An Efficient Biosurfactant by *Pseudomonas stutzeri* Z12 Isolated from an Extreme Environment for Remediation of Soil Contaminated with Hydrocarbons

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https://doi.org/10.15255/CABEQ.2019.1718

Original scientific paper
Received: August 10, 2019
Accepted: March 31, 2020

Capability of a biosurfactant produced by *Pseudomonas stutzeri* Z12 for the removal of hydrocarbons from oily sludge contaminated soil was investigated. The effect of operating parameters, including pH, critical micelle concentration (CMC), salinity, and contact time were studied. The chemical structure of produced biosurfactant was characterized using FTIR and LC-MS-MS analysis, which revealed that the extracted biosurfactant was a combination of both mono- and di-rhamnolipid congeners. The main three congeners $RhaC_{12:1}C_{10}$ (529.9 m z^{-1}), $RhaC_{12}C_{10}$ (531 m z^{-1}), and $RhaC_{10}C_{10}$ (503.2 m z^{-1}) were associated to mono-rhamnolipid, while five congeners, $RhaRhaC_{10}C_8$ (621.2 m z^{-1}), $RhaRhaC_{12}C_{12}$ (707.7), $RhaRhaC_{10}C_{12}$ (677.1), $RhaRhaC_{10}C_{12:1}$ (675.3 m z^{-1}), and $RhaRhaC_{10}C_1$ (649.5 m z^{-1}) were associated to di-rhamnolipid structures. The critical micelle concentration (CMC) was 80 mg L^{-1}, and emulsification index (E_{24}) values for *n*-hexadecane, *n*-hexane, kerosene, diesel oil, xylene, and crude oil were 62.1, 57.6, 54.4, 41.5, 46.9, and 30.2 %, respectively.

Keywords:

soil washing, biosurfactant, TPH (total petroleum hydrocarbons), oily sludge, soil, *Pseudomonas stutzeri* Z12

Introduction

Oily sludge contains toxic compounds, such as hydrocarbons that exert carcinogenic and mutagenic effects on humans, and present environmental risks due to their persistency in the environment.[1–6] The US Environment Protection Agency (USEPA) has included oily sludge on the list of priority pollutants (K series).[7,8] Therefore, the removal and treatment of these wastes before their emission into the environment is vital.[9] The common treatment methods for the removal of oil pollutants are often biological, physical or chemical processes, such as bioremediation, incineration, chemical extraction, chemical oxidation, sonication, etc.[10,11] These methods are limited due to their inefficiency in some cases, and for being time-consuming, costly and causing by-product pollutants.[12,13] Also, it should be noted that the biodegradation of petroleum hydrocarbons is limited due to their low solubility and recalcitrant nature.[14] Soil washing with different agents is a widespread and proved technology for enhancement of the desorption and solubility of hydrocarbons in soil.[15,16] In soil washing, the contaminants are separated from the solid matrix by providing a deep contact between washing agent, contaminant, and soil.[17] Soil washing can be performed with different washing solutions such as solvents, hot water, emulsifiers, biosurfactants, and chemical surfactants.[18,19] Synthetic surfactants and biosurfactants are amphiphilic compounds, which can enhance the solubility of hydrophobic organic compounds (HOCs) by reducing surface tension at the soil/water interface.[20,21] However, the problem related to chemical surfactants is their toxicity and persistence.[22] Hence, the use of biosurfactants having lower toxicity, higher biodegradability, technical efficiency, and cost-effectiveness has increased.[23] Biosurfactants are natural compounds that are synthesized by microorganisms. Basically, biosurfactants are classified as lipoproteins, phospholipids, glycolipids, lipopeptides, and fatty acids.[24,25] Biosurfactants can reduce

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surface tension through aggregation and formation of micelles, and thus cause an increase in solubility and mobility of oil in water. In this study, isolation and screening of *Pseudomonas stutzeri* Z12 as a biosurfactant-producing strain were carried out and, after characterization of extracted biosurfactant, the effect of extracted biosurfactant on desorption of petroleum hydrocarbons from soil was investigated and compared with that of a chemical surfactant. The results of this study are expected to provide a useful tool for introducing an efficient biosurfactant in desorption of hydrocarbons from contaminated soil.

Materials and methods

Chemical reagents

Methanol (CH_3OH, 96 %), chloroform ($CHCl_3$, 99.5 %) and *n*-hexane ($CH_3(CH_2)_4CH_3$, ≥ 95.0 %) were all of analytical grade and supplied by Sigma-Aldrich, USA. Constituents of phosphate mineral salt (PMS), nutrient broth, nutrient agar, and Tween 80 ≥ 98.0 % were purchased from Merck, Germany, and used with no additional purification.

Soil contaminated with oily-sludge

The soil contaminated with oily sludge was collected near surface impoundments of oil industries in Khuzestan province, Iran. To determine the main elements present in the soil, X-ray fluorescence spectroscopy analysis (XRF, PW1410, Holland) was performed. The soil moisture content was measured according to ASTM method (ASTM D422-63, 1998).[26] The pH was measured using a digital pH meter (Cyberscaneutech-instruments 5500). Also, a qualitative GC-MS analysis (GC Agilent 7890 – MS Agilent 5975) was performed to determine the type and amount of hydrocarbons in the contaminated soil.

Isolation of biosurfactant-producing strain

Isolation of biosurfactant-producing bacterial strains was performed by the modified enrichment culture method described by Jorfi *et al*.[27] Briefly, a sample of soil contaminated with oil sludge (20 g) with salinity of 30–40 g kg^{-1} was transferred to a 250-mL Erlenmeyer flask containing 100 mL phosphate mineral salt (PMS), and stirred vigorously for 30 min with a shaker incubator (IKM 4000ci, Germany). The flask was the left to rest until complete settling of the solids. In the next step, an aliquot of 5 mL of supernatant was added to a 250-mL flask containing 95 mL of sterilized PMS. The composition of the PMS medium was as follows: (g L^{-1}), K_2HPO_4 (6.3), KH_2PO_4 (1.8), $CaCl_2{\cdot}H_2O$ (0.1), $MgSO_4{\cdot}7H_2O$ (0.1), and $FeSO_4{\cdot}7H_2O$ (0.1). The trace element solution consisted of (g L^{-1}): H_3BO_3 (0.03), $ZnSO_4{\cdot}7H_2O$ (0.01), $CoCl_2{\cdot}6H_2O$ (0.02), $NaMoO_4$ (0.006), $CuSO_4{\cdot}2H_2O$ (0.001). The pH of culture media was adjusted to 7 ± 0.5 using hydrochloric acid (HCl) and sodium hydroxide (NaOH) solutions. Crude oil (2 % v v^{-1}) was used as the only source of carbon and energy for bacterial growth. The flask was placed in a shaker incubator (IKM 4000ci, Germany) at 35 °C for 7 days at 180 rpm. Variations of bacterial growth were determined by measuring the optical density of the samples at 600 nm ($OD_{600\ nm}$). After one week of incubation, 5 mL of culture solution was transferred into a fresh PMS medium with same conditions. This procedure was repeated for seven weeks. Afterwards, 1 mL of the culture broth was serially diluted to 10^{-4} – 10^{-6}, transferred to nutrient agar plates, and incubated at 31 °C for 24 h. In order to obtain pure isolates, bacterial colonies of different morphology were carefully chosen. The isolates were preserved in 30 % glycerol stored at –80 °C. For assessing biosurfactant production, pure strains with the same optical density (OD_{600} = 2) were prepared in nutrient broth (NB). Then 5 mL of nutrient broth medium was transferred to a 250-mL Erlenmeyer flask containing 100 mL PMS supplemented with 2 % (v v^{-1}) crude oil, and placed in a shaker incubator for one week at 35 °C at 180 rpm to monitor the possibility of biosurfactant production.

