Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments

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Abstract

Amongst the total forty-seven bacterial isolates, eleven potent biosurfactant producing and concomitant hydrocarbon degraders were obtained after primary screening involving drop collapse method (DCM) and Oil-spreading method (OSM) followed by secondary screening comprising of Haemolytic assay (HA), Cetyl trimethyl ammonium bromide (CTAB) assay, Surface tension (ST), Emulsification index (E_{24}) and Emulsification activity (EA). 16S-rRNA sequencing and phylogenetic analysis revealed the presence of *Achromobacter, Bacillus*, *Citrobacter*, *Lysinibacillus, Ochrobactrum* and *Pseudomonas*. Two genera, *Achromobacter* (PS1) (observed for the first time) and *Bacillus* (SLDB1) were found to be glycolipid producers as evident by TLC, FT-IR and GC-MS chromatograms. The surface tension values were 30.43 mN/m and 31.10 mN/m with (E_{24}) of 69.90% and 65.23% respectively. Similarly the TLC, FT-IR and GC-MS results of the other two genera *Ochrobactrum* (GREW1) and *Bacillus* (SB2) confirmed them as lipopeptide biosurfactant producers with surface tension values of 31.14 mN/m and 28.16 mN/m and (E_{24}) of 59.51% and 61.35% respectively. Qualitative 2,6 - Dichlorophenol Indophenol (2,6 - DCPIP) and quantitative methods for hydrocarbon degradation revealed that *Achromobacter* sp. (PS1) showed a maximum degradation (46.32%) of 2% (w/v) crude oil with 70.77% and 77.17% reduction in peak area of aliphatic and aromatic fractions respectively with simultaneous lowering of surface tension from 59.27 mN/m (control) to 32.43 mN/m in 7 days. In case of *Achromobacter* sp. (PS1) and *Bacillus* sp. (SB2 and SLDB1), glucose supported biosurfactant production, whereas in *Ochrobactrum* sp. (GREW1) glucose along with 1% diesel enhanced biosurfactant production. This signifies the role of substrate in the nature of biosurfactants produced.

Keywords: Biosurfactant; Hydrocarbon degradation; Microbial Diversity; Glycolipid; Lipopeptide.

1. Introduction

Keeping in view of the increasing awareness towards environmental safeguards, stringent policies, volatile petroleum prices and simultaneous increase in consumer's demand, in recent years, the focus has been directed at the use of microbially produced surface active amphiphilic compounds known as the biosurfactants. They are promising substitutes for chemically – synthesized surfactants because of their unique properties like higher biodegradability, low toxicity, ecological acceptability, increased surface activities, higher foaming, low critical micelle concentrations (CMC), high selectivity and specificity at extreme temperatures, pH and salinity ranges. Biosurfactants constitute a heterogeneous group of biomolecules ranging from low molecular weight glycolipids, lipopeptides, flavolipids, phospholipids to high-molecular-weight polymers as lipoproteins, lipopolysaccharide-protein complexes and polysaccharide protein fatty acid complexes with wide structural and functional variability. These factors make them a multifunctional material of the 21st century with many commercial applications as cosmetics, personal care, textile processing, food, agricultural formulations, pharmaceutical industries, soil remediation, hydrocarbon degradation and oil recovery. For a bioremediation application, where solubility is an important criterion, biosurfactants prove to be promising vehicles for the removal of toxic polyaromatic hydrocarbons / dyes from contaminated soil / aquifers by lowering the surface tension and increasing the solubility of these compounds, thereby enhancing their bioavailability. In heavy metal-contaminated environments, biosurfactant enhances metal desorption from soils by forming complexes with free non-ionic forms of metals in solution. It also makes direct contact with absorbed metals at solid-solution interface under conditions of reduced interfacial tension with subsequent sequestration of metals into micelles [1].

