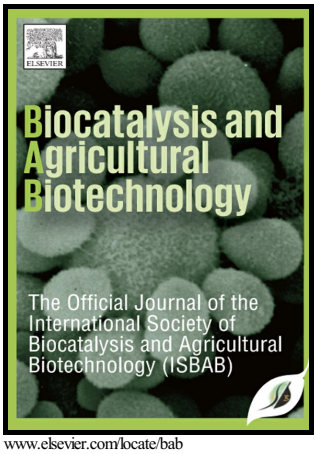


www.elsevier.com/locate/bab

Isolation, characterization, and optimization of biosurfactant production by an oil-degrading *Acinetobacter junii* B6 isolated from an Iranian oil excavation site

Mandana Ohadi, Gholamreza Dehghan-Noudeh, Mojtaba Shakibaie, Ibrahim M. Banat, Mostafa Pournamdari, Hamid Forootanfar

PII: S1878-8181(17)30174-3
DOI: http://dx.doi.org/10.1016/j.bcab.2017.08.007
Reference: BCAB602

To appear in: *Biocatalysis and Agricultural Biotechnology*

Received date: 22 March 2017
Revised date: 6 August 2017
Accepted date: 10 August 2017

Cite this article as: Mandana Ohadi, Gholamreza Dehghan-Noudeh, Mojtaba Shakibaie, Ibrahim M. Banat, Mostafa Pournamdari and Hamid Forootanfar, Isolation, characterization, and optimization of biosurfactant production by an oil-degrading *Acinetobacter junii* B6 isolated from an Iranian oil excavation site, *Biocatalysis and Agricultural Biotechnology,* http://dx.doi.org/10.1016/j.bcab.2017.08.007

Isolation, characterization, and optimization of biosurfactant production by an oil-degrading *Acinetobacter junii* B6 isolated from an Iranian oil excavation site

Mandana Ohadi[a], Gholamreza Dehghan-Noudeh[a,*], Mojtaba Shakibaie[b], Ibrahim M. Banat[c], Mostafa Pournamdari[d], Hamid Forootanfar[b,**]

[a] *Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran*

[b] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran*

[c] *Faculty of Life & Health Sciences, University of Ulster, Coleraine BT52 1SA, N. Ireland, UK*

[d] *Department of Medicinal Chemistry, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran*

---

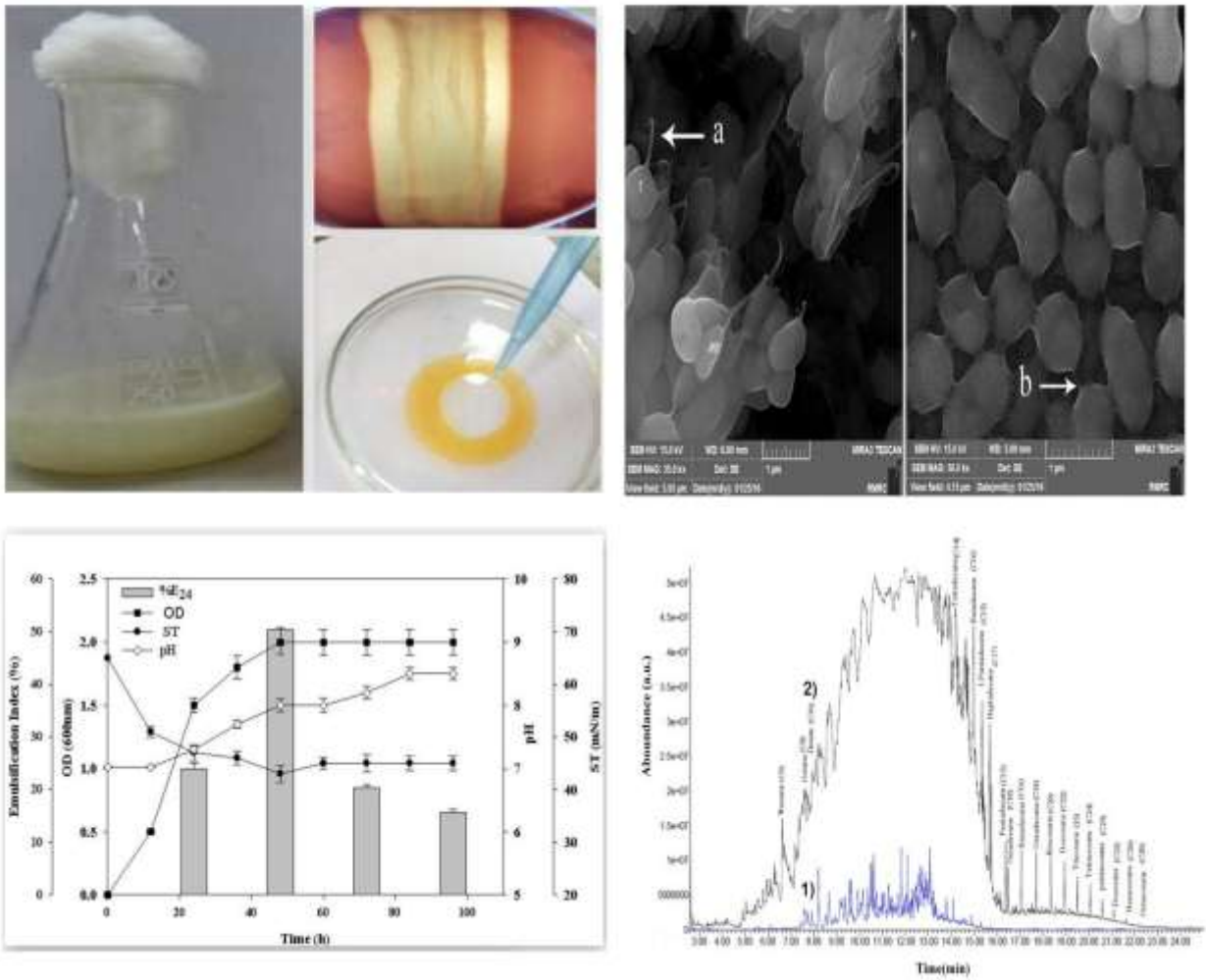
*Corresponding author for biosurfactant-producer isolation: Tel.: +98-34-31325015; fax: +98-34-31325003. E-mail: addresses: ghr_dehghan@kmu.ac.ir (G. Dehghan-Noudeh) gholamreza.dehghannoudeh@utoronto.ca.

**Corresponding author for biosurfactant characterization, optimization, and application: Tel: +98-34-31325238; Fax: +98-34-31325003; E-mail: h_forootanfar@kmu.ac.ir (H. Forootanfar).

Abstract

An enrichment program for hydrocarbon-utilizing bacteria capable of biosurfactant production isolated from soil samples was carried out on mineral salt medium supplemented with Iranian light crude oil (ILCO, 1%). The most promising isolate was identified as *Acinetobacter junii* B6 using 16S rDNA sequencing and biochemical characterization. Time course profile of biosurfactant production using *A. junii* B6 showed surface tension reduction in the culture broth to 45 mN/m after 48 h incubation and a 51% emulsification of ILCO. Scanning electron microscopy (SEM) analysis of *A. junii* B6 showed the tendency of bacterial cells to adhere to each other and to the oil droplets. Application of two-level fractional factorial design showed that surface tension of culture broth was maximally reduced to 38 mN/m in the presence of $NaNO_3$ (2 g/L), ILCO (5%), temperature of 25 °C, aeration rate of 300 rpm, and inoculum size of 2%. GC-MS analysis of the culture broth showed the ability of *A. junii* B6 to degrade most of the alkanes' components of ILCO when used as sole carbon source. Both strain and product has potential applications in various oil industries including bioremediation and enhanced oil recovery.

Graphical Abstract



Key words: Biosurfactant; Optimization; *Acinetobacter junni*; Surface tension; Experimental design

1. Introduction

Microbial derived biosurfactants are amphipathic molecules consisting of both the hydrophobic and hydrophilic domains which allow them to be aggregate at interfaces of immiscible liquids such as water and oil (Zou et al., 2014). In addition, these surface-active metabolites reduce surface tension (ST) and interfacial tension (IFT) within different phases of matters like gas, liquid, and solid (Chandankere et al., 2013). Such properties lead to several potential applications of biosurfactants in cosmetics (anti-cellulite products), pharmaceutics (nano sized drug delivery systems), food (emulsifying and stabilizing additives), and petrochemical (microbial enhanced oil recovery (MEOR) process) industries (Ekpenyong et al., 2017; Li et al., 2016). According to the biosurfactants's origin and the nature of their chemical structures they are categorized into five main groups of lipopeptides, lipoproteins, glycolipids, phospholipids, and polymeric ones (Banat et al., 2010). The lower toxicity, higher biodegradability, mild production conditions, and environmental compatibility of biosurfactants as well as their high selectivity and stable activity at extreme pH, salinity, and temperature make biosurfactants as credible alternative for their petroleum-derived chemically synthesized counterparts (Marchant and Banat, 2012). Despite all these advantages, the commercial success of biosurfactants' applications has not been fully achieved due to the difficulty in their efficient and economical bioprocess development and downstream production technologies (Mukherjee et al., 2006). Optimization of biosurfactant production conditions and the development of cost-effective downstream recovery procedures are therefore among the most important approaches to maximize the biosurfactant production (Banat et al., 2014). In this regards, the restrictions of the classical one-factor-at-a-time process such as time and cost -consuming, as well as inability to predict the effect of the total interactions between the different factors/parameters could be overcome by applying statistical design

approaches (Dos Santos et al., 2016). The relationship between response(s) and the vital input factors involved in building a reliable model to find the optimal medium conditions for maximum biosurfactant yield are usually carried out using factorial designs (FD) and response surface methodology (RSM) (Deepika et al., 2016). Biosurfactant producers mostly exist in oil contaminated soils, where they are able to facilitate the uptake of hydrocarbons through the formation and release of surface active molecules (Chen et al., 2012). There are many reports about the isolation of *Bacillus* and *Pseudomonas* species as biosurfactant producers from oil-contaminated areas (Chandankere et al., 2013; Sharma et al., 2015). However, such ability in the genus of *Acinetobacter* has been seldom described. For example, Zou et al. (2014) isolated *Acinetobacter baylyi* from oil contaminated soil and applied it for MEOR process. In another study, the produced biosurfactant of *Acinetobacter* sp. was optimized using RSM design to reach final 57.5% increase in biosurfactant production (Chen et al., 2012). The main objective of this work was screening and identification of a biosurfactant-producing bacterial (BPB) strain from oil contaminated soils followed by optimization of cultural conditions to reach maximum production of biosurfactant. We also evaluated the selected strain's ability to grow on crude oil and degrade petroleum alkanes.