Screening of biosurfactant production

The oil spreading method is a preliminary test for detection of biosurfactant production. The procedure was performed by adding 50 μL of crude oil to a 150-mm diameter plate containing 40 mL of distilled water. Fifteen μL of culture solution was then added to the oil surface, and the diameter of clear zone on the oil surface was measured and compared with the negative test control. The existence of the clear zone was an indicator of biosurfactant production.[28] Drop collapse test is a quick test for screening biosurfactant production. This test is based on the destabilization of liquid droplets by biosurfactants, and was done in microwell plates; 40 mL of biosurfactant solution was poured on the surface of paraffin. If the solution contains no biosurfactant, the polar water molecules would be repelled from the hydrophobic surface, and the drops would remain stable. Otherwise, the drops spread or collapse, because the force or interfacial tension between the liquid drop, and the hydrophobic surface is declined.[29] Also, the blood agar plate test was performed as a primary method for determination of biosurfactant activity. A small amount of culture broth was added to blood agar medium and the

plate incubated at 37 °C for 48 – 72 hours.[30] In addition, measurement of surface tension of culture medium was done as a valid test for the presence of biosurfactant.

Extraction and characterization of biosurfactant

The culture broth was centrifuged at 4 °C for 15 min to remove bacterial mass. For biosurfactant precipitation, the pH of cell-free supernatant was adjusted to 2 by using 2 N HCl, and the precipitate settled overnight at 4 °C. The biosurfactant was then extracted according to method of Joy *et al.* with chloroform:methanol solution of 2:1 w w^{-1}, the solvent was evaporated under vacuum, and the remaining solid identified as crude biosurfactant.[31]

The emulsification activity of the biosurfactant solution was evaluated by measuring the emulsification index (E_{24}) according to the method described by Cooper and Goldenberg.[32] Briefly, 2 mL of each hydrocarbon (crude oil, *n*-hexadecane, *n*-hexane, diesel, and kerosene) was added into 15-mm-diameter test tubes containing 2 mL of the cell-free culture broth. The contents of the tubes were then vortexed for 2 min, and left to stand for 24 h to determine the stability of the emulsion. The E_{24} was calculated according to Eq. (1).

$$\text{Emulsion Index } (E_{24}\ \%) = \text{Height of emulsion layer/Total height} \cdot 100 \quad (1)$$

The surface tension (ST) was measured using the Du-Nouy ring method,[33] with a tensiometer (Sigma 700, Khushboo Sci. Co., Mumbai). In addition, for the determination of critical micelle concentration (CMC), various concentrations of biosurfactant solution (0 – 200 mg L^{-1}) were prepared by dissolving the crude biosurfactant in distilled water. The CMC was determined by plotting ST versus concentration of biosurfactant in the solution.

Functional groups such as alkyl, carbonyl, ester, etc., in the biosurfactant structure, were identified by Fourier transform infrared (FTIR) spectroscopy analysis (Spectrum 400 FTIR Spectrophotometer, USA). Analysis of the spectra was performed in a range of 400 – 4000 cm^{-1}. In addition, the mass spectrum and chemical structure of the components of pure biosurfactant were identified by liquid chromatography – tandem mass spectrometry (LC-MS-MS) analysis (Q-trap 3200, USA). Purification of the biosurfactant was performed using column chromatography. One gram of crude biosurfactant was dissolved in 5 mL of chloroform and poured onto silica gel (60 – 120 mesh size). The loaded column was washed with chloroform and then chloroform:methanol mobile phases were applied in sequence; 50:3 v v^{-1} (300 mL), 50:5 v v^{-1} (200 mL), and 50:50 v v^{-1} (100 mL) at a flow rate of 1 mL min^{-1}. A final wash with 50:50 chloroform/methanol removed all the remaining rhamnolipid from the column.[34] For quantification of total carbohydrates, rhamnose test was performed according to the Nayak *et al.* method.[35] Briefly, 0.5 mL of culture supernatant was mixed with 2.5 mL of sulfuric acid (98 %) and 0.5 mL phenol solution (5 %, v v^{-1}) in thick-wall glass test tubes, and then the solution was incubated for 15 min before calibration of absorbance at 490 nm. Sugar concentration was measured versus glucose standards. Protein concentration was determined by the Lowry method.[36] Bovine serum albumin (BSA) was used as calibration standard. The lipid content was determined according to Manocha *et al.*[37] Thus, 0.5 g of the isolated material was extracted with chloroform:methanol in different proportions (1:1 and 1:2, v v^{-1}). The organic extracts were then evaporated under vacuum conditions, and the lipid content determined gravimetrically.

Identification of bacterial strain

Cell morphology was assessed by Gram stain method and microscopy. The biochemical profile of the isolate was determined by conventional biochemical tests.[38] The genomic bacterial DNA was extracted using boiling method as described by Theron *et al.*,[39] and 16s rDNA was amplified using universal primers: fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′).[40]

PCR amplifications were performed in 25 µL reactions using Master Mix RED 2x (Ampliqon Odense, Denmark), containing 0.5 µL of each primer (10 µM) and 3 µL of cDNA under the following parameters: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 60 sec and 72 °C for 90 sec, as well as a final extension at 72 °C for 10 min. PCR products were visualized in 1 % agarose gel. Clean-up and bi-directional sequencing were performed at Bioneer Corporation (Daejeon, South Korea). Original sequence fragments were edited and assembled using DNA Sequence Assembler v4 (2013). The 16S rDNA consensus sequence was compared with available sequences in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and Ezbiocloud (www.ezbiocloud.net). Highly similar sequences were then found and chosen in Ezbiocloud to construct a phylogenetic tree. The phylogenetic tree was constructed using MEGA6 with the neighbor-joining algorithm,[41] and the topological accuracy of the trees was evaluated with 1,000 bootstrap replicates.

Oily sludge contaminated soil recovery

Soil washing experiments using biosurfactant for desorption of total petroleum hydrocarbons (TPH) from contaminated soil were conducted in a lab-scale reactor. Effects of operating parameters, including pH (3 – 9), contact time (4 – 96 h), biosurfactant concentration (1, 2, 3, 4, 5, 6, 7, 8 CMC), and salinity (0.5, 1, 2, 3, 4 %) on soil washing efficiency were investigated according to one-factor-at-a-time design. For experimental runs, 20 g of contaminated soil was poured in 250-mL Erlenmeyer flasks, the pH and salinity were adjusted to a desired value, and a desired amount of biosurfactant was added to the flasks, which were then shaken at a fixed agitation speed of 200 rpm by a shaker (Rotator R430, Iran). TPH were extracted according to the USEPA method 418.1.[42] The solid-liquid phase separation was performed before the analysis. For this purpose, 2 g of the soil sample was mixed with 10 mL of *n*-hexane:acetone solution of 1:1 w w^{-1}. Sonication (Hielscher: UP 400S, Germany) was then conducted at a power of 400 W for 10 min. The amount of TPH was determined using a gas chromatograph equipped with a flame ionization detector (GC-FID, GC Agilent 7890 – MS Agilent 5975, USA), and HP5-MS capillary column (30 m length, 0.32 mm inner diameter and 0.2 mm film thickness). Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1}. The temperature program was as follows: column temperature was held at 40 °C for 1 min, then ramped at 10 °C min^{-1} to 280 °C, and then held for 5 min. The injector and detector temperatures were set at 280 °C and 300 °C, respectively. The removal efficiency of TPH from soil was calculated using Eq. (2):

$$R\ (\%) = (1-\gamma_{res}/\gamma_{ini}) \cdot 100 \qquad (2)$$

where, R is the TPH removal efficiency, γ_{ini} (mg L^{-1}) and γ_{res} (mg L^{-1}) denote the initial and residual TPH concentrations in the soil before and after the washing process.

