Biosurfactant Production using Egyptian Oil Fields Indigenous Bacteria for Microbial Enhanced Oil Recovery

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ABSTRACT

Biosurfactant production is one of the most efficient mechanisms in microbial enhanced oil recovery (MEOR) processes. This work investigates the production of biosurfactants by indigenous bacteria isolated from Egyptian oil fields, and how to optimize these produced biosurfactants for MEOR. 59 Egyptian oil reservoirs were screened to evaluate the potential applicability of MEOR processes, based on their rock and fluid properties. Results showed that 8 reservoirs from the Gulf of Suez and 3 reservoirs from the Western Desert had the potential to MEOR. Combined analysis of morphological, and biochemical characterization was performed on the 11 bacterial strains isolated from different crude oil samples collected from the reservoirs that have the potential to MEOR process to identify their types. Bacillus spp, a bacilli species that can produce biosurfactants, was selected for further studies. To optimize the surface activity of the produced biosurfactant, ten different reported nutrient media, and a new proposed nutrient media were examined. *Bacillus spp* has shown the ability to produce a very active biosurfactant that reduced the surface tension of water from 71.8±1.9 mN/m to 25.7±1.2 mN/m,

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and the interfacial tension of water against kerosene from 48.4±2.1 mN/m to 0.38±0.07 mN/m at Critical Micelle Concentration (CMC) of 0.04±0.01 g/l, in a medium supplemented by the new proposed nutrient medium H. The growth rate of Bacillus spp was studied, and it was found it reached its maximum (OD600nm 2.59±0.16) after 24 hours of incubation. Biosurfactant production has no significant change in its surface activity over a wide range of temperature range up to 120ºC, which means the studied species *Bacillus spp* is a thermophilic bacterium. *Bacillus spp* grew well in the presence of high salt concentration up to 20% (w/v) NaCl, the optimal surface activity was obtained in the range of 0-2% (w/v) NaCl, and at pH 7. The emulsification activity of the produced biosurfactant was examined, and it reached the maximum (69.6±1.5%) against kerosene at temperature 25ºC, Salinity 0% (w/v) NaCl (distilled water), and pH 7. The produced biosurfactant was purified and extracted by the acid precipitation method, and the biosurfactant yield of the purified compound was found to be 2.8±0.3 g/l. Finally, the core-flooding experiments were conducted to investigate the effect of produced biosurfactants by *Bacillus subtilis* in oil recovery. The obtained results reveal the potential of *Bacillus spp* to grow in the new proposed medium H and produce effective and efficient biosurfactants that enhanced oil recovery by 25.19-39.35% of additional oil over the water flooding residual oil saturation in the studied cores and retain more than 60% of its surface activity under harsh conditions and that are relevant to Microbial Enhanced Oil Recovery, MEOR.

KEYWORDS

Microbial Enhanced Oil Recovery; Indigenous Bacteria; Biosurfactant; Bacillus Subtilis; Surface Activity; Critical Micelle Concentration

List of Abbreviations:

MEOR, Microbial Enhanced Oil Recovery; CMC, Critical Micelle Concentration; EIA, Energy Information Agency; EOR, Enhanced Oil Recovery; WD, Western Desert; BHMS, Bushnell Hass Mineral Salts; ST, Surface Tension; IFT, Interfacial Tension; OD, Optical Density; E_{24}, emulsification index; BV; Bulk Volume; PV, Pore Volume; OOIP, Original oil in place; RF, Recovery Factor; AOR, Additional Oil Recovery.

50 **1. INTRODUCTION**

51 The US Energy Information Agency (EIA) predicts that hydrocarbons will remain the primary energy
52 source to satisfy the surging energy demands in the near future. However, due to the high demand for
53 hydrocarbon and the lack of exploration of new massive hydrocarbon reservoirs, optimization of the
54 existing reserves to improve their recovery could be the solution to this problem (Gouda et al., 2021;
55 Haghighi et al., 2020; Shahat et al., 2021). Microbial Enhanced Oil Recovery MEOR is a cost-effective
56 and eco-friendly technique that exhibits many advantages comparing with the other conventional
57 Enhanced Oil Recovery EOR techniques where it does not need huge amounts of energy, like thermal
58 techniques, and it does not depend on the oil price, like many chemical techniques. On other hand,
59 MEOR could be easily applied after simple adjustments to the existing field facilities, and also it can
60 produce up to 50% of the remaining oil (Mahran et al., 2019, 2018; Omoniyi, 2015).

61 The concept of using bacteria to improve oil recovery is not a new issue. In 1926, J. W. Beckman made
62 a breakthrough in terms that he proposed developing a method to enhance oil recovery based on the use
63 of bacteria. The actual laboratory experiment was performed by Zobell in 1946 to confirm Beckman's
64 hypothesis. The first field trial of MEOR was performed in Arkansas, the USA in 1954 by Lisbon field
65 Union County. There was an interest fluctuation in terms of MEOR as in the 1970s this area of research
66 lost its interest. In 1995, more than 322 MEOR projects were conducted in the USA and it has been
67 proven that 81% of the projects effectively improved oil recovery, and there was no reduction in oil
68 production at any project. In the present decade, many field tests have been applied in many countries
69 such as the USA, Russia, Germany, Malaysia, China, Romania India, Norway, UK, Venezuela, China,
70 Iran, and Trinidad (Omoniyi, 2015), (Cooper et al., 1981).

71 Many bio-products could be obtained from bacteria such as biosurfactants, biopolymers, bioacids, and
72 biogases, and bioalcohols. Biosurfactants can improve the mobility of trapped oil because they are
73 surface-active materials that reduce the interfacial tension between crude oil and water, and wettability
74 alteration. Biopolymers can improve the mobility ratio and sweep efficiency because they increase water
75 viscosity which will lead to a decrease in its mobility(AN et al., 2018; Attia M and Musa, 2015; Soliman
76 et al., 2020). Bioacids can dissolve some rock and clean its pores; hence, it escalates the porosity and

77 permeability. Biogas has two functions, which are decreasing the oil viscosity and increasing the
78 reservoir pressure (Omoniyi, 2015).
79 Biosurfactant production is one of the most efficient mechanisms in MEOR processes. Biosurfactants
80 are surface-active agents produced by distinct groups of bacteria. Biosurfactants reduce surface tension
81 and interfacial tension and increase the mobilisation of residual oil. The interfacial tension between two
82 immiscible phases is caused by the interaction force between the molecules of the two phases at the
83 interface (Al-Anssari et al., 2020). Biosurfactants can aggregate at interfaces between fluids having
84 different polarities, such as water and oil, leading to the reduction in interfacial tension. Several authors
85 have reported how reduced interfacial tension IFT has a positive effect on capillary forces, and oil
86 recovery (Al-Anssari et al., 2021, 2019; Ali et al., 2017, 2015; Haghighi et al., 2020). Because of their
87 efficiency in lowering interfacial tension, biosurfactants have been employed for the enhancement of
88 oil production especially in microbial enhanced oil recovery (Aiad et al., 2015).
89 Surfactants are generally classified into four categories, which are anionic, cationic, amphoteric, and
90 non-ionic surfactants. Among these types, anionic surfactants are widely used in EOR processes due to
91 their lower adsorption on reservoir rocks (sandstones) as compared to other types of surfactants. When
92 a surfactant is dissolved in water, its molecules form aggregates called micelles. Each surfactant
93 molecule is composed of a non-polar moiety (lyophilic) and a polar moiety (hydrophilic) (Lazar et al.,
94 2007)**.** Surfactants have shown promising results in reducing the interfacial tension and altering the rock
95 wettability (Jha et al., 2020, 2019). However, due to the risks of oil spills and injection fluid leakage,
96 injection of synthetic surfactants into oil wells may be toxic to shallow waters in onshore locations and
97 marine environments in offshore locations (Nazarahari et al., 2021).
98 The significance of biosurfactants in MEOR is based on many factors. Biosurfactants can reduce the
99 interfacial tension, they are less adsorbed on the rock surface, and they have low toxicity,
100 biodegradability, and cost-effectiveness. Several bacteria could produce several types of biosurfactants
101 such as glycolipids, phospholipids, fatty acids, amino-acid-containing compounds (i.e. proteins and
102 lipopeptides) and neutral lipids. The production of biosurfactants is affected by the bacterial strain, and
103 the fermentation conditions, e.g. nutrient composition, temperature, pH, and presence of metal ions.
104 Many biosurfactant-producing bacteria require oxygen for growth and are unsuitable for in-situ

105 production. Recently, however, microorganisms that produce surfactants under anaerobic conditions
106 have been isolated. several studies indicate that the production of relatively large amounts of
107 biosurfactant within the reservoir is feasible, hence the possibility to use biosurfactant instead of
108 synthetic surfactant could be applied to enhance oil recovery and reduce the production cost (S.
109 Mukherjee et al., 2009).
110 Selected bacteria for biosurfactant production applications must meet the most important requirement,
111 which is the ability to survive and produce biosurfactants in the reservoir. Ideal candidates that meet
112 these requirements are the indigenous bacteria in the reservoir. Indigenous bacteria have a selective
113 advantage over exogenous bacteria because they are adapted to the reservoir conditions. The main
114 adaptations that bacteria must have are high tolerance to high temperatures, salinity, as well as be active
115 under anaerobic conditions (Al-Maghrabi et al., 1999; Lazar et al., 2007; Omoniyi, 2015). Indigenous
116 microbial community structures in oil reservoirs are expected to vary as each reservoir is different in
117 terms of depth, temperature, pressure, salinity, and other characteristic features. Most studies exploring
118 microbial communities use culture-based methods to recover and identify individual microbial isolates
119 and do not provide complete information on how these communities are structured (Lazar et al., 2007).
120 However, these bacteria are likely introduced into the reservoir during drilling and water flooding.
121 Bacteria could be categorized based on their oxygen intake into three types, aerobic bacteria (oxygen is
122 needed for growth), strictly anaerobic bacteria (no need for oxygen for growth), and facultative
123 anaerobic bacteria (can grow either in the presence or absence of oxygen). Successful field trials
124 commonly use anaerobic or facultative anaerobic bacteria, because they have a small cell size that makes
125 it easier for them to move through the porous media of petroleum reservoirs. They can tolerate the harsh
126 conditions that could exist in the petroleum reservoir such as temperature, salinity, pressure, and pH,
127 and they produce useful metabolic bio-products. The required bacterial species for MEOR could be
128 isolated from many sources. Depending on the place of extraction, bacteria sources are divided into
129 indigenous, which is from the reservoir itself, and exogenous, which is from external sources (Lazar et
130 al., 2007; Omoniyi, 2015).
131 Nutrients are considered the major expense in MEOR projects because they could cost almost 30% of
132 the total cost of the project. A successful MEOR project requires selecting a suitable nutrient in terms

133 of types, concentrations, and the nutrients components supplemented for bacterial growth and
134 metabolism of the bacteria. Furthermore, it was noticed that there is a relation between nutrient
135 concentrations and bacterial growth rate (Lazar et al., 2007; Omoniyi, 2015). For bacterial growth,
136 nutrients must contain organic carbon sources, nitrogen sources, and salt sources. These nutrients are
137 usually transported in the aqueous phase. Fermentative bacteria usually use molasses, glucose, or
138 sucrose as nutrients (Donaldson, 1991). Generally, molasses has been used as a carbon source in many
139 MEOR field applications, since it is cheap and provides vital vitamins and minerals (Bryant et al., 1994),
140 (Makkar and Cameotra, 1997), (Joshi et al., 2008b).
141 The selection of nutrients is important because the types of bio-products that are also produced by
142 several types of bacteria are dependent on the types, concentrations, and components of the nutrients
143 provided. Generally, molasses has been used in many field applications as a carbon source because of
144 its low price and presence of essential minerals and vitamins. Updegraff and Wren (1954) were
145 considered the first users of molasses as a substrate. Furthermore, some bacteria use oil as a carbon
146 source, which is excellent for heavy oil production, since it can reduce the carbon chain of heavy oil
147 and increase the quality (Cooper et al., 1980). Under anaerobic conditions, however, the use of
148 petroleum components as food is thought to be not effective at least within a period required for
149 economic recovery. Even though growth can occur, the growth can be very slow and hardly detected
150 for several months (Moses et al., 1993).
151 In this study, 59 Egyptian oil field reservoirs were screened to evaluate the potential applicability of
152 MEOR processes, based on their main properties (reservoir temperature, salinity, reservoir depth, rock
153 permeability, API gravity, and crude oil viscosity). Combined analysis of morphological, and
154 biochemical characterization was performed on the 11 bacterial strains isolated from different Egyptian
155 crude oil samples to identify their types. To optimize the surface activity of the produced biosurfactant,
156 bacterial strains were grown in 10 different reported nutrient media, and a new proposed nutrient
157 medium H. The growth rate of selected bacterial strains was studied using the optical density method.
158 Furthermore, the emulsification activity of the produced biosurfactants was examined by measuring the
159 emulsification index. The produced biosurfactant was purified and extracted by acid precipitation
160 method. Moreover, stability studies of the produced biosurfactants were done under different conditions

161 (temperature, salinity, and pH). Finally, the effect of produced biosurfactants on oil recovery was
162 evaluated by performing core-flooding experiments.

163 **2. MATERIALS AND METHODS**

164 **2.1 Sampling**

165 MEOR screening parameters of 59 Egyptian oil reservoirs representing the two main Egyptian oil
166 concessions areas (the Gulf of Suez, and the Western Desert), were summarized in Table 1 and Table
167 2. These data were statistically analysed, based on each screening criterion mentioned in Table 3. The
168 crude oil samples were collected from Egyptian oil fields located in the Gulf of Suez and the Western
169 Desert to isolate biosurfactant-producing bacteria that can improve oil recovery. The crude oil samples
170 were preserved in a fridge at 4°C in 50 ml sterilized bottles for further studies.

171 Table 1. MEOR screening parameters for Gulf of Suez Oil Reservoir.

#	Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
1	EL-MORGAN	N. KAREEM	79	60,000	1,829	190	29.5	0.55
2	EL-MORGAN	S. KAREEM	79	70,000	1,859	300	29.5	1.17
3	EL-MORGAN	N. BELAYIM	71	66,000	1,615	150	26.0	3.40
4	GS-382	NUBIA	138	370,000	3,200	60	33.0	0.49
5	GS-315	KAREEM	79	70,000	1,829	1,400	30.4	0.94
6	GS-315	BALEYIM	72	65,000	1,615	1,000	28.0	1.96
7	JULY	L. RUDIES	116	50,000	2,713	158	29.0	0.62
8	JULY	S. NUBIA	138	250,000	2,926	360	29.9	0.59
9	RAMADAN	NUBIA	146	184,000	3,627	83	31.3	0.72
10	OCTOBER	NUBIA	121	172,000	3,231	450	27.0	1.20
11	OCTOBER	L. SENONIAN	116	144,000	3,048	700	25.2	1.50
12	GS-173	NUKUL	110	39,600	2,957	660	31.0	0.72
13	GS-404	NUBIA	138	170,000	3,139	87	31.6	0.70
14	GS-404	NUKHUL	135	41,300	3,048	300	31.0	0.40

15	GS-336	U. RUDEIS	85	126,000	2,103	300	28.0	1.36
16	GH-376	KAREEM	113	236,000	2,576	187	24.5	0.87
17	RAS FANAR	N. LIMESTONE	46	100,800	642	100	27.7	2.35
28	ZEIT BAY	CARBONATE S.S.	67	220,000	1265	370	38	0.90
19	RAS BADRAN	NUBIAN S.S.	124	143,000	3,277	45	27.3	1.71
20	WEST BAKR	RUDES S.	60	15,000	1,049	3,000	20	30

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173 Table 2. MEOR Parameters for Western Desert Oil Reservoir.

