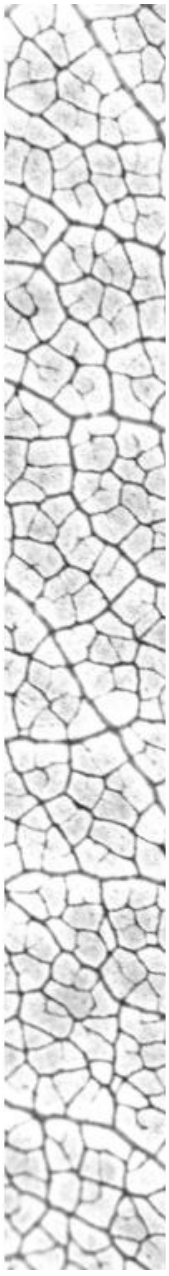

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Screening, enhance production and characterization of biosurfactant produced by *Pseudomonas aeruginosa* isolated from hydrocarbon contaminated soil

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Abstract
A wide variety of biosurfactant-producing microorganisms were isolated from hydrocarbon-contaminated soil and were screened for biosurfactant production using conventional methods including oil spreading test, emulsification index, emulsification activity and CTAB agar test. Among the isolated bacteria, A2 isolate, a Gram negative bacterium was selected for further studies based on its highest activity and was identified by 16S rDNA sequencing as *Pseudomonas aeruginosa.* The presence of specific genes responsible for the biosynthesis of mono-rhamnolipid (rhlB) and dirhamnolipid (rhlC) were detected. Optimization of different cultural conditions (carbon source, carbon concentration, nitrogen source, nitrogen concentration, pH, incubation time, and inoculum concentration) were performed to achieve maximum production of biosurfactant. Production of biosurfactant was estimated in terms of oil spreading test, emulsification index, emulsification activity and biomass as 15 cm, 60 %, 1.831 ± 0.025 and 2.851 ± 0.043 g/l respectively. The obtained results demonstrated that the maximum rhamnolipid production (5.42 ± 0.475 g/l) happened using olive oil at a concentration of 2% as carbon source, 2 g/l of urea as nitrogen source, inoculum size of 3 %, pH: 7, and 6 days incubation period at 30°C. The analysis of the extracted biosurfactant by TLC, FTIR spectra and GC-MS analysis confirmed that the biosurfactant nature was rhamnolipid. The rhamnolipid could decrease the surface tension of water to 28.49 mN/m and exhibited good stabilities at high temperatures (up to autoclaving at 121°C), salinities (up to 10 % NaCl), and pH values (up to pH: 10 except 4 and 2 pH).

Keywords: Rhamnolipid, *Pseudomnas aeruginosa*, enhance production, rhlB, rhlC

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INTRODUCTION

Biosurfactants are surface-active amphipathic compounds possessing hydrophobic and hydrophilic parts that have ability to reduce surface and interfacial tension of different fluids. Hydrophobic part 'tail' is a hydrocarbon chain containing saturated/unsaturated and hydroxylated fatty alcohols or fatty acids and the hydrophilic part 'head' is a polar group that contains mono, oligo or polysaccharides and peptides (Nayarisseri et al. 2019). A wide variety of microorganisms have been shown to synthesize biosurfactants with various types of structures (Banat et al. 2010). Microorganisms that synthesize biosurfactants inhabit in water (fresh water, groundwater, and sea), land (soil, sediment and sludge) and extreme environments (oil reservoirs and hot springs) (Chirwa and Bezza 2015). The bacteria are considered the main biosurfactant producers, however, many bacterial genera such as *Bacillus*, *Pseudomonas, Acinetobacter, Burkholderia*, *Stenotrophomonas* and *Flavobacterium* are reported to produce biosurfactants (Femi-Ola et al. 2015).

The biosurfactants are classified according to origin and chemical composition into six main groups: lipopeptides, lipoproteins, glycolipids, phospholipids, neutral lipids, and polymeric biosurfactants (Zhao et al. 2017). The most abundant of biosurfactants are glycolipids and lipopeptides of which the most investigated types are the rhamnolipid (RL) synthesized by *Pseudomonas aeruginosa* and surfactin synthesized by *Bacillus subtilis* strains (Desai and Banat 1997, Abdel-Mawgoud et al. 2010, Twigg et al. 2018). Rhamnolipids biosurfactants are composed of one or two rhamnose molecules (mono- or di-rhamnolipids,

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respectively), linked in a 1,2-glycosidic linkage to 1, 2 or 3 β-hydroxy fatty acids of varied chain lengths ranging from 8 to 16 carbons (Abdel-Mawgoud 2011).

Pseudomonas aeruginosa main producer of the rhamnolipid, is an opportunistic human pathogen and ubiquitous in the environment and can be found in many habitats such as water, soil, human hosts and plants, where it survives due to its remarkable physiological abilities (Jarvis and Johnson 1949). *P. aeruginosa* is able to grow and produce rhamnolipid (RL) biosurfactants but in order to obtain maximum yield, the optimization of fermentation medium is needed. Different variables have been reported to affect RL yield including carbon source, nitrogen source, pH, cultivation time, agitation, and temperature (El-Housseiny et al. 2016, Câmara et al. 2019).

Biosurfactants have some unique properties compared to chemical synthesized surfactants are having low toxicity, higher biodegradability, environmental compatibility, stable activity under extreme temperature, salinity and pH and easy to synthesize from low cost substrates (Marchant and Banat 2012). Such properties pay an attention and interest to biosurfactants and have become an important product of biotechnology in recent years in numerous potential applications such as cosmetics, pharmaceutics, food processing, environmental management, bioremediation, microbial enhanced oil recovery industries and agriculture (Li et al. 2016).

The aim of the study was screening, molecular detection, isolation, enhance production of biosurfactant by *P. aeruginosa* isolated from hydrocarbon contaminated soil in different cultivation conditions including various carbon sources, nitrogen source, pH, cultivation time and inoculum size and characterization of the produced biosurfactant using TLC, FTIR and GC-MS.

MATERIALS AND METHODS

Sample collection

The soil samples contaminated with hydrocarbon were collected from the area around the electricity generators in Al-dair district, Basrah governorate south of Iraq (30.536242°N 47.815819°E). Soil samples were collected from five points under depth 5 cm using a sterile shovel and placed in sterile containers and transported in cool box to laboratory for the investigation.

Isolation of biosurfactant-producing bacteria

Isolation of biosurfactant-producing bacterial strain was conducted by enrichment method using modified mineral salt medium (MSM) adopted from (Deng et al. 2014). The MSM containing (g/l) NaCl (5), Na_2HPO_4 (3), KH_2PO_4 (2), NH_4NO3 (1), $MgSO_4.7H_2O$ (0.7) and 1 ml /l trace salt solution with 1% (v/v) olive oil as the sole carbon source and pH of 7. The trace salt solution containing (mg/l) $CaCl_2$ (20), $FeCl_3$ (30), $CuSO_4$ (0.5), $MnSO_4.H_2O$ (0.5), and $ZnSO_4.7H2O$ (10). The MSM was sterilized by autoclaving at 121 °C for 20 min. Two grams of soil sample was enriched with 50 ml of MSM in 250-ml Erlenmeyer conical flasks incubated at 30 °C and 150 rpm for 7 days. After 7 days, the enrichment culture products were sequentially diluted, and spread on the nutrient agar medium (Himedia) for incubation at 30 °C for 24 hrs. The colonies with different morphologies were picked out and purified based on their Gram staining characteristic, cell shape, and colony morphology. The bacteria were maintained on nutrient agar slants and stored at 4 °C.

Preparation of the bacterial inoculum

The isolated bacteria were activated in fifty ml of autoclaved Nutrient broth in 250-ml flask by inoculating loop full of the isolated bacteria and incubating at 30 °C for 24 hrs. The uninoculated nutrient broth was used as a negative control. Then, 5 % of the prepared bacterial inoculum was transferred to 250-ml Erlenmeyer's flasks containing 50 ml MSM.

Screening for biosurfactant production

For screening biosurfactant production by the isolated bacteria, 50 ml MSM with 1 % of olive oil as carbon source in 250 ml Erlenmeyer flasks was inoculated with 5 % bacterial inoculum and incubated in a rotary shaker at 30 °C and 150 rpm for 7 days. After 7 days of fermentation, bacterial cells were removed by centrifuging flasks at 5000 rpm with temperature of 4 °C for 20 min (Xiangsheng et al. 2010). The cell-free supernatant was subjected to below provide various screening methods to obtain biosurfactant-producing strains.

1. **Oil spreading test**

The oil spreading test was carried out by adding 40 ml of distilled water to a 15 cm diameter petri dish. Subsequently 20 µl of crude oil was added onto the surface of the water, which formed a thin layer over water. 10 µl of culture supernatant was added in center of the crude oil layer. The area of the clear zone on the oil surface was measured and compared with 10 µl of distilled water as negative control (Satpute et al. 2010).

