Archive of SID

http://dx.doi.org/10.22037/afb.v%vi%i.23663

Research Article

APPLIED FOOD BIOTECHNOLOGY, 2019, 6 (2):127-138
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Production and Characterization of Biosurfactants Using Bacteria Isolated from Acidic Hot Springs

Somayeh Ebrahimi[1], Hasan Jalili[1*], Khosro Khajeh[2], Kambiz Akbari Noghabi[3], Maryam Tajabadi Ebrahimi[4], Abdeltif Amrane[5]

1- Life Science Engineering Department, Faculty of New Sciences and Technologies, Tehran University, Tehran, Iran.

2- Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.

3- Division of Industrial & Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

4- Department of Biology, Faculty Member of science, Islamic Azad University, Central Tehran Branch, Tehran, Iran.

5- Ecole Nationale Supérieure de Chimie de Rennes / Université de Rennes 1, CNRS, UMR 6226, 11 allée de Beaulieu, CS 50837, 35708 Rennes cedex 7, France.

Abstract

Background and objective: Biosurfactants are increasingly used by food industries due to their low toxicities and unique structures. In this study, biosurfactants were produced and characterized for the first time using acidic bacteria isolated from acidic hot springs in Bushehr Province, Iran.

Material and methods: Screening and identification of the most efficient species for biosurfactant production were carried out on 12 bacterial species using several experiments such as hemolysis, surface tension, emulsification index and diameter of clear zone. In addition to biosurfactant production, kinetics, stability and structural and thermal analysis were carried out for the bacterial strains using thin layer chromatography, Fourier Transform Infrared, nuclear magnetic resonance and differential scanning calorimetry.

Results and conclusion: The biosurfactant from the selected bacteria (0.1 g l^{-1}) was thermally stable at 120°C for 30 min. Stability at temperatures up to 140°C was confirmed using differential scanning calorimetry. The most significant novelty included the fact that the surface property was preserved until an osmolarity of 4% w v^{-1}. Decreased surface tension and the emulsification potential were only reported at concentrations higher the highlighted concentration. Biological assay showed that *Staphylococcus aureus* was susceptible to produced biosurfactants, while no susceptibility was seen in *Escherichia coli*. Degeneration of SW480 cell line exposed to 0.601 µg μl^{-1} of the biosurfactant was detected after 24 h. The structural analysis showed that the biosurfactant was similar to surfactin as a food bioemulsifier.

Conflict of interest: The authors declare that they have no conflict of interest.

Article Information

Article history:

Received	17 Jun 2018
Revised	23 Dec 2018
Accepted	30 Jan 2019

Keywords:
- Acidic hot spring
- Biosurfactant production
- Characterization
- Extremophile

***Corresponding author:**
Hasan Jalili,
Faculty Of New Sciences and Technologies, North kargar St. Tehran, Iran, P.O. Box: 14395-1561.

Tel: +98-2188618431-3
Fax: +98-2188617087
E-mail: hjalili@ut.ac.ir

1. Introduction

Surfactants are surface-active agents accumulated at the interfacial surfaces. Many organisms such as bacteria, algae, yeast, fungi and animal cells include the ability of producing these molecules which are found either attached to the cell surfaces or in secreted forms [1]. As amphipathic molecules, surfactants include hydrophilic and hydrophobic moieties, including glycolipids, lipopeptides, fatty acids and polymeric and particulate biosurfactants [2]. Biosurfactants include multiple biotechnological uses in petroleum, food, pharmaceutical, agriculture and cosmetic industries [3]. Relating chemical synthetic surfactants, these biomolecules include several advantages. Examples of these advantages include biodegradation capability, low toxicity, bio-compatibility, availability, environmental cleanup usage, exclusivity linkage to specific functional groups, detoxification of pollutants, special usage in pharmaceutical and cosmetic industries, resistance to temperature, pH and ionic strength and higher surface and interfacial activities [4,5].

www.SID.ir

Archive of SID

Therefore, attentions to these biomolecules as alternatives to synthetic surfactants are rising. The biosurfactant production in microorganisms is influenced by various physiochemical factors, including carbon and nitrogen sources, carbon-nitrogen ratio, pH, temperature and agitation rate [1]. Literature reviews show no current reports on the biosurfactant production ability of microbial strains isolated from Iran hot springs. Therefore for the first time in the current study, 12 bacterial species isolated from acidic hot springs in Bushehr's Province (south west of Iran) were assessed for the biosurfactant production capability. Furthermore, characteristics and uses of these molecules were investigated.

2. Materials and methods

2.1. Bacteria and media

Bacterial species previously isolated and reported by Kazemi et al. from Bushehr's acidic hot springs were investigated for the biosurfactant production [6]. Luria-Bertani (LB) media, containing 1% tryptone (Merck, Germany), 1% NaCl (provided by Dr. Mojallali, Iran) and 0.5% yeast extract (Liofilchem, Italy) was used for the bacterial strain culture [6]. To assess ability of bacterial growth on a hydrophobic source, the culture media included 2% v v^{-1} of corn oil (Bahar, Iran) as unique carbon source; sterilized using filtration through 0.45-µm membrane filters. The media components were as follows: 2.2 g l^{-1} of $NaHPO_4$, 1.4 g l^{-1} of KH_2PO_4, 0.6 g l^{-1} of $MgSO_4.7H_2O$, 3 g l^{-1} of $(NH_4)_2SO_4$, 1 g l^{-1} of yeast extract, 0.05 g l^{-1} of NaCl, 0.02 g l^{-1} of $CaCl_2.7H_2O$, $FeSO_4.7H_2O$ and 1% v v^{-1} of trace elements. The trace element solution included the following composition: 3 g l^{-1} of $MnSO_4.H_2O$, 0.1 g l^{-1} of $ZnSO_4.7H_2O$, 0.01 g l^{-1} of $CuSO_4.5H_2O$, 0.01 g l^{-1} of $NaMoO_4.2H_2O$, 0.01 g l^{-1} of H_3BO_3, 0.003 g l^{-1} of $NiCl_2$ $6H_2O$ and 0.5 g l^{-1} of EDTA [7]. For the optimal biosurfactant production, glucose mineral salt medium was used. The composition of the medium was as follows: 36.5 g l^{-1} of glucose, 4 g l^{-1} of KH_2PO_4, 7.119 g l^{-1} of Na_2HPO_4, 4.5 g l^{-1} of NH_4 (NO_3), 0.197 g l^{-1} of $MgSO_4$, 0.01 g l^{-1} of $CaCl_2.7H_2O$, 0.004 g l^{-1} of $FeSO_4.7H_2O$, 0.275 g l^{-1} of $MnSO_4$ and 0.0014 g l^{-1} of EDTA. Phosphate buffer saline (PBS) and carbon source were autoclaved separately and added to the medium shortly before inoculation. Moreover, pH of the medium was adjusted 6.5-7 [8].

2.2. Inoculum preparation and biosurfactant production

A loopful of the bacterial colonies from 1-day-old cultures on LB-agar (Merck, Germany) was transferred into 50 ml of mineral salt medium (MSM) with 4% (w v^{-1}) of glucose, incubated at room temperature and shaken at 180 rpm for 48 h. Then, 2 ml of the culture were inoculated into 20 ml of MSM with 1% (w v^{-1}) glucose and shaken at 180 rpm at 30°C for 10-12 h. This culture was used as seed culture for the biosurfactant production as 2% v v^{-1} of the inoculum were added to a 500-ml flask containing 100 ml of the production medium and incubated at 30°C for 72 h with 180 rpm [8,9].

