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Microbial Enhanced Oil Recovery (MEOR) Surfactant from *Pseudomonas aeruginosa* isolated from Automobile Garage soil.

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Abstract: ***Microbial enhanced oil recovery (MEOR), is gaining attention today for being environmentally friendly, economically attractive, demonstrating improvement in recovery of oil entrapped in porous media. It is considered to be more efficient than other EOR methods when applied to carbonate oil reservoirs. In the current study oil degrading bacteria was isolated from soil of automobile garage. The biosurfactant producing organism was screened and characterized. 16SrRNA sequence of the most potent bacterial strain suggests it to be belonging to the genus Pseudomonas and species aeruginosa. The biosurfactant produced by the bacteria was detected as rhamnolipid with emulsification index of 69%, foaming of 57.69% and interfacial surface tension of 0.6 mN/m. The MEOR column assay revealed that the additional oil recovery for sand1 and sand2 was 62.142% and 52.173 respectively.***

Keywords: Microbial enhanced oil recovery, Pseudomonas aeruginosa rhamnolipid, biosurfactant, emulsification index, interfacial surface tension

I. INTRODUCTION

The current global energy production and consumption from fossil fuels is about 80-90% that contribute 60% of total oil and gas production [1]. Though primary oil recovery during the oil production recovers 30-40% oil and additional 15-25% can be recovered by secondary oil recovery methods like water injection, steam injection etc., 35-55% of oil is left as residual oil in the reservoirs [2].Thus this residual oil becomes the target of enhanced oil recovery technologies and it amounts about 2-4 trillion barrels that is about 67% of the total oil reserves [3,4]. The techniques used for recovery of residual oil is commonly termed as Enhanced Oil Recovery (EOR). Enhanced oil recovery (EOR) is a tertiary recovery process that involves application of different thermal, chemical, and microbial processes to recover additional 7–15% of Original oil in place (OOIP) at an economically feasible production rate from poor performing and depleted oil wells [5]. EOR can significantly impact oil production, as increase in the recovery rate of oil by even a small margin could bring significant revenues without developing unconventional resources [6]. Microbial enhanced oil recovery (MEOR), the use of microorganism and their surface active metabolites in recovery of residual oil after secondary recovery is an alternative oil recovery approach which is claimed in the literature to be very promising in recovering up to 50% of residual oil and also reported to improve petroleum extraction efficiency [7-10]. In 1926, it was suggested for the first time that microorganisms could be used to release oil from porous media [11]. This technology has some advantages. Economically attractive for marginally producing oil fields; a suitable alternative before the abandonment of marginal Cells. According to a statistical evaluation (1995 in U.S.), 81% of all MEOR projects demonstrated a positive incremental increase in oil production and no decrease in oil production. The implementation of the process needs only minor modifications of the existing field facilities. It is less expensive to install and more easily applicable than another EOR method. MEOR products are all biodegradable and will not be accumulated in the environment, so environmentally friendly [12].

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Biosurfactants produced by microorganisms from various taxonomic groups and diverse habitats, have attained the special status of a chemical in demand in recent years, have many potential applications including enhanced oil recovery [13,14]. Soil of the automobile garages are contaminated with oil. Contaminants usually found at the garages include brake fluid, engine/dirty oil, petrol, kerosene and bitumen [15]. In the current study the microorganisms were isolated from soil of automobile garage in Udhana area of Surat, Gujarat. The isolates were screened for their ability to produce biosurfactant. The most potent microorganism and the chemical nature of the biosurfactant produced by it was identified. The applicability of the surfactant in enhanced oil recovery was evaluated by analysing its emulsification capacity, foaming characteristics and its ability to reduce the water surface tension. Finally, the oil recovery ability of the biosurfactant was assessed.

II. MATERIALS AND METHODS

The soil sample was collected from automobile garage in Udhana, Surat-Gujarat. Bushnell Hass Mineral Salts (BHMS) medium was used for the isolation of hydrocarbon degrading bacteria. The pH was adjusted to 7. The BHMS medium was supplemented with 1% (v/v) used engine oil as the sole carbon source [16]. After a series subcultures, inoculums from the flask were streaked on nutrient agar plates, and phenotypically different colonies were isolated. The pure isolates were stored in stock media with glycerol at -20℃ for further Characterization [17,18].

A. Screening of Biosurfactant producing Organisms

Each isolate was screened for Biosurfactant production by the methods described below:
Oil Displacement test [19], Drop collapse test [20], Hemolytic assay [21].

