲۷ درجه سلسیوس، pH ۱۱ و دور همزن ۱۷۰ rpm به عنوان شرایط بهینه انتخاب شدند.

نتیجه گیری: نتایج این پژوهش پتانسیل سویه *میکروباکتریوم* تولیدکننده بیوسورفکتانت به منظور استفاده کاربردی در پاکسازی زیستی آب و اکوسیستم خاک را نشان می دهد.

واژگان کلیدی: بیوسورفکتانت، تحمل کننده نمک، اکتینوباکتریا، میکروباکتریوم، خاک نمکی.

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Original Article

Production and characterization of biosurfactant by indigenous halotolerant *Microbacterium* sp., isolated from Qom saline soils lake

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Abstract

Background & Objectives: Biosurfactants are biological surface-active compounds produced by molds, yeasts and bacteria. These amphiphilic compounds can reduce surface tension and interfacial tension between individual molecules. The aim of this investigation was screening of biosurfactant (BS) producing halotolerant *Actinobacteria* species from the unexplored regions of Qom saline lake.

Materials & methods: About 110 soil *Actinobacteria* isolated strains were initially screened and then tests for their ability to BS production. Conventional screening methods of BS carried out using blood hemolysis, drop collapse method, oil spreading and surface tension measurements. *16S rRNA* sequencing for the best biosurfactant producing strain, was done. The partially purified BS identified by TLC (Thin Layer Chromatography), FTIR (Fourier-Transform Infrared Spectrophotometery) and compositional analysis. BS production was optimized using different carbon & nitrogen sources and optimized by different culture conditions such as temperature, pH and stirring rate.

Results: 15 out of 110 isolates were able to tolerate high salt concentrations up to 10%. 8 isolated strains were BS producer. Isolate No.9 showed 99% similarity to *Microbacterium paraoxidans* by *16S rRNA* gene sequencing method. Compositional analysis methods proved a glycolipid structure of BS. Sucrose and yeast extract identified as the most appropriate carbon and nitrogen source, respectively. Maximum production of BS obtained at pH 7, temperature 27 ^{o}C and stirring speed 170 rpm.

Conclusion: These findings emphasize that such bacterial strains with superior BS production may find their potential application in bioremediation of marine and soil ecosystems.

Keywords: Biosurfactant, Halotolerant, *Actinobacteria, Microbacterium* sp., saline soil.

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Introduction

Biosurfactants (BS) are biological surface-active compounds produced by bacteria, yeasts, and fungi (1, 2). These amphipathic molecules have some advantages compared to synthetic surfactants, including low toxicity, high biodegradability, better environmental compatibility, high foaming capability, and specific activity at extreme temperature, pH, and salinity (3, 4). Biosurfactants are classified as glycolipids, lipopeptides, lipoproteins, phospholipids, and polymeric biosurfactants structurally (4, 5). However, glycolipids and lipopeptides are the most extensively studied and characterized (6). Bacteria are the major group of BS-producing organisms. However, information on the chemical structure of biosurfactants produced by *Microbacterium* is limited. *Microbacterium* is a genus in *Actinobacteria* phylum. *Actinobacteria* are Gram positive, filamentous, free-living and saprophytic bacteria widely distributed in soil, water, and other ecological niches (7). At first, they considered as an intermediate group between bacteria and fungi and known to produce chemically diverse compounds with a wide range of biological activities, including antibiotics, enzymes, pigments, and biosurfactants (8, 9). There are few reports on the BS production using *Actinobacteria*, for example, lipopeptide BS production by *Streptomyces* Sp.VITDDK3-a (10), glycolipid BS by *Nocardia otitidiscaviarum* (11), and glycolipid BS by *Rhodococcus erythropolis* (12). However, there are no prior findings of biosurfactant production by halotolerant *Microbacterium* strains. Here, we report for the first time the isolation and identification of a local halotolerant strain of *Microbacterium* with the considerable capability of biosurfactant production that may be appropriate for various industrial applications. This accomplished by a comprehensive screening of halotolerant *Actinobacteria* isolated from Qom saline lake. This accomplished by (1) comprehensive examinations of bacterial strains isolated from Qom saline lake for their ability to produce biosurfactant, (2) isolation of halotolerant Actinobacteria from isolated samples (3) purification of biosurfactant and testing its stability at different temperatures and pH (4) preliminary identification and characterization of the produced biosurfactant and (5) growth and BS production kinetic studies.

Materials and methods

Sampling and selective isolation of Actinobacteria

Soil samples collected from various locations of saltpan of Qom saline lake (345734N, 505413E), Qom province, Iran. The samples collected in plastic bags and immediately transported to the Laboratory and stored in an appropriate condition for further processing. Collected soil samples suspended in sterile water. 2-3 droplet of tween 80 added and mixed on rotary incubator shaker at 150 rpm for 20 minutes. Then soil samples were serially diluted and 0.1 ml of each dilution was plated on Starch Casein Agar (1 % (w.v) starch, 0.1 casein, 0.03 KNO3, 0.02 NaCl, 0.02 K2HPO4, 0.02 MgSO4.7H2O, 0.005 CaCO3,

0.002 FeSO4, 0.001 agar, 1.8 with 1 % v.v of olive oil) by spread plate method (13, 14). To isolate halotolerant Actinobacteria, different concentrations of NaCl added to the medium. The plates incubated at 28-30oC for 7-10 days. The colonies with morphological differences were selected and purified by streak plate techniques and were maintained for further studies (15).

