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Production of Biosurfactants by Crude Oil Biodegrading Bacterial Isolates and its Potential on Enhanced Crude Oil Recovery

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ABSTRACT:

Background The present work attempts to investigate production of biosurfactants, the four bacterial isolates were isolated from mud contaminated soil and formation water. The ability of these bacterial isolates to produce a biosurfactant were detected, Surface properties of the produced biosurfactant were confirmed by determining the emulsification power $E_{24}\%$, surface tension, and the mutants bacteria were tested for viscosity.

Methods The expected biosurfactants were tested for antibacterial effect, oil dispersion test, the crude biosurfactant has been extracted from supernatant culture growth, and the yield of crude biosurfactant was evaluated where the chemical structure of the produced biosurfactant was confirmed using FTIR analysis, and microbial enhanced oil recovery (MEOR) was calculated.

Results Eighteen bacterial isolates were isolated from formation water samples and mud-contaminated soil, four bacterial isolates were selected after screening for biosurfactants production and testing the optimum biosurfactants production conditions. it was found that the bacterial biosurfactant has the ability to recover more crude oil and the additional oil recovery was found to be (16.4, 13.7, 21.2 and 22.7) % for *Stenotrophomonas maltophilia* (isolate 6) , *Alcanivorax dieselolei* (isolate 8) , *Pseudomonas aeruginosa*(10) and *Staphylococcus aureus* (isolate 11) respectively, FTIR test were done to the crude biosurfactants, and the produced chemical groups such as COOH and OH, tell us that the produced biosurfactants are almost rhamnolipid and glycolipids.

Conclusion Results demonstrate the ability of bacteria to produce biosurfactants, that enable bacteria to enhance oil recovery, by reducing surface tension and inducing emulsification power.

INTRODUCTION

Biosurfactants, synthesized by microorganisms, are gaining significant attention as a sustainable and effective alternative to conventional chemical surfactants, which often pose environmental and health risks [1,2]. These amphipathic compounds contain both hydrophilic and hydrophobic moieties, allowing them to reduce surface and interfacial tension (IFT) at the interfaces of different phases, such as oil, water, and gas [3]. This unique characteristic gives them a wide range of applications, especially in industries seeking eco-friendly solutions.

The key advantages of biosurfactants over their synthetic counterparts include their biodegradability, low toxicity, and effectiveness under extreme conditions of temperature, pH, and salinity [4]. These properties make them highly versatile for use in the petroleum industry, bioremediation, and various other sectors like cosmetics, pharmaceuticals, and food production [5].

In the petroleum sector, biosurfactants play a crucial role in Enhanced Oil Recovery (EOR). A significant portion of crude oil remains trapped in reservoirs even after conventional recovery methods [6]. Biosurfactants, whether produced *in situ* by microbes or injected into the reservoir, reduce IFT, mobilizing the residual oil and improving recovery efficiency [7]. This technique, known as Microbial Enhanced Oil Recovery (MEOR), is often more cost-effective and environmentally benign than traditional chemical or thermal EOR methods [4].

Furthermore, biosurfactants are used for oil waste treatment, including the cleaning of oil storage tanks and the remediation of oily sludge. Their ability to emulsify hydrocarbons makes it easier to remove and biodegrade pollutants [8]. They are also effective de-emulsifiers, helping to separate water from oil in crude oil emulsions, a critical step before refining [9]. Beyond this, certain biosurfactants exhibit anti-corrosion properties, protecting industrial equipment, and can inhibit the growth of corrosive bacteria like sulfate-reducing bacteria (SRBs) [10].

Biosurfactants are a diverse group of compounds classified based on their chemical structure and molecular weight. Low-molecular-mass biosurfactants are highly effective at reducing surface tension. Glycolipids are composed of a carbohydrate head group and a fatty acid tail. Examples include rhamnolipids produced by *Pseudomonas aeruginosa* and sophorolipids synthesized by yeast species [11]. Lipopeptides consist of a short peptide chain linked to a fatty acid. Surfactin from *Bacillus subtilis* is a well-known example with potent surface-active and antimicrobial properties [12]. High-molecular-mass biosurfactants are polymeric biopolymers that primarily act as emulsifiers. Emulsan, a prominent example, is synthesized by *Acinetobacter calcoaceticus* and is effective at stabilizing oil-in-water emulsions [13].

The synthesis of biosurfactants by microorganisms is often linked to the presence of specific genes and enzymes that are activated in the presence of hydrophobic substrates like hydrocarbons [14]. While bacteria like *Pseudomonas* and *Bacillus* are well-known producers, many other genera, including *Candida*, *Rhodococcus*, and *Serratia*, also synthesize these valuable compounds [15]. Research efforts are focused on optimizing biosurfactant production by using inexpensive substrates, controlling fermentation conditions, and enhancing downstream processing to reduce costs [16]. The economic viability of biosurfactant applications is often improved by reducing the need for costly purification steps, particularly for large-scale industrial uses like MEOR [17].

Biosurfactants represent a significant advancement in 21st-century biotechnology. Their unique properties, including low toxicity and high biodegradability, make them a superior alternative to synthetic surfactants. From enhancing oil recovery to facilitating environmental bioremediation and improving industrial processes, biosurfactants demonstrate their potential to drive sustainable and innovative solutions across multiple sectors. Continued research into their genetic pathways and production optimization will further unlock their full potential.

This study aims to investigate the potential of microbial enhanced oil recovery (MEOR) as a sustainable and economically viable method for increasing the recovery of heavy crude oil. It also focusses on identifying and characterizing specific microbial strains with superior capabilities for interfacial tension reduction, emulsification, and hydrocarbon degradation. The ultimate goal is to develop an ecofriendly, cost-effective bioprocess that significantly improves oil mobilization and elevates the economic and environmental benefits of heavy oil extraction globally.

METHODS

Sample Collection

This study utilized natural materials collected from oil drilling sites. A three-liter sample of heavy crude oil was collected in November 2020 from the Qarun Petroleum Company's oil field, situated in the C region west of the Nile River in Beni Suef, Egypt. This sample served as the carbon source for the experiment and the subject of testing. In September 2020, three mud-contaminated soil samples were collected: two from Petroshahd Company and one from Khalda Company, both in Egypt's Western Desert. Additionally, three formation water samples were obtained in September 2020; two from a Red Sea oil well operated by Petrozanema Company (Abu Rudeis, South Sinai) and one from Agiba Petroleum Company's Meleiha field in the Western Desert. The soil and formation water samples were used as a source of potential biosurfactant-producing bacteria. All samples were collected and transported in sterile plastic bags and bottles and were subsequently stored under refrigerated conditions.

Enrichment and Isolation of Bacterial Isolates

Bacteria were enriched and isolated from formation water and mud-contaminated soil samples. Five mL of each sample were inoculated into 100-mL flasks of three liquid media: Luria-Bertani (LB) [18], Mineral Salt Medium (MSM) [19], and Bushnell-Haas Medium (BHM) [20], with each condition performed in duplicate. Heavy crude oil served as the sole carbon source for the MSM and BHM, while glucose was used for the LB medium. All flasks were incubated at 30°C for three days on a rotary shaker at 150 rpm. Following enrichment, isolates were obtained using the serial dilution plate method. One mL from each dilution was spread on LB agar plates and incubated for 24 hours at 30°C. Individual colonies were then isolated and purified through three successive restreaking cycles to ensure purity.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted and used as a template for polymerase chain reaction (PCR) to amplify the full-length 16S rRNA gene. A 1-μL aliquot of the extracted genomic DNA was used in the amplification. The approximately 1500-bp fragment, corresponding to positions 8 to 1509 of the *E. coli* 16S rRNA gene, was amplified using the universal primer pair 27F (5'-AGA GTT TGA TC[A/C] TGG CTC AG-3') and 1492R (5'-G[C/T]T ACC TTG TTA CGA CTT-3')[21].

The PCR was performed in a 50-μL reaction volume containing 5 μL of 10× Taq buffer, 1.25 mM MgCl₂, 200 μM dNTPs, 1.2 μM of each primer, 1 U Taq DNA polymerase, and approximately 5 ng of template DNA. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Amplified products were separated by electrophoresis on a 1% agarose gel alongside a 1-kb DNA ladder for size estimation.

Nucleotide sequence analysis was performed using the automated fluorescent dye-terminator sequencing method [22]. Sequencing was performed with a DYEnamic ET Terminator Cycle Sequencing Kit on an ABI Prism® 310 Genetic Analyzer. The resulting sequences were analyzed using the Genetyx-Win MFC application software.

For sequence identification, a BLAST search was performed against the National Center for Biotechnology Information (NCBI) database. Sequence alignments were conducted using Clustal W software [23]. Phylogenetic analysis was performed by constructing a phylogenetic tree with the neighbor-joining method [24] using MEGA6 software. The 16S rRNA gene sequences determined in this study have been deposited in GenBank with accession numbers ON980773.1 – ON980775.1 – ON972426.1 – ON920704.1.

