Environmental Comparative Study of Biosurfactants Production and Optimization using Bacterial Strains Isolated from Egyptian oil Fields

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ABSTRACT

Biosurfactants have recently gained popularity because they have numerous benefits over chemical synthetic surfactants, including higher biodegradability, lower toxicity, higher foaming, environmental compatibility, and effective properties under harsh conditions. This study aimed to produce effective biosurfactants by selected bacterial strains isolated from Egyptian oil fields to improve oil recovery and investigate their environmental aspects for microbial enhanced oil recovery. The selected strains were incubated in a new proposed nutrient medium H to produce biosurfactants with optimum surface and emulsification activities. Stability studies were conducted to examine the tolerance of produced biosurfactants in harsh reservoir conditions. Core flooding tests were performed to investigate the potential of produced biosurfactants in enhancing oil recovery. The environmental risk assessment was conducted to investigate if there are any possible threats of the selected bacterial strains. Results showed that selected bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* could produce effective

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biosurfactants that reached their maximum surface activity after 24 hrs of incubation by reducing the surface tension from 71.8 mN/m to 27.13 mN/m and 25.74 mN/m, and the interfacial tension against kerosene from 48.4 mN/m to 1.27 mN/m and 0.38 mN/m at critical micelle concentration of 0.06 g/l and 0.04 g/l, respectively. The produced biosurfactants by *Bacillus licheniformis* and *Bacillus subtilis* showed significant emulsification activity against crude oil with emulsification indices of 50.2% and 63.7%, respectively. High stability was observed at high temperatures for a long-time period and more than 60% of their surface and emulsification activities were maintained over a wide range of pH and salinity. It was also found that 31.41-39.35% of additional oil could be recovered by the produced biosurfactants. Finally, *Bacillus licheniformis* and *Bacillus subtilis* are environmentally safe, have no potential for toxicity, and no risk could occur for MEOR.

KEYWORDS

Biosurfactant; Microbial Enhanced Oil Recovery; Bacillus Licheniformis; Bacillus Subtilis; Core Flooding Micromodel; Environmental Risk Assessment

1. INTRODUCTION

Surfactants are widely employed in a variety of industries, including pharmaceuticals, cosmetics, soap & detergents, textiles, petroleum industry, agrochemicals, and food [1,2]. Three main limitations affect the robustness of the synthetic surfactant flooding in oil reservoirs, which are environmental impacts, synthetic surfactant cost, and oil price. Recently, the increase in ecological concerns, the rise of more rigorous environmental laws, and the development of biotechnology have encouraged biosurfactants to be a potent alternative to synthetic surfactants existing in the market due to their biodegradability, low toxicity, and cost-effectiveness [1,3–6]. Currently, the petroleum industry is considered the major market for biosurfactants, since they could be used in microbial enhanced oil recovery, oil spill clean-up, oil residue removal from storage tanks, and soil and water bioremediation [7–9].

Microorganisms provide a unique opportunity to make hydrocarbon production economically and environmentally considerate in a technique known as microbial enhanced oil recovery (MEOR). Microbial Enhanced Oil Recovery (MEOR) is a biological-based technology that utilizes microorganisms to produce metabolic bioproducts such as biosurfactants that are used to improve oil

recovery. MEOR is considered the most eco-friendly and cost-effective Enhanced Oil Recovery (EOR) technique that demonstrates several advantages compared with the conventional EOR Technique. In 2012, MEOR technique was examined by Zahner et al. according to its economical, technological and environmental standpoint compared with other EOR techniques [10,11]. This study was based on a successful history of field trials. MEOR methods have shown positive results when applied in many countries around the world not only economical but also environmentally [6,11,12]. MEOR has many unique advantages such as minimum energy consumption during operation compared with thermal EOR, oil price independence compared with chemical methods because microbial growth occurs at exponential rates, and the ability to produce multiple useful by-products from cheap and renewable resources [13–15]. Generally, the cost of a thermal EOR project could be 25$ per barrel of additional oil, and the cost of carbon dioxide and surfactant injection projects could be as high as 30$ and 50$ per barrel of additional oil, respectively [16,17]. On other hand, the MEOR process could cost $6 to $10 per barrel of additional oil [16,18], However, The MEOR process could cost $6 to $10 per barrel of additional oil [16,18], making it the most cost-effective EOR technique because it only requires low-cost nutrient brine solutions and minor modifications to existing secondary recovery facilities. As a result, MEOR offers huge potential as a competitive alternative to conventional EOR chemical techniques. Furthermore, The most significant features of bioproducts are their nontoxicity and biodegradability, which means no accumulation or hazard in the environment when utilised in the MEOR process, which reveals that they are environmentally friendly [19–21]. The bacterial activity impacts improve with time in the reservoir, whereas the effects of additives in EOR technologies tended to decline with time and distance from the injection well.

There are two types of bacteria responsible for MEOR metabolites productions such as biosurfactants, which are indigenous bacteria and exogenous bacteria. MEOR selected bacteria must have the potential to survive in harsh reservoir conditions and produce the required bioproducts. Indigenous bacteria are considered the ideal candidates for this MEOR process compared with exogenous bacteria since they are compatible with their reservoir conditions [13,22]. Biosurfactants produced by indigenous bacteria have been proposed as offering an effective mechanism to increase the oil recovery from low productive

reservoirs [23,24]. Many researchers have used different types of bacteria to produce biosurfactants in growth media. Bacterial species that are biosurfactants production candidates could be isolated from several sources. The majority of these bacteria are found in contaminated areas that contain petroleum hydrocarbon by-products and/or industrial wastes [25,26]. Lazar et al. (2007) suggested Crude oil, formation water, sediments from formation water purification facilities (collecting stations), sludge from biogas operations and effluents from sugar refineries are the four primary sources of bacterial isolation [22].

The biosurfactant amphipathic structure generally consists of hydrophobic moiety (tail) and hydrophilic moiety (head). The hydrophobic moiety (tail) may be a hydroxy fatty acid or a long-chain fatty acid of different lengths. the hydrophilic moiety (head) may be a peptide, phosphate, carboxylic acid, amino acid, carbohydrate, or alcohol. Structurally, Biosurfactants are a diverse surface-active molecules group mostly produced by microorganisms [27–29]. Glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids are examples of microbial biosurfactants. Biosurfactants are classified based on their microbiological origin and chemical composition, whereas synthetic chemical surfactants are generally categorized by the nature of their polar grouping. biosurfactants produced from bacterial strains are classified mainly into two groups based on their molecular mass, which are the “low-molecular-weight biosurfactants” group and the “high-molecular-weight biosurfactants” group. Glycolipids, lipopeptides, fatty acids, neutral lipids, and phospholipids are considered examples of the low-molecular-weight biosurfactants group. Glycolipids and lipopeptide compounds are associated with the potential of reducing Surface and interfacial tensions in liquids. the other Low-molecular-weight biosurfactants that have low critical micelle concentration could increase the hydrocarbon's apparent solubility by integrating them into micelles' hydrophobic cavities [30]. The group of high-molecular-weight biosurfactants like polysaccharides, Liposans, Alasans, Emulsans, and protein complexes are associated with the potential of producing stable emulsions, even if they do not have significant potential in reducing surface tension. However, the production of stable emulsions allows bacteria to strongly adhere to hydrophobic surfaces, which indicates their high biodegradation potential [31].

Table 1. summarizes a list of biosurfactants produced by several bacteria [1,4,14,32–35].

Biosurfactant			Microorganism
Group	Sub-group	Class	
Low-Molecular-Weight Biosurfactants	Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa* *Pseudomonas sp.*
		Trehalose lipids	*Rhodococcus erithropolis* *Arthobacter sp.*
		Sophorolipids	*Candida bombicola* *Candida apicola* *Candida lipolytica* *Candida bogoriensis*
		Mannosylerythritol lipids	*Candida antartica*
		Cellobiolipids	*Ustilago zeae* *Ustilago maydis*
	Lipopeptides	Surfactin/Iturin/Fengycin	*Bacillus subtilis*
		Viscosin/tolaasin/syringomycin	*Pseudomonas fluorescens* *Pseudomonas sp.*
		Putisolvin/Amphisin	*Pseudomonas spp.*
		Lichenysin	*Bacillus licheniformis*
		Serrawettin	*Serratia marcescens*
	Fatty acids, Neutral lipids, and Phospholipids	Fatty acids	*Corynebacterium Lepus*
		Corynomicolic acids	*Corynebacterium insidibasseosum*
		Neutral lipids	*Nocardia erythropolis*
		Phospholipids	*Acinetobacter sp.* *Corynebacterium Lepus* *Thiobacillus thiooxidans*
High-Molecular-Weight Biosurfactants	Polymeric surfactants	Emulsan	*Acinetobacter calcoaceticus*
		Biodispersan	*Acinetobacter calcoaceticus*
		Alasan	*Acinetobacter radioresistens*
		Liposan	*Candida lipolytica*
		Lipomanan	*Candida tropicalis*
	Particulate biosurfactants	Vesicles and Fimbriae	*Acinetobacter calcoaceticus*
		Whole cells	*Cyanobacteria*

Several authors reported the results of their produced biosurfactants on oil recovery using different porous micromodel systems as summarized in Table 2. It became obvious that when micromodel tests are carried out under standard laboratory conditions and the reservoir conditions are neglected, much more oil is recovered [24,36,37]. Unfortunately, the conducted micromodel tests without mimicking reservoir conditions will significantly reduce the accuracy of the simulations of the microcosm, and will almost certainly drive to field failures, as seen many times before [38]. On the other hand, the other studies that mimic reservoir conditions reported that the most typically simulated parameters that mimic

reservoir conditions are temperature, crude oil density, gravity, and formation water composition. In addition, a few reported studies mimicked additional parameters like porosity and well pressure. Until now, model systems that mimicked all reservoir parameters (temperature, porosity, crude oil formation water, and pressure) had resulted in lower oil recovery than less demanding laboratory studies [39,40]. Sand-pack column is the most utilized model in micromodels studies since it is an easy, rapid and inexpensive construction. The increased cost of core flooding micromodels tests in terms of both time and money, as well as the difficulties of core acquisition, make core floods an unfeasible technique. However, core flooding micromodels offer a more accurate and precise evaluation of MEOR potential since they can simulate reservoir conditions, resulting in more realistic microcosm simulations [41–46].

Table 2. The reported Additional oil recovery studies in different porous micromodel systems and their reservoir simulated conditions.

