Egypt. J. Chem. Vol. 65, No. 12 pp. 1 - 11 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Characterization of rhamnolipids produced by *Pseudomonas putida* ON763757 isolated from petroleum contaminated soils

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Abstract

Sixteen biosurfactant-producing bacteria were isolated from multiple petroleum-contaminated soils in Kaluybia Governorate, Egypt. Six screening tests (emulsification index (EI24%), oil spreading assay, emulsification activity, cetyltrimethylammonium bromide (CTAB), hemolytic and lipolytic activities) were conducted to select the high producer isolate. Out of 16 isolates, only one was chosen for molecular identification as the most efficient biosurfactant-producer and was found to be Pseudomonas putida ON763757. The type of biosurfactant was determine via three chromatography analytical methods, High-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FT-IR) and Gas chromatography–mass spectrometry analysis (GC-MS). HPLC analysis showed that chlorogenic acid (1409.20 µgg-1) was the main component, followed by ellagic acid (530.12 µgg-1). FTIR and GC-MS analyses indicated that the biosurfactant of Ps. putida was rhamnolipid, which is an exciting product for environmental and industrial applications.

Keywords: Biosurfactants, Emulsification, Chromatographic Technique, Pseudomonas putida, Rhamnolipid.

1. Introduction

Petroleum hydrocarbons have become the most common pollutant in different regions of the world, and oil spills are the primary source of this pollution. It occurs when crude oil is extracted, transported, and stored because it leaks into the surrounding environment (Liu et al., 2018). Surfactants are amphiphilic chemical compounds discovered to clean areas contaminated with hydrocarbons and heavy metals through their hydrophobic and hydrophilic moieties (Gomaa and El-Meihy, 2019). Microbial surfactants are the biological origin surfactants manufactured using a wide variety of microorganisms, especially microorganisms considered secondary metabolites or a part of the cell membrane (Mian, 2021). In comparison with synthetic surfactants, microbial surfactants have some benefits like low toxicity, high biodegradability, efficiency in adverse conditions (multiple pH levels, salinities, and temperatures), stability, being less harmful and eco-friendly, as well as being easy to produce (Mian, 2021; Bagheri et al., 2022).

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DOI: 10.21608/EJCHEM.2022.148123.6406

These features distinguish surfactants by allowing them to work to reduce surface tension and generate a microemulsion where hydrocarbons can dissolve in water or where water can dissolve in hydrocarbons (Deshmukh and Kadam, 2019). Microbial surfactants can be categorized based on these features, including molecular weight and chemical composition. They are divided into different types based on their molecular weight: low-molecular-weight (LMW), including Lipopeptides, glycolipids, and phospholipids, and high-molecular-weight (HMW) like polymeric and particulate biosurfactants (Shekhar et al., 2015). Biosurfactants are grouped into glycolipids (e.g., rhamnolipids, sophorolipids, trehalose triglycerides), lipopeptides (e.g., surfactin, iturin, fengycin), fatty acids/phospholipids/neutral lipids (phosphatidylethanolamine, spiculisporic acid), and polymers biosurfactants (emulsan, alasan, and biodisp (vesicles, whole microbial (Gayathiri et al., 2022).

Generally, bacteria, actinobacteria, fungi, and yeasts are munificent biosurfactant producers, especially *Bacillus*, *Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Streptomyces*, and *Candida* genera, are excellent producers of biosurfactants (Fenibo et al., 2019). Previous researchers represented *Pseudomonas putida* as a valuable biosurfactant source (Janek et al., 2013; Camara et al., 2019). Additionally, some bacterial species contain advantageous properties for cleaning up polluted mines tailings. To degrade pollutants, bacteria produce many metabolites like biosurfactants, and they also have many endurance mechanisms to help them survive in this environment. Hence, because of their ability to transform hazardous compounds into non-toxic forms, they can be used to remove and mobilize pollutants (Abou-Aly et al., 2019).