#	Field	Reservoir	Temperature (°C)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
1	RAZZAK1	APT. DEL.	82	152,000	2,207	5.3	37.8	1.30
2	RAZZAK2	APT. DEL.	71	84,000	1,698	400	37.5	0.93
3	CYG	UPPER Bah	85	80,000	1,981	132	33	1.50
4	CYG	LOWER Bah	87	143,000	2,286	131	33	1.50
5	BED15	ARC	111	88,000	3,200	307	34	0.65
6	BED3C9	ARG	116	93,000	3,400	40	42.2	0.25
7	BED3-8/3-13	ARC	112	88,000	3,000	303	35	0.25
8	BED3-6/3-11	ARC	107	89,000	2,875	305	38	0.32
9	BED-1	ARC	109	90,000	2,852	304	36.3	0.68
10	BED-1	ARD	113	122,000	2,960	185	33.8	0.80
11	BED-1	KHARITA	128	93,000	3,475	200	40.6	0.50
12	BED-1	Bahariya	121	100,000	3,390	131	39.9	0.22
13	BED-9	Bahariya	121	102,000	3,230	132	36.2	0.36
14	BAHGA	A.R.G	114	93,000	2,812	40	33.2	1.14
15	BAHGA	L. Bah	121	143,000	3,113	131	35.9	0.63
16	AL MAGD	North AR G″	114	97,000	3,048	40	33.2	0.95

17	AL MAGD	South A/R″G″	114	138,000	3,044	40	33.2	0.95
18	AL BARQ	A/R″E″	112	85,000	2,990	239	38.7	0.48
19	AL BARQ	A/R ″G″	112	94,000	3,182	40	31.8	2.10
20	KARAM	ARC	149	90,000	4,220	307	38.7	0.32
21	ASSIL	A/R ″G″	124	92,000	3,870	40	38.6	0.45
22	ASSIL	Bahariya	128	101,000	4,074	132	9	0.45
23	AL FADL	Bahariya	64	122,000	1,200	131	40.4	1.82
24	AL QADR	Bahariya	64	122,000	1,200	133	41.3	1.5
25	NEAG2	Bahariya	96	152,879	2,600	132	42.8	0.87
26	NEAG2 EAST	Bahariya	97	154,720	2,600	132	42.4	0.87
27	NEAG3	Bahariya	92	144,936	2,513	132	41	0.87
28	JG (JD-2BLOCK)	LSA-East	113	133,000	3,235	216	42	0.60
29	JG	LSA- West	114	207,420	3,235	198	37.5	0.77
30	JG (JD- 7BLOCK)	LAC- West	112	185,000	3,235	207	38	0.70
31	JG (JD-10BLOCK)	LSC- East	112	173,00	3,235	195	33	0.50
32	JG	LSB	112	207,420	3260	231	38	0.60
33	SHIBA	Bahariya	96	124,350	2,054	131	36	0.60
34	SITRA-8 BLOCK	A.R.C	109	93,000	2,860	307	29	0.40
35	SITRA-8 BLOCK	A.R.C	110	89,000	2,895	305	39	0.32
36	SITRA-8 BLOCK	ARG Sand1	127	113,000	3,270	100	31	1.29
37	SITRA-8 BLOCK	ARG Sand2	127	107,000	3,270	100	31	1.29
38	SITRA-8 BLOCK	ARE	114	94,000	3,070	163	39	0.50
39	SITRA-8 BLOCK	UPPER Bah	124	91,000	3,410	114	40	0.36

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180 Table 3. MEOR reservoir screening parameters (She et al., 2019).

Parameter	Value range	Optimum
Formation Temperature (°C)	20– 80	30 – 60
Salinity (ppm)	≤ 300,000	≤ 100,000
Depth (m)	≤ 2,377	≤ 2,134
Permeability (mD)	≥ 50	≥ 150
Crude Oil Type (API)	≥ 15°	30° - 40°
Crude Viscosity (mPa.s)	≤ 500	30 -150

181 **2.2 Media**

182 Bushnell Hass Mineral Salts BHMS media were used to isolate the biosurfactant-producing bacteria
183 from crude oil samples. BHMS media were composed of, in g/l distilled water: KH_2PO_4, 1; Dipotassium
184 phosphate K_2HPO_4, 1; NH_4NO_3, 1; $MgSO_4$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, 0.05. The pH was adjusted to 7 at
185 25°C. For the preparation of agar plates (solid medium), 15.0 g/l agar (strength 1300) were added.
186 The isolated strains were examined for their potential of producing metabolic biosurfactants using a
187 new proposed nutrient medium Nominated H and other ten different reported nutrients media for bacilli
188 species, nominated N1 (Makkar and Cameotra, 1997), N2 (Al-bahry et al., 2013), N3 (Joshi et al.,
189 2008b), N4 (Soumen Mukherjee et al., 2009), N5 (Joshi et al., 2008a), N6 (Landy et al., 1948), N7
190 (Jenny et al., 1991), N8 (Cooper et al., 1981), N9 (Joshi et al., 2008c), and N10 (Youssef et al., 2007),
191 for making comparative analysis to maximize the surface activity of produced biosurfactant. The
192 chemical composition of the nutrients media was listed in Table 4. The reported nutrients media used
193 different carbon sources at different concentrations, which were date molasses, cane molasses, glucose,
194 and sucrose. The carbon source for the new proposed nutrient medium H was glucose (20 g/l). Media
195 were sterilized (120 ^{o}C for 20 min) in an autoclave, then adding sterilized trace elements, and adjusting
196 the pH value to 7 by adding sterilized 6 N NaOH.

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200 Table 4. Different Nutrients compositions used for Bacilli species

Composition (g/l)	Nutrient										
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	H
Cane Molasses	80	-	50	-	-	-	-	-	-	-	-
Date Molasses	-	80	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	10	20	-
Glucose	-	-	-	10	20	20	34	40	-	-	20
NH_4NO_3	-	3.3	-	-	-	-	1	4	-	3.3	5
Na- Glutmate	-	-	-	-	-	5	-	-	-	-	4
$NaNO_3$	-	-	-	2.8	-	-	-	-	-	-	3
$K2HPO_4$	-	2.2	-	-	-	-	-	-	13.9	2.2	-
KH_2PO_4	1.4	0.14	-	-	-	1	6	4.08	2.7	0.14	4
$Na2HPO_4$	2.2	-	-	-	3.8	-	2.7	7.12	-	-	6
$MgSO_4.7H_2O$	0.6	0.6	-	0.2	0.7	0.5	0.1	0.2	0.25	0.6	0.3
$FeSO_4.7H_2O$	0.02	0.2	-	0.0003	8	0.15	0.00165	0.0011	0.1	0.2	0.1
$MnSO_4.4H_2O$	-	-	-	0.0002	2.2	0.005	0.001	0.00067	3	-	1.78
KCl	-	-	-	0.5	-	-	-	-	-	-	-
$CaCl_2$	0.04	0.04	-	0.03	-	-	0.0012	0.00077	0.1	0.04	0.1
Na-EDTA	-	-	-	0.2	30	-	0.000745	0.00148	1	-	-
H_3PO_4 (85.4%)	-	-	-	2ml	-	-	-	-	-	-	-
$CuSO_4$	-	-	-	-	-	0.16	-	-	-	-	-
Yeast Extract	-	-	-	-	0.5	1	-	-	1	-	1
NaCl	0.1	0.01	-	-	-	-	-	-	50	0.01	-
$(NH_4)_2SO_4$	3	-	-	-	7	-	-	-	1	-	1
KNO_3	-	-	-	-	-	-	-	-	-	-	3
Urea	-	-	-	-	-	-	-	-	-	-	1
Trace elements	-	0.5ml[a]	-		10ml[b]	-	-	-	10ml[c]	0.5ml[a]	1ml[d]

[a]$ZnSO_4.7H_2O$, 2.32; $MnSO_4.4H_2O$, 1.78; Na-EDTA, 1.0; $CuSO_4.5H_2O$, 1.0; KI, 0.66; H_3BO_3, 0.56; $CoCl_2.6H_2O$, 0.42; $NiCl_2.6H_2O$, 0.004; and $Na_2MoO_4.2H_2O$, 0.39.

[b]$CoCl_2$, 1.0; $CuSO_4$, 0.6; Na_2MoO_4, 0.5; H_3BO_3, 0.25; and $ZnSO_4$, 6.0.

[c] $ZnSO_4.7H_2O$, 0.1; Na_2MoO_4, 0.1; $CuSO_4.5H_2O$, 0.01; AlK $(SO_4)_2$, 0.01; Na_2MoO_4, 0.01; H_3BO_3, 0.01; Na2MoO4, 0.1; Na_2SeO_4, 0.005, and $NiCl_2.6H_2O$, 0.003.

[d] $ZnSO_4.7H_2O$, 2.28; $CuSO_4.5H_2O$, 1.14; H_3BO_3, 0.61; $CoCl_2.6H_2O$, 0.47; and $Na_2MoO_4.2H_2O$, 0.42.

201 **2.3 Isolation of Bacteria**

202 The enrichment culture technique was used for isolating bacteria (Liu et al., 2014). 100ml of BHMS
203 broth medium was prepared in a conical flask for each crude oil sample and autoclaved. 1ml of each
204 crude oil sample was added to the prepared conical flasks, and then flasks were incubated for three days
205 on a rotary shaker at 30°C and 180 rpm. the bacteria cultures were streaked out from the conical flasks
206 using a sterile swab or loop and spread on sterile agar (solid medium) plates. The inoculated plates were
207 incubated at 30°C for 24 hours. A successive streaking was executed by the quadrant pattern method on
208 sterile agar plates to separate single colonies.

209 **2.4 Identification of Bacteria**

210 Bacteria can be described and classified in three major ways, namely, microscopic examination,
211 morphological characteristics, and biochemical characteristics based on Bergey's manual of systematic
212 bacteriology (Bergey et al., 2012). Morphology means the systematic study of external characters of
213 bacteria. The identification of the unknown bacteria could be done by examination of seven
214 characteristics of the unknown bacteria is necessary. These seven characteristics are colony
215 morphology, cell morphology, gram stain reaction, presence of endospores in a culture, motility, oxygen
216 intake, and biochemical tests (Bergey et al., 2012). The identification of isolated bacteria in this research
217 was performed based on these seven characteristics. The AmScope 40X-1000X Compound Microscope
218 was used for observation of the size and morphology of the bacterial colonies with a 1000×
219 magnification objective.

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222 **2.5 Surface Activity**

223 The isolated strains were examined for their potential of producing biosurfactants by studying their
224 Surface activity by measuring their Surface Tension ST and Interfacial Tension IFT, by applying the
225 rod method using the EZ Tensiometer (Model 201, USA). All measurements were performed in
226 triplicate at ambient temperature (25±2 ºC) and atmospheric pressure (1 atm) and the average values
227 were reported.

228 **2.6 Bacterial Growth**

229 The bacterial growth rate was estimated using the optical density method. A JASCO spectrophotometer
230 (V-630, Japan), was used to estimate the bacterial growth rate in culture media. The optical density was
231 measured at wavelength 600 nm, which is recommended for estimating the bacteria concentration (Joshi
232 et al., 2008a).

233 **2.7 Biosurfactant Extraction and Purification**

234 There are several methods for biosurfactant extraction and purification. The method used for extracting
235 and purifying the produced biosurfactant from bacteria culture media was based on acid
236 precipitation(Joshi et al., 2008a). 100 ml of culture media was centrifuged using a Sigma centrifuge
237 machine (Model 2-16KL, Germany), at 10,000 rpm for 20 minutes to separate bacterial cells. Then, the
238 pH of the cell-free supernatant was adjusted to 2by adding 6N HCl to precipitate the produced
239 biosurfactant, and then the cell-free supernatant was kept at 4ºC for 24 hours. The precipitates were
240 collected by centrifugation at 12,000 rpm for 30 minutes at 4ºC, and then dissolved in distilled water
241 and the pH was adjusted to 8 by using 6N Na/OH, and finally lyophilized and weighed.

242 **2.8 Critical Micelle Concentration CMC**

243 Critical Micelle Concentration (CMC) is simply the biosurfactant concentration above which micelles
244 aggregates initially start to form. surface tension reduces by increasing surfactant concentration until
245 surfactant molecules saturate the surface of the solution at which no more reduction in surface tension
246 is observed. When the formation of micelles is desirable, the CMC is a measure of the efficiency of a
247 surfactant. CMC is an essential characteristic for surfactants because once reaching CMC, there is no
248 more reduction in surface tension even after adding any further amount of biosurfactant. CMC was
249 estimated by plotting a graph between the surface tension versus the concentration of biosurfactant.

250 Consequently, several solutions of extracted biosurfactant with concentrations ranging from 0.01 to 0.1
251 g/l were prepared in distilled water, and then the change of surface tension was observed using the Rod
252 Tensiometer (Joshi et al., 2008a).

253 **2.9 Emulsification Activity**

254 Emulsification activity is the ability of surface-active molecules to form a stable emulsion. The ability
255 of produced biosurfactants to emulsify different hydrocarbons such as hexane, heptane, hexadecane,
256 kerosene, and crude oil was estimated by measuring the emulsification index E_{24}. The emulsification
257 index E_{24} was measured by adding 2 ml of hydrocarbon to 2 ml of cell-free supernatant in a test tube,
258 and then vertexing for 2 minutes, and kept at 25°C for 24 hours. E_{24} was calculated by dividing the
259 emulsion layer's height (mm) over the total mixture's height (mm) (Pereira et al., 2013).

260 **2.10 Stability Studies**

261 The effect of temperature, salinity, and pH was investigated to examine the stability of the produced
262 biosurfactant, and the optimum condition for the studied biosurfactant-producing bacteria (Joshi et al.,
263 2008a). The stability of the produced biosurfactant was examined over a wide range of temperatures
264 (30–120°C) at pH 7, and 0% (w/v) NaCl by measuring ST, IFT, and E_{24} of the cell-free supernatant at
265 different temperatures to determine the optimum temperature that maximizes the biosurfactant activity.
266 The salinity effect on surface activity was studied by measuring ST, IFT, and E_{24} of the cell-free
267 supernatant at different NaCl concentrations (0–20% w/v) at 25°C, and pH 7. A pH range (2 – 12) was
268 adjusted, using Jenway pH meter (Model 3505, UK), by adding 6N NaOH or 6N HCl to investigate
269 the pH effect on the stability of the biosurfactant by measuring ST, IFT, and E_{24} the cell-free supernatant
270 at 25°C, and 0% (w/v) NaCl concentration.