Microorganism	System Model/ Inoculum	Reservoir Simulated Conditions	Oil Type	Additional Oil Recovery (%)
Pseudomonas aeruginosa sp. [44]	Sand-Pack Column Model/ Crude Biosurfactant	Temperature-Porosity	N/A	30
Bacillus licheniformis AC01 [39]	Sand-Pack Column Model/ Bacteria & Nutrient Medium	Temperature-Crude Oil-Formation Water-Porosity-Pressure	Light Oil	22
Bacillus licheniformis AC01 [39]	Sand-Pack Column Model/ Bioemulsifier	Temperature-Crude Oil-Formation Water-Porosity-Pressure	Light Oil	< 1
Bacillus licheniformis TT42 [47]	Sand-Pack Column Model/ Crude Biosurfactant	Formation Water-Pressure	Synthetic	35
Bacillus licheniformis K125 [47]	Sand-Pack Column Model/ Crude Bioemulsifier	Formation Water-Pressure	Synthetic	43
Bacillus mojavensis JF-2 [47]	Sand-Pack Column Model/ Crude Bioemulsifier	Formation Water-Pressure	Synthetic	29
Bacillus subtilis 20B [48]	Sand-Pack Column Model/ Crude Biosurfactant	Formation Water-Pressure	Light Oil	25-33
Bacillus subtilis 20B [48]	Glass Packed Column Model/ Crude Biosurfactant	Crude Oil-Formation Water-Porosity	Light Oil	30
Bacillus licheniformis TT33 [40]	Sand-Pack Column Model/ Microbial Biomass in a Nutrient Medium (Selective Plugging)	Temperature-Crude Oil-Formation Water-Pressure	Heavy Oil	25-32
Bacillus sp. [49]	Glass Etched Micromodels	Temperature-Crude Oil		13
Enterobacter sakazakii/ Bacillus subtilis fusion [50]	Sand-Pack Column Model/ Engineered Bacteria & Nutrient	Temperature-Formation Water-Pressure	N/A	17-25

Bacillus subtilis W19 [51]	Berea Sandstone Core Model/ Crude Biosurfactant	Crude Oil-Formation Water-Porosity	Light oil	23
Bacillus licheniformis sp. [52]	Sand-Pack Column Model/ Bacteria &Nutrient	Temperature-Crude Oil	Light Oil	6-25
Bacillus licheniformis sp. [52]	Sand-Pack Column Model/ Bacteria & Nutrient	Temperature-Crude Oil	Heavy Oil	15-17
Bacillus licheniformis R1 [43]	Sand-Pack Column Model/ Crude Biosurfactant	Formation Water-Porosity	N/A	32
Fusarium sp. BS-8 [37]	Sand-Pack Column Model/ Crude Biosurfactant	Formation Water	Light Oil	46
Bacillus subtilis W19 [53]	Berea Sandstone Core Model/ Cell-free Biosurfactant	Crude Oil-Formation Water-Porosity	Light Oil	13-28
Bacillus subtilis B30 [54]	Berea Sandstone Core Model/ Cell-free Biosurfactant	Crude Oil-Formation Water-Porosity	Light Oil	17-26
Bacillus subtilis B30 [54]	Berea Sandstone Core Model/ Cell-free Biosurfactant	Crude Oil-Formation Water-Porosity	Heavy Oil	31
Bacillus subtilis R2 [54]	Berea Sandstone Core Model/ Cell-free Biosurfactant	Crude Oil-Formation Water-Porosity	Heavy Oil	37
Candida albicans IMRU 3669 [41]	Sand-Pack Column Model/ Crude Biosurfactant	Crude Oil-Formation Water	Light Oil	9
Bacillus subtilis MTCC 2422 [55]	Sand-Pack Column Model/ Bacteria & Nutrient	-	Synthetic	9
Bacillus licheniformis ATCC 14580 [41]	Sand-Pack Column Model/ Crude Biosurfactant	Crude Oil-Formation Water	Light Oil	17
Bacillus licheniformis W16 [56]	Berea Sandstone Core Model/ Cell-free Biosurfactant	Temperature-Crude Oil-Formation Water	Light Oil	24-26
Bacillus licheniformis L20 [57]	Sandstone Core Model/ Cell-free Biosurfactant	Temperature-Crude Oil	Heavy Oil	14

- No reservoir properties were simulated in the micromodel system.
N/A Not Applicable

Herein, the potential of microbiology in petroleum engineering was investigated through many phases. Firstly, the potential of biosurfactant production by selected bacterial strains isolated from crude oil samples collected from Egyptian oil fields located in the Western Desert was examined. Secondly, the optimization of produced biosurfactants was examined by incubating the selected bacterial strains in the new proposed nutrient medium H. furthermore, the effect of the produced biosurfactants in improving oil recovery was studied using simulated core flooding micromodels. In addition, the Stability of the produced biosurfactants under different conditions (temperature, were Finally, a comprehensive environmental risk assessment was performed to investigate if there is any possible

hazard that could be occurred by the selected biosurfactants producing bacteria for microbial enhanced oil recovery.

2. MATERIALS AND METHODS

2.1 Media

The selected bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* were isolated and identified by Aboelkhair et al. (2022) from Egyptian oil fields called AL FADL and Al QADR, respectively, and located in the Western Desert [58]. the new proposed nutrient medium H was used to examine the potential of selected bacterial strains for producing biosurfactants and making a comparative analysis between the produced biosurfactants. The chemical composition of the new proposed medium H were, in g/l distilled water: Glucose, 20; Na2HPO$_4$, 6; NH$_4$NO$_3$, 5; Na- Glutamate, 4; KH$_2$PO$_4$, 4; NaNO$_3$, 3; KNO$_3$, 3; MnSO$_4$.4H$_2$O, 1.78; Yeast Extract, 1; (NH$_4$)$_2$SO$_4$, 1; Urea, 1; MgSO$_4$.7H$_2$O, 0.3; FeSO$_4$.7H$_2$O, 0.1; CaCl$_2$, 0.1, and Trace elements 1ml/l distilled water. The chemical composition of the trace elements, in g/l distilled water: ZnSO$_4$.7H$_2$O, 2.28; CuSO$_4$.5H$_2$O, 1.14; H$_3$BO$_3$, 0.61; CoCl$_2$.6H$_2$O, 0.47; and Na$_2$MoO$_4$.2H$_2$O, 0.42Media were sterilized in an autoclave at 120^{o}C for 20 min, then the sterilized trace elements were adding, and the pH value was adjusted to be 7 using sterilized 6 N NaOH.

2.2 Examination of Surface Activity

ST and IFT were measured as an indication of the potential of the selected bacterial strains to produce biosurfactants using the EZ Tensiometer rod method (Model 201, USA). At ambient temperature (252 ^{o}C) and air pressure (1 atm), all obtained measurements were made in triplicate and the average values were reported [58].

2.3 Bacterial Growth

The optical density approach was implemented to evaluate the growth profile of the selected bacterial strains. The bacterial growth profile was estimated using a JASCO spectrophotometer (V-630, Japan). The optical density was determined at 600 nm, which is the optimal wavelength for determining bacteria concentrations [48].

2.4 Biosurfactant Extraction and Purification

Extraction and purification of biosurfactants can be conducted by many methods. Acid precipitation was used to extract and purify the biosurfactant that was produced in the new proposed medium H [48]. To separate bacterial cells, 100 ml of culture media was centrifuged (10,000 rpm for 20 min) in a Sigma centrifuge machine reaching cell-free supernatant (Model 2-16KL, Germany). The pH was then adjusted to two by adding 6N HCl to the cell-free supernatant and maintained at 4oC for 24 hrs to precipitate the biosurfactants that had been produced. After that, it was centrifuged (12,000 rpm for 30 min) at 4°C, and the precipitates were recovered. then, the recovered precipitates were dissolved in distilled water. Finally, the pH value was adjusted to eight using 6N NaOH, lyophilized, and weighed.

2.5 Determination of Critical Micelle Concentration

The critical micelle concentration (CMC) is the biosurfactant concentration at which micelles begin to aggregate. Simply, the increase in surfactant concentration leads to a reduction in ST. when the surfactant molecules completely saturate the solution's surface, no further decrease in ST will occur. CMC is considered an indicator of a surfactant's efficiency. The critical micelle concentration is an important feature of surfactants because once they achieve it, no further drop in ST and IFT, even after adding more biosurfactants. CMC was determined by graphing ST versus biosurfactant concentration. Therefore, different solutions of extracted biosurfactant in distilled water were prepared with different ranges of concentrations from 0.01 to 0.1 g/l, and the change in ST and IFT were detected by the EZ Tensiometer rod method (Model 201, USA) [48].

2.6 Examination of Emulsification Activity

The potential of surfactants to create a stable emulsion is known as emulsification activity. The produced biosurfactant's emulsification activity was evaluated by measuring the emulsification index (E24) against different hydrocarbons including hexane, heptane, hexadecane, kerosene, and crude oil. E24 was obtained by mixing 2 ml of each hydrocarbon in a separate test tube with 2 ml cell-free supernatant, vertexing for 2 min, and storing at 25°C for 24hr. The emulsification index E24 was measured by dividing the emulsion layer height (mm) by the whole mixture height (mm) [59].

2.7 Stability Studies

The stability of the produced biosurfactant under harsh conditions was studied by measuring ST, IFT, and E24 of the cell-free supernatant over a wide range of temperatures, salinities, and pH values [48]. the produced biosurfactant stability was investigated at different temperatures (30-100ºC) at pH 7, and 0% (w/v) NaCl. Similarly, the salinity effect was studied at variable concentrations of NaCl (0-20% w/v), temperature 25ºC, and pH 7. The pH effect on the stability of the produced biosurfactants was also studied at different pH values range 2-12). pH value was altered by adding 6N NaOH or 6N HCl at a temperature of 25ºC and 0% (w/v) NaCl concentration using a Jenway pH meter (Model 3505, UK). The thermal stability of biosurfactants is a significant property for its commercial application at extreme temperatures. The long-term thermal stability of the produced biosurfactants at high temperature was also examined by measuring emulsion stability (ES, %) of the produced biosurfactants at the temperature of fields of interest at time intervals (1, 24, 48, 72, 96, 120, 144 and 168 hrs). Emulsions were formed in sterile test tubes by adding 2 ml of biosurfactant aqueous solution to 2 ml of crude oil of the fields of interest and then vertexing for 2 mins. The crude oil and formation water aqueous solution was prepared using the crude oil and formation water of the field of interest and containing the produced biosurfactant at CMC. The sterile tubes were then kept in the oven at the temperature of fields of interest for 1 hr before measuring the relative emulsion volume (EV, %) and emulsion stability (ES, %) were measured at time intervals (1, 24, 48, 72, 96, 120, 144 and 168 hrs). The EV and ES were then calculated using Eqs. (1) and (2), respectively [60].

$$EV\ (\%) = \frac{Emulsion\ volume\ (ml)}{Total\ liquid\ Volume\ (ml)} \times 100 \quad (1)$$

$$\mathrm{ES}\ (\%) = \frac{\mathrm{EV_t}}{\mathrm{EV_0}} \times 100 \quad (2)$$

where EV_t is the emulsion volume after time (t) and EV_0 is the emulsion volume at time (0).

2.8 Core Flooding

Simulated core flooding micro modelling tests were conducted to examine the potential of biosurfactants produced by *Bacillus licheniformis* as a tertiary stage in recovering a significant additional amount of crude oil after the stage of water flooding. Sandstone core plugs used in core flooding tests were extracted from Al FADL and AL QADR oil fields (fields of interest), which are the

same fields of the selected bacteria strains to make simulated micromodels, respectively. Table 3 summarises the general properties (Core length, diameter, Bulk-volume, pore-volume, porosity, and absolute permeability) of the used sandstone core plugs. The core flooding tests were performed by using crude oil samples and formation water samples collected from the same fields of interest at the reservoir temperatures (60 ºC) of the fields of interest for mimicking reservoir conditions. Table 4 and Table 5 summarize the crude oil and formation water compositions of Al FADL and AL QADR, respectively. Millipore Filtration Unit by (0.45 µm), was used to filter the Formation water samples before use, [61,62].

Table 3. Sandstone core plugs basic properties for core-flooding tests.