B. Morphological and Biochemical and Characterization

Standard procedure for gram staining, motility test and Colony Characterization of isolates was carried out by prescribed methods. Biochemical test viz., Indole production test, Methyl red test, Voga Proskauer, Citrate utilization test, Gelatin liquefaction test, H_2S production test, Catalase test, Urease, Nitrate reductase test, was performed for characterization of isolates [22].

C. Molecular identification of Isolates

Molecular identification of isolates was carried out by 16s rRNA sequencing. 16S rRNA gene sequence of the purified single colony of the selected isolate, G2N15 was obtained for precise confirmation about the identification of these strain. The obtained sequence was searched for its similarity using nucleotide BLAST (blast) software available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The identification was made by evaluating maximum score obtained, coverage, expected Value, and percentage identity.
For recovery and determination of dry weight of biosurfactant the culture was inoculated into medium BHM (1000ml) using glucose as sole carbon source (0.1M) and the pH value was adjusted to 7.5. Incubation was carried out at 30℃, 150 rpm, for 72 hours. For extraction the combination of acid precipitation and solvent extraction method is utilized. The broth culture sample of the broth was centrifuged (at 4 ^{0}C using 12000rpm for 15 min). The obtained supernatant was acidified with 6M HCl to pH 2.0 and was left overnight at 4 ^{0}C for complete precipitation of the biosurfactants. Supernatant was removed to obtain the pellet. The pellet was extracted with methanol for 2 hours while stirring continuously and then evaporated to dryness [23]. Dry weight of Biosurfactant was measured in mg/ml. The weight of Biosurfactant was measured by subtracting the Initial weight of the plate from weight of the plate containing biosurfactant.
Dry weight of Biosurfactant =Weight of the plate containing biosurfactant – Initial weight of the plate
The biosurfactant was characterized by determination of its the emulsification index (E24) [24], Foaming characteristics [25], Interfacial tension [26]. For determination of the emulsification power, the isolate was grown in BHMS Broth containing 0.1M glucose for 48 h at 30℃. After incubation the broth was centrifuged at 12000rpm for 20min.In next step 2ml of supernatant was added in test tube along with 2 ml of oil. The mixture was vortexed at high speed for 2 minutes. The tubes were maintained static for 24 h to stabilize the emulsion, and the emulsification index (E24) was calculated as

$$E24 = \text{height of the emulsified layer (mm)} / \text{total height [of the liquid column (mm)]} \times 100$$

Foaming of the biosurfactant in the culture medium was determined by shaking the supernatant (10 ml) for 2 min after 96 h of incubation period and then foaming was calculated according to the following equation calculated as

$$\text{Foaming} = (\text{height of foaming} / \text{total height}) \times 100$$

Interfacial tension of biosurfactant was estimated using a ring tensiometer (*Krüss* T9 Du *Nouy tensiometer.* Interfacial tension at different concentrations of biosurfactant solutions was carried out and measured against paraffin oil.

D. Optimization of pH, Temperature for Production of Biosurfactants

For optimization of pH the BHM Broth supplemented with 0.1M glucose as a sole carbon source was used with the pH ranging from 5-10 with 1N HCl and 1N NaOH. The broth was inoculated with 1ml of activated culture and was incubated at 30°C for 3-4 days and on each day parameter like biomass, oil displacement, growth rate, E24 was estimated to determine optimum pH for production of biosurfactants. After the pH optimization, temperature optimization was carried out at pH 7.0 with temperature range from 20°C-60°C. Biomass and growth rate was determined under optimum condition of pH and temperature up to eight day [27,28].

For determination of the chemical nature of the biosurfactant CTAB Method was employed [29]. This method is usually used to detect bacteria that produce rhamnolipid biosurfactants in agar plates. Blue agar plates were prepared using Bushnell-Haas medium (HI Media) (3.27 g/l) added with glucose (0.1 M) as a carbon source; 2 ml of trace element solution (acidified with 37% HCl) containing $FeSO_4 \cdot 7H_2O$ (7.1 mM), $MnSO_4 \cdot H_2O$ (8.9 mM), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.5 mM); CTAB (0.5 mM); methylene blue dye (0.02 mM); and agar (15 g/l). Rhamnolipids combined with the cationic substances that were present in the medium (CTAB and methylene blue) formed dark blue halos around the colonies from rhamnolipid-producing strains.

