

**ISOLATION AND CHARACTERIZATION OF
BIOSURFACTANTS FROM BACTERIAL STRAINS
FOUND IN MARINE OIL POLLUTED-WATER SAMPLES.**

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ABSTRACT

Petroleum hydrocarbons are the most common environmental pollutants and oil spills pose a great hazard to terrestrial and marine ecosystems. In the current study two oil-contaminated water samples from Gemsa Company and Adabiya Suez Port were collected. Some different physico-chemical properties of the contaminated water samples were characterized. The extraction and characterization of oil from studied samples were performed. Nine bacterial strains were isolated from the contaminated samples. The bacterial isolates were screened for the production of biosurfactants. Two promising bacterial strains (B3 and B4) were selected from bacterial isolates according to the highest oil displacement test, emulsification power and the lowest surface tension results. The biosurfactants were recovered from the cultural media of (B3 and B4) at 1.1 and 1.3 g/L respectively. These bacterial isolates were identified using molecular identification performed by 16S rRNA gene sequences.

The results indicated that the two isolated bacteria (B3 and B4) belong to *Bacillus flexus* and *Gelidibacter japonicus* respectively. The characterization of the produced biosurfactants were determined by FTIR analysis. The result of FTIR analysis, indicated that the biosurfactants obtained from *Bacillus flexus* and *Gelidibacter japonicus* are mainly a complex lipopeptides with a small fraction of glycolipids called polymeric biosurfactants.

Key words: Oil pollution, Biodegradation, Biosurfactants, Fourier transform infrared (FTIR) analysis.

INTRODUCTION

Crude oil, also called black gold, is the most important natural resource of the industrialized countries. It is a fact that oil-related companies cause potentially major hazards for the environment (Tian *et al.*, 2018). Oil spills are one of the main factors with long-term adverse effects on marine life. Oil spills may be caused by releasing the crude oil from tankers transportation, offshore Platforms, drilling rigs and wells. Additionally the oil spills resulted from refined petroleum products and their by-products, heavier fuels used by large ships such as Bunker fuel, or the spill of any oily refuse or waste oil (Mathew *et al.*, 2020).

The U.S. EPA has defined bioremediation agents as “microbiological cultures, enzyme additives, or nutrient additives that significantly increase the rate of biodegradation to mitigate the effects of the discharge” (Office of Technology Assessment, 1991). Bioremediation has several potential advantages over conventional technologies, such as being cost effective, less intrusive to the contaminated site, and more environmental friendly in terms of its end products.

Biosurfactants can be defined as the surface-active biomolecules produced by microorganisms with wide-range of applications. In recent years, due to their unique properties like specificity, low toxicity and relative ease of preparation, these surface-active biomolecules have attracted wide interest.

Due to their unique functional properties, biosurfactants were used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others (Nooman *et al.* 2017). They can be used as emulsifiers, as well as, demulsifies wetting agents, foaming agents, spreading agents, functional food ingredients and detergents. At present, the biosurfactants are produced by different microbial strains. Franzetti *et al.* (2006) reported that the ability of biosurfactants made them to play important role in oil recovery and bioremediation of heavy crude oil. They were used to increase the surface area of hydrophobic substrates. Biosurfactants also are used to increase the bioavailability of hydrophobic substrates through solubilization/desorption. They also regulate the attachment and removal of microorganisms from the surfaces. The water samples in this study were collected from Gemsa Company and Adabiya Suez port, these areas were to expose high petroleum oil contaminated. These hydrocarbons are carcinogens and mutagens, they cause harm to animals, plants, and humans.

In the present study, the extraction and characterization of crude oil from two contaminated sites were performed. The physico-chemical characterization of water samples was investigated. The total bacterial counts and bacterial degraders of crude oil from two contaminated sites were tested. The production of biosurfactants by different oil degrading bacterial isolates and some surface properties of the produced biosurfactants were

studied. The molecular identification of the bestpromising bacterial isolates for production of biosurfatants was also determined by 16S rRNA.

MATERIALS AND METHODS

Samples collection: The crude oil and water samples were collected from Egyptian Gulf Suez (Gemsa Company and Adabiya Suez port).

Physicochemical properties of water samples: pH value, electrical conductivity, Total Dissolved Solids (TDS), density and specific gravity and salinity value were studied according to standard tests methods (The American Society of Testing and Materials, ASTM).

Extraction of oil from water samples: The sample was acidified with HCl, and the pH was adjusted at 2. Then the contaminated water samples were treated with carbon tetrachloride to extract all oil from these samples. The oil content was calculated from the following equation (Khalil, 2007):

$$\text{Oil content} = ((A-B) * 1000 / \text{mL of water sample}) \text{ mg/mL}$$

Where A and B are the weights of the empty flask and containing the extracted oil respectively. The separation of crude fractions was carried out into maltene and asphaltene by reflux methods using (n- heptane). The separation of the fractionation of maltene fraction into its components, saturates, aromatics and resins were carried out using liquid column chromatography for the two samples (Gemsa, Adabiya Suez port) using aluminum oxide neutral (70-230 mesh) activated by heating to 300 °C for 24 h, n-heptane, benzene, methylene chloride, most of these solvents were

produced from (Honeywell specialty chemicals seeleze GmbH). The process of separation was carried out according to the method used by Zewen *et al.* (2006).