2. **Determination of Emulsification Index**

A mixture of 2 ml supernatant and 2 ml kerosene was vertically stirred by vortex for 2 min and the height of the emulsion layer was measured after 24 h to determine the emulsification index (Ozdal et al. 2017). The equation used to determine the emulsification index (E24 %) is as follows:

$$E24\ (\%) = \frac{\text{the height of emulsion layer}}{\text{the height of total solution}} \times 100\%$$

3. **Determination of emulsification activity**

Cell free supernatant (0.5 ml) of the sample was added to a screw-capped tube containing 7.5 ml of Tris-Mg [20mM Tris HCl (pH 7.0) and 10mM $MgSO_4$] and 0.1 ml of kerosene. The tubes were vortexed for 2 min and

Table 1. Primers used for PCR amplification of 16s rRNA and genes involved in rhamnolipids biosynthesis

Genes	Primers	Sequence	Reference
16S rRNA	27 F	5-AGAGTTTGATCCTGGCTCAG-3	(Miyoshi et al. 2005)
	1492 R	5-GGTTACCTTGTTACGACTT-3	
rhlB	rhlB F	5- CACGCCATCCTCATCGCC-3	(Perfumo et al. 2013)
	rhlB R	5-GGTCAGTTCGTCGCTCAGC-3	
rhlC	rhlC F	5-AACTGGCGGCGGCGTTTCC-3	(Perfumo et al. 2013)
	rhlC R	5-AGTCCTGGTCGAGCAGCAGCA-3	

allowed to sit for 1 hour. Absorbance was measured at 540 nm. Emulsification activity (EA) was defined as the measured optical density (Sifour et al. 2005).

4. Cetyl trimethyl ammonium bromide (CTAB) agar test

Approximately 50 µl of cell-free culture supernatant was loaded into pre-cut wells in CTAB-methylene blue agar plates and incubated at 30 °C for 72 h. The appearance of a dark blue halo zone around the well was considered positive for anionic biosurfactant production (Siegmund and Wagner 1991).

Bacterial Identification by 16S rRNA

Promising biosurfactant producer was identified up to species level by targeting 16S rRNA gene. Chromosomal DNA was extracted according to the procedure of Presto™ Mini g DNA bacteria kit from the (Geneaid) company. DNA was amplified by the polymerase chain reaction (PCR) using universal primers (**Table 1**). PCR reactions were made in a total volume of 50 µl. An initial denaturation step of 96 °C for 3 min was followed by 27 cycles of 96 °C for 30 s, annealing temperature of 56 °C for 25 s and extention at 72 °C for 15 s and final extention at 72 °C for 10 min (Miyoshi et al. 2005). PCR products were separated based on molecular weight using a 1% (w/v) agarose gel made with TBE buffer (Thermo Fisher Scientific). DNA was visualized under UV light using ethidium bromide DNA stain (Thermo Fisher Scientific). Amplified DNA was purified and sequenced by Macrogen Company (South Korea).

Detection of genes coding for rhamnolipid biosurfactant

Screening for presence of the rhamnolipid genes including rhlB gene and rhlC gene was carried out using specific primers (**Table 1**). PCR Reactions of rhlB gene were made in a total volume of 50 µl. An initial denaturation step of 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, annealing temperature of 56 °C for 30 s and extention at 72 °C for 1 min and final extention at 72 °C for 5 min. while, PCR Reactions of rhlC gene were also made in a total volume of 50 µl with PCR program including an initial denaturation step of 95 °C for 2 min followed by 29 cycles of 95 °C for 30 s, annealing temperature of 64 °C for 30 s and extention at 72 °C for 20 s and final extention at 72 °C for 5 min.

Screening of cultural conditions for enhanced biosurfactant production

For the optimum biosurfactant production, the effect of different cultural conditions (carbon source, nitrogen source, carbon concentration, nitrogen concentration, pH, incubation time, and inoculum concentration) on the growth and the ability of the selected bacterial isolates to produce biosurfactant was optimized. Seven carbon sources (corn oil, glucose, glycerol, lactose, mannitol, olive oil and sunflower oil) were chosen to select optimum carbon source. Seven different carbon concentrations were examined (1 %, 1.5 %, 2 %, 2.5 %, 3 %, 3.5 % and 4 %) to choose optimum concentration keeping NH_4NO_3 as nitrogen source in production medium and incubating at 30 °C for 7 days in a shaker incubator. Eight different nitrogen sources (glutamic acid, KNO_3, $NaNO_3$, NH_4Cl_2, NH_4NO_3, $(NH_4)_2SO_4$, urea and yeast extract) were experimented to choose the optimum nitrogen source. Five increasing concentrations of nitrogen (0.2 %, 0.4 %, 0.6 %, 0.8 % and 1 %) were examined to find out the optimum nitrogen concentration using optimized carbon source in production medium and incubated at 30 °C for 7 days in a shaker incubator. The optimum pH for biosurfactant production was determined by differing the pH (5, 6, 7, 8 and 9) of the production medium. The optimum inoculum size was determined by differing inoculum sizes (1 %, 2 %, 3 %, 4 % and 5 %) of the production medium. Subsequently, the optimum incubation time for biosurfactant production was determined by varying the incubation time (1, 2, 3, 4, 5, 6 and 7 days) of the culture medium. The optimum parameters for biosurfactant production were determined by emulsification activity and oil spreading test.

Biomass determination

Biomass was determined by centrifuging 10 ml samples at 5000 rpm for 15 min at 5 °C and the cell pellet was dried in an oven at 105 °C for 24 h (Santos et al. 2018).

Surface Tension (ST) determination

The surface tension of the optimized cell free supernatant was measured at 25 °C with a surface tensiometer (sigma, Germany) working on the principle of Du Nouy ring method. An un-inoculated MSM was used as control. The instrument was calibrated by using MSM as control (72 mN/m) prior to use (Wei et al. 2005).

Biosurfactant stability determination

The stability of biosurfactant activity was determined by studying the effect of environmental factors including temperature, pH, and NaCl concentration on the cell-free broth obtained by centrifuging the cultures at 5000 rpm for 15 min. To determine the stability of the biosurfactant at different temperatures, four milliliter of the cell-free broth was maintained at a constant temperature (4, 30, 70, 100, and 120 °C) for 30 min and cooled to room temperature, after which emulsification index (E24 %) was measured. Effect of pH on E24% was evaluated by adjusting pH of the cell free broth in the range 2.0−12.0 with HCl (6 N) and NaOH (6 N) and measuring E24%. The effect of salinity on the biosurfactant stability was determined by measuring E24% after adding NaCl concentrations (2−20 % w/v) to the cell free supernatant (Aparna et al. 2012).

Recovery of the biosurfactant

Extraction of biosurfactant

The optimized parameters were used in setting up the biosurfactant production media. The production was carried out in a 250 ml Erlenmeyer flask containing 100 ml of the production media. The crude biosurfactant compounds produced by inoculating 3 % of fresh culture of the bacterium into mineral salt medium with optimum conditions (pH=7, olive oil (2 %) as carbon source, and urea (0.4 %) as a nitrogen source) and incubated in a shaker incubator (150 rpm) at temperature 30 °C for 6 days. After incubation period, bacterial cells were removed from culture broth by centrifugation at 5000 rpm for 15 min to obtain the supernatant. Equal volume of acetone was added to cell-free culture broth and kept for 24 hrs at 4 °C. The resulting precipitate was collected by centrifugation (5000 rpm) for 15 min, at 4 °C and freeze dried. The extracted brownish semisolid biosurfactant was weighted as g/l as done in studies by (Paraszkiewicz et al. 2002).

Thin layer chromatography

The extracted brownish semisolid biosurfactant (10 mg) was dissolved in 1 ml chloroform. Approximately 100 μg biosurfactant solution was loaded on to a TLC plate (Merck, Germany). The plates were developed in the solution of chloroform: methanol: water at a ratio of 65:15:2 and visualized with different TLC reagents. Iodine vapor was used for detecting lipids. Ninhydrin reagent (0.2 % ninhydrin in ethanol) was used to detect lipopeptide biosurfactant as red-pink spots. Molish agent (5 % 1-naphthol in alcohol) was used for detecting carbohydrate. Plates were heated at 110 °C for 10 min after application of the spraying agents.

Column chromatography

The extracted biosurfactant was purified by dissolving 1 g in 5ml chloroform and loaded to a column (1.5 cm × 35 cm) for the separation of biosurfactant by column chromatography. The column was packed with activated silica gel (200−400 mesh, Himedia) in chloroform slurry. This was followed by washing the column with chloroform till the neutral lipids eluted completely. The mobile phases consisted of chloroform/methanol and were applied in following sequence: 50:3 v/v (300 ml), 50:5 v/v (200 ml) and 50:50 v/v (100 ml) at a flow rate of 1 ml min^{-1} followed by collection of 15 ml fractions. The fractions were checked using TLC as done in studies by (Saikia et al. 2011).