2.3. Screening of the most efficient strain for the biosurfactant production

Cell-free supernatants from the production medium were screened for the biosurfactant production through assessment of surface tension, emulsification index and oil spreading. Surface tension was assessed using Kruss tensiometer and Du Nuoy ring method [10]. Emulsification index (E24) of the cell-free samples was assessed based on the Cooper and Goldenberg method. Briefly, 4 ml of the cell-free supernatant were added to a test tube and mixed with 6 ml of hexadecane. The mixture was vigorously vortexed for 2 min and the emulsion stability was calculated after 24 h based on the following formula [11]:

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Total height of the mixture}} \times 100$$

For the assessment of oil spreading, 10 µl of crude oil were added to 40 ml of distilled water (D.W.) in a petri dish for thin oil layer formation. Then, 10 µl of the supernatant were placed at the center of oil layer. Diameter of the clear zone on the oil surface can be linked to the biosurfactant concentration [12]. To assess the bacterial ability of glycolipid biosurfactant production, plates of cetyl-trimethylammonium bromide (CTAB) and methylene blue were used. Bacteria were grown in this media at 30°C for 48 h and the productive colonies were surrounded by dark blue halos [13]. Furthermore, each strain was streaked on sheep blood agar plates and incubated at 30°C for 48 h. The β-hemolysis indicated bisurfactant production by the bacteria [10].

2.4. 16S rRNA sequence analysis

The bacterial cell wall was lysed using lysozyme, proteinase K and sodium dodecyl sulfate (SDS) solution. Saturated solution of NaCl and organic solvents were used for the extraction and purification of bacterial DNA. The bacterial 16S rRNA sequence was amplified using the following primers of 9F-AGGAGGTGATCCAACCGCA and 1541R-AAGAGTTTGATCATGGCTCAG.

Polymerase chain reaction (PCR) was carried out in a 100-µl volume, containing 10 µl of 10× PCR buffer, 2 µl of 10 mM dNTP, 3 µl of 1.5 mM $MgCl_2$, 3 µl of 20 pM of each primer, 1 µl of the template DNA, 0.7 µl of 5 U l^{-1} Taq DNA polymerase and 77.3 µl of sterile D.W. The PCR was carried out using an initial denaturation cycle at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 40 s and a final elongation cycle of 72°C for 7 min. The PCR products were electrophoresed on agarose gels and stained with ethidium bromide [9,10]. The PCR products with a single band on the gels were

www.SID.ir

Archive of SID

sequenced using ABI 3730xl DNA analyzer (Applied Biosystems, USA). The bacterial sequence homologies were compared to each other using BLAST-N and a phylogenetic tree was constructed using neighbor-joining methods of MEGA software v.5.05 [9].

2.5. Bacterial adhesion to hydrocarbon (BATH) assay

This method which is based on the degree of adherence of cells to liquid hydrocarbon allows assessment of the bacterial cell hydrophobicity. In general, bacterial cells grown on the production medium were collected using centrifugation at 286 ×g for 20 min and then washed three times using PBS. Suspensions of washed bacterial cells with OD_{600} of 1 (A0) in BATH buffer (7.3 g l^{-1} of KH_2PO_4, 16.9 g l^{-1} of K_2HPO_4 and 0.2 g l^{-1} of $MgSO_4 \cdot 7H_2O$) were mixed with hexadecane at a ratio of 4:1. The mixture was vortexed for 60 s and left for 30 min to separate the two phases. Then, OD of the aqueous phase was measured at 600 nm (A1). Turbidity reduction linked to hydrophobicity of the cells and proportion of the attached cells to hydrophobic phase (H) were calculated using the following formula [9]:

$$H = \left(1 - \frac{A}{A_{\circ}}\right) \times 100$$

2.6. Stability of the biosurfactants

Bacterial cells were collected using centrifugation at 2,861 ×g for 20 min and the biosurfactant stability was analyzed using supernatants. To study temperature stability of the biosurfactants, cell-free broth was heated at various temperatures, including 40, 70, 100 and 121°C for 30 min and cooled down at room temperature. The biosurfactant stability was assessed using measurement of E_{24} and surface tension. Moreover, effects of low temperatures on biosurfactants were assessed after exposure of biosurfactants to 4°C for 30 min. The pH stability of biosurfactants was assessed using adjustment of pH of the supernatants between 2 and 12 with 6 M HCl and 6 M NaOH. The E_{24} index and surface tension were assessed using various concentrations of NaCl (1-12% w v^{-1}) to report effects of osmolarity on E_{24} and surface activity of the supernatants [9,14].

2.7. Extraction of biosurfactants

Selected bacteria (bacteria with high biosurfactant production potentials) were cultured in the production medium for 72 h. Cultures were centrifuged at 10,000 ×g for 20 min. The pH value of cell-free broth was adjusted to 2.0 using 6 M HCl. This was stored at 4°C overnight to allow precipitation of the biosurfactants. Precipitates were then harvested using centrifugation at 20,000 ×g for 30 min at 4°C. The precipitates in centrifuge tubes were dried using heating at 37°C in oven. Dried materials were dissolved in deionized water and extracted three times with dichloromethane. Briefly, equal volumes of dichloromethane and deionized water were used and the two phases were vigorously mixed. Mixture was centrifuged at 6,000 ×g for 2 min to accelerate phase separations. The organic solvent phases, containing the biosurfactants, were collected and evaporated at room temperature [15].

2.8. Bacterial growth and biosurfactant production kinetics

Bacterial growth was assessed through measuring OD_{600} of the culture and dry cell weight (DCW) at intervals. To assess DCW, bacterial cells were harvested using centrifugation at 10,000 ×g for 20 min. Cell pellets were washed twice with 0.1 M phosphate buffer (pH 7.0) and dried through heating at 105°C until achieving a constant weight. Time course samples of the culture medium were drawn within fixed intervals and then assessed for the biosurfactant production. The assessment included surface tension, oil spreading, emulsification activity and bio-surfactant production [14,16].

2.9. Effects of environmental factors on bacterial growth

Effects of culture temperature, initial pH, salinity and agitation on biosurfactant production, biomass concentration, surface tension and final culture pH were assessed. To assess effects of the culture temperature and initial pH on the highlighted factors, the culture temperature was set at 30, 37 and 45°C and the initial pH was adjusted to 5, 7 and 9. To assess effects of medium osmolarity on the highlighted factors, NaCl was added to the medium at concentrations of 2 and 4% w v^{-1}. To assess effects of the agitation speed on the highlighted factors, selected bacterial species were incubated at various agitation rates of 100, 180 and 250 rpm. All cultures were carried out in 1 l flasks, containing 250 ml of the production medium at 37°C and 180 rpm [14,17,18].

2.10. Chemical characteristics of biosurfactants

Biosurfactants were analyzed on thin layer chromatography (TLC) plates using $CHCl_3$:CH_3OH:H_2O (65:15:1) as solvent system [19]. Chromatograms were imagined using TLC reagents such as iodine vapors for lipid spots and molisch reagent (10% solution of α-naphthol in 96% ethanol) with 5-8 drops of H_2SO_4 followed by heating at 110°C for sugar detection. Ninhydrin reagent (0.5 g of ninhydrin in 100 ml of acetone) AND heating at 110°C ere used to detect peptides to see red spots [20]. Fourier-Transform Infrared (FTIR) spectra of the biosurfactants were recorded using Bruker, TENSOR 27 FTIR spectrometer, (USA). Biosurfactant samples were studied directly in dried states without further preparations using attenuated total reflection (ATR) in a wave-number range of 4000-600 cm^{-1}. The ^1H nuclear magnetic resonance (NMR) data were collected using Bruker AC 250 MHz spectrometer (USA) with deuterated chloroform as solvent. Differential

www.SID.ir

Archive of SID

scanning calorimetry (DSC) (Mettler Toledo, Switzerland) was used to assess thermal characteristics of the biosurfactants. Therefore, biosurfactants were heated at a rate of 10°C min^{-1} from 30 to 450°C [9].