Gas chromatographic analysis (GC): The oil extracted from the studied water samples was monitored using Agilent 6890 plus, Gas chromatograph attached to computerized system with ChemStation software condition of operation according to the standard test method IP 318/75 Institute of Petroleum, 1995. The component separation was completed on HP-1 capillary column (100% methyl silicone siloxane, 30 m length, 0.35 mm internal diameter and 0.25 mm thickness film). For a typical chromatogram, a 0.5 μ L crude oil sample was introduced into a splitter injector which was previously heated at 350 C. The oven temperature was programmed from 100–320 °C at a fixed rate of 3 °C/min. The nitrogen (oxygen-free) was used as a carrier gas with a flow rate of 2 ml/min. A mixture of pure n-paraffins was used as standard. The peak area of each resolved component (consisting of either n- and iso- paraffins) was determined individually. While, the unresolved complex mixtures (humps) free from of non-paraffins presumably mainly cycloalkanes and aromatics with long side chains, were determined only as a total.

High Performance Liquid Chromatographic analysis (HPLC): The oil extracted from the studied water samples was analyzed using a (HPLC) instrument model Waters 600E, equipped with dual UV absorbance detector Waters 2487 and auto sampler Waters 717 plus attached to a computerized

system with Millennium 3.2 software. PAHs standards were obtained from Supelco. The conditions of separation (Chen et al., 2006) are as follows: Column: Supelcosil. LC-PAH, 5 µm particles, 15 cm length and 4.6 mm ID, Mobile phase: gradient acetonitrile: water 60–100 % acetonitrile (v/v) over 45 min. Flow rate: 0–2 min. 0.2 ml/min., 2–45 min. 1.0 ml/min. Detector set at 254 nm.

Enumeration of total bacterial counts from contaminated water samples:

The total bacterial isolates were determined from the two different contaminated water samples. The bacterial counts were implemented by using the plate count technique where the polluted sea water samples (1 mL) were serially diluted in a sterile saline. Then inoculation in Luria Broth (LB) plates medium containing g/L: NaCl 10.0; tryptone 10.0; yeast extract 5.0. The medium was adjusted to pH 7.2±2. The cultures were then incubated at 30 °C for 24 h. Thereafter, plate count in the range of 30 and 300 colonies was recorded. The experiments were conducted in three independent replicates. The bacterial count per mL was calculated from the following equation:

Bacterial count/mL = colony count per plate x dilution factor

The colonies with different morphological features were selected and purified on LB plates medium (Benson, 2001).

Oil degrading isolation of bacteria: Mineral Salts Medium (MSM) was used for the isolation of crude oil degrading bacteria. MSM medium (g/L) contains the following: MgSO₄·7H₂O, 0.5; KH₂PO₄, 0.5; KCl, 0.1; NH₄NO₃, 4.0; K₂HPO₄, 1.0; NaCl, 2.0; CaCl₂, 0.01 and FeSO₄·7H₂O, 0.01,

and contained trace elements solution (g/L): H₃BO₃, 0.26; CuSO₄·5H₂O, 0.5; MnSO₄·H₂O, 0.5; (NH₄)₆ (Mo₇O₂)₄·4H₂O, 0.06 and ZnSO₄·7H₂O, 0.7, were autoclaved separately. PH of the medium was adjusted to 7.0 ± 0.2 , using crude oil as a sole carbon source. 250 mL conical flasks containing with 100 mL MSM broth were inoculated. Then, sample flasks were incubated at an agitation of 150 rpm in a shaking incubator at 30 ± 2 °C, for 7 days. The media was taken as a control sample at constant culture conditions without bacterial strain (Larik et al., 2019). The bacterial colonies formed on the plates were selected for further study.

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Examination of biosurfactants production from oil degrading bacteria:

One loop of each isolate was inoculated to nutrient broth media and shaken 150 rpm at 30 °C for 18-24 h. Five mL of each bacterial culture were transferred to 100 mL of MSM supplemented with 2 % of glucose as a sole carbon source separately. The inoculated flasks were agitated in a shaking incubator for 30 °C at different time intervals (24, 48, 72 and 96 h). After different incubation period the culture supernatants were obtained by centrifugation of different inoculated flasks at 10.000 xg for 15 min. The screening of biosurfactant-producing bacterial isolates was carried out by a drop displacement test, surface tension, and emulsification assay (El-Sheshtawy and Doheim, 2014). The highest biosurfactant-producing bacterial isolates were selected for further identification.

Determination of some surface properties:

A drop oil displacement test: In this technique, 40 mL of distilled water were placed into a petri-dish with the diameter at 15 cm, then dropped 2–3mL of the crude oil. After the stable oil film was formed, then 500 µL of the bacterial culture supernatant was added to the center of the film center. After that, the diameter of the clear zones on the oil surface was measured. (Nayarisseri *et al.*, 2018).