E. Particle size Distribution Analysis

For particle size distribution, water and paraffin light oil was homogenized using homogenizer. (T 10 basic ULTRA-TURRAX ®) at 6500 rpm. The droplet size distribution for the microemulsions at different salinities and biosurfactant was measured by a laser diffraction method of Zetasizer Ver. 6.00 (Malvern Instruments Ltd., Worcestershire, UK). The droplet size distribution of the dispersed particles can be obtained by the build in software of the instrument. The software uses a reflective index (RI) of paraffin liquid oil of 1.48 and a dispersant RI of 1.33 (water) during the measurement. Drops of microemulsions were introduced into the cell until the volume reached the optimum one, indicated by the instrument. All the experiments were conducted at 298 K [30].

F. MEOR Column Assay

Sand pack column assay [31] with modification was used for Enhanced oil recovery. Two different sand (calcite and silicate) was used for this purpose. Four sets were prepared for two different sample among them one served as control for each sand sample. The experiment was carried out at 30°C.

The system was flooded with water at a constant flow rate 1ml/min. Pore volume (PV, ml) was calculated using following formula,

PV= (weight of core 100% saturated with water - weight of dry core) / density of water

Porosity(%) of the system was measured by dividing the PV by the total volume of packed syringed with sand(20ml).In the Second step, the paraffin oil was injected into the system to replace water in all the syringe, until there was no water coming out from the nozzle in effluent. Original Oil in Place (OOIP) was calculated as the volume of paraffin oil retained in the system. Thus from this Initial Oil saturation (S_{oi}, %) and initial water saturation (S_{wi}, %) were calculated by following formula:

$S_{oi} = (OOIP / PV) \times 100$

$S_{wi} = [(PV – OOIP) / PV] \times 100$

Following step was to stabilize the system for 24 h and was flooded again to remove out the excess oil until no more paraffin oil was observed in the water effluent. Oil recovered after flooding (S_{orwf} ,ml) was determined using volumetric flask. Residual oil saturation was calculated as follow:

$S_{or} = [(OOIP - S_{orwf)} / OOIP] \times 100$

Finally, the residual oil in the system was supplemented with the supernatant containing biosurfactant and for control only BHM broth was utilized. This system was maintained at 30°C for 48 h at shaker with 100rpm. After incubation the system was again flooded to recover the oil. The recovered oil was measured volumetrically and denoted as S_{orbf} (ml).

The Additional oil recovery (MEOR) was calculated as follow:

MEOR (%) = $[S_{orbf} / (OOIP - S_{orwf)}] \times 100$

III. RESULTS AND DISCUSSIONS

Total 14 different bacterial strains were isolated from the soil of automobile garage located in Surat, Gujarat. Among them the isolate designated as G2N15 was found to be the most potential candidate. This Isolate was further studied for different parameter.

An oil displacement test is a preliminary screening method used to determine biosurfactant production ability of the isolated strain[32] (Deng et al.2020). All 14 isolates were screened for oil displacement on used engine oil. Maximum oil displacement was shown by isolate G2N15 with halo diameter 72mm (Figure 1) indicating that the strain with potentials for production of biosurfactant. The isolate G2N15 showed β hemolysis, representing complete lysis of the red blood cells surrounding the colonies (Figure 2). In the drop collapse test the supernatant collapsed as it touches the oil coated slide in the drop collapse test.

This showed that active biosurfactant to be present in the supernatant which is responsible for lowering the interfacial surface tension between the oil drop and the hydrophobic surface and did not form a stable drop on the oily surface. Drop collapse test is a sensitive and rapid method for screening bacterial colonies that produce surfactants [33].

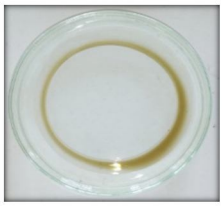


Figure:1 Oil displacemnt zone of halo



Figure: 2 Blood hemolysis plate



Figure:3Microscopic view of Gram-stained bacteria

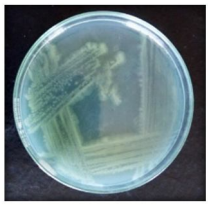


Figure:4 Isolate G2N15 on McConkey agar plates

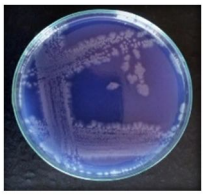


Figure:5 Isolate G2N15 on Nutrient Agar plate

TABLE I
BIOCHEMICAL CHARACTERIZATION OF ISOLATES

TEST	**RESULT**
Pigmentation	Green
Oxidase	+
Citrate	+
Indole	-
Methyl Reduction	-
VP	-
Catalase	+
Urease	-
H_2S Production	-
Nitrate Reductase	+
Gelatin Hydrolysis	+
Coagulase	-
McConkey	Lactose non fermenter