Rhamnolipids are an LMW biosurfactant, of glycolipid type, and they have a critical micelle concentration (CMC) of 20 (mg/ml) (Sobrinho et al., 2013). They have two moieties: rhamnose (also known as the glycon portion) and lipid (also known as the aglycon portion) (Thakur et al., 2021). Rhamnose moiety is hydrophilic in the natural world consisting of mono or di (L)-rhamnose moleculesthe natural world and consists of mono or di (L)-rhamnose molecules linked together with α-1,2-glycosidic linkage. The lipid moiety is hydrophobic in the natural world and includes one or more saturated/unsaturated β-hydroxy fatty acids chains of C8−C24 lengths, linked together with an ester bond (Thakur et al., 2021). Using varieties of sugar or hydrocarbons, Gram-negative bacteria *Pseudomonas aeruginosa* is the most predominant species producing four common rhamnolipids, viz. 3-[3-(2-*O*-α-L-Rhamnopyranosyl-α-L-rhamnopyranosyloxy) decanoyloxy] decanoic acid (Rha2-C10-C10), 3-[(6-Deoxy-α-L-mannopyranosyl)oxy]decanoic acid (Rha-C10), 3-[3-(α-L-Rhamnopyranosyloxy) decanoyloxy] decanoic acid (Rha-C10-C10) and 3-[(2-*O*-α-LRhamnopyranosyl- α-L-rhamnopyranosyl)oxy] decanoic acid (Rha2-C10). Other *Pseudomonas* species that have been reported to produce rhamnolipids are *P. chlororaphis*, *P. plantarii*, *P. putida*, and *P. fluorescens*. Rhamnolipid has many applications in bioremediation and enhanced oil recovery (EOR). Rhamnolipids show exceptional emulsification properties, effectively removing crude oil from contaminated soil and facilitating bioremediation of oil spills (Sekhon Randhawa and Rahman, 2014). In pharmaceuticals and therapeutics, rhamnolipids have low toxicity, surface-active properties, and antimicrobial activities against several microorganisms (Magalhaes and Nitschke, 2013). Finally, in agriculture, rhamnolipids are now used for soil remediation for developing soil quality and are now supplementary being explored for plant pathogen removal, for supporting the absorption of fertilizers and nutrients through roots, and as biopesticides (Sachdev and Cameotra, 2013).

This study aimed to isolate, screen, and identify biosurfactant-producing bacteria from petroleum-contaminated soils. Then, chromatography and analytical techniques are used to characterize the biosurfactants.

2. Material and methods

2.1. Soil samples collection

Seven contaminated samples of soil (**Table 1**) were collected from auto repair shops in Kaluybia Governorate (latitude 30.3541° north and longitude 31.201° east) at 5 cm from the surface and kept in sterile plastic bags at 4℃ for the following steps.

Agricultural soil samples with the addition of diesel oil and gasoline (Nineteen grams of agricultural soil samples were transferred to a plastic pot with the addition of 10 % of diesel oil and gasoline and 50% moisture, then left for one week before isolation).

Table 1. Sources of isolation soils at Kaluybia Governorate, Egypt.

Soil No.	Source of soil
Soil 1	Auto repair shop (1) at Elmorig
Soil 2	Auto repair shop (2) at Elmorig
Soil 3	Auto repair shop (3) at Elmorig
Soil 4	Auto repair shop (4) at Elmorig
Soil 5	Auto repair shop (5) at Shibin El-Qanater
Soil 6	Auto repair shop (7) at Shibin El-Qanater
Soil 7	Auto repair shop (8) at Shibin El-Qanater
Soil 8	Agricultural soil amended with diesel oil
Soil 9	Agricultural soil amended with gasoline

2.2. Isolation and purification of bacterial isolates

Ten g of each soil sample was added to 150 ml Erlenmeyer flasks containing 90 ml of Luria Bertani (LB) broth (pH 7.0) (HIMEDIA, Co., Germany) and incubated at 352°C with shaking (150 rpm) for 48 hours. The suspension was then serially diluted from 10^{-1} to 10^{-6} in sterile distilled water. One ml from each dilute was moved to a sterile Petri plate, and 20 ml of (LB) agar was poured over it before being incubated at 35±2°C for 48 hours. Following incubation, the single-formed colonies were selected, purified, and stored at 4°C on (LB) agar slants for further studies (Chioma et al., 2013).