Biosurfactant Production and Screening

Bacterial isolates were screened for biosurfactant production in Mineral Salt Medium (MSM) supplemented with 2% glucose as the sole carbon source. The glucose was sterilized separately before aseptic addition to the medium. Cultures were incubated with shaking at 30°C for 96 hours. Surface activity was assessed after 24 hours of incubation. The emulsification index (E₂₄%) was determined at 96 hours by mixing 4 mL of culture broth with 2 mL of kerosene. The mixture was vortexed for 2 minutes and allowed to stand undisturbed for 24 hours. E₂₄% was calculated using the ratio of the emulsified layer height to the total mixture height [25]. Biosurfactant activity was qualitatively assessed on Petri dishes containing distilled water overlaid with 10 mL of crude oil. A 10-μL drop of cell-free supernatant (CFS) was applied to the oil surface, and the diameter of the resultant clear zone was measured and compared to negative controls [26]. Biosurfactant production was

confirmed by hemolytic activity on blood agar plates prepared with 5% (v/v) sheep's blood. Approximately 150 μ L of CFS was loaded into 6.5-mm diameter wells. Plates were incubated at 30°C for 24–48 hours, and the presence and diameter of a clear lysis zone indicated biosurfactant presence [27]. The effectiveness of the crude biosurfactant was quantified by measuring the surface tension of 50-mL broth supernatants at room temperature using a ring tensiometer (Krüss-tensiometer K6) [28]. The critical micelle concentration (CMC) of a crude biosurfactant was determined using the surface tension method. Various concentrations of the biosurfactant, ranging from 50 to 1200 ppm were prepared. The CMC value was identified from a semilog plot of surface tension versus biosurfactant concentration, which shows the point at which micelles begin to form. All measurements were conducted in duplicate.

Biosurfactant production was scaled up to 1-liter volumes in optimized MSM. A starter culture (25 mL) was incubated overnight at 30°C and then transferred to the 1-liter flask, followed by incubation for an additional 48 hours with shaking at the determined optimal temperature. Cultures were stored at 4°C overnight prior to processing. The crude biosurfactant was isolated through acid precipitation and solvent extraction. The culture broth was centrifuged at 10,000 rpm for 20 minutes at 4°C to collect the CFS. The CFS was then acidified to a pH of 2 using 6N HCl and stored at 4°C overnight to promote biosurfactant precipitation. The resulting precipitate was separated by centrifugation at 8,000 rpm for 20 minutes. Biosurfactant was subsequently extracted from both the precipitate and the remaining supernatant using a chloroform–methanol (2:1 v/v) mixture. The combined organic phases were subjected to rotary evaporation under vacuum to remove the solvent and yield the crude biosurfactant [29].

Optimization of Cultural Conditions for Biosurfactant Production

A series of experiments were conducted to determine the optimal cultural conditions for maximizing biosurfactant production followed a one-variable-at-a-time (OVAT) approach. Cultures were grown in 100-mL volumes of Mineral Salt Medium (MSM), and the efficiency of biosurfactant production was monitored throughout the incubation period. The effects of initial pH (5, 7, and 9), temperature (22°C & 30°C), and agitation (static vs. 150 rpm) were evaluated. Different carbon sources, including sucrose, paraffin oil, and sunflower oil, were tested. The impact of three nitrogen sources—urea, potassium nitrate, and sodium nitrate—was also investigated. The optimal inoculum volume was determined by testing 1 mL, 5 mL, and 10 mL of a pre-cultured inoculum.

Antimicrobial Activity

The antimicrobial properties of a rhamnolipid compound were evaluated using the agar well diffusion method. The compound's *in vitro* antibacterial activity was tested against Gram-positive bacteria (*Staphylococcus aureus* and *S. hemolyticus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *E. coli*). Its antifungal activity was also screened against the fungus *Aspergillus niger* and the yeast *Saccharomyces cerevisiae*. Erythromycin and Metronidazole served as antibacterial and antifungal positive controls, respectively. All substances were dissolved in dimethylformamide (DMF). The efficacy was determined by measuring the diameter of the zone of inhibition, which indicates the area where microbial growth was suppressed.

Microbial Enhanced Oil Recovery (MEOR) Evaluation

A 100-mL acrylic sand-pack column was utilized to simulate an oil reservoir and evaluate microbial enhanced oil recovery (MEOR). Experiments were conducted at 40°C, a temperature reflective of the source oil reservoir. The experimental procedure was divided into three phases. First, the column's pore volume (PV) and porosity were determined by water saturation, followed by the injection of a hydrocarbon mixture to establish the original oil in place (OOIP). The column was then flooded with water to measure oil recovery after water flooding (Sorwf) and to determine the residual oil saturation (Sor).

In the second phase, the column was inoculated with 50 mL of a microbial solution from selected isolates, diluted to an optical density of 0.2 at 600 nm. This solution, prepared in a Mineral Salt Solution (MSS) containing a nitrate source, supported microbial growth under the oxygen-limited conditions of the column. Control columns received only the MSS. Both inoculated and control columns were sealed and incubated for 14 days at 40°C.

Finally, the columns were flooded with water a second time to measure the oil recovered after microbial flooding (Sormf). Any resulting emulsions were broken by centrifugation. The additional oil recovery (AOR) percentage was then calculated, with all experiments performed in triplicate.

Statistical Analysis

All experiments were performed in triplicate, with data presented as the mean $\pm$ standard deviation (SD). Statistical significance was set at a p-value < 0.05 . Data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 16 for Windows. The unpaired Student's t-test was used to compare the means of two groups, while a one-way ANOVA was utilized for comparisons involving more than two groups. To assess the normality of the data, the Shapiro-Wilk test was performed [30].

RESULTS

Characterization of Biosurfactant-Producing Bacterial Isolates

The emulsification power (E24%) and surface tension of the four selected bacterial isolates were evaluated at 24, 48, 72, and 96 hours, with the results presented in Fig.1. All data are expressed as the mean $\pm$ standard deviation. One-way ANOVA was used for statistical analysis, with a significance level of $P < 0.05$ and a high significance level of $P < 0.001$.

The results indicate a variation in emulsification power and surface tension across the isolates and over time. Isolate No. 9 demonstrated the highest emulsification power at 96 hours with a value of $50.0 \pm 0.01\%$, followed by Isolate No. 6 at $53.1 \pm 1.70\%$ at 72 hours. In contrast, Isolate No. 2 showed the lowest emulsification power at 96 hours, with a value of $59 \pm 2.6\%$.

Regarding surface tension, a decrease was observed in all isolates over the incubation period. Isolate No. 8 exhibited the lowest surface tension at 72 hours with a value of 52 ± 2.6 mN/m, while Isolate No. 6 recorded the lowest surface tension at 96 hours with a value of 54 ± 1.0 mN/m.

The statistical analysis showed significant differences ($P < 0.05$) among the values, as denoted by the different letters in Fig. 1.

The results suggest that both isolates 6 and 9 could be considered for further investigation as potential biosurfactant producers due to their high emulsification power and ability to significantly reduce surface tension.

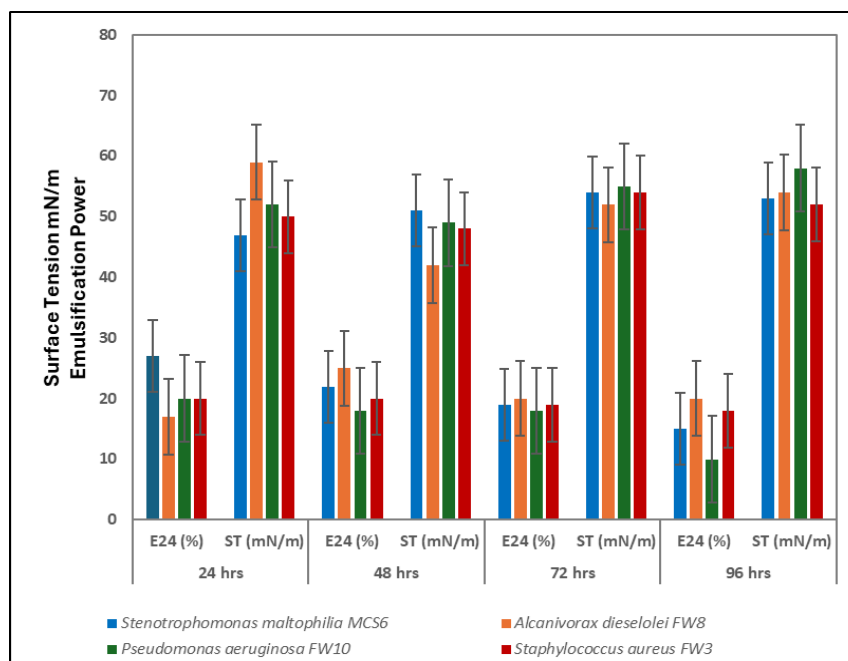


Fig. 1: Emulsification Power E24% and surface tension

Optimal Biosurfactant Production Parameters for Isolates

Optimization experiments were carried out at 30 °C under shaking conditions 150 rpm for all isolates, The optimal parameters for biosurfactant production for four different isolates (No. 6, 8, 10, and 11) were determined, as shown in Table 1. For all isolates, the optimal carbon and nitrogen sources were identified as sucrose and KNO₃, respectively, inoculum size

of 10% were also found to be optimal for all four isolates. The optimal pH and incubation time, however, varied among the isolates. Isolate No. 6 was found to produce the best results at a pH of 7, while isolate No. 8, isolate No. 10 and isolate No.11 performed best results at a pH 9.