Surface tension: The surface tension of the biosurfactant produced by different bacterial isolates was measured on a ring tensiometer (Krüss tensiometer K6) using the broth supernatant solution (20 mL) at 30 °C (Nitschke and Pastore, 2006).

Emulsification index: For emulsification assay, the selected bacterial isolates were culture in MSM broth for 72 h. The culture supernatants were collected by centrifugation at 10,000 xg for 15 min 4 mL of culture supernatant and 6 mL kerosene were introduced in a test tube. The prepared mixture was vortexed for 2 min at room temperature and left at 30 °C for 24 h and the emulsification index percentages were calculated according to the following equation (Cooper and Goldenberg, 1987): $(E_{24}) = 100(\text{height of the emulsion layer}/\text{the total height})$.

Molecular identification of the selected bacterial isolates: The selected biosurfactant-producing bacterial isolates were genomic identified using 16S rRNA in Sigma Scientific Services Co., Egypt. The bacterial cells were incubated for 72 h at 30 °C on MSM agar supplemented with 2 % glucose as a carbon source. For 16S rRNA gene amplification, DNA was extracted using a protocol of Gene Jet genomic DNA purification Kit (Thermo) (Sigma Scientific Services Co., Egypt). The 16S rRNA genes were amplified using a polymerase chain reaction (PCR) with (5'-AGA GTT TGA TCC TGG CTCAG-3') (5'-GGT TAC CTT ACG ACT T-3') as universal bacterial forward and reverse primers respectively. The PCR Purification Kit GeneJET™ applied for the cleanup of PCR to the production of pure PCR. A

45 μL of binding buffer was added to the completed PCR mixture. This mixture was fully transferred from step 1 to the Gene JET™ cleaning column. After that, the mixture was centrifuged for 30-60 s at $>12000 \text{ xg}$, then the flow was discarded. A 100 μL wash buffer was added to the Gene JET™ cleaning column, centrifuged for 30-60 s, neglected the flow, and place the purification column back into the collection tube. The blend was centrifuged at the empty Gene JET™ purification column for a supplementary 1 min. to eliminate any residual wash buffer. The purification column was transferred to a clean 1.5 mL micro-centrifuge tube. A 25 μL of elution buffer was then added to the center of the column membrane, which was then centrifuged for 1 min, discard the column and store the purified DNA at $-20 \text{ }^\circ\text{C}$. After the purification of the PCR products, the DNA sequence of the positive clone was subjected to a similar search, BLAST on the NCBI website (<http://www.ncbi.nlm.nih.gov>), and deposited into Gen Bank. Many appropriate 16S rRNA gene sequences with validly exported names were chosen as references from the Gen- Bank.

Extraction of the produced biosurfactant: The chosen bacterial strains were cultured in (MSM) medium for the production of biosurfactant at 72 h, followed by centrifugation at 10,000 xg for 15 min at $30 \text{ }^\circ\text{C}$. The culture supernatant was acidified to pH 2 with 6 N HCl and then kept at $4 \text{ }^\circ\text{C}$ overnight for precipitation. To produce the biosurfactant, the precipitate was centrifuged at 10,000 rpm for 15 min at $5 \text{ }^\circ\text{C}$. It's dissolved to a final pH of 7 in deionized water (pH 8.0). The biosurfactant was extracted using

chloroform: methanol (2:1) (Nogueira et al., 2020; Tripathi et al., 2020). The organic phase was transferred to a round-bottom flask connected to a rotary evaporator to remove the solvent, yielding a pure biosurfactant product (El-Sheshtawy and Doheim, 2014).

Characterization of extracted biosurfactants by FT-IR analysis: The chemical structures of the extracted biosurfactants were determined by Fourier transform infrared (FTIR) spectroscopic analysis. The samples were prepared by homogeneous dispersal of 1 mg of biosurfactant sample in pellets of potassium bromide and measured using a Nicolet IS-10 FTIR spectrometer. The spectra were obtained in the range of 400–4000 cm^{-1} with a resolution of 1 cm^{-1} (Ferreira et al., 2017).

RESULTS AND DISCUSSION

The production of biosurfactants had received great attraction in the last decades due to their widerange of applications. In this investigation, we report on the production and characterization of biosurfactants from bacterial strains isolated from oil polluted water samples collected from two sites namely, Gemsa and Adabiya Suez port.

Physico-chemical properties of water samples: The obtained results (Table 1) indicate that higher electrical conductivity of tested water Gemsa sample than Adabiya sample, also high total dissolved solids, density and salinity. The density ofwater Adabiya sample is less than Gemsa sample; although the concentration of dissolved solids in Gemsa is much higher than that in

Adabya, also from Table (2) the concentration of Asphaltene for Gemsa and Adabiya were observed to be 11.1 and 19.94 % respectively.

El-Sheshtawy *et al.* (2014) reported that the physic-chemical properties of water sample were studied according to standard tests methods (The American society of testing and Materials, ASTM).