2.3. Screening for biosurfactant production

For screening, each isolate was separately cultured in (LB) broth medium (HIMEDIA, Co., Germany) for 3-5 days at 35±2°C under shaking. The cultures were then centrifuged at 8000 rpm for 20 minutes while cooling at 4°C to yield bacterial cell-free supernatants. The obtained supernatants were refrigerated for further experiments. Six tests were conducted to estimate the biosurfactant production ability by the isolated bacteria as follows:

2.3.1. Emulsification index (EI24), 2 mL of cell-free bacterial supernatant was transferred to a clean glass tube, and 2 mL of toluene was added before vertexing the mixture for 2 minutes (2500 rpm) and storing the resulting emulsion for 24 hours. The EI24 index was calculated using the following equation (Phulpoto et al., 2020):

$$EI24 = \frac{\text{Height of emulsion after 24 h}}{\text{Height of total liquid}} \times 100$$

2.3.2. Oil spreading assay, 30 mL of distilled water was placed in the middle of a petri dish (60 mm), and then 15 µl of olive oil (commercial) was added to the surface of the water, and above the oil layer, 50 µl of the bacterial cell-free supernatant was added. A drop of distilled water was used as a negative control instead of the bacterial cell-free supernatant. The diameter of the formed area of displacement in the oil was measured (cm) directly and compared to the negative control (Datta et al., 2018).

2.3.3. For emulsification activity, 3 ml of the cell-free supernatant was added to 0.5 ml of toluene, vortexed rapidly for 2 minutes, and incubated at 30 °C for 1 hour. The aqueous phase was carefully separated in a clean test tube and using a spectrophotometer (Sco. Tech, SP UV-19), the absorbance was read at 450 nm. The blank was made by replacing the cell-free supernatant with a sterile broth medium (LB) (Patil and Chopade, 2001).

2.3.4. Hemolytic activity, all isolates were tested on blood agar plates (HIMEDIA, Co., Germany) amended with 5% (v/v) blood and cultured for 48 h at 35±2°C. The presence of a clear area around the growth is indicative of hemolytic activity (Carrillo et al., 1996), the activity was recorded as α, β, and, then the net area was measured and recorded as cm.

2.3.5. Lipolytic activity, Luria Bertani (LB) agar medium (HIMEDIA, Co., Germany) was mixed with 1% of olive oil (commercial) and poured into Petri dishes, then spot inoculated with freshly cultured isolates (48 h) and incubated at 35±2°C for 5 days. Creating a hydrolysis area around the colonies was a positive result (Deepa et al., 2015).

2.3.6. CTAB agar plate assay, 1.0 L of mineral salts medium (MSM) (containing gL^{-1}: To make CTAB agar, 0.03 g KH2PO4, 0.03 MgSO4, and 0.3 $NaNO_3$) was combined with 0.2 g cetyltrimethylammonium bromide, 0.005 g methylene blue, and 15 g agar (Difco). Two wells (5 mm) were made in each agar plate containing 20 ml CTAB medium, and each well was filled with 50 µL of 24 h old bacterial culture (Siegmund and Wagner, 1991). For control plates, wells in CTAB agar were filled with distilled water instead of bacterial culture. All prepared plates were incubated at 35±2°C for 5-7 days, then examined for the appearance of dark blue halos surrounding the well, which confirmed the

existence of extracellular anionic surfactants and was considered a positive result (Shoeb et al., 2015).

2.4. Identification of the more potent isolates

The most influential biosurfactant producer was identified as the most active bacterial isolate by 16S rDNA sequencing. The DNA was extracted, replicated using PCR (Khedr et al., 2017), and sequenced using Sanger Sequencing Technology on an Applied Biosystems automated DNA sequences, model ABI 3730XL DNA Analyzer (Applied Biosystems, USA; service provided by Macrogen Inc., South Korea). Then the resulted sequence was aligned with other identified strains in the Gene Bank database using an online BLAST tool to determine the similarity score (http://www.blast.ncbi.nlm.nih.gov/Blast). The phylogenetic trees were constructed using the MEGA-X program and the neighbor-joining method.