Oil Field	Core ID	Length (cm)	Diameter (cm)	Bulk Volume (cm3)	Pore Volume (cm3)	Porosity (%)	Absolute Permeability (mD)
AL QADR	C-F-Q	5.32	3.51	51.63	9.24	20.64	205.97
AL FADL	C-F-F	5.42	3.51	52.49	8.24	15.69	133.26

Table 4. Al QADR and ALFADL crude oil composition by Chromatograph up to C_{12}^{+}

Component	AL QADR Oil Field		AL FADL Oil Field		Liquid Density (g/cm^3)	Molecular Weight
	Stock Tank Oil		Stock Tank Oil			
	Mole %	Wt.%	Mole %	Wt.%		
Methane	0	0	0	0	0.30	16.04
Ethane	0.14	0.03	0.12	0.02	0.36	30.07
Propane	1.13	0.30	0.797	0.20	0.51	44.10
I-Butane	1.34	0.47	0.887	0.29	0.56	58.12
n-Butane	2.33	0.81	1.418	0.46	0.58	58.12
I-pentane	2.66	1.15	2.340	0.94	0.62	72.15
n-Pentane	2.59	1.12	2.415	0.97	0.63	72.15
Hexane	6.96	3.60	4.832	2.31	0.66	86.18
Benzene	0.89	0.42	0.884	0.38	0.88	78.11
Heptanes	6.94	4.18	5.355	2.98	0.69	100.20
Toluene	1.34	0.74	1.535	0.78	0.87	92.14
Octane	10.25	7.03	8.476	5.37	0.71	114.23
Ethyl-benzene	0.40	0.26	0.233	0.14	0.87	106.16
P, m-xylene	0.91	0.58	0.979	0.58	0.87	106.16
o-xylene	0.32	0.21	0.375	0.22	0.88	106.16
Nonanes C9	7.66	5.90	7.235	5.14	0.72	128.26
Decanes C10	6.33	5.41	6.315	4.98	0.73	142.29
Undecanes C11	5.99	5.28	6.329	5.16	0.79	147.00
dodecanes C12+	41.80	62.51	49.474	69.11	0.90	248.96
Total	100	100	100	100		

Table 5. Al QADR and AL FADL formation water compositions.

Ion Composition	AL QADR Oil Field	AL FADL Oil Field
	Concentration (mg/l)	Concentration (mg/l)
Na^+	38,906.56	41,359.89
K^+	8,310.51	9,225.17
Ca^{2+}	1,353.45	1,678.23
Mg^{2+}	373.57	190.78
Br^+	2.00	1.0
Cl^-	66,222.35	63,022.28
S^{2-}	5,503.71	2,774.83
HCO_3^-	1,250.57	2,636.27
Total	121,922.73	12,0888.40

In this experiment, the initial reservoir condition needs to be applied to the core samples to determine oil recovery. Initially, the Soxhlet extraction method was applied for cleaning the core plugs before use by using an azeotropic mixture consisting of chloroform and methanol (75:25) and then core plugs were dried at 65°C for 24 hrs [63]. Then, the core plugs were saturated with the filtered formation water of the fields of interest after the cleaning and drying using vacuum desiccators for 24 hrs. the pore volume was calculated using the dry weight and wet weight of the core plugs. After that, the core plugs were flooded with the crude oil of the fields of interest, using the core flooding system illustrated in Figure 1, until no more water was recovered, achieving the irreducible water saturation state that originally existed in the oil reservoirs. The original oil in place (OOIP) was determined by the volume of water displaced.

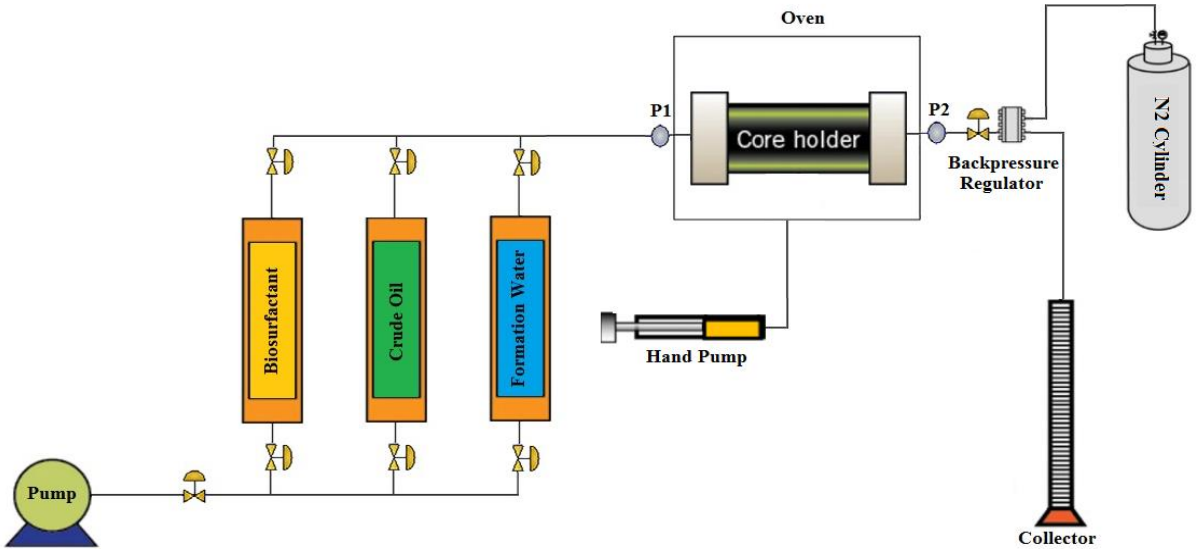

Figure 1. The core flooding system Schematic diagram.

The initial oil saturation (S_{oi}, %) and initial water saturation (S_{wi}, %) were calculated using Eqs. (3) and (4), respectively.

$$S_{oi}\ \% = \frac{OOIP}{PV} \times 100 \quad (3)$$

$$S_{wi}\ \% = \frac{PV - OOIP}{PV} \times 100 \quad (4)$$

For simulation of the stage of water flooding, the core plugs were flooded by many pore-volumes PV of formation water, until no more oil was recovered in the collector. The amount of recovered oil, so-called oil recovery after water flooding ($S_{orwf,}$ cm^3) was volumetrically measured. Then, the oil recovered by the water flooding ($OR_{wf,}$ %) and the saturation of oil that remained ($S_{or,}$ %) were calculated using Eqs. (5) and (6), respectively.

$$OR_{wf}\ \% = \frac{S_{orwf}}{OOIP} \times 100 \quad (5)$$

$$S_{or}\ \% = \frac{OOIP - S_{orwf}}{OOIP} \times 100 \quad (6)$$

Finally, the remaining oil was subjected to biosurfactants flooding, to simulate the biosurfactants flooding stage. The core plugs were flooded by many cell-free supernatant pore-volume s PV until no more oil was recovered in the collector achieving the residual oil saturation after biosurfactant flooding (S_{orbf}, cm^3) and its volumetric amount was determined in the collector. The biosurfactant flooding

additional oil recovery (AOR_{bf}, %) was calculated using Eq. (7). These simulated tests were performed at the average reservoir temperatures of the fields of interest and a flow rate of 0.25 cm^3/min.

$$AOR_{bf}\% = \frac{S_{orbf}}{OOIP - S_{orwf}} \times 100 \quad (7)$$

2.9 Environmental Risk Assessment

In this work, the risk assessment including both ecological and human risk assessments will be examined. This evaluation aims to determine the effects and likelihood of environmental hazards that may arise as a result of a certain activity, such as bacteria culture in an insufficiently controlled environment or wastewater that has not been treated. There are four steps to these evaluations s starting with hazard identification, which is the first and most important step since it establishes the environmental risk assessment scope by defining what needs to be protected from hazards and identifying potential harmful effects. The second step is hazard characterisation, in this step the potential hazards and the consequences of potential harm are examined. The third step is exposure characterization, which considers the likelihood and level of exposure to the hazards, as well as the likelihood of harm. The fourth step is risk characterisation, which estimates the level of risk by combining both consequences and likelihood of harm are combined. In some scenarios, risk mitigation strategies are included in the environmental risk assessment. These strategies seek to mitigate risk to an acceptable level. [64]. This environmental risk assessment is performed to assure that the biosurfactants produced will not cause unacceptable environmental harm when utilised in an actual field in-situ operation.

the main emphasis of this study is the environmental impact of utilising produced biosurfactants for enhancing oil recovery. Consequently, this study will follow the guidelines of the European Federation of Biotechnology, the Canadian Environmental Protection Agency (CEPA), the US Environmental Protection Agency (EPA), and others [65–67]. The produced biosurfactants will be reviewed to assess the probability of harm, define the risks of exposure, and propose measures to mitigate such risks to prevent them.

Risk matrices are possibly one of the most widely utilised risk assessment tools. They're mostly utilised to figure out the size of risk and whether it's well-controlled or not. For this assessment, a bowtie diagram was used to describe the three most common areas of risk matrices, which are low probability impact, medium likelihood impact level, and high or extreme likelihood impact level (Figure 2). The low likelihood impact level (typically green) implies that an event's risk has been adequately addressed or is not high enough. This generally necessitates no action. The medium likelihood impact level (typically yellow) requires efforts to reduce the risk within a set timeframe. Events should be monitored and controlled to the extent that is practically possible, implying that if the risk is maintained at that level, it will be acceptable. Nevertheless, the prevention and control expenses must be considered carefully. The high or extreme likelihood, high impact level (typically red) is unacceptable and advises that work must not begin or continue until mitigating the risk. In this case, large resources or additional control actions will be required to reduce the impact or chance [64,68].

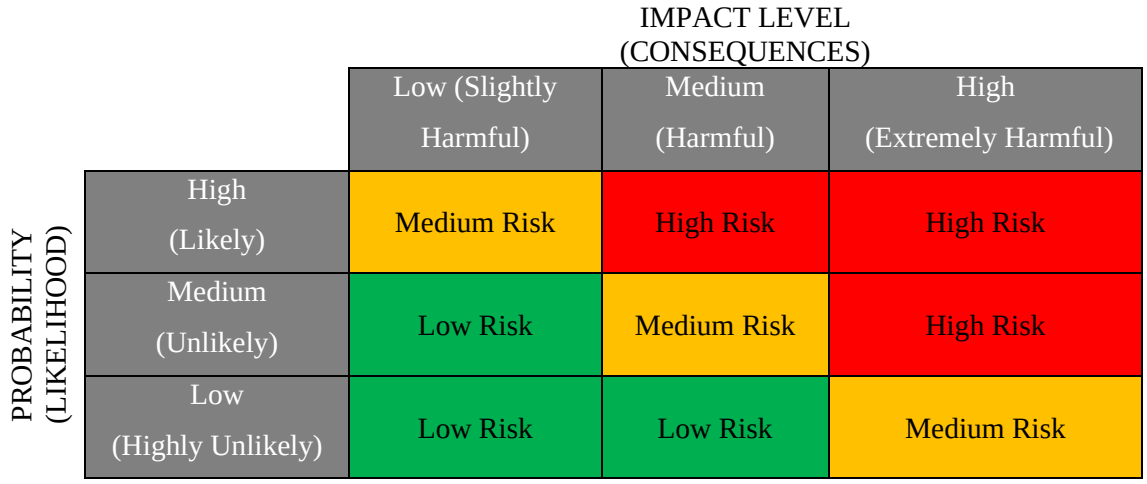

Figure 2. (3×3) Probability Impact matrix [69].