23 Globally, in 2015 the biosurfactant market was estimated at 370.5 kilo tons, which is expected to
24 reach 476.5 kilo tons equivalent to 2.21 billion USD by 2018 and to a further 2.69 billion USD by
25 2023 with a compound annual growth rate (CAGR) of 4.2% [2]. However, the successful
26 commercialization of the biosurfactants is hindered by the high raw material and processing costs,
27 lower product concentrations and severe stable foam formation under aerated and agitated
28 conditions. These factors can be overcome by the selection of efficient strains, optimized medium
29 composition, use of cost-free agro-industrial wastes, development of novel economical and
30 efficient downstream processing methods. This study is designed to isolate and screen potent
31 biosurfactant producing bacteria with hydrocarbon degrading potential.

32 ## 2. Materials and methods

33 ### 2.1. Chemicals

34 All chemicals, solvents and reagents used in the present study were of analytical grade. The crude
35 oil was provided by IOCL Mathura refinery, Uttar Pradesh, India. Glycolipid (Rhamnolipid)
36 standard (JBR 215, 15% solution in water) was obtained from Jeneil biosurfactant Company
37 (Saukville, WI, USA), Lipopeptide standard (Surfactin) of 99.0% purity from Sigma.

38 ### 2.2. Sampling sites

39 Hydrocarbon contaminated samples as oil spilled soil, raw oil effluent, activated sludge, refinery
40 tank settled sludge, sludge with oil recovered, dry sludge, waste drain, refinery tank cleaning water,
41 surge pond water and bioremediation site soil were collected from various sections of petroleum
42 refineries as these are known to be excellent sources for isolating biosurfactant producing
43 microbes. Also, the samples from extreme environments as desert soil and hot spring water were
44 collected. The samples were collected in sterile Duran bottles and stored at 4°C until use.

45 ### 2.3. Enrichment and isolation

For isolation of pure microbial colonies, collected samples were subjected to three successive cycles of enrichment in erlenmeyer flasks containing 50 mL of minimal salt medium (MSM), pH 6.5 supplemented with 1% (v/v) of sterile diesel as carbon source and incubated at 30ºC, 120 rpm for 7 days. The composition of minimal salt medium (g/L) is as follows: $NaNO_3$ (7.5); KCl (1.1); NaCl (1.1); $FeSO_4.7H_2O$ (0.00028); K_2HPO_4 (4.4); KH_2PO_4 (3.4); $MgSO_4.7H_2O$ (0.5); yeast extract (0.5); glucose ($C_6H_{12}O_6$) (10.0) and trace element composition (g/L): $ZnSO_4.7H_2O$ (0.29); $CaCl_2.4H_2O$ (0.24); $CuSO_4.5H_2O$ (0.25); $MnSO_4$. H_2O (0.17) [3].

The final enrichment culture broth was plated on Bushnell Haas (BH) agar plates supplemented with (1% v/v) diesel and incubated at 30ºC for 24 h. BH agar is a recommended medium for studying the microbial utilization of hydrocarbons. The composition of BH agar (g/L) is, $MgSO_4.7H_2O$ (0.2); $CaCl_2.4H_2O$ (0.02); KH_2PO_4 (1.0); K_2HPO_4 (1.0); NH_4NO_3 (1.0); $FeCl_3$ (0.05) and agar (20.0) [4]. Phenotypically different colonies were picked and pure colonies were maintained on nutrient agar (NA) plates with subsequent sub-culturing for further studies.

2.4. Screening of biosurfactant producing isolates

The phenotypically different colonies obtained were initially screened using drop collapse and oil spread method as these methods give more precise response based on the surfactant property. The stability of the drops and the displacement of oil are dependent on the surface tension and correlated to the biosurfactant concentration [5]. Subsequently, the colonies found positive in primary screening were further selected for secondary screening.

2.4.1. Primary screening:

2.4.1.1. **Drop collapse method (DCM):** Approximately 40 µL of culture filtrate was placed on the hydrophobic surface of parafilm strip to observe the shape of the drop. The presence of surfactant leads to a reduction in the interfacial tension between the hydrophobic film and

69 hydrophilic drop which cause the appearance of flat/collapsed drops on the film. Un-inoculated
70 filtrate served as the negative control [5].