2.11. Antibacterial activity

Antibacterial activity of the biosurfactants was assessed in *Staphylococcus* (*S.*) *aurous* PTCC 1113 and *Escherichia* (E.) *coli* PTCC 1338 using disc diffusion and minimum inhibitory concentration (MIC) methods. A bacterial inoculum (0.5 McFarland standard) of *E. coli* or *S. aureus* was spread on Mueller-Hinton (MH) plates and then discs with various concentrations of biosurfactants (10, 15 and 20 mg ml^{-1}) were placed on the plates. Plates were incubated at 37°C for 18 h and then assessed for growth inhibition zones [21]. The MIC is the lowest concentration of antimicrobial agents which visibly inhibits the bacterial growth. In this study, MIC was assessed using 96-well microplates and serial dilution techniques. Briefly, various concentrations of the biosurfactants in MH broth (0.078-5 mg ml^{-1}) were prepared and inoculums (0.5 McFarland standard) of *E. coli* or *S. aureus* were used in 96-well microplates. Microplates were incubated at 37°C for 18 h and then OD_{600} of the biosurfactants was measured [20].

2.12. Cytotoxicity assay

Cytotoxicity of the biosurfactants was assessed using MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and SW480 cell line. Briefly, SW480 cells were cultured in 96-well microplates. Cells were attached to the wells after 24 h incubation at 37°C with 5% CO_2. Then, old media were replaced by fresh media, containing various concentrations of the biosurfactants (1.25-320 µg ml^{-1}). Microplates were incubated at 37°C in 5% CO_2 for various times (24, 48 and 72 h) to assess inhibitory effects of the biosurfactants on cells. Cells were treated with 20 µl of MTT solution (5 mg ml^{-1} in PBS) and incubated at 37°C for 4 h. Then, media was removed and cells were soaked in 150 µl of DMSO for 15 min to dissolve the resulting formazan crystals as MTT metabolic products. Cell viability was assessed through measurement of OD_{570} [22].

3. Results and discussion

3.1. Screening and identification of the most efficient bacterial species for the biosurfactant production

All the 12 bacterial species were able to grow on oil as the unique carbon source. Results of the red blood cell lysis, surface tension, emulsification index and oil spreading are shown in Table 1. None of the species were able to form dark blue halos on CTAB agar plates; a specific sign of the rhamnolipid biosurfactant production. Based on the results, isolate No. 12 was identified as the most efficient biosurfactant-producing bacterial species. After sequencing 16S rRNA of the isolate, homology of the sequence with other sequences was analyzed using BLAST online tool. The isolate showed the maximum similarity to *Bacillus* (*B.*) *subtilis* (Fig. 1).

Table 1. Hemolysis activity, surface tension, emulsification index and oil spreading assay for the 12 isolated species

Bacterial species	1	2	3	4	5	6	7	8	9	10	11	12
Hemolysis	+	+	-	+	-	+	-	+	+	-	-	+
Surface tension (mN m^{-1})	33.8	46.7	40.65	60.65	43.05	33.5	31.05	31.45	31.35	44.6	43.05	30.75
E_{24}%	60	20	33	6	26	35	53	60	40	13	20	53
Diameter of clear zone (mm)	8	2	4	1	3	4	5	6	5	2	3	8

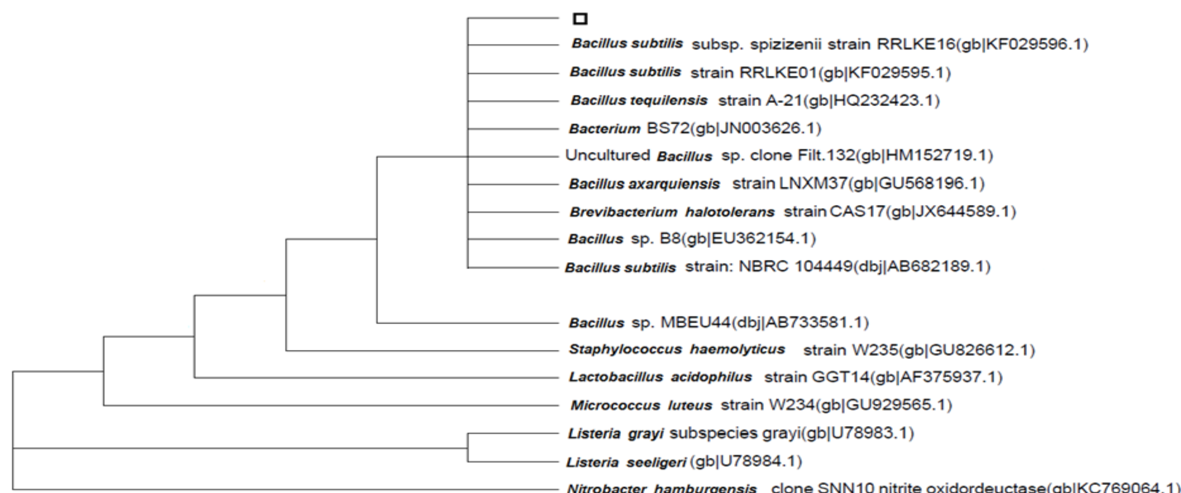


Figure 1. Phylogenetic tree of the selected biosurfactant-producing bacterial species

www.SID.ir

Archive of SID

3.2. Time course profile of the bacterial growth, biosurfactant activity and BATH test

Bacterial growth, surface tension, clear zone diameter on oil surface, E_{24} and biosurfactant production were assessed at various times after inoculation (Fig. 2). The highest biosurfactant production was recorded in the bacterial stationary growth phase (Fig. 2a). To assess the preference of cells for the aqueous or organic phase, cell surface hydrophobicity was calculated for the selected species. Based on the results (Fig. 2b), H value for the isolate No. 12 was estimated as 9.0%.

3.3. Biosurfactant stability

Results of the supernatant thermal stability, containing biosurfactants, indicated stability of the biosurfactants from isolate No. 12 at a wide range of temperatures (up to 120°C). As seen, effects of the temperature were limited; however, a mild decrease was seen in emulsifying capacity of supernatants at 120°C (Fig. 3a). An increase in concentration of NaCl negatively affected the surface tension and emulsifying capacity with a significant rate. This caused a drastic decrease in emulsifying capacity of the cell-free broth, containing biosurfactants, and a low increase in the surface tension (Fig. 3b). Regarding pH, stability of the biosurfactants at alkaline pH should be emphasized (Fig. 3c). Acidic pH negatively affected emulsifying capacity and surface tension of the biosurfactants, owing to decreased emulsifying capacity and increased surface tension (Fig. 3c).