3. RESULTS AND DISCUSSION

3.1 Examination of Surface Activity

Screening of the selected bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* for biosurfactant production using the new proposed medium H exhibited a substantial increase in the surface activity when inoculated in the new proposed nutrient medium broth H after 72 hrs. The comparison between the effect of the biosurfactants produced by *Bacillus licheniformis* on the surface tension ST and

interfacial tension IFT during 72 hrs of incubation in the new proposed medium broth H is shown in Figure 3. The biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* showed the maximum surface activity after 24 hrs of incubation when grown in the new proposed nutrient medium H, since the surface tension ST was reduced from 71.81 mN/m to 24.13 mN/and 25.74 mN/m, respectively. Similarly, IFT was significantly reduced from 48.43 to 0.27 mN/m and 0.38 mN/m, respectively, by the same nutrient medium. Many authors confirmed the potential of *Bacillus licheniformis* and *Bacillus subtilis* to produce biosurfactants [36,70–75]. However, when the biosurfactants producing bacteria incubated in the new proposed medium H, the produced biosurfactants reached their maximum surface activity compared with all reported studies.

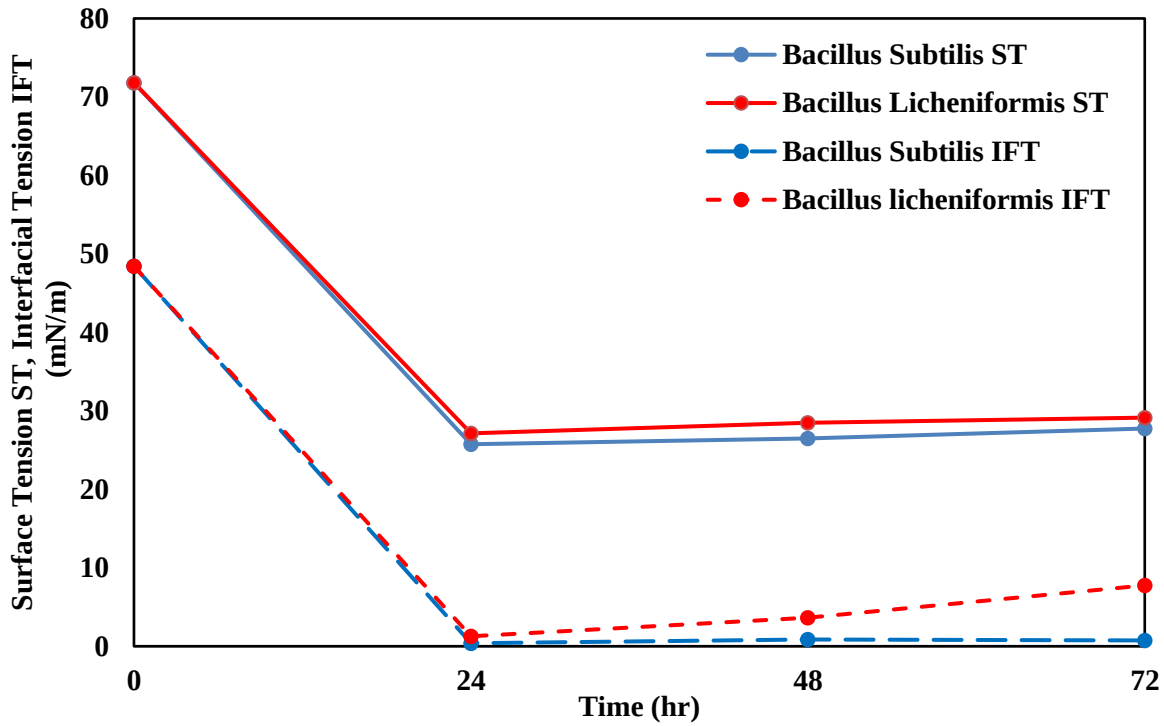

Figure 3. The comparison between the effect of the biosurfactants produced by *Bacillus licheniformis* on ST and IFT during 72 hrs of incubation.

3.2 Bacterial Growth Profile

Figure 4 shows the comparison between the growth profile of the selected bacterial strains during 72 hrs of incubation in the new proposed nutrient medium H. During 3 to 6 hrs of incubation, A lag phase was observed. After 24 hrs, exponential growth was observed. Finally, during the period of 24-72 hrs, the stationary phase and death phase was noticed. The maximum bacterial concentrations of the selected

bacterial strains *Bacillus licheniformis* and *Bacillus subtilis* were 2.072 x 10^9 CFU/ml and 2.237 x 10^9 CFU/ml (1 OD_{600} =8 x 10^8 CFU/ml), Respectively, before growth decreased and the optical density began to decrease.

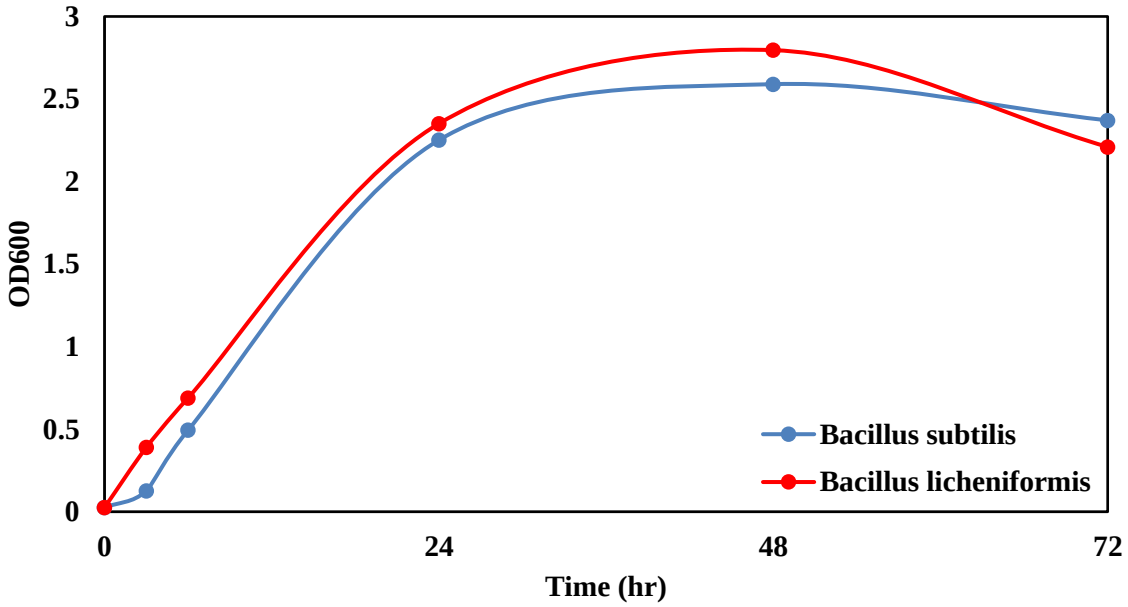

Figure 4. The comparison between the growth profile of the selected bacterial strains *Bacillus licheniformis and Bacillus subtilis* during 72 hrs of incubation.

3.3 Biosurfactant Yield and Critical Micelle Concentration

The yields of the biosurfactants produced from *Bacillus licheniformis* and *Bacillus subtilis* were determined to be 2.974 and 2.853 g/l, respectively. Pereira et al. (2013) reported that the produced surfactin by *Bacillus* isolate has a yield of 2.56 g/l after 3 g/l of yeast extract has been added to the culture media [59]. The extracted powder of biosurfactant was progressively dissolved in distilled water, and surface tension and interfacial tension against kerosene were measured. It was found that the biosurfactant produced by *Bacillus licheniformis* and *Bacillus subtilis* reached their maximum decrease in ST and IFT as shown in Figure 5, at biosurfactant concentrations of 0.04 g/l, and 0.03 g/l, respectively. It was also noticed that no change occurred even after adding more biosurfactants; therefore, these values were considered the critical micelle concentrations of the purified biosurfactants. These critical micelle concentrations are a little greater than the critical micelle concentrations reported by Cooper et al. (1981) and lower than the critical micelle concentrations reported by Makkar et al.

(1997), which were 0.023 and 0.160 g/l, respectively [72,76]. It was reported that the CMC values for biosurfactants utilized in microbial enhanced oil recovery were generally in the range of 0.001 to 2.0 g/l [77]. therefore, these produced biosurfactants are promising candidates for the microbial enhanced oil recovery process.

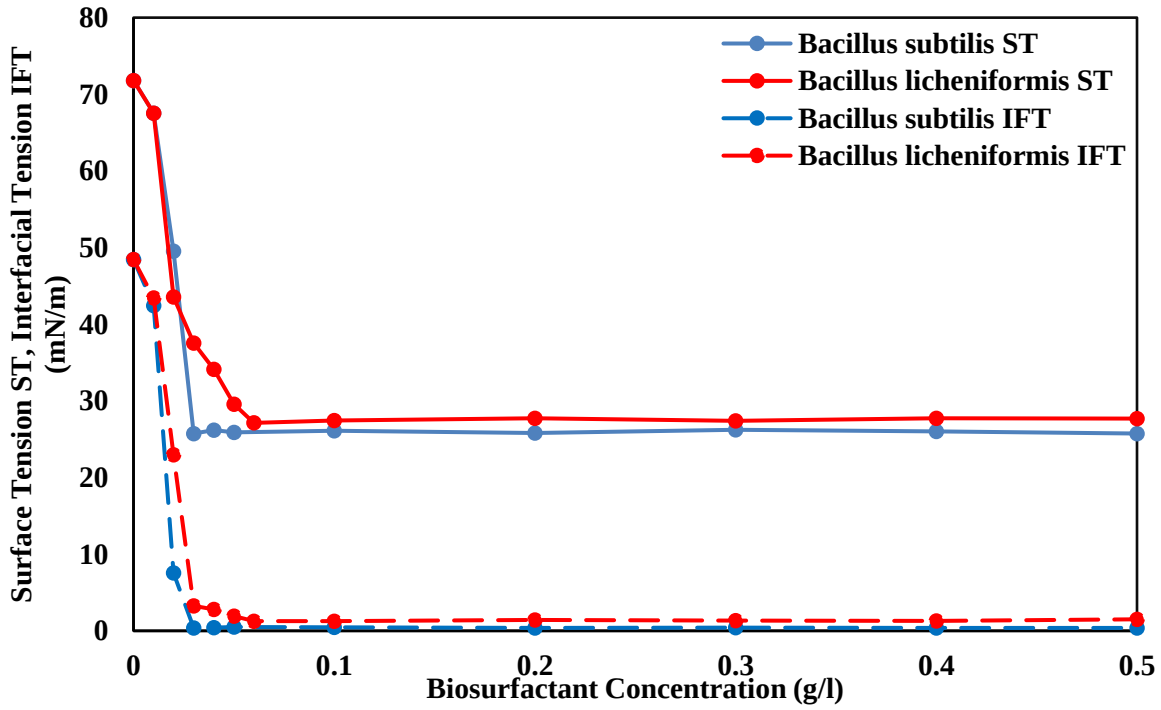

Figure 5. The comparison between the critical micelle concentration of the biosurfactants produced by *Bacillus licheniformis and Bacillus subtilis*.