Effect of pH

The data at Fig. (2) showed that the bacterial isolate (6) obtained the best results at pH 7. Where the optimal highest emulsification power E₂₄% was 44% after 48 h and the optimal lowest surface tension (mN/m) was 38 after 24 hr, and 39 after 48 hr.

While the isolate (8) *Alcanivorax dieselolei*, the best results were obtained at pH 9, where the optimal highest emulsification power E₂₄% was 40% after 72 h and the optimal lowest surface tension (mN/m) was 38 after 48 hr, and 39 after 72 hr. Otherwise, For isolate (10) *Pseudomonas aeruginosa*, the best results were obtained at pH 9 where the optimal highest emulsification power E₂₄% was 45 % after 48 h and the optimal lowest surface tension was 35 mN/m after 48 hrs, and 39 mN/m after 24 hr.

On the other hand, For isolate (11) (*Staphylococcus aureus*), the best results were obtained at pH 9 where the optimal highest emulsification power E₂₄% was 44 % after 72 hr and the optimal lowest surface tension (mN/m) was 33 after 72 hr, and 34 after 48 hr.

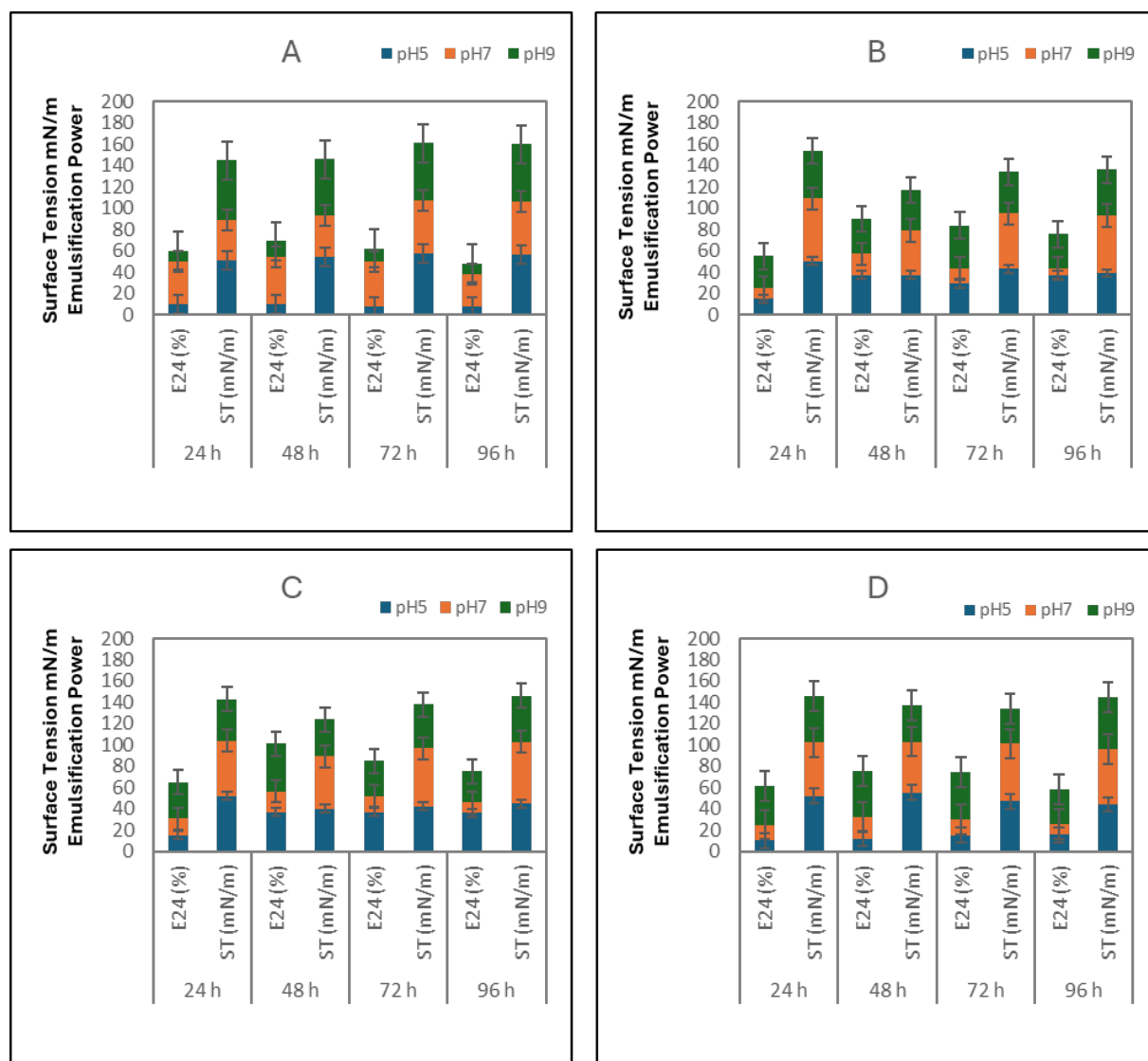


Fig.2: Effect of different PH levels for Isolate No. 6 (A), 8 (B), 10 (C) and 11 (D)

Effect of nitrogen source

The results illustrated that the bacterial isolate (6) (*Stenotrophomonas maltophilia*), was obtained the best results with NaNO_3 as nitrogen source where the highest emulsion was 44% after 24 hr and 45% after 48 hr, and the lowest surface tension was 36 mN/m after 24 hr and 38 mN/m after 48 hr Fig.(3).

On the other hand, the best results were obtained with KNO_3 as nitrogen source, where the highest emulsion was 34% after 48 hr and 41% after 72 hr, and the lowest surface tension (mN/m) was 36 after 24 hr and 38 after 48 hr in the microcosm contain isolate (8) *Alcanivorax dieselolei*.

For bacterial isolate (10) *Pseudomonas aeruginosa*, the best results were obtained with NaNO_3 as nitrogen source where the highest emulsion was 47 % after 48 hr and 38% after 24 hr, and the lowest surface tension (mN/m) was 33 after 48 hr and 39 after 24 hr. Additionally, the best results were obtained with NaNO_3 as nitrogen source where the highest emulsion % was 46 % after 72 hr and 42 % after 96 hr, and the lowest surface tension (mN/m) was 30 after 72 hr and 36 after 96 hr with bacterial isolate (11) (*Staphylococcus aureus*).