271 The pH of the medium is also considered the most significant factor that affects zeta potential. The
272 significance of zeta potential is that its value could be related to the stability of the system.
273 Consequently, the zeta potential was measured over a wide range of pH (2-12) to examine the electrical
274 stability of the produced biosurfactant in aqueous media. Zetasizer Nano Series ZS instrument
275 (Malvern, UK) was used for measuring zeta potential (Ali et al., 2021; Awan et al., 2021; Dupraz et al.,
276 2009).

277

278 **2.11 Core-Flooding Experiments**

279 Core-flooding experiments were performed to investigate the effect of produced biosurfactants by
280 *Bacillus subtilis* in improving the oil recovery when compared with conventional water flooding.
281 Sandstone cores used in these experiments were extracted from the producing reservoir of Al QADR
282 oil field (field of interest) located in the Western Desert, Egypt. Porosity, absolute permeability, and
283 general properties of the sandstone cores used in this study were represented in Table 5. The crude oil
284 and formation water used in these experiments were obtained from the same field of interest (Al QADR
285 oil field), which has an average reservoir temperature of 64°C. The API, density, and viscosity of crude
286 oil were 41.3°, and 1.5 mPa.s, respectively. The compositions of the crude oil and the formation water
287 are also listed in Table 6 and Table 7, respectively. Formation water was filtered before use, by
288 Millipore Filtration Unit (0.45 µm) (Alramahi et al., 2005; Alshibli et al., 2006).

289 Table 5. Basic Properties of the Sandstone Cores used in Core-flooding Experiments

Core ID	Length (cm)	Diameter (cm)	Bulk Volume (cm^3)	Pore Volume (cm^3)	Porosity (%)	Absolute Permeability (mD)
C-F-1	5.32	3.51	51.63	10.65	20.64	205.97
C-F-2	5.42	3.51	52.60	10.60	20.15	179.68
C-F-3	5.43	3.51	52.67	10.33	19.62	187.03

290

291 Table 6. Composition of Al QADR Crude oil by Chromatograph up to C_{12}^{+}

<table>
<tr><th rowspan="2">Component</th><th colspan="2">Stock Tank Oil</th><th rowspan="2">Liquid Density (g/cm³)</th><th rowspan="2">MW</th></tr>
<tr><th>Mole %</th><th>Wt.%</th></tr>
<tr><td>Methane</td><td>0</td><td>0</td><td>0.30</td><td>16.04</td></tr>
<tr><td>Ethane</td><td>0.14</td><td>0.03</td><td>0.36</td><td>30.07</td></tr>
<tr><td>Propane</td><td>1.13</td><td>0.30</td><td>0.51</td><td>44.10</td></tr>
<tr><td>I-Butane</td><td>1.34</td><td>0.47</td><td>0.56</td><td>58.12</td></tr>
<tr><td>n-Butane</td><td>2.33</td><td>0.81</td><td>0.58</td><td>58.12</td></tr>
<tr><td>I-pentane</td><td>2.66</td><td>1.15</td><td>0.62</td><td>72.15</td></tr>
</table>

n-Pentane	2.59	1.12	0.63	72.15
Hexane	6.96	3.60	0.66	86.18
Benzene	0.89	0.42	0.88	78.11
Heptanes	6.94	4.18	0.69	100.20
Toluene	1.34	0.74	0.87	92.14
Octane	10.25	7.03	0.71	114.23
Ethyl-benzene	0.40	0.26	0.87	106.16
P, m-xylene	0.91	0.58	0.87	106.16
o-xylene	0.32	0.21	0.88	106.16
Nonanes C9	7.66	5.90	0.72	128.26
Decanes C10	6.33	5.41	0.73	142.29
Undecanes C11	5.99	5.28	0.79	147.00
dodecanes C12+	41.80	62.51	0.90	248.96
Total	100	100		
Molecular weight	152.86			

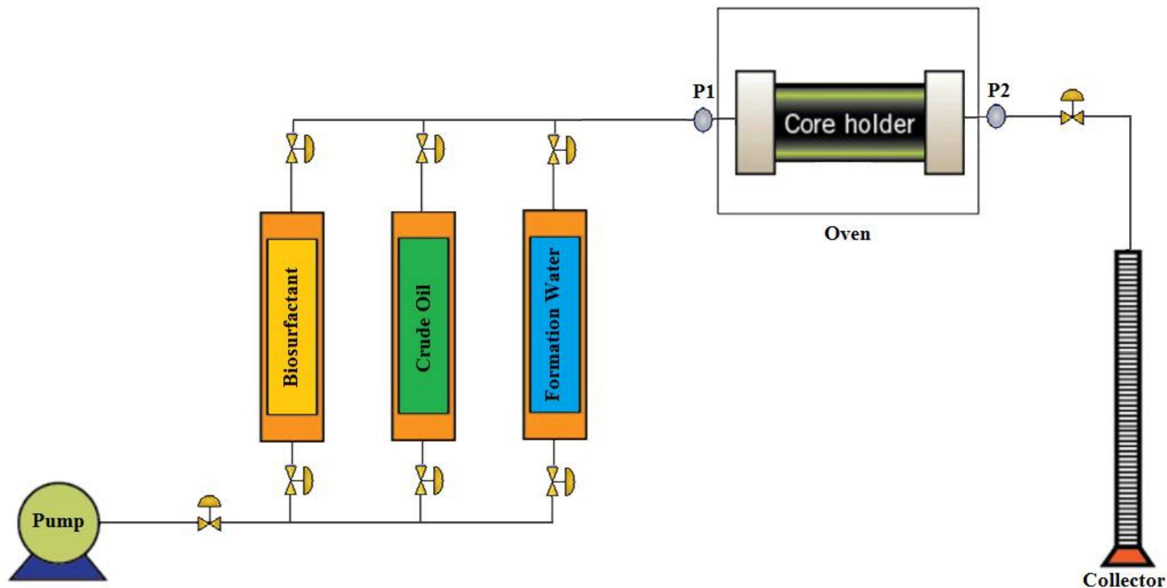
304 Table 7. Composition of Al QADR Formation Water.

Ion Composition	Concentration (mg/l)
Sodium, Na^{+}	38,906.56
Potassium, K^{+}	8,310.51
Calcium, Ca^{2+}	1,353.45
Magnesium, $Mg2^{+}$	373.57
Bromine, Br^{+}	2.00
Chloride, Cl^{-}	66,222.35
Sulfide, $S2^{-}$	5,503.71
Bicarbonate, HCO_3^{-}	1,250.57
Total	121,922.73

305

306 Initially, the core was cleaned by using the Soxhlet extraction method, using chloroform and methanol
307 as an azeotropic mixture in the proportion of (75:25), and then dried at 65°C for 24 h before use (Al-
308 Sulaimani et al., 2011). After the cleaning and drying, the cores were saturated with filtered formation
309 water using vacuum desiccators for 24 h and pore volume PV was determined using the dry-weight and
310 wet-weight of the cores. Then, the core was flooded with crude oil until no more water was produced
311 to achieve the connate water saturation condition as it is found in the oil reservoirs (Figure 1). The
312 original oil in place OOIP, which was indicated by the volume of water displaced was determined. To
313 simulate the process of water flooding as a secondary oil recovery stage, the core was injected by several
314 pore volumes of the formation water until no more oil was produced to achieve the residual oil saturation
315 after water flooding S_{orwf}. The oil recovered by the water flooding was determined. Finally, to simulate
316 the process of biosurfactants flooding as a tertiary recovery stage, the cores were injected by several
317 pore volumes of the cell-free supernatant until no more oil was produced to achieve the residual oil
318 saturation after biosurfactant flooding S_{orbf}. The additional oil recovered by the biosurfactant flooding
319 was determined. core-flooding experiments trials were conducted at different flow rates of 0.25

320 cm^3/min, 0.50 cm^3/min, and 0.75 cm^3/min to obtain the optimum flow rate that could maximize the oil
321 recovery. All core-flooding experiments were conducted at 64°C to simulate the average reservoir
322 temperature of Al QADR oil field.



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324 Figure 1. Schematic Diagram of Flooding System.

325 **3. RESULTS AND DISCUSSION**

326 The activity of bacteria employed in MEOR applications depends on the physical and chemical
327 conditions they encounter in the reservoirs, such as temperature, salinity, pH, permeability, and
328 nutrients. although these reservoir conditions vary a great deal from one reservoir to another. All these
329 factors, which are generally physical and environmental can affects bacterial growth, proliferation,
330 metabolism, and survival, and limit their ability to produce desired quantities of metabolites such as
331 biosurfactants that are needed for enhanced oil recovery. Consequently, the data ranges of these physical
332 and chemical conditions of Egyptian reservoirs in the Gulf of Suez and the Western Desert and the
333 number of Egyptian reservoirs that fit each MEOR screening criterion are presented in Table 8 and
334

335

336 Table 10, respectively. It was found that the number of oil reservoirs from the Gulf of Suez and the
337 Western Desert that has the potential to MEOR based on available data is equal to 8 and 3 oil reservoirs,

respectively, which are listed in Table 9 and Table 11. Moreover, the statistical analyses of these data are plotted in Figure 2 and Figure 3. It was also found that the main factors, which are considered as limiting for successful application of MEOR in Egyptian oil fields, are reservoir temperature and depth. Temperature plays a significant role in bacteria metabolism. With increasing depth, the temperature increases. Consequently, bacterial growth and their metabolism will certainly be affected as the increasing temperature can exert negative effects on enzyme function by disruption of important cell activities. However, The effects of temperature on enzyme function are generally accepted, but it is also to be observed that the temperatures at which these phenomena occur vary widely between organisms (Lazar et al., 2007; Marshall, 2008). Marshall et al. (2008) reported that bacteria could be classified according to their optimum temperature range as psychrophilic bacteria (<25°C), mesophilic bacteria (25-45°C), and thermophilic bacteria (>45°C), based on the temperature ranges for microorganisms survival (Marshall, 2008). They also reported that in most developed petroleum reservoir conditions, temperatures are expected to vary greatly but can be as high as 70°C, and even 100°C in some cases. To survive such high temperatures, thermophilic bacteria are often spore-forming and possess thermally stable enzymes that allow the normal functioning of cellular processes under such harsh conditions (Marshall, 2008).

Table 8. Data Ranges and Number of Egyptian Oil Reservoirs in Gulf of Suez fitting MEOR Screening Criteria

Reservoir Property	Data Ranges for Egyptian Reservoirs in Gulf of Suez	Number of Egyptian Reservoirs in Gulf of Suez Fitting MEOR Screening Criteria
Formation Temperature (^{o}C)	46 - 146	8
Formation Water Salinity (ppm)	15,000 - 370,000	19
Reservoir Depth (m)	642 - 3,627	9
Reservoir Rock Permeability (mD)	45 - 3,000	19
Crude Oil Type (API)	20 - 38	20

Crude Oil Viscosity (mPa.s)	0.4 - 30	20

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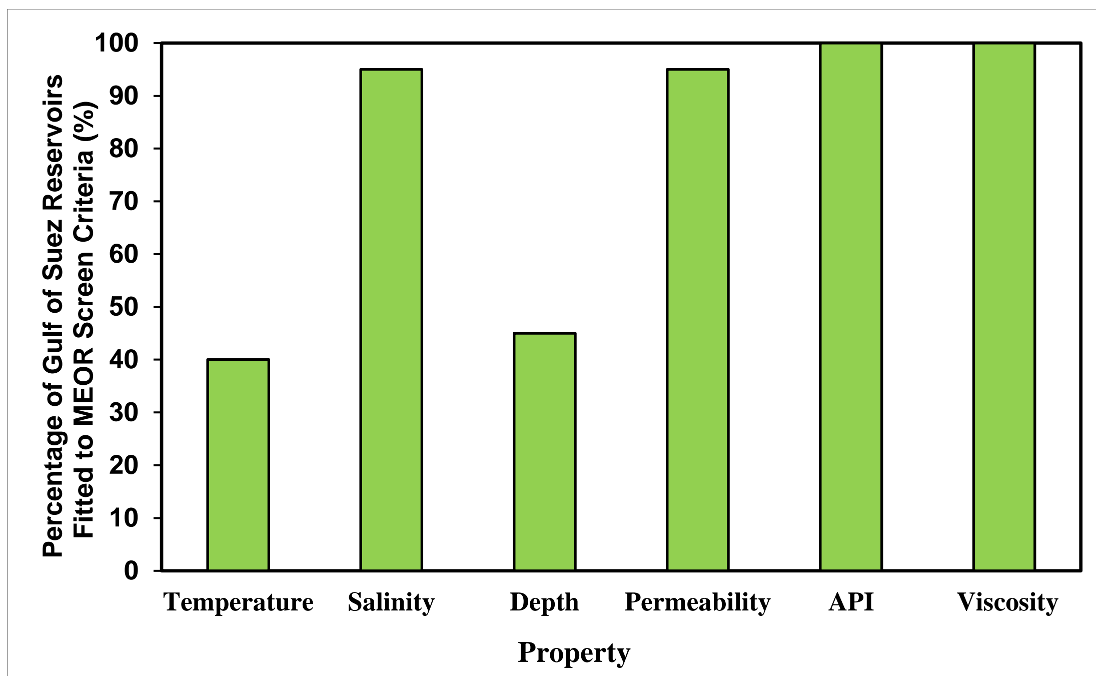

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358 Figure 2. Frequency Diagram of Gulf of Suez Screening Criteria Analysis.