Liquid column chromatography for fractionation of extracted crude oil

samples: Liquid column chromatography is a useful tool for the separation of petroleum components, namely saturates, aromatics, resins and asphaltenes. It is used as a marker for the characterization and identification of Gemsa and Adabiya crude oil samples through its hydrocarbon type's distribution. Table (2) shows that saturates content are 50.4 and 36.45 % for Gemsa and Adabiya crude oil respectively. On the other hand, the aromatic hydrocarbons content was estimated at 31.3 and 31.81 % of Gemsa and Adabiya crude oil respectively. In addition, resin components which considered as a polar fraction, 7.2 and 18.8 % of Gemsa and Adabiya crude oil respectively. Indeed, asphaltene was observed to be 11.1 and 19.94 % of Gemsa and Adabiya crude oil respectively.

Table (1): Physico-chemical characteristics of the collected water samples

Properties	Water Adabiya sample	Water Gemsa sample
Density @ 60 F	1.02594 g/mL	1.09840 g/mL
Specific gravity	1.02696	1.09949
pH @ 25 °C	7.066	6.364
Conductivity	51.8 ms/cm @17.5 °C	167.2 ms/cm @17.5 °C
Salinity	31820 mg/L	139850 mg/L
Total Dissolved Solids	32020 mg/L	139940 mg/L

Table (2): Hydrocarbons type analysis of crude oil of Gemsa and Adabiya samples

Type crude oil	Saturates %	Aromatics %	Resins %	Asphaltene %	Total %
Gemsa	50.4	31.3	7.2	11.1	100
Adabiya	36.45	31.81	18.8	12.94	100

Determination of chemical composition of the extracted crude oil samples

Gas chromatographic analysis: The gas chromatograms for two saturates fractions from crude oil samples that extracted from polluted sea water are shown in Tables (3 and 4). The shorter chain length of paraffins range from C11–C20, while, the middle chain length of paraffins range from C21–C30. On the hand the long chain length of paraffins range from C31–C40 of the two different crude oil sample. The contents of n-paraffins are 62.15 and 61.31 for Gemsa and Adabiya respectively, while the contents of iso- paraffins are 37.85 and 38.69 of Gemsa and Adabiya respectively. The percentage paraffins of total samples at 59 % and 35.81 % of Gemsa and Adabiya respectively. The presence of a prominent UCM is normally taken to indicate

the presence of petroleum hydrocarbons. The UCM percentages were 41 % and 54.19 % of Gemsa and Adabiya respectively. From these results, it was confirmed that the crude oil sample from Gemsa was mainly as a paraffinic oil, while the crude oil sample from Adabiya was fundamentally has high molecular weight hydrocarbons. Additionally from the result proven the petrogenic pollution type of these sites (Zaghden *et al.*, 2007).

Table (3): Carbon numbers distribution in gas chromatogram of Gemsa crude oil sample

Carbon No.	N-paraffins	Iso-paraffins
C11	0.67	0.3
C12	1.8	1.2
C13	3.08	2.46
C14	3.67	3.45
C15	4.26	3.64
C16	4.28	2.52
C17	3.87	4.73
C18	3.87	5.81
C19	3.87	2.5
C20	3.62	1.72
Total	32.99	25.05
C21	3.55	1.34
C22	3.03	1.41
C23	2.89	1.39
C24	2.7	1.31
C25	2.44	0.75
C26	2.04	0.58
C27	1.79	0.63
C28	1.91	1.03

Cont. table (3):

Carbon No.	N-paraffins	Iso-paraffins
C29	1.59	1.27
C30	1.44	0.48
Total	23.38	10.19
C31	1.2	0.66
C32	1.05	0.67
C33	0.86	0.55
C34	0.75	0.59
C35	0.71	0.14
C36	0.31	0
C37	0.34	0
C38	0.25	0
C39	0.17	0
C40	0.14	0
Total	5.78	2.61
Σ Total area	62.15	37.85

Table (4): Carbon numbers distribution in gas chromatogram of Adabiya crude oil sample

Carbon No.	N-paraffins	Iso-paraffins
C11	0.31	0
C12	1.2	0.55
C13	2.29	1.11
C14	3.26	2.8
C15	0.4	2.91
C16	4.15	2.53
C17	3.89	5.0
C18	3.9	6.2
C19	4.17	1.09
C20	3.68	1.74
Total	30.85	23.93

Cont. table (4):

Carbon No.	N-paraffins	Iso-paraffins
C21	3.64	1.05
C22	2.93	1.5
C23	2.6	1.24
C24	2.63	1.53
C25	2.26	0.66
C26	2.42	1.44
C27	2.28	1.44
C28	2.27	1.13
C29	1.73	1.61
C30	1.57	0.61
Total	24.33	12.21
C31	1.29	0.75
C32	1.13	0.48
C33	0.84	0.5
C34	0.82	0.66
C35	0.67	0.16
C36	0.34	0
C37	0.35	0
C38	0.33	0
C39	0.19	0
C40	0.17	0
Total	6.13	2.55
Σ total area	61.31	38.69

High Performance Liquid Chromatography (HPLC): There are many natural and anthropogenic sources of polyaromatic hydrocarbons (PAHs) in the environment. These hydrocarbon compounds have a potential risk to animals, plants and human health, as many of them are carcinogens and mutagens (Deziel *et al.*, 1996).