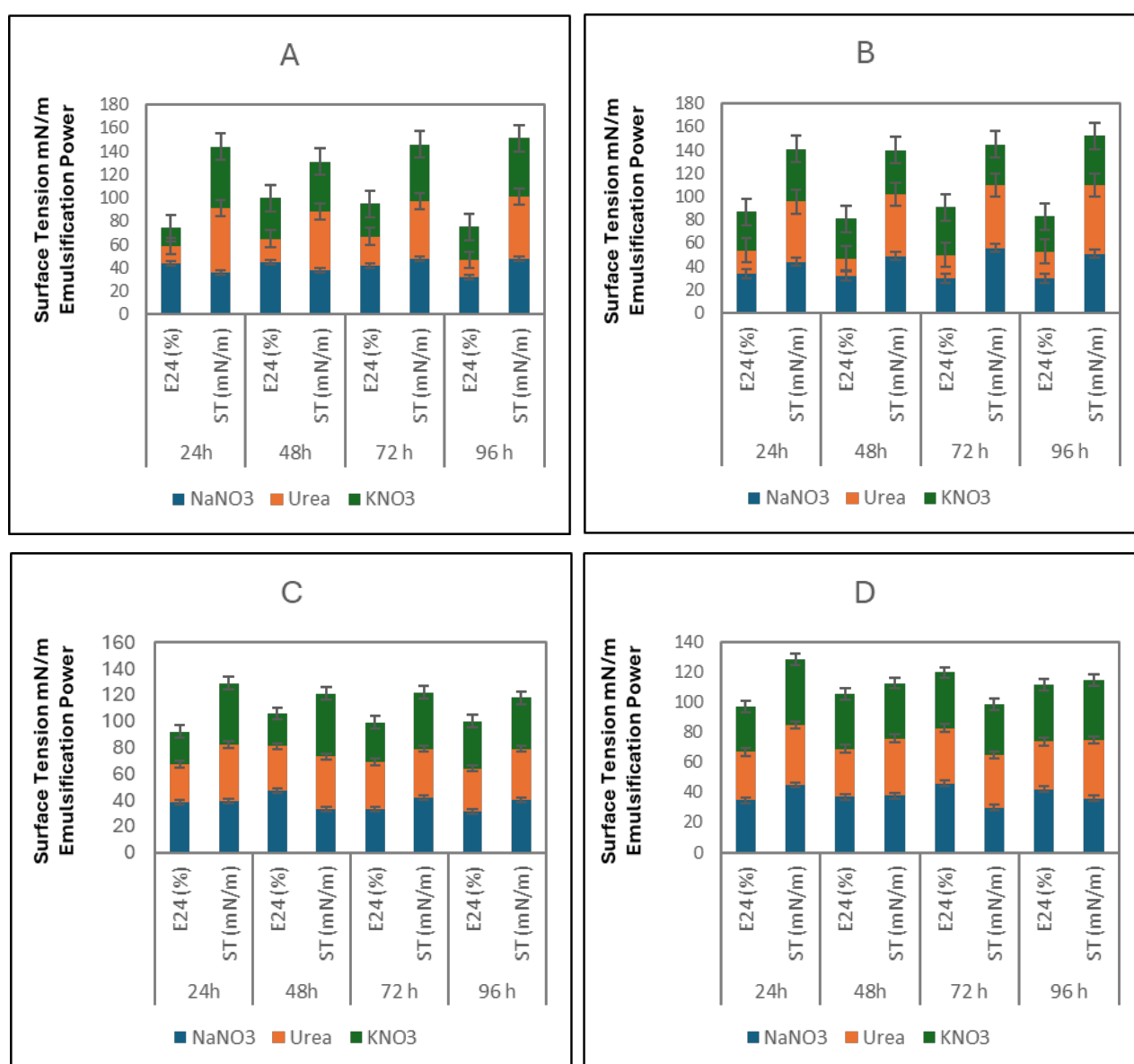


Fig. 3: Effect of different nitrogen sources for the production of biosurfactants For isolate No 6 (A), 8 (B), 10 (C) and 11 (D)

Effect of carbon source

The results investigated that for isolate 6 (*Stenotrophomonas maltophilia* (6), , the best results were obtained with sucrose as carbon source where the highest emulsion % was 43 % after 72 hr and 41% after 48 hr, and the lowest surface tension (mN/m) was 36 after 48 hr and 38 after 72 hrs. For isolate 8 *Alcanivorax dieselolei*, the best results were obtained with sucrose as carbon source where the highest emulsion % was 42 % after 72 hrs and 36% after 48 hr, and the lowest surface tension (mN/m) was 34 after 72 hr and 37 after 48 hr. Also, the best results were obtained with sucrose as carbon source where the highest emulsion percent was 45 % after 48 hr and 37% after 24 hr, and the lowest surface tension was 37 mN/m after 48 hr and 38mN/m after 24 hr for isolate (10) *Pseudomonas aeruginosa* Fig.(4).

Additionally, for bacterial isolate (11) *Staphylococcus aureus*, the best results were obtained with sucrose as carbon source where the highest emulsion percent was 45 % after 72 hr and 40% after 48 hr, and the lowest surface tension (mN/m) was 40 after 48 hr and 45 after 72 hr.

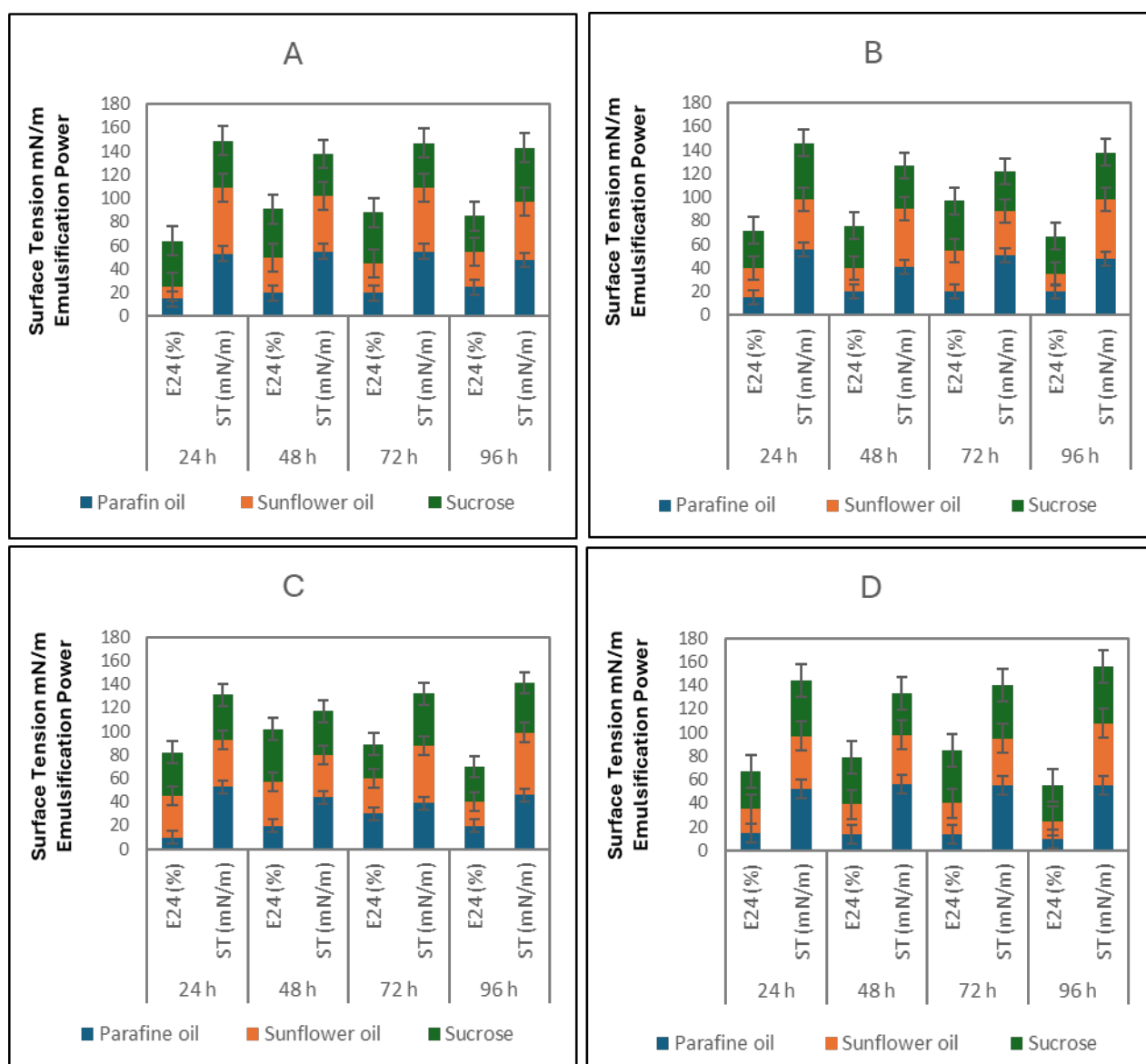


Fig.4: Effect of different carbon sources on the production of biosurfactants For Isolate No 6 (A), 8 (B), 10 (C) and 11 (D).

Effect of inoculum size

The results demonstrated that the microcosm contains bacterial isolate (6) *Stenotrophomonas maltophilia*, the best results were obtained with inoculum size 10 ml (1.25×10^8 CFU/ml) where the highest percentage of the emulsification power was 45 % after 24 hr and also 45% after 48 hr. Also, the lowest surface tension (mN/m) was 35 after 24 h and 36 after 48 hr. on the other hand, for isolate (8) *Alcanivorax dieselolei*, the highest emulsion % was 48 % after 72 h and 37% after 48 hr, and the lowest surface tension was 37 mN/m after 72 hr and 38 mN/m after 48 hr were obtained with inoculum size 10 ml (2.0×10^8 CFU/ml). While, for bacterial isolate (10) *Pseudomonas aeruginosa*, the best results were obtained with inoculum size 10 ml (1.39×10^8 CFU/ml) with the highest percentage of emulsion was 47 % after 48 hrs and 34% after 24 hrs, and the lowest surface tension (mN/m) was 36 after 48 hrs and 39 after 24 hrs. also, for isolate (11) *Staphylococcus aureus*, the best results were obtained with inoculum size 10 ml (1.58×10^8 CFU/ml) where the highest emulsion % was 48 % after 72 hr and 38% after 48 hr, and the lowest surface tension was 34 mN/m after 48 hr and 31 mN/m after 72 hr Fig (5).