359

360 Table 9. Gulf of Suez oil reservoirs that have the potential to MEOR.

Field	Reservoir	Temperature (ºC)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
EL-MORGAN	N. KAREEM	79	60,000	1,829	190	29.5	0.55
EL-MORGAN	S. KAREEM	79	70,000	1,860	300	29.5	1.17
EL-MORGAN	N. BELAYIM	71	66,000	1,615	150	26.0	3.40
GS-315	KAREEM	79	70,000	1,829	1,400	30.4	0.94
GS-315	BALEYIM	72	65,000	1,615	1,000	28.0	1.96
ZEIT BAY	CARBONATE S.S.	67	220,000	1,264	370	38.0	0.90
RAS FANAR	N. LIMESTONE	46	100,800	642	100	27.7	2.35
WEST BAKR	RUDES S.	60	15,000	1,049	3,000	20	30

361

362

363

364 Table 10. Data Ranges and Number of Egyptian Oil Reservoirs in Western Desert fitting MEOR

365 Screening Criteria

Reservoir Property	Data Ranges for Egyptian Reservoirs in the Western Desert	Number of Egyptian Reservoirs in Western Desert Fitting MEOR Screening Criteria
Formation Temperature (°C)	64 - 149	3
Formation Water Salinity (ppm)	34,000 - 207,420	39
Reservoir Depth (m)	1,200 - 4,220	7
Reservoir Rock Permeability (mD)	5.3 - 400	32
Crude Oil Type (API)	9 - 42.8	38
Crude Oil Viscosity (mPa.s)	0.17 - 2.10	39

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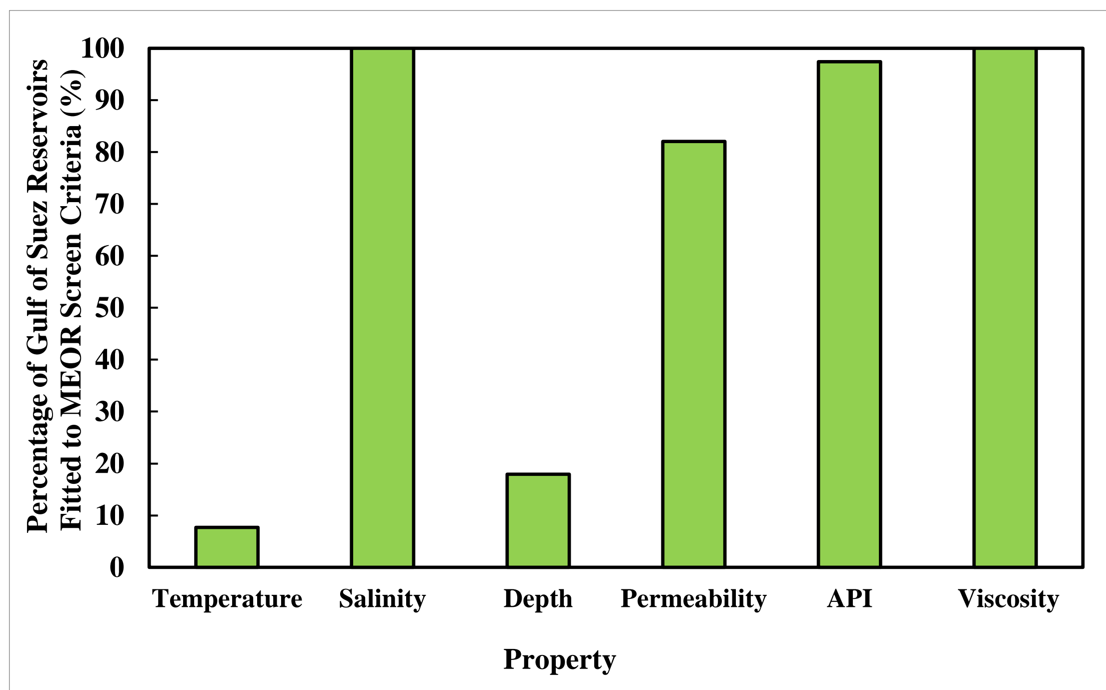


367

368 Figure 3. Frequency Diagram of Western Desert Screening Criteria Analysis.

369 Table 11. Western Desert oil reservoirs that have the potential to MEOR

Field	Reservoir	Temperature (ºC)	Salinity (ppm)	Depth (m)	Permeability (mD)	API Gravity (API)	Viscosity (mPa.s)
RAZZAK2	APT. DEL.	71	84,000	1,698	400	37.5	0.93
AL FADL	Bahariya	64	122,000	1,200	131	40.4	1.82
AL QADR	Bahariya	64	122,000	1,200	133	41.3	1.50

370 For the isolation of crude oil indigenous bacteria, 11 crude oil samples were collected from Egyptian
371 reservoirs that have the potential to MEOR, and nominated as G1, G2, G3, G4, G5, G6, G7, G8, WD1,
372 WD2, and WD3, as listed in Table 12. The collected crude oil samples represented the main two
373 different Egyptian oil concessions areas (Gulf, and the Western Desert). They were collected and
374 preserved at the fridge at 4°C in a 50 ml sterilized bottle for further studies.

375 Table 12. Collected samples of Egyptian oil fields that have the potential to MEOR

Sample	Location	Field	Reservoir
G1	Gulf	EL-MORGAN	N. KAREEM
G2	Gulf	EL-MORGAN	S. KAREEM
G3	Gulf	EL-MORGAN	N. BELAYIM
G4	Gulf	GS-315	KAREEM
G5	Gulf	GS-315	BALEYIM
G6	Gulf	ZEIT BAY	CARBONATE S.S.
G7	Gulf	RAS FANAR	N. LIMESTONE
G8	Gulf	WEST BAKR	RUDES S.
WD1	WD	RAZZAK2	APT. DEL.
WD2	WD	AL FADL	Bahariya
WD3	WD	AL QADR	Bahariya

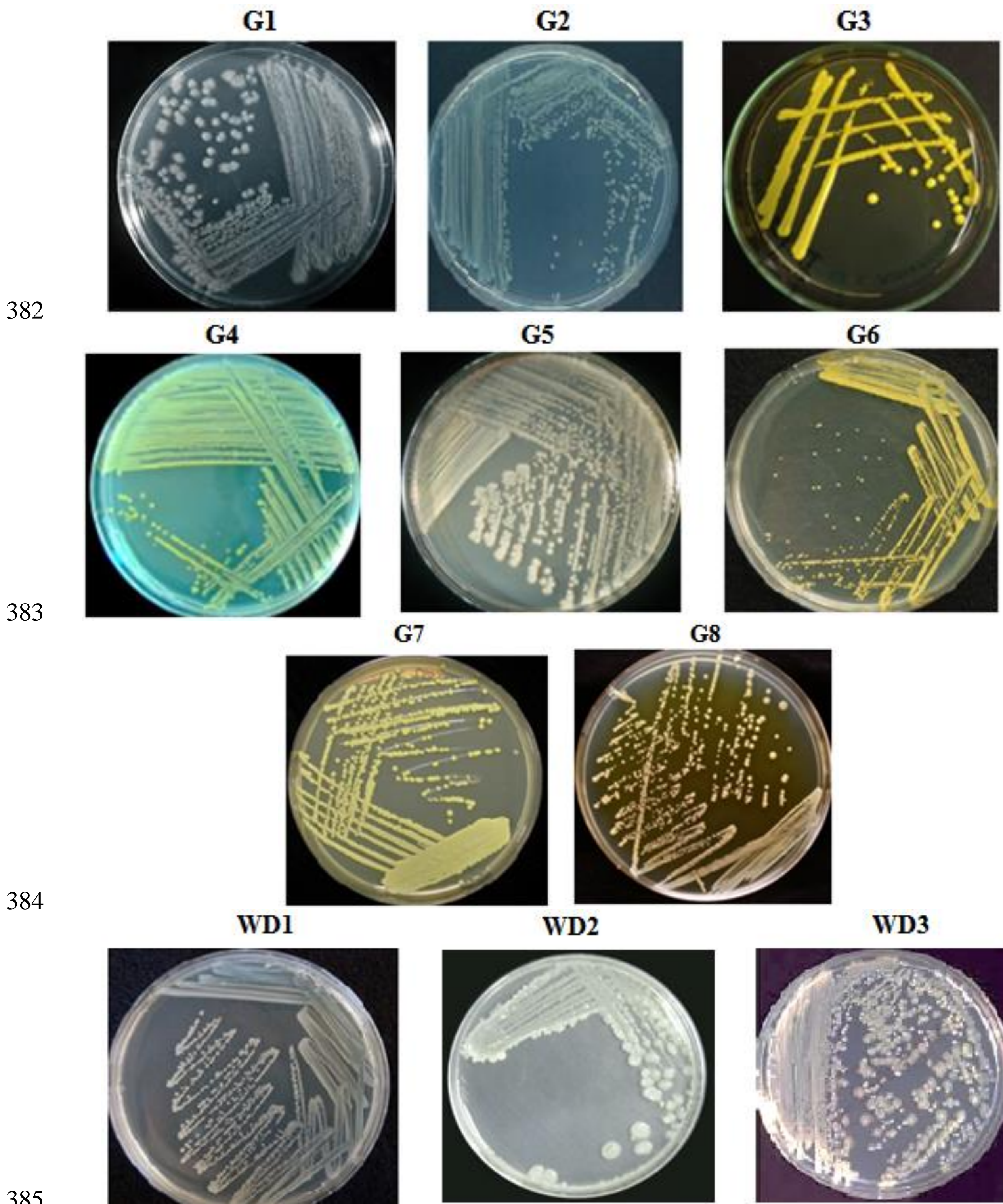
376 **3.1 Isolation of Bacteria**

377 The laboratory studies show the existence of bacterial strains in collected crude oil samples. The
378 turbidity of broth media after incubation in a rotary shaker for three days at 180 rpm is confirming the

379 existence of microorganisms in these samples. Figure 4 shows the colony morphology of isolated strains

380 from the studied crude oil sample. Initially, 11 pure bacteria cultures were isolated from collected crude

381 oil samples.



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386 Figure 4. Colony morphology of isolated bacteria that collected from Gulf of Suez (G1-G8), and

387 Western Desert (WD1-WD3) oil fields in agar plates.

388 **3.2 Identification of Bacteria**

389 Morphological and biochemical analysis was conducted to identify the 11 studied bacteria isolated from
390 different Egyptian oil fields collected samples.

391 ***3.2.1 Colony Morphology***

392 The colony morphology of isolated colonies has been studied and listed in Table 13. It was found that
393 the colony morphology of isolated strain G1 is circular, small, circular, light yellow fluorescent, convex,
394 and smooth colonies with irregular edges. Isolated strain G2 is large, circular, light grey, convex, and
395 smooth colonies with irregular edges. Isolated strain G3 is large, circular, yellow fluorescent, flat, and
396 smooth colonies with irregular edges. Isolated strain G4 is small, circular, greenish-yellow fluorescent,
397 convex, and smooth colonies with regular edges. Isolated strain G5 is small, irregular, shiny white,
398 convex, and smooth colonies with irregular edges and distinctive cheese odour Isolated strain G6 is
399 small, circular, shiny yellow, and convex colonies with irregular edges. Isolated strain G7 is small,
400 circular, light yellow fluorescent, convex, and smooth colonies with irregular edges. Isolated strain G8
401 is large, circular, slightly yellow, convex, and smooth colonies with irregular edges. Isolated strain WD1
402 is small, small, circular, white, raised, and smooth colonies with irregular edges. Isolated strain WD2 is
403 large, circular, white, flat, and finely wrinkled colonies with irregular edges. Isolated strain WD3 is
404 medium, circular, fuzzy white, flat, and mucoid colonies with irregular edges.

405 Table 13. Colony Morphology of Isolated Strains.

Isolates	Colony Size	Colony Shape	Colony Pigmentation Colour	Colony Elevation	Colony Surface	Colony Margin
G1	Small	Circular	Light yellow fluorescent	Convex	Smooth	Irregular
G2	Large	Circular	Light grey	Convex	Smooth	Irregular
G3	Large	Circular	Yellow fluorescent	Flat	Smooth	Irregular
G4	Small	Circular	Greenish-yellow fluorescent	Convex	Smooth, Shiny	Regular
G5	Small	Irregular	White	Convex	Smooth, Shiny	Irregular
G6	Small	Circular	Yellow	Convex	Shiny	Irregular
G7	Small	Circular	Medium yellow fluorescent	Convex	Smooth	Irregular

G8	Large	Circular	light yellow	Convex	Smooth	Irregular
WD1	Small	Circular	White	Raised	Smooth	Irregular
WD2	Large	Circular	White	Flat	Finely wrinkled	Irregular
WD3	Medium	Circular	fuzzy white	Flat	Mucoid	Irregular

406 ### *3.2.2 Cell Morphology*

407 Figure 5 shows the cell morphology of isolated strains under the microscope after gram staining. It was
408 found that all isolated strains are bacilli (rod-shaped) species. Each of G1, G3, G4, G7, G8 are gram-
409 negative bacteria. However, G2, G5, G6, WD1, WD2, and WD3 are gram-positive bacteria.

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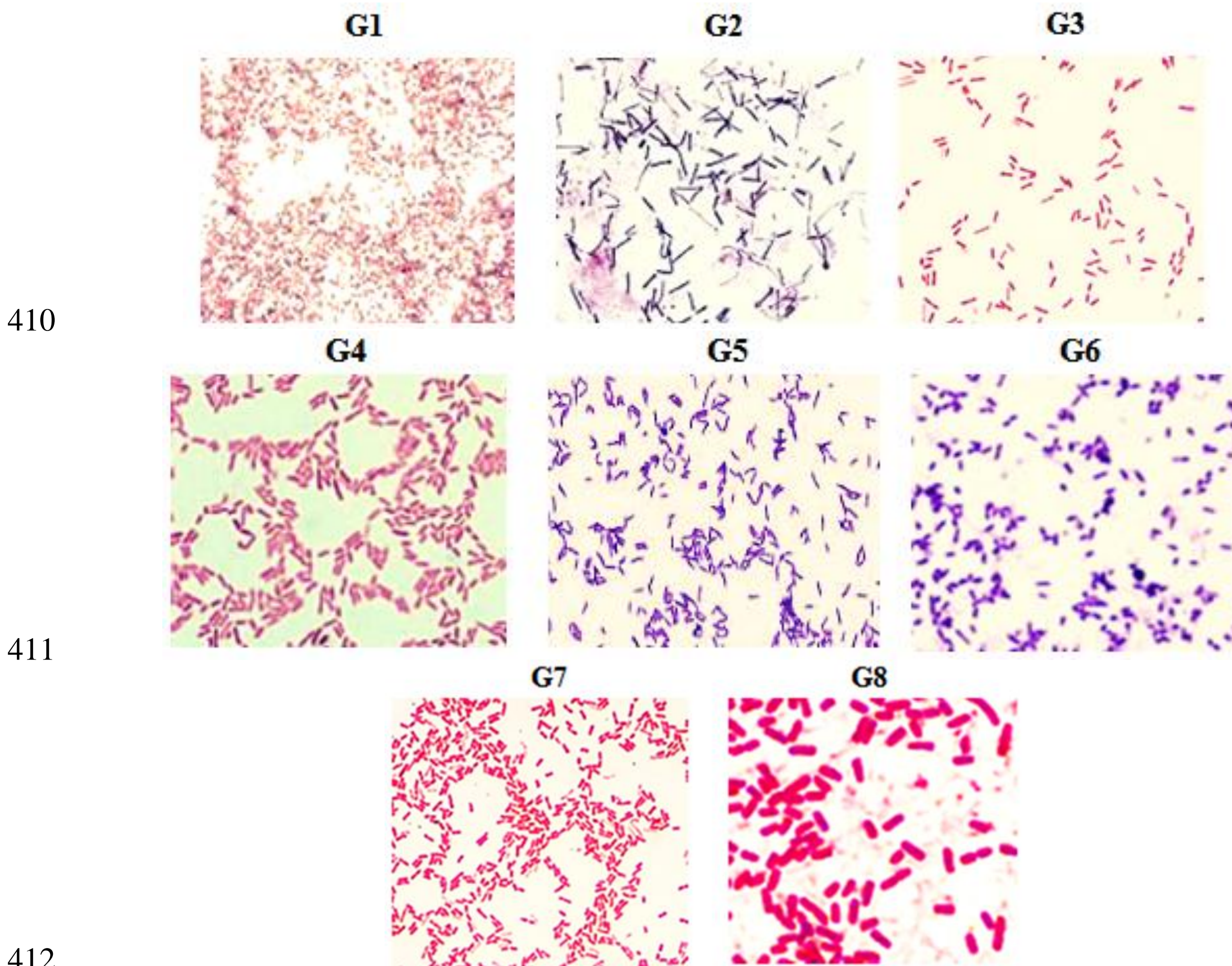
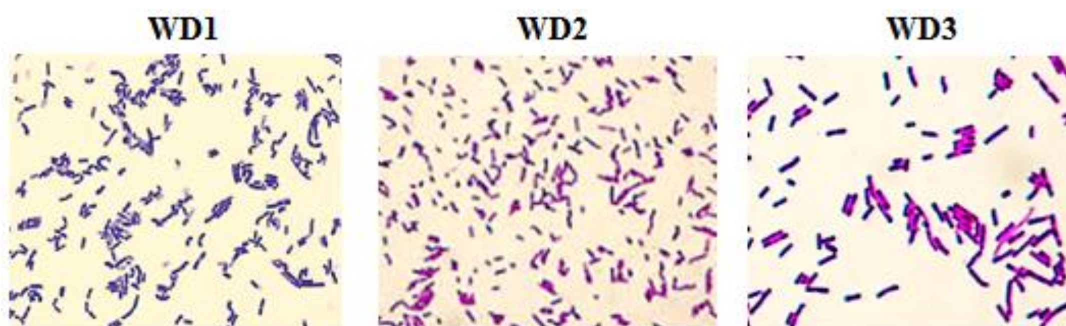


413 

414 Figure 5. Cell morphology of isolated bacterial strains collected from the Gulf of Suez (G1-G8), and
415 the Western Desert (WD1-WD3) oil fields under Microscope (1000× magnification objective).