Screening methods for biosurfactant activity

Various methods including hemolytic assay, drop-collapsing test, oil spreading test, emulsification activity as well as surface tension analysis employed to identify and screen BS producer bacteria among isolated *Actinobacteria*.

Hemolytic activity: Pure culture of *Actinobacteria* isolates streaked on the freshly prepared blood agar (with 5% human blood) and incubated at 28-30 C for 7-10 days. The plates were then examined for the zone of clearance around the colonies (16).

Oil spreading test (OST): The standard procedure of oil spreading test done by adding 50 ml of distilled water to a Petri dish with a diameter of 15 cm. Then, 20 µl of crude oil dropped onto the surface of the water, followed by the addition of 10 µl of the broth containing a biosurfactant onto the surface of the oil. The diameters of the clear zones were determined for an averaged value of the clear zone diameter of triplicate experiments (17).

Drop collapsing test: 2µl of Crude oil poured in 96-well microtitre plate. This left to equilibrate for 1 hour at 37 C. 5µl of the culture supernatant added on the surface of the oil in the well. The shape of the drop on the oil surface observed after 1 min. The result was positive when the drop was flat, indicating the production of BS. It scored as negative for rounded drops, indicating the lack of BS presence (18).

Lipase activity: Lipase production by the Actinobacteria pure culture was determined using tributyrin agar plates. The cultures were inoculated on the plates and incubated at 28-30oC for seven days. After this, the plates examined for clear zone around the colonies (19).

Emulsification activity: The emulsification activity of produced biosurfactant was measured, according to Cooper and Goldenberg (20). Briefly, 4 ml of cell-free supernatant added to 6 ml kerosene and vortexes at high speed for 2 min. The emulsification index (E24) is the height of the emulsion layer divided by the total height that measured after 24 h and expressed as a percentage.

Surface tension (ST): ST was measured using Krüss K100MK2 tensiometer (Krüss GmbH, Hamburg, Germany) using Wilhelm plate technique. The tensiometer calibrated against double distilled water. Before each measurement, the probe rinsed several times with double distilled water and acetone followed by flaming on Bunsen burner.

Biochemical and molecular identification of selected isolates

Gram staining, oxidase, catalase, gelatinase and cellulase tests, starch hydrolysis, casein hydrolysis, nitrate reduction as well as sugar assimilation tests were employed for initial biochemical identification of selected isolate

(21). Molecular identification of selected bacterial isolate referred for *16S rRNA* gene sequence analysis. In order to the extraction of DNA, selected isolate initially grown in 10 ml SCA with agitation at 30 OC for 2 days. After centrifugation (7500×g, 2 min) and elimination of supernatant, the pellet washed with 500 ml of 10 mM Tris-HCl.1 mM EDTA (TE) buffer (pH 7.7) and resuspended in 500 ml TE buffer (pH 7.7). The sample heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500×g, 3 min). The supernatant (300 ml) was transferred to a clean tube and stored at 4 ◦C (22). The *16S rRNA* gene was amplified by the PCR method using universal primers 27F (5'-AGAGTTTGATYMTGGCT CAG- 3') and 1492R (5'-GGTTACCTTGTTASGACTTC-3'). The reaction was carried out in a 25 µl volume containing 1x PCR buffer, 1.5 mM MgSO4, 2mM dNTP mixture, 1 µM of each primer, 1 µl of Pfu DNA polymerase (Fermentas, St. Leon-Rot, Germany) and 1 ng of template DNA. PCR amplification performed as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles each of 94 °C for 35 s, 57 °C of annealing for 35 s, and a 150 s extension at 72 °C. The 203 and 124 bp PCR products amplified from the bacterial isolates which appeared as a single band were purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Germany) and sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). Sequence homologies were examined using BLAST version 2.2.12 of the National Center for Biotechnology Information (23). Multiple sequence alignments were carried out using ClastalW, and a consensus neighbor-joining tree was constructed using molecular evolutionary genetics analysis (MEGA) software (version 4.0) (23).

Extraction and purification of biosurfactant

The produced BS extracted from the culture broth of strain with the method of acid precipitation followed by solvent extraction reported elsewhere (24). Briefly, the culture broth was centrifuged (6000×g, 10 min, 4 ˚C) to remove the cells. Then pH was adjusted to 2 by 3N HCl in order to precipitate the biosurfactant. After keeping, it overnight, the crude BS obtained by centrifugation (18000 g, 30 min, 4 °C). The crude BS partially purified using several times extraction by ethyl acetate. Purification of BS was performed using preparative layer chromatography (PLC) (silica gel 60, 20 cm x 20 cm, Merck), as reported by Hajfarajollah et al. (6). For this purpose, partially purified biosurfactant dissolved in chloroform and then spotted on the plate. The plate later developed with the solvent system of chloroform.methanol.distilled water (65:45:4). Visualization carried out by UV transilluminator. In preparative mode, visualized spots scraped off and extracted more than six times with 10-15 ml of ethyl acetate. Fractions separated and evaporated under vacuum. The oil spreading test revealed that only one of the spots (among three fractions obtained) was biosurfactant. This compound was stored as a pure biosurfactant for further studies.