2.5. Production and extraction of biosurfactants

250 mL Erlenmeyer flasks with 50 mL of LB medium (pH 7.0) were inoculated with 1.0 mL of the dominant strain (24 h old) and shaken at 352°C (150 rpm) for 3-5 days. The culture was centrifuged at 8000 rpm for 20 minutes under refrigeration at 4°C at the end of the incubation period for biosurfactant extraction. The bacterial cell-free supernatant was collected into a glass jar, adjusted to pH 2.0 with HCl (6 M), and refrigerated overnight. Next, the jar was moved to room temperature to add an equal volume of an aqueous mixture of chloroform and methanol (2:1) and directly centrifuged (8000 rpm/ 20 min), then transferred to an oven at 50°C for complete dryness. The white precipitate is considered a biosurfactant (Sivasubramani and Selvaraj, 2017).

2.6. Characterization of the produced biosurfactant

The biosurfactant was characterized at the Central Laboratories Network, National Research Centre, Cairo, Egypt (https://www.nrc.sci.eg/centrel-labs/).

2.6.1. High-performance liquid chromatography (HPLC)

A 5 µL of biosurfactant sample solution was injected into an HPLC machine (an Agilent 1260 series) at 40°C. The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A); 15-16 min (82% A) and 16-20 (82%A). The multi-wavelength detector was monitored at 280 nm.

2.6.2. Gas chromatography–mass spectrometry analysis (GC-MS)

Before GC analysis, the extracted biosurfactant sample was dried at 50°C in an oven to constant weight, then washed in 50 L of silylation reagent and 50 L pyridine for derivatization of sample functional groups to trimethylsilyl groups (abbreviated TMS). The silylation reagent was made by combining 99:1 bis (trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchloro-silane (TMCS) (Moldoveanu and David, 2018). A 2 µL of biosurfactant solution was fed into the GC-MS machine (Agilent Technology 7890B gas chromatograph with a split detector and a mass spectrometer detector (5977A). Hydrogen was employed as the carrier gas, with a constant flow rate of 2 mL/min at a splitless. The temperature protocol is 50°C for 5 minutes, rising at 5°C/min to 100°C and holding for 0 minutes, and rising at 10°C/min to 320°C and holding for 10 minutes. The injector and detector were held at 280°C-320°C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 25-700 and solvent delay 6 min. The mass temperature was 230°C and Quad 150°C. Different constituents were identified by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

2.6.3. Fourier-transform infrared spectroscopy (FT-IR)

The infrared spectra (with wavenumbers spanning from 4000 to 400 cm^{-1}) were recorded in a Shimadzu FT-IR-8400 spectrometer using 2 mg of the crude extracted biosurfactant combined with 200 mg KBr (Spectroscopic Grade). The collected data reflected an average of 50 scanning across the entire range (Anaukwu et al., 2020).

3. Result and discussion

3.1. Isolation of biosurfactant-producing bacteria

A total of 16 bacterial colonies were isolated from different oil-contaminated soils. All isolates were examined for their abilities to produce biosurfactants through the emulsification of toluene and the emulsification index (EI24%) was calculated. Emulsification Index (EI) is a measure used to evaluate the efficiency of the emulsification process,

along with the stability of the emulsion formed for 24 hours; in this case, it is known as (EI_{24}).

Based on the result of the EI_{24} test, 16 isolates were classified into four categories (20-29), (30-39), (40-49), and (50-60%). The last two highest categories have the same (lowest) number of isolates that belonged to the categories with EI24ranging from 40-49 and 50 to 60 %. According to (Phulpoto et al., 2020), the best bacterial isolate exhibited great emulsification activity after 24 hours (EI24) for kerosene oil of 55%. BSs-producing bacteria can be found in various environments, while areas touched by hydrophobic contaminants such as petroleum products yield more than uncontaminated areas (Soltanighias et al., 2019).