Table 1 – *Characteristics of oily sludge contaminated soil*

Characteristics	Value (%)	Characteristics	Value (%)
Soil type	Silty clay loam	L.O.I	32.1
Sand	30.7	Al_2O_3	6.233
Clay	28.5	MgO	4.195
Silt	40.8	Fe_2O_3	4.004
Moisture content (%)	11	K_2O	1.52
pH	6.4	MnO	0.067
Conductivity (μS cm^{-1})	1230	Na_2O	0.622
Organic matter (%)	1.1	TiO_2	0.377
SiO_2	36.53	P_2O_5	0.105
CaO	26.015	TPH (mg kg^{-1})	3200

Results and discussion

Soil characteristics

The characteristics of oily sludge contaminated soil are shown in Table 1. As may be seen, the soil has TPH concentration of 3200 mg kg^{-1}. According to the results obtained from the soil sample analysis, the soil type used in this study was silty clay loam with 30.7 % sand, 28.5 % clay and 40.8 % silt. In addition, the XRF analysis showed that the major elements in the soil were CaO, SiO_2, Al_2O_3, MgO, Fe_2O_3, and K_2O, with moisture content of 11 %. The main hydrocarbons present in the sample were hexadecane, benzaldehyde dimethyl acetal, dodecane, 1,2-benzenedicarboxylic acid, etc., as presented in Fig. 1.

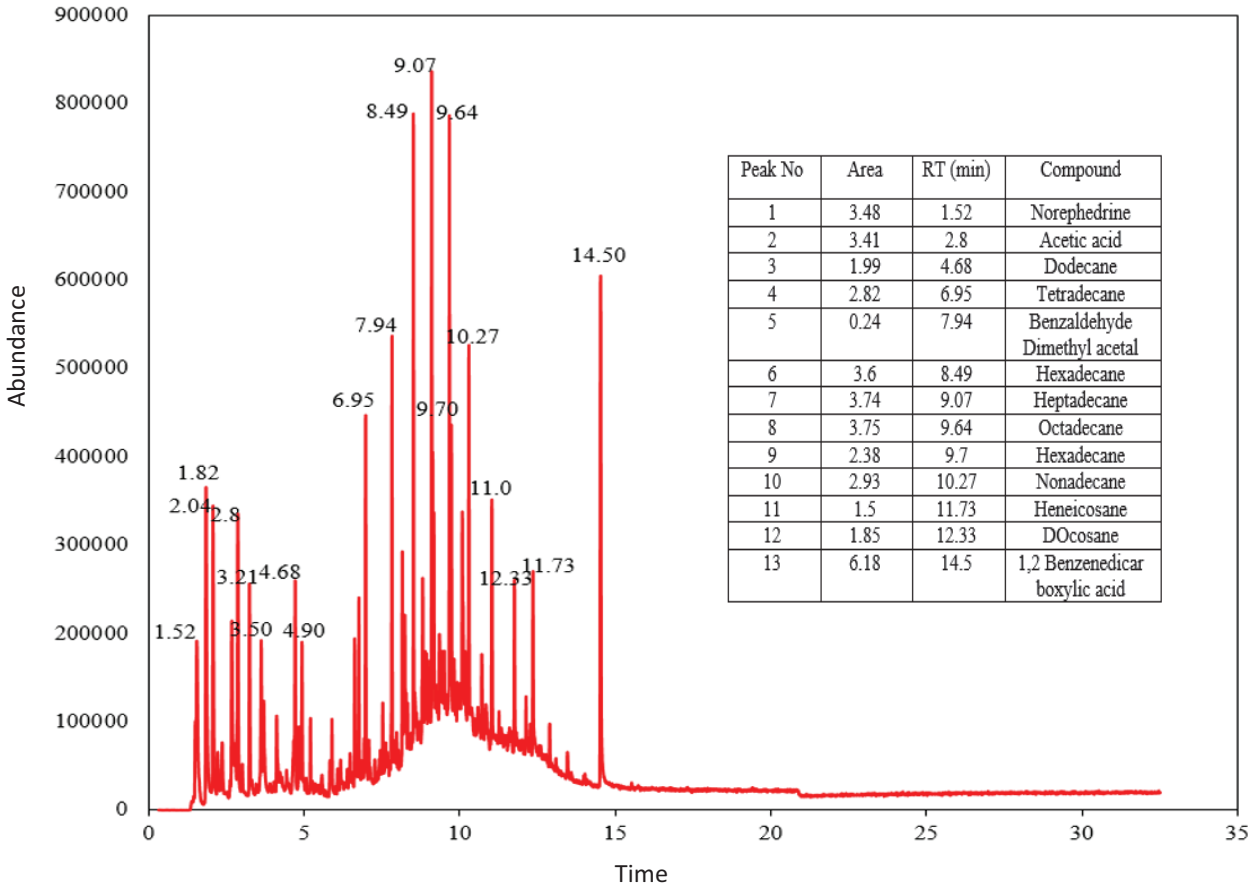
Biosurfactant-producing strain

Among all pure isolates, the best biosurfactant-producing strain was selected according to the best results of the oil spreading test, drop collapse test, and ability to lower solution surface tension.[43] Results are presented in Table 2. The pure strain was identified as *Pseudomonas stutzeri* Z12 based on microscopic, biochemical, and genetic analysis. The sequence data was submitted to the NCBI GenBank database under the accession number MN053965 (Fig. 2).

The oil spreading test showed a hole with a diameter of about 5.1 cm versus 0 for distilled water

Table 2 – *Initial screening of isolated strains for the possibility of biosurfactant production*

Bacterial isolate	Oil spreading test (diameter)	Drop collapse test[38]	Blood agar test	Surface tension of solution (mN m^{-1})
Pseudomonas stutzeri Z12	>5 cm (++++)	completely flat (+++)	(+)	35.3
Isolate S1	1 – 3 cm (++)	flat (+)	(–)	48.7
Isolate S1	<1 cm (+)	completely spherical (–)	(–)	58.2
Isolate S1	no displacement (–)	completely spherical (–)	(–)	64.8



Peak No	Area	RT (min)	Compound
1	3.48	1.52	Norephedrine
2	3.41	2.8	Acetic acid
3	1.99	4.68	Dodecane
4	2.82	6.95	Tetradecane
5	0.24	7.94	Benzaldehyde Dimethyl acetal
6	3.6	8.49	Hexadecane
7	3.74	9.07	Heptadecane
8	3.75	9.64	Octadecane
9	2.38	9.7	Hexadecane
10	2.93	10.27	Nonadecane
11	1.5	11.73	Heneicosane
12	1.85	12.33	DOcosane
13	6.18	14.5	1,2 Benzenedicar boxylic acid