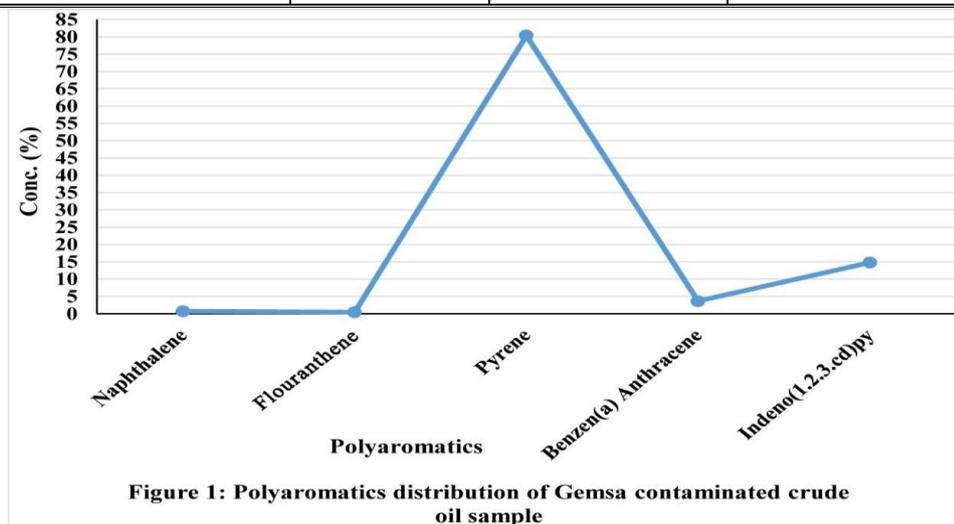
The percentages of different membered rings polyaromatics were determined for two aromatic fractions of two crude oil samples from the studied polluted areas shown (Tables 5 and 6) and (Figs. 1 and 2). The Gemsa crude oil sample has naphthalene as two aromatic rings, also, fluoranthene, pyrene and benzo (a) anthracene as four aromatic rings, besides, Indeno (1,2,3 cd) pyrene as six aromatic rings. Additionally, it has high percentage of pyrene polyaromatic which is considered highly carcinogenic polyaromatic hydrocarbons (Patel *et al.* 2020). On the other hand, the Adabiya crude oil sample has the highest percentage of Acenaphthene (three member ring polyaromatics) at 65 % from the total polyaromatics.

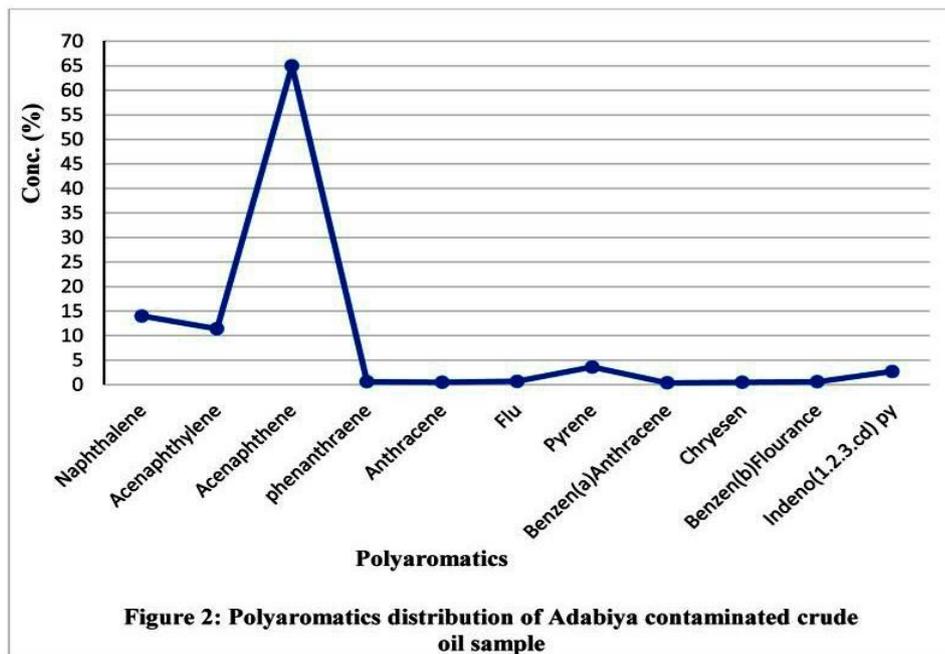
Table (5): Polyaromatics distribution of Gemsa contaminated crude oil sample