416 ***3.2.3 Oxygen Intake, Motility, and Endospore Tests***

417 Table 14 shows the results of Oxygen Intake, Motility, and Endospore Tests of all isolated strains. Based
418 on the Oxygen intake test, it was found that isolated strains G1, G3, G4, G5, G7, and WD1 are strictly
419 aerobic, while G6, G8, WD2, and WD3 are facultatively anaerobic, and G2 is strictly anaerobic. It was
420 also found that all isolated strains are non-spore-forming, except G2, WD1, WD2, and WD3. Based on
421 the motility test, all isolated strains are motile, except G5 and G6.

422 Table 14. Oxygen Intake, Motility, and Endospore Tests of isolated bacteria.

Sample	Oxygen Intake Test	Endospore Staining Test	Motility Test
G1	Strictly aerobic	Non-spore forming	Motile
G2	Strictly anaerobic	Spore forming	Motile
G3	Strictly aerobic	Non-spore forming	Motile
G4	Strictly aerobic	Non-spore forming	Motile
G5	Strictly aerobic	Non-spore forming	Nonmotile
G6	Facultatively anaerobic	Non-spore forming	Nonmotile
G7	Strictly aerobic	Non-spore forming	Motile
G8	Facultatively anaerobic	Non-spore forming	Motile
WD1	Strictly aerobic	Spore forming	Motile
WD2	Facultatively anaerobic	Spore forming	Motile
WD3	Facultatively anaerobic	Spore forming	Motile

423 ***3.2.4 Biochemical Tests***

424 The flow diagram that was shown in Figure 6 summarized the identification process of studied bacilli
425 bacteria based on their morphological and biochemical characteristics. The results of several
426 biochemical characteristics of all isolated strains are represented in Table 15. The first step in bacteria
427 identification was to determine the Gram staining of studied bacteria. After Gram testing, the
428 observation of bacterial cell morphology is observed. Bacilli (rod-shaped) bacteria were divided into
429 two categories based on gram staining reaction results to bacilli gram-positive, and bacilli gram-
430 negative. Each of the isolated bacterial strains G2, G5, G6, WD1, WD2, and WD3 were bacilli gram-
431 positive bacteria, and G1, G3, G4, G7, G8 were bacilli gram-negative bacteria.

432 Table 15. Biochemical characteristics of isolated bacteria.

Sample	Biochemical Tests											
	Catalase	Oxidase	Indole	H_2S	MR	VP	Citrate	Nitrate	Urease	OF	Gelatin	Starch
G1	+	+	-	-	-	-	+	+	-	O	+	+
G2	-	-	-	+	-	-	+	-	-	F	-	-
G3	+	+	+	-	-	+	+	+	-	O	+	-
G4	+	+	-	-	+	-	+	+	-	O	+	-
G5	+	-	-	-	-	-	+	-	+	O	+	+
G6	+	+	+	+	+	-	+	-	+	F	+	+
G7	+	+	-	-	-	-	+	+	+	O	-	+
G8	+	-	-	-	-	+	+	+	-	F	-	-
WD1	+	-	-	+	-	-	+	+	+	O	+	+
WD2	+	-	-	-	-	-	+	+	-	F	-	+
WD3	+	-	-	-	-	+	+	+	-	F	+	+

433 + = Positive, - = Negative, O = Oxidative, F Fermentative

Sample	Fermentation Tests			Genus
	Mannitol	Glucose	Lactose	
G1	+	-	-	*Pseudomonas stutzeri*

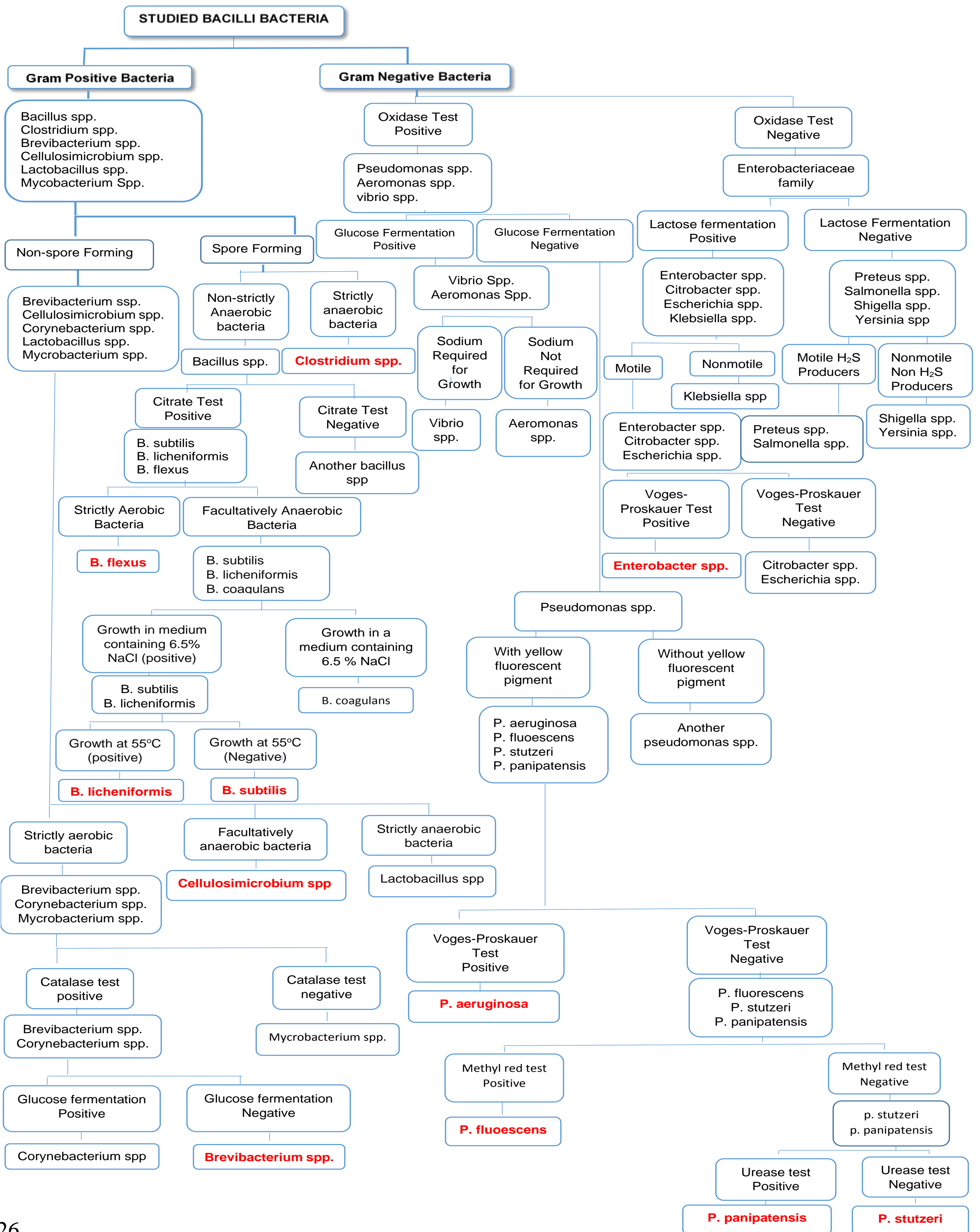
G2	+	+	-	*Clostridium spp*
G3	+	-	-	*pseudomonas aeruginosa*
G4	-	-	-	*pseudomonas fluorescens*
G5	-	-	-	*Brevibacterium spp*
G6	+	+	+	*Cellulosimicrobium spp*
G7	-	-	-	*Pseudomonas panipatensis*
G8	+	+	-	*Enterobacter spp*
WD1	+	+	+	*Bacillus flexus*
WD2	+	+	+	*Bacillus licheniformis*
WD3	+	+	+	*Bacillus subtilis*

Bacilli gram-positive bacteria (G2, G5, G6, WD1, WD2, and WD3) are evaluated to distinguish their ability to form spores. If they are spore-forming, they can be *Bacillus spp* or *Clostridium spp*. It was found that each of the isolated bacterial strains G2, WD1, WD2, and WD3 are spore-forming. In this step, isolated bacterial strains G2, WD1, WD2, and WD3 are evaluated to distinguish whether they are strictly anaerobic or not. If they are strictly anaerobic, they are Clostridium spp. It was found that isolated strain G2 is strictly anaerobic, which means it is Clostridium spp. If they are not strictly anaerobic, they are *Bacillus spp*. It was found that isolated strains WD1, WD2, and WD3 are not strictly anaerobic, which means they are *Bacillus* spp. In this step, a citrate test was applied on isolated strains WD1, WD2, and WD3. If they are citrate positive, they can be *B. subtilis*, *B. licheniformis, B. flexus, or B. coagulans*. It was found that isolated strains WD1, WD2, and WD3 are citrate positive, which means they can be *B. subtilis*, *B. licheniformis, B. flexus, or B. coagulans*. In this step, bacteria are evaluated to distinguish whether they are strictly aerobic, or facultatively anaerobic. If they are strictly aerobic bacteria, they are *B. flexus.* It was found that isolated strain WD1 is strictly aerobic, which means it is *B. flexus*. If they are facultatively anaerobic bacteria, they can be *B. subtilis*, *B. licheniformis, or B. coagulans*. It was found that isolated strains WD2 and WD3 are facultatively anaerobic, which means they can be *B. subtilis*, *B. licheniformis, or B. coagulans*. In this step, these bacteria can be categorized further by determining their growth ability in a medium containing 6.5% (w/v) NaCl. To

451 determine this growth ability, bacteria are added to a sterile test tube containing 6.5% (w/v) NaCl broth
452 (which is a mixture of nutrient broth and 6.5% (w/v) NaCl) and incubated for 24 h. A positive test is
453 indicated by the presence of turbidity. If they can grow in a medium containing 6.5% (w/v) NaCl, they
454 can be *B. subtilis or B. licheniformis*. Otherwise, they are *B. coagulans*. It was found that isolated strains
455 WD2 and WD3 can grow in a medium containing 6.5% (w/v) NaCl, which means they can be *B. subtilis*
456 or *B. licheniformis*. In this step, *B. subtilis and B. licheniformis* bacteria can be easily distinguished
457 from each other by evaluating their ability to grow at 55°C. If the bacteria can grow at 55°C, they are
458 identified to be *B. licheniformis*. Otherwise, they are *B. subtilis*. It was found that isolated strain WD2
459 can grow at 55°C, which means it is *B. licheniformis*. It was also found that isolated strain WD3 is not
460 able to grow at 55°C, which means it is *B. subtilis*.
461 If Bacilli gram-positive bacteria are non-spore-forming, they can be *Brevibacterium spp*,
462 *Cellulosimicrobium spp, Corynebacterium spp, Lactobacillus spp* or *Mycobacterium spp. It was found*
463 *that each of the isolated bacterial strains* G5 and G6 *are* non-spore-forming, which means they can be
464 *Brevibacterium spp*, *Cellulosimicrobium spp, Corynebacterium spp, Lactobacillus spp* or
465 *Mycobacterium spp.* In this step, bacteria are evaluated to distinguish whether they are strictly aerobic,
466 facultatively anaerobic, or strictly anaerobic. If they are strictly aerobic, they can be *Brevibacterium*
467 *spp*, *Corynebacterium spp,* or *Mycobacterium spp*.
468 If they are facultatively anaerobic, they are *Cellulosimicrobium* spp. If they are strictly anaerobic
469 bacteria, they are *Lactobacillus spp. It was found that isolated strain* G6 is facultatively anaerobic,
470 which means it is *Cellulosimicrobium* spp. *It was also found that isolated strain* G5 is strictly aerobic,
471 which means it can be *Brevibacterium spp*, *Corynebacterium spp,* or *Mycobacterium spp.* In this step,
472 a catalase test was applied on isolated strain G5. If they are catalase-positive, they can be
473 *Brevibacterium spp* or *Corynebacterium spp*. *It was found that isolated strain* G5 is catalase-positive,
474 which means it can be *Brevibacterium spp* or *Corynebacterium spp.* In this step, isolated strain G5 is
475 evaluated to distinguish its ability to ferment glucose by applying a glucose fermentation test. If they
476 are glucose fermenters, they are *Corynebacterium spp*. If they are not glucose fermenters, they are
477 *Brevibacterium spp*. It was found that isolated strain G5 is a glucose fermenter, which means it is
478 *Brevibacterium spp.*

479 It was found that each of the isolated strains G1, G3, G4, G7, and G8 are Bacilli gram-negative bacteria.
480 In this case, an oxidase test was applied on isolated strains G1, G3, G4, G7, and G8. If they are oxidase-
481 positive, they can be *Pseudomonas spp*, *Aeromonas spp,* or *Vibrio spp*. It was found that each of G1,
482 G3, G4, and G7 are oxidase-positive, which means they can be *Pseudomonas spp*, *Aeromonas spp,* or
483 *Vibrio spp.* In this step, bacteria are evaluated to distinguish their ability to ferment glucose by applying
484 a glucose fermentation test. If they are glucose fermentation positive, they can be *Aeromonas spp* or
485 *Vibrio spp*. If they are glucose fermentation negative, they are *Pseudomonas spp*. It was found that each
486 of G1, G3, G4, and G7 are glucose fermentation negative, which means they are *Pseudomonas spp.* In
487 this step, isolated strains G1, G3, G4, and G7 can be categorized further by observing their colony
488 pigmentation colours in the agar plate. If they are yellow fluorescent pigment, they can be *P.*
489 *aeruginosa, P. fluorescens, P. stutzeri, or P. panipatensis.* It was found that the colony pigmentation
490 colours of isolated strains G1, G3, G4, and G7 are yellow fluorescent, which means they can be *P.*
491 *aeruginosa, P. fluorescens, P. stutzeri, or P. panipatensis.* In this step, Voges-Proskauer test was
492 applied on isolated strains G1, G3, G4, and G7. If they are Voges-Proskauer positive, they are *P.*
493 *aeruginosa.* it was found that isolated strain G3 is Voges-Proskauer positive, which means it is *P.*
494 *aeruginosa.* If they are Voges-Proskauer negative. They can be *P. fluorescens, P. stutzeri, or P.*
495 *panipatensis*. it was found that isolated strains G1, G4, and G7 are Voges-Proskauer negative, which
496 means they can be *P. fluorescens, P. stutzeri, or P. panipatensis.* In this step, the methyl red test is
497 applied on isolated strains G1, G4, and G7. If they are methyl red positive, they are *P. fluorescens.* It
498 was found that isolated strain G4 is methyl red positive, which means it is *P. fluorescens*. If they are
499 methyl red negative, they can be *P. stutzeri or P. panipatensis*. It was found that isolated strains G1 and
500 G7 are methyl red negative, which means they can be *P. stutzeri or P. panipatensis*. In this step, a urease
501 test was applied on isolated strains G1 and G7. If they are urease positive, they are *P. panipatensis.* It
502 was found that isolated strain G7 is urease positive, which means it is *P. panipatensis*. If they are urease
503 negative, they are *P. stutzeri*. It was found that isolated strain G1 is urease negative, which means it is
504 *P. stutzeri*.
505 If Bacilli gram-negative bacteria are oxidase negative bacteria, they belong to the Enterobacteriaceae
506 family. It was found that isolated strain G8 is oxidase negative, which means it belongs to the