Fig. 1 – *GC-MS analysis of oily sludge contaminated soil*

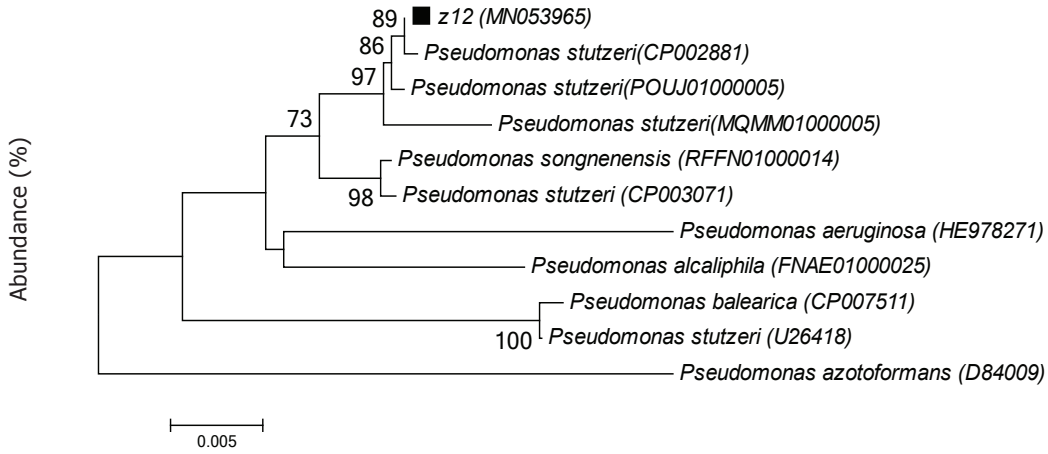


Fig. 2 – *Phylogenetic tree constructed using the neighbor-joining method based on the 16S rRNA gene sequences*

(Fig. 3a, b). The diameter of clear zone depends on the concentration of biosurfactant produced by the bacterial colonies. In addition, the drop collapse test showed positive results. In blood agar test, creation of a clear zone around biosurfactant-producing bacterial colonies indicated the presence of biosurfactant in the culture medium (Fig. 3c).

Characterization of biosurfactant

CMC, yield, and growth conditions

Results of bacterial growth versus variations of culture broth ST is shown in Fig. 4a. After an initial lag phase of 12 h, the growth conditions changed to an exponential phase that lasted 48 h. In accordance

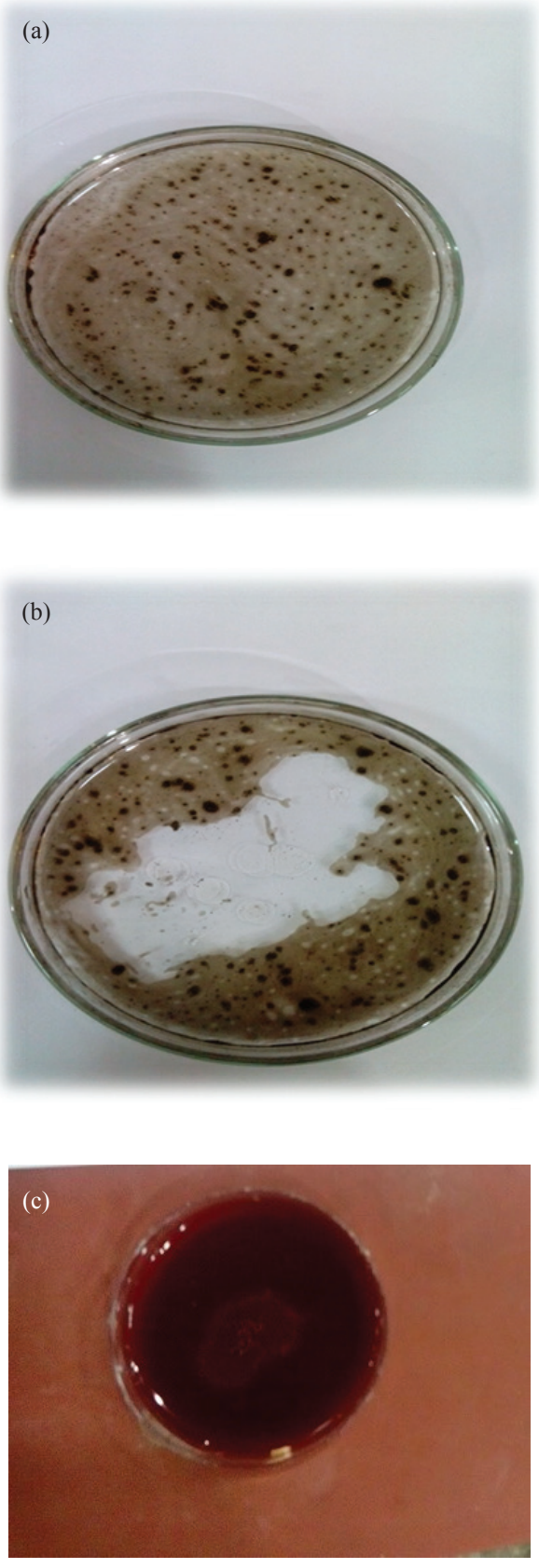


Fig. 3 – *Results of a, b) oil spreading test, and c) the blood agar test for initial screening of biosurfactant production by Pseudomonas stutzeri Z12*

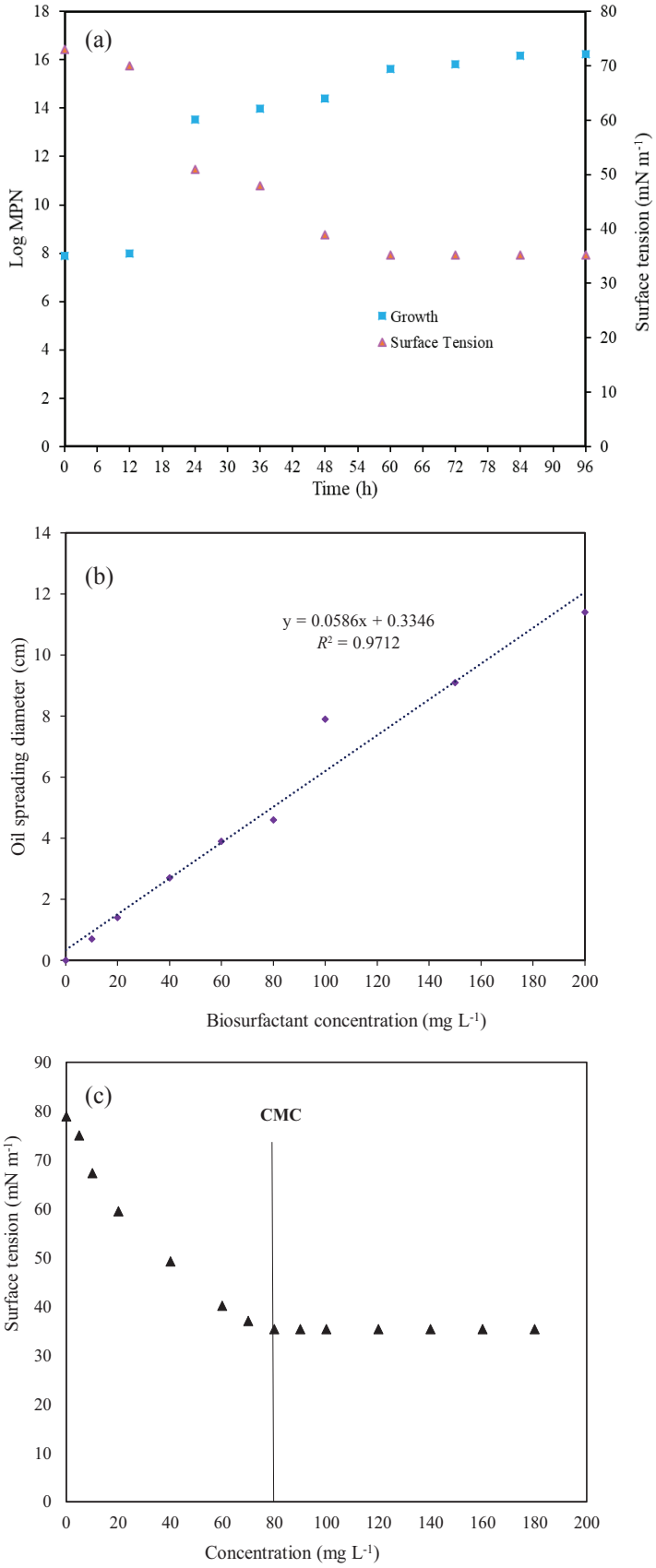


Fig. 4 – *a) Variations of surface tension (ST) of culture broth versus bacterial growth, b) oil spreading test for evaluation of biosurfactant yield, and c) critical micelle concentration (CMC) value of crude biosurfactant*