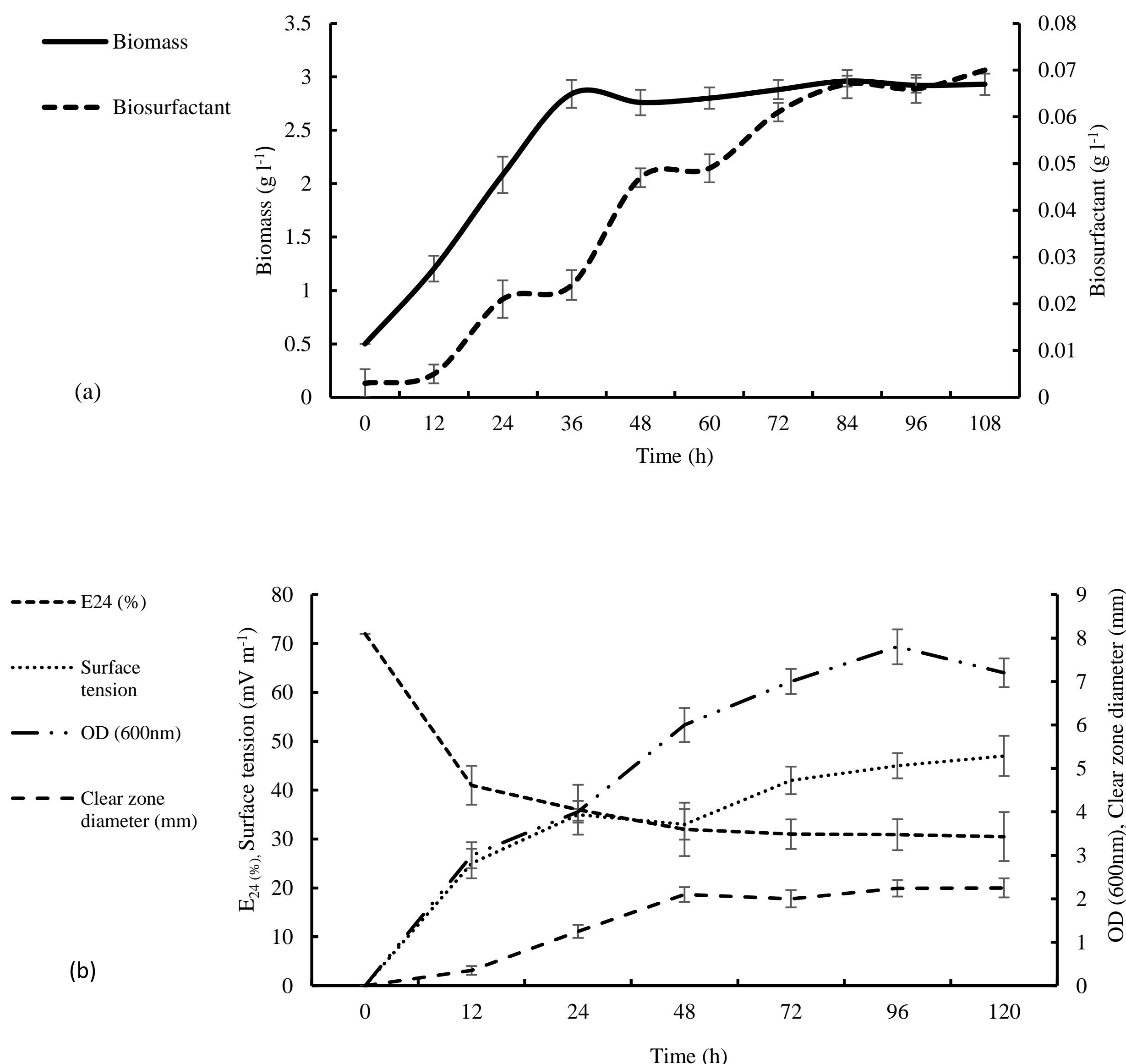


Figure 2. Kinetic of biosurfactant production and bacterial growth (a), time course profiles of surface tension, emulsification capability, clear zone increase and OD600 (b)

www.SID.ir

Archive of SID

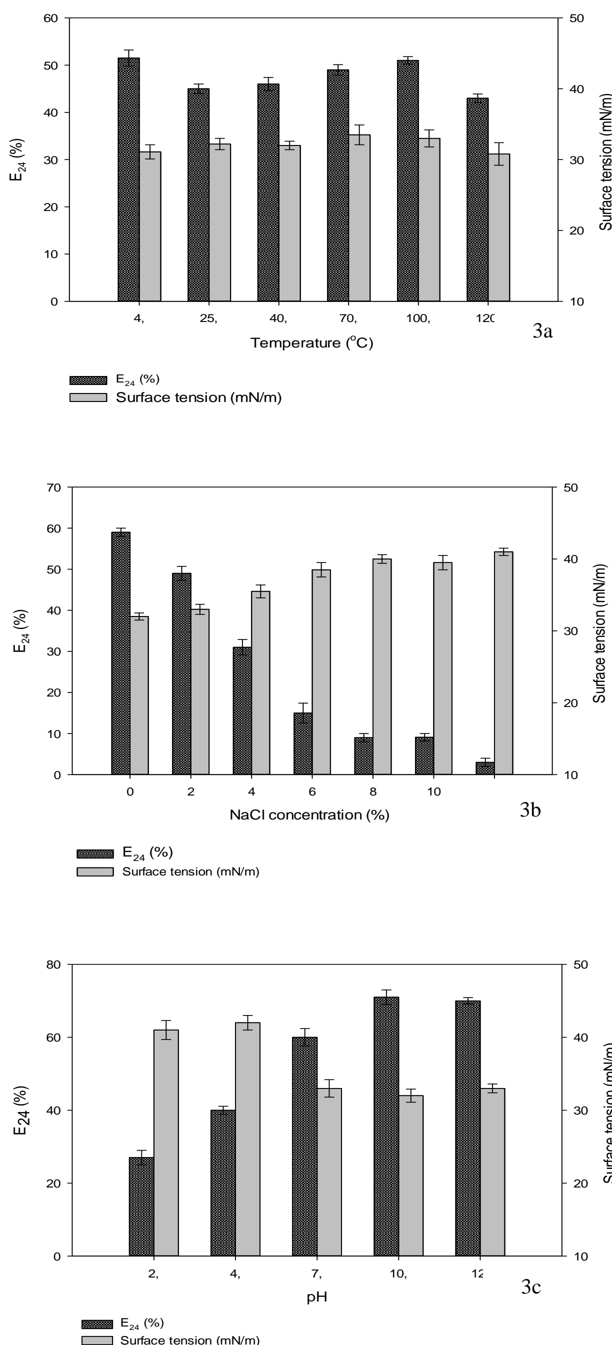
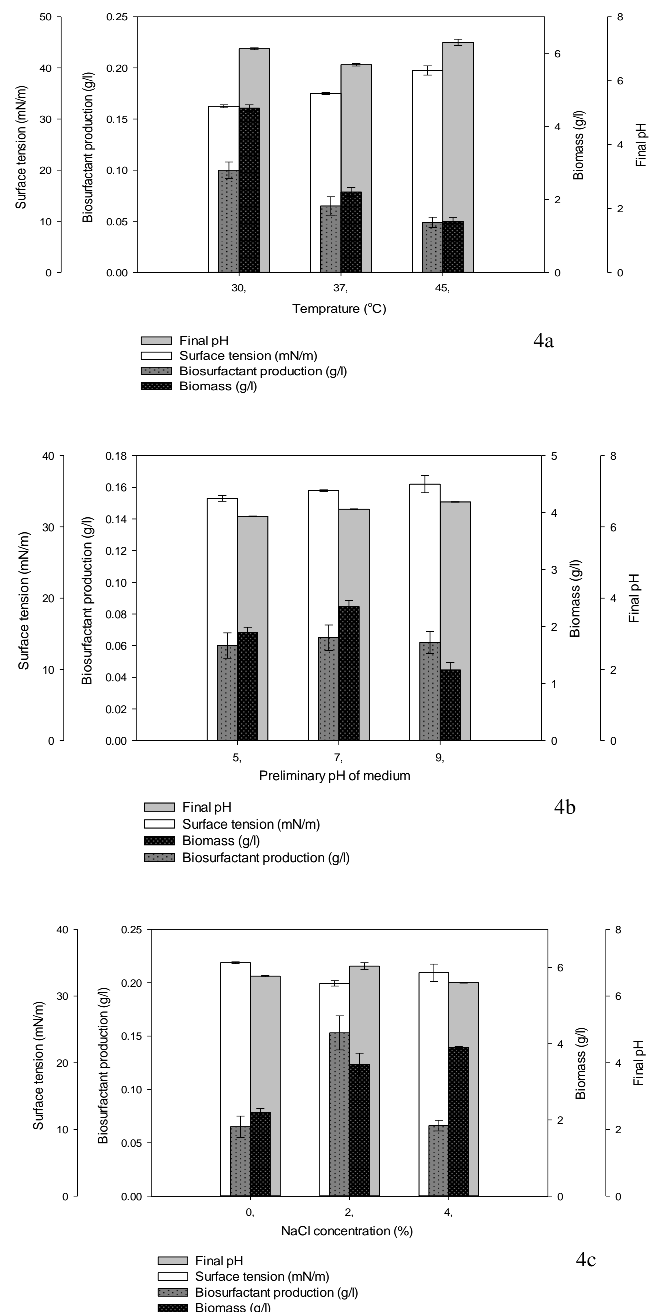