3.4 Examination of Emulsification Activity

The produced biosurfactants emulsified hexane, heptane, hexadecane, kerosene, and crude oil with different values as shown in Figure 6. The emulsification indices of biosurfactants produced by *Bacillus licheniformis* against these different hydrocarbons were in the range of 47-61%, while the emulsification indices of biosurfactants produced by *Bacillus subtilis* against the same different hydrocarbons were in the range of 64-70%. Moreover, the produced biosurfactants show their maximum emulsification activity against kerosene, followed by Hexadecane, Heptane, Hexane, and crude oil. The length of the alkyl chain of hydrocarbons is the reason for the exhibited increase in emulsification activity against hexane, heptane, and hexadecane. While the emulsification activity of kerosene and crude oil varies based on the hydrocarbon's composition. Nitschke and Pastore (2006)

reported that *Bacillus subtilis* LB5 produced a biosurfactant on cassava medium, and this biosurfactant showed emulsification activity in the range of 67-71% against different hydrocarbons including kerosene, hexadecane, heptane, and crude oil [78]. Ali et al. (2019) reported that the biosurfactant produced by *B. Licheniformis* Ali5 shows emulsification activity against different hydrocarbons such as kerosene, heptane, diesel, tetradecane, tridecane, hexadecane, pristane, and crude oil in the range of 50-64% [79]. De Faria et al. (2011) reported that the produced biosurfactant by *Bacillus species* emulsified hydrocarbons including kerosene, benzene, hexadecane, diesel, and petrol in the range of 30-80% [80]. In summary, the biosurfactant produced by *Bacillus subtilis* showed higher emulsification activity than the biosurfactant produced by *Bacillus licheniformis*. However, the obtained findings confirmed that the biosurfactants produced by either *Bacillus licheniformis* or *Bacillus subtilis* effectively emulsified long-chain hydrocarbons like crude oil, which reveal their potential in improving oil recovery.

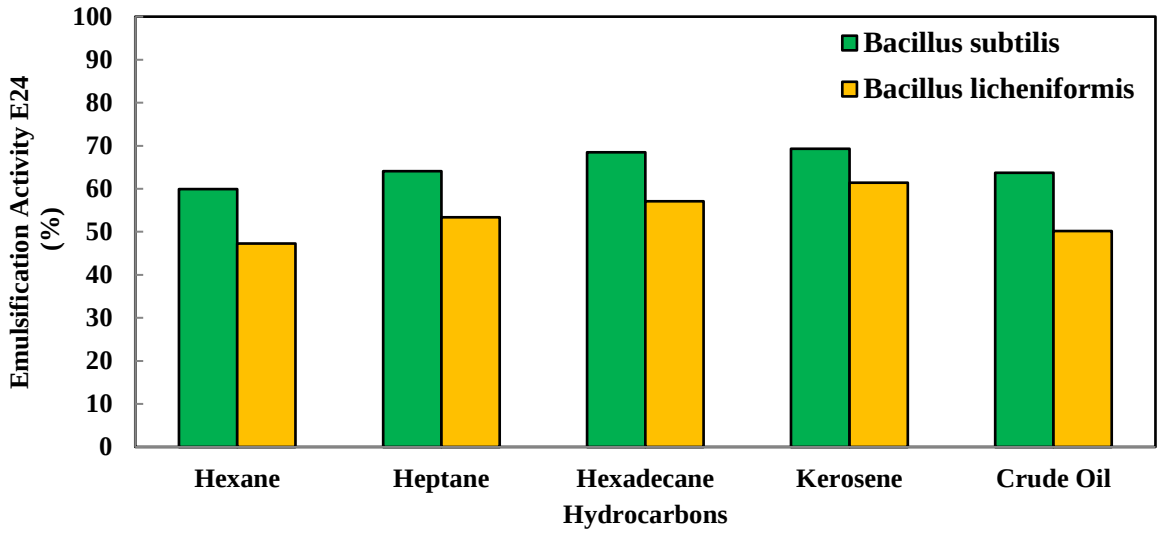

Figure 6. The comparison between the emulsification indices of the biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* against different hydrocarbons.

3.5 Stability Studies

The comparative stability studies of the biosurfactants produced by selected bacterial strains show that there are insignificant changes in surface activity and emulsification activity at different temperatures up to 100°C as shown in Figure 7. The obtained result is confirmed by several reported temperature stability studies [42,70,76]. Regarding the effect of salinity, there was a slight effect on surface activity

and emulsification activity, when the salinity increased up to 2% NaCl (w/v) as shown in Figure 8. However, the salinity effect was significantly noticed at NaCl concentrations range of 4–10%, then the surface activities were stabilized again at NaCl concentrations range of 12-20%. The optimum surface activity and emulsification activity were observed at pH 7 (neutral value), as well as no significant changes were noticed at pH values range 8-12 (alkaline range) as shown in Figure 9. These results are associated with several reports that approved the biosurfactant stability in an alkaline medium [42,71,81,82]. However, the surface activity was suddenly reduced in pH values range 2-4 due to settling out of the biosurfactants solution as a precipitate under acidic conditions (not soluble). Therefore, the biosurfactants lose their potential to increase the surface activity by decreasing of ST and IFT because of the occurrence of precipitation and structural distortion. The biosurfactant's behaviour under acidic conditions was described by Gudina et al. (2010). They reported the presence of negatively charged groups at the polar ends of the molecules could be causing the drop in surface activity [83]. however, the obtained results reveal that the produced biosurfactants could maintain more than 60% of their surface activity and emulsification activity and tolerate the oil reservoir's harsh conditions, which means they are promising candidates for MEOR.

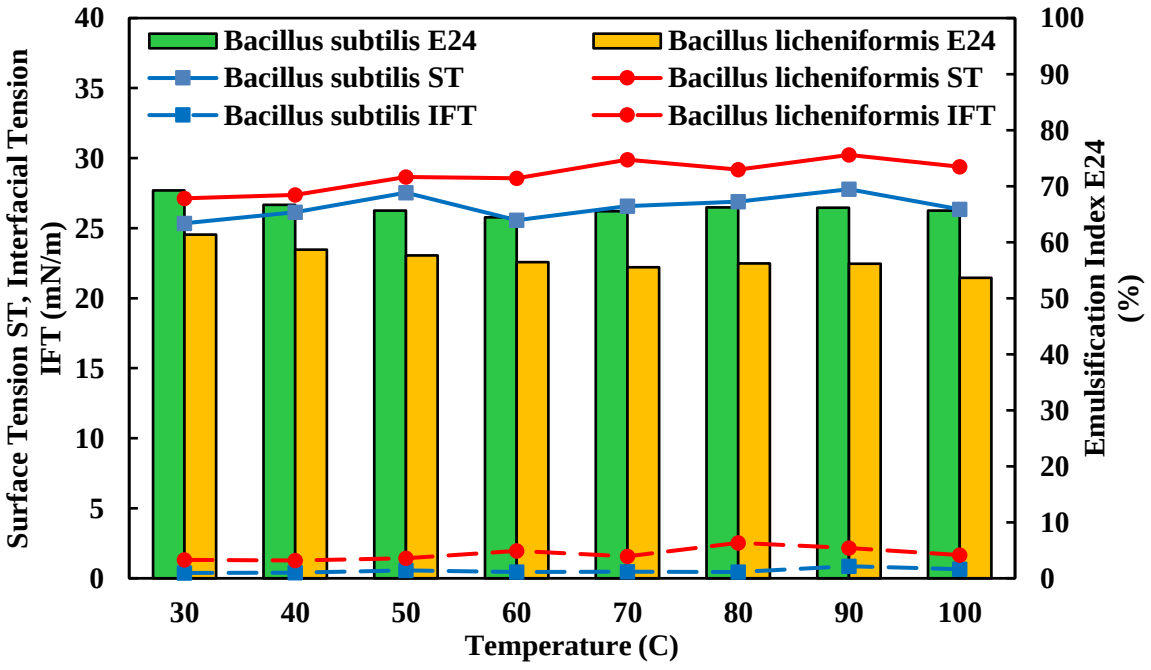

Figure 7. The comparison between the stability of biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* over a wide range of temperatures (30-100°C).

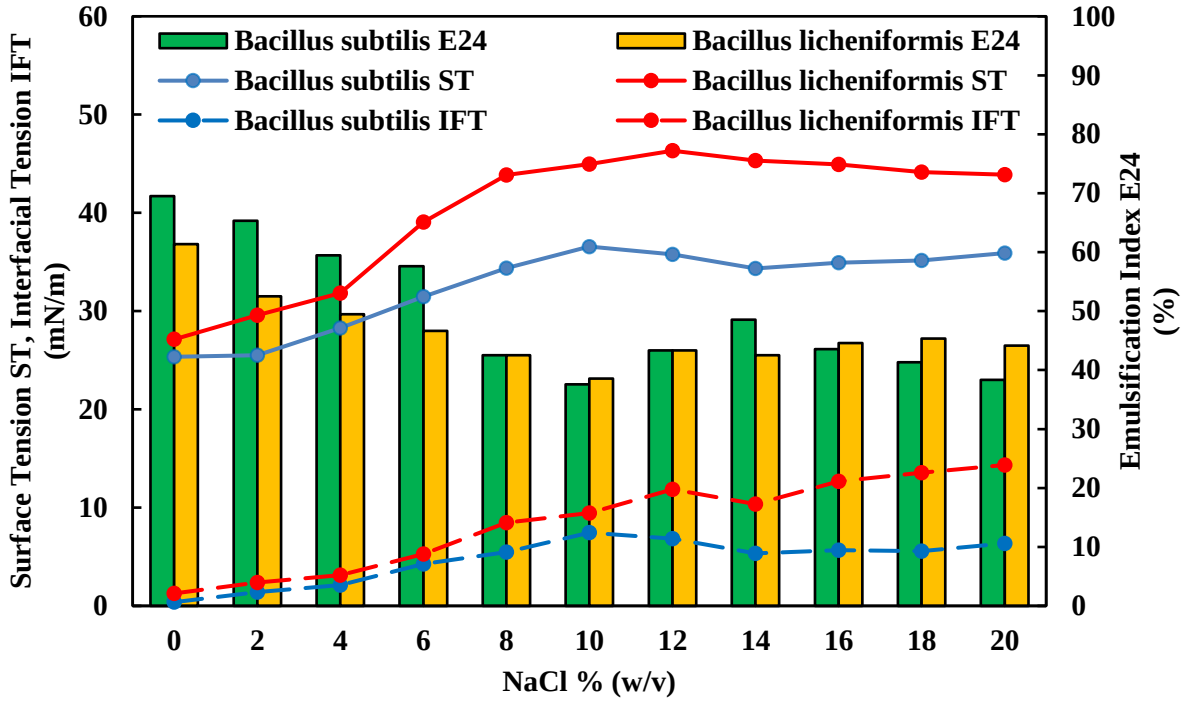

Figure 8. The comparison between the stability of biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* over a wide range of salinities (0-20% (w/v) NaCl concentration).