Characterization of dried biosurfactant

Thin-layer chromatography (TLC): TLC is a simple method for primary characterization of biosurfactants. The purified biosurfactant dissolved in ethyl acetate and applied to TLC plate (silica gel 60, Merck) to develop in various solvent systems. Chloroform. methanol.water (65:45:4) and hexane. ethyl acetate (8:2) were taken as solvent systems. After development, one of the plates put into a jar saturated with iodine vapors to detect lipids. Two other plates sprayed evenly with the ninhydrin reagent (0.5 g ninhydrin (Sigma-Aldrich) in 100 ml anhydrous acetone) and molisch reagent (a solution of α-naphthol in 95% ethanol) to detect the presence of peptide as red spots or carbohydrate as blue spots (6, 25).

Fourier Transform Infrared spectroscopy (FTIR): The purified BS was analyzed using FTIR spectroscopy by Perkin-Elmer Spectrometer (FTIR GX 2000). Biosurfactant samples dried first and then ground before performing FTIR analysis. The dried powder finely mixed with a KBr matrix (Sigma). The spectra were in the range of 400–4000 cm−1 with 8 cm−1 resolution.

Composition analysis: Carbohydrate content of the produced biosurfactant was determined by the Dubois method of phenol–sulfuric acid using D-glucose as a standard (26). Lipid content was estimated according to the Folch et al. (27) Protein content was determined by the method of Bradford (28) using coomassie brilliant blue with bovine serum albumin as a standard.

Effects of various carbon and nitrogen sources

In order to examine the effect of some essential components of the medium on the growth of strain and BS production, different carbon and nitrogen sources evaluated. The examined carbon sources were glucose, sucrose, fructose, xylose, glycerol, starch, and sunflower oil. Nitrogen sources, on the other hand, include potassium sulfate, ammonium sulfate, ammonium chloride, peptone yeast extract, and beef extract. The maximum diameter of the clear zone in oil spreading test during cultivation reported as an indicator of BS production. The maximum optical density also measured during cultivation to determine bacterial growth.

Optimization of culture condition

The selected strain was cultivated in different pH (5.5, 6.5, 7 and 7.5), Temperature (28, 30, 37) and stirring speed (150, 200, 250) to evaluate the optimum cultural condition for BS production. Maximum diameter of the clear zone in oil spreading test and optical density measured as indicators of BS production and bacterial growth, respectively.

Growth kinetics and biosurfactant production

A 4% cell suspension (in physiological saline) from an overnight seed culture medium inoculated in 2000 ml flasks containing 500 ml medium and incubated at 30 ◦C while shaking at 200 rpm. Sucrose used as carbon source. During fermentation, samples aseptically retrieved from the liquid culture at different time intervals to monitor the kinetic parameters, including cell growth, biosurfactant concentration, surface activity (diameter of the clear zone (cm)), and pH of culture medium. Bacterial growth was determined by measuring

the optical density of the culture broth at 600 nm by UV spectrophotometer (PerkinElmer, Lambda 25, USA).

Stability of biosurfactant

To evaluate the stability of the biosurfactant at different environmental conditions, 50 mg.ml solution of the biosurfactant was prepared and maintained at temperature range of 20–90 °C for 120 h. After cooling to room temperature, ST measured as an activity indicator of the biosurfactant. To determine the effect of pH on the biosurfactant activity, the ST was measured after adjusting the pH between 4-13 using 3 N NaOH or 3 N HCl. The effect of the addition of different concentrations of NaCl on the activity of the biosurfactant studied as well.

Results

Sampling and isolation of BS producers

Among many numbers of soil samples collected from saline soil of Qom saline lake, 110 *Actinobacteria* strains were isolated after a series of serial dilution steps and plating procedures. Fifteen isolates were able to tolerate high salt concentrations, up to approximately *10%* of NaCl. These 15 isolates tested for their ability to produce BS. Table 1 shows the results of hemolytic activity, oil spreading test, emulsification activity as well as surface tension analysis for 15-selected halotolerant *Actinobacteria*.

Biochemical and molecular identification of the selected isolate

Strain No. 9 selected as a BS producer for further studies. The selected strain identified by PCR method followed by *16S rRNA* gene sequencing, according to section 2.3. The biochemical tests also performed for a better understanding of the strain. Table 2 shows biochemical characteristics of this strain. The

Table 1. Hemolytic activity (+,-), Emulsification activity (%), oil spreading test (mm) and surface tension (mN/.m) results for 15 Actinobacteria isolated from saline soils.

Isolate NO.	Beta hemolysis	E24%			OST (mm)	ST (mN/.m)
		kerosene	Gasoline	Crude oil		
1	+	16	16	60	10	50.2
2	-	0	0	0	0	71.5
3	+	5	5	18	41	44.6
4	-	12	0	0	2	66.6
5	-	42	5	8	4	69.8
6	+	20	25	48	22	38.1
7	+	15	20	23	43	44.6
8	+	32	25	30	12	34.8
9	+	72	80	84	71	28.1
10	-	18	11	5	9	51.2
11	+	25	5	12	13	45.2
12	+	2	2	37	24	37.8
13	+	12	12	37	55	35.4
14	-	15	0	5	3	70.7
15	+	20	18	42	50	38.5

Table 2. Biochemical identification of the selected isolate.