Characterization of biosurfactant

FTIR measurements

The infrared spectra useful for identification of chemical bonds and functional groups of partially purified unknown biosurfactants were performed using Shimadzu FTIR instrument (chemistry department, science college, University of Basrah). About 1mg of dried biosurfactant was ground with 100mg of KBr to form powder and pressed into a thin pellet which could be measured by FT-IR in the wave number range of 4000-400 cm^{-1}.

Gas chromatography-Mass spectroscopy (GC-MS) analysis

GC-MS is an analytical method comprising of gas chromatography coupled with mass spectroscopy for analyzing the lipid part of biosurfactant into their fatty acids components that were performed in GC-MS lab at Nehran Omar field, Basrah Oil Company. GC-MS model (Agilent Technologies, USA) column type used was HP-5MS with 5 % phenyl methyl siloxane (30m × 250 µm × 0.25 mm). One µl of sample was injected by split mode at 75:1 ratio. The carrier gas was Helium, and the flow rate was set at 1 ml/min with injection temperature set at 260 °C. The gradient temperature was set as ranging from 40 °C to 310 °C at a speed of 10 °C min^{-1}, through an isothermal phase of 5 min at the end of the analysis. The mass spectra were obtained with an m/z range: 30−600. The identification of fatty acids components was done in scan mode and the target mass spectra obtained from sample were compared with mass spectra obtained from the library.

Statistical analysis

One way ANOVA was performed to evaluate the differences among the different optimization parameters using SPSS software (version 20). $P < 0.05$ were considered as statistically significant. The average values presented for emulsification activity and biomass were estimated using 3 replications and expressed as mean ± standard deviation.

RESULTS

Screening for biosurfactants production

Six bacterial isolates were isolated from soil sample. The isolates were screened in MSM medium supplemented with 1 % olive oil as sole carbon source

Table 2. Screening results of bacterial isolates for the biosurfactant production

Isolates	Gram staining	Emulsification activity/ 540 nm	Emulsification index (E24%)	oil spreading/ cm	biomass gm/l
A1	**-**	0.741±0.050*****	53.3	12	2.787±0.002*
A2	**-**	1.678±0.050	56.6	14	2.787 ± 0.100
A3	**+**	0.353±0.055	-	-	1.142±0.344
A4	**+**	0.048±0.006	10	4	3.268±0.583
A5	**-**	0.038±0.038	-	-	0.942±0.363
A6	**+**	0.050±0.038	-	0.4	1.413±0.163

* Mean ± SD, n=3

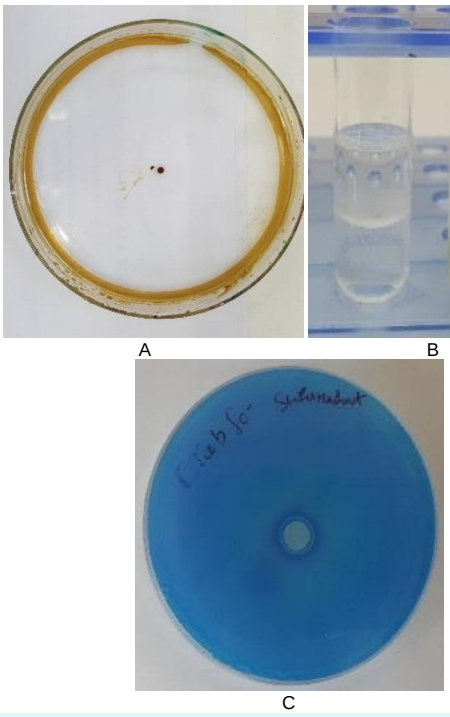


Fig. 1. Tests of biosurfactant detection A-oil spreading test B-emulsification index

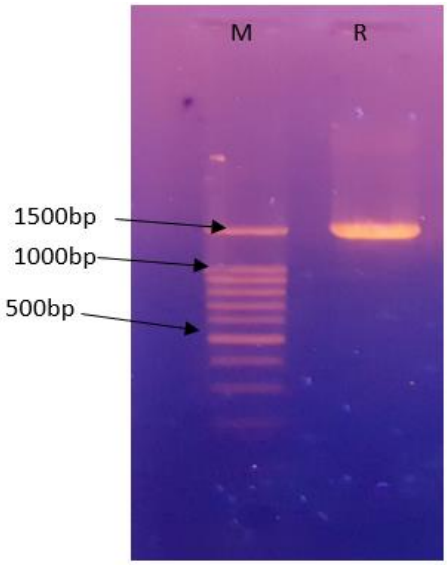


Fig. 2. Agarose gel electrophoresis of PCR product for 16S rRNA gene. Lane M: 100 bp DNA ladder. Lane R: 16 sRNA gene

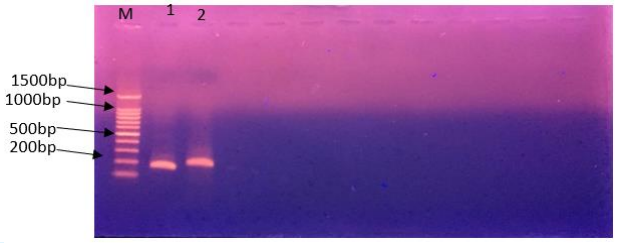


Fig. 3. Agarose gel electrophoresis of PCR product for rhamnolipid genes. Lane M: 100 bp DNA ladder. Lane 1: rhlB gene. Lane 2: rhlC gene

to assess an ability of isolates to produce biosurfactants. The results demonstrated (**Table 2**) efficient bacterial isolate designated as A2 among the bacterial isolates. A2 isolate gave positive results to all the screening methods employed (**Fig. 1 A, B, C**). It was observed that A2 isolate was gram negative, rod-shaped and produced light green colonies when cultured on nutrient agar.

Bacterial isolate identification by 16S rRNA

For precision identification of A2 isolate, 16S ribosomal DNA gene was amplified (**Fig. 2**) and sequenced. The 16S rRNA sequence obtained was compared and aligned with sequences available in the NCBI Gen-Bank database using BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov). The results showed 100 % similarity with *Pseudomonas aeruginosa* strain PA2 (accession number: MN636758.1). Therefore, A2 isolate was identified as *P. aeruginosa*.

Detection of genes coded for rhamnolipid biosurfactant

From the results obtained, the molecular detection of genes that responsible for synthesizing rhamnolipid biosurfactant demonstrated (**Fig. 3**) the presence of amplified partial fragment of rhlB and rhlC genes in *P. aeruginosa* (A2 isolate). These results indicate the ability of *P. aeruginosa* (A2 isolate) to produce rhamnoipid biosurfactant.

Screening of cultural conditions for enhanced biosurfactant production

In the present study, several variables were assessed to determine the optimal conditions for biosurfactant production by *P. aeruginosa* (A2 isolate). Different carbon sources were investigated for biosurfactant production. The results provided in (**Table 3**) showed that the olive oil was the best carbon source for biosurfactant production with E24% (56.6 %),

Table 3. Effect of various carbon sources for rhamnolipid production by *P. aeruginosa*

Carbon sources	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
Olive oil	1.678 ± 0.050*	56.6	14	2.787 ± 0.100*
Sunflower oil	1.535 ± 0.013	56.6	14	1.453 ± 0.060
Corn oil	1.429 ± 0.103	56.6	14	1.424 ± 0.026
Glycerol	1.091 ± 0.36	53.3	14	1.918 ± 0.024
Lactose	0.054 ± 0.011	Nil	Nil	0.917 ± 0.037
Glucose	1.440 ± 0.013	56.6	13	1.177 ± 0.006
Mannitol	1.515 ± 0.006	56.6	13	1.914 ± 0.006

* Mean ± SD, n=3

Table 4. Effect of change in olive oil concentration on rhamnolipid production by P. aeruginosa

Olive oil (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
1.0	1.614 ± 0.006*	60.0	14.5	2.575 ± 0.045*
1.5	1.640 ± 0.007	60.0	14.5	2.883 ± 0.045
2.0	1.648 ± 0.028	60.0	14.5	2.836 ± 0.037
2.5	1.617± 0.006	46.6	14.5	2.623 ± 0.055
3.0	1.481± 0.008	46.6	14.5	2.040 ± 0.158
3.5	1.439± 0.014	36.0	14.5	1.856 ± 0.032
4.0	1.421 ± 0.007	36.0	14.5	1.764 ± 0.058

* Mean ± SD, n=3

Table 5. Effect of various nitrogen sources on rhamnolipid production by *P. aeruginosa*

Nitrogen sources	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
Glutamic acid	1.374 ± 0.024*	50.0	14.2	2.316 ± 0.096*
KNO_3	0.862 ± 0.043	53.3	13.0	1.342 ± 0.101
$NaNO_3$	1.542 ± 0.039	53.3	13.5	2.122 ± 0.109
NH_4Cl_2	0.628 ± 0.031	40.0	13.5	1.324 ± 0.206
NH_4NO_3	1.638 ± 0.038	60.0	14.5	2.495 ± 0.074
$(NH_4)_2SO_4$	1.350 ± 0.022	40.0	12.5	1.875 ± 0.046
Urea	1.744 ± 0.059	60.0	14.5	2.390 ± 0.072
Yeast extract	1.453 ± 0.043	50.0	14.0	2.205 ± 0.076

* Mean ± SD, n=3

emulsification activity (1.678 ± 0.050), oil spreading 14 cm and biomass 2.787 ± 0.100. Lactose proved to be the poorest carbon source for biosurfactant production with no values recorded for E24 % and oil spreading, emulsification activity (0.054 ± 0.011), and biomass 0.917 ± 0.037.