3.2. Screening of isolated biosurfactant-producing bacteria

To choose the efficient BS-producing isolates, multiple tests were applied for screening the recovered 16 isolates, such as emulsification index (EI24) %, emulsification activity (OD_{450}), and oil spreading ability (cm), and the hemolytic and lipolytic activities besides the CTAB agar plate assay. The results showed that the emulsification index (EI24) percent ranged between 20.0 and 57.7 percent, while the emulsification activity ranged between 1.297 and 3.231. It is worth noting that the isolate (SB164) gave the lowest emulsification index (EI24) value while giving the greatest emulsification activity value. This trend of results proved that the length of the formed column is not an indicator of strain efficiency and biosurfactant quality, but the density is the best evidence of its strength (**Table 2**). The emulsification index measures a biosurfactant's ability to improve contact between oil and water. When a biosurfactant's emulsification index is high, it indicates that the interaction between oil and water is additionally high (Sumiardi et al., 2018).

Table 2. Screening of the recovered bacterial isolates from various petroleum-contaminated soils.

Source of isolates	Isolate No.	Emulsification index (EI24) %	Emulsification activity (OD_{450})	Diameter of oil spreading (cm)	Lipolytic activity as clear zone (cm)	Hemolytic activity		CTAB agar plate
						Type	Clear zone (cm)	
Soil 1	SB 2	44.0	2.291	7.0	1.0	β	1.3	+++
	SB 5	28.6	2.480	7.5	0.7	β	0.7	+
	SB 7	36.7	2.773	7.2	0.8	β	0.7	+
	SB 11	46.4	1.297	2.5	0.0	β	0.2	-
	SB 14	57.7	1.730	6.5	0.0	β	0.8	+
Soil 2	SB 27	37.1	2.920	6.8	0.8	α	0.7	-
	SB 30	53.6	1.766	6.7	0.0	β	0.2	-
	SB 31	44.0	1.827	0.0	0.0	β	0.2	-
Soil 3	SB 39	31.8	1.691	5.0	0.0	γ	0.0	-
Soil 4	SB 52	21.7	2.949	6.5	0.0	β	0.2	+
Soil 5	SB 57	24.0	2.583	6.2	0.0	γ	0.0	-
Soil 6	SB 84	22.7	2.854	7.0	0.5	β	0.8	+
Soil 7	SB 99	25.0	1.836	7.0	0.7	γ	0.0	+
Soil 8	SB 143	55.0	2.822	7.0	0.7	γ	0.0	+
	SB 145	27.3	1.728	6.5	0.0	β	0.3	-
Soil 9	SB 164	20.0	3.231	6.5	0.8	β	0.9	+

Biosurfactant has polar and nonpolar parts when entering the water and oil system. The polar part will leave the water phase, and the nonpolar part will exit the oil phase. The polar component of the biosurfactant is dominant, and it will tend to produce an oil-in-water emulsion. Additionally, the non-polar component of the biosurfactant will seek to produce water in an oil emulsion. The hydrophilic-lipophilic balance (HLB) of biosurfactants produced by microorganisms is high, indicating that the polarity properties of the emulsion formed are also high. The HLB is the ratio of a molecule that is oil soluble (lipophilic) to water-soluble (hydrophilic) (Rosen and Kunjappu, 2012).

In the oil-spreading experiment, fifteen isolates (93.75 %) gave positive results, ranging from 2.5 to 7.5 cm. In this approach, Shoeb et al., (2015) demonstrated that only 44% of isolates produced positive findings in the oil-spreading assay. The oil spreading test findings are positive when a clear zone forms on the supernatant droplets in the oil layer. The clear zone is formed when the hydrophobic portion of the oil and the hydrophilic portion of the biosurfactant combine, resulting in pressure between the two portions. As a result of this state, the interface tension drops, the oil layer breaks apart, and a clear zone forms.

The lipolytic activity was estimated because lipase is an enzyme that functions at the oil-water interface and is involved in forming bio-emulsifiers and surfactants (Appaiah and Karanth, 1995). In this regard, 8 isolates showed good activity with varied values that ranged from 0.5 to 1.0 cm as a clear zone

(**Table 2**). Isolate SB2 was the best and showed lipolytic activity of 1.0. Moreover, the hemolytic test is used to screen bacteria for their ability to produce biosurfactants. It is used to examine bacteria for their potential to create biosurfactants. As a result, microorganisms that show positive hemolytic tests are considered biosurfactant producers.