Tests	Results
Oxidase	-
Catalase	+
Range of NaCl (%)	0 - 10
pH	11
Gelatinase	-
Casein hydrolysis	+
Starch hydrolysis	+
Tween 80	-
celloluse	-
Urease	-
DNase	-
Lysosyme	+
Nitrate reduction	+
Anaerobic growth	-
Xylose	-
Glucose	-
Sucrose	+
Raffinose	-
Rhamnose	-
Manitol	-
Fructose	-

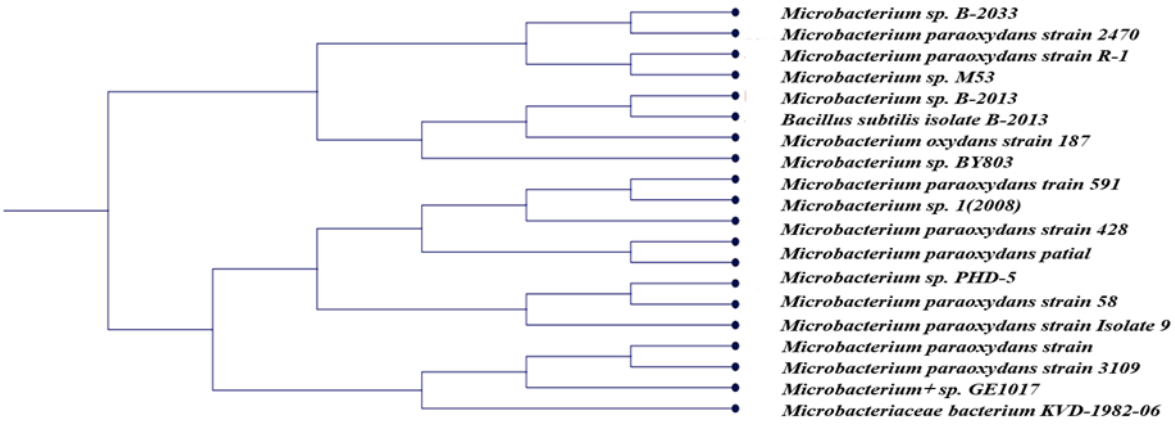

Fig. 1. Phylogenetic tree based on *16S rRNA* gene sequence analysis of isolate No. 9, showing homology with different sources of *Microbacterium*

Table 3. Optical density (OD) and (OST) for Carbon and Nitrogen sources.

Carbon source	OST-Carbon	OD-Carbon	Nitrogen sourse	OST-Nitrogen	OD-Nitrogen
Glucose	0.00	0.20	potassium Nitrate	5.00	1.00
Sucrose	6.60	4.00	Ammonium Sulfate	2.00	0.80
Fructose	0.00	0.45	Ammonium Chloride	2.10	0.70
Xylose	2.00	1.10	Peptone	5.00	1.40
Starch	6.70	2.00	yeast Extract	6.00	1.70
Glycerol	3.40	0.30	Beef extract	6.00	3.70
Sunflower	1.10	0.80	--	--	--

bacterium is gram-positive, aerobic, non-motile, and often form a mycelium, which at maturity form chains of spores. The sequence examined using BLAST version 2.2.12 of the National Center for Biotechnology Information. Multiple sequence alignment carried out using ClastalW, and a consensus neighbor-joining tree was constructed as indicated in Fig 1. The *16S rRNA* gene sequences showed 99% similarity with *Microbacterium paraoxydans* in the existing NCBI database.

Effect of various carbon and nitrogen sources on surface activity

The isolate grown on the various carbon and nitrogen sources, as indicated in Table 3, Fig 2, and 3. The diameter of the clear zone as an indicator of BS production and optical density

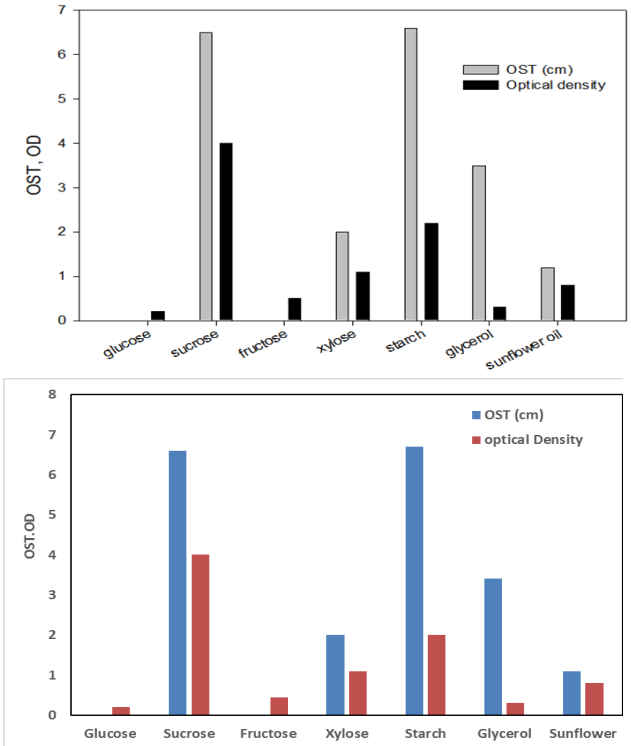

Fig. 2. Effect of various carbon sources on cell growth and surface activity in terms of optical density and diameter of clear zone (OST).