Figure 3. Effect of the temperature (a), the osmolarity (b), and the pH (c) on the stability of the produced biosurfactant

3.4. Effects of environment factors on biosurfactant production and bacterial growth

The highest biomass concentration included 4.5 g l^{-1} observed at 30°C which decreased beyond this temperature (Fig. 4a). However, bacteria were able to grow at 45°C even the final biomass concentration included only 1.5 g l^{-1}. Moreover, biosurfactant production decreased by increased incubation temperature. The highest biosurfactant production included 0.1 g l^{-1} at 30°C, decreasing to 0.05 g l^{-1} at 45°C. However, it is noteworthy that the ratio of biosurfactant production to biomass was higher at 45°C, compared to other culture temperatures. Following increased biosurfactant productions, surface tension of the culture media was lower at decreased temperatures (Fig. 4a). Bacterial growth and biosurfactant production were affected by initial pH of the culture media; optimally at pH 7, 2.3 and 0.07 g l^{-1}. However bacteria were able to grow and produce biosurfactant at pH 5 and 9, they showed a greater resistance to acidic conditions, compared to alkaline conditions. Therefore, no significant differences were seen in bacterial growth and biosurfactant production in acidic and neutral conditions (Fig. 4b). As shown in Fig. 4c, biomass and biosurfactant productions are affected by the concentration of NaCl in culture media. The highest biomass production of 4 g l^{-1} was recorded at 4% of NaCl concentration.



www.SID.ir

Archive of SID

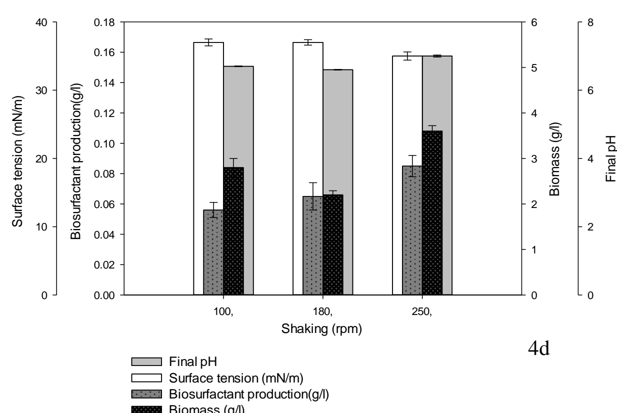
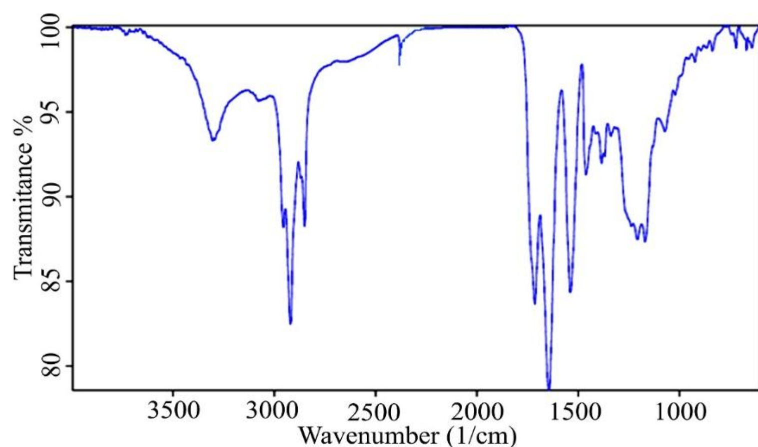


Figure 4. Effect of different cultivation temperature (a), initial pH (b), osmolarity (c), and aeration rates (d) on biosurfactant production, biomass, surface tension and final pH of the production medium.

This decreased at lower concentrations of NaCl. An optimal biosurfactant production of 0.15 g l^{-1} was observed in 2% NaCl. A weak minimum was observed in surface tension at 2% of NaCl (Fig. 4c). Figure 4d shows that biomass and biosurfactant productions were optimal at the 250-rpm aeration rate with 3.8 and 0.08 g l^{-1}, respectively. Biosurfactant production decreased at lowest aeration rates. Accordingly, a limited decrease was observed in surface tension.

3.5. Characterization of biosurfactants

The TLC analysis showed that the biosurfactant produced by the selected *Bacillus* spp. included lipopeptide structure since RF values of 0.4 and 0.5 were recorded for the lipopeptide spots on TLC plates. Figure 5a shows FTIR spectra of the biosurfactants produced by *Bacillus* spp. The broad peak at 3,390 cm^{-1} shows hydroxyl and amine functional groups. Peaks at 2,925 and 2,850 cm^{-1} could be linked to asymmetric and symmetric stretching of CH_2 and CH_3 groups, respectively. Moreover, in FTIR spectra of the biosurfactants, absorption peaks of the carbonyl were observed at 1,730 cm^{-1} for the ester and 1,650 cm^{-1} for the C=O bond of the amide group. Peaks of 1,450-1,460 cm^{-1} referred to the bending of CH_2 and CH_3 groups. Absorption at 1,190 cm^{-1} was seen due to the stretching vibration of C-O bond in ester or carboxylic acid structure. Figure 5b shows thermogram of the biosurfactant samples. The peak temperature of 50°C is most likely attributed to moisture evaporation of the samples. Based on the thermogram, the corresponding biosurfactant showed thermal stability up to 140°C. In H NMR spectrum (Fig. 5c), peaks at 1.002-0.827 ppm (with an area of 52.61under the peak) may be linked to the CH_3 group. Moreover, peaks at 1.257-1.376 ppm (with an area of 99.60 under the peak) belonged to the hydrogen atoms in CH_2 group. Peak at 1.542 ppm was associated to the hydrogen atom attached to the carbon atom near the carbonyl group (with an area of 16.29 under the peak). Peaks at 2.312-2.371 ppm (with an area of 4.36 under the peak) were linked to the hydrogen atoms attached to the carbon atoms near the amide group. Furthermore, a small peak was seen at 5.2 ppm (with an area of 0.98 under the peak) which could be associated to the hydrogen atoms near to the ester group. In this spectrum, the peak at 7.268 ppm was linked to the sample solvent (deuterated chloroform) and peaks at 6.986-7.962 ppm to the hydrogen atoms of the amide group.