The effect of different concentrations of olive oil as a sole carbon source on biosurfactant production was determined and the results were provided (**Table 4**). The optimum olive oil concentration to maximize production of biosurfactant was determined by gradually increasing the concentration of olive oil in the present study. Olive oil concentration of 2 % seemed to provide the best results with E24% (60 %), Emulsification activity (1.648 ± 0.028), oil spreading 14.5 cm and biomass 2.836 ± 0.037 with significant differences with all other olive oil concentration ($P < 0.05$).

Nitrogen sources affected the biosurfactant production in current study. Eight nitrogen sources (glutamic acid, KNO_3, $NaNO_3$, NH_4Cl_2, NH_4NO_3, $(NH_4)_2SO_4$, Urea and Yeast extract) were evaluated. The results (**Table 5**) show that urea was the best nitrogen source for biosurfactant production with E24 % (60 %), Emulsification activity (1.744 ± 0.059), oil spreading 14.5 cm and biomass 2.390 ± 0.072 with significant differences in evaluated parameters ($P < 0.05$). The lowest emulsification activity was obtained by using NH4Cl as nitrogen source (**Table 5**).

Since urea provided the best biosurfactant production, different concentrations of urea were further examined for their effect on biosurfactant production (**Table 6**). The optimum nitrogen concentration was determined by gradually increasing urea concentrations till the optimum concentration was reached at 0.2 % with values of E 24% (60 %), emulsification activity (1.834 ± 0.048), oil spreading 15 cm and biomass (2.898 ± 0.202) with significant differences with all values ($P < 0.05$).

MSM was adjusted to different pH values (5, 6, 7, 8 and 9) to see effect of different pH values on biosurfactant production. The obtained results (**Table 7**) demonstrated that the optimum pH for biosurfactant production was 7 with values of E24% (60 %), emulsification activity (1.846 ± 0.023) and oil spreading 15 cm and biomass (3.186 ± 0.059) with significant differences recorded with all values ($P < 0.05$).

The effect of incubation period on biosurfactant production was examined and presented (**Table 8**). Maximum biosurfactant activity was observed at incubation period of 6 days with values of E24% (60 %), emulsification activity (1.856 ± 0.015), oil spreading 15 cm and biomass (2.876 ±0.087) along with significant differences recorded with all values ($P < 0.05$). Emulsification activity, oil spreading, and biomass increased with increasing number of incubation days until 6. While biomass kept on increasing with days of

Table 6. Effect of urea concentration on rhamnolipid production by *P. aeruginosa*

Urea concentration (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
0.1	1.621 ± 0.023*	60.0	14.5	2.317 ± 0.172*
0.2	1.834 ± 0.048	60.0	15.0	2.898 ±0.202
0.4	1.727 ± 0.049	60.0	15.0	2.303 ± 0.194
0.6	1.642 ± 0.016	60.0	14.0	2.343 ± 0.073
0.8	1.630 ± 0.008	53.3	13.0	2.148 ± 0.111

* Mean ± SD, n=3

Table 7. Effect of adjusting pH values on rhamnolipid production by *P. aeruginosa*

pH	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
5	0.419 ± 0.016*	30.0	8	2.262 ± 0.053*
6	1.576 ± 0.135	40.0	13	2.605 ± 0.042
7	1.846 ± 0.023	60.0	15	3.186 ± 0.059
8	1.631 ± 0.011	40.0	13	3.821 ± 0.029
9	0.367 ± 0.004	3.3	9	1.706 ± 0.022

* Mean ± SD, n=3

Table 8. Effect of incubation period on rhamnolipid production by *P. aeruginosa*

Incubation period	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
1	0.257 ± 0.013*	3.3	10	0.572 ± 0.051*
2	0.381 ± 0.017	30.0	13	0.923 ± 0.013
3	1.389 ± 0.028	53.3	14	1.494 ± 0.039
4	1.485 ± 0.040	60.0	14.5	1.832 ± 0.100
5	1.679 ± 0.057	60.0	14.5	2.393 ± 0.156
6	1.856 ± 0.015	60.0	15	2.876 ± 0.087
7	1.723 ± 0.032	60.0	15	3.345 ± 0.116

* Mean ± SD, n=3

Table 9. Effect of inoculum size on rhamnolipid production by *P. aeruginosa*

Inoculum size (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass gm/l
1	1.638 ± 0.021*	53.3	11	2.047 ± 0.072*
2	1.663 ± 0.034	60.0	14.5	2.440 ± 0.170
3	1.831 ± 0.025	60	15	2.851 ± 0.043
4	1.825 ± 0.019	53.3	15	3.518 ± 0.083
5	1.748 ± 0.030	53.3	14	3.778 ± 0.038

* Mean ± SD, n=3

incubation until 7, meanwhile E24%, emulsification activity and oil spreading decreased at seven days of incubation.

The effect of inoculum size on biosurfactant production was examined and presented (**Table 9**). Increasing inoculum size above 3 % didn't have any significant effect on the biosurfactant production, but increase in biomass was observed with increase in inoculum upto 3 %, therefore 3 % was chosen as the optimum inoculum size with value of E24% (60 %), emulsification activity (1.831 ± 0.025), oil spreading 15 cm and biomass (2.851 ± 0.043) with significant differences recorded in all values ($P < 0.05$).

Surface tension

The surface tension of the cell free supernatant produced under optimum condition by *P. aeruginosa* was 28.49 mN/m as compared to MSM (72 mN/m).

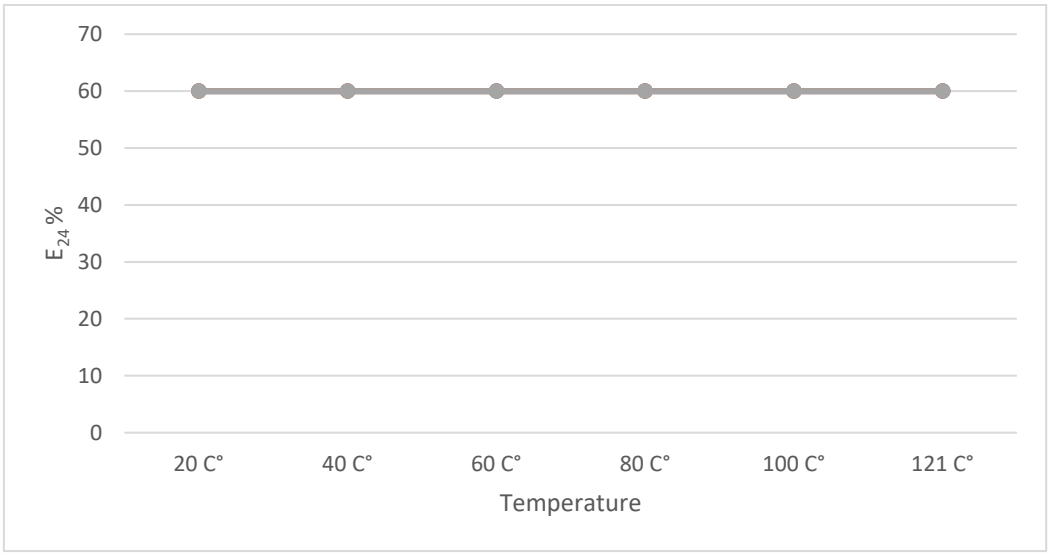
Biosurfactant stability

The results showed that biosurfactant was well stabilized when subjected to different temperatures (20, 40, 60, 80, 100 and 121 °C) with emulsification index E24% at 60 % (**Fig. 4A**) revealing heat stability of this biosurfactant. The results of pH effect on emulsification index E24% of biosurfactants activity are presented in (**Fig. 4B**). The emulsification index E24% remained stable (60%) at pH values 6, 8 and 10, followed by lower emulsification index E24% at acidic pH values of 4 (40 %) and 2 (6.66 %). **Fig. (4C)** shows the effect of NaCl concentrations (2 %, 6 %, 10 %, 14 % and 20 %) on the emulsification index E24%. The results showed that the emulsification index E24% was stable at concentration 2 %, 6 % and 10 %. The emulsification index E24% decreased at concentration of 14 % and 20 % recording 50 % and 20 % respectively.