507 Enterobacteriaceae family. In this step, isolated strain G8 is evaluated to distinguish its ability to ferment
508 lactose by applying a lactose fermentation test. If it is lactose fermentation positive, it can be
509 *Enterobacter spp, Citrobacter spp, Escherichia spp, or Klebsiella spp.* It was found that isolated strain
510 G8 is lactose fermentation positive, which means it can be *Enterobacter spp, Citrobacter spp,*
511 *Escherichia spp, or Klebsiella spp*. In this step, isolated strain G8 is evaluated to distinguish its ability
512 to motile by applying a motility test. If they are motile, they can be *Enterobacter spp, Citrobacter spp,*
513 *or Escherichia spp*. If they are nonmotile, they are Klebsiella. It was found that isolated strain G8 is
514 motile, which means it can be *Enterobacter spp, Citrobacter spp, or Escherichia spp*. In this step, the
515 Voges-Proskauer test was applied on isolated strain G8. If it is Voges-Proskauer positive, it is
516 *Enterobacter spp.* If it is Voges-Proskauer negative. It can be *Citrobacter spp or Escherichia spp*. it
517 was found that isolated strain G8 is Voges-Proskauer positive, which means it is *Enterobacter spp.*
518 Based on the above-combined analysis of isolated bacteria colony morphology results, cell morphology
519 results, Oxygen Intake, and Motility, Endospore characterization results, and finally biochemical
520 characterization results, it can be concluded that the 11 types of isolated indigenous bacterial strains,
521 which were coded G1, G2, G3, G4, G5, G6, G7, G8, WD1, WD2, and WD3, are *Pseudomonas stutzeri,*
522 *Clostridium spp, pseudomonas aeruginosa, pseudomonas fluorescens, Brevibacterium spp,*
523 *Cellulosimicrobium spp, Pseudomonas panipatensis, Enterobacter spp, Bacillus flexus, Bacillus*
524 *licheniformis*, and *Bacillus subtilis*, respectively.
525



526

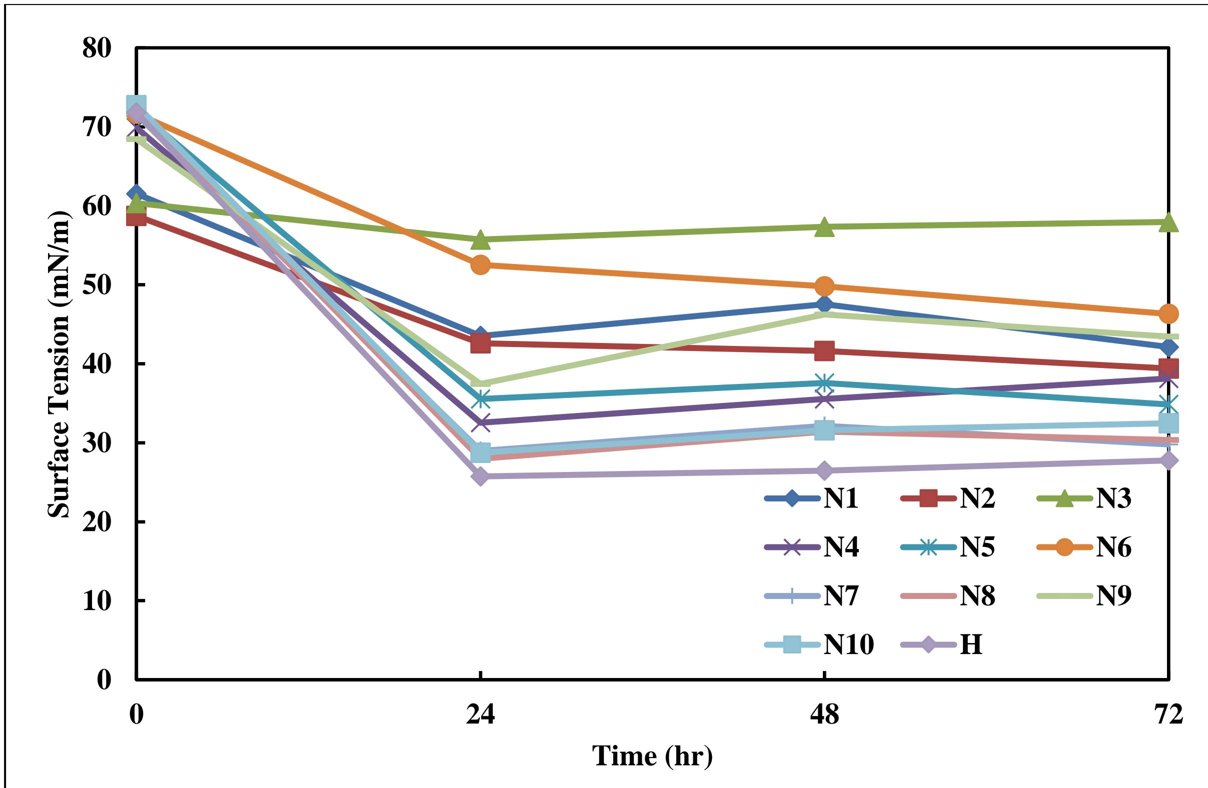
527 Figure 6. A flow diagram summarising the test for classification and identification of studied bacilli

528 bacteria based on their morphological and biochemical characteristics.

529 ### 3.3 Surface Activity

530 Isolated strain *Bacillus subtilis* was selected out of all isolated strains for further studies. The
531 biosurfactant produced from isolated strain *Bacillus subtilis* showed a significant increase in surface
532 activity in most of the nutrients after 72 hours. The ability of the produced biosurfactant to increase the
533 surface activity by decreasing ST and IFT against kerosene are shown in Figure 7, and Figure 8
534 respectively. The produced biosurfactant showed excellent surface activity when supplemented by
535 nutrients media N7, N8, N10, and the new proposed nutrient medium H. However, no significant
536 increase in surface activity was shown from nutrient medium N3, which was supplemented only by 50
537 g/l cane molasses, and that could be due to the missing of effective nitrogen sources and trace elements
538 that are required for reaching acceptable surface activity. The maximum surface activity was observed
539 in the new proposed nutrient medium H because ST was reduced from 71.8±1.9 mN/m to 25.7±1.2
540 mN/m. Similarly, IFT was significantly reduced by the same nutrient type from 48.4±2.1 mN/m to
541 0.38±0.07 mN/m after 24 hours of incubation. The ability of *Bacillus subtilis* to produce biosurfactants
542 have been confirmed by several reports (Makkar and Cameotra, 1997), (Hossein Ghojavand et al.,
543 2008), (Cooper et al., 1981), (H Ghojavand et al., 2008)(Alsharhan, 2003)(Makkar et al., 1997) (Amani
544 et al., 2010). Cooper et al. (1981) reported that *Bacillus subtilis* can decrease ST and IFT against
545 hexadecane to 27 mN/m, and 1 mN/m, respectively (Cooper et al., 1981). On the other hand, after
546 activating the isolated bacteria by the new proposed medium H, the maximum surface activity was
547 achieved comparing with the other media. This great result was due to adding sodium nitrate, potassium
548 nitrate and urea, which are reported that they are the best nitrogen sources for *Bacillus subtilis* (Makkar
549 and Cameotra, 2002). Moreover, the added trace elements have an important influence on producing
550 biosurfactants. Makkar et al. (2002) reported that the most effective trace elements used for
551 biosurfactant production are Zn, Cu, B, Co, and Mo (Makkar and Cameotra, 2002).

552



553 Figure 7. The surface tension of different nutrients broth media inoculated with *Bacillus subtilis*.

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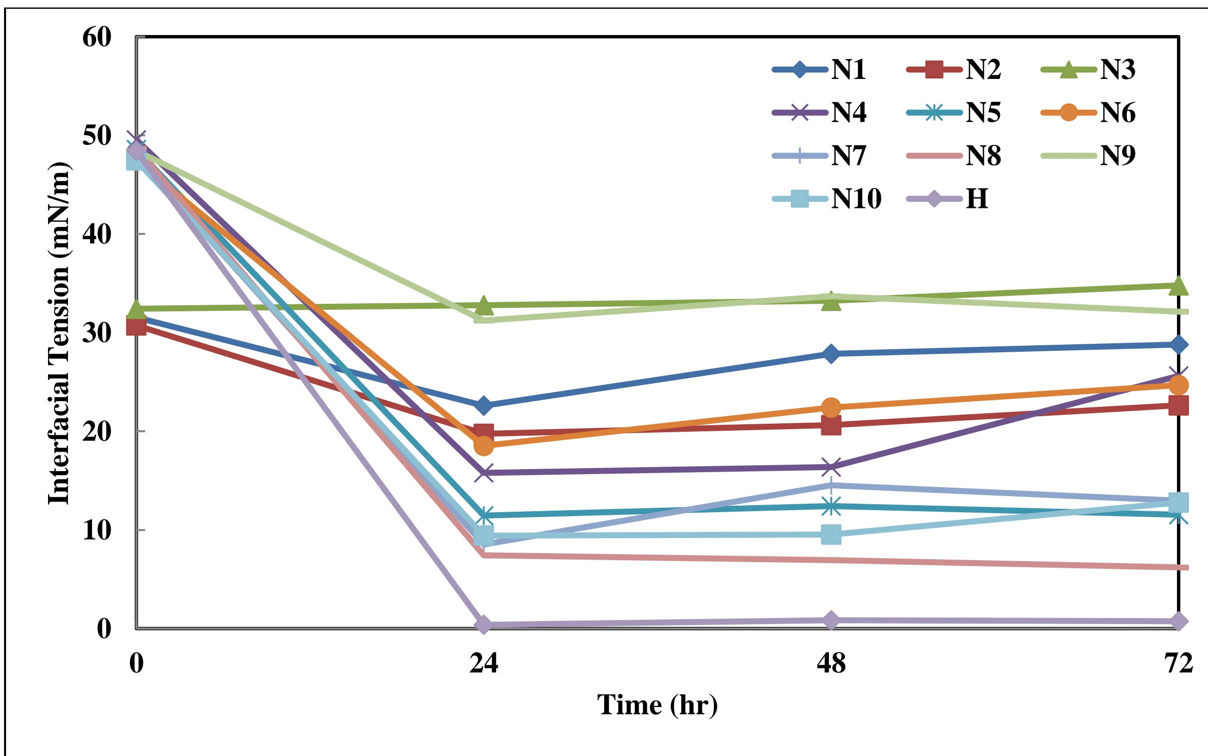


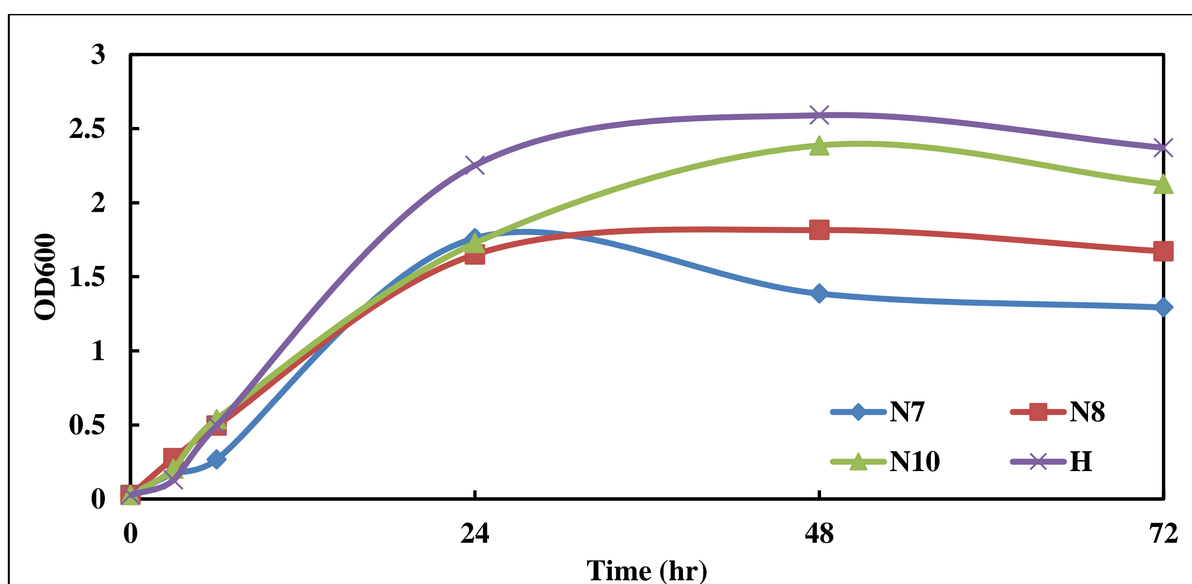
555 Figure 8. The interfacial tension of different nutrients broth media inoculated with *Bacillus subtilis*

556 against kerosene.

557

558 ### 3.4 Bacterial Growth Profile

559 Further analysis for the growth and Decay rates of *Bacillus subtilis* during 72 hours of incubation in
560 N7, N8, N10, and the proposed nutrient medium H was implemented as shown in Figure 9. A lag phase
561 occurred during 3 to 6 hours of incubation, after that the exponential growth was observed up to 24
562 hours, then the stationary phase and death phase was observed during the period of 24-72 hours. A
563 maximum bacterial concentration (OD600nm 2.59±0.16) was obtained before growth decreased, as
564 indicated by the optical density decrease. Since the maximum surface activity and growth rate results
565 were obtained by the new proposed nutrient medium H, it was used for further studies.