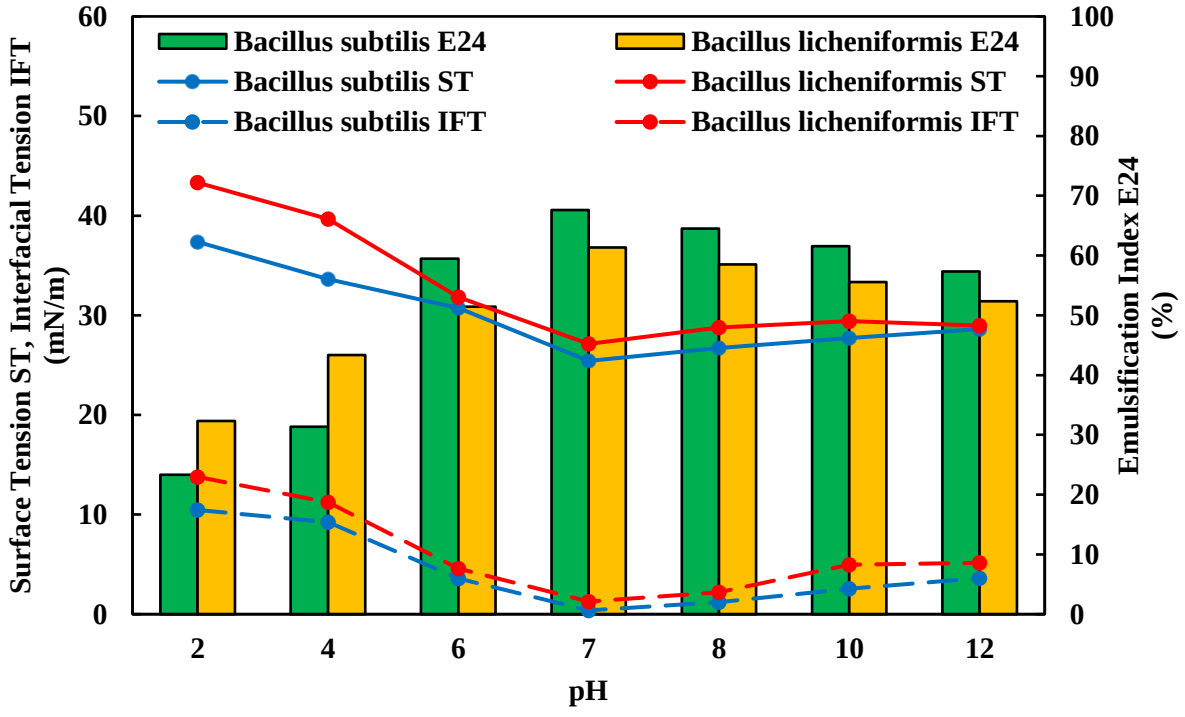

Figure 9. The comparison between the stability of biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* over a wide range of pH values (2-12).

The long-term thermal stability of the produced biosurfactants at high temperatures was examined at 60°C (temperature of fields of interest). the effect of time on the values of emulsion volume and emulsion stability of the aqueous solution containing the produced biosurfactant was shown in Figure 10 and Figure 11, respectively. It was found that no significant effect of high temperature on the emulsification activity of the biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis*, where they retained 62.41% and 70.62% of the original emulsion volume after 168 hrs of formation, respectively. Willumsen and Karlson reported that biosurfactants show a significant emulsification power when they could maintain at least 50 % of the original emulsion volume for 24 hours after formation [84]. Consequently, the long-term thermal stability study reveals that the produced biosurfactants are thermostable and have high emulsification power for stabilizing crude oil-formation water aqueous solution at high temperatures.

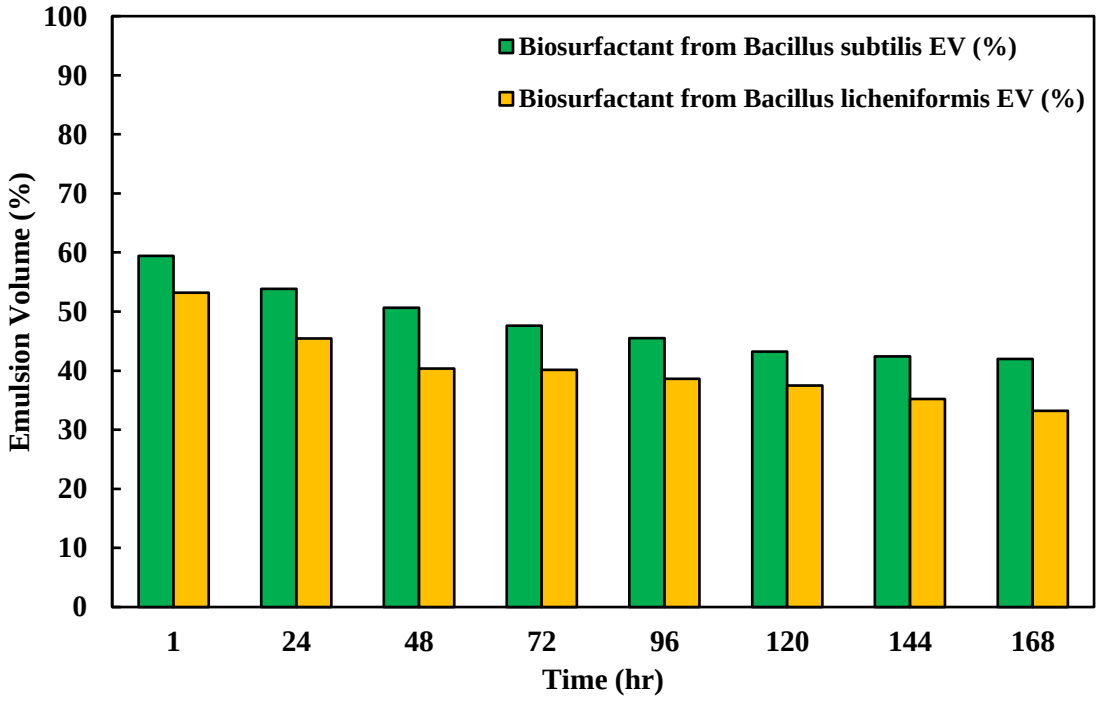

Figure 10. The comparison between the emulsion volume of aqueous solutions containing biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* at 60°C for a long-term time interval.

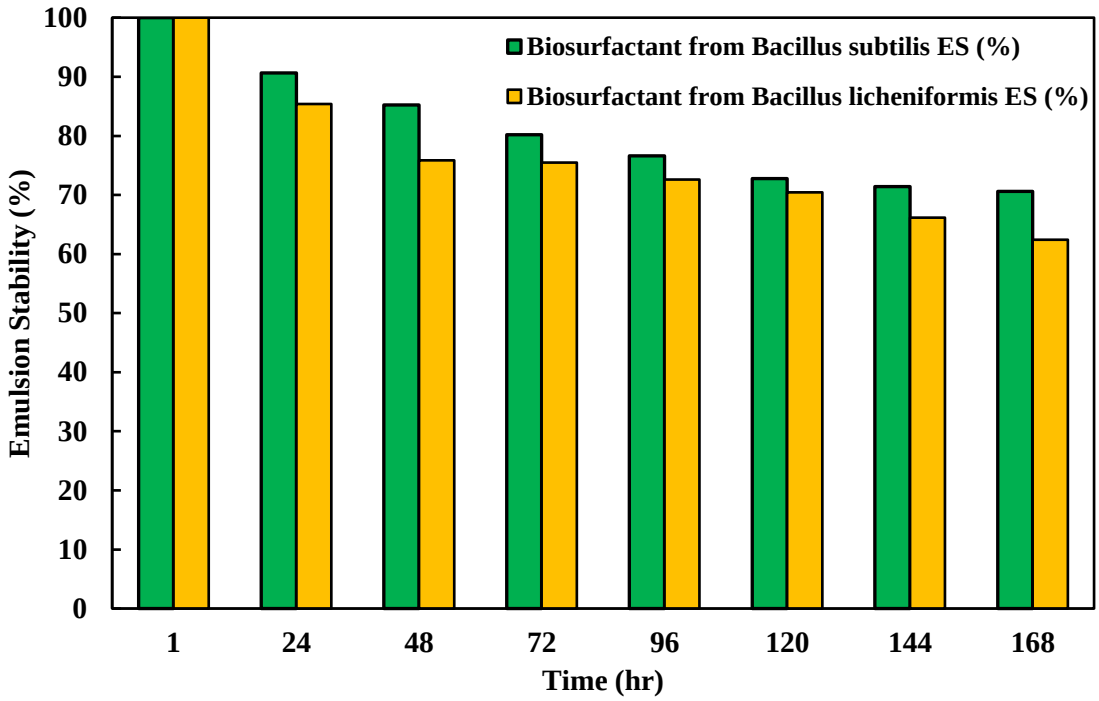

Figure 11. The comparison between the long-term thermal emulsion stability of aqueous solutions containing biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* at the temperature of the field of interest.

3.6 Core Flooding

The core flooding tests were conducted to examine the effectiveness of produced biosurfactants by *Bacillus licheniformis* and *Bacillus subtilis* in improving oil recovery. Figure 12 shows the water flooding as a secondary stage followed by biosurfactant flooding as a tertiary stage using biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis*. It was found that the oil recovery increased dramatically at the early stage of the water flooding, and after injecting 3 pore-volume PV of formation water, the water flooding stage reached the oil recovery rate plateau. However, no more oil recovered, even after injecting an additional 2 pore-volume PV of formation water.

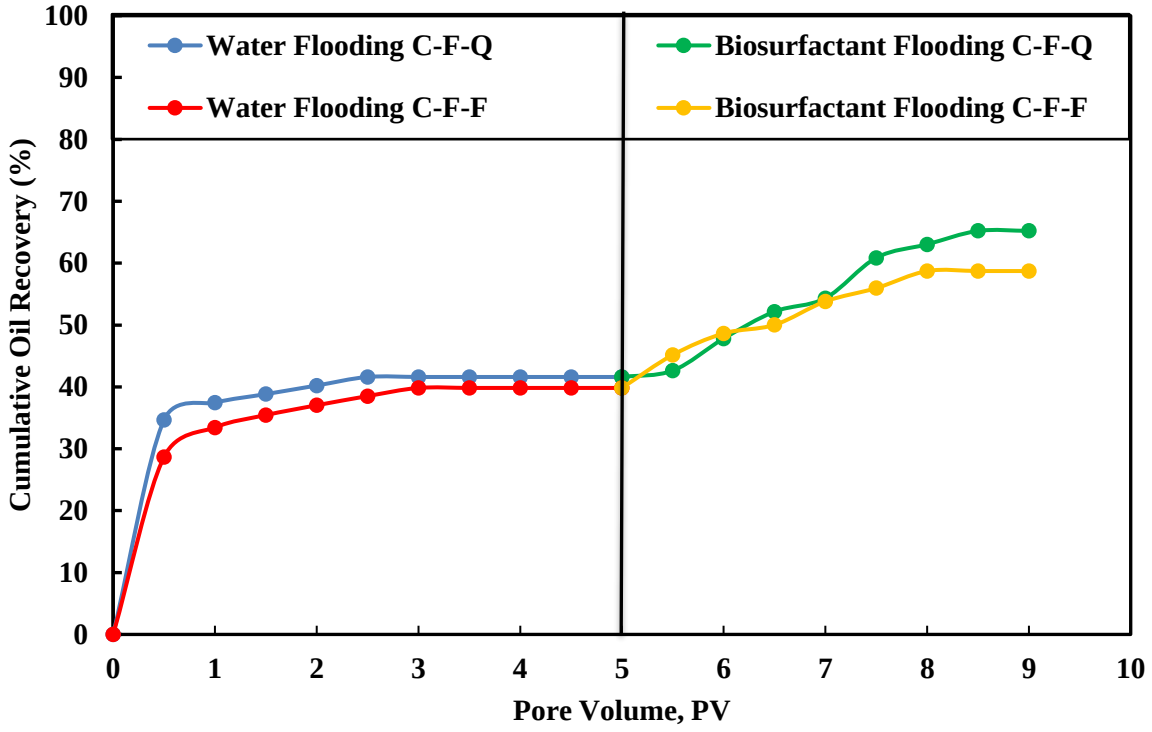

Figure 12. The comparison between the cumulative oil recovery of biosurfactants produced by Bacillus licheniformis and *Bacillus subtilis* during the water flooding and followed by biosurfactant flooding.