with the growth increase, the surface tension decreased gradually, reached the lowest value of 35.2 mN m^{-1} after 72 h, and then remained constant until 96 h. The rhamnose analysis was considered to determine rhamnolipid as an equivalent of rhamnose. The glycolipid-type biosurfactant produced by *Pseudomonas stutzeri* Z12 comprised 52 % lipids (w w^{-1}) and 44 % carbohydrates (w w^{-1}). A low fraction of protein (4 %) was observed, possibly due to co-precipitation with cell debris from the supernatant during the extraction of biosurfactant. The yield of biosurfactant production incubation was 318 mg L^{-1} after 96 h (Fig. 4b). According to Fig. 4c, by increasing of biosurfactant concentration, the ST of the solution reduced from 78.9 mN m^{-1} to 35.2 mN m^{-1}, and there was no change even after raising the biosurfactant concentration. The CMC of extracted biosurfactant was about 80 mg L^{-1}.

Emulsifying index

Emulsifying index (E_{24}) of the biosurfactant produced by *Pseudomonas stutzeri* Z12 was determined using different hydrocarbons. As may be seen in Table 3, results for all hydrocarbons were positive, comparable with the studies mentioned below. The maximum E_{24} of 62.1 % was recorded for *n*-hexadecane, followed by 57.6 % for *n*-hexane.

Table 3 – *E_{24} value of the extracted biosurfactant against hydrocarbons*

Hydrocarbon	E_{24}/%
Diesel oil	41.5
Crude oil	30.2
n-Hexadecane	62.1
n-Hexane	57.6
Kerosene	54.4
Xylene	46.9

The results of a study conducted by Kaur *et al.*,[44] showed that the maximum E_{24} value was related to biosurfactant produced by *Pseudomonas* sp. GBS.5, and E_{24} emulsification values of 51.61 % were observed for crude oil after 24 hours.[45] Also, Patowary *et al*., used *Pseudomonas aeruginosa* SR17 in degradation of hydrocarbons using crude oil as the source of carbon,[46] and E_{24} index for hexadecane, olive oil, kerosene, diesel oil, engine oil, and crude oil was 83, 88, 81, 92, 86, and 100 %, respectively.

FTIR analysis

The structure of biosurfactant was characterized by FTIR analysis. As shown in Fig. 5, the strong adsorption peaks at 1219 cm^{-1} and 3435 cm^{-1}

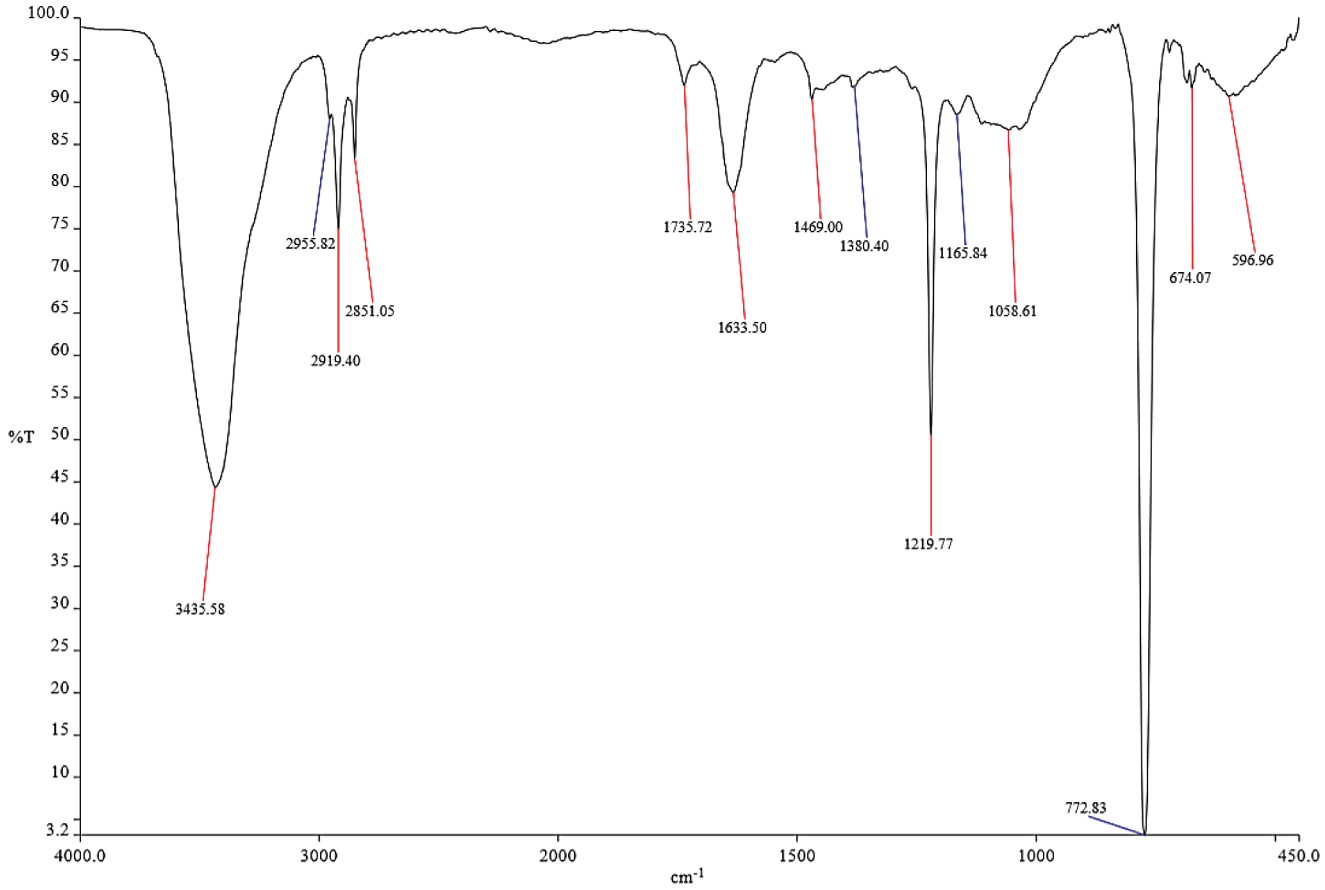


Fig. 5 – *FTIR spectrum of the purified biosurfactant produced by Pseudomonas stutzeri Z12*