+ = Positive, - = Negative

The bacterial isolate G2N15 was found to be Gram negative, rod shaped (Figure 3) and motile
Biochemical characterization of isolate G2N15 is summarized in Table I

A. Molecular Identification

The forward sequence of isolate G2N15 is mentioned below:

GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTG
GGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTC
AGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
GTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGA
GGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA
GTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCTCCAGAAGTAGCTAGTCTAACCGCAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAAC
AAGGGTAGCCGTAGGGGTGGGAGCCGGGCCCGG

The sequence was searched for match using blast and following result was obtained.

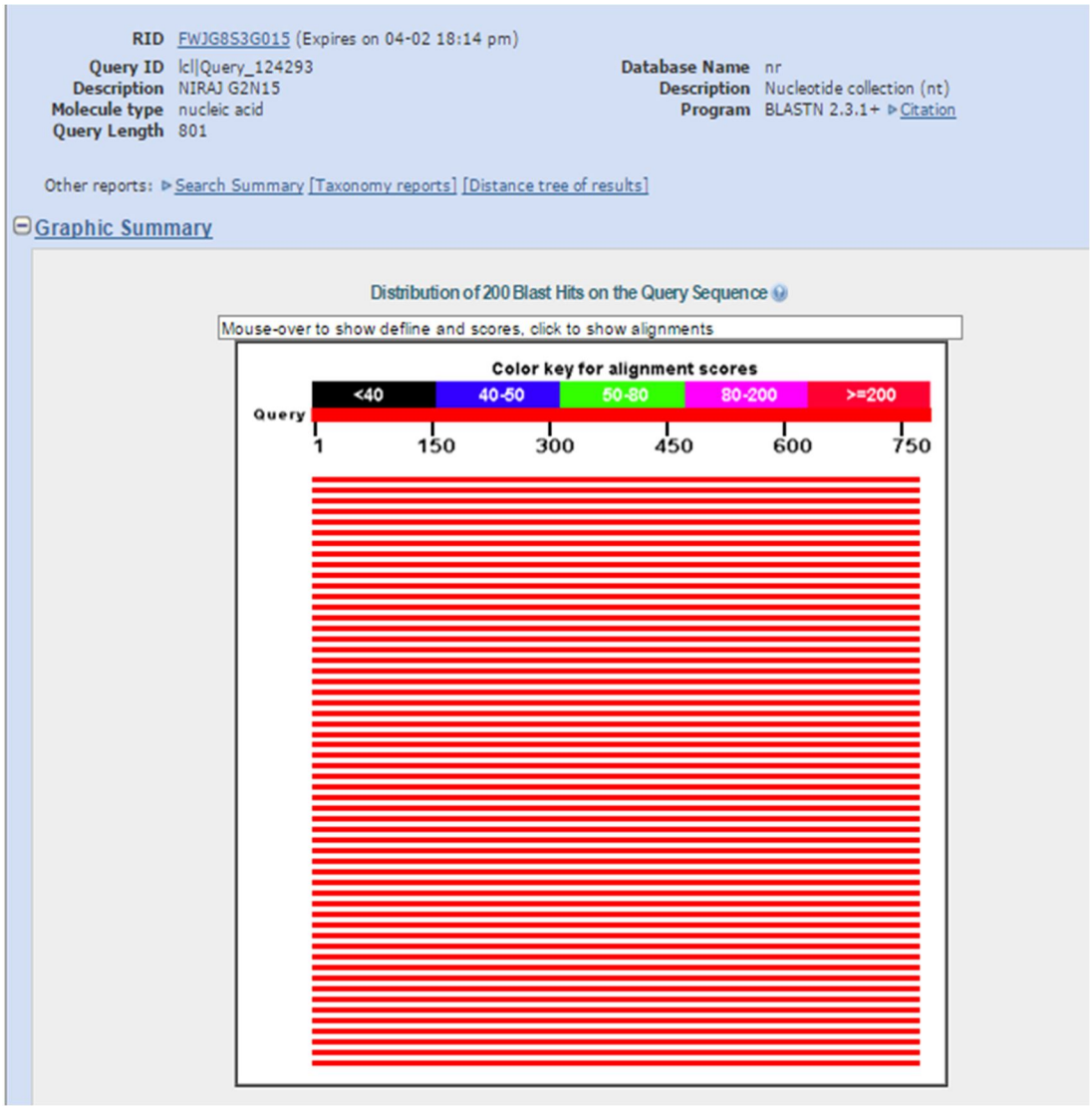


Figure: 6 Graphical Result of blast For Sequence of isolate G2N15

TABLE II
RESULT OF BLAST FOR SEQUENCE OF ISOLATE G2N15 FOR MAXIMUM IDENTITY

Description	Max score	Total score	Query	E value	Identity	Accession
Pseudomonas aeruginosa strain KU genome	1439	3393	98%	0	99%	CP014210.1
Pseudomonas aeruginosa strain F9670, complete genome	1439	5758	98%	0	99%	CP008873.1
Pseudomonas aeruginosa strain X78812, complete genome	1439	5753	98%	0	99%	CP008872.1
Pseudomonas aeruginosa DHS01, complete genome	1439	5753	98%	0	99%	CP013993.1
Pseudomonas aeruginosa strain USDA-ARS-USMARC-41639, complete genome	1439	5758	98%	0	99%	CP013989.1
Pseudomonas aeruginosa strain W45909, complete genome	1439	5758	98%	0	99%	CP008871.1
Pseudomonas aeruginosa strain W36662, complete genome	1439	5758	98%	0	99%	CP008870.1
Pseudomonas aeruginosa strain W16407, complete genome	1439	5758	98%	0	99%	CP008869.1
Pseudomonas aeruginosa strain T63266, complete genome	1439	5758	98%	0	99%	CP008868.1
Pseudomonas aeruginosa strain T52373, complete genome	1439	5753	98%	0	99%	CP008867.1
Pseudomonas aeruginosa strain T38079, complete genome	1439	5758	98%	0	99%	CP008866.1