2. Materials and methods

2.1. Sample collection and isolation of microorganisms

Soil samples were collected from the oil exploration areas of Ahvaz, BiBi Hakimeh (N, 50°24′0″ E, 30°10′48″) in the southwest part of Iran. Five grams of each collected sample was then

suspended in 100 mL sterile normal saline and mixed well. Samples (2 mL) were taken from the upper phase of the suspension to inoculate a 100 mL mineral salt medium (MSM) containing (g/L): $MgSO_4$, 0.1; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.001; $NaNO_3$, 1; and K_2HPO_4, 0.5 supplemented with Iranian light crude oil (ILCO) (1%, v/v) as the sole carbon source in a conical flask. Cultures were then incubated in a rotary shaker incubator (DAIKI SCIENCES Co., Ltd., Seoul, South Korea) (160 rpm, 37 °C) for 72 h as an enrichment method to select hydrocarbon utilizing bacteria. Pure cultures were then obtained through serially diluting the culture and spread plating 500 µL on MSM agar plates incubated at 37 °C for 24 h. Pure bacterial cultures were subsequently preserved at -80°C using glycerol 15%.

2.2. Preliminary screening of the biosurfactant producers

Potential biosurfactant producers were screened based on the hemolytic activity measurement according to Carrillo et al. (1996). In brief, the isolated bacteria were streaked on blood agar plate containing (g/L); trypticase (10), beef extract (3), NaCl (5), and agar (15) supplemented by sterile sheep blood (5%, v/v). The plates were then incubated at 30 °C for 24 h and observed visually for zone of clearance around the colony and the bacterial strains with highest hemolysis zone were selected for further investigations.

2.3. Biosurfactant profile

Seed cultures were prepared by transferring a loop-full of the selected isolate to a 250-mL Erlenmeyer's flask containing 50 mL nutrient broth (NB) medium and incubation at 37 °C for 12 h. Then, 1% of the prepared bacterial inoculum (OD_{600}, 0.8) was transferred to 250-mL Erlenmeyer's flasks containing 100 mL MSM supplemented by ILCO (1% v/v) and incubated

for 96 h. Interval samples (each 12 h up to 96 h) were aseptically taken and analyzed for OD_{600} by UV/VIS spectrophotometer (UV-1800, Shimadzu Co., Tokyo, Japan) followed by centrifugation (7168 ×g for 20 min) and determination of ST (section 2.4.2), emulsification index (EI, section 2.4.3), and pH alteration.

2.4. Evaluation of biosurfactant activity

2.4.1. Oil spreading assay

Oil spreading technique was performed according to Morikawa et al. (2000). Firstly, 10 µL of the supernatant (as mentioned above) was added to the surface of the mineral oil (20 µL), which was previously spread on the distilled water surface (50 mL) and the flatness of the mineral oil droplet in water was observed after 1 min compared to the negative control (MSM without bacteria).

2.4.2. Surface activity measurement

The ST of the culture supernatant (at room temperature) was measured using a duNouy tensiometer apparatus (Tensiometer K100, KRUSS, Germany) working on the principles of Wilhelmy plate method compared to that of the negative control. Pure water was used for calibration of the instrument (70.1 mN/m) (Dehghan–Noudeh et al., 2007). The ST reduction percent was then calculated by the equation (1) as following:

$$\text{ST reduction (\%)} = (\gamma_c - \gamma_s)/\gamma_c \times 100 \quad (1)$$

Where γ_c is the ST of control and γ_s is the ST of the sample obtained from culture supernatant. All measurements were conducted in triplicate and mean of the obtained results was reported.

2.4.3. Emulsification index (EI)

In order to demonstrate the EI of the culture broth, 2 mL ILCO was added to equal volume of the supernatant and vigorously mixed for 2 min and incubated for 24 h, 48 h, and 72 h to obtain the E24, E48, and E72, respectively. EI% was then calculated using the following equation (2) (Chandankere et al., 2013):

EI%= Height of emulsion layer (mm)/Total height of solution (mm) ×100 (2)

2.4.4. Bacterial adhesion to hydrocarbons (BATH) assay

The method of Rosenberg (2006), which is based on a simple photometrical assay, was applied in order to evaluate the bacterial adhesion to various liquid hydrocarbons. For measuring this trait, a turbid aqueous suspension of washed microbial cells (2.5 mL, OD_{600} 2.8) was mixed with the same volume of ILCO, vigorously shaken for 2 min, and allowed time for the two phases to be separated. Thereafter, OD_{600} of the lower phase (1.3) containing bacterial biomass was recorded. The percentage of cells bound to the hydrophobic phase (H %) was then calculated using the following formula:

$$H\% = [1-A/A_0] \times 100 \quad (3)$$

where A_0 is the OD_{600} of the bacterial suspension without hydrophobic phase added and A is the OD_{600} after mixing with hydrophobic phase.

2.5. Identification and morphological characterization of the selected bacterial isolate

The morphological characteristics of the selected isolate were determined according to the Bergey's manual of determinative bacteriology (Vos et al., 2011). For further identification of the isolate analysis of 16S rDNA gene was performed after amplification of ribosomal gene using universal bacterial primers of 27F (AGAGTTTGATCCTGGCTCAG) and rp2 (ACGGCTACCTTGTTACGACTT) (Hasan-Beikdashti et al., 2012). PCR master mix was prepared by addition of 1 µL $MgCl_2$ (2 mM), dNTP mixture (250 µM of each), *Taq* DNA polymerase (1 U), and 10 pmol of each primer followed by insertion of DNA template (40–60 ng) and transferring to FlexCycler thermal cycler (Analytik Jena, Germany). The reaction was run as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 45 s, and 72 °C for 90 s, and the final extension at 72 °C for 5 min. The samples were consequently electrophoresed to check the appearance of the related amplified gene. PCR products were subsequently purified from 1% agarose gel by GeneJET gel extraction kit (Thermo Scientific, California, USA) and sent for automated sequencing (Bioneer, South Korea). The obtained DNA sequence was then matched with deposited genes of GenBank using the basic local alignment search tool (BLAST). Then, the phylogenetic tree was constructed using the MEGA 4.0 software (MEGA, Tempe, AZ, USA).

2.6. Scanning electron microscopy (SEM) analysis

The surface and cross sectional morphology of the selected bacterial isolate were analyzed using field emission scanning electron microscopy (FE-SEM, MIRA 3 XM, Tescan Inc., USA). Briefly, cultures of bacteria were withdrawn at the late exponential growth phase (48 h), filtered by 0.22 µm membranes (Millipore, Bedford), and fixed with 2.5% glutaraldehyde in 0.2 M PBS for 60 min at 4 °C and post-fixed in osmium tetroxide (1%) for 2 h at 4 °C. The cells were

washed with PBS again and dehydrated using increasing concentration (50, 75, 95, and 99.9%) of ethanol. Samples were consequently dried at room temperature and then mounted on aluminium stubs and sputter-coated with gold for 5 min. All samples were examined at an acceleration voltage of 15 kV (Marin et al., 1996).

2.7. Statistical optimization of biosurfactant production

Two-level fractional factorial design was employed to identify the most important medium components and physical factors affecting on biosurfactant production. Five parameters including aeration rate, temperature, ILCO percentage (v/v %), inoculum size, and $NaNO_3$ concentration were selected. The specified codes for each parameter and its levels are presented in Table 1. According to the fractional factorial design, nineteen experiments were performed regarding one replicate and three center point (2^{5-1}). Thereafter, analysis of variance (ANOVA) of the obtained responses was carried out using Design Expert 7.0.0 package (Stat-Ease, Inc., Minneapolis, MN, USA). The mathematical relationship between the five independent variables was estimated by the two-factor interaction (2FI) model equation:

$$y=\beta_0 + \Sigma_{i}^{5}{}_{=1}\,\beta i Xi + \Sigma_{i}^{5}{}_{=1}\,\Sigma_{j}^{5}{}_{=1}\,\beta ijxixj \qquad (4)$$

where y is the predicted response (ST), X represents the coded levels of the independent variables, β_0 is the model constant, βi is the linear coefficient, and βij is the cross-product coefficient.