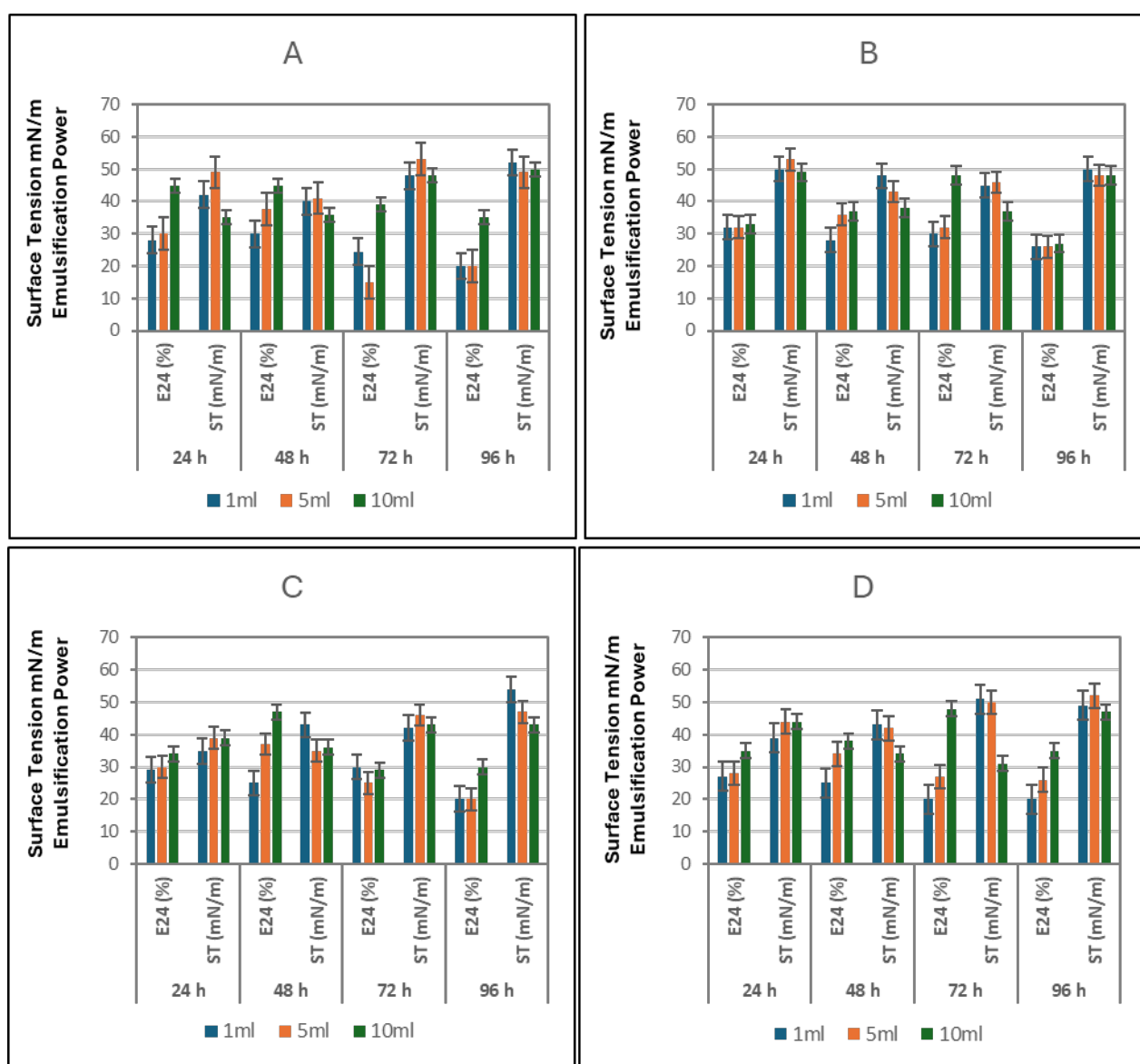


Fig. 5: Effect of different inoculum size for the production of biosurfactants using different bacterial isolates For Isolate No 6 (A), 8 (B), 10 (C) and 11 (D)

Table (1): Optimal parameters for biosurfactants productions

Parameter	6	8	10	11
PH	7	9	9	9
C source	Sucrose	Sucrose	Sucrose	Sucrose
N source	NaNO ₃	KNO ₃	NaNO ₃	NaNO ₃
Inoculum size	10 %	10 %	10 %	10 %

Optimal Biosurfactant Production by Bacterial Isolates

The results of the biosurfactant production evaluation are summarized in Table 1, which details the emulsification index (E24%) and surface tension (ST) for four different bacterial isolates over a 72-hour period.

All four isolates demonstrated the ability to produce biosurfactants, with significant reductions in surface tension and notable emulsification activity observed over time. The data for both E24% and ST were statistically significant ($P < 0.01$), indicating a strong effect from the isolates.

At 24 hours, the E24% for all isolates was relatively low, but this increased consistently. By 72 hours, isolate 11 reached the highest emulsification index, achieving a value of $45 \pm 38\%$.

Regarding surface tension, all isolates effectively reduced the value over the incubation period. The lowest surface tension values were achieved at 72 hours by two isolates: Isolate 6 at 38 ± 0.001 mN/m and Isolate 11 at 38 ± 0.002 mN/m. These results highlight the high efficacy of these particular isolates in producing a potent biosurfactant.

Bacterial Isolate Identification and Phylogenetic Analysis

The identity of the selected bacterial isolates was confirmed through both molecular and phenotypic analysis. Genomic DNA was extracted and the 16S rRNA gene was amplified using universal primers. The resulting PCR products were then sequenced to determine their evolutionary history via the Maximum Likelihood method and the Tamura-Nei model. This phylogenetic analysis involved 29 nucleotide sequences, with a final dataset of 1542 positions, and was conducted using the MEGA X software.

The findings are visually represented in a phylogenetic tree Fig. (6) with a log likelihood of -7474.71. A separate evolutionary analysis, conducted in MEGA11, used 21 nucleotide sequences and a final dataset of 1491 positions, resulting in a tree with a log likelihood of -13616.31.

In addition to the molecular approach, phenotypic identification was performed using a Vitek instrument. Based on these combined methods, the four isolates were definitively identified as follows:

Isolate 6: Sourced from mud-contaminated soil, this isolate was identified as *Stenotrophomonas maltophilia*, a multidrug-resistant, Gram-negative bacillus.

Isolate 8: Isolated from formation water, this strain was identified as *Alcanivorax dieselolei*, a motile, Gram-negative, rod-shaped bacterium known for its ability to degrade alkanes.

Isolate 10: Also from formation water, this isolate was confirmed to be *Pseudomonas aeruginosa*, a motile, Gram-negative rod that can grow at both 25–27 °C and 42 °C.

Isolate 11: Also from formation water, this isolate was identified as *Staphylococcus aureus*, a well-known human pathogen.

Table (2): Blast search results of the study isolates with their best matched species from the GenBank database including their sequences accession numbers and similarities.

Sample Name	Best match (BLAST search)	GenBank Accession	e-value	Identity (%)
FW10	<i>Pseudomonas aeruginosa</i> strain AR 120	KX650652.1	0.0	99.88
MCS6	<i>Stenotrophomonas maltophilia</i> strain gc-N49	KU726007.1	0.0	99.80
FW8	<i>Alloalcanivorax dieselolei</i> strain NMRL6	MW709915.1	0.0	99.70
FW3	<i>Staphylococcus aureus</i> strain NR_118997.2	MW453042.1	0.0	100