According to our results in (**Table 2**), of 16 isolates, only one isolate, SB27, showed partial lysis, while four isolates were unable to induce hemolysis and were considered non-hemolytic bacteria. The other 11 isolates, on the other hand, were able to lysis the red cells in the medium surrounding and below the colonies. The technique to screen method is valid because biosurfactants would cause lysis of erythrocytes. Although the test expects the surface activity of biosurfactant-producing microorganisms, as reported by Sumiardi et al., (2018), multiple studies have shown that this is not a useful approach for detecting biosurfactants due to the effect of bioproducts on causing red blood cell lysis, so being the surface-active molecule is not enough (Youssef et al., 2004; Rashedi et al., 2005). Based on the previous studies, isolate SB2 demonstrated the most significant hemolytic activity compared to other isolates; hence, it was chosen for further testing and identification.

According to the CTAB assay (**Fig.1**), the isolate SB2 was more productive for producing anionic extracellular surfactants. In a previous study (Shoeb et al., 2015), 52.8 % of isolates tested positive for CTAB. In addition, anionic biosurfactants generate an insoluble ion couple with CTAB and methylene blue, creating a blue ring (Walter et al., 2010); this method was conducted by (Siegmund & Wagner, 1991).

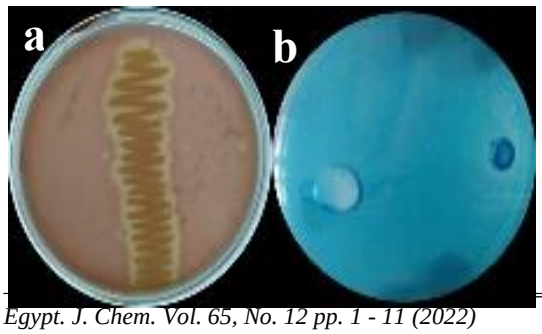

Fig. 1. Activities of the highest biosurfactants-producing isolate: a) hemolytic activity, b) CTAB agar plate.

3.3. Identification

The obtained result confirmed a high similarity of the 16S rDNA gene sequence for isolate number (SB2) with more than 99.89% homology of *Pseudomonas putida* strain ICMP 2758 partial 16S rDNA sequences (**Fig. 2**). Most microorganisms isolated from petroleum-contaminated areas have been proven capable of hydrocarbon degradation. The isolate SB2 was recognized as *Pseudomonas putida*; its entry number in the Gene Bank is ON763757. It was determined that the isolate SB2 might use petroleum oil as its sole carbon source. Because *Pseudomonas* is a well-known hydrocarbon degrader, it's easy to find them in petroleum-contaminated areas (Johnsen et al., 2005).

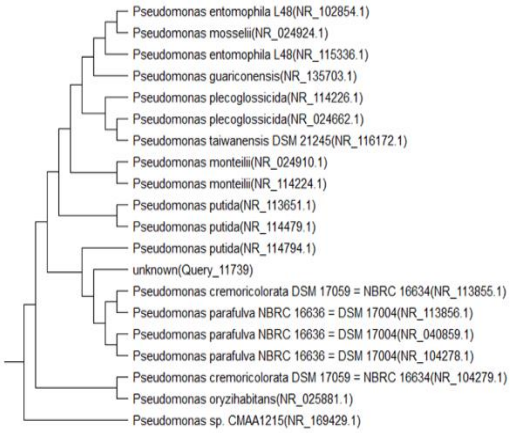

Fig. 2. Phylogenetic tree showing relationship of the isolate SB2 with other related bacterial species retrieved from Gen Bank based on their sequences homologies of 16S rRNA.

3.4. Production, extraction and recovery of biosurfactants

The results shown in **Fig. 3(a)** show that Pseudomonas putida (SB2) is the most prolific producer of biosurfactants. According to scientific research, *Pseudomonas* dominates bacterial PAH degradation in soil by synthesizing a variety of secondary metabolites such as biosurfactants (Johnsen et al., 2005). Also, *Pseudomonas* sp. is considered one of the best-known bacteria that gets

its carbon and energy from various aliphatic and aromatic compounds (Pacwa-Płociniczak et al., 2014). Because of the presence of various metabolic and physiological features in this genus, it has a wide range of metabolic and physiological properties due to the presence of a complex enzyme system. This genus has a wide range of metabolic and physiological features. For example, *Pseudomonas aeruginosa* UKMP-14T was discovered to degrade a significant percentage of total petroleum hydrocarbons and have biosurfactant activity (Ainon et al., 2013).