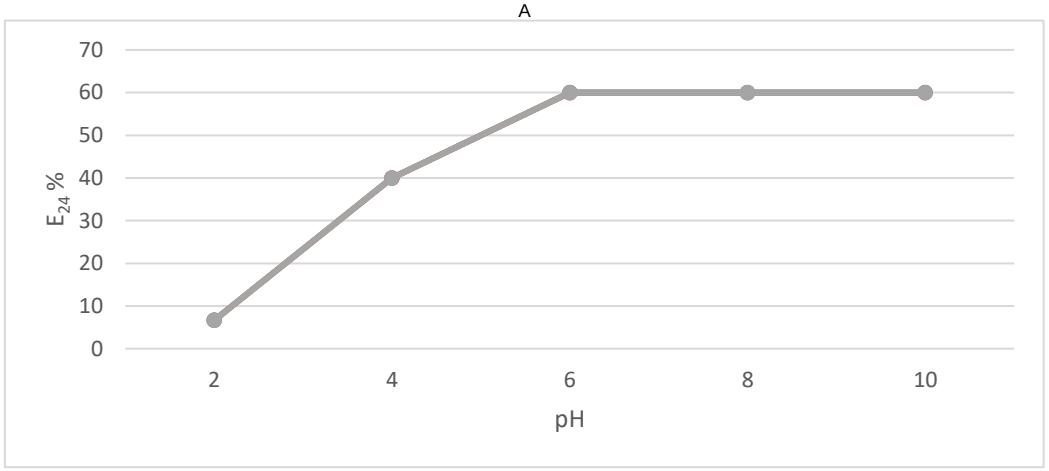
Recovery and detection of the biosurfactant

P. aeruginosa produced 5.42 ± 0.475 g/l of biosurfactant when grown under optimum condition in the biosurfactant production media. The biosurfactant was extracted using acetone precipitation method. The analysis of extracted biosurfactant by TLC was confirmed as glycolipid by observing two spots on TLC plate when sprayed with α-naphthol reagent (**Fig. 5A**). Lower spot with a Rf value of 0.34 (**Fig. 5B**) and upper spot with an Rf value of 0.63 (**Fig. 5C**). The Lower spot was the more polar one that appeared as the less mobile spot. While, upper spot was the less polar one that appeared as the more mobile spot on TLC plates.

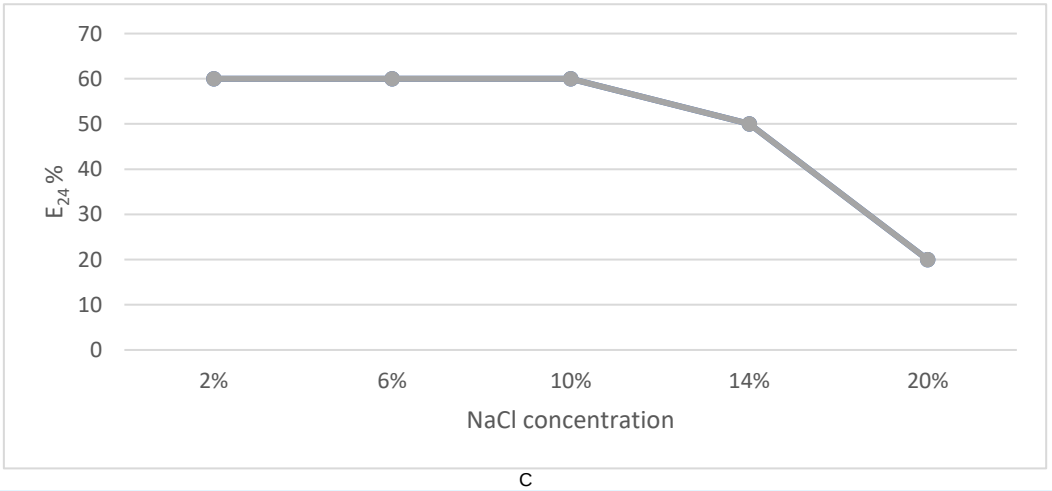
The rhamnolipid biosurfactant was purified by column chromatography using Silica gel 60. The



A



B



C

Fig. 4. A. Effect of temperature on biosurfactant stability **B.** Effect of pH on biosurfactant stability **C.** Effect of NaCl concentration on biosurfactant stability.

fractions were collected from the column and tested using TLC in order to monitor the separation of the components. The fractions showed TLC spots at Rf 0.34 and 0.63 combined. Several studies indicated the efficiency of silica gel column chromatography to purify rhamnolipid biosurfactant.

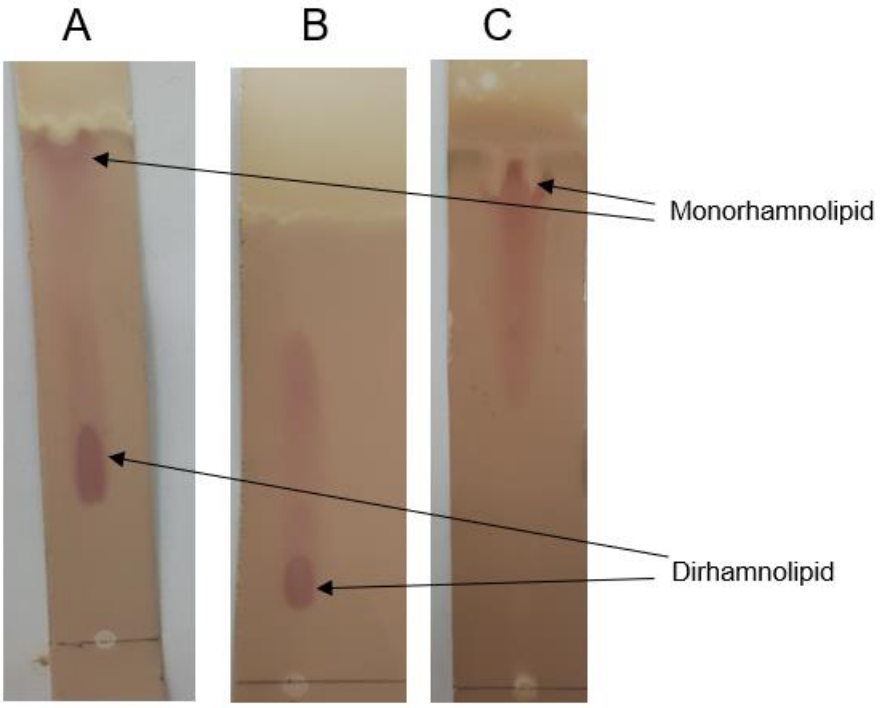


Fig. 5. TLC plates of A-Mixed rhamnolipid, B-Dirhamnolipid, C-Monorhamnolipid

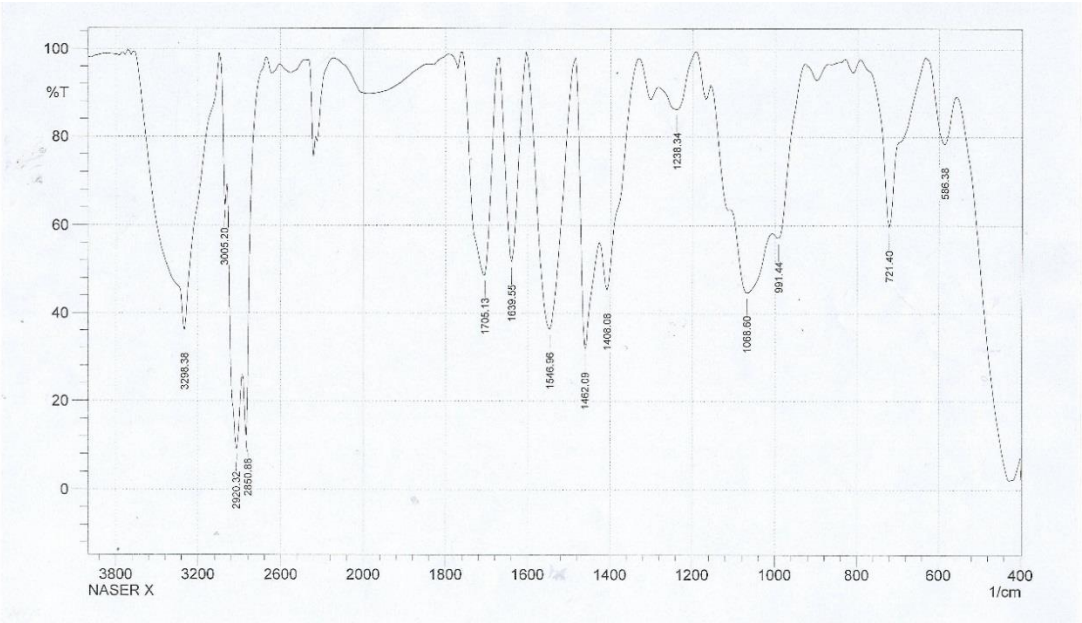


Fig. 6. FTIR spectrum of biosurfactant produced by *P. aeruginosa*

Characterization of biosurfactant

FTIR measurements

The infrared spectra of partial purified biosurfactant produced by *P. aeruginosa* (**Fig. 6**) showed structural features of biosurfactant. The band at 3298.38 cm^{-1} is attributed to free -OH stretching groups. The peaks observed at 3005.20 cm^{-1}, 2920.32 cm^{-1} and 2850.88 cm-1 are characteristics of several C-H stretching bands of CH, CH2 and CH3 groups. Absorption bands around 1705.13 cm-1 and 1639.55 cm-1 represent ester and carbonyl groups (C=O). Absorption bands around 1238.34 cm-1, 1068.60 cm-1 and 991.44 cm-1 are attributed to (C−O−C) stretching of polysaccharides. The results of the infrared spectra taken together prove that this biosurfactant is a rhamnolipid.