The water flooding stage recovered 40-43% of the original oil in place OOIP, whereas the biosurfactant flooding stage additionally recovered 31.41-39.35% of crude oil over S_{orwf} as represented in Table 6, after injecting 2-4 pore-volume PV of the cell-free biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis*, respectively. Furthermore, the obtained results reveal that the biosurfactants produced could increase the mobilization of the crude oil by reducing ST, IFT, and increasing E24. The decrease in IFT leads to an increase in capillary number, which is an essential factor in improving oil recovery. The residual oil saturation was decreased as the capillary number increased, allowing more oil to be extracted [85–88].

Table 6. The Results of Core flooding tests

Oil Field	AL QADR	AL FADL
Core ID	C-F-Q	C-F-F
Flow Rate, Q (cm3/min)	0.25	0.25
Pore Volume, PV (cm3)	9.24	8.24
IOIP (cm3)	4.60	4.40
S_{oi} (%)	49.78	53.42
S_{wi} (%)	50.22	46.58
S_{orwf} (cm3)	2.64	2.65

S_{orwf} (%)	28.55	32.14
OR_{wf} (%)	42.65	39.83
S_{orbf} (cm3)	1.60	1.82
S_{orbf} (%)	34.78	22.05
ORF_{bf} (%)	22.57	18.90
AOR_{bf} (%)	39.35	31.41

Al-Sulaimani et al. (2012) reported that *Bacillus subtilis* W19 biosurfactant produced improved oil recovery by producing 23% of additional oil at a flow rate of 0.5 cm^3/min using core flooding micromodel [51]. Al-Wahaibi et. al (2014) reported that the *Bacillus subtilis* B30 produced biosurfactant improved oil recovery of light and heavy crude oil by 26% and 31%, respectively, at a flow rate of 0.4 cm^3/min using core flood micromodel [54]. Souayeh et al. (2014) reported that *Bacillus subtilis* W19 produced a biosurfactant that additionally improve oil by 13-28% over S_{orwf} from sandstone core in a core flooding micromodel at a flow rate of 0.4 cm^3/min under 60°C [53]. Joshi et al. (2016) reported that the *Bacillus licheniformis* W16 produced a biosurfactant that was able to improve oil recovery by 24–26% over S_{orwf} at a flow rate of 0.4 cm^3/min using core flooding micromodel from sandstone core [56]. Liu et al. (2021) reported that *Bacillus licheniformis* L20 produced biosurfactant enhance oil recovery by 14.18% of additional oil from sandstone core in a core-flood study under 80°C at a flow rate of 0.5 cm^3/min [57]. All reported core flooding tests summarized in Table 2 recovered 6–31% of additional oil when injecting biosurfactants produced by either *Bacillus licheniformis* or *Bacillus subtilis* in sandstone cores [51,53,54,56,57]. In this study, the maximum additional oil recovery over S_{orwf} was achieved by the biosurfactants produced by *Bacillus licheniformis* that incubated in the new proposed medium H at a flow rate of 0.25 cm^3/min compared with all reported core flooding tests that used the biosurfactants produced by either *Bacillus licheniformis* or *Bacillus subtilis*. The comparison between the reported core flooding results and all reported core flooding tests study were summarized in Figure 13. It could be concluded that the produced biosurfactants are effective alternatives to synthetic biosurfactants and promising candidates for MEOR.

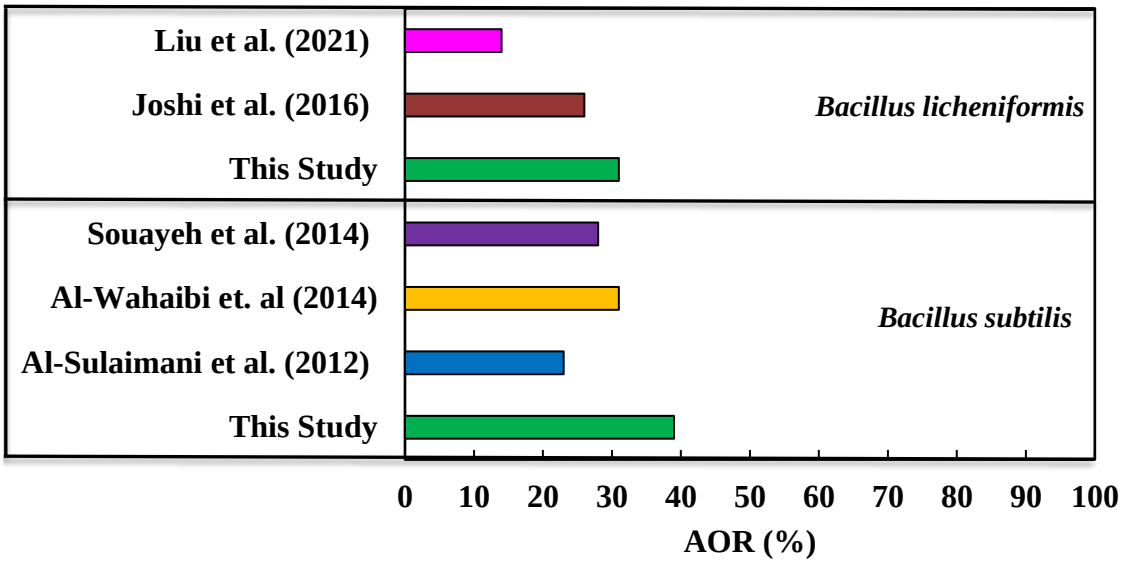

Figure 13. The comparison between the obtained result in this study and the results of reported core flooding studies using biosurfactants produced by either *Bacillus licheniformis* or *Bacillus subtilis*.

3.7 Environmental Risk Assessment

3.7.1 ***Bacillus licheniformis***

Bacillus licheniformis is a saprophytic bacteria found throughout nature that is hypothesised to aid in a nutrient cycle and has antifungal properties [89]. For more than a decade, it was utilised in the fermentation facility to produce antibiotics, proteases, amylases, and speciality chemicals, with no known detrimental effects on the environment or human health. This species can easily be distinguished from other pathogenic genus members in people and animals.

To see if any negative effects from *Bacillus licheniformis* have been reported, a series of literature searches were conducted. *Bacillus licheniformis* does not appear to be pathogenic to plants or estuarine marine organisms. Human infections with *Bacillus licheniformis*, on the other hand, occurred in immunocompromised people or after trauma. There have also been indications of a relationship between livestock abortions *and Bacillus licheniformis*. In most reported cases, few cases contributed to the affected animals' immunosuppression. Since *Bacillus licheniformis* is common in the environment and seems to be an unprincipled pathogen in cooperated hosts, the possible risk connected with using this microorganism in fermentation industries is in the low range.

Bacillus licheniformis does not release any extracellular enzymes that may make it susceptible to infection. *Bacillus licheniformis*, unlike numerous other microorganisms in the genus, does not produce toxins. Generally, *Bacillus licheniformis* has a low level of pathogenicity. Although human infection is not impossible, it is unlikely in an industrial environment where very immunocompromised persons are not present. Infection after trauma is a possibility, however in the industrial environment, with basic safety precautions, good laboratory procedures, and proper protective equipment (PPE) including protective clothes, masks, and safety goggles, the risk of employees becoming infected should be in the low range.

Similarly, the risks of *Bacillus licheniformis* use to the environment are in the low range. *Bacillus licheniformis* is suspected to be a reason for livestock abortion. However, these hypotheses have yet to be proven correct in proving that this bacterium was the causative agent. However, *Bacillus licheniformis* infections in most of these cases occurred in animals that were already immunocompromised due to infection with other pathogens or inadequate nutrition. Immunosuppression is linked to maternal and foetal placentas in pregnant animals, allowing opportunistic bacteria to infect and cause lesions in the foetus. Even though *Bacillus licheniformis* is not the cause of animal abortion, it has been linked to several cases. Despite this, the number of abortions caused by *Bacillus licheniformis* is insignificant compared with the overall number of livestock abortions caused by all other microbes, including fungi and viruses.

The utilization of *Bacillus licheniformis* for the industrial production of enzymes could not be harmful to the environment because the amount of microorganisms released by the fermentation facility is insignificant. Furthermore, *Bacillus licheniformis* is common in the environment, and the expected releases from fermentation industries under these exemption terms would not considerably expand the population of this microorganism in the environment. Consequently, even if *Bacillus licheniformis* might be linked to livestock abortions, utilization of this bacterium in fermentation industries could not significantly raise this occurrence possibility. Even if a high-exposure scenario of releasing *Bacillus licheniformis* from the fermentation industry to livestock might be imagined.

493 It could be concluded *Bacillus licheniformis* is safe to employ in fermentation industries to produce
494 biosurfactants, enzymes, and speciality chemicals. Bacillus licheniformis, while not fully harmless,
495 poses a low risk of harming the environment or human health. As a result, the utilization of *Bacillus*
496 *licheniformis* in the production of biosurfactants is justified without creating any harm. As shown in
497 Table 7, the likelihood of risk occurrence is performed to evaluate the risk occurrence probability and
498 its impact on positive events, as well as to reduce the chance of risk occurrence and negative events in
499 a project. Table 8 and Figure 14 represent the risk matrix rating provided in specific for this study.

500 Table 7. Probability of occurrence of *Bacillus licheniformis*.

Descriptor	Frequency
Low	• The infection could happen to someone who is immunocompromised or after a traumatic event. • Could only happen as a result of a series of separate system or control failures. • Occurrence is extremely unlikely, and no poisons are produced. • There has never been such an incidence. • An environmental hazard is unlikely to occur because the amount of microorganisms emitted from the fermentation facility is in the low range • Would most likely occur as a result of a high amount of exposure during the fermentation process. • Infection reports similar to this have been reported in the past.
Medium	• Could be caused by predisposing factors in the affected animals' immunosuppression. • It may happen in animals who are already immunocompromised due to infection by other bacteria or inadequate nutrition. • The number of reports concerning animal abortion is quite low.
High	• Occurrence is quite unlikely.

501 Table 8. Risk rating matrix of *Bacillus licheniformis*

Risk Code	Risk Description	Probability (Likelihood)	Consequences (Impact level)
R1	Environment	Low	Low
R2	Human Health	Low	Low
R3	Water	Low	Low
R4	Hazard to animal	Low	Medium
R5	Air	Low	Low
R6	Soil	Low	Low
R7	Worker Exposure	Low	Low
R8	Hazard to plant	Low	Low
R9	Virulence	Low	Low

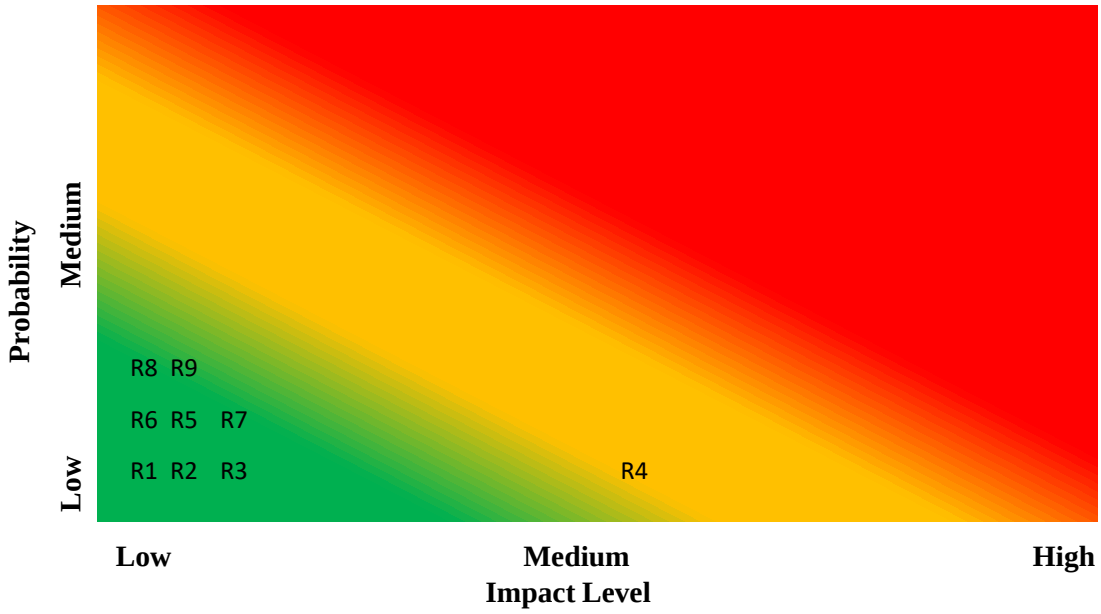

Figure 14. Risk assessment chart of *Bacillus licheniformis*.