2.8. Gas chromatographic mass spectroscopy (GC-MS) analysis of residual hydrocarbons after cultivation of the selected isolate in optimized culture media containing ILCO

After cultivation of the selected isolate in optimized culture medium for 7 days, the obtained culture broth (50 mL) was harvested using centrifugation (7168 ×g for 20 min) and subjected to extraction (twice) by the same volume of *n*-hexane. Thereafter, samples were concentrated using rotary evaporator and nitrogen vapour to reach the final volume of 2 mL. Then, the biodegradation of predominant alkanes and the aromatic fractions were analyzed using Agilent gas chromatograph (7890A) apparatus coupled with the mass spectrometer system (Agilent 5975) equipped with DB-1 MS none polar capillary column (30 m×0.25 mm, 0.25 µm film thickness) with helium as the carrier gas at flow rate of 0.9 mL/min. The oven temperature was kept initially at 50 °C for 2 min, followed by an increase to 280 °C at a rate of 4 °C/min. The degradation of selected peaks was identified by searching for closest matches in the Wiley mass spectral libraries.

2.9. Determination of the bacterial growth in ILCO

After preparation of a seed culture (OD_{600}, 0.8) by transferring of bacterial colony into NB medium, one mL of this pre-culture was inserted into 4 mL ILCO and incubated at 37 °C. Interval samples were then taken after 24 h, 48 h, 96 h, and 120 h and the related colony count was measured based on the method previously described (Bezza et al., 2015). One additional flask containing only the ILCO without bacterial inoculum was used as control.

3. Results and discussion

3.1. Isolation and screening for biosurfactant-producer

Four morphologically distinct bacterial colonies grew and were isolated on MSM media supplemented with ILCO as a sole source of carbon and energy. Taking into account the employed enrichment and isolation strategy together with the emulsification ability of biosurfactant producer in the presence of hydrophobic compounds (Fig. 1a), only one efficient BPB was isolated in the present study. It has been shown that enrichment cultures with hydrophobic substrates is a very promising technique for the isolation of BPB strains (Ismail et al., 2013). For example, in the study of Zou et al. (2014) a BPB strain, *A. baylyi* ZJ2, was isolated from crude oil-contaminated soil sample in China. In another study, a BPB strain identified as *Bacillus methylotrophicus* USTBa was isolated from petroleum reservoir in northeast China with a high ability to produce an anionic biosurfactant on MSM supplemented by crude oil (2%, v/v) (Chandankere et al., 2013). Literature review pointed out that biosurfactant production could be determined via additional examinations such as hemolytic activity, oil displacement, and the BATH method (Joy et al., 2017; Mouafi et al., 2016). In an earlier report, Carrillo et al. (1996) have recommended the blood agar lysis procedure as a primary method to screen biosurfactant production and they have found an association between blood hemolysis and biosurfactant production. In this study, all of four above mentioned isolates were initially screened for their ability to produce biosurfactant based on hemolysis. According to its highest hemolytic activity (~ 15 mm, Fig. 1b) around the colony and related growth on the MSM supplemented with ILCO, an efficient BPB strain (designated as isolate B6) was selected for further studies. To confirm these results, oil displacement tests were also performed which exhibited clear diameter of 20 mm with size area of 314 mm^2 (Fig. 1c). Similar results were observed by Chandankere et al. (2013) and Sharma et al. (2015) who isolated *B. methylotrophicus* USTBa and *P. aeruginosa* DSVP20, respectively as BPB from petroleum-

contaminated soil samples. Furthermore, the hydrophobicity of the cell surface of isolate B6 was examined using the BATH method. The obtained results showed an adhesion percentage of 52% for isolate B6 cells to ILCO. The direct relationship between the cell surface hydrophobicity and the level of biosurfactant production was also reported by Pruthi and Cameotra (1997). Among 348 microbial isolates in the study of Sharma et al. (2015) five bacterial strains were found to be positive for BATH examination among which *P. aeruginosa* DSVP20 exhibited the BATH percent of 12% and 14% for hexadecane and pristane, respectively.

3.2. Identification of biosurfactant producing isolate B6

Morphological characteristics and biochemical tests of the isolate B6 were determined using standard protocols. The morphological observations showed that bacterial colonies were circular, convex, and smooth with entire margins. It was characterized as Gram-negative, methyl red and Voges–Proskauer negative, catalase positive, and oxidase negative, which was not able to reduce nitrate to nitrite. In addition, acid was not produced from glucose, maltose, sucrose, and lactose while the bacteria utilized citrate. Gelatin and starch were not hydrolyzed by the isolate. Molecular identification showed that isolate B6 was closely related to *A. junii* with similarity of 93% (Fig. 1d). The 16S rDNA gene sequence was then deposited in GenBank under the accession number of KT946907.

3.3. Profile of biosurfactant production by A. junii B6

The biosurfactant production and growth profiles of *A. junii* B6 were established by time course study of bacterial growth (OD_{600}), pH, ST, and EI_{24}% determination (Fig. 2). Maximum emulsifying activity (51%) and minimal surface tension (45.0 mN/m) were reached in the same way to the cell growth (OD_{600}, 2) that coincided with the exponential growth phase before 48 h (Fig. 2). Medium pH slowly increased from 7 to 8.5 during 96 h incubation, suggesting that a slightly alkaline condition occurred during *A. junii* B6 growth (Chen et al., 2012; Lotfabad et al., 2009). Such alteration in the pH of the cultivation medium could be probably ascribed to the production of alkaline metabolite(s) elaborated out of microbial cell (Padan et al., 2005). It was reported that *Acinetobacter* strains generally grew optimally in the medium with the pH range of 5.0–8.5 (Chen et al., 2012; Cao et al., 2009). However, it merits further investigations to know about the related reason of such pH increasing. The obtained results (Fig. 2) indicated that the biosurfactant biosynthesis occurred predominantly during the exponential growth phase and suggesting that the biosurfactant was produced as primary metabolite accompanying cellular biomass formation (Chen et al., 2012; Khopade et al., 2012; Rodrigues et al., 2006). Growth-associated biosurfactant production was also previously described for the production of Biodispersan by *A. calcoaceticus* (Desai and Desai, 1993). In another study performed by Bento et al. (2005) it was shown that *A. junii*, was able to reduce the surface tension of culture medium to 46.5 mN/m. The air–water surface tension was reduced to 38.4 mN/m by crude biosurfactant produced by *A. calcoaceticus* (Zhao and Wong, 2009).

3.4. Scanning electron microscopy (SEM) analysis

After 48 h of incubation, hydrocarbon dispersion in the aqueous phase of *A. junii* B6 cultures was evident from emulsification of culture broth (Fig. 1a). SEM studies showed that *A. junii* B6 form aggregates in liquid cultures containing hydrocarbons, adhering to each other through an extracellular polymeric material (Fig. 3A and Fig. 3B). Anchor-like appendages were identified on *A. junii* B6 cells as could be seen in Fig. 3C and Fig. 3D. Bacterial cells use the anchor-like appendages to attach themselves to the substratum on a string at distances of several hundred nanometers (Fig. 3A). The anchor-like appendages also functions as a long distance connector between two bacterial cells, extending more than 500 nm in many cases (Fig. 3D). The production of surface-active agents and decreasing the ST of culture media (which was evident from emulsified culture broth, Fig. 1a) also participated in adherence of bacterial cells to the oil droplets for further metabolizing. Similar result was reported by Marin et al. (1996) where *A. calcoaceticus* MM5 aggregated on the crude oil droplets through extracellular appendages and bioemulsifier synthesis. In another survey, Ishii et al. (2004) showed that two morphological types of appendages, the anchor-like appendage and the peritrichate fibril-type appendage, were formed on cells of *Acinetobacter* sp. strain Tol5 after cultivation in basal salt medium containing toluene as carbon source.

3.5. Optimization of medium and data analysis

Medium components such as carbon and nitrogen sources, as well as physical factors like aeration rate, temperature, and inoculum size showed strong influences on biosurfactant production in the present study. To determine the effects of the independent variables the

experimental design was carried out as represented in Table 2. The response (ST) was calculated using the following equation (5):

$$ST = +43.41 - 0.5\,(A) + 0.81\,(B) - 0.98\,(C) - 0.71\,(D) + 1.19\,(E) + 1.92\,(A \times B) + 0.56\,(A \times D) - 1.89\,(A \times E) + 0.29\,(B \times C) - 0.88\,(B \times E) - 0.74\,(C \times E) - 0.34(D \times E) \quad (5)$$

where, ST is surface tension (mN/m) of culture broth, A, B, C, D, and E are coded values pertaining to the aeration rates, temperatures, additives oil percentage, inoculum size, and amount of $NaNO_3$ concentration, respectively (Table 1).

To validate the R^2, an analysis of variance (ANOVA) of the response (ST) was performed (Table 3). According to the ANOVA results, the model of the study was significant ($p \leq 0.0001$) while the lack of fit of the model was not significant ($p \geq 0.6551$). A very high degree of precision (30.173) and a good deal of reliability of the experimental values were indicated by a very low value of the coefficient of variation (CV = 1.22 %). Therefore, the suggested model was able to predict the biosurfactant production efficiently by using the above mentioned conditions. According to the results the 'Pred R^2 of 0.8981 was in reasonable agreement with the 'Adj R^2 of 0.9768 that suggests an excellent correlation between the predicted values and the experimental results (R^2 = 0.9932) (Fig. 4).