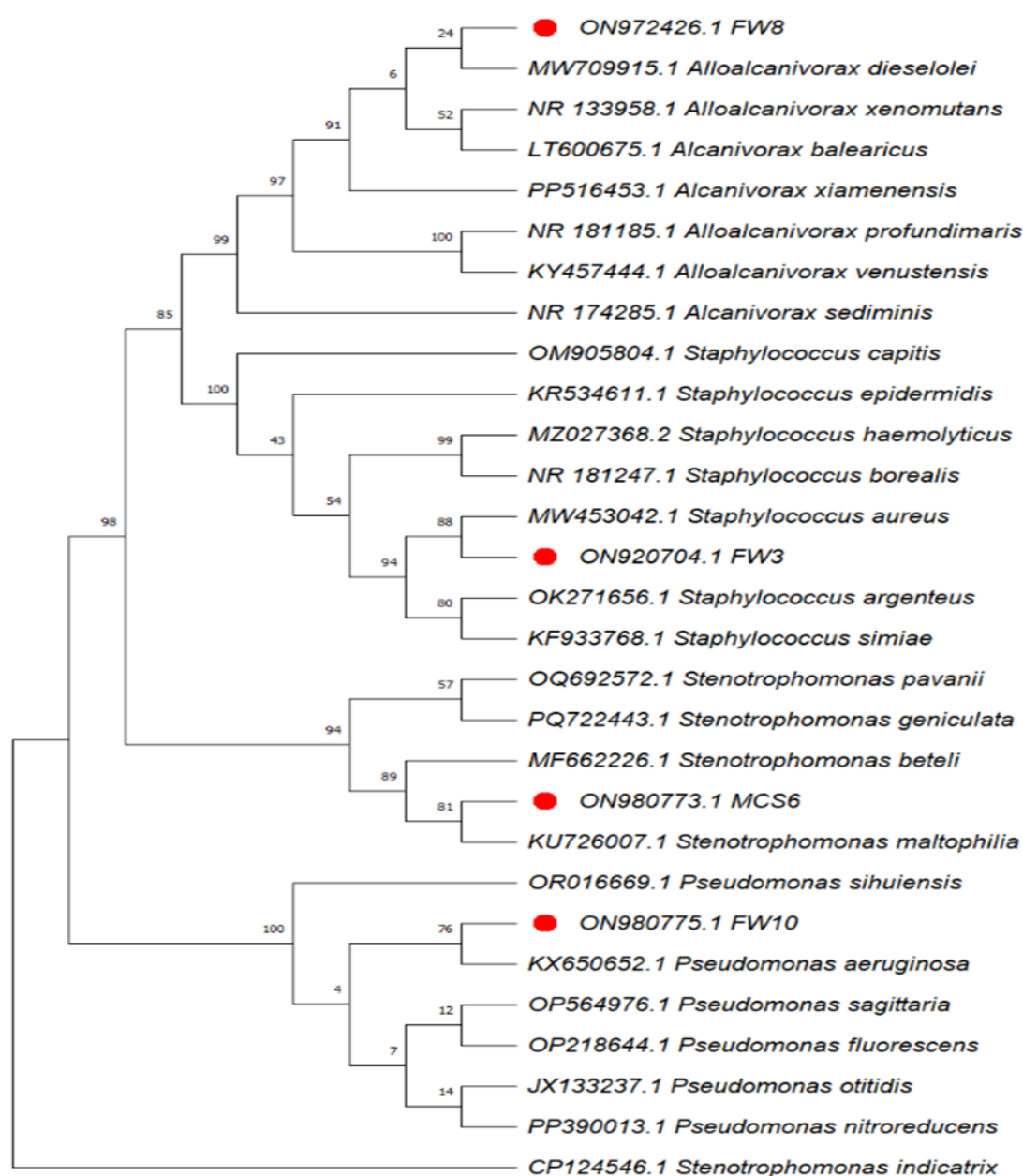


Fig 6: Phylogenetic tree of the four identified bacterial isolates

Table (3): Antimicrobial Activities of different biosurfactants concentration against gram +ve & gram – ve bacteria

Isolate no.				6	8	10	11
Gram - ve	<i>Pseudomonas aeruginosa</i>	Clear zone/ Bios. Concentration ppm	600	15±1 ^{Na}	14±1. ^{Na}	13±1 ^{Na}	15±1.7 ^{Na}
			500	6±1 ^{Na}	5±1.73 ^{Na}	5±0.001 ^{Na}	5±1 ^{Na}
			300	0	0	0	0
			100	0	0	0	0
			50	0	0	0	0
	<i>E.Coli</i>	Clear zone/ Bios. Concentration ppm	600	19±1 ^{a**}	14±0.001. ^{c**}	9±1 ^{c**}	15±1 ^{b**}
			500	15±1 ^{b**}	8±1.73 ^{b**}	0 ^{c**}	9±1.7 ^{b**}
			300	0	0	0	0
			100	0	0	0	0
			50	0	0	0	0
Gram +ve	<i>Staphylococcus aureus</i>	Clear zone/ Bios. Concentration ppm	600	20±1 ^{b*}	14±1. ^{c**}	13±1 ^{c**}	22±0.001 ^{a**}
			500	14±1 ^{a**}	0	0 ^{d**}	15±1.7 ^{c**}
			300	0	0	0	0
			100	0	0	0	0
			50	0	0	0	0
	<i>Staphylococcus hemolyticus</i>	Clear zone/ Bios. Concentration ppm	600	14±1 ^{a**}	15±1.73 ^{a**}	6±0.001 ^{b**}	15±1 ^{a**}
			500	11±1 ^{a**}	7±10.001 ^{c**}	0 ^{d**}	9±1.7 ^{c**}
			300	0	0	0	0
			100	0	0	0	0
			50	0	0	0	0

Determination of Surface Tension and Critical Micelle Concentration (CMC) of Crude Biosurfactants

The effect of various biosurfactant concentrations on surface tension was investigated for different bacterial strains. The control group, with no biosurfactant, maintained a surface tension of approximately 73 mN/m across all tested concentrations, which served as the baseline Fig. (7).

The results for bacterial strain 6 showed a significant reduction in surface tension, with the lowest value of 39 mN/m observed at a biosurfactant concentration of 1200 ppm. This strain's CMC, however, was determined to be 500 ppm, at which point the surface tension was 44 mN/m. For strain 8, the lowest surface tension recorded was 40 mN/m at 1200 ppm, with its CMC being 500 ppm at a surface tension of 41 mN/m.

Strain 10 exhibited its lowest surface tension of 37 mN/m at 1200 ppm. Its CMC was found to be 400 ppm, where the surface tension was 42 mN/m. Finally, strain 11 achieved its lowest surface tension of 38 mN/m at 1200 ppm, and its CMC was identified at 900 ppm, with a corresponding surface tension of 38 mN/m.

Overall, all tested bacterial strains effectively reduced the surface tension of the medium compared to the control. The data is expressed as mean ± SD (n=6). A one-way ANOVA was conducted for statistical analysis, with a p-value of <0.001 indicating a highly significant difference, a p-value of <0.05 showing a significant difference, and a p-value of >0.05 considered non-significant. Different letters indicate a significant difference at p<0.05 by Duncan test.

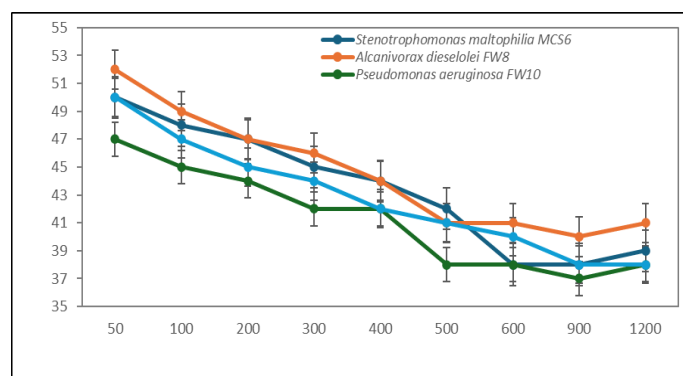


Fig. 7: Determination of critical micelle concentration (CMC) of different crude biosurfactants

Molecular Characterization of Bacterial Biosurfactants

Analysis of the molecular composition of the freeze-dried biosurfactants from isolates 6, 8, 10, and 11 was performed using Fourier-transform infrared (FTIR) spectroscopy. The FTIR spectrum for isolate 6 Fig.(8A) revealed distinct stretching peaks. The peaks at 2495 and 3383.243 cm^{-1} correspond to the presence of a carboxylic acid ($-\text{COOH}$) group. Additionally, an absorption peak at 1720 cm^{-1} suggests a carbonyl (C=O) or ester (C-O) group, while a peak at 1287 cm^{-1} indicates a carbon-nitrogen double bond (C=N). For isolate 8 Fig.(8B), the FTIR spectrum showed an absorption peak at 3420 cm^{-1} , which is characteristic of hydroxyl ($-\text{OH}$) or amine ($-\text{NH}$) groups. A prominent peak at 1642 cm^{-1} points to the presence of a carbonyl (C=O) group. The FTIR analysis of the crude biosurfactant from strain 10 Fig.(8C) exhibited a peak at 3402 cm^{-1} , confirming the presence of a carboxylic acid ($-\text{COOH}$) group. Furthermore, a peak at 1729.45 cm^{-1} corresponds to a carbonyl (C=O) group. The FTIR spectrum for strain 11 Fig. (8D) showed absorption bands between 2800 and 3500 cm^{-1} , which are indicative of a carboxylic acid ($-\text{COOH}$) group. A peak at 1731 cm^{-1} also confirms the presence of a carbonyl (C=O) group.