as a growth indicator measured. Fig 2 shows the effect of different carbon sources on the surface activity of the produced biosurfactant. As shown, biosurfactant production varied significantly from one source to another. Sucrose was the most effective amongst water-soluble carbon substrates with a clear zone diameter of more than 6 cm. A similar manner observed over starch as a carbon source. Surprisingly, poor results both, in terms of bacterial growth and BS production, obtained when the strain was grown on glucose. The clear zone diameter over fructose was also zero, showing the biosurfactant production on these carbon sources is insignificant. Maximum clear zone diameters obtained on sucrose and starch. Therefore, it concluded that these carbon sources are more efficient in the production of biosurfactant by selected strain. So, fructose selected as a carbon source for further experiments. Among different nitrogen sources, beef extract and yeast extract found to be the most suitable nitrogen sources for BS production. Fig. 3 presents the effect of nitrogen sources on growth and BS production. Ammonium sources showed poor results compared to other nitrogen sources. According to the obtained results, yeast extract selected as a nitrogen source for further experiments. Statistical analysis with the SPSS software ver. Twenty-three confirmed a positive Pearson and Spearman correlation coefficient between the OD and OST of different carbon and nitrogen sources. In the next series of experiments, the culture condition optimized with the method of one factor at a time. The diameter of the clear zone and bacterial growth considered responses. The overall results of this step of the study showed that the temperature of 30 oC, pH 7, and stirring rate of 200 would be the most appropriate amounts for BS production and growth of selected strain. Therefore these amounts will be used in further experiments.

Kinetics of the growth and biosurfactant production

Table 4 and Fig. 4 show the variation of bacterial growth, the diameter of clear zone, surface tension (ST), and pH over the cultivation period. Sucrose used as a carbon

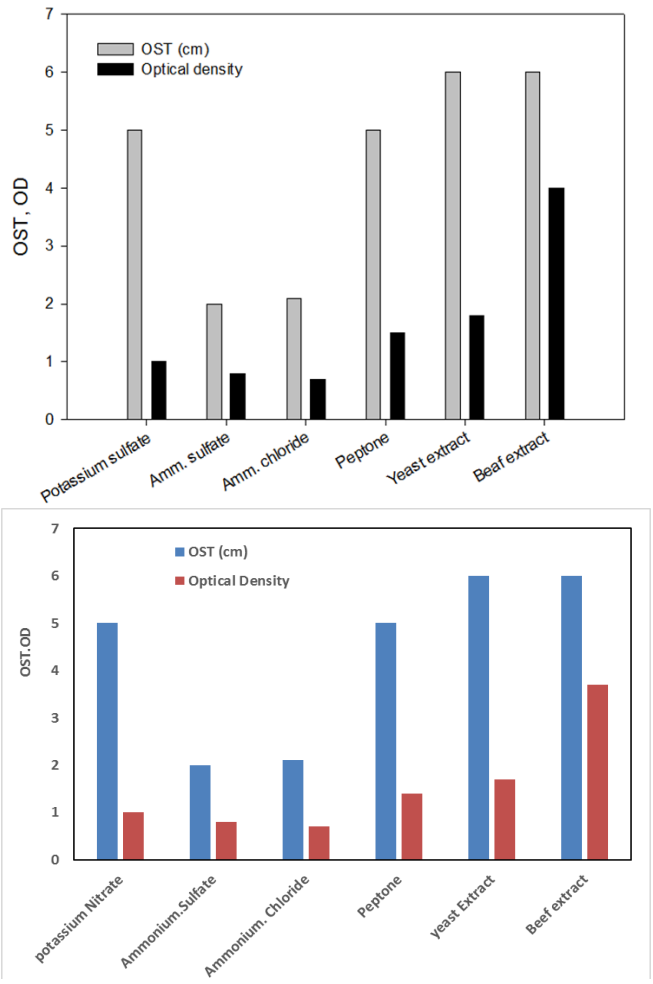

Fig. 3. Effect of various nitrogen sources on cell growth and surface activity in terms of optical density and diameter of clear zone (OST).