GC-MS analysis

GC-MS chromatogram of the biosurfactant showed major peaks indicating the presence of different compounds (**Table 10**). The major peaks of compounds at different retention time of 13.996, 16.678, 17.894

Table 10. Peak report of lipid part of rhamnolipid using GC-MS

Retention time/min	Compound name	MFG Formula
13.996	2-Octenoic acid	C8H14O2
16.678	trans-2-Decenoic acid	C10H18O2
17.894	3-Hydroxydecanoic acid	C10H20O3
18.763	trans-2-Dodecenoic acid	C12H22O2
22.167	Palmitoleic acid	C16H30O2
22.605	n-Hexadecanoic acid	C16H32O2
23.307	n-Hexadecanoic acid	C16H32O2

18.763, 22.167, 22.605 and 23.307 minutes were identified from the standard library compound as 2-Octenoic acid, trans-2-Decenoic acid, 3-Hydroxydecanoic acid, trans-2-Dodecenoic acid, Palmitoleic acid, n-Hexadecanoic acid. Therefore, the results of GC-MS analysis indicate the presence and structure of the major rhamnolipid components.

DISCUSSION

The current study is aimed to screen, detect, isolate and characterize the biosurfactant by bacteria isolated from hydrocarbon contaminated soil sample. The biosurfactant producing bacteria are widely distributed in hydrocarbon contaminated soil (Zou et al. 2014). Biosurfactants were used by microorganisms to increase the bioavailability of the hydrophobic substrates and facilitate the attachment of microbial cells to the surfaces (Cameotra and Makkar 2004).

The screening methods employed were oil spreading test, emulsification index method, emulsification activity and CTAB−methylene blue agar method. These methods have constituted a quick and easy methods to screen and predict biosurfactant production by bacteria. The principle of oil spreading test, emulsification index and emulsification activity methods depend on the reduction of interfacial tension of the liquids and emulsifying capacity of biosurfactants regardless of biosurfactant structure (Chandran and Das 2011). The dark blue halo formed around the well indicates the ability of isolate for producing glycolipid biosurfactant using CTAB−methylene blue agar method. Anionic surfactants like glycolipids form dark blue-purple, insoluble ion pair with CTAB and methylene blue (Siegmund and Wagner 1991).

The A2 isolate was selected as a potential producer of biosurfactant based on the positive results to all the screening methods and identified as *P. aeruginosa* based on rRNA gene sequence. *P. aeruginosa* is a Gram-negative, motile with polar flagellum and nonspore forming bacterium that can be isolated from many environmental habitats. It is well-known as potent biosurfactant producer, with rhamnolipid as most common among the biosurfactants are produced (Saikia et al. 2011, El-Housseiny et al. 2016).

The results reported the presence of amplified partial fragment of rhlB and rhlC genes in *P. aeruginosa* (A2 isolate). These two genes responsible for synthesizing rhamnolipid biosurfactant and therefore, indicated the ability of *P. aeruginosa* (A2 isolate) to synthesize rhamnoipid biosurfactant. The rhlB and rhlC genes carried on two separate operons, code for the enzymes responsible for synthesis of rhamnolipid (Abdel-Mawgoud et al. 2010). Ochsner et al. (1994) mentioned that the rhlB gene expresses rhamnosyltransferase 1 enzyme which conjugates HAA with dTDP-L-rhamnose to form mono-rhamnolipid, both rhlA and rhlB genes located on one operon alongside an AHL-mediated quorum sensing system (rhlRI). The rhlC gene of *P. aeruginosa* expresses rhamnosyltransferase 2 enzyme which conjugates mono-rhamnolipid to a second dTDP-Lrhamnose to form di-rhamnolipid. The rhlC gene is located on separate operon approx. 2.5 Mb downstream of the rhl operon (Ochsner et al. 1994, Rahim et al. 2011).

The effect of various cultural conditions (carbon source, carbon concentration, nitrogen source,nitrogen concentration, pH, incubation time, inoculum size) on biosurfactant production by *P.aeroginosa* (A2 isolate) were determined. The effect of various carbon sources (olive oil, sunflower oil, corn oil glycerol, lactose glucose and mannitol) on biosurfactant production by *P.*aeroginosa (A2 isolate) were investigated and revealed that the olive oil was the best carbon source with concentration 2 % to provide the best results for biosurfactant production. The tendency of *P. aeruginosa* for using olive oil as carbon source for biosurfactant production than other carbon sources may be due to three reasons, first being ability of *P. aeruginosa* to produce lipase enzyme which facilitates assimilation of fatty acids contained in olive oil, second being olive oil as water insoluble material that stimulates bacteria to produce biosurfactant to emulsify these water insoluble material for utilization and third reason being growth of bacteria on carbohydrate substrates like glucose, lactose and manittol causing reduction in pH of the medium and leading to inhibition of biosurfactant production (Abouseoud et al. 2008, Rashedi et al. 2006). The increasing olive oil concentration above 2 % was accompanied by an inhibitory effect on microbial growth and activity. This effect was due to the solubility of olive oil and the difficulty of *P. aeruginosa* to gain access to the nutrients in the culture medium (Eraqi et al. 2016). Similar results were reported by Abouseoud et al. (2008) that olive oil was the best carbon source for biosurfactant production by *Pseudomonas fluorescens* which decreased the surface tension to 38 dyne/cm, and the emulsifying activity as 49 %. Khopade et al. (2012)

reported enhanced biosurfactant production by *Nocardiopsis* sp. B4 using olive oil as the carbon source with 2 % concentration. El-Housseiny et al. (2016) found the optimum carbon source for maximum rhamnolipid production was 2 % glycerol. The optimum glycerol concentration enhanced the rhamnolipid production threefold with maximum rhamnolipid concentration reaching 7.54 g/l. Ozdal et al. (2017) reported use of waste frying oil as carbon source on rhamnolipid production by *P. aeruginosa* OG1 as a low-cost substrate. The optimized medium containing 5.2 % frying oil enhanced the rhamnolipid production twofold compared to preliminary cultivations up to 13.31 g/l.

The effect of various nitrogen sources (glutamic acid, KNO_3, $NaNO_3$, NH_4CL_2, NH_4NO_3, $(NH_4)_2SO_4$, urea and yeast extract) on the biosurfactant production by *P.*aeroginosa (A2 isolate) were investigated and revealed that the urea was the best nitrogen source with concentration 0.2 % to provide the best results for biosurfactant production. The nitrogen source is essential for the synthesis of bacterial enzymes that required for microbial growth and completing its metabolic pathways (Okoliegbe and Agarry 2012). The difference reported in the biosurfactants production when by *P.*aeroginosa (A2 isolate) was grown in the presence of different nitrogen sources may be due to the preferential demand for a particular nitrogen source for growth and the synthesis of enzymes by the bacterium. Subasioglu and Cansunar (2008) demonstrated that the production of rhamnolipid by *P. aeruginosa* when growing on 6 different nitrogen sources such as $(NH4)_2SO_4$, $NH_4H_2PO_4$, NH_4NO_3, KNO_3, $NaNO_3$ and peptone, but the maximum production was 589.3 mg/l, achieved with 0.2 % $NaNO_3$ as the nitrogen source. While Fazli and Hertadi (2018) demonstrated the rhamnolipid was optimally produced by *Pseudomonas stutzeri* BK-AB12 in urea 0.175 % as nitrogen source.