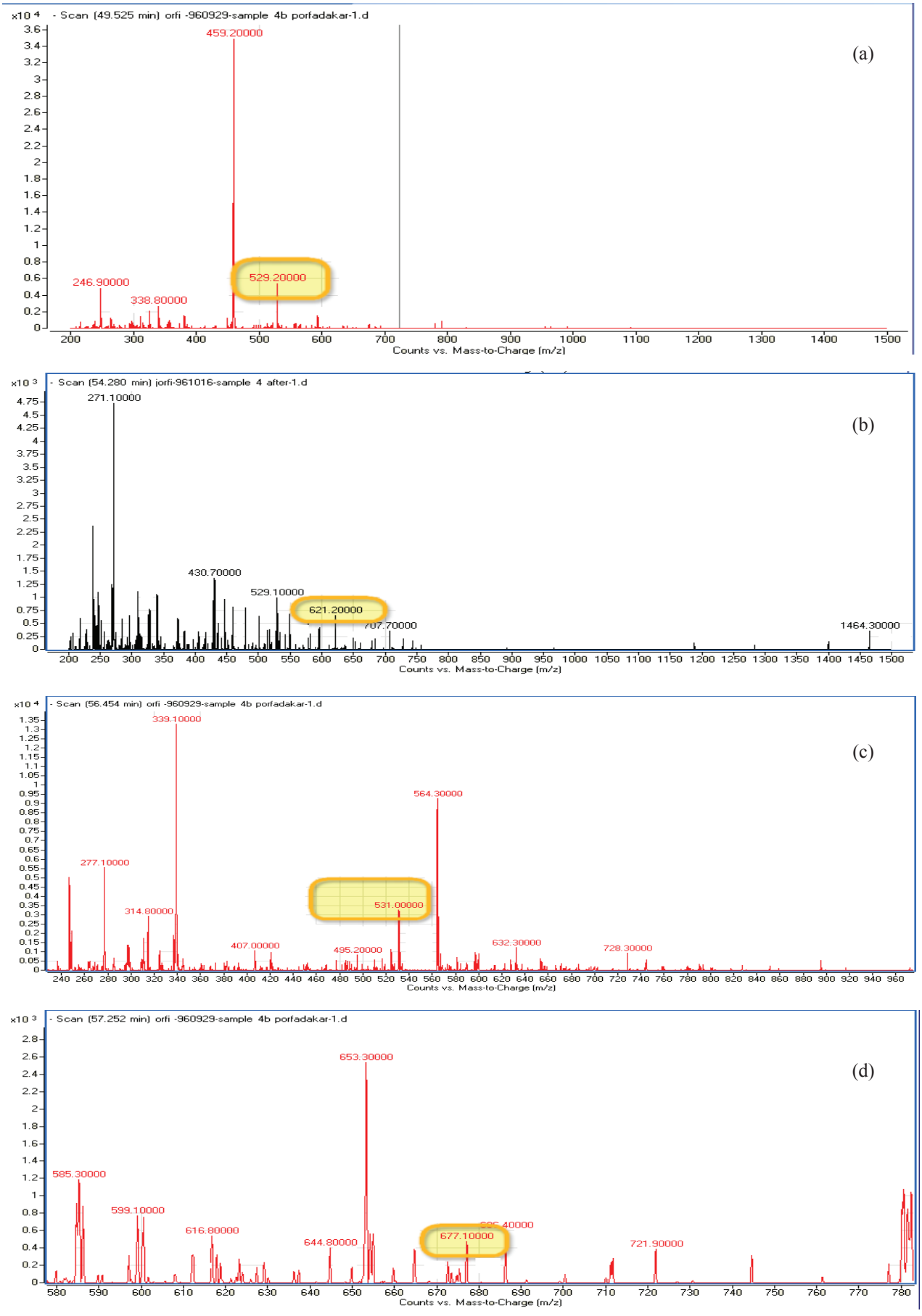
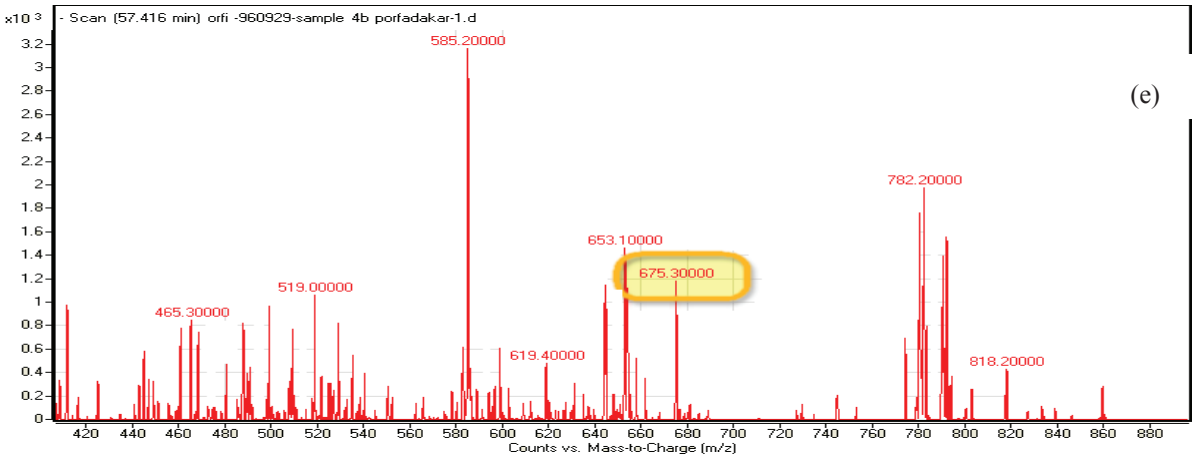
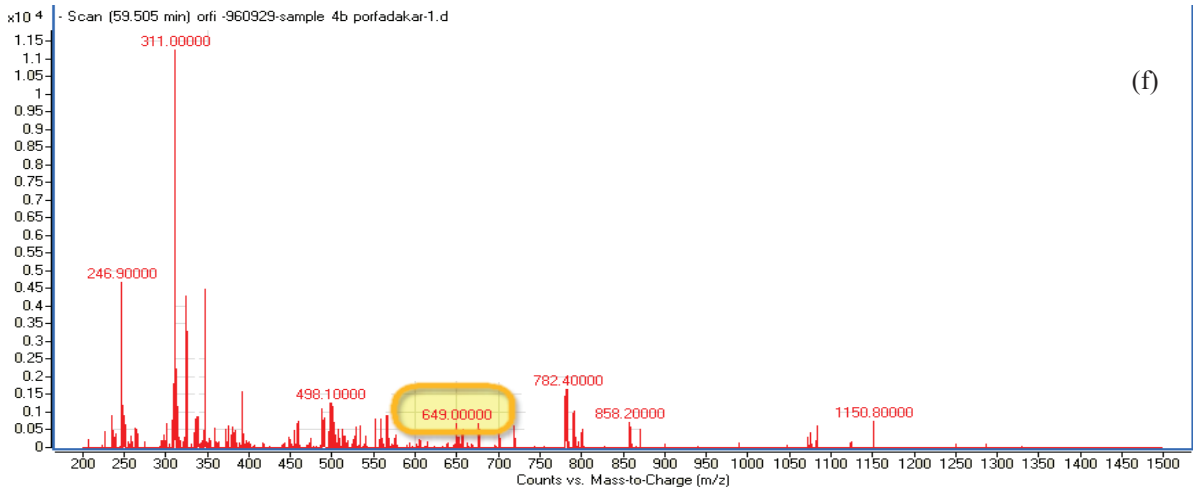