The effects of $NaNO_3$ and ILCO concentrations on the ST of culture broth are presented in the three-dimensional (3D) contour plot (Fig. 5a). As it obvious from the mentioned figure and according to its contour, the area with the smallest circle indicates the most appropriate conditions for maximum biosurfactant production (Fig. 5a). Both the ILCO and $NaNO_3$ concentration had the most significant ($p \leq 0.05$) influence on the production of biosurfactant where at the highest concentration of ILCO (5%, v/v) and $NaNO_3$ (2 g/L), the ST of culture broth

was at its lower (38 mN/m) (Fig. 5a). It has been previously reported that types and concentration of the carbon substrates markedly affected the production yield of most biosurfactant (Wu et al., 2008). The carbon sources applied in the microbial culture could be mainly divided into three categories of carbohydrates, vegetable oils, and hydrocarbons (Pornsunthorntawee et al., 2008). Crude oil is a mixture of hydrocarbons identified as a desirable source of carbon supply for biosurfactant production (Ilori et al., 2005; Zou et al., 2014). Wei et al. (2005) reported that hydrophobic substrates like crude oil induce biosurfactant production by *P. aeruginosa* J4 more than did glucose or glycerol as carbon sources. There are many reports that indicated hydrocarbon-degrading microorganisms do not require other carbon sources (Da Silva et al., 2009; Joy et al., 2017; Souza et al., 2014). For instance, Acinetobacter-related bacteria like *A. beijerinckii* had the ability to utilize crude oil when used as sole carbon source (Huy et al., 1999). Our results indicated that the addition of ILCO (5%, v/v) showed a positive effect on the production of the biosurfactant by *A. junii* B6 (decreasing ST from 65 to 38 mN/m), which was in accordance with the results of the Zou et al. (2014) who observed that crude oil affected the biosurfactant production of *A. baylyi* ZJ2 (reducing ST from 65 to 35 mN/m). However, Jagtap et al. (2010) reported that bioemulsifier production by *Acinetobacter* sp. was not influenced by the addition of crude oil. In addition to carbon source, nitrogen sources are other critical factors that regulate the biosurfactant production (Abouseoud et al., 2008; Burkovski, 2003; Najafi et al., 2011; Wu et al., 2008). In this study, it was found that using $NaNO_3$ at concentration of 2 g/L positively affected biosurfactant production (ST of 38 mN/m) (Fig. 5a) which was in agreement with the results of the Lotfabad et al. (2009) and Wu et al. (2008) who showed that the highest biosurfactant production yield (2.1 g/L) and (8.6 g/L), respectively by *P. aeruginosa* was obtained when sodium nitrate applied as the sole nitrogen

source. In contrast, Desai and Banat (1997) and a host of other reports suggested that nitrogen limitation often increased the biosurfactant production. Our results also confirmed the critical role of temperature on biosurfactant production by *A. junii* B6 as showed in Fig. 5b. Maximum production of biosurfactant occurred at 25 °C which was similar to those reported by Cao et al. (2009) who observed that the surface tension of culture medium of *Acinetobacter* sp. reduced to 28.6 mN/m at temperature range of 25 °C to 30 °C. *A. junii* has been reported to grow at the temperature range of 15−37 °C (Vos et al., 2011). Aeration and oxygen supply also have a key role in biosurfactants biosynthesis (Oliveira et al., 2009). The minimum surface tension in the present study was achieved at the lowest temperature (25 °C) and the highest aeration rate (300 rpm) (Fig. 5b). Fig. 5c shows the effects of the $NaNO_3$ concentration and aeration rate on the biosurfactant production indicated that surface tension was decreased when $NaNO_3$ concentration and aeration rate were at the highest levels (Fig. 5c). The relation between temperature and $NaNO_3$ concentration (Fig. 5d) showed the production of the biosurfactant increased when temperature was lowest (25 °C) while the $NaNO_3$ concentration was highest (2 g/L). The validation of the model was carried out by shake flask experiments using the optimized medium. The results showed a reduction in the ST value to 38 mN/m in the predicted optimal conditions ($NaNO_3$, 2 g/L; ILCO, 5%; temperature of 25 °C; aeration rate of 300 rpm; and inoculum size of 2%) compared to the control (65 mN/m), which was confirmed by the prediction point CI (95%) by the software.

3.6. Biodegradation capacities of crude oil by A. junii B6

A. junii B6 was cultured in MSM medium containing ILCO (5%) as the carbon source, and GC-MS analysis was carried out in order to determine the degradation ability of this bacterial strain. According to the Fig. 6a (1) *A. junii* B6 preferentially degraded almost all *n*-alkanes (C8–C28) compared to the aromatic hydrocarbons which are usually much more difficult to degrade within the 7 days duration of the experiment (Huang et al., 2013). GC-MS examination of un-inoculated control culture media showed the presence of all aliphatic and aromatic hydrocarbons (Fig. 6a (2)) without any change. Crude oil as organic pollutants is mainly composed of *n*-alkanes (Hassanshahian et al., 2012; Huang et al., 2013) and several studies have indicated that hydrocarbon removal was closely dependent on their molecular composition (Nkem et al., 2016; Verma et al., 2006). Microorganisms could degrade aliphatic alkanes more easily compared to those of aromatic ones due to their simpler structure (Nkem et al., 2016). *Acinetobacter* spp. isolated from hydrocarbon-contaminated sites have been reported to have a good ability to grow on crude oil because of their capacity to produce biosurfactant (Huang et al., 2013; Lee et al., 2012; Luo et al., 2013; Nkem et al., 2016; Saimmai et al., 2012; Verma et al., 2006). Biosurfactant can enhance hydrocarbon biodegradation by two mechanisms of i) increasing substrate bioavailability for microorganisms and ii) interaction with the microbial cell surface which increases the hydrophobicity of the surface, allowing hydrophobic substrates to associate more easily with bacterial cells surface (Bezza et al., 2015; Elshafie et al., 2015; Tahseen et al., 2016). Hasanshahian and Emtiazi (2008) described a direct relationship between cell surface hydrophobicity and alkane degradation in crude oil-degrading bacteria that were isolated from the Persian Gulf. Mishra et al. (2004) reported that alkanes were degraded by *A. baumannii*. Other studies showed that *A. beijerinckii* (Huang et al., 2013), *A. baumannii* (Nkem et al., 2016),

A. baylyi (Zou et al., 2014), and *A. radioresistens* (Toren et al., 2001) were also able to degrade crude oil hydrocarbon.

3.7. Determination of A. junii B6 *growth in ILCO*

In order to evaluate the growth of *A. junii* B6 in sole ILCO a set of colony count experiment were performed after insertion of *A. junii* B6 pre-culture into ILCO. The attained results (Fig. 6b) showed a significant increase ($p \leq 0.05$) of cell density (log CFU/mL) in the ILCO from 7 to 8.6 during 48 h incubation (Fig. 6b). In contrast, no visible changes were observed in the control flask. Due to the highest production of biosurfactant, bacteria could utilize ILCO as the sole carbon and energy source (Bezza et al., 2015; Bezza and Chirwa, 2015). Bezza et al. (2015) examined the colony count of *Ochrobactrum intermedium* on the amended and unamended medium with biosurfactant. Results showed that colony count was lower in unamended medium compared to the amended medium, because of the biosurfactant activities.

4. Conclusion

An indigenous bacterial strain; *A. junni* B6 was successfully isolated from the contaminated soil samples taken from oil field in the southwest of Iran. *A. junni* B6 was able to grow well on media containing ILCO as sole carbon source because of their ability to produce biosurfactant, and emulsifying these non-hydrophilic hydrocarbons. Furthermore, *A. junii* B6 preferentially degraded almost all the aliphatic hydrocarbons present in the crude oil. Such biosurfactant would

have great potentials in several oil related industries and applications including microbial enhanced oil recovery and bioremediation related activities.

Acknowledgments

The present work was part of a Ph.D. thesis financially (Grant No. 94/417) supported by the Kerman University of Medical Sciences (Kerman, Iran).

References

Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S., Nabi, A., 2008. Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination 223, 143-151.

Banat, I.M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M.G., Fracchia, L., Smyth, T.J., Marchant, R., 2010. Microbial biosurfactants production, applications and future potential. Appl. Microbiol. Biotechnol. 87, 427-444.

Banat, I.M., Satpute, S.K., Cameotra, S.S., Patil, R., Nyayanit, N.V., 2014. Cost effective technologies and renewable substrates for biosurfactants' production. Frontiers Microbiol. 5, 697.

Bento, F.M., de Oliveira Camargo, F.A., Okeke, B.C., Frankenberger, W.T., 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol. Res. 160, 249-255.

Bezza, F.A., Beukes, M., Chirwa, E.M.N., 2015. Application of biosurfactant produced by *Ochrobactrum intermedium* CN3 for enhancing petroleum sludge bioremediation. Process Biochem. 50, 1911-1922.

Bezza, F.A., Chirwa, E.M.N., 2015. Production and applications of lipopeptide biosurfactant for bioremediation and oil recovery by *Bacillus subtilis* CN2. Biochem. Eng. J. 101, 168-178.

Burkovski, A., 2003. Ammonium assimilation and nitrogen control in *Corynebacterium glutamicum* and its relatives: an example for new regulatory mechanisms in actinomycetes. FEMS Microbiol. Rev. 27, 617-628.

Carrillo, P., Mardaraz, C., Pitta-Alvarez, S., Giulietti, A., 1996. Isolation and selection of biosurfactant-producing bacteria. World J. Microbiol. Biotechnol. 12, 82-84.

Chandankere, R., Yao, J., Choi, M.M., Masakorala, K., Chan, Y., 2013. An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir. Biochem. Eng. J. 74, 46-53.

Chen, J., Huang, P., Zhang, K., Ding, F., 2012. Isolation of biosurfactant producers, optimization and properties of biosurfactant produced by *Acinetobacter* sp. from petroleum-contaminated soil. J. Appl. Microbiol. 112, 660-671.