Component of polyaromatics	Aromatic Ring	Total of concentration (mg/L)	% Concentration of component
Naphthalene	2	0.04	0.73
Flouranthene	4	0.024	0.44
Pyrene	4	4.39	80.40
Benzen (a) Anthracene	4	0.2	3.66
Indeno (1.2.3.cd)pyrene	6	0.81	14.77
Total		5.46	100

Table (6): Polyaromatics distribution of Adabiya contaminated crude oil sample

Component of polyaromatics	Aromatic Ring	Total of conc. (mg/L)	% conc. of component
Naphthalene	2	6.71	14
Acenaphthylene	3	5.36	11.4
Acenaphthene	3	31.5	65
phenanthraene	3	0.02	0.6
Anthracene	3	0.27	0.5
Flu	4	0.34	0.7
Pyrene	4	1.75	3.6
Benzen(a)Anthracene	4	0.14	0.4
Chrysen	4	0.24	0.5
Benzen(b)Flourance	5	0.3	0.6
Indeno(1.2.3.cd) py	6	1.3	2.7
Total		47.93	100





Isolation of biosurfactant-producing bacteria: The bacterial population of oil-polluted water Gemsa sample showed count at 4.5×10^6 CFU/mL using (LB) medium. On the other hand, the total bacterial count of oil-polluted water Adabiya sample at 3×10^8 CFU/mL using (LB) medium. Additionally, after 7 days of incubation, the bacterial count on MSM was 6×10^{10} and 9×10^9 CFU/mL of oil-polluted water Adabiya and Gemsa samples, respectively. As mentioned before the results of physico-chemical parameters of the water samples indicated that higher electrical conductivity, total dissolved solids, density and salinity of Gemsa water sample than Adabiya sample, therefore,

the Gemsa water sample is less suitable for the bacterial growth than Adabiya sample.

Different bacterial isolates (B1-B9) were detected from oil-contaminated water samples (Adabiya and Gemsa water samples) by different culture media (Table 7). El-Sheshtawy *et al.* (2014) established that fifteen bacterial strains were isolated from Gemsa, Red sea, Egypt.

The screening of bacterial isolates for producing biosurfactants and selecting of potential biosurfactant producer were listed in Table 8. The bacterial isolates were screening the production of biosurfactants are The highest oil displacement test was observed at 45 and 60 mm with B3 and B4 bacterial strains respectively. Additionally, the highest percentage of the emulsification power was obtained at 40 and 60 % with the same bacterial strains. While, the lowest surface tension at 38 and 29 mN/m was obtained by the collected of culture supernatant from bacterial isolates B3 and B4 respectively after 72 h. Morikawa *et al.* (2000) stated that the oil displacement test is directly proportional to the biosurfactant compound in the solution. The measurement of surface activity of cultural supernatant showed a reduction in surface tension. There was a direct correlation found between drop collapse, oil displacement test and surface tension assays (Nayariseri *et al.*, 2018). The emulsification index is an indirect correlation between the oil displacement test and surface tension used to screen biosurfactant production. The emulsification index > 30 % indicates the high activity of microbial strain for production of biosurfactants (Nayariseri *et al.*, 2018). In the present

study, the bacterial isolates (B3 and B4) were selected for further study due to their high potential of biosurfactant production after 72 h.

Table (7): Morphological characteristics of the different bacterial isolates

Code of isolate	Morphological characteristic						
	Media	Color	Edge	Elevation	Shape	Transparency	Abundance
B1	MSM (Gemsa)	Yellow	Entire	Raised	Circular, large	Opaque	Abundant
B2	LB (Gemsa)	Creamy	Entire	Flat	Circular, medium	Opaque	Moderate
B3	MSM (Gemsa)	Buff	Branched	Raised	Irregular	Opaque	Moderate
B4	MSM (Adabiya)	Buff	Entire	Flat	Circular, medium	Transparent	Moderate
B5	LB (Adabiya)	Creamy	Entire	Flat	Irregular	Opaque	Moderate
B6	LB (Adabiya)	Creamy	Branched	Flat	Irregular	Opaque	Moderate
B7	MSM (Gemsa)	Creamy	Entire	Raised	Circular, medium	Transparent	Abundant
B8	MSM (Adabiya)	Mucous	Entire	Raised	Circular, large	Transparent	Moderate
B9	MSM (Adabiya)	Buff	Entire	Raised	Circular, medium	Opaque	Moderate

Table (8): Screening for biosurfactants production from different bacterial isolates after 72 h

Bacterial isolate	A drop oil displacement test (mm) at different time				Surface tension (mN/m) at different time				Emulsification index (E24)(%) at different time			
	24	48	72	96	24	48	72	96	24	48	72	96
B1	5	10	25	10	44	44	46	40	10	16	20	20
B2	10	10	35	30	51	50	50	51	10	25	23	20
B3	20	40	45	40	40	41	38	42	10	15	40	30
B4	30	40	60	50	43	32	29	30	20	43	60	50
B5	30	35	25	20	50	48	44	43	3	20	20	25
B6	20	25	31	25	49	45	45	47	10	25	30	25
B7	25	30	35	30	50	51	48	50	2	20	25	20
B8	40	42	40	40	47	46	44	45	5	10	10	15
B9	32	30	32	32	48	48	45	47	10	35	36	30

Identification of the selected bacterial isolates: The bacterial isolate (B3) is a Gram positive, rod-shaped motile bacterium. On the other hand, the bacterial isolate (B4) is a Gram negative, rod shaped, non-motile bacterium.