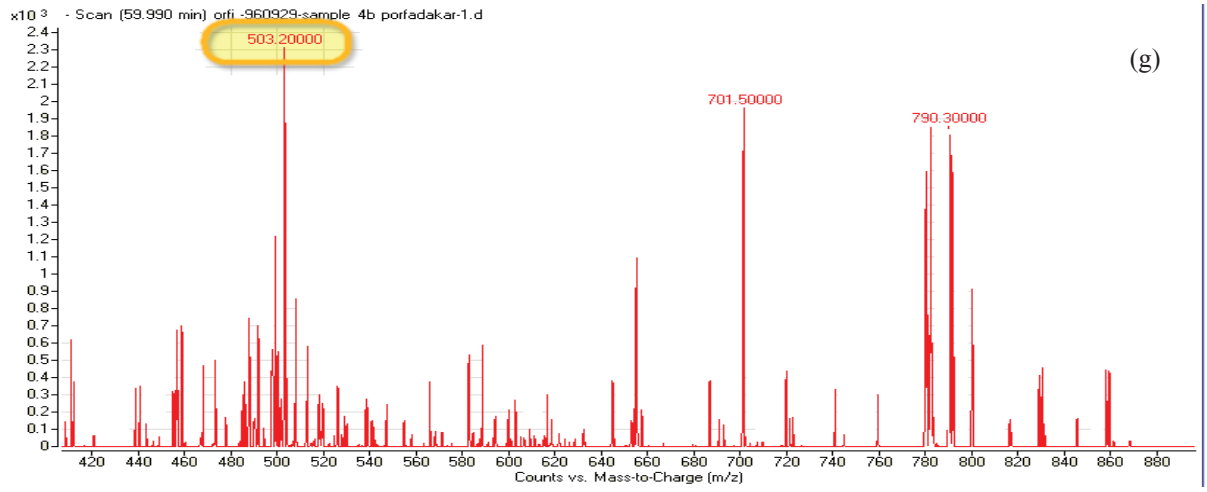
Fig. 6 – *LC-MS-MS analysis of the rhamnolipids produced by Pseudomonas stutzeri Z12*



(e)



(f)



(g)

Fig. 6 – *Continued.*

were related to carbonyl (COO^-) group and O–H stretching vibrations of hydroxyl group of rhamnolipid rings. The small peaks were located at 2955, 2919, 2851 cm^{-1} which belonged to the asymmetric C–H stretch of CH_2 and CH_3 groups of aliphatic chains. In addition, C–O–C stretching peak was observed at 1058 cm^{-1}. The peak at 1735 cm^{-1} indicated the presence of –C=O stretching vibrations of the carbonyl groups. The weak peaks around 1469–1380 cm^{-1} showed bending of the hydroxyl (O–H). In addition, the N–H bond stretching was identified at 1633 cm^{-1}. The peak at 674 and 596 cm^{-1} belonged to CH_2 group. Similar peaks were observed in previous studies.[47,48] Therefore, FTIR analysis proved the biosurfactant production by *Pseudomonas stutzeri* Z12. The findings are consistent with the study of Shekhar *et al.*[49]

LC-MS-MS analysis

LC-MS-MS analysis of the purified biosurfactant was performed in the m z^{-1} range of 200 – 1500 at retention times of 22.50 – 59.90 min. According to the obtained results (Fig. 6), it was specified that biosurfactant contained a mixture of both mono-rhamnolipid and di-rhamnolipid congeners. Eight main congeners are shown in Table 4, in which three congeners $RhaC_{12:1}C_{10}$ (529.9 m z^{-1}), $RhaC_{12}C_{10}$ (531 m z^{-1}), and $RhaC_{10}C_{10}$ (503.2 m z^{-1}) were associated to mono-rhamnolipid structure and five congeners $RhaRhaC_{10}C_8$(621.2 m z^{-1}), $RhaRhaC_{12}C_{12}$(707.7), $RhaRhaC_{10}C_{12}$(677.1), $RhaRhaC_{10}C_{12:1}$(675.3 m z^{-1}) and $RhaRhaC_{10}C_1$(649.5 m z^{-1}) were associated to di-rhamnolipid structure.[48,50] It was revealed that di-rhamnolipid congeners were prevalent as compared with mono-rhamnolipid congeners.

Table 4 – *Chemical structure of rhamnolipids produced by Pseudomonas stutzeri Z12*

Fraction	Retention time (min)	Chemical structure	Molecular ion peak (m z^{-1})
A	49.52	$RhaC_{12:1}C_{10}$	529.2
B	54.28	$RhaRhaC_{10}C_8$	621.2
		$RhaRhaC_{12}C_{12}$	707.7
C	56.45	$RhaC_{12}C_{10}$	531
D	57.25	$RhaRhaC_{10}C_{12}$	677.1
E	57.416	$RhaRhaC_{10}C_{12:1}$	675.3
F	59.50	$RhaRhaC_{10}C_{10}$	649.5
G	59.90	$RhaC_{10}C_{10}$	503.2

Soil washing

Effect of pH

Effect of pH on TPH washing from contaminated soil by biosurfactant produced by *Pseudomonas stutzeri* Z12 was investigated for pH range of 3 – 9, initial TPH concentration of 3200 mg kg^{-1}, concentration of 2 CMC, and contact time of 4 h. As seen in Fig. 7a, no significant change was observed for TPH desorption along with pH variations. This could be due to the existence of different organic compounds, including aromatic and aliphatic hydrocarbons in oily sludge, each of which has its particular physical and chemical characteristics and behavior.[6] However, by decreasing pH to 3, the removal efficiency reduced to 10.31 %, and in alkaline pH, the removal efficiency reached 11.93 %. Therefore, neutral pH of 7.0 was considered as optimum pH for continued experiments. The results of the study are similar to the study of Zhang.[51] In neutral pH, the interfacial tension between the aqueous and non-aqueous phases decreases, so that reduced interfacial tension leads to mobilization of residual TPH from soil. In addition, the increase in pH causes the release of soil organic matter into aqueous phase, and as a result, leads to a decrease in TPH adsorption.

Effect of biosurfactant concentration

The biosurfactant concentration is an important factor for assessment of biosurfactant activity. The results presented in Fig. 7b show that by increasing the concentration value from 1 to 8 CMC, the removal efficiency increased from 1.25 % to 20.93 %. However, due to slight difference between the concentration values of 7 and 8 CMC, the former was considered as the selected level. At lower concentration values, the reduction in interfacial tension between the oil and water is the cause for oil mobilization, and desorption happens by capillary force. At higher concentrations, biosurfactant starts to form micelles. Micelle formation in turn reduces the surface and interfacial tension, and raises the solubility and bioavailability of hydrophobic organics.[52,53] The results of the studies performed by Bezza *et al*.,[54] and Sarubbo *et al*.,[55] are similar to the results obtained in this study.

Effect of salinity

Fig. 7c shows the relationship between the salinity (0.5 – 4 %) and the TPH desorption from the soil. By increasing NaCl concentration from 0.5 % to 4 %, the desorption efficiency increased from 22.4 % to 36.7 %. It was revealed that the rise in salinity had a positive effect on the TPH desorption.[53] The results of the study conducted by Zhang *et al*., showed that salinity positively impacted the removal of TPH compounds from soil. By increas-