Based on the FTIR analysis, the presence of functional groups such as carboxyl ($-\text{COOH}$), carbonyl (C=O), hydroxyl ($-\text{OH}$), and amine ($-\text{NH}$) was confirmed for all four bacterial strains (6, 8, 10, and 11). These results suggest that the produced biosurfactants are complex molecules consistent with the structures of glycolipids, rhamnolipids, and lipopeptides.

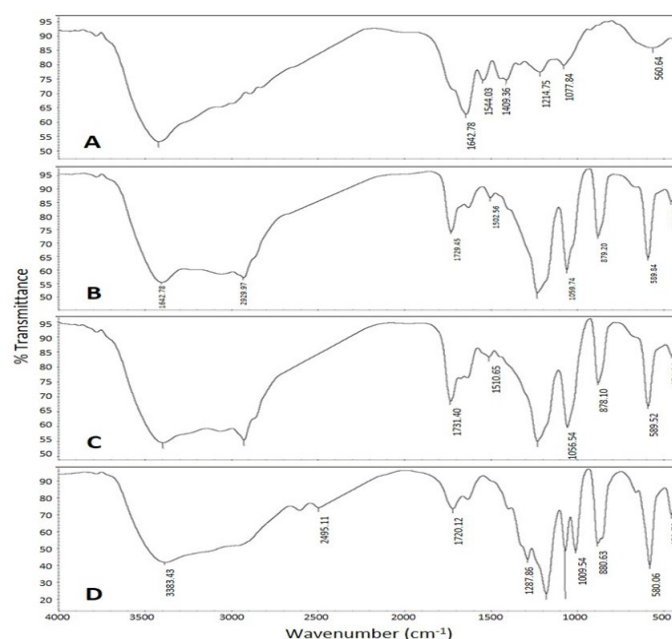


Fig. 8: FTIR spectra of biosurfactants produced by *Stenotrophomonas maltophilia* A, *Alcanivorax diesolei* B, *Pseudomonas aeruginosa* C, and *Staphylococcus aureus* D

Evaluation of Bacterial Strains for Enhanced Crude Oil Recovery

The efficacy of four different bacterial strains (*Stenotrophomonas maltophilia* 6, *Alkanivorax dieseldolei* 8, *Pseudomonas aeruginosa* 10, and *Staphylococcus aureus* 11) in crude oil recovery was evaluated in a sand-pack column, Fig. (9).

The control group, which had no bacterial treatment, yielded an additional oil recovery (AOR) of 0.7 %. In contrast, all four bacterial strains demonstrated a statistically significant increase in AOR. *Staphylococcus aureus* (11) exhibited the highest additional oil recovery, at 22.7%. This was followed by *Pseudomonas aeruginosa* (10) at 21.2 %, *Stenotrophomonas maltophilia* (6), at 16.4 %, and *Alkanivorax dieseldolei* (8), at 13.7 %. The overall performance of the strains in improving oil recovery was ranked as: *Staphylococcus aureus* > *Pseudomonas aeruginosa* > *Stenotrophomonas maltophilia* > *Alkanivorax dieseldolei*.

Further analysis revealed that the Control group had an initial oil in place (OOIP) of 36 ml and a final OOIP of 16 ml, with a residual oil saturation (Sor) of 45.7%. After treatment with *Stenotrophomonas maltophilia*, the final OOIP was reduced to 17 ml and the Sor was 47.2%. *Staphylococcus aureus* reduced the final OOIP to 18 ml and the Sor to 51.4 %. Similarly, *Alkanivorax dieseldolei* reduced the final OOIP to 17.5 ml and the Sor to 46.01 %, while *Pseudomonas aeruginosa* reduced the final OOIP to 16 ml and the Sor to 47.05 %.

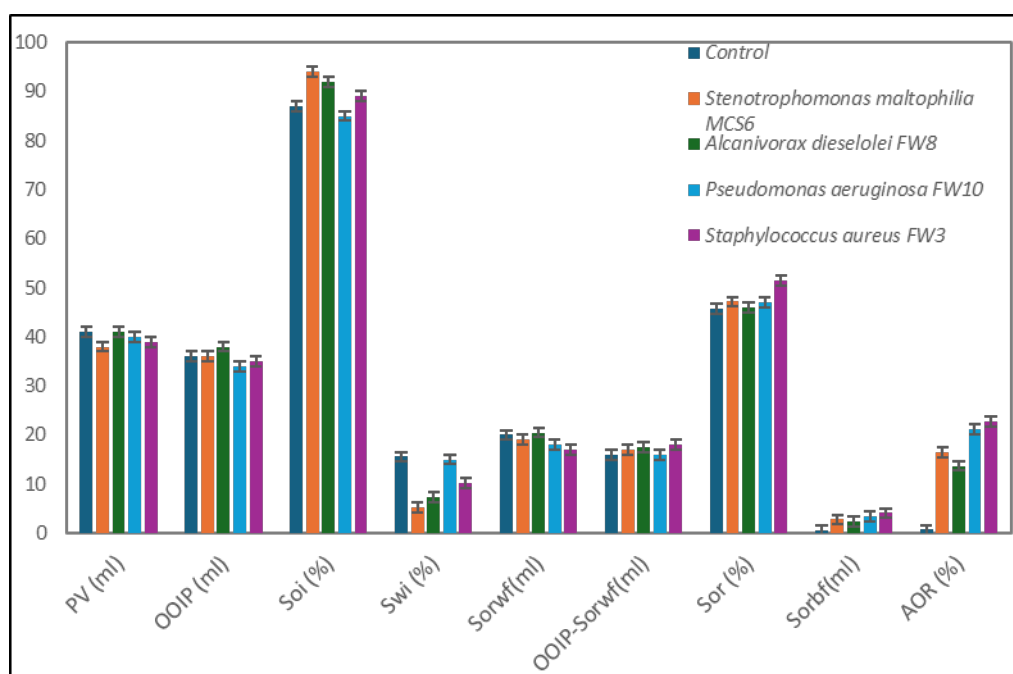


Fig. 9: Summary of the results obtained in sand-pack column for crude oil recovery

The statistical analysis, expressed as mean $\pm$ SD (n=6), showed that a p-value of less than 0.001 was considered highly significant. A one-way ANOVA was performed, and different letters indicate a significant difference at $p < 0.05$ by Duncan's test.

DISCUSSION

In this study, the ability of various bacteria isolated from contaminated environments to produce biosurfactants and to biodegrade hydrocarbons was demonstrated. Kanwal et al., [31]. demonstrated that the biodegradation of such polluted environments is carried out either through bioaugmentation procedure (where the contaminated environment is supplemented with the bacteria or bacterial consortium that has high capabilities to degrade the target pollutant) or through bio-stimulation (in which the environmental conditions of the indigenous bacteria is optimized) to enhance biodegradation process.

Microbial enhanced oil recovery (MEOR) has been a topic of increasing academic and industrial interest owing to the fact that it has the potential to simultaneously be environmentally friendly and cost-effective, [32].

One of the most efficient mechanisms in MEOR processes are biosurfactants [33]. They are secondary metabolic amphipathic substances produced by a variety of microorganisms consisting of two parts, a polar head (hydrophilic) moiety may be ionic (cationic or anionic), non-ionic or amphoteric, and other parts nonpolar tail (hydrophobic) is often a chain of hydrocarbons [34].

The principal of sequential isolation used in this study was the pattern of crude oil susceptibility to reducing viscosity and surface tension by bacteria by emulsification and also the probability to degrade the crude hydrocarbon [35].

The bacterial isolates were screened for their biosurfactant producing ability with blood hemolysis test, oil displacement technique, and emulsification index [36].

The emulsification power Fig. (1) demonstrated the variety and ability of the different bacterial isolates, where some of these isolates result in no emulsion, and the others can give emulsion with different levels, also the bacteria which produce biosurfactants can disperse the crude oil with different abilities shows different clear zone diameters, that agreed with Gomaa et al., [37], which showed the oil spreading capacity of different biosurfactant producing strains which ranged in between 7 to 30 mm that were considered positive for screening test for biosurfactant production.

Stenotrophomonas maltophilia (6) is a halophilic bacterium, *Pseudomonas aeruginosa* (10) is a thermophilic bacterium it can grow at 42 °C.

Alcanivorax dieselolei (8) is a halophilic bacterium, these extreme environmental conditions assure the ability of these bacteria to produce biosurfactants [38]. Also, the most bacterial isolates are gram negative, that indicates that G^{-ve} bacteria are dominate the surface and oil-contaminated environment, and this agreed with Shoeb et al., [39], where the researcher showed that G^{-ve} bacteria have adapted to live in a hydrocarbon mixture such as mono- and poly-aromatics.