Da Silva, G.P., Mack, M., Contiero, J., 2009. Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol. Adv. 27, 30-39.

Deepika, K., Kalam, S., Sridhar, P.R., Podile, A.R., Bramhachari, P., 2016. Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium *Pseudomonas aeruginosa* KVD-HR42 using response surface methodology. Biocatal Agric. Biotechnol. 5, 38-47.

Dehghan–Noudeh, G., Moshafi, M.H., Sharififar, F., Masoumi, M., 2007. Studies on biosurfactant production by *Acinetobacter calcoaceticus* (PTCC 1318). Jundishapur J. Nat. Pharm. Prod. 2007, 116-123.

Desai, J.D., Banat, I.M., 1997. Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61, 47-64.

Desai, J.D., Desai, A.J., 1993. Production of biosurfactants, in: Kosaric, N. (Ed.), Biosurfactants Production, Properties, Application. Marcel Dekker, New York, pp. 65-97.

Dos Santos, L.F.M., Coutte, F., Ravallec, R., Dhulster, P., Tournier-Couturier, L., Jacques, P., 2016. An improvement of surfactin production by *B. subtilis* BBG131 using design of experiments in microbioreactors and continuous process in bubbleless membrane bioreactor. Bioresour. Technol. 218, 944-952.

Ekpenyong, M., Antai, S., Asitok, A., Ekpo, B., 2017. Response surface modeling and optimization of major medium variables for glycolipopeptide production. Biocatal. Agric. Biotechnol. 10, 113-121.

Elshafie, A.E., Joshi, S.J., Al-Wahaibi, Y.M., Al-Bemani, A.S., Al-Bahry, S.N., Al-Maqbali, D.A., Banat, I.M., 2015. Sophorolipids production by *Candida bombicola* ATCC 22214 and its potential application in microbial enhanced oil recovery. Frontiers Microbiol. 6.

Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M., Ameri, A., Ghahremani, M., Khoshayand, M., Faramarzi, M., 2012. Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. J. Taiwan Inst. Chem. Eng. 43, 670-677.

Hasanshahian, M., Emtiazi, G., 2008. Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. Int. Biodeterior. Biodegrad. 62, 170-178.

Hassanshahian, M., Emtiazi, G., Cappello, S., 2012. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Mar. Pollut. Bull. 64, 7-12.

Huang, L., Xie, J., Lv, B-Y., Shi, X-F., Li, G-Q., Liang, F-L., Lian, J-Y., 2013. Optimization of nutrient component for diesel oil degradation by *Acinetobacter beijerinckii* ZRS. Mar. Pollut. Bull. 76, 325-332.

Huy, N.Q., Jin, S., Amada, K., Haruki, M., Huu, N.B., Hang, D.T., Ha, D.T.C., Imanaka, T., Morikawa, M., Kanaya, S., 1999. Characterization of petroleum-degrading bacteria from oil-contaminated sites in Vietnam. J. Biosci. Bioeng. 88, 100-102.

Ilori, M., Amobi, C., Odocha, A., 2005. Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere 61, 985-992.

Ishii, S.I., Koki, J., Unno, H., Hori, K., 2004. Two morphological types of cell appendages on a strongly adhesive bacterium, *Acinetobacter* sp. strain Tol 5. Appl. Environ. Microbiol. 70, 5026-5029.

Ismail, W., Al-Rowaihi, I.S., Al-Humam, A.A., Hamza, R.Y., El Nayal, A.M., Bououdina, M., 2013. Characterization of a lipopeptide biosurfactant produced by a crude-oil-emulsifying *Bacillus sp*. I-15. Int. Biodeterior. Biodegrad. 84, 168-178.

Jagtap, S., Yavankar, S., Pardesi, K., Chopade, B., 2010. Production of bioemulsifier by *Acinetobacter* species isolated from healthy human skin. Indian J. Exp. Biol. 48, 70-76.

Joy, S., Rahman, P.K.S.M., Sharma S., 2017. Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments. Chem. Eng. J. 317, 232-241.

Cao, J., Xu, Z.H., Li, L.Z., Shen, B., 2009. Biosurfactant-producing petroleum-degrader-*Acinetobacter* BHSN. J. Ecol. Rural Environ. 25, 73-78.

Khopade, A., Ren, B., Liu, X.-Y., Mahadik, K., Zhang, L., Kokare, C., 2012. Production and characterization of biosurfactant from marine *Streptomyces* species B3. J. Colloid Interface Sci. 367, 311-318.

Lee, M., Woo, S.-G., Ten, L.N., 2012. Characterization of novel diesel-degrading strains *Acinetobacter haemolyticus* MJ01 and *Acinetobacter johnsonii* MJ4 isolated from oil-contaminated soil. World J. Microbiol. Biotechnol. 28, 2057-2067.

Li, J., Deng, M., Wang, Y., Chen, W., 2016. Production and characteristics of biosurfactant produced by *Bacillus pseudomycoides* BS6 utilizing soybean oil waste. Int. Biodeterior. Biodegrad. 112, 72-79.

Lotfabad, T.B., Shourian, M., Roostaazad, R., Najafabadi, A.R., Adelzadeh, M.R., Noghabi, K.A., 2009. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloids Surf. B. Biointerfaces 69, 183-193.

Luo, Q., Zhang, J-G., Shen, X-R., Fan, Z-Q., He, Y., Hou, D-Y., 2013. Isolation and characterization of marine diesel oil-degrading *Acinetobacter* sp. strain Y2. Ann. Microbiol. 63, 633-640.

Marchant, R., Banat, I.M., 2012. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol. 30, 558-565.

Marin, M., Pedregosa, A., Laborda, F., 1996. Emulsifier production and microscopical study of emulsions and biofilms formed by the hydrocarbon-utilizing bacteria *Acinetobacter calcoaceticus* MM5. Appl. Microbiol. Biotechnol. 44, 660-667.

Mishra, S., Sarma, P.M., Lal, B., 2004. Crude oil degradation efficiency of a recombinant *Acinetobacter baumannii* strain and its survival in crude oil-contaminated soil microcosm. FEMS Microbiol. Lett. 235, 323-331.

Mouafi, Abo Elsoud, M.M., Moharam, M.E., 2016. Optimization of biosurfactant production by Bacillus brevis using response surface methodology. Biotechnol. Reports 9, 31–37.

Morikawa, M., Hirata, Y., Imanaka, T., 2000. A study on the structure–function relationship of lipopeptide biosurfactants. Biochim. Biophys. Acta (BBA)-Mol. Cell Biol. Lipids. 1488, 211-218.

Mukherjee, S., Das, P., Sen, R., 2006. Towards commercial production of microbial surfactants. Trends Biotechnol. 24, 509-515.

Najafi, A., Rahimpour, M., Jahanmiri, A., Roostaazad, R., Arabian, D., Soleimani, M., Jamshidnejad, Z., 2011. Interactive optimization of biosurfactant production by *Paenibacillus alvei* ARN63 isolated from an Iranian oil well. Colloids Surf. B. Biointerfaces. 82, 33-39.

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M.P., Medipally, S.R., Kannan, N., 2016. Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. Mar. Pollut. Bull. 107, 261-268.

Oliveira, F., Vazquez, L., De Campos, N., De Franca, F., 2009. Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. Process Biochem. 44, 383-389.

Padan, E., Bibi, E., Ito, M., Krulwich, T.A., 2005. Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta. 1717, 67-88.

Pornsunthorntawee, O., Arttaweeporn, N., Paisanjit, S., Somboonthanate, P., Abe, M., Rujiravanit, R., Chavadej, S., 2008. Isolation and comparison of biosurfactants produced by *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery. Biochem. Eng. J. 42, 172-179.

Pruthi, V., Cameotra, S.S., 1997. Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity technique. Biotechnol. Tech. 11, 671-674.

Rodrigues, L., Teixeira, J., Oliveira, R., Van Der Mei, H.C., 2006. Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. Process Biochem. 41, 1-10.

Rosenberg, M., 2006. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol. Lett. 262, 129-134.

Saimmai, A., Kaewrueng, J., Maneerat, S., 2012. Used lubricating oil degradation and biosurfactant production by SC-9 consortia obtained from oil-contaminated soil. Ann. Microbiol. 62, 1757-1767.

Sharma, D., Ansari, M.J., Al-Ghamdi, A., Adgaba, N., Khan, K.A., Pruthi, V., Al-Waili, N., 2015. Biosurfactant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization. Environ. Sci. Poll. Res. 22, 17636-17643.

Souza, E.C., Vessoni-Penna, T.C., de Souza Oliveira, R.P., 2014. Biosurfactant-enhanced hydrocarbon bioremediation: An overview. Int. Biodeter. Biodegr. 89, 88-94.

Tahseen, R., Afzal, M., Iqbal, S., Shabir, G., Khan, Q.M., Khalid, Z.M., Banat, I.M., 2016. Rhamnolipids and nutrients boost remediation of crude oil-contaminated soil by enhancing bacterial colonization and metabolic activities. Int. Biodeterior. Biodegrad. 115, 192-198.

Toren, A., Navon-Venezia, S., Ron, E.Z., Rosenberg, E., 2001. Emulsifying activities of purified alasan proteins from *Acinetobacter radioresistens* KA53. Appl. Environ. Microbiol. 67, 1102-1106.

Verma, S., Bhargava, R., Pruthi, V., 2006. Oily sludge degradation by bacteria from Ankleshwar, India. Int. Biodeterior. Biodegrad. 57, 207-213.