The current study showing highest biosurfactant production at pH 7, with decreasing activity of rhamolipid in acidic (pH of 5 or 6) or basic (pH of 8 or 9) conditions. The pH affects production of biosurfactant by bacteria through its effect on activity and growth of bacterial cells. Literature shows that most bacteria produce maximum biosurfactant at neutral pH. Saikia et al. (2011) reported maximum production of biosurfactant with pH range of 7−8. In studies documented by (Khopade et al. 2012), highest yield of rhamnolipid was obtained at pH of 6 (1977 mg/l), followed by pH 7 and pH 8 (1406 mg/l and 818 mg/l respectively) with undetectable rhamnolipid production at pH 4 and 10.

The effect of incubation period (1, 2, 3, 4, 5, 6 and 7 days) on the ability of *P.*aeroginosa (A2 isolate) to produce biosurfactant was investigated. The current study showing highest biosurfactant production was observed at incubation period of 6 days. The effect of the long incubation period may be due to the interference between secondary metabolites and biosurfactant which led to drop in biosurfactant activity. Eraqi et al. (2016) reported rhamnolipid yield produced by *P. aeruginosa* increasing with incubation time and reaching maximum after 54 hrs of incubation period. Fazli and Hertadi (2018) showed optimally produced rhamnolipid by *P. stutzeri* BKAB12 at 90 hrs of incubation period.

The effect of Inoculum size (1%, 2%, 3%, 4% and 5%) on the ability of *P.aeroginosa* (A2 isolate) to produce biosurfactant was investigated. The current study showing highest biosurfactant production was observed at 3% inoculum size. Al-Waely (2013) found the optimum inoculum size for maximum yield of rhamnolipid to be 1%. Sarachat *et al.* (2010) reported the best inoculum size at 3%. Neto et al. (2009) found the optimum inoculum size for maximum yield of rhamnolipid at 4-5 %.

The surface tension of cell free supernatant is decreased as the biosurfactant concentration increased in the production medium. Biosurfactants absorb at the air-water interface due to their amphiphilic nature and break interactions between the water molecules and the intermolecular forces between water molecule and biosurfactant are much lower than between two water molecules and therefore, surface tension will decrease. El-Housseiny et al. (2016) reported the ability of rhamnolipid to reduce water surface tension from 72.02 to 35.26 mN m−1. Tiwary and Dubey (2018) reported that the rhamolipid produced by *Pseudomonas aeruginosa* ADMT1 reduced surface tension of water 72 to 28.4 mN/m.

The results showed that biosurfactant was well stabilized when subjected to different temperatures, pH and NaCl concentrations. These results were supported by Saikia et al. (2011) and Anyanwu (2010) where they showed rhamnolipid produced by *P. aeruginosa* as thermostable even at an autoclaving temperature. Yin et al. (2009) and Techaoei et al. (2011) reported rhamnolipid activity remained stable at neutral and basic pH due to the structural nature of rhamnolipid. In similar studies by Lan et al. (2015), rhamnolipid activity remained relatively stable at NaCl concentration up to 8% then the change in rhamnolipid activity occurred with rising NaCl concentration. Helvaci et al. (2004) reported that the carboxylic groups of rhamnolipid perform the functional control of their amphipathic properties by giving the molecule its anionic character that are strongly affected by the pH and the presence of NaCl.

The obtained results demonstrated that the maximum biosurfactant production was (5.42 ± 0.475 g/l) happened using olive oil at a concentration of 2% as carbon source, 2 g/l of urea as a nitrogen source, inoculum size of 3 %, pH: 7, and 6 days incubation period at 30°C. The produced biosurfactant was purified using silica gel column chromatography. The results of TLC, FTIR and GC-MS analysis indicate the presence

and structure of the major rhamnolipid components (Abbasi et al. 2012, Abdel-Mawgoud et al. 2009, Rath et al. 2016), as result the biosurfactant produced by *P.aeroginosa* (A2 isolate) was characterized as both mono-rhamnolipids and di-rhamnolipids. Rhamnolipids are produced by various *Pseudomonas* species such as *P. aeruginosa*, *P. cepacia*, *P. chlororaphis*, *P. putida* and *P. fluorescens* (Twigg et al. 2018) and *Burkholderia* species such as *B. pseudomallei, B. thailandensis*, *B. plantarii* and *B. glumae* (Costa et al. 2011, Irorere et al. 2017). Nalini and Parthasarathi (2013) reported the production of rhamnolipoid by *Serratia rubidaea* SNAU02. Rhamnolipids are important biosurfactants, with a variety of industrial and environmental applications due to their unique properties such as emulsification, detergency, wetting, dispersing or solubilization, foaming, antimicrobial and antiadhesive activities in various industries including food, pharmaceutical, cosmetic, paper, agricultural, enhanced oil recovery and bioremediation of pollutants (Banat et al. 2000, Randhawa and Rahman 2014). The application of rhamnolipid biosurfactant holds great promise, but the commercial production of rhamnolipid is associated with economically challenging. Rhamnolipid biosurfactant can be produced in large amounts when optimized cultural conditions, an inexpensive substrates and effective bacteria used in fermentation processes. The results provided from the current study demonstrate the ability of *P. aeruginosa* isolated from hydrocarbon contaminated soil to produce significant amounts of rhamnolipid biosurfactant that can be applied in medical applications as antimicrobial and anticancer agents in further studies.

CONCLUSIONS

The current study demonstrated that bacterium *P. pneumonia* isolated from hydrocarbon contaminated soil was efficient biosurfactant producing bacterium and that the biosurfactant it produced was a rhamnolipid based on the result obtained from TLC, FTR and GC–MS analyses. The Biosurfactant produced by this isolate under optimum conditions has effective surface tension in addition to their strong stability over wide range of temperature, pH and NaCl. The bacterium produced (5.42 ± 0.475 g/l) crude rhamnolipid biosurfactant under optimum conditions that can be applied in medical applications as antimicrobial and anticancer agents in further studies.

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REFERENCES

Abbasi, H, Hamedi, M M, Lotfabad, T B, Zahiri, H S, Sharafi, H, Masoomi, F, Moosavi-Movahedi, A A, Ortiz, A, Amanlou, M and Noghabi, K A. (2012). Biosurfactant-producing bacterium, Pseudomonas aeruginosa MA01 isolated from spoiled apples: Physicochemical and structural characteristics of isolated biosurfactant. J Biosci Bioeng, Volume 113, Issue 2, P: 211-219.

Abdel-Mawgoud AM, Aboulwafa MM, Hassouna, NA-H (2009) Characterization of Rhamnolipid produced by Pseudomonas aeruginosa isolate Bs20. Appl Biochem Biotechnol 157(2):329–345. doi: 10.1007/s12010-008-8285-1

Abdel-Mawgoud AM, Hausmann R, Le ´pine F, Mu¨ller MM De ´ziel E 2011 Rhamnolipids: detection, analysis, biosynthesis, genetic regulation, and bioengineering of production. In: Sobero ´n-Cha ´vez G (ed) Biosurfactants: from genes to applications. Springer, Berlin, pp 13–55.

Abdel-Mawgoud AM, Lépine F, Déziel E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86: 1323–1336. https://doi.org/10.1007/s00253-010-

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.

Al-waely WAS (2013) Production, purification and characterization of rhamnolipid from a local isolate of Pseudomonas aeroginosa. Dissertation, College of Agriculture. University of Basrah. (In Arabic).

Anyanwu CU (2010) Surface activity of extracellular products of a Pseudomonas aeruginosa isolated from petroleum¬ contaminated soil. Inter. J Environ Sci 1 :(2):225-235.

Aparna A, Srinikethana G, Smitha H (2012) Production and characterization of biosurfactant produced by a novel Pseudomonas sp. 2B. Colloid Surface B 95:23–29. doi:10.1016/j.colsurfb.2012.01.043

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444. https://doi.org/10.1007/s00253-010-2589-0.

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53 (5): 495–508.

Câmara JMDA, Sousa MASB, Neto ELB (2019) Optimization and Characterization of Biosurfactant Rhamnolipid production by Pseudomonas aeruginosa isolated from an artificially contaminated soil. J Surfactants Deterg doi:10.1002/jsde.12287.

Cameotra SS, Makkar RS (2004) Recent applications of biosurfactants as biological and immunological molecules. Curr Opin Microbiol 7:262–266

Chandran P, Das N (2011) Characterization of sophorolipid biosurfactant produced by yeast species grown on diesel oil. Inter J Sci Nature 2(1):63–71.

Chirwa EMN, Bezza FA (2015) Petroleum hydrocarbon spills in the environment and abundance of microbial community capable of biosurfactant production. J Pet Environ Biotechnol 6:237

Costa SGVAO, Déziel E, Lépine F (2011) Characterization of rhamnolipid production by Burkholderia glumae. Lett Appl Microbiol 53:620–627. doi:10.1111/j.1472 765X.2011.03154.x

Deng M-C, Li J, Liang F-R, Yi M, Xu X-M, Yuan J-P, Peng J, Chou-Fei Wu C-F, Wang J-H (2014) Isolation and characterization of a novel hydrocarbon-degrading bacterium Achromobacter sp. HZ01 from the crude oil-contaminated seawater at the Daya Bay, southern China. Mar Pollut Bull 83:79–86.