The sequential emulsification percent in the present study at the different experiments along the study agreed with Ehinmitola et al., [40]. that showed *P. aeruginosa* to emulsify kerosene resulting in emulsification percentage of 60 %.

Also, the similar range of the emulsification percentage has been shown by Abouseoud et al., [41] at (45% - 50%) Aparna et al., [42] at (67%–73%), Chen et al., [43]. (%53–%49) in different species under genus *Pseudomonas*. In this work the emulsification in this range is considered significant for screening and assessment of quality of biosurfactant. Optimal conditions are playing an important role in biosurfactants production. Carbon and nitrogen content and their ratio in the medium strictly affect the productivity and yield of biosurfactants by the microbe and hence require optimization [44].

In the present study, the bacterial isolate (10) was identified as *Pseudomonas aeruginosa* which could be utilized KNO₃ as the sole nitrogen source and can produce a proper biosurfactants (BIOs) percent, that can reduce the surface tension to 33 mN/m Fig.(3) and this agreed with Ahmed L Elazzazy et al., [38] that previously demonstrated the ability of various species under genus *pseudomonas* to utilize inorganic salts as sole nitrogen source

Also, the present study agreed Abouseoud et al. [44] where NH₄NO₃ were the best nitrogen sources for *Pseudomonas fluorescens*, this indicate the ability of genus *pseudomona* to utilize a cheap inorganic source of nitrogen for BIOs production. However, showed the difference and verities of bacterial ability to consume nitrogen in feeding.

For carbon source, the present study disagreed with Moussa et al, [45] where they showed glucose to be the most agreeable carbon source as compared to others like glycerol and sucrose that lowered the surface tension of water to 34 mN/m in *Pseudomonas aeruginosa*. On the other hand, in the present study sucrose was the most effective carbon source with all of the four bacterial strains. The yield of biosurfactant was assessed by obtaining the dry weight of extracted crude biosurfactant. Several groups of authors that have shown the synthesis of biosurfactants by newly isolated microbial strains have much emphasis on their stability to extremes of conditions, specifically in terms of pH, temperature and salinity, like Tripathi et al. [46] and Purwasena et al. [47].

the best results were obtained with inoculum size 10 ml , it can be concluded that biosurfactant that is produced by the bacterium can be considered to be a growth-associated metabolite, the result concurs with the previous report by Nayak et al., [48].

In the present investigation values of emulsion power are lower but consistent with Amani et al., [49] that reached emulsion rate of 68% and Amani [50] with emulsion rate of 85% .

The effect of crude biosurfactants against the Gram (-ve) & Gram (+ve) and against fungal strains were determined. Crude biosurfactants applied against the prepared pseudomonas spp showed the ability of crude biosurfactants to eliminate gram negative bacteria at different concentrations. Where at 600 ppm concentration the clear zone increased with the four bacterial strains till 15 mm diameter with organism 6 & 11. Gram-negative bacteria *E. coli* was tested, and the same results were almost obtained with little difference. Where at 600, 500 ppm concentration the highest clear zones were obtained till 15 and 19 mm diameter. But the organism 10 gave its effect at 600 ppm only.

In the present study agreed with Purwasena et al., [47] that showed that biosurfactant produced by indigenous oil reservoir bacteria. *Bacillus* sp is a good candidate for a new antibacterial agent.

Antifungal effect of crude biosurfactants appeared at against *Aspergillus niger* as a multicellular form and *Saccharomyces cerevisiae* as a unicellular form where after 48 hr and 72 hr no clear zones appeared for all isolates at different concentrations, at 30 °C for all isolates. The present study disagreed with Soffritti, et al., [51], where all of the biosurfactants produced by the four bacterial isolates give no effect as antifungal but they demonstrate the ability of biosurfactants produced from bacteria of genera *Bacillus* to be used as antifungal.

The CMC for bacterial strain (11) *Staphylococcus aureus* is 900 ppm and the surface tension is 38 mN/m. In general, in the present study the CMCs are higher and disagree with Varjani and Upasani [52], where they found that biosurfactant produced by *Pseudomonas aeruginosa* strains can reduce the surface tension of water from 72 to 30 mN/m at CMC ranging from 5 to 200 mg/l.

Biosurfactant that is produced by bacteria in the microbial enhanced oil recovery (MEOR) mechanism may increase the amount of recovered oil by reducing the IFT between oil and water inside the reservoirs, which modifies the pore-rock wettability by changing the rock properties from oil-wet to water-wet, and by emulsifying hydrocarbons [7]. These surface-active molecules can disperse oil components in water by establishing a bridge between fluids with different polarities, such as oil–water, by reducing free energy per unit area by forming micelles that are surrounded the oil droplets, and by increasing the oil pseudosolubilization in water [53].

In the present study the bacterial isolates could decrease the IFT between oil and water from 14 to 6 dyne/cm with strain (6)

Stenotrophomonas maltophilia, from 14 to 7 dyne/cm with strain (8) *Alcanivorax dieselolei*, from 12 to 5 dyne/cm with bacterial strain (10) *Pseudomonas aeruginosa* and from 14 to 7 dyne/cm with strain (11)

Staphylococcus aureus. A little difference between the reported CMC value in this experiment and previous studies may result from some inherent properties of the biosurfactant, such as the hydrophobic chain length, purity, growth-media composition, and strain of producing bacteria [54].

For bacterial strain (6) *Stenotrophomonas maltophilia*, (Figure 8A) illustrated that the stretching peak at 2495 and 3383.243 cm⁻¹ suggested the presence of the COOH group. The absorption peak at 1720 suggest the presence of C=O group and 1287 suggest the presence of C=N group.

For strain (8) *Alcanivorax dieselolei*, Figure (8B) determined the absorption at 3420 lead to presence of OH or NH group, and 1642 lead to presence of C=O. For bacterial strain (10) *Pseudomonas aeruginosa*, Figure (8C) the peak at 3402 lead to presence of COOH group, and the peak 1729.45 lead to C=O group, and finally. For bacterial strain (11)

Staphylococcus aureus, Figure (8D), the absorption at 2800 and 3500 lead directly to COOH group, and peak at 1731 lead to C=O group. The resulted mixture of chemical groups such as (carboxyle, carbonyle, ketone, hydroxyle and hydrogen bonding N-H) that produced from the FTIR analysis for all isolates 6, 8, 10 & 11 indicated the nature of the produced surfactin to be glycolipid, rhamnolipid and lipopeptide in nature that is supported by the study of Chen et al., [43] and Sharma et al., [55].

The data represented in Fig. (9) demonstrated the results of microbial enhanced oil recovery (MEOR) in sand-pack columns by *Stenotrophomonas maltophilia* (strain 6), *Alcanivorax dieselolei* (strain 8), *Pseudomonas aeruginosa* (strain 10) and *Staphylococcus aureus* (strain 11). The additional oil recovery was found to be (16.4, 13.7, 21.2 and 22.7%) for strains 6, 8, 10, and 11 respectively. So, in the present study we agreed with Zhao et al. [56], that used indigenous biosurfactant produced by *Pseudomonas aeruginosa* for core flooding tests revealed that an extra 5.22% of the oil was displaced. This increasing of oil recovery can be due to several factors, the bacterial strains used in this study produced biosurfactants, which increased the

surface tension and decreased the emulsify hydrocarbons, which can contribute to reduce the interfacial tension at the oil-water interface and contribute to enhance the mobilization of the hydrocarbon into the column. Where water flooding was unable to recover this residual oil, but biosurfactant flooding was used as a tertiary oil recovery process to retrieve this oil.

In the present study we can conclude that many bacterial producing biosurfactant at optimal conditions can produce biosurfactants that can clearly increase the additional oil recovery. This result agrees with that of (Gudiña et al., [20]. and Suthar et al., [57].

Also, In the present study, agreed with a Previous study, where a sand-packed column experiment that used crude biosurfactant flooding produced by different species of *Bacillus* spp. and *Fusarium* sp. resulted in 24.5 - 46 % additional oil recovery [58]. Also, the present study agreed with Gudina et al., [59] and Sun et al., [60] . where they reported that for the MEOR experiment, the oil recovery from OOIP was below 50%, where the oil recovery from OOIP was also below 50% .

Many studies compared between bacterial and fungal biosurfactants, El-Sheshtawy et al., [61] compared between biosurfactant produced by *Bacillus licheniformis* and *Candida albicans* introduced into two sand-pack column the oil recovery rate was (16.6%), (8.6%) respectively, they concluded that bacterial biosurfactant is more efficient than biosurfactant produced from yeastm, [62] . Sophorolipids biosurfactant produced by *Candida bombicola* ATCC 22214 was further used in MEOR using oil reservoir core-plugs where it showed additional 9-13% extra oil recovery.

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