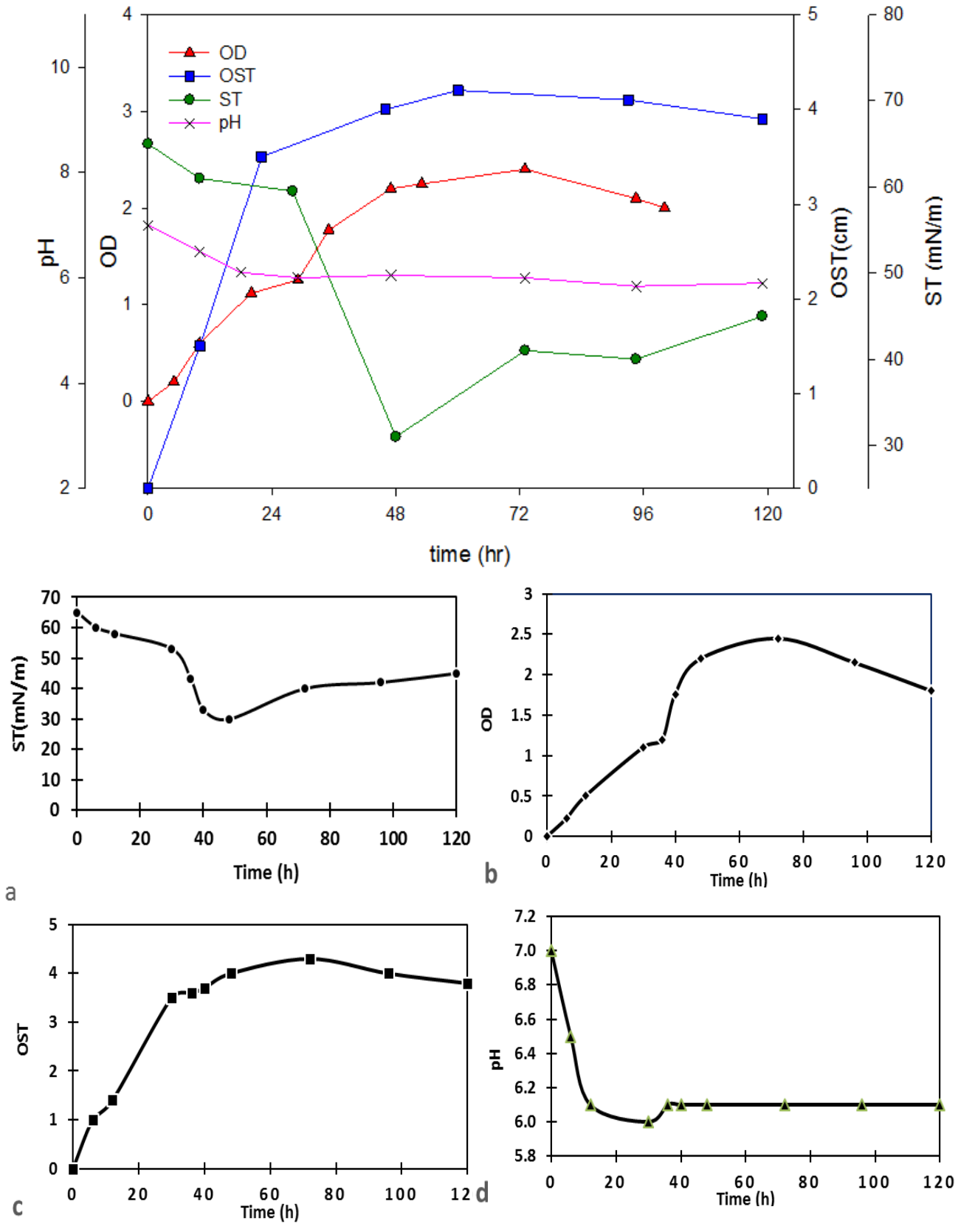

Fig. 4. (a,b,c,d). Kinetics of bacterial growth and BS production by the selected strain, a) ST; b) OD; c) OST; d: pH

source, while yeast extract used as a nitrogen source. As observed in the Figure, bacterial growth initiated from the first hours of cultivation and reached to its maximum level after about 48 h.

Characterization of biosurfactant

The chemical composition analysis revealed that the biosurfactant produced by *Microbacterium* sp. belongs to glycolipid type. Primary characterization of the biosurfactant done by thin-layer chromatography. After air-drying, the developed plate was placed in a tank containing iodine vapors, and a yellow spot was appeared indicating the presence of lipid. In order to detect carbohydrate, another TLC plate treated with molisch reagent. A positive reaction in the molisch test indicated by green-blue color on the substance tested. These results partially confirmed the glycolipid nature of the biosurfactant. For further complementary characterization, the FTIR spectroscopy did. Fig. 5 shows the FTIR spectral peaks of purified glycolipid biosurfactant. The IR spectrum of the produced biosurfactant compared with the IR spectra da-ta of some known biosurfactants, and it was very similar to glycolipid BS. Peaks at 2920-2930 cm-1 indicates C-H stretching band of CH2 and CH3. The absorption band at 3387 related to carboxylic acids group O-H stretch. The peak at 1642 cm-1 assigned to ring stretching, which related to rhamnose ring. The ester carbonyl also proved from the band at about 1120. Other important bands observed on the figure. Thus, all these observations confirmed that the biosu factant produced by the isolate has a glycolipid structure (6).

Stability of the produced biosurfactant

The biosurfactant produced by *Microbacterium* species shown to be thermo-stable (table 5 and Fig. 6). The heating of the biosurfactant up to 100 °C caused no significant effect on the BS performance. Therefore, it concluded that the BS produced from the selected strain preserves its surface

Table 4. Optical Density (OD), Clear zone Diameter (OST), pH and Surface tension versus Time (hour).

Time	OD	OST	PH	ST
0	0	0	7	65
6	0.22	1	6.5	60
12	0.5	1.4	6.1	58
30	1.1	3.5	6	53
36	1.2	3.6	6.1	43
40	1.76	3.7	6.1	33
48	2.2	4	6.1	30
72	2.45	4.3	6.1	40
96	2.15	4	6.1	42
120	1.8	3.8	6.1	45

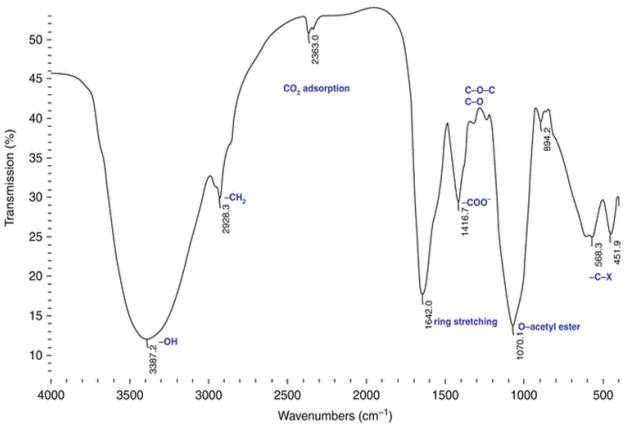

Fig. 5. FTIR spectrum of the produced biosurfactant by *Microbacterium paraoxidans*. Biochemical composition analysis revealed that the produced biosurfactant is composed of 18.6 % carbohydrate, 66% lipid. The presence of minor fraction of other components in the purified sample possibly arising from the residual cell debris's in broth co-precipitated with biosurfactant during its extraction process.