71 ***2.4.1.2.*** **Oil-spreading method (OSM)**: Approximately 30 mL of distilled water was poured to a
72 petri dish of 90 mm diameter followed by the addition of 30 µL of diesel on the water surface to
73 form a thin oil layer. Subsequently, 10 µL of culture supernatant obtained after seven days of
74 incubation was gently placed on the center of the oil layer surface. The diameter of displaced oil
75 was chosen as the criteria for the selection of potent biosurfactant producers [5].

76 **2.4.2. Secondary screening:**

77 ***2.4.2.1.*** **Haemolytic assay (HA):** Haemolytic assay was carried out by incubating the streaked 5%
78 blood agar plates (Hi-Media) with overnight grown culture at 30ºC for three days [5]. Appearance
79 of dark green zones under the colonies indicate alpha haemolysis or partial haemolysis. A yellow
80 transparent zone around the colony indicate beta or complete hemolysis of the blood cells. No
81 change in the blood agar plates indicates gamma or no hemolysis. Alpha and beta haemolysis were
82 considered positive for biosurfactant production.

83 ***2.4.2.2.*** **Cetyl trimethyl ammonium bromide (CTAB) agar test**: Approximately 30 µL of each
84 72h cell-free culture supernatant was loaded into pre-cut wells in CTAB-methylene blue agar
85 plates and incubated at 30°C for 72 h. The appearance of a dark blue halo zone around the well
86 was considered positive for anionic biosurfactant production [6].

87 ***2.4.2.3.*** **Emulsification index (E_{24}):** In a 15 mL clear glass tube, 1 mL each of diesel and seventh
88 day culture filtrate was added and vortexed vigorously for 2 min. The emulsified mixture was
89 allowed to stand undisturbed at room temperature for 24 h to separate the aqueous and oil phases.
90 The emulsification index (E_{24}) was calculated as the percentage of height (cm) of the emulsion
91 layer divided by the total height (cm) [5].

92 *2.4.2.4.* **Emulsification activity (EA):** For emulsification activity, an emulsification mixture
93 comprising of 0.3 mL diesel, 1 mL of buffer and 0.5 mL of cell-free supernatant was incubated for
94 1h at room temperature and the absorbance of the aqueous layer was determined
95 spectrophotometrically at 400 nm. This constitutes the test sample (A_T) as against the control (A_C)
96 in which the cell-free supernatant was replaced by 0.5 mL distilled water. The dilution factor was
97 kept into consideration [7].

98 $$\text{EU/mL} = \text{Absorbance at 400nm x dilution factor}/0.01 \qquad \textbf{Eq (A.1)}$$

99 **2.***4.2.5.* **Surface Tension (ST)**: The surface tension of the culture filtrate obtained after seven days
100 of incubation was measured with a digital surface tensiometer (SEO, Instruments, Korea) working
101 on the principle of Du Nouy ring method [8]. An un-inoculated flask served as the control. The
102 lowering of surface tension is dependent on the surfactant property. All surface tension readings
103 were taken in triplicates.

104 **2.5. Identification of the biosurfactant producers**

105 **2.5.1. Biochemical characterization**

106 The bacterial isolates were characterized and identified using the results of following biochemical
107 tests - IMViC, catalase, coagulase, triple sugar iron (TSI), arginine dihydrolase, casease, gelatin
108 hydrolysis as recommended by Bergey's manual of determinative bacteriology and as described
109 in "Laboratory exercises in microbiology" [9,10].