www.SID.ir

Archive of SID

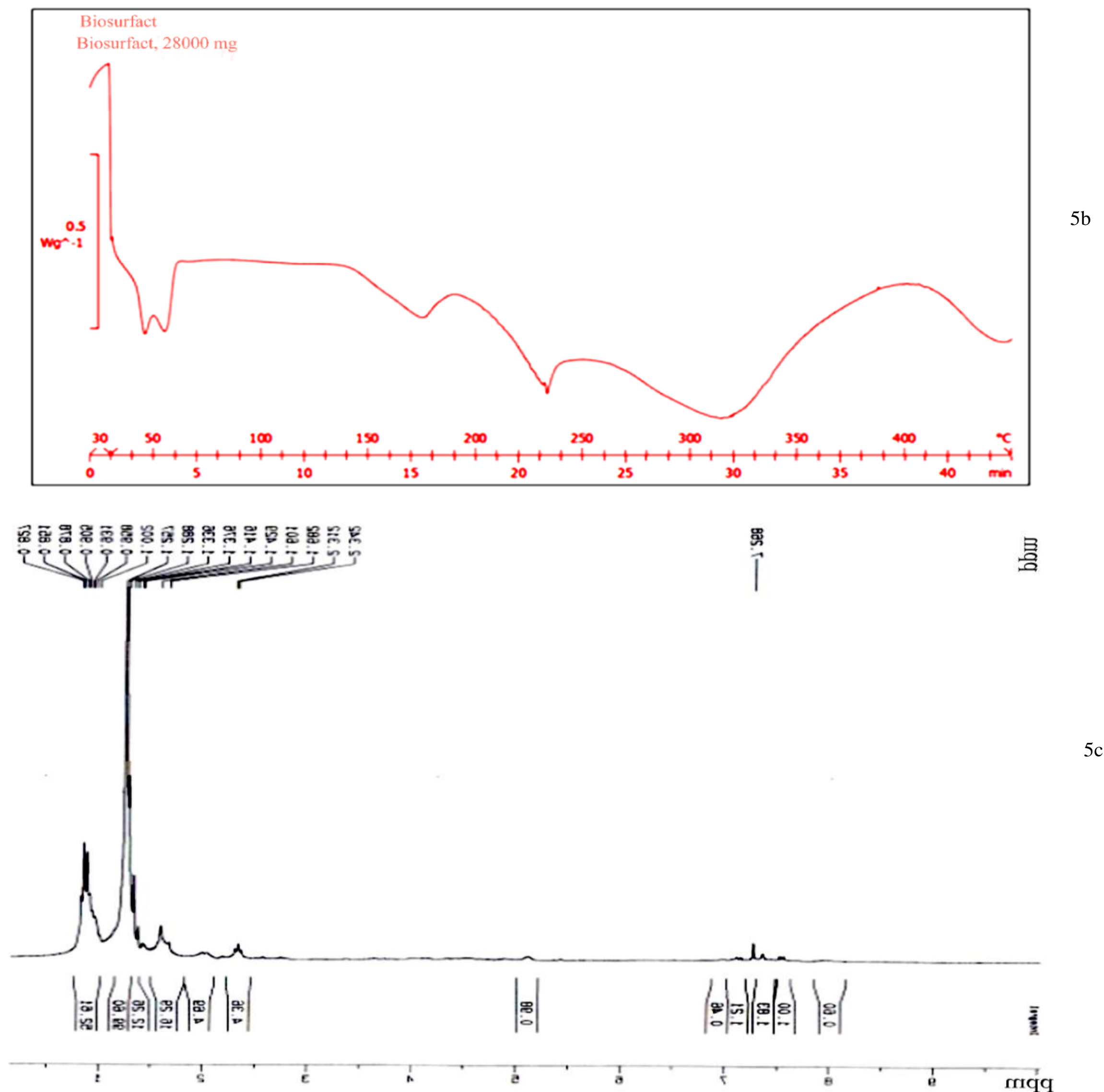


Figure 5. FTIR spectrum (a), DSC thermogram (b) and H NMR spectrum (c) of the biosurfactant produced by *Bacillus subtilis.*

3.6. Biological assay

Disc diffusion assay showed that *S. aureus* was susceptible to biosurfactants produced by the selected bacteria. Diameters of the growth inhibition zones of *S. aureus* for various biosurfactant concentrations of 10, 15 and 20 mg ml^{-1} included 4, 4 and 5 mm, respectively. Disc diffusion assay of *E. coli* indicated no susceptibility to the biosurfactants at tested concentrations. In MIC assay of *S. aureus*, a concentration of 1.52 mg ml^{-1} was reported as the MIC of biosurfactants. For *E. coli*, MIC could not be determined for the bacteria at the highlighted concentrations. The survival yields of cells at various exposure times to biosurfactants are shown in Fig. 6. Concentrations of biosurfactants; to which, 50% of the cancer cells were degenerated due to 48 and 72 h of exposures, could not be calculated. This was reported as 0.601 µg μl^{-1} for the samples exposed to the biosurfactants for 24 h. Due to biosurfactant potential uses in various industries, great attentions have been paid to these biomolecules in recent years. Various approaches are described for increasing production of the biosurfactants with unique characteristics such as screening micro-organisms from various environments, genetic engineering of microorganisms and optimizing conditions for microbial growth. The current study focused on screening and optimizing bacterial growth conditions for biosurfactant production. Since surface tension reduction is the intrinsic characteristic of the biosurfactants, screening methods for biosurfactant producing bacteria were validated based on the results from the surface tension assay. Similar to studies by Youssef et al. and Plaza et al., the present study showed that screening biosurfactant producing bacteria using red blood cell (RBC) lysis was not a reliable method and was error prone [7,23]. This was due to the presence of

www.SID.ir

Archive of SID

hemolytic compounds with no roles in reducing surface tension and the lack of hemolysis by biosurfactants. However, the current results were not similar to those by Carrillo et al. They showed that haemolysis and biosurfactant production were possibly associated [24]. In some cases, no direct relationships existed between the results of surface tension and E_{24} assays; as reported by Willumsen et al. [25]. The presence of extracellular molecules, such as proteins that cause emulsification with no decrease in surface tension, could be a reason for this phenomenon.

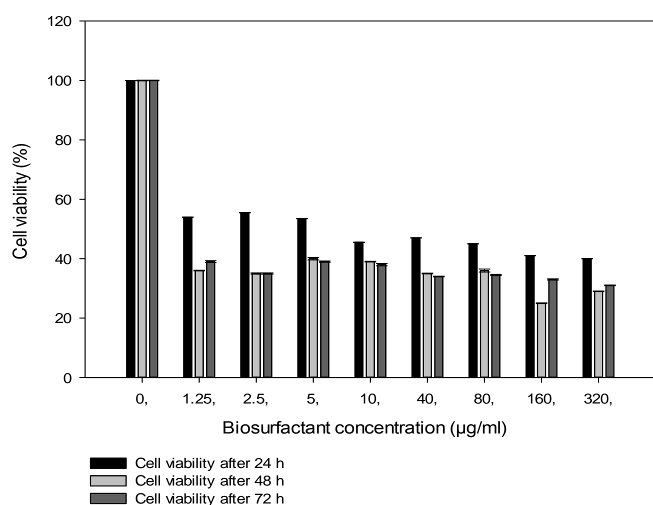


Figure 6. Cell viability percentage at different concentration of biosurfactant after 24, 48 and 72 h.