3.7.2 Bacillus subtilis

Bacillus subtilis is a saprophytic soil microorganism that is considered to have a role in nutrient cycling due to its potential of producing several kinds of enzymes. For more than a decade, this property of the microorganism has been economically exploited, and it has been used to produce antibiotics, proteases, amylases, and speciality chemicals. The US Environmental Protection Agency (EPA) investigated the production of the enzymes by genetically modified *Bacillus subtilis* in fermentation facilities and found no unacceptable risks to the environment or human health. It is not pathogenic and does not have disease-causing characteristics [65].

Historically, the term *Bacillus subtilis* was used to refer to all endospore-forming aerobic bacilli bacteria. Later, *Bacillus subtilis* was taxonomically grouped with two closely similar species, *Bacillus licheniformis* and *Bacillus pumilus*, to form what was called the "*subtilis* group". Recently, *Bacillus subtilis* can be distinguished from these other species, due to the development of new methods. Even though it is not a frank human pathogenic microorganism, it is isolated in various cases from human infections [65,90].

The Infections linked to *Bacillus subtilis* include endocarditis, septicaemia, bacteraemia, pneumonia, and bacteraemia. Nevertheless, these reported infections were detected in immunocompromised

patients cases. Before infection with *Bacillus subtilis* could occur, the host must be immunosuppressed and then inoculated in large numbers. Several cases of food poisoning have also been linked to excessive amounts of food contaminated by *Bacillus subtilis*. The amounts of extracellular enzymes or other components produced by *Bacillus subtilis* are not that large to make it susceptible to infection. *Bacillus subtilis* is not toxigenic, unlike the other several species in the genus. *Bacillus subtilis* produces the subtilisin (extracellular enzyme), which was linked to allergy or hypersensitive reactions in people who have been exposed to it frequently [65].

Workers should have a low risk of infection in an industrial environment if the basic safety precautions, suitable laboratory practices, and proper protective equipment (PPE), including protective clothes, masks, and safety goggles are used, as described in Table 9. The likelihood of allergic reactions from prolonged exposure to subtilisin is the only human health hazard for fermentation industry workers. Generally, Bacillus subtilis has a low level of pathogenicity [65,91]. However, the infection in humans is not impossible, it is unlikely in an industrial environment where bacterial exposure is believed to be in the low range if immunocompromised workers are not present.

Table 9. Probability of occurrence of *Bacillus subtilis*

Descriptor	Frequency
Low	• The infection could happen to immunocompromised people or people who have been exposed to the bacteria. It has a low pathogenicity level. • Could only happen as a result of a series of separate system or control failures. • Occurrence is extremely unlikely, and no toxins are produced. • There has never been such an incidence. • An environmental hazard is unlikely to occur because the amount of released microorganisms from fermentation facilities is in the low range • The risk of allergic responses or hypersensitivity with a high degree of exposure is low, which is a concern for personnel in the fermentation facility. • There have been similar instances of food poisoning linked to many Bacillus subtilis contaminated foods.
Medium	• Could be caused by predisposing factors in the affected animals' immunosuppression. • It may happen in animals who are already immunocompromised due to infection with other microorganisms or inadequate nutrition. • There aren't many comparable reports concerning livestock abortion.
High	• Occurrence is quite unlikely.

Similarly, the environmental risks linked to the utilization of *Bacillus subtilis* are in the low range. There have been various reports linked *Bacillus subtilis* to livestock abortions [92]. Nevertheless, these few reported cases show that this linking was rare, and in most cases the animals were immunocompromised. *Bacillus subtilis* is also not a causative agent and is not classified as an animal pathogen [93]. Similarly, Bacillus *subtilis* isn't considered a plant pathogen. Even though some of their produced enzymes like cellulose and polygalacturonase are linked sometimes to cause soft rot in plant tissue, several microorganisms can cause soft rot when inoculated below the layers of protective epidermal. Unreasonable risk should not be posed to the environment or human health when utilising *Bacillus subtilis* in the industrial environment as evaluated in Table 10. Firstly, *Bacillus subtilis* poses a low risk to the environment or human health. Secondly, the amount of microorganisms released by the fermentation facility is insignificant. Furthermore, *Bacillus subtilis* is common in the environment, and the fermentation industry expected released amount will not considerably expand the bacterium's population in the environment.

Table 10. Risk rating matrix of *Bacillus subtilis*

Risk Number	Risk Description	Probability (Likelihood)	Impact level (Consequences)
R1	Environment	Low	Low
R2	Human Health	Low	Low
R3	Soil	Low	Low
R4	Hazard to animal	Low	Low
R5	Food poison	Low	Medium
R6	Worker Exposure	Low	Low
R7	Hazard to plant	Low	Low
R8	Water	Low	Medium
R9	Virulence	Low	Low

Currently, there are no available specific data approving the ability of released *Bacillus subtilis* to survive in the atmosphere. Due to point source releases, fermentor off-gas released air could cause nonoccupational inhalation exposures. The soil is considered the natural habitat of Bacillus subtilis. Consequently, long-term survival in soil could happen.

Finally, the risk associated with utilizing *Bacillus subtilis* to produce biosurfactants or speciality chemicals in fermentation industries is in the low range. Even though it is not harmless, the industrial

utilization of *Bacillus subtilis* poses a low risk of harm to the environment or human health, and that was shown in Figure 15.

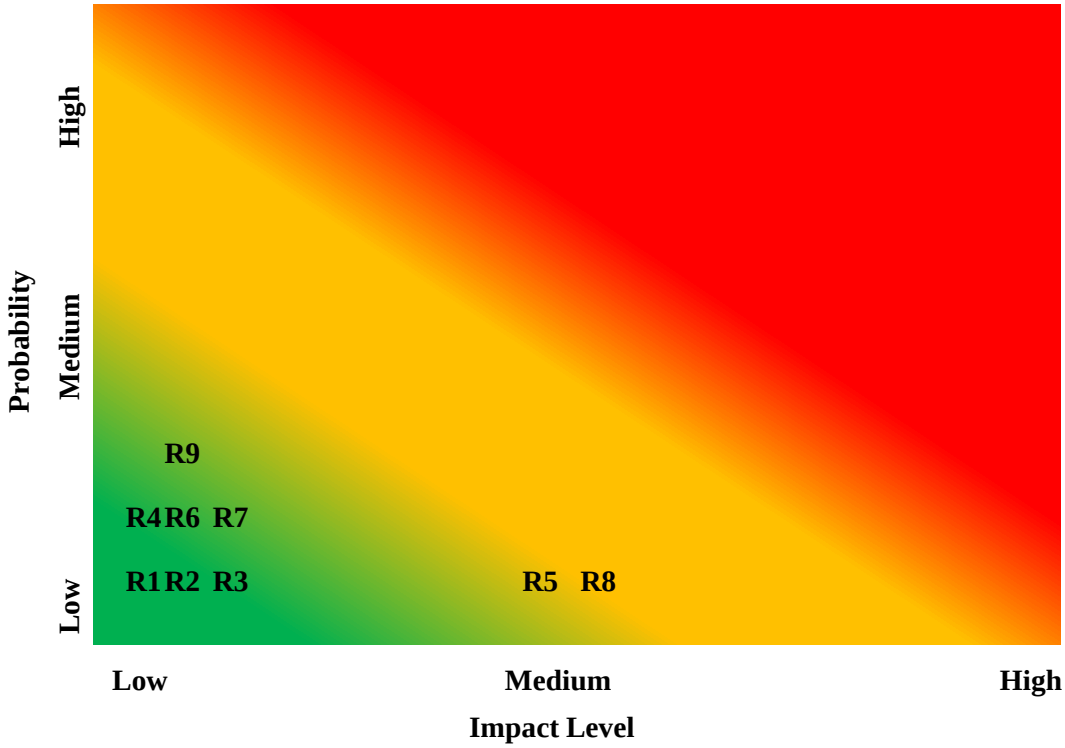

Figure 15. Risk assessment chart of *Bacillus subtilis*

However, if a risk is likely to occur, a mitigation plan should be implemented to avoid the risk to prevent and mitigate the possibility of such risk. This planned response is summarized in Table 11 as a planned response against any possible risk of producing biosurfactants from selected indigenous bacterial strains.

Table 11. Mitigation Plan

Risk Probability	Mitigation Plan
Low	• Basic safety precautions, suitable laboratory practices, and proper protective equipment (PPE), including protective clothes, masks, and safety goggles could provide the necessary protection against impact hazards. • Actions of Reducing the risk are unlikely to be cost-effective.
Medium	• Avoid high levels of inhalation exposure that can result from breathing air that could be contaminated with the source of the release. • Actions of risk reduction will be roughly cost-neutral.

4. CONCLUSIONS

In summary, *Bacillus licheniformis* and *Bacillus subtilis* are indigenous bacterial strains isolated from crude oil samples obtained from Egyptian oil fields located in the Western Desert. These selected bacterial isolates *Bacillus licheniformis* and *Bacillus subtilis* produced effective biosurfactants after 24 hrs of incubation in the new proposed medium H, where the surface tension of water decreased from 71.8 mN/m to 27.13 mN/m and 25.74 mN/m, and similarly, the interfacial tension of water against kerosene decreased from 48.4 mN/m to 1.27 mN/m and 0.38 mN/m at critical micelle concentration of 0.06 g/l and 0.04 g/l, respectively. The emulsification activity of biosurfactants produced by *Bacillus licheniformis and Bacillus subtilis* show a significant emulsification activity against long-chain hydrocarbons, which are 50.2% and 63.7%, respectively. Moreover, the stability studies show insignificant changes in the surface and emulsification activity of produced biosurfactants at high temperatures up to 100^{o}C for a long period, and more than 60% of the surface activity and emulsification activity of produced biosurfactants were retained over a wide range of salt concentration up to 20% (w/v) NaCl, and pH values range 5-12. In addition, core flooding tests by biosurfactants produced by *Bacillus licheniformis* and *Bacillus subtilis* resulted in 31.41% and 39.35% of additional oil over the water flooding residual oil saturation, respectively. Besides the beneficial effects of the selected indigenous bacterial strains in producing effective biosurfactants, the performed environmental risk assessment reveals that it could be an outstanding tool to be used in enhanced oil recovery schemes and could lead to promoting environmental sustainability.

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