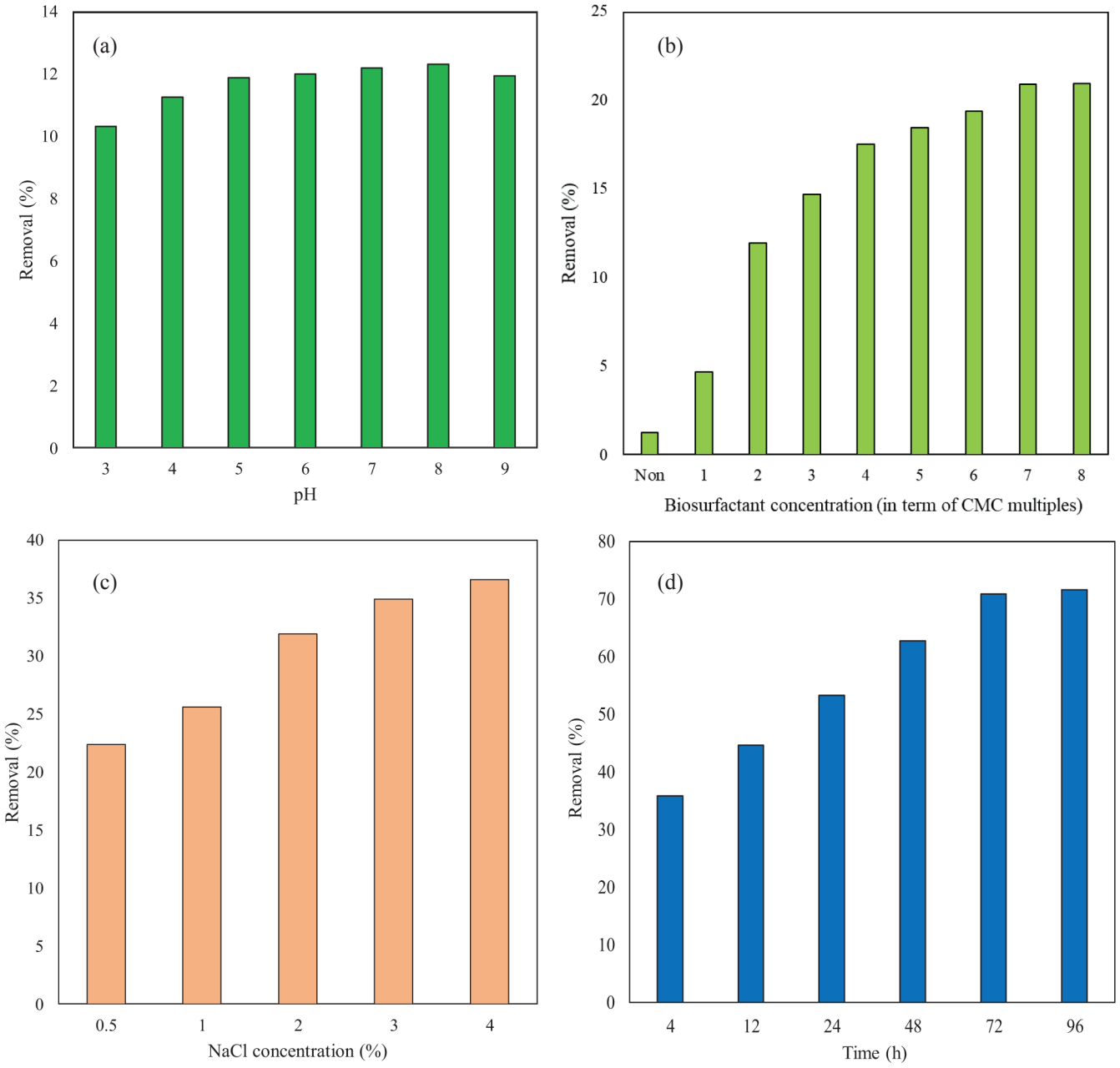


Fig. 7 – *Effects of operating parameters on PAHs desorption from contaminated soil; a) effect of pH (contact time 4 h, concentration = 2 CMC), b) effect of concentration (pH = 7.0, contact time 4 h), c) effect of salinity (pH = 7.0, concentration = 7 CMC, contact time 4 h), and d) effect of contact time (pH = 7.0, concentration = 7 CMC, NaCl = 4 %)*

ing the solution salinity, the electrochemical double layer between soil particles and surface density of emulsified petroleum hydrocarbon decreases, which leads to increased attractive force between TPH compounds and soil particles.[56]

Effect of contact time

Contact time is one of the most important parameters influencing desorption of TPH from soil. According to Fig. 7d, by increasing the contact time from 4 h to 96 h, with the concentration value of 7 CMC and NaCl concentration of 4 %, the removal efficiency increased from 35.6 % to 71.9 %. However, because of a slight difference between reaction times of 72 and 96 h, the contact time of 72 h was selected for desorption of TPH from soil. In other words, contact time of 72 h is enough for solubilization of TPH from soil to the liquid phase.

Alternative washing solutions

In order to evaluate the performance of biosurfactant produced by *Pseudomonas stutzeri* Z12 in TPH removal, biosurfactant was compared with synthetic surfactant (Tween 80) and distilled water

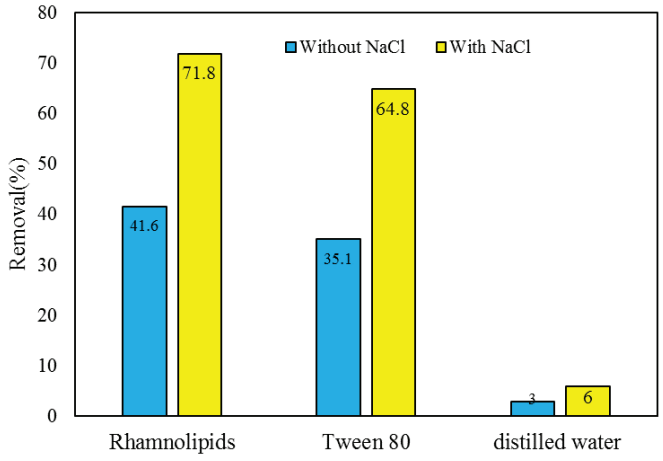


Fig. 8 – *Soil washing efficiency using rhamnolipid-based biosurfactant solution, Tween 80 solution and distilled water under selected conditions (pH = 7, concentration = 7 CMC, contact time = 72 h, and NaCl = 4 %)*

in similar conditions obtained in previous steps. Tween 80 is a nonionic surfactant with CMC value of 50 mg L^{-1}. According to results obtained, TPH desorption from soil was higher by the produced rhamnolipid biosurfactant in comparison with Tween 80 and distilled water (Fig. 8). Desorption percentages of TPH for biosurfactant, Tween 80 and distilled water without addition of NaCl were 41.6 %, 35.1 %, and 3 %, respectively. With addition of NaCl to system, desorption percentages of TPH increased and reached 71.8 %, 64.8 %, and 6 %, respectively. In other words, distilled water could not remove TPH from soil, due to the differences between nonpolar hydrocarbons and highly polar water. In a study by Chaprão *et al.*,[57] biosurfactants were more efficient in removal of motor oil than were the commercially available surfactants.[58] Efficient desorption of hydrophobic hydrocarbons from polluted soil, therefore, needs addition of more synthetic surfactants. Beside the operating costs, adverse effects, including toxicity due to disruption of bacterial cell membranes and accumulation of inhibitory by-products of incomplete degradation, are possible with synthetic surfactants.[59] Rhamnolipid-based biosurfactants have high biodegradability (are easily decomposed by microorganisms) which makes them less harmful to the environment and less toxic than chemical synthetic surfactants.

Conclusion

The results showed that the production of rhamnolipid-based biosurfactant could reduce surface tension from 78.9 mN m^{-1} to 35.3 mN m^{-1}. The glycolipid-type biosurfactant produced by *Pseudomonas stutzeri* Z12 consisted of lipids with a relative percent of 52 % (w w^{-1}), and 44 % (w w^{-1}) of carbohydrates. The yield of biosurfactant obtained was 318 mg L^{-1}. The results showed that the addition of salt to the system had a positive effect on desorption of TPH from soil. Comparing rhamnolipid-based biosurfactant solution with Tween 80 solution and distilled water, the highest efficiency was obtained with biosurfactants. Therefore, the results of this study could be valuable for remediation of soils contaminated with high molecular weight hydrophobic organic compounds using *Pseudomonas stutzeri* Z12.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by Student Research Committee, Ahvaz Jundishapur University of Medical Sciences (Grant No. ETRC- 98S39).

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