The oil spreading technique is an appropriate technique for the screening of biosurfactant producing bacteria. In the present study, results from this technique were similar to results from the surface tension assay. Previously, a study by Plaza et al. verified the validity of this test [7]. In the current study, surface tension was maximum at the beginning of the bacterial growth. Despite a low biosurfactant production rate in logarithmic growth phase, surface tension of the culture media decreased greatly due to the high efficiency of biosurfactants produced by *B. subtilis*. Makkar et al. and later Suwansukho et al. reported similar results for surface tension reduction during growth of *B. subtilis* [26,27]. According to El-Sersy, biosurfactants from *B. subtilis* were able to preserve their surface properties at high temperatures [16]. Acidic conditions or high salt concentrations increased surface tension and decreased E_{24} coefficient. Furthermore, El-Sersy [16] and Suwansukho et al. [26] reported decreased surface activity in acidic conditions. No susceptibility of biosurfactants produced by *B. subtilis* to alkaline pH was reported by Suwansukho et al. [26], in contrast that reported by El-Sersy [16]. Similar to the findings by Abdel-Mawgoud et al., the optimal growth and biosurfactant production were observed at 30°C. The most appropriate growth conditions were recorded at neutral pH and salt concentrations of 4%; similar to reports by Makkar et al. [17,28]. In another study by Makkar et al. [17], biosurfactant production decreased with increased salt concentration; similar to that seen in the present study. However, no similar results of association between biosurfactant production and NaCl concentration were seen in previous reports. Furthermore, the lower susceptibility of bacterial isolates to acidic conditions in the current study was not surprising as these bacteria were isolated from acid springs. Increased aeration resulted in increased bacterial growth and biosurfactant production; as previously reported by Jokari et al. and Ghribi et al. [18,29]. The two lipopeptide spots seen in TLC analysis were possibly linked to surfactin isomers. Moreover, FTIR and NMR spectra of the biosurfactants showed similarities with surfactin due to their close chemical structures. The biosurfactants were able to inhibit growth of Gram-positive bacteria such as *S. aureus*. However, biosurfactant concentrations did not include antimicrobial effects on Gram-negative bacteria such as *E. coli*. Possibly, the outer membrane of Gram-negative bacteria was the reason for this resistance. Several reports have been published on anti-cellular effects of biosurfactants. For example, Nozhat et al. [22] assessed surfactin from Sigma (USA) on the Hella cell line and reported an IC_{50} of 5,000 ng ml^{-1}. The commercial biosurfactant was able to degenerate half of the SW480 colon cancer cells at a concentration of 6,000 ng ml^{-1}. Biosurfactants possibly interact with membrane, creating holes in cells, and hence lead to cell death.

4. Conclusion

Acidic hot springs are potential sources of bacteria with specific metabolites. In this study, 12 bacterial species isolated from acidic hot springs in Bushehr Province, Iran, were assessed for biosurfactant production and one species was reported as the most potential bacterial species. The bacterial biosurfactant was thermally stable up to 140°C. The interesting novelty of this study included the fact that the chemical surface property was preserved to an osmolarity of 4% w v^{-1}. The biosurfactant structure was similar to surfactin structure and hence useful as bioemulsifier for osmolarity solutions.

5. Acknowledgements

The authors thank the Agricultural Engineering Research Institute for providing facilities in experimental analyses.

6. Conflict of interest

The authors declare that they have no conflict of interest.

www.SID.ir

Archive of SID

References

1. Varjani SJ, Upasani VN. Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. Bioresour Technol. 2017; 232: 389-97.
   doi: 10.1016/j.biortech.2017.02.047.

2. Mukherjee AK, Das K. Microbial Surfactants and Their Potential Applications: An Overview. In: Sen R, editor. Biosurfactants. New York, NY: Springer New York; 2010: pp. 54-64.
   doi: 10.1007/978-1-4419-5979-9_4.

3. Rahman PKSM, Gakpe E. Production, characterisation and applications of biosurfactants - Review. Biotechnol. 2008; 7 (2):360-370.
   doi: 10.3923/biotech.2008.360.370.

4. Souza EC, Vessoni-Penna TC, de Souza Oliveira RP. Biosurfactant-enhanced hydrocarbon bioremediation: An overview. Int Biodeter Biodegr. 2014; 89: 88-94.
   doi: 10.1016/j.ibiod.2014.01.007.

5. Singh P, Patil Y, Rale V. Biosurfactant production: Emerging trends and promising strategies. J Appl Microbiol. 2018; 126(1): 2-13.
   doi: 10.1111/jam.14057.

6. Kazemi GA, Tajabadi Ebrahimi M, Jalili H, Dameshqyan M, Sharifpoor L. Isolation, identification and considering the antagonistic properties of bacteria from hot water acidic spring in Boushehr province. New Cell Mol Biotechnol J. 2014; 4(14): 65-72.

7. Płaza GA, Zjawiony I, Banat IM. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils. J Petro Sci Eng. 2006; 50(1): 71-77.
   doi: 10.1016/j.petrol.2005.10.005.

8. Sen R. Response surface optimization of the critical media components for the production of surfactin. J Chem Technol Biotechnol. 1997; 68(3): 263-270.
   doi: 10.1002/(Sici)1097-4660(199703)68:3<263::Aid-Jctb63-1>3.0.Co; 2-8.

9. Lotfabad TB, Shourian M, Roostaazad R, Najafabadi AR, Adelzadeh MR, Noghabi KA. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloid Surface B. 2009; 69(2): 183-193.
   doi: 10.1016/j.colsurfb.2008.11.018.

10. Jain RM, Mody K, Mishra A, Jha B. Isolation and structural characterization of biosurfactant produced by an alkaliphilic bacterium *Cronobacter sakazakii* isolated from oil contaminated wastewater. Carbohyd Polym. 2012; 87(3): 2320-2326.
    doi: 10.1016/j.carbpol.2011.10.065.

11. Cooper DG, Goldenberg BG. Surface-Active Agents from Two *Bacillus* Species. Appl Environ Microbiol. 1987; 53(2): 224-229.
    doi: 0099-2240/87/020224-06.

12. Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y, Imanaka T. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. J Bacteriol. 1993; 175(20): 6459-6466.
    doi: 10.1128/jb.175.20.6459-6466.1993.

13. Siegmund I, Wagner F. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnol Tech. 1991; 5(4): 265-268.
    doi: 10.1007/BF02438660.

14. Abbasi H, Hamedi MM, Lotfabad TB, Zahiri HS, Sharafi H, Masoomi F, AkbarMoosavi-Movahedi A, Ortiz A, Amanlou M, AkbariNoghabi K. Biosurfactant-producing bacterium, *Pseudomonas aeruginosa* MA01 isolated from spoiled apples: physicochemical and structural characteristics of isolated biosurfactant. J Biosci Bioeng. 2012; 113(2): 211-219.
    doi: 10.1016/j.jbiosc.2011.10.002.

15. Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NA. Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol. 2008; 150(3): 289-303.
    doi: 10.1007/s12010-008-8153-z.

16. El-Sersy NA. Plackett-Burman design to optimize biosurfactant production by marine *Bacillus subtilis* N10. Rom Biotech Lett. 2012; 17(2): 7049-7064.

17. Makkar RS, Cameotra SS. Effects of various nutritional supplements on biosurfactant production by a strain of *Bacillus subtilis* at 45°C. J Surfactants Deterg. 2002; 5(1): 11-17.
    doi: 10.1007/s11743-002-0199-8.

18. Jokari S, Rashedi H, Amoabediny G, Dilmaghani SN, Assadi MM. Optimization of surfactin production by *Bacillus subtilis* ATCC 6633 in a miniaturized bioreactor. Int J Environ Res. 2013; 7(4): 851-858.
    doi: 10.22059/ijer.2013.666.