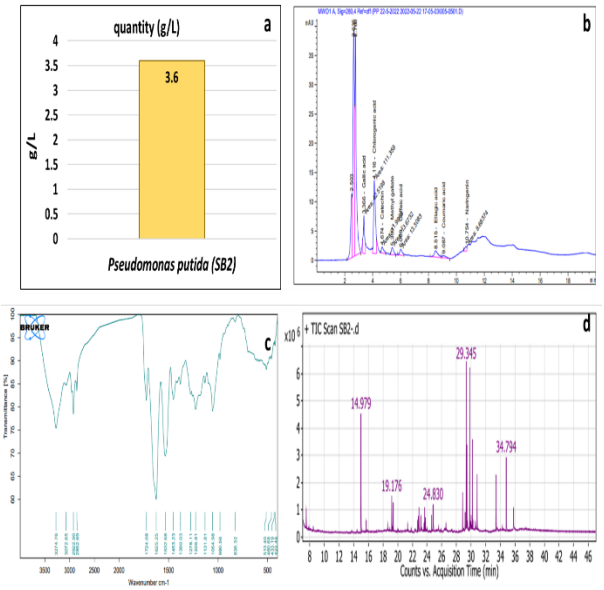

Fig. 3. a) Amounts (g) of biosurfactants produced by *Pseudomonas putida* SB2; Three methods were used to characterize the produced biosurfactant., **b)** HPLC, **c)** FT-IR and **d)** GC-mass.

3.5. Characterization of the produced biosurfactants

Three methods were used to characterize the produced biosurfactant *Pseudomonas putida* (SB2), HPLC, FT-IR, and GC-mass. Our investigation used HPLC to detect the purity check and validation of the biosurfactant molecule. In this regard, results illustrated in (**Fig. 3b**) showed that the produced biosurfactant by *Ps. putida* contained 8 components. Among them, chlorogenic acid was the main one at 1409.2 µg/g. Also, ellagic and gallic acids were observed at high amounts compared to other components (**Table 3**).

Table 3. HPLC analysis of main components identified in biosurfactants produced by *Ps. putida* (SB2)

Components	Area (%)	Conc. (µg/g)
Gallic acid	42.52	329.24
Chlorogenic acid	111.4	1409.2
Catechin	12.00	284.39
Methyl gallate	13.67	81.42
Coffeic acid	13.31	107.78
Ellagic acid	20.84	530.12
Coumaric acid	7.72	22.00
Naringenin	8.68	88.77

The FTIR spectra were recorded between 500 to 3500 $cm^{-1,}$ as shown in Fig. 3(c). Concerning the analysis of biosurfactants produced by *Ps. putida*, a typical absorption band appeared at 3274.76 cm^{-1} due to C-H bonds and O-H groups of carboxyl OH stretching referred to carbohydrate fragments and hydrogen vibration of amide N-H functions. Another distinct band in the range of 2922.90 cm^{-1} indicated the presence of -CH_2- and -CH_3- aliphatic stretching, which corresponds to symmetrical and asymmetrical -CH- vibrations in lipids. The existence of absorption bands connected to protein sections between 1625.25 and 1724.08 cm^{-1} is evidence of the -CO- bond, which demonstrates that crude biosurfactant contains polypeptides, owing to impurities caused by cell debris during the biosurfactant purification process. A single band near 1453.23 cm-1 was caused by vibration bending (CH). A peak of about 1380.03 cm-1 was caused by -COC- anti-symmetric stretching, which also corresponded to groups characteristic of carbonyl groups in unsaturated aliphatic carboxylic acids, and C-H bending of the CH_2 and CH_3 groups was detected.