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K. H., Whitman, W., 2011. Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. New York, USA, Springer-Verlag.

Wei, Y-H., Chou, C-L., Chang, J-S., 2005. Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biochem. Eng. J. 27, 146-154.

Wu, J-Y., Yeh, K-L., Lu, W-B., Lin, C-L., Chang, J-S., 2008. Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. Bioresour. Technol. 99, 1157-1164.

Zhao, Z., Wong, J.W., 2009. Biosurfactants from *Acinetobacter calcoaceticus* BU03 enhance the solubility and biodegradation of phenanthrene. Environ. Technol. 30, 291-299.

Zou, C., Wang, M., Xing, Y., Lan, G., Ge, T., Yan, X., Gu, T., 2014. Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude

oil-contaminated soil sample toward microbial enhanced oil recovery applications. Biochem. Eng. J. 90, 49-58.

Figure Captions:

Fig. 1. Cultivation of *A. junii* B6 in a) MSM medium supplemented with ILCO (5%) and b) Blood agar medium showing erythrocyte hemolysis. c) Oil dispersion ability of the *A. junii* B6 culture broth and d) Phylogenic tree of the 16S rDNA gene sequence of *A. junii* B6 derived by MEGA 4.0 software.

Fig. 2. Time course profile of the growth (OD_{600}), surface tension (ST) reduction, $\%E_{24}$, and pH alteration of *A. junii* B6 during 96 h cultivation in desired culture medium.

Fig. 3. Scanning electron micrograph (SEM) of *A. junii* B6 cells adhering to emulsion droplets after cultivation in the presence of ILCO. A) Aggregation of bacterial cells to the oil droplets and B-D) to each other via a) peritrichate fibrils and b) anchor-like appendages could be clearly seen on *A. junii* B6 cell surface.

Fig 4. The related plot of predicted vs. actual of surface tension (ST) reduction for *A. junni* B6 derived from Design Expert software.

Fig. 5. The related 3D counter plots of a) $NaNO_3$ concentration and ILCO percent, b) temperature and aeration rate, c) $NaNO_3$ concentration and aeration rate, and d) $NaNO_3$ concentration and temperature versus surface tension of the culture broth of *A. junni* B6 drawn by Design Expert software.

Fig. 6. a) Gas chromatogram of the residual ILCO (1) after 7 days cultivation of *A. junii* B6 in the optimized medium at 25 °C and (2) that of the abiotic negative control (optimized medium in the absence of *A. junii* B6) and b) bacterial colony count (n=3) of *A. junii* B6 during 120 h

cultivation in the ILCO. The significant values were symbolized as * on each bar (compared to that of the control group, $p \leq 0.05$) after Tukey Post Hoc analysis.

Table 1. Parameters used and level applied in the fractional factorial design (FFD) for maximizing biosurfactant production by *A. junni* B6 in shake flasks.

Variables	Symbol	Unit	Test levels of each parameter		
			-1	0	+1
Aeration rate	(A)	(rpm)	150	225	300
Temperature	(B)	(^{o}C)	25	31	37
ILCO*	(C)	(v/v %)	1	3	5
Inoculum size	(D)	(%)	1	2	3
$NaNO_3$	(E)	(g/L)	0.2	1.1	2

* Iranian light crude oil

Table 2. Experimental design and results of the fractional factorial design (FFD).

Run	Coded value of independent factor					ST (mN/m)	
	A	B	C	D	E	Experimental	Predicted
1	-1	-1	-1	-1	1	52.5±0.07	52.62
2	1	-1	-1	-1	-1	40.4±0.04	40.33
3	-1	1	-1	-1	-1	41.1±0.09	41.46
4	1	1	-1	-1	1	45.8±0.09	45.97
5	-1	-1	1	-1	-1	41.8±0.05	41.44
6	1	-1	1	-1	1	39±0.07	38.83
7	-1	1	1	-1	1	45.3±0.02	45.18
8	1	1	1	-1	-1	47±0.09	47.07
9	-1	-1	-1	1	-1	40.6±0.08	40.67
10	1	-1	-1	1	1	42±0.02	41.88
11	-1	1	-1	1	1	45±0.04	44.83
12	1	1	-1	1	-1	47.7±0.07	47.34
13	-1	-1	1	1	1	45.2±0.04	45.37
14	1	-1	1	1	-1	39.3±0.07	39.66
15	-1	1	1	1	-1	39.8±0.09	39.73
16	1	1	1	1	1	42±0.06	42.12
17	0	0	0	0	0	45±0.04	44.40
18	0	0	0	0	0	43.8±0.05	44.40
19	0	0	0	0	0	44.4±0.05	44.40

Table 3. Analysis of variance (ANOVA) [partial sum of squares-type III] of medium components in relation to surface tension as per two-level factorial design.

Source	**Sum of Squares**	**Degree of freedom**	**Mean square**	**F-value**	**P-value Prob > F**
Model	206.47	12	17.21	60.72	0.0001
A-Aeration rate	4.10	1	4.10	14.47	0.0126
B-Temperature	10.40	1	10.40	36.70	0.0018
C-ILCO	15.41	1	15.41	54.36	0.0007
D-Inoculum size	7.98	1	7.98	28.16	0.0032
E-$NaNO_3$	22.80	1	22.80	80.46	0.0003
AB	58.91	1	58.91	207.87	< 0.0001
AD	4.95	1	4.95	17.47	0.0087
AE	57.38	1	57.38	202.46	< 0.0001
BC	1.38	1	1.38	4.87	0.0784
BE	12.43	1	12.43	43.85	0.0012
CE	8.85	1	8.85	31.23	0.0025
DE	1.89	1	1.89	6.67	0.0493
Curvature	2.49	1	2.49	8.80	0.0313
Residual	1.42	5	0.28		
Lack of Fit	0.70	3	0.23	0.65	0.6551
Pure Error	0.72	2	0.36		
Corrected total	210.38	18			

R^2 = 0.9932; Adj R^2= 0.9768; Pred R^2=0.8981; Adeq Precision= 30.173

Highlights:

- Biosurfactant producer *A. junni* was isolated from Iranian oil contaminated soils.
- Experimental design approach was applied for optimization of medium components.
- SEM micrograph revealed the formation of anchor-like appendages on *A. junni* surface.
- *A. junni* was able to grow in the presence of crude oil as sole carbon source.
- Almost all *n*-alkanes of crude oil were eliminated by *A. junni* after 7 days.