Pseudomonas aeruginosa strain S86968, complete genome	1439	5774	98%	0	99%	CP008865.1

The results of the 16S rDNA sequence analysis of the isolate G2N15 suggest that it possesses 99% sequence similarity with the strain belonging to genus *Pseudomonas* and species *aeruginosa.*
The bacterial strain G2N15 is recommended to be *Pseudomonas aeruginosa.* In a previous work *Pseudomonas aeruginosa* isolated from a soil that was artificially contaminated by petroleum was able to produce a biosurfactant with adequate characteristics to be used in the MEOR [34].

TABLE III
DRY WEIGHT OF CRUDE BIOSURFACTANT

Weight of petri plate containing crude biosurfactant	Weight of petri plate	Dry weight of crude biosurfactant
60.915g	60.675g	0.24g/100ml

The dry mass of crude recovered biosurfactant was measured about 0.24g/100ml broth (Table III). Same method was utilized in a previous work for recovery of biosurfactant [25].



Figure:7 Emulsification index for isolate G2N15



Figure:8 Foaming % for isolate G2N15

Emulsification index E24 gives the measure of the emulsification capacity of any surface active molecule with different hydrocarbons [35]. Stabilizing the emulsion is a function of the biosurfactant concentration [36,37] The emulsification index was found to be 69% that represent a great ability of biosurfactant for formation of emulsion. Total height was 42mm and emulsion height was found to be at 29mm. This method provides better option for screening microorganisms that produce biosurfactant. This result was in accordance with other publications [34,38]. In the present study, foaming was observed after shaking that shows presence of surface active compound. Foaming was found to be 57.69%.The foaming characteristic of surfactant solutions is one of the most important property. Occurrence of foaming, indicates the presence biosurfactant [39,25].

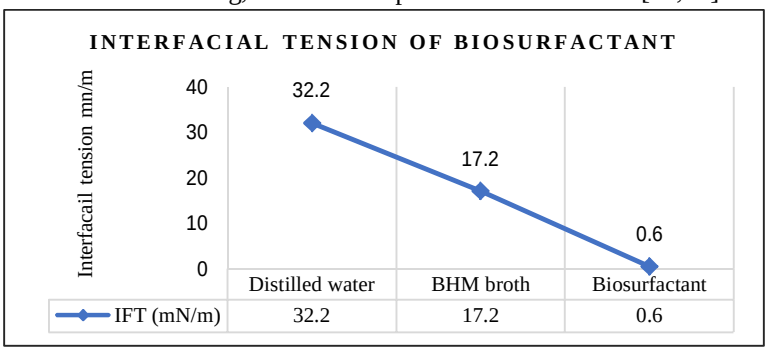
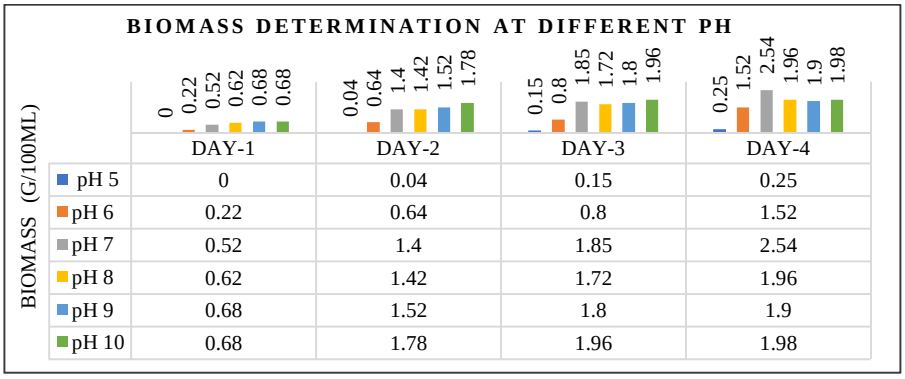