19. Makkar RS, Cameotra SS. Biosurfactant production by a thermophilic *Bacillus subtilis* strain. J Ind Microbiol Biotechnol. 1997; 18(1): 37-42.
    doi: 10.1038/sj.jim.2900349.

20. Lotfabad TB, Abassi H, Ahmadkhaniha R, Roostaazad R, Masoomi F, Zahiri HS, Ahmadian Gh, Vali H, Noghabi KA. Structural characterization of a rhamnolipid-type biosurfactant produced by *Pseudomonas aeruginosa* MR01: Enhancement of di-rhamnolipid proportion using gamma irradiation. Colloid Surface B. 2010; 81(2): 397-405.
    doi: 10.1016/j.colsurfb.2010.06.026.

21. Fernandes PAV, de Arruda IR, dos Santos AFAB, de Araujo AA, Maior AMS, Ximenes EA. Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. Braz J Microbiol. 2007; 38(4): 704-709.
    doi: 10.1590/S1517-83822007000400022.

www.SID.ir

Archive of SID

22. Nozhat Z, Asadi A, Zahri S. Properties of surfactin C-15 nanopeptide and Its cytotoxic effect on human cervix cancer (HeLa) cell line. J Nanomater. 2012; 2012: 16. doi: 10.1155/2012/526580.

23. Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ. Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Methods. 2004; 56(3): 339-347. doi: 10.1016/j.mimet.2003.11.001.

24. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM. Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol. 1996; 12(1): 82-84. doi: 10.1007/bf00327807.

25. Willumsen PA, Karlson U. Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. Biodegradation. 1996; 7(5): 415-423. doi: 10.1007/BF00056425.

26. Suwansukho P, Rukachisirikul V, Kawai F, H-Kittikun A. Production and applications of biosurfactant from *Bacillus subtilis* MUV4. Sonklanakarin J Sci Technol. 2008; n30 (1): n87. doi: 10.1016/j.jbiosc.2011.10.002.

27. Makkar RS, Cameotra SS. Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. J Ind Microbiol Biotechnol. 1998; 20(1): 48-52. doi: 10.1038/sj.jim.2900474.

28. Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NA. Optimization of surfactin production by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol. 2008; 150(3): 305-325. doi: 10.1007/s12010-008-8155-x.

29. Ghribi D, Ellouze-Chaabouni S. Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. Biotechnol Res Int. 2011; 2011. 1-6 doi: 10.4061/2011/653654.

www.SID.ir

Archive of SID

http://dx.doi.org/10.22037/afb.v%vi%i.23663

Research Article

Applied Food Biotechnology

APPLIED FOOD BIOTECHNOLOGY, 2019, 6 (2): 127-138
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

تولید و تعیین ویژگی‌های ماده فعال سطحی زیستی با استفاده از باکتری‌های جدا شده از چشمه‌های آب گرم اسیدی

سمیه ابراهیمی[۱]، حسن جلیلی[۱*]، خسرو خواجی[۲]، کامبیز اکبری نوقابی[۳]، مریم تاج‌آبادی ابراهیمی[۴]، عبدالطیف عامران[۵]

۱- گروه مهندسی علوم زیستی، دانشکده علوم و فنون نوین، دانشگاه تهران، تهران، ایران.
۲- گروه بیوشیمی، دانشکده علوم زیستی، دانشگاه تربیت مدرس، تهران، ایران.
۳- پژوهشکده زیست فناوری صنعت و محیط زیست، پژوهشگاه ملی مهندسی ژنتیک و زیست فناوری، تهران، ایران.
۴- گروه زیست شناسی، دانشکده علوم، دانشگاه آزاد اسلامی، واحد تهران مرکز، تهران، ایران.
۵- دانشکده ملی رن، دانشگاه رنوس ۱، CNRS، UMR 6226، 11 کوچه بیلیو، CS 50837، 35708 Rennes cedex 7، فرانسه.

تاریخچه مقاله

دریافت ۱۷ ژوئن ۲۰۱۸
داوری ۲۳ دسامبر ۲۰۱۸
پذیرش ۳۰ ژانویه ۲۰۱۹

واژگان کلیدی

- چشمه آب گرم
- اکسترموفیل
- تولید زیست‌سطح‌فعال
- تعیین ویژگی‌ها

نویسنده مسئول*

حسن جلیلی، دانشکده علوم و فنون نوین، خیابان کارگر شمالی، تهران، ایران.

تلفن: ۳-۹۸۲۱۸۸۶۱۸۴۳۱+
نمابر: ۹۸۲۱۸۸۶۱۷۰۸۷+
پست الکترونیک: hjalili@ut.ac.ir

چکیده

سابقه و هدف: زیست‌سطح‌فعال‌ها[۱] به طور فزاینده‌ای در صنایع غذایی به علت سمیت پایین و ساختار منحصر به فرد مورد استفاده قرار می‌گیرند. در این مطالعه، برای اولین بار زیست‌سطح‌فعال‌ها توسط باکتری‌های اسید دوست جدا شده از چشمه‌های آب گرم اسیدی استان بوشهر ایران تولید و مشخصات آنها بررسی شد.

مواد و روش ها: غربال‌گری و شناسایی گونه‌هایی با بالاترین توانایی تولید زیست‌سطح‌فعال با استفاده از آزمون‌هایی مانند خون‌کافت[۲]، کشش سطحی، شاخص نامیزه‌سازی[۳] و قطر منطقه روشن بر روی ۱۲ گونه باکتری انجام شد. علاوه بر تولید زیست‌سطح‌فعال، سینتیک، پایداری آن و تجزیه و تحلیل ساختاری و حرارتی با استفاده از کروماتوگرافی لایه نازک، طیف بینی مادون قرمز تبدیل فوریه[۴]، تشدید مغناطیسی هسته‌ای[۵] و آزمون تجزیه ای گرماسنجی روبشی افتراقی[۶] انجام شد.

یافته‌ها و نتیجه گیری: زیست‌سطح‌فعال حاصل از باکتری‌های انتخابی ($۰/۱\ g\ l^{-1}$) در ۱۲۰°C به مدت ۳۰ دقیقه پایداری حرارتی داشتند. پایداری در درجه حرارت‌های بالاتر از ۱۴۰°C با استفاده از آزمون تجزیه ای گرماسنجی روبشی افتراقی مورد تایید قرار گرفت. نوآورانه‌ترین یافته این مطالعه حفظ خواص سطحی تا اسمولاریتی $w\ v^{-1}$ ۴٪ بود. کاهش کشش سطحی و توانایی نامیزه‌سازی فقط در غلظت‌های بیشتر از غلظت مورد اشاره مشاهده شد. بررسی بیولوژیکی نشان داد که *استافیلوکوکوس اورئوس* به زیست‌سطح‌فعال تولید شده حساس است، در حالی که در مورد *اشرشیا کلی* حساسیتی مشاهده نشد. تخریب سلول SW480 پس از ۲۴ ساعت تماس با زیست‌سطح‌فعال به میزان $۰/۶۰۱\ \mu g\ \mu l^{-1}$ روی داد. تجزیه و تحلیل ساختاری زیست‌سطح‌فعال مشابه سورفاکتین، نوعی زی‌نامیزه[۷] غذایی می‌باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] biosurfactant
[2] hemolysis
[3] emulsification index
[4] Fourier transform infrared spectroscopy or FTIR
[5] Nuclear Magnetic Resonance
[6] Differential scanning calorimetry or DSC
[7] bioemulsifier

www.SID.ir