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64. https://doi.org/10.1016/S0140-6701 (97)84559-6.

El-Housseiny S, Aboulwafa M.M, Aboshanab KA, Hassouna NA (2016) Optimization of Rhamnolipid production by P. aeruginosa isolate P6. J Surfact Deterg 19:943–955. doi 10.1007/s11743-016-1845 4.

Eraqi WA, Yassin AS, Ali AE, Amin MA (2016) Utilization of crude glycerol as a substrate for the production of Rhamnolipid by Pseudomonas aeruginosa. Biotech Research Intern http://dx.doi.org/10.1155/2016/3464509.

Fazli RR, Hertadi R (2018) Optimization of rhamnolipid production from bioconversion of palm oil mill effluent (POME) waste by Pseudomonas stutzeri BK-AB12 using response surface methodology. IOP Conf. Ser.: Earth Environ Sci 209 012024. doi:10.1088/1755-1315/209/1/012024.

Femi-Ola T, Oluwole O, Olowomofe T, Yakubu H (2015) Isolation and screening of biosurfactant producing bacteria from soil contaminated with domestic waste water. BJES 3: 58-63.

Helvaci SS, Peker S, Özdemir G (2004) Effect of electrolytes on the surface behavior of rhamnolipids R1 and R2. Colloids Surf B Biointerfaces 35:225–233

Irorere VU, Tripathi L, Marchant R, McClean S, Banat IM (2017) Microbial rhamnolipid production: a critical re-evaluation of published data and suggested future publication criteria. Appl Microbiol Biotechnol DOI 10.1007/s00253-017-8262-0.

Jarvis FG, Johnson MJ (1949) A glycolipide produced by Pseudomonas aeruginosa. J Am Chem Soc 71:4124–4126. doi: 10.1021/ja01180a073.

Khopade A, Biao R, Liu X, Mahadik K, Zhang L, Kokare C (2012) Production and stability studies of the biosurfactant isolated from marine Nocardiopsis sp. B4. Desalination 285:198–204. doi:10.1016/j.desal.2011.10.002.

Lan G, Fan Q, Liu Y, Chen C, Li G, Liu Y, Yin X (2015) Rhamnolipid production from waste cooking oil using Pseudomonas SWP-4. Biochem Eng J 101:44–54.

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.

Marchant R, Banat IM (2012) Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol. Lett. 34, 1597–1605. doi:10.1007/s10529- 012-0956-x

Miyoshi T, Iwatsuki T, Naganuma T (2005) Phylogenetic characterization of 16S rRNA gene clones from deep-groundwater microorganisms that pass through 0.2-micrometer-pore-size filters. Appl Environ Microbiol 71(2):1084-1088.

Nalini S, Parthasarathi R (2013) Biosurfactant production by Serratia rubidaea SNAU02 isolated from hydrocarbon contaminated soil and its physicochemical characterization. Bioresour Technol 147: 619e622

Nayarisseri A, Singh P, Singh S (2019) Screening, isolation and characterization of biosurfactant-producing Bacillus tequilensis strain ANSKLAB04 from brackish river water. Int J Sci Environ Technol.16 (11):7103–12.

Neto DC, Meira JA, Tiburtius E, Zamora PP, Bugay C, Mitchel DA, Krieger N (2009) Production of rhamnolipids in solid-state cultivation: Characterization, downstream processing and application in the cleaning of contaminated soils. Biotechnol J 4(5):748-755.

Ochsner UA, Fiechter A, Reiser J (1994) Isolation, characterization, and expression in Escherichia coli of the Pseudomonas aeruginosa rhlAB genes encoding a rhamnosyl transferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 269:19787-19795.

Okoliegbe IN, Agarry OO (2012) Application of microbial surfactant (a review). Scholarly J Biotech 1(1):15-23.

Ozdal M, Gurkok S, Ozdal OG (2017) Optimization of rhamnolipid production by Pseudomonas aeruginosa OG1 using waste frying oil and chicken feather peptone. Biotech 7:117. Doi: 10.1007/s13205-017-0774-x.

Paraszkiewicz K, Kanwal A, Długon´ski J (2002) Emulsifier production by steroid transforming filamentous fungus Curularia lunata. Growth and product characterization. J Biotechnol 92:287–294.

Perfumo A, Rudden M, Smyth TJP, Marchant R, Stevenson PS, Parry NJ, Banat IM (2013) Rhamnolipids are conserved biosurfactants molecules: implications for their biotechnological potential. Appl Microbiol Biotechnol doi: 10.1007/s00253-013-4876-z.

Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, Soberon-Chavez G (2001) Cloning and functional characterization of the Pseudomonas aeruginosa rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 40:708-718.

Randhawa KKS, Rahman PKSM (2014) Rhamnolipid biosurfactants-past, present, and future scenario of global market. Front Microbiol 5:454.

Rashedi H, Assadi M, Jamshidi E, Bonakdarpour B (2006) Production of rhamnolipids by Pseudomonas aeruginosa growing on carbon sources. Int J Environ Sci Tech 3 (3): 297-303.

Rath, K, Singh, AB, Chandan, S, Vatsala, RS (2016) Isolation and characterization of a biosurfactant producing strain Pseudomonas aeruginosa SMVIT 1 from oil contaminated soil. J Sci. Ind. Res., vol. 75: 681-686.

Saikia RR, Deka S, Deka M, Sarma H (2011) Optimization of environmental factors for improved production of rhamnolipid biosurfactant by Pseudomonas aeruginosa RS29 on glycerol. J Basic Microbiol 51:1-12.

Santos APP, Silva MDS, Costa EVL, Rufino RD, Santos VA, Ramos CS, Sarubbo LA, Porto ALF (2018) Production and characterization of a biosurfactant produced by Streptomyces sp. DPUA 1559 isolated from lichens of the Amazon region. Braz J Med Biol Res 51(2): e6657. doi: 10.1590/1414-431X20176657.

Sarachat T, Pornsunthorntawee O, Chavadej S, Rujiravanit R (2010) Purification and concentration of a rhamnolipid biosurfactant produced by Pseudomonas aeruginosa SP4 using foam fractionation. Bioresour Technol 101:324–330.

Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM, Chopade BA (2010) Methods for investigating biosurfactants and bioemulsifiers: A review. Crit Rev Biotechnol 30 (2): 127-144. DOI: 10.3109/07388550903427280

Siegmund I, Wagner F (1991) New method for detecting rhamnolipids excreted by Pseudomonas sp. during growth on mineral agar. Biotechnol Tech 5(4):265-268.

Sifour M, Ouled-Haddar H, Aziz GM (2005) Production of biosurfactants from two Bacillus species. Egypt J Aquat Res 31:142-148.

Subasioglu T, Cansunar E (2008) Nutritional Factors Effecting Rhamnolipid Production by a Nosocomial Pseudomonas aeruginosa. Hacettepe J Biol Chem 36 (1):77-81.

Techaoei S, Lumyong S, Prathumoai W, Santiarwarn D, Leelapornpisid P (2011) Screening characterization and stability of biosurfactants produced by Pseudomonas aeruginosa SCMU106 isolated from soil in northern Thailand. Asian J Biol Sci 4:4:340-351. doi: 10.3923/ajbs.2011.340.351.

Tiwary M, Dubey AK (2018) Characterization of Biosurfactant Produced by a Novel Strain of Pseudomonas aeruginosa, Isolate ADMT1. J Surfact Deterg doi: 10.1002/jsde.12021.

Twigg MS, Tripathi L, Zompra A, Salek K, Irorere VU, Gutierrez T, Spyroulias GA, Marchant R, Banat IM (2018) Identification and characterisation of short chain rhamnolipid production in a previously uninvestigated, non-pathogenic marine pseudomonad. Appl Microbiol Biotechnol 102:8537–8549. https://doi.org/10.1007/s00253-018-9202-3.

Wei YH, Chou CL, Chang JS (2005) Rhamnolipid production by indigenous Pseudomonas aeruginosa J4 originating from petrochemical wastewater. Biochem Eng J 27:146.

Xiangsheng Z, Miao L, Tingsheng X (2010) Genetic modification of MEOR bacterium Bacillus licheniformis H strain by low energy ion beam irradiation. Open Biotechnol J 4:14-17.

Yin H, Qiang J, Jia Y, Ye J, Peng H, Qin H, Zhang N, He B (2009) Characteristics of biosurfactant produced by Pseudomonas aeruginosa S6 isolated from oil-contaminated wastewater. Process Biochem 44:302-308.

Zhao H, Shao D, Jiang C, Shi J, Li Q, Huang Q, Rajoka, MSR, Yang, H, Jin, M (2017) Biological activity of lipopeptides from Bacillus. Appl. Microbiol. Biotechnol. 101, 5951–5960.

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 14:49–58