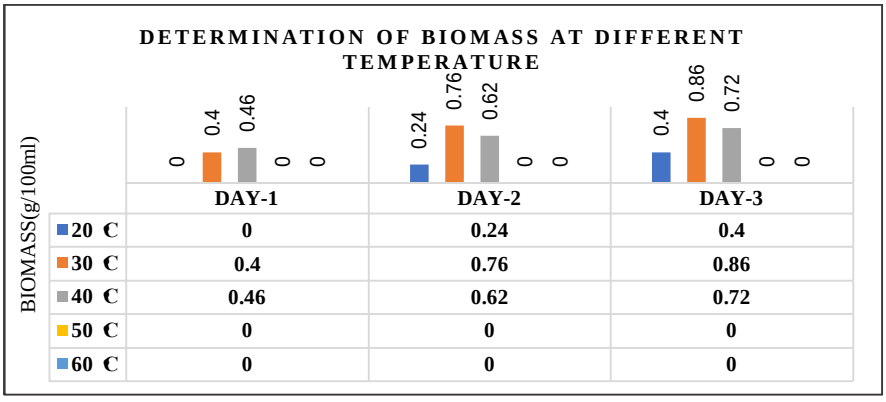
Figure 9 Interfacial tension

The interfacial tension was measured against paraffin oil. The interfacial tension for distilled water against water was found to be 32.2 mN/m and of BHM broth was found to be 17.2mN/m and this served as a blank for measuring interfacial tension for supernatant containing biosurfactant. The interfacial tension was found to be 0.6mN/m (Figure 9). This showed that the biosurfactant produced by isolated strain identified as *P. aeruginosa* is capable of reducing interfacial surface tension. A higher biosurfactant concentration in the test sample provides a lower surface tension until the critical micelle concentration (CMC) is reached [40]. The ability of the biosurfactant to reduce the surface tension of the water and a good emulsifying capacity indicates a promising application in microbial enhanced oil recovery (MEOR) [34].



	DAY-1	DAY-2	DAY-3	DAY-4
pH 5	0	0.04	0.15	0.25
pH 6	0.22	0.64	0.8	1.52
pH 7	0.52	1.4	1.85	2.54
pH 8	0.62	1.42	1.72	1.96
pH 9	0.68	1.52	1.8	1.9
pH 10	0.68	1.78	1.96	1.98

Figure 10: Biomass determination at different pH



	DAY-1	DAY-2	DAY-3
20 ℃	0	0.24	0.4
30 ℃	0.4	0.76	0.86
40 ℃	0.46	0.62	0.72
50 ℃	0	0	0
60 ℃	0	0	0

Figure 11: Biomass determination at different temperature

In the current study maximum biomass production was observed at pH 7.0 (Figure10) and temperature 30^{0}C (Figure 11). Temperature is a critical environmental factor affecting microbial growth and reproduction [41]. Similar result for optimal growth conditions of temperature and pH for strain belonging to the genus *Achromobacter* was reported [32]. In our study slow growth was observed at 20^{o}C which gradually increases and maximum biomass production (0.86/100ml) was observed at 30^{o}C (Figure 11). Production was found to decline at temperature beyond 30^{o}C.

The characteristic of the biosurfactant observed in this study with respect to Emulsification index (E24),Foaming,Interfacial tension was found to be in co-relation with previous study by [42] where biosurfactant rhamnolipid was isolated from *Pseudomonas aeruginosa*. In the present study also the biosurfactant was detected to be rhamnolipid.