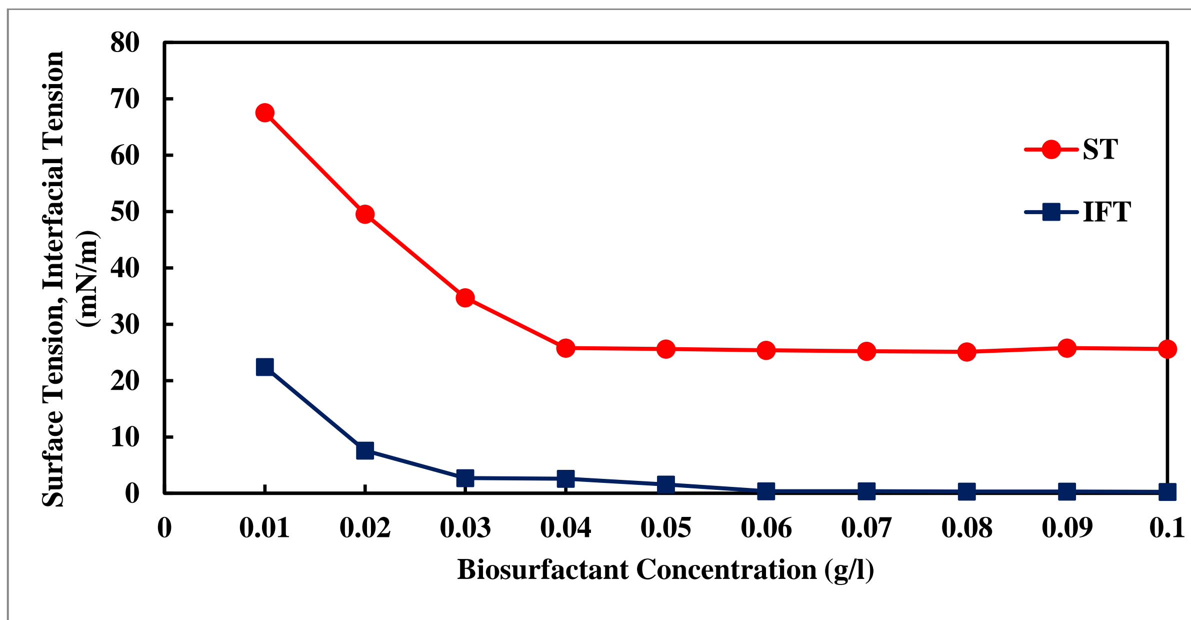
566 

567 Figure 9. The growth curve of biosurfactant-producing bacteria *Bacillus subtilis*.

568 ### 3.5 Biosurfactant Yield and Critical Micelle Concentration

569 The observed yield of the purified biosurfactant was 2.8±0.3 g/l. Pereira et al. (2013) produced 2.56 g/l
570 of surfactin from Bacillus isolate by adding 3 g/l of yeast extract to the culture media (Production and
571 Overview, 2018). The extracted biosurfactant was dissolved gradually in distilled water to increase
572 concentration, and ST and IFT were measured. The maximum reduction in surface tension and
573 interfacial tension were observed at 25.7±1.2 mN/m, 0.38±0.07 mN/m, respectively, at biosurfactant
574 concentration 0.04±0.01 g/l, as shown in Figure 10, even after adding more biosurfactant no change
575 occurred, therefore, this value was considered the CMC of the purified biosurfactant. This CMC value
576 is slightly higher than the CMC value reported by Cooper et al. (1981) and less than the CMC value
577 reported by Makkar et al. (1997), which were 0.023 and 0.160 g/l, respectively (Cooper et al., 1981),

578 (Makkar et al., 1997). Santos et al. (2016) reported that the value of critical micelle concentration for
579 biosurfactant applied in MEOR was usually from 0.001 to 2.0 g/l (Santos et al., n.d.). Consequently,
580 this produced biosurfactant was effective and efficient.



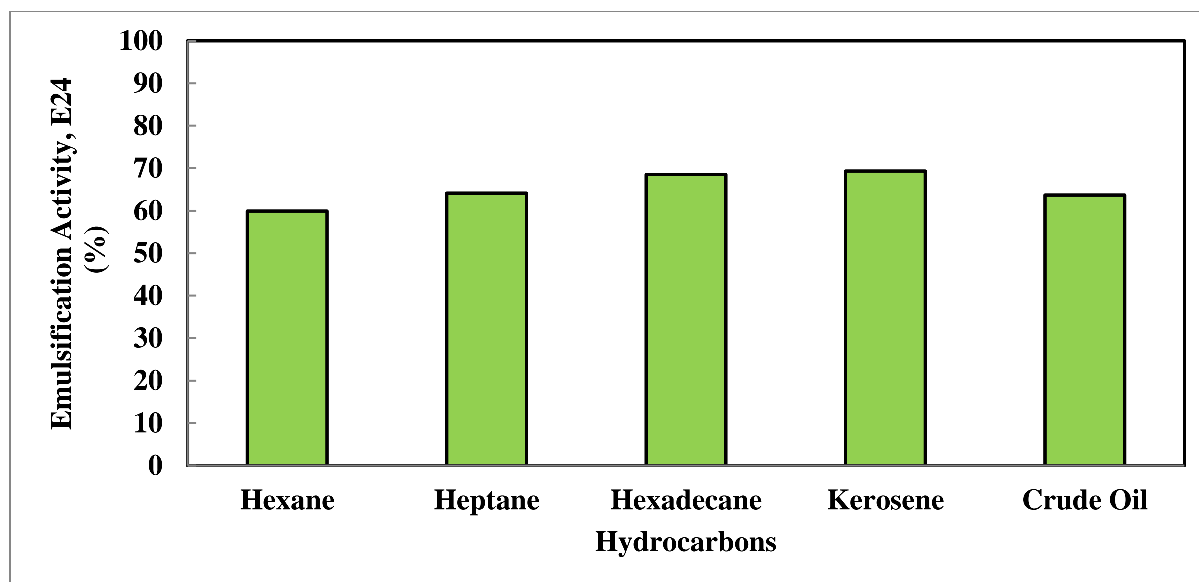
581

582 Figure 10. The critical micelle concentration CMC of the produced biosurfactant by *Bacillus subtilis*.

583 **3.6 Emulsification Activity**

584 The ability of produced biosurfactants to emulsify different hydrocarbons (hexane, heptane,
585 hexadecane, kerosene, and crude oil) was examined. It was found that all hydrocarbons were emulsified
586 with different values (60-70%) as shown in Figure 11. It was also found that the highest emulsification
587 activity was obtained against kerosene (69.6±1.5%), followed by Hexadecane (68.5±1.9%), Heptane
588 (64.1±0.4%), Hexane (59.9±1.3%), and crude oil (63.7±2.3%). The increase of biosurfactants
589 emulsification activity against hexane, heptane, and hexadecane, due to the increase in length of the
590 alkyl chain of hydrocarbons. While emulsification activity against kerosene and crude oil could be
591 varied depending on the composition of the hydrocarbon. Das et al. (2008) reported that biosurfactant
592 production by *Bacillus circulans* emulsified different hydrocarbons such as kerosene, diesel, benzene,
593 hexadecane, and petrol in the range of 30-80% (Das et al., 2008). Khopade et al. (2012) reported that
594 the emulsification index by biosurfactant produced by a marine *Nocardiopsis species* against
595 hydrocarbon was almost 80% E24, within 8–9 days (Khopade et al., 2012). However, the produced

596 biosurfactant showed a significant emulsification activity against long-chain hydrocarbons such as
597 crude oil (63.7±2.3%), which could significantly play an effective role in enhancing oil recovery.



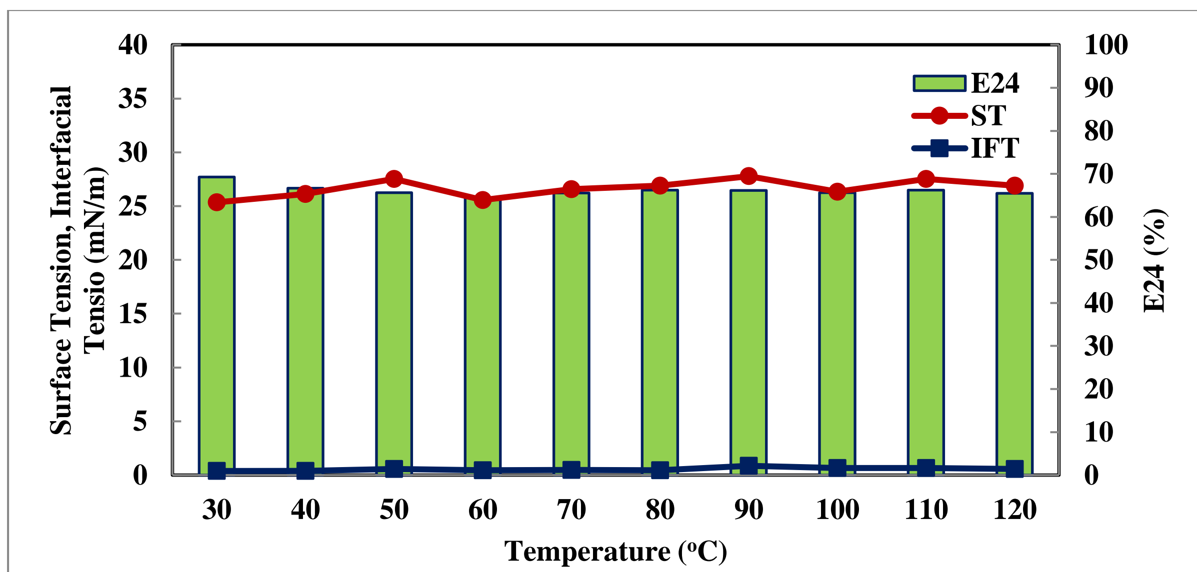
598

599 Figure 11. Emulsification index E24 of the produced biosurfactant by *Bacillus subtilis* against
600 different hydrocarbons.

601 **3.7 Stability Studies**

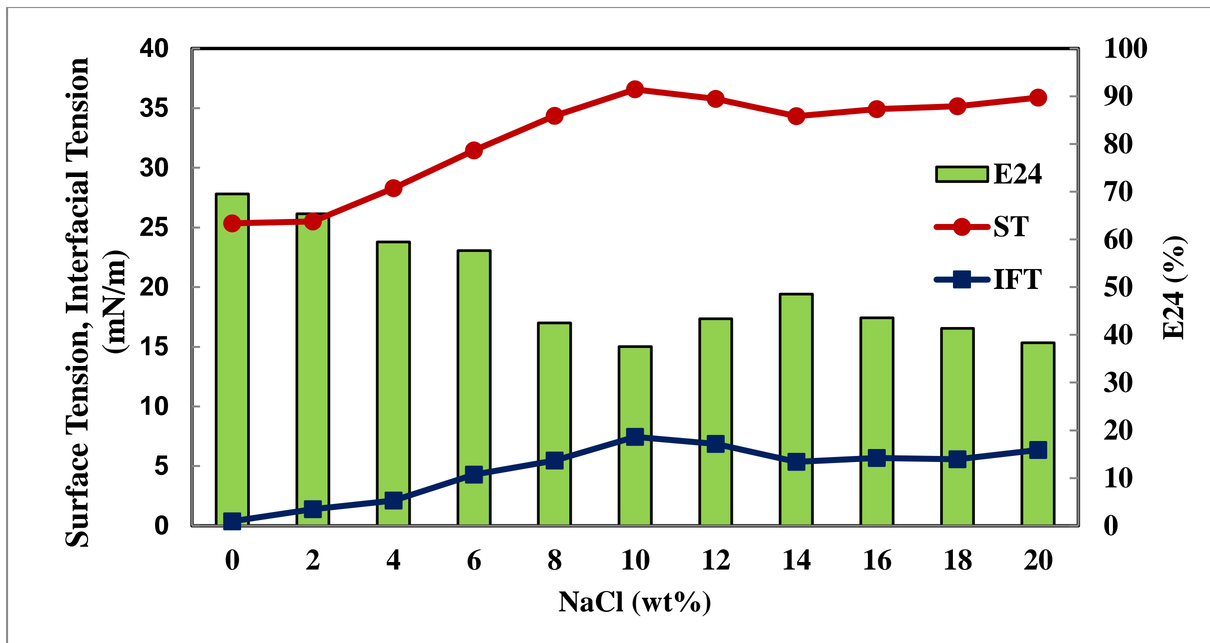
602 The cell-free supernatant was exposed to different conditions (temperature, salinity, and pH), to study
603 the stability of the produced biosurfactant. It was noticed that no significant change in surface activity
604 over a wide range of temperatures up to 120ºC as shown in Figure 12. This result is aligned with
605 temperature stability reports (Makkar et al., 1997), (Joshi et al., 2008b), (Makkar and Cameotra, 1997).
606 The surface activity was marginally affected by increasing salinity up to 2% (w/v) NaCl as shown in
607 Figure 13. The effect of salinity was more significant at higher NaCl concentrations (4–10% (w/v)), but
608 then the surface activity was stabilized at higher salinity (10-20% (w/v)). However, the produced
609 biosurfactant can retain more than 60% of its surface activity at the highest salt concentrations.
610 The surface activity was significantly decreased at pH range 2-4 because the biosurfactant settles out of
611 solution as a precipitate at acidic conditions. Consequently, the biosurfactant loses its ability to increase
612 the surface activity by decreasing each ST, IFT, and emulsification activity due to the precipitation and
613 structural distortion that occurred. Gudina et al. (2010) described the behaviour of biosurfactants in
614 acidic conditions due to the presence of negatively charged groups at the polar ends of the molecules

615 (Gudiña et al., 2010). It was also noticed that the produced biosurfactant solution has the optimum
616 surface activity at pH 7 as shown in Figure 14, where ST, IFT and E_{24} were 25.7±1.2 mN/m, 0.38±0.07
617 mN/m, and 69.6±1.5%, respectively. No significant change in the surface activity of the produced
618 surfactant at pH range 8-12, which was in agreement with several reports that confirmed the stability of
619 biosurfactant in an alkaline medium (Hossein Ghojavand et al., 2008), (Joshi et al., 2008b), (Batista and
620 Mounteer, 2006), (Gudiña et al., 2010).
621 Generally, high zeta potentials (more negative than -30 or more positive than +30), means there are
622 sufficient repulsive forces that exceed the attractive forces, which results in a relatively stable system,
623 whereas low zeta potentials are leading to aggregation, coagulation, or flocculation, and may lead to
624 poor physical stability (Ali et al., 2021; Awan et al., 2021). The effect of pH on the electrical stability
625 of the produced biosurfactants was also determined by measuring its zeta potential over a wide range
626 of pH (2-12). In this study, Low zeta potential values were observed at pH range 2-4 as shown in Figure
627 15. However, it was also found that the zeta potential values were more negative than -30 mV at pH
628 ranges 4-12. Such high zeta potential reveals the stability of the produced biosurfactant in aqueous
629 media. It could be concluded that the biosurfactants produced by *Bacillus subtilis* can tolerate the harsh
630 conditions of oil reservoirs, while it retains more than 60% of its surface activity and shows physical
631 stability in aqueous media, which means it is a suitable candidate for MEOR.