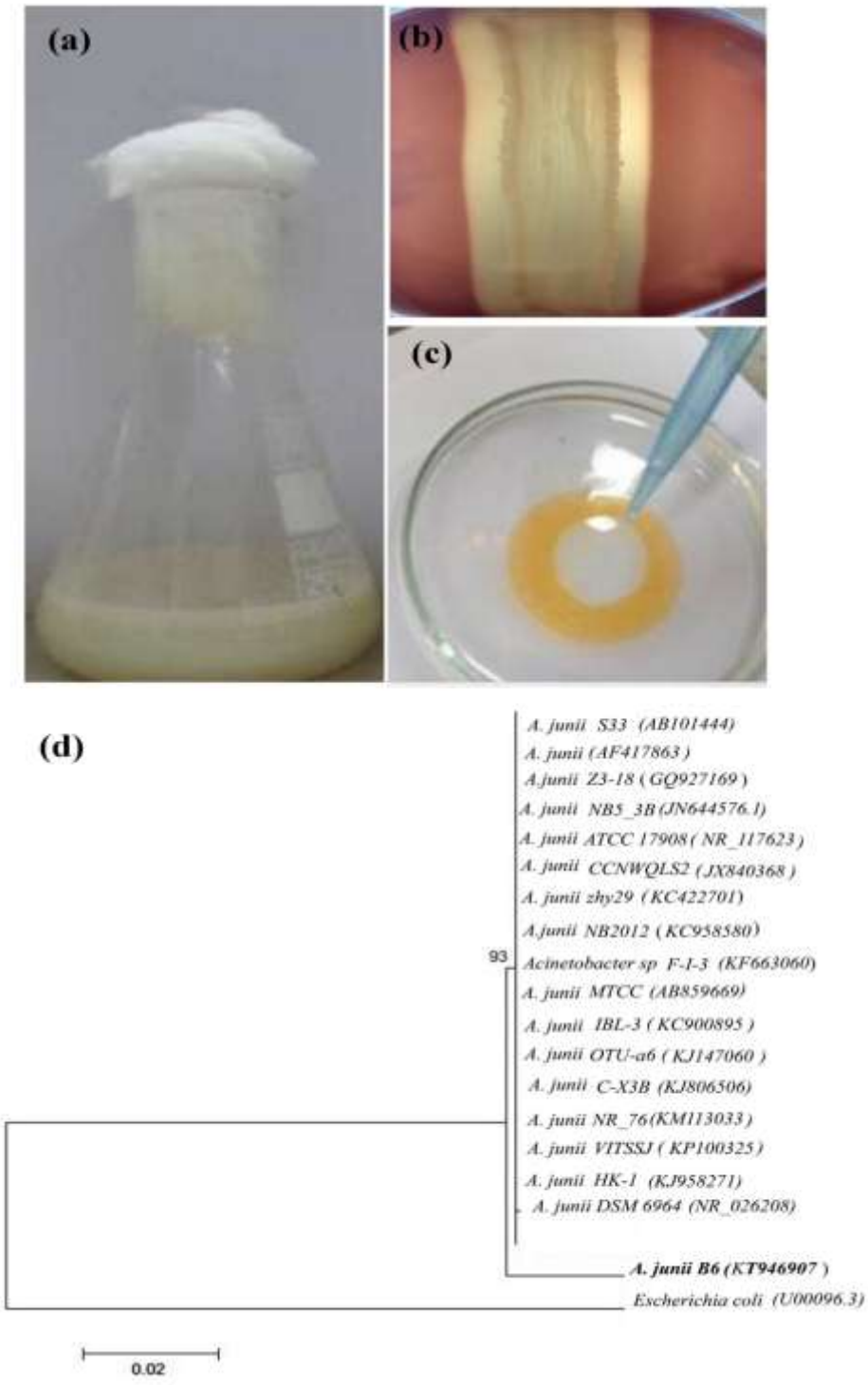

(a)
(b)
(c)
(d)
A. junii S33 (AB101444)
A. junii (AF417863)
A.junii Z3-18 (GQ927169)
A. junii NB5_3B (JN644576.1)
A. junii ATCC 17908 (NR_117623)
A. junii CCNWQLS2 (JX840368)
A. junii zhy29 (KC422701)
A.junii NB2012 (KC958580)
93
Acinetobacter sp F-I-3 (KF663060)
A. junii MTCC (AB859669)
A. junii IBL-3 (KC900895)
A. junii OTU-a6 (KJ147060)
A. junii C-X3B (KJ806506)
A. junii NR_76(KM113033)
A. junii VITSSJ (KP100325)
A. junii HK-1 (KJ958271)
A. junii DSM 6964 (NR_026208)
A. junii B6 (KT946907)
Escherichia coli (U00096.3)
0.02

Fig. 1.

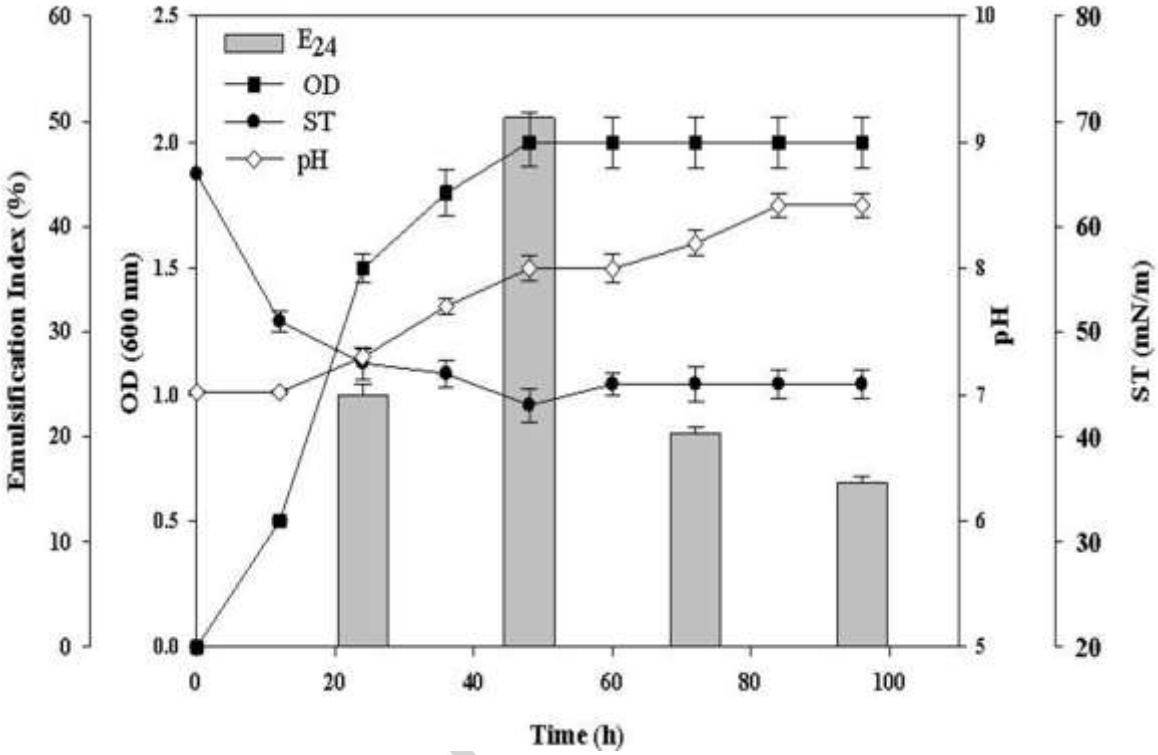


Fig. 2.

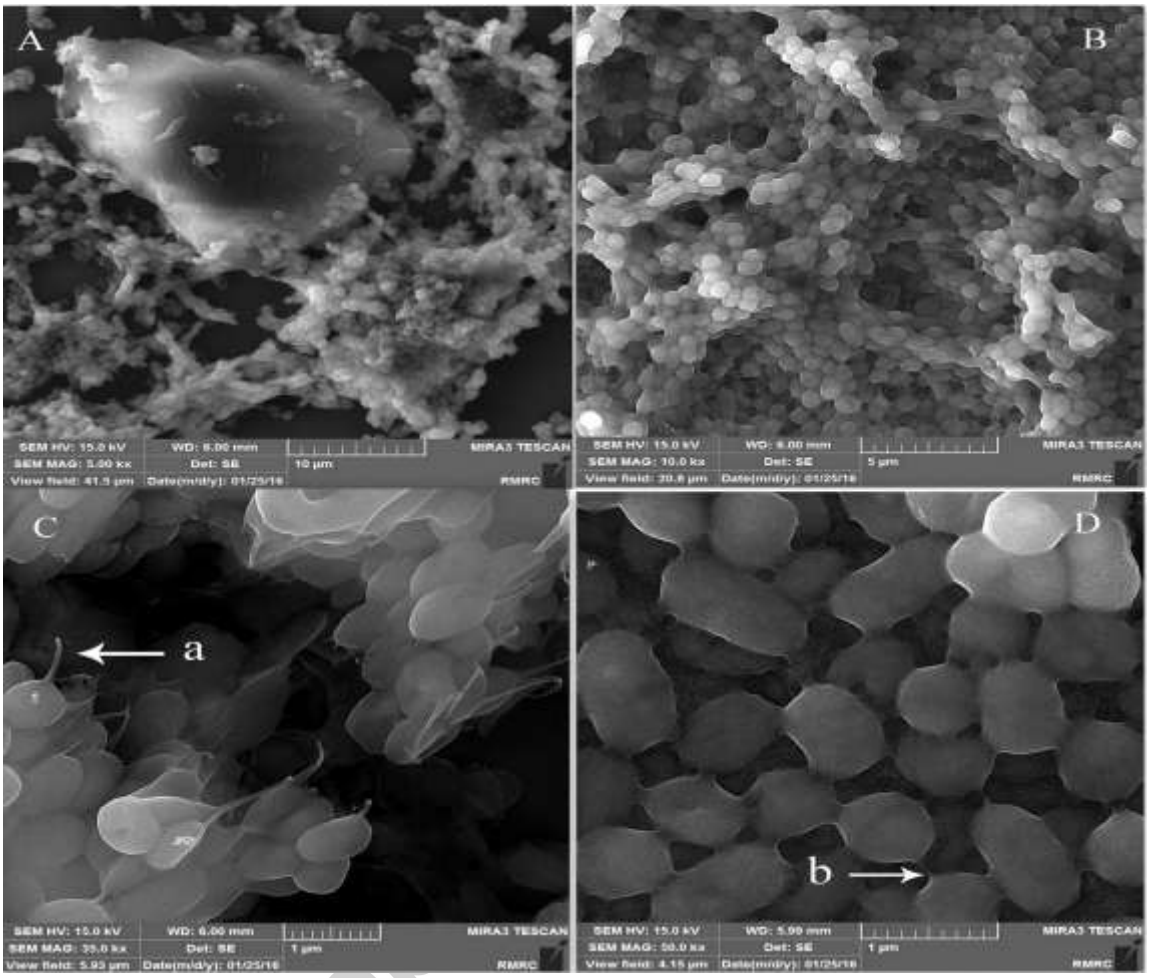


Fig. 3.

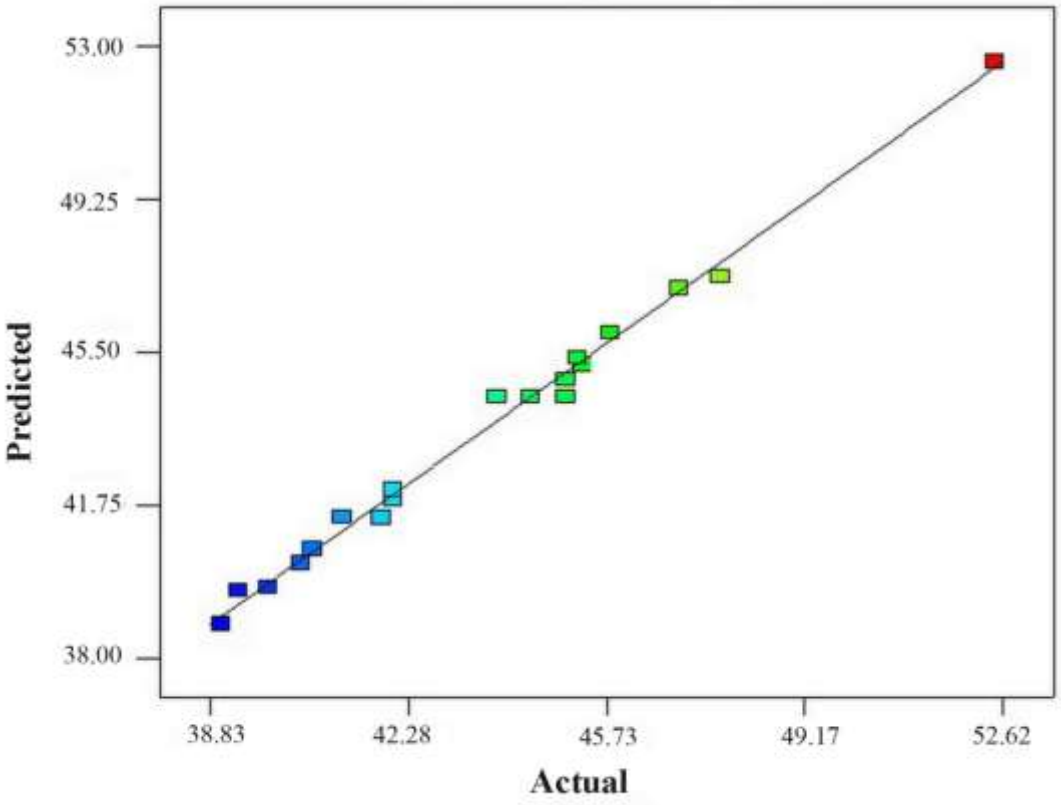


Fig. 4.