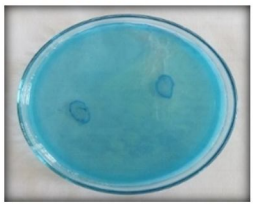


Figure: 12 Rhamnolipid detection for isolate G2N15

After incubation of 48 hours, blue coloured ring surrounding the colony was observed, indicating biosurfactants have the ability to form a coloured complex with the cationic indicator methylene. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue- purple, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Accordingly, rhamnolipid-producing strains can be recognized by the dark blue halos formed around the colonies and, ideally, the amounts of rhamnolipids produced could be correlated with the areas of the halos [29]. The dark blue halo around the colonies in the present study (Figure 12) confirmed the presence of rhamnolipid compound produced by the isolate identified as *Pseudomonas aeruginosa*.

It was reported that the biosurfactants produced by *Pseudomonas aeruginosa* are mainly rhamnolipids[43].

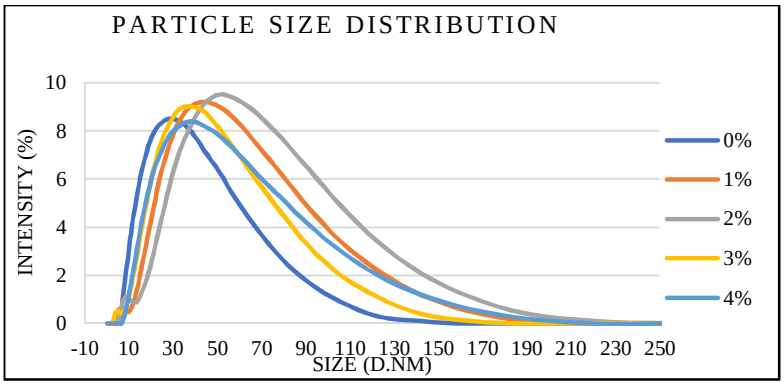


Figure 13: Particle size distribution

Table IV
Z average and particle size at different concentration

% NaCl	z-average	Diameter (nm)
0%	22.98	0.26
1%	33.5	0.326
2%	39.52	0.281
3%	28.06	0.324
4%	29.14	0.354

Particle size distributions in microemulsions at 0-4 wt% NaCl solutions are shown in Figure 13. The mean particle diameter and poly dispersity have been calculated from intensity, mass and number bimodal distribution. Size of the dispersed particles in microemulsions plays an important role in EOR because as the size decreases, the colloidal interaction between the dispersed particles increases.

The Z average diameter of dispersed droplet is the mean hydrodynamic diameter and is calculated according to the International Standard on dynamic light scattering ISO13321. The Z-average diameter is intensity weighted and is therefore sensitive to the presence of large particles. Z-average diameter is found to be strong function of the salinity of the microemulsion. Particle size and IFT of the microemulsion is dependent on the salinity and hence oil recovery by microemulsion flooding is also function of salinity of microemulsion [30]. In our study Z-average diameter of the microemulsion was detected at optimal salinity of 3 % NaCl with particle size about 0.324 nm diameter (Table IV). Which is in accordance with previous report [30].

Table V
Summary of results obtained in sand-pack column for oil recovery using isolate G2N15

PARAMETER	**SAND-1**		**SAND-2**	
	Paraffin oil	Control	Paraffin oil	Control
PV	5.523	5.395	7.157	7.902
Porosity	27.615	26.975	35.785	39.51
OOIP	3	3.1	4.5	4.8
PrS_{oi} (%)	10.863	11.492	12.572	12.14
S_{wi} (%)	89.136	88.5	87.424	87.85
S_{orwf} (ml)	1.6	1.5	2.2	2.8
OOIP-S_{orwf} (ml)	1.4	1.6	2.3	2
S_{or} (%)	46.66	51.61	51.11	41.66
S_{orbf} (ml)	0.87	0.21	1.2	0.28
MEOR(%)	62.142	13.125	52.173	14

The biosurfactant produced from the isolated strain of *Pseudomonas auregiona* was subjected to MEOR Column assay. The additional oil recovery for sand1 and sand2 was measured after nullifying the control volume (Table V). The additional oil recovered for sand1 and sand2 is 49.0% and 38% respectively. This result is in agreement with that of previous work [16,44].
Previous investigations have reported that the biosurfactants rhamnolipid isolated from *P aeruginosa* can be efficiently used for MEOR applications, since it presented expressive results when compared with other biosurfactants including synthetic surfactants [34].