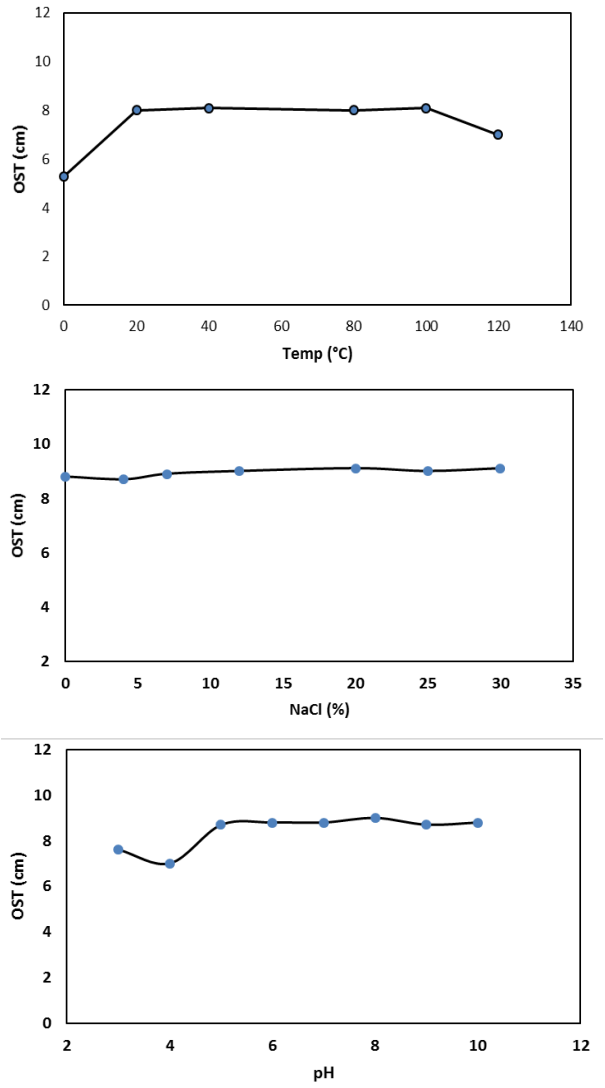

Fig.6. Stability of biosurfactant as a function of temperature, pH and Salinity.

properties unaffected in the wide range of temperatures between 20 – 100 °C. At 120 hours after bacterial culture, four variables of OD, OST, pH, ST measured at different times, and the results plotted in two different diagrams. In the first method, the changes of all four variables plotted on a graph, and in the second, the ST variable plotted as a separate graph from the other three variables. The reason for this change is the large difference in the amplitude of ST changes with the other three variables. Statistical analysis with the SPSS software ver. 23 confirmed that there is a high positive Pearson and Spearman correlation coefficient between the pH and NaCl, low positive between NaCl and temperature and between pH and temperature.

Table 5. Clear zone diameter (OST) versus temperature, pH and NaCl concentration.

PHOST	OSTPH	TempOST	OSTtemp	naclOST	OSTnacl
3.00	7.60	0.00	5.30	0.00	8.80
4.00	7.00	20.00	8.00	4.00	8.70
5.00	8.70	40.00	8.10	7.00	8.90
6.00	8.80	80.00	8.00	12.00	9.00
7.00	8.80	100.00	8.10	20.00	9.10
8.00	9.00	120.00	7.00	25.00	9.00
9.00	8.70	-	-	30.00	9.10
10.00	8.80	-	-	-	-

Table 6. Correlation Coefficient and Sinificant 2-tail (P value) of relationship between four variable: OD, OST, pH and ST .

Correlations

		OD	OST	pH	ST
OD	Pearson Correlation	1	**.933****	**-.680***	**-.894****
	Sig. (2-tailed)		.000	.030	.000
	N	10	10	10	10
OST	Pearson Correlation	**.933****	1	**-.828****	**-.870****
	Sig. (2-tailed)	.000		.003	.001
	N	10	10	10	10
pH	Pearson Correlation	**-.680***	**-.828****	1	**.653***
	Sig. (2-tailed)	.030	.003		.041
	N	10	10	10	10
ST	Pearson Correlation	**-.894****	**-.870****	**.653***	1
	Sig. (2-tailed)	.000	.001	.041	
	N	10	10	10	10