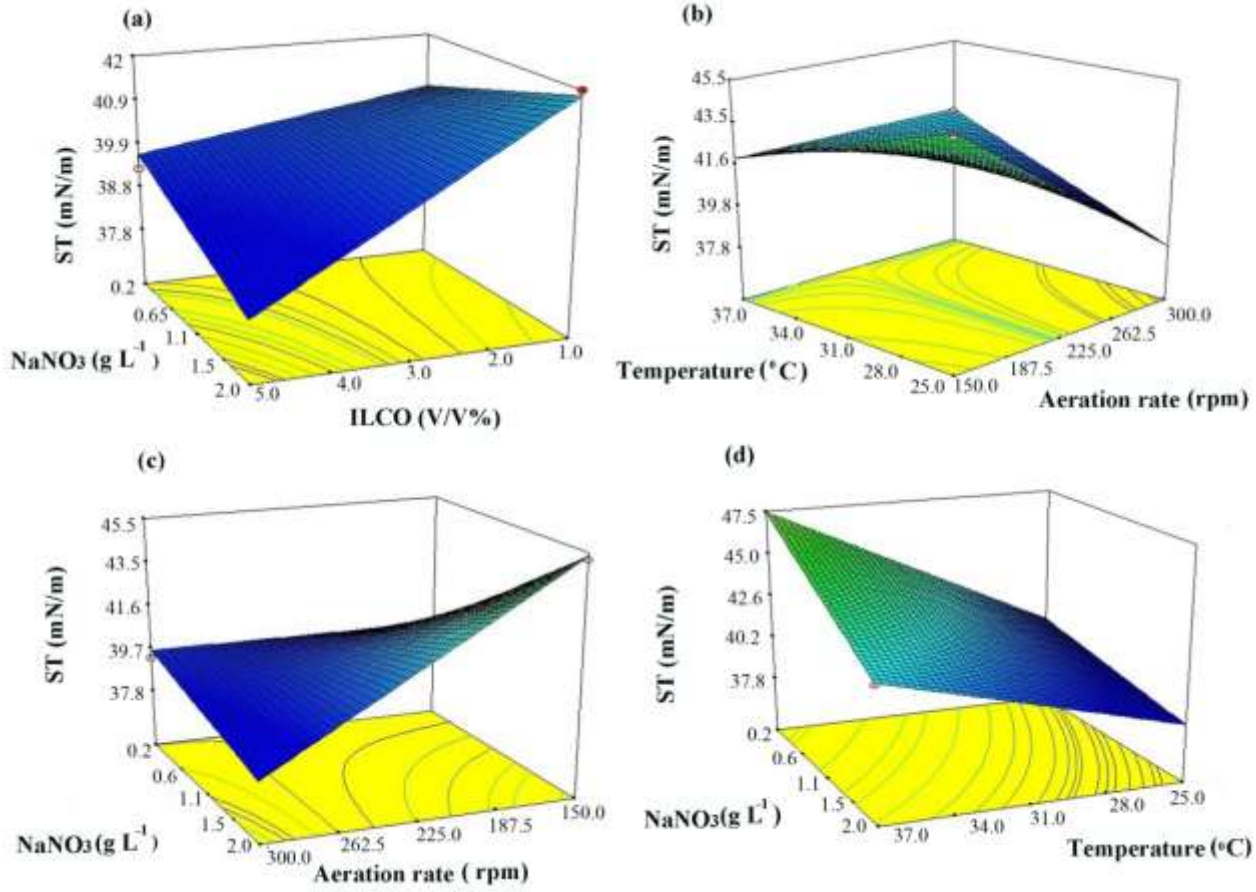


Fig. 5.

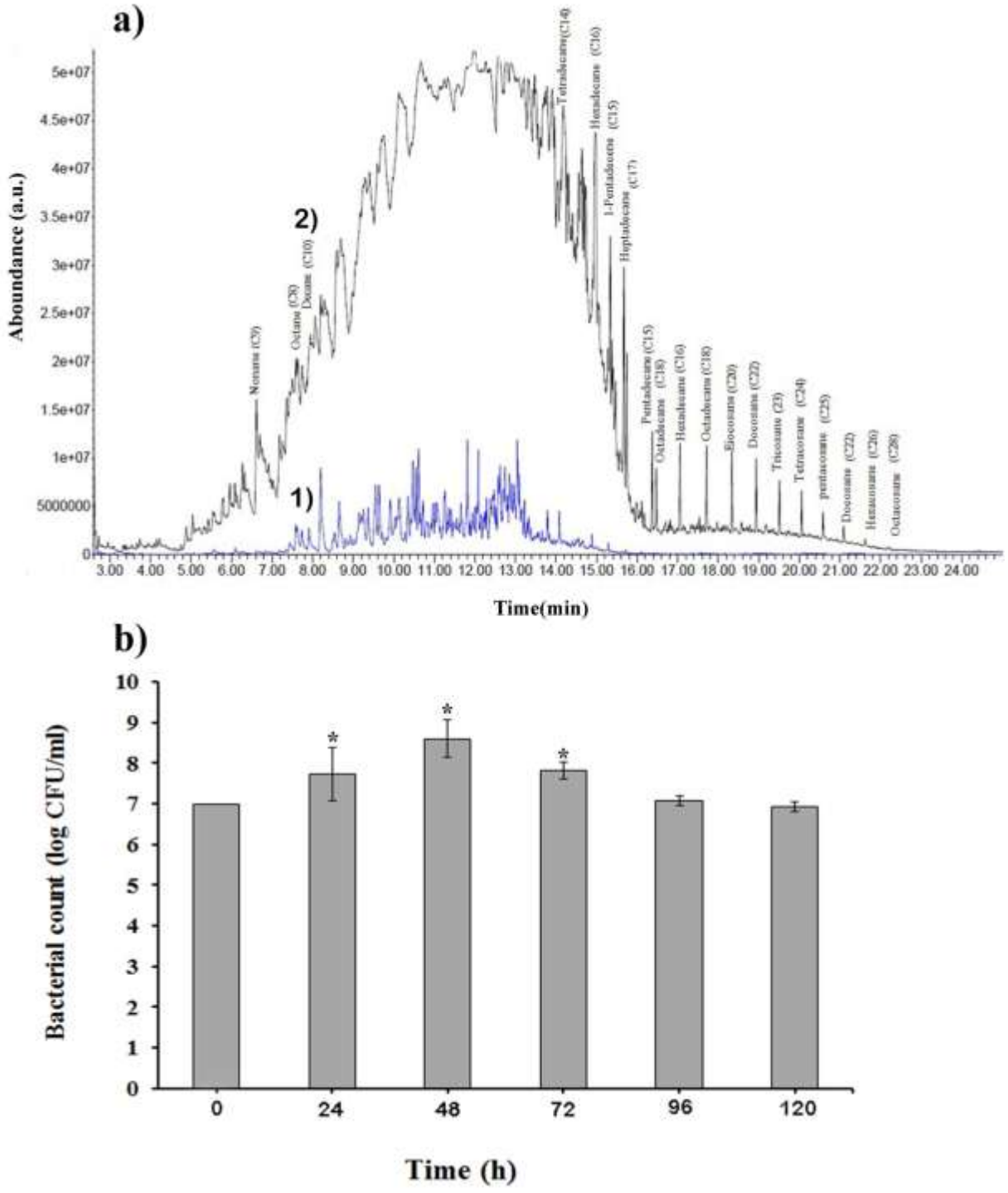

a)
Abundance (a.u.)
5e+07
4.5e+07
4e+07
3.5e+07
3e+07
2.5e+07
2e+07
1.5e+07
1e+07
5000000
0
2)
1)
Nonane (C9)
Octane (C8)
Decane (C10)
Tetradecane(C14)
Hexadecane (C16)
1-Pentadecene (C15)
Heptadecane (C17)
Pentadecane (C15)
Octadecane (C18)
Hexadecane (C16)
Octadecane (C18)
Eicosane (C20)
Docosane (C22)
Tricosane (23)
Tetracosane (C24)
pentacosane (C25)
Docosane (C22)
Hexacosane (C26)
Octacosane (C28)
3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.00 20.00 21.00 22.00 23.00 24.00
Time(min)
b)
Bacterial count (log CFU/ml)
10
9
8
7
6
5
4
3
2
1
0
*
*
*
0
24
48
72
96
120
Time (h)

Fig. 6.