IV. CONCLUSION

In the current study oil degrading bacterial strain designated as G2N15 was isolated from the contaminated soil of automobile garage located in Surat, Gujarat. Based on the results of Gram staining, biochemical and 16s rRNA sequence analysis the strain was suggested to be belonging to genus *Pseudomonas* and species *aeruginosa.* The biosurfactant produced by the strain was detected as rhamnolipid possessing excellent surface activities and emulsifying properties. The MEOR column assay predicted the efficiency of the biosurfactant for MEOR. Our current study demonstrated the possibility of developing an easy and inexpensive technique for MEOR using biosurfactant produced by *Pseudomonas aeruginosa* isolated from the contaminated soil of automobile garage.

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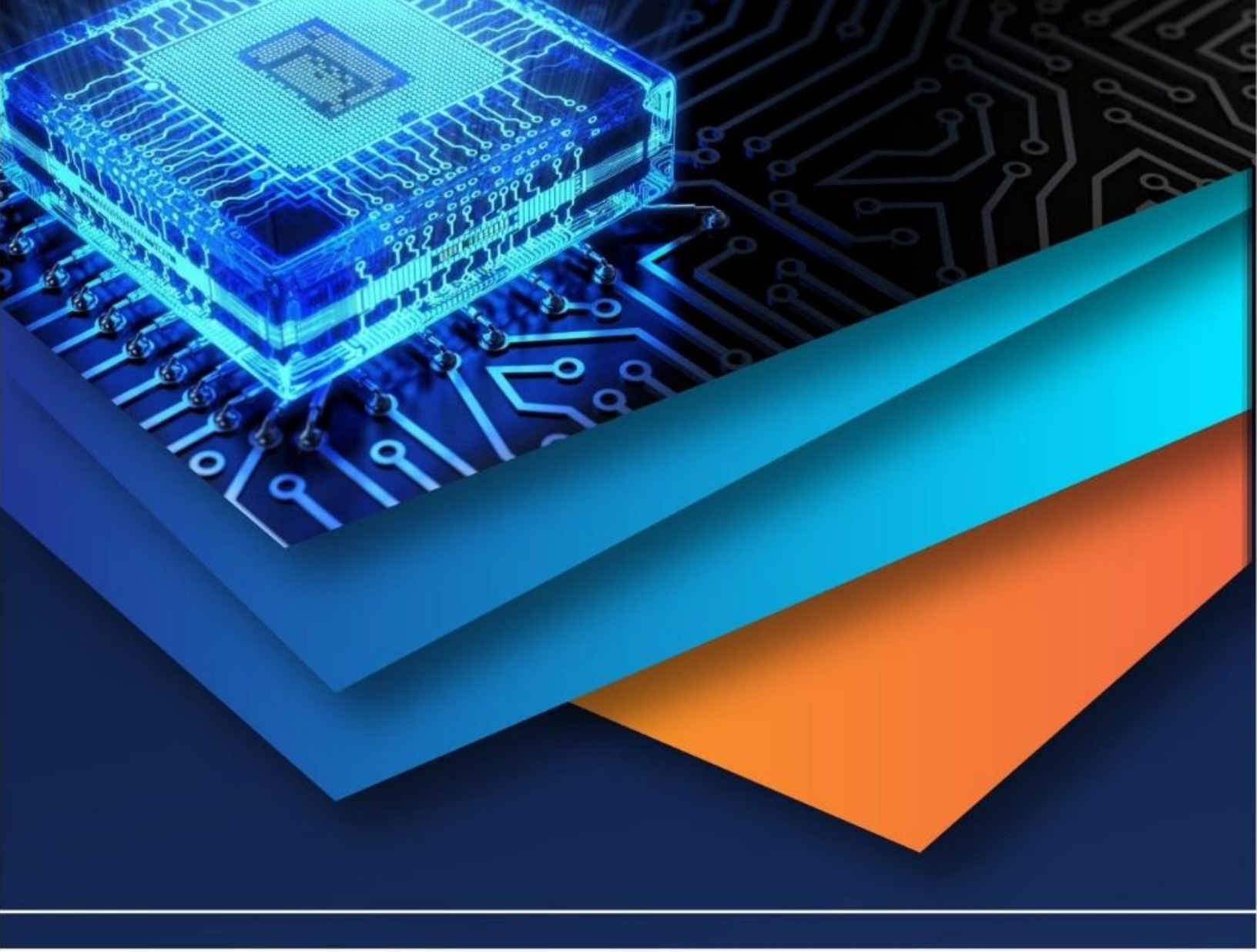
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