The identification of the selected bacteria was determined by 16S rRNA gene sequence analysis to be *Bacillus flexus* strain NBRC 15715 (B3) with similarity at 100 %. While, the bacterial isolate (B4) was identified to *Gelidibacter japonicus* strain Bio7-1 16S with similarity at 98.66 %. Jebeliet al. (2017) reported that the *Bacillus flexus* is a Gram-positive, aerobic, motile, rod-shaped and endospore-forming bacterium which belongs to the family of Bacillaceae. Doi and Osawa (2019) investigated the *Gelidibacter japonicus* and showed that it is a Gram negative, halophilic, oxidase-negative, catalase-

positive, aerobic, non-motile, but gliding, rod-shaped bacterium without flagella.

Extraction and characterization of the biosurfactant: In the present study, the recovery of biosurfactant from supernatant broth culture by selected bacterial strains was done by solvent extraction and precipitation method (Nogueira *et al.*, 2020; El-Sheshtawy and Doheim, 2014). The yield of biosurfactants at 1.1 and 1.3 g/L were obtained respectively from bacterial strains (B3 and B4). In the current study, some surface properties of the recovered biosurfactants from culture media were determined. The biosurfactants of B3 and B4 showed the emulsification index at 50 and 75 % and surface tension at 35 and 28 mN/m respectively. Microbial surfactants have the same advantages over their chemical analogue to their best physical and chemical characteristics, e.g. foaming, environmental compatibility, surface tension, emulsification activity and higher biodegradability. Also, they can be used in extreme temperatures, acidity and salt concentrations (Banat, 1995). Yadav *et al.* (2016) reported that the emulsification power of biosurfactant from *Bacillus flexus* was 88.65 %. The biosurfactants extracted from cultural *Bacillus flexus* (B3) and *Gelidibacter japonicas* (B4) media were characterized by FTIR analysis (Fig. 3, 4). In their spectrum a broad band at 3400 cm^{-1} was detected elucidating OH group. The absorption peaks at 1540.26 and 1542.83 cm^{-1} correspond to stretching mode of the C-N bond. The peaks at 1650.79 cm^{-1} indicated the presence of carbonyl functional group C=O revealing the formation of lipopeptide biosurfactant (Chittepu

2019). The Peaks recorded in the range of 1200–1085 cm^{-1} indicate the presence of bonds between carbon atoms and the hydroxyl groups found in the rhamnose units according to Hassen *et al.* (2018). Theregion between 720-557 cm^{-1} is attributed to aromatic C-H absorption of the bending vibration. From the results of spectrum, it is evident that the obtained biosurfactants are mainly a complex of lipopeptides with a small fraction of glycolipids suggesting a polymeric molecule (Nogueira *et al.*, 2020).