An absorption band about 1054.98 cm^{-1} corresponds to -COC- group vibrations in carbohydrate cyclic structures and indicates the presence of bonds between carbon atoms and hydroxyl groups in rhamnose ring chemical structures. The glycolipid nature of the biosurfactant produced by *Ps. putida* was confirmed by comparing the FTIR spectra of this fraction with the previous results obtained by (Lotfabad et al., 2010; Janek et al., 2013; Deepika et al., 2015; Deepika et al., 2017). The biosurfactants produced by the *Ps.putida* PP021 isolate were extracted and characterized, and their potential to enhance oil recovery was established. It was found that the cell-free biosurfactant-containing supernatant decreased the air-water interface tension from 74 to 28 mNm−1. Using TLC and FTIR methods, the biosurfactants produced by the isolate

were classified as mono- and di-rhamnolipid mixtures (Biktasheva et al., 2022).

Regarding the GC-mass profile, the isolated biosurfactant from *Ps. putida* revealed different types of free and bounded compounds (**Fig. 3 d)**. The detected compounds ranged between C10 and C42 and included fatty acids, sugars, amino acids, and phenols. However, the predominant component has the molecular formula $C_{12}H_{32}O_3Si_3$ and the compound is Glycerol, also lactulose, octakis(trimethylsilyl) ether (isomer 1) and D- (+)-turanose, octakis(trimethylsilyl) ether found in high amounts compared to other components. Among the detected sugars, maltose, sucrose, lyxose, and galactose, as well as amino acids (Oxoproline) were found in low amounts Fig. 3(d) & Table (4). Overall, compared to the previous studies, the analysis of HPLC, FT-IR, and GC-mass profiles of the produced biosurfactants by *Ps. putida* confirmed their nature as glycolipid (rhamnolipid).

6. Conclusions

In our study, only one of the 16 bacterial isolates collected from petroleum-contaminated soils showed good biosurfactant activities as measured by the emulsification index E24, emulsification ability, oil spreading, lipolytic activity, hemolytic activity, and CTAB agar plate assays. Based on the molecular identification of their 16S rRNA gene, this isolate was recognized as *Pseudomonas putida*. HPLC, FT-IR, and GC-mass were used to characterize the biosurfactants produced by *Ps. putida*. In sum, the analysis of the generated

Table 4. GC-mass profile of main components identified in biosurfactants produced by *Ps. putida* (SB2)

Peak	RT	Name	Formula	Area (%)
1	7.52	2,3-Butanediol, 2TMS derivative	C10H26O2Si2	1.96
2	14.979	Glycerol, 3TMS derivative	C12H32O3Si3	14.21
3	15.693	Butanedioic acid, 2TMS derivative	C10H22O4Si2	1.24
4	19.176	L-5-Oxoproline, 2TMS derivative	C11H23NO3Si2	3.52
5	19.381	2,4-Di-tert-butylphenoxytrimethylsilane	C17H30OSi	2.17
6	22.894	L-(-)-Sorbofuranose, pentakis(trimethylsilyl) ether	C21H52O6Si5	2.67
7	23.16	Mannofuranoside, methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-, α.-D-	C19H46O6Si4	1.48
8	23.676	D-Galactose, 5TMS derivative	C21H52O6Si5	2.05
9	23.775	D-Lyxose, 4TMS derivative	C17H42O5Si4	1.16
10	24.83	Palmitic Acid, TMS derivative	C19H40O2Si	3.20
11	28.852	1-Monoferuloylglycerol, tris(trimethylsilyl) ether	C22H40O6Si3	2.61
12	29.193	2-Palmitoylglycerol, 2TMS derivative	C25H54O4Si2	1.93
13	29.345	D-(+)-Turanose, octakis(trimethylsilyl) ether	C36H86O11Si8	11.73
14	29.444	1-Monopalmitin, 2TMS derivative	C25H54O4Si2	6.07
15	29.815	Lactulose, octakis(trimethylsilyl) ether (isomer 1)	C36H86O11Si8	11.58
16	30.005	Maltose, 8TMS derivative, isomer 2	C36H86O11Si8	1.43
17	30.187	Sucrose, 8TMS derivative	C36H86O11Si8	7.01
18	30.582	2-Monostearin, 2TMS derivative	C27H58O4Si2	1.25
19	30.825	Glycerol monostearate, 2TMS derivative	C27H58O4Si2	4.83
20	33.412	10,12-Tricosadiynoic acid, TMS derivative	C26H46O2Si	5.12
21	34.794	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	C42H63O3P	6.62

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