632

633 Figure 12. The effect of temperature on surface activity of the produced biosurfactant by *Bacillus*
634 *subtilis*.



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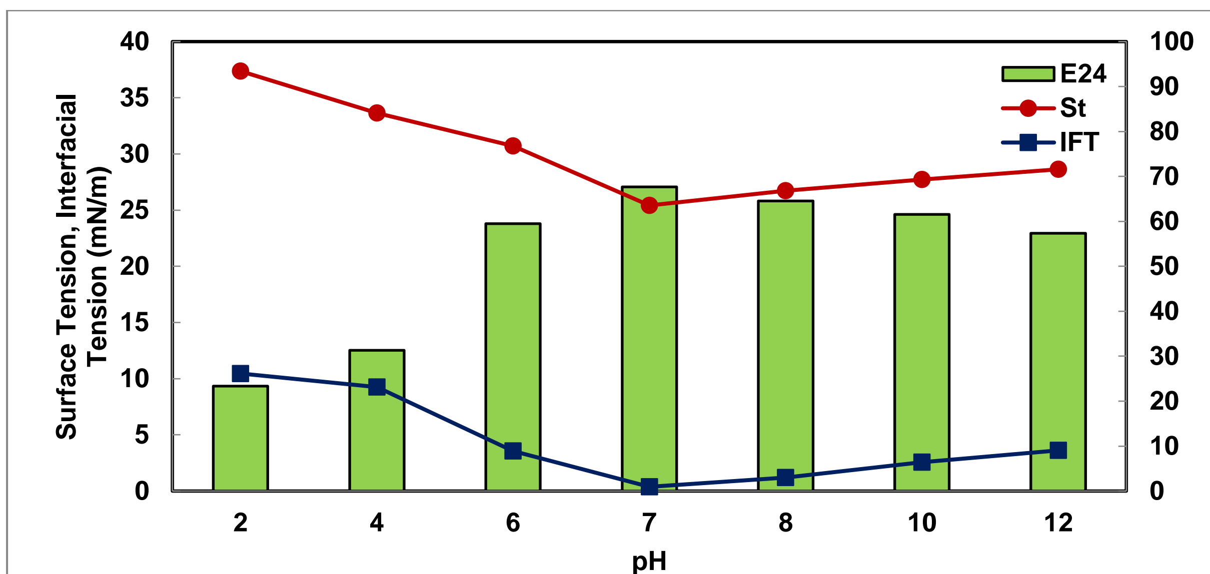
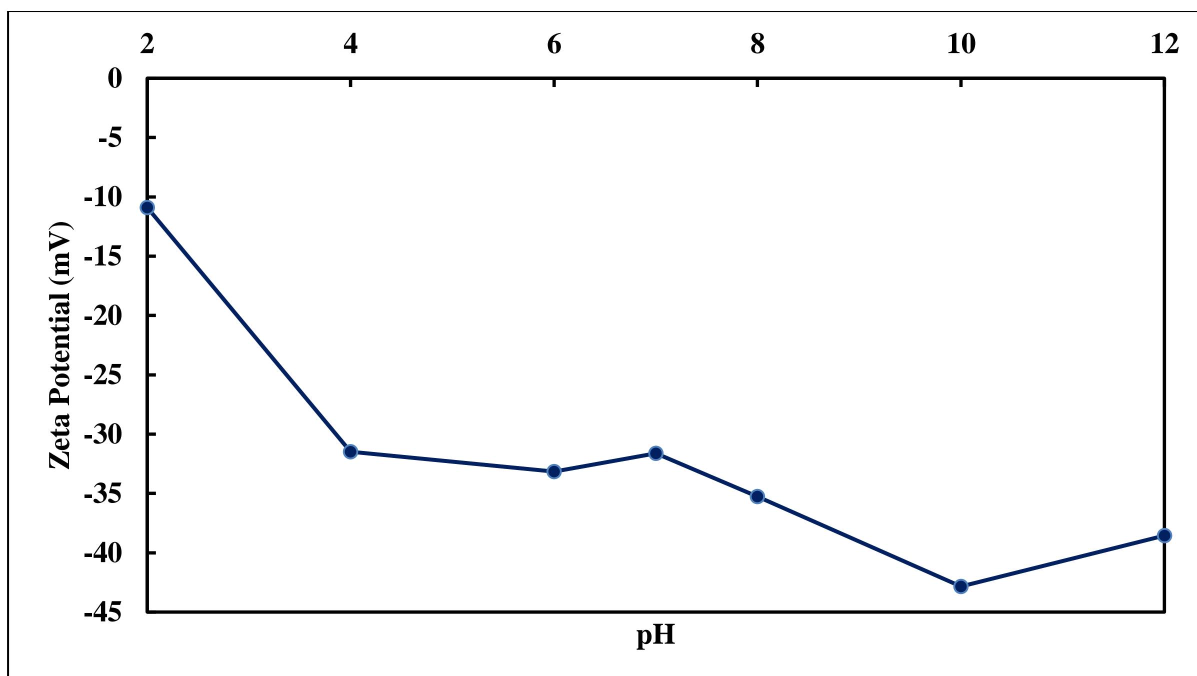
636 Figure 13. The effect of salinity on surface activity of the produced biosurfactant by *Bacillus subtilis*.



637

638 Figure 14. The effect of pH on surface activity of the produced biosurfactant by *Bacillus subtilis*.



639

640 Figure 15. Zeta potential of the produced biosurfactant by *Bacillus subtilis* as a function of pH.

641 **3.8 Core Flooding Experiments**

642 The core flooding experiments were performed to investigate the effect of produced biosurfactants by
643 *Bacillus subtilis* on oil recovery. The effect of water flooding followed by biosurfactant flooding in the
644 studied core was shown in Figure 16. It was observed that at the early stage of the water flooding, the
645 oil recovery increased dramatically, and the oil recovery rate reached the plateau of the water flooding
646 stage after injecting 3 PV. Then, the water flooding was continued until the injected pore volume was
647 5 PV, no more oil was produced and the oil recovery after water flooding was obtained (41-43%) of
648 original oil in place. During the biosurfactant flooding, after injecting 2-4 PV of the cell-free
649 supernatant, 25.19-39.35% of additional oil over the water flooding residual oil saturation was
650 recovered after biosurfactant flooding. several researchers reported that additional oil from 10–40% had
651 been recovered when injecting the cell-free supernatant in sandstone core plugs (Al-Wahaibi et al.,
652 2014; Mcinerney et al., 2004; Souayeh et al., 2014; Xu and Lu, 2011; Yakimov et al., 1997).
653 Furthermore, the obtained results indicated that the produced biosurfactant by *Bacillus subtilis* can
654 mobilize the crude oil by lowering its surface tension ST, interfacial tension IFT, and causing
655 emulsification. The reduction of the interfacial tension played an important role in enhance oil recovery
656 because it leads to an increase in the capillary number. The increase in a capillary number lowered the

657 residual oil saturation, and hence additional oil could be recovered (Al-Anssari et al., 2019; Ali et al.,

658 2017, 2015; Haghighi et al., 2020).

659 Table 16. Summary of Core-Flooding Experiments Results

Core ID	C-F-1	C-F-2	C-F-3
Flow Rate (cm^3/min)	0.25	0.50	0.75
Pore Volume, PV (cm^3)	9.24	10.65	10.60
Original Oil in Place, OOIP (cm^3)	4.60	6.30	5.20
Initial Oil Saturation, S_{oi} (%)	49.81	59.13	49.07
Initial Water Saturation, S_{wi} (%)	50.19	40.87	50.93
Residual Oil Saturation after Water Flooding, S_{orwf} (cm^3)	2.64	3.68	3.07
Recovery Factor after Water Flooding, RF_{wf} (%)	42.65	41.61	40.88
Residual Oil Saturation after Water Flooding, S_{orbf} (cm^3)	1.60	2.60	2.30
Recovery Factor after Biosurfactant Flooding, RF_{bf} (%)	22.57	17.12	14.89
Additional Oil Recovery over S_{orwf}, AOR (%)	39.35	29.32	25.19

660

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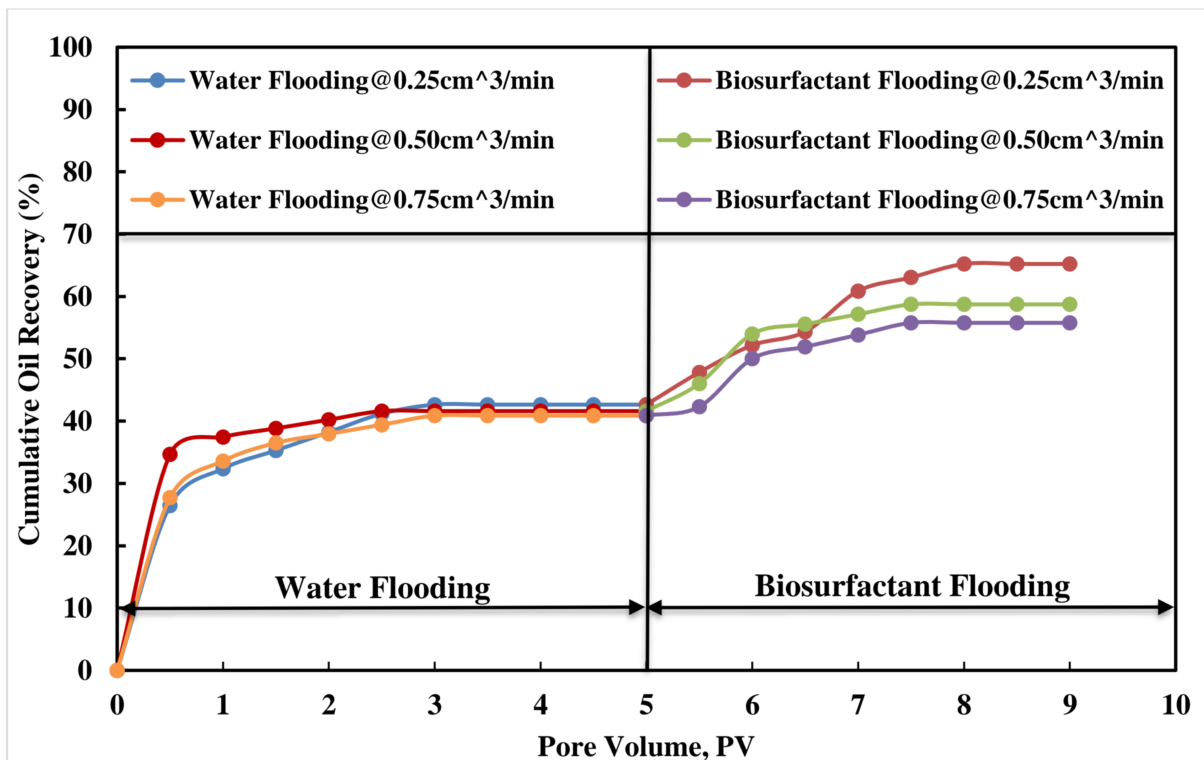

662 Figure 16. Cumulative oil recovery by produced biosurfactants by *Bacillus subtilis*.

663 Furthermore, the additional oil recovery over S_{orwf} reached its maximum at a flow rate of 0.25 cm^3/min
664 (Table 16). The obtained results indicate that the longer the saturation period is, the more the time for
665 the biosurfactant to redistribute in the core, and thus the more the crude oil that could be produced. Al-
666 Sulaimani et al. (2012) reported that 23% of residual oil has been recovered by the biosurfactant in core-
667 flood studies at a flow rate of 0.5 cm^3/min (Al-Sulaimani et al., 2012). Al-Wahaibi et. al (2014) reported
668 that the crude biosurfactant enhanced light oil recovery by 26% and heavy oil recovery by 31% in core-
669 flood studies at a flow rate of 0.4 cm^3/min (Al-Wahaibi et al., 2014). In this study, the produced
670 biosurfactant by *Bacillus subtilis* showed a significant improvement in oil recovery (39.35% of
671 additional oil over S_{orwf} at a flow rate of 0.25 cm^3/min), which reveals that it could be an effective and
672 promising candidate for MEOR.

673 **4. CONCLUSIONS**

674 This study has investigated the potential of producing biosurfactants in-situ by indigenous bacteria
675 isolated from Egyptian oil fields. MEOR screening parameters of 59 Egyptian oil reservoirs
676 representing the two main Egyptian oil concessions areas (the Gulf of Suez, and the Western Desert)
677 were summarized and statistically analysed. it was found that the minimum number of Gulf of Suez and
678 western desert oil reservoirs that have the potential to MEOR based on available data is equal to 8 and
679 3 oil reservoirs, respectively. Therefore, 11 oil samples were collected from these Egyptian oil
680 reservoirs to isolate, identify, and select the suitable bacteria for the in-situ production of biosurfactants.
681 Combined analysis of morphological, and biochemical characterization results showed that the 11 types
682 of isolated indigenous bacterial strains, which were coded G1, G2, G3, G4, G5, G6, G7, G8, WD1,
683 WD2, and WD3, are *Pseudomonas stutzeri, Clostridium spp, pseudomonas aeruginosa, pseudomonas*
684 *fluorescens, Brevibacterium spp, Cellulosimicrobium spp, Pseudomonas panipatensis, Enterobacter*
685 *spp, Bacillus flexus, Bacillus licheniformis, and Bacillus subtilis,* respectively. In this study, *Bacillus*
686 *subtilis* isolated from Egyptian oil fields located in the Western Desert was selected for producing
687 metabolic biosurfactants to improve oil recovery. It was found that *Bacillus subtilis* has the ability to
688 produce a highly active biosurfactant. For reaching the optimal surface activity of this biosurfactant, a
689 comparative analysis by 10 different reported nutrient media for bacilli species, and a new proposed
690 medium nominated H was performed. It was found that the maximum surface tension (25.7±1.2 mN/m),

691 and interfacial tension against kerosene (0.38±0.07 mN/m) was observed after 24 hours of incubation
692 in the new proposed nutrient medium H. The growth profile of *Bacillus subtilis* was investigated, the
693 maximum growth rate (OD600nm 2.59±0.16) was observed after 24 hours of incubation. The produced
694 biosurfactant was extracted and purified from culture media, and the biosurfactant yield was about
695 2.8±0.3 g/l. the critical micelle concentration CMC was also determined, it was 0.04±0.01 g/l at minimal
696 surface tension 25.7±1.2. The stability of produced biosurfactant in different conditions (temperature,
697 salinity, and pH) was investigated. There was no notable change in surface activity over a wide range
698 of temperatures up to 120ºC, which means *Bacillus subtilis* is thermophilic and could tolerate the harsh
699 temperature of oil reservoirs. The surface activity of produced biosurfactant exhibited high stability
700 against salt concentration, even at high NaCl concentration up to 20% (w/v), it retains more than 60%
701 of its surface activity, which means it could tolerate the harsh salinity of oil reservoirs. The optimum
702 salinity of the produced biosurfactant was in the range of 0 to 2% (w/v) NaCl concentration. The
703 optimum pH value of the produced biosurfactant was observed at neutral values. The emulsification
704 activity of the produced biosurfactant was confirmed, and it was noticed that the maximum
705 emulsification power against kerosene was 69.6±1.5%. finally, the core-flooding experiments were
706 conducted. The oil recovery after water flooding was 41-43% of original oil in place, whereas 25.19-
707 39.35% of additional oil over the water flooding residual oil saturation using biosurfactant produced by
708 *Bacillus subtilis* was recovered.

709 **ACKNOWLEDGMENT**

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