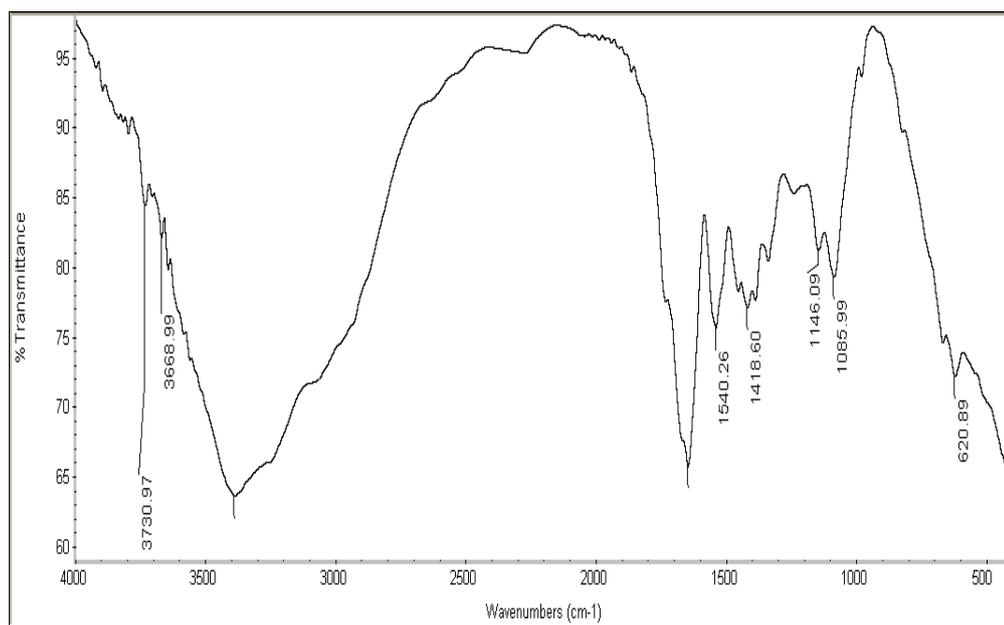


Fig. (3): FTIR spectrum of the produced biosurfactant by *Bacillus flexus*

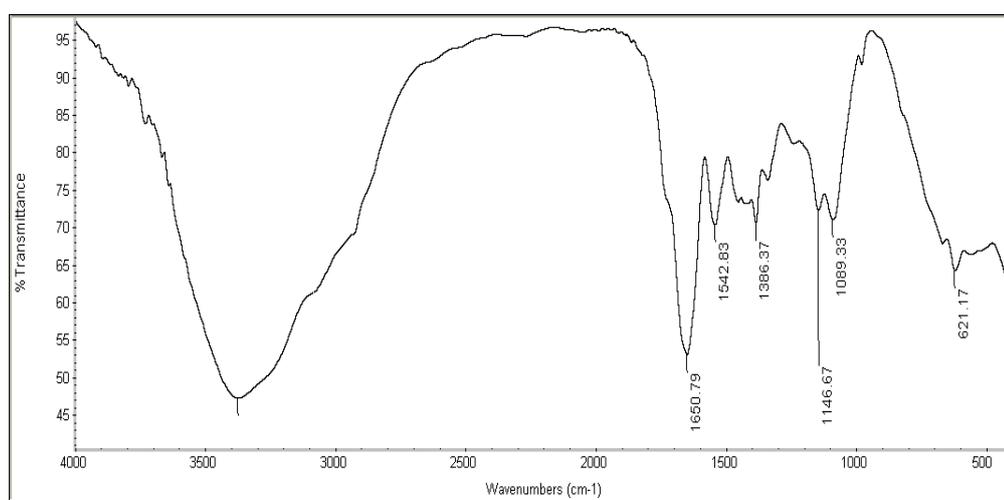


Fig. (4): FTIR spectrum of the produced biosurfactant by *Gelidibacter japonicus*

CONCLUSION

In the present study, the crude oil sample from Gemsa was mainly as a paraffinic oil, while the crude oil sample from Adabiya was fundamentally has high molecular weight hydrocarbons. Additionally the result proven the petrogenic pollution type of these sites. The bacterial isolates (B3 and B4) were selected for high potential of biosurfactant production after 72 h. The most potent bacterial isolates (B3 and B4) were identified by 16S rRNA gene sequence to *Bacillus flexus* strain NBRC 15715 and *Gelidibacter japonicus* strain Bio7-1 16S. The biosurfactants were recovered from cultural media of (B3 and B4) at 1.1 and 1.3 g/L respectively. The evaluation and characterization by FTIR analysis of biosurfactants were performed. The

Bacillus flexus and *Gelidibacter japonicus* proved to have their potency in the production of polymeric biosurfactants with excellent bioemulsifier properties.

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عزل وتوصيف المواد الخافضة للتوتر السطحي من السلالات البكتيرية الموجودة في عينات المياه الملوثة بالزيت البحري

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المستخلص

الهيدروكربونات البترولية هي أكثر الملوثات البيئية شيوعًا وانسكابات النفط تشكل خطرًا كبيرًا على النظم البيئية الأرضية والبحرية. وفي الدراسة الحالية تم جمع عينتين من المياه الملوثة بالزيت من شركة جمصة للبترول وميناء الأدبية بالسويس. تم توصيف بعض الخواص الفيزيائية والكيميائية المختلفة لعينات المياه الملوثة. كما تم استخلاص وتوصيف الزيت من العينات المدروسة وعزل تسع سلالات بكتيرية من العينات الملوثة وفحص العازلات البكتيرية لإنتاج المواد الحيوية ذات النشاط السطحي. تم اختيار سلالتين من السلالات البكتيرية الواعدة (B3 و B4) من العازلات البكتيرية وفقاً لأعلى اختبار إزاحة زيت وقوة استحلاب وأقل نتائج توتر السطحي وتم استخلاص المواد الخافضة للتوتر السطحي من الوسائط المغذية (B3 و B4) عند ١,١ و ١,٣ جم / لتر على التوالي وتم التعرف على هذه العازلات البكتيرية باستخدام التعريف الجزيئي الذي تم إجراؤه بواسطة تسلسل الجين 16S rRNA.

وأشارت النتائج إلى أن البكتريا المعزولة (B3 و B4) تنتمي إلى *Bacillus flexus* و *Gelidibacter japonicus* على التوالي وتم تحديد توصيف للمواد الحيوية ذات النشاط السطحي الناتجة عن طريق تحليل FTIR وأشارت النتائج الى ان هذه المواد هي أساساً بيتيدات شحمية معقدة مع جزء صغير من الجليكوليبيدات كمواد حيوية بوليميرية. الكلمات الدالة: تلوث الزيت، التحلل البيولوجي، المواد ذات النشاط الحيوي.