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Discussion

Saltpans are one of the hypersaline extreme environments, which are a reservoir of hundreds of microorganisms. Under the stressing condition, the organisms forced to produce several secondary metabolites like biosurfactants to survival. One-hundred and ten *Actinobacteria* strains were isolated from Qom saline lake which 15 isolates among them were able to tolerate high salt concentrations, up to approximately 10%NaCl. These 15 halotolerant *Actinobacteria* examined for biosurfactant production by standard tests. According to the results, 68% of these 15 isolates were positive for hemolytic activity, and 33% considered highly positive based on the oil spreading method and showed a diameter of the clear zone more than 40 mm. The latter screening stage included surface tension (ST) and emulsion activity measurements. As it is known, the less the ST, the more efficient is the produced BS. A good BS can reduce the ST to the amounts less than 35 mN.m. As can be observed from the results of Table 1, only two out of 15 halotolerant isolates could reduce the ST below 35 mN.m. Emulsification activity (E24%) using three different hydrocarbons, including crude oil, gasoline, and kerosene were also measured as another critical test for BS producer screening. As an overall conclusion, according to the results obtained, strain No. 9 showed the best results in biosurfactant screening tests. The strain No. 9 gave also beta hemolysis on a blood agar plate. Researches show that there is an association between hemolytic activity and biosurfactant production (17). Oil spreading test results in a clear zone diameter of about 70 mm, which is a reliable way indicating the surface activity. Morikawa et al. reported that the area of oil displacement in oil spreading assay is directly proportional to the concentration of the biosurfactant in the solution (17). Isolate No. 9 also showed excellent positive results in emulsification activity. As can be observed in table 1, the emulsification activity of more than 70% obtained using kerosene and over 80% using crude oil. The most meaningful result, i.e. surface tension showed the produced BS in the culture of strain No. 9, can reduce surface tension to about 28 mN.m. Drop collapse analysis also confirmed the production of BS by strain No. 9 (data not shown). All these results confirmed the ability of strain No. 9 to produce surface-active agents. *16S rRNA* revealed the strain No. 9 showed 99% similarity with *Microbacterium paraoxidans* in the existing NCBI database. In another stage, Different environmental, as well as nutritional factors, optimized for biosurfactant production. pH 11, Tem 300C, and 5% (w.v) NaCl were selected as the best environmental factors. When the initial pH of the medium was set in the range of 8-11, the best biosurfactant production observed. Similar studies reported by Manivasagan *et al* in 2013, that at pH 13, surface tension decreased up to 28 mN.m, whereas at pH 7, activities decreased to 41 mN.m (14). These results revealed that an elevated pH is having a positive effect on reducing surface tension and emulsion stability, which could cause by better stability of fatty acids surfactant micelles in the presence of sodium hydroxide. One of the advantages of the biological surface-active

compounds to surfactants is resistance to ionic strength. While 2 % of salts is enough for inactivation of most chemical surfactants, biosurfactants can remain activated in solution with 10 % (w.v) NaCl (atlas 1991). In this study, the isolate 9 tolerated 10 % (w.v) NaCl, but the best biosurfactant production was observed at 5% (w.v) NaCl. Similar results obtained by Yakimov et al. in 1995, for resistant biosurfactant produced by Bacillus strain, which remained activated at 13 % (w.v) NaCl. Among different carbon and nitrogen sources, sucrose and yeast extract found to be the most suitable sources for biosurfactant production. BS production increased over time and reached a constant level after about 48 h. All these observations shows that BS production is growth associated. The ST changes over cultivation time can confirm previous results. ST decreased from 72 to about 30 mN.m after about 48 h. ST started to increase after 48 h may be due to BS degradation in the broth by the strain itself. Similar manner observed by Hajfarajollah *et al.* (6). Different concentration of oils, surfactants, and hydrocarbons added to the optimized medium of strain 9. It observed that olive oil, tween 80, and crude oil had the greatest impact on biosurfactant activity and selected as the best sources for biosurfactant production. Khopade et al. in 2012 have reported similar results for Streptomyces species B3 (15). Structural analysis done by using TLC and FTIR. It was observed three glycolipid spots in TlC analysis, and FTIR spectrum showed structural similarity of the resulting bisourfactant with other glycolipid biosurfactant. Manivasagan et al. in 2013 obtained similar results, which FTIR analysis of biosurfactant produced by *Streptomyces* sp. MAB36 confirmed the glycolipid structure (14). This Biosurfactant showed thermo-stable feature up to 100 °C. This activity was indicated the usefulness of the BS in food, pharmaceutical, and cosmetics industries as well as its application in high-temperature bioremediation. Recently, it was reported that bioremediation in the presence of biosurfactant at high temperatures can lead to better results. The surface activity of the crude biosurfactant remained relatively stable over pH range 5-10. The BS also remains its stability at NaCl concentrations between 10-30%.

Conclusion

In the present work, the ability of strains separated from saltpans investigated for biosurfactant production. Among 110 isolated colonies, 15 strains tolerate high salt concentration. These isolates assayed by screening methods to identify the strains with capability for BS production. Hemolytic activity, oil spreading test, drop collapse assay as well as surface tension measurements revealed that strain No. 9 has a significant capability to produce BS. The strain identified as *Microbacterium* sp. By PCR method followed by *16S rRNA* gene sequencing (Accession number KX499494). The extracted BS from culture broth of the selected strain purified and identified as glycolipid using TLC, FTIR and compositional analysis. Effect of various carbon and nitrogen sources evaluated and environmental culture condition (temperature, pH, and stirring rate) optimized.

Kinetics evaluation of bacterial growth and BS production revealed that BS production is growth associated. At the final step, the stability of BS in different temperature, pH, and salinity investigated.

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Ethical Consideration

Authors of all ethics including non-plagiarism, Dual publishing has complied with data distortions and data making in this article.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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