110 **2.5.2. Molecular identification**

111 *2.5.2.1.* **DNA extraction and PCR amplification of 16S-rRNA gene fragment**

112 The bacterial isolates were identified by 16S-rRNA gene sequencing. Genomic DNA of the
113 bacterial isolates was extracted from overnight grown culture, the extracted genomic DNA was
114 used as the template for PCR amplification of the 16S-rRNA sequence of the isolates using two

115 universal primers. These were BS1F 5′-GAGTTTGATCCTGGCTCA-3′ and BS1R
116 5′ACGGCTACCTTGTTACGACTT-3′, which are complementary to the conserved regions at the
117 5′- and 3′- ends of the 16S-rRNA gene corresponding to positions 9-27bp and 1477-1498bp of the
118 *Escherichia coli* 16S-rRNA gene [11]. The thermal cycle amplification program was performed
119 on a Bio-Rad PCR system 2400 (Bio-Rad laboratories, USA) with temperature program as: 94ºC
120 for 5 min, 94ºC for 1 min, 54ºC for 1 min, 72ºC for 1 min, 30 cycles; then final extension at 72ºC
121 for 10 min and finally storage at 4ºC. The purity and size of each PCR product was examined by
122 resolving the amplified product by gel electrophoresis on 2.0% agarose gel in 1× TAE (Tris acetate
123 EDTA) buffer for 1–2 h at 80V. The gel profiles were visualized in UV gel documentation system
124 (Bio-Rad laboratories, USA).
125 The sequencing of the amplified 16S-rRNA gene was outsourced from Eurofins genomics India
126 Pvt. Ltd. Bangalore. The amplified 16S-rRNA gene was sequenced through Sanger sequencing
127 method in Applied Biosystems 3730xL Genetic Analyzers, USA. The sequenced nucleotides were
128 searched for 16S-rRNA gene sequence homology using BLAST algorithm with NCBI database
129 [12].
130 Multiple sequence alignment was performed using CLUSTAL W and the phylogenetic tree was
131 constructed using the Neighbor-Joining method. The evolutionary distances were computed using
132 the maximum composite likelihood method and were expressed in the units of the number of base
133 substitutions per site. Phylogenetic analyses were conducted using MEGA4 [13].
134 The 16S-rRNA sequences of isolates from this study have been submitted to the NCBI Genbank
135 database and the accession numbers have been provided for the same.
136 **2.6. Evaluation of hydrocarbon degrading potential**

137 One of the numerous adaptations of microorganisms induced by presence of hydrocarbons is
138 biosurfactant production, which helps in metabolizing and degrading hydrocarbons thereby
139 representing a physiological response [14]. Hence, the eleven selected isolates obtained after
140 secondary screening were also evaluated for hydrocarbon degradation potential both qualitatively
141 and quantitatively.

142 **2.6.1. Qualitative degradation using Di-chlorophenol indophenol (DCPIP) method**

143 Hydrocarbons ranging from simple to complex with varying chain lengths were selected to
144 evaluate the hydrocarbon degrading potential of the isolates using DCPIP as redox indicator in W
145 medium with hydrocarbon source at 1% (v/v) [15]. These were hexane, decane, benzene, diesel
146 (C10 - C19 hydrocarbons) and crude oil (mixture of straight or branched chain aliphatics ranging
147 from C4 - C40 and aromatics from benzene to multi-ring polycyclic compounds). The acceptance
148 of electrons by an electron acceptor (DCPIP) results in the change of redox state from oxidized
149 (blue) to reduced state (colourless) thereby indicating the use of the hydrocarbon as carbon source.

150 **2.6.2. Quantitative degradation using gravimetric method and GC-FID**

151 **Gravimetric method**

152 Quantitatively the degradation of the hydrocarbon, in crude oil was evaluated by inoculating
153 overnight grown culture (OD_{600} equivalent to 1.0) of these respective eleven isolates separately in
154 50 mL of MSM supplemented with 2% (w/v) crude oil as sole carbon source at 30°C, 120 rpm for
155 seven days. Un-inoculated medium was treated as control. After seven days of incubation, the
156 culture broth was filtered through a porous layer of non-absorbent cotton to separate the crude oil
157 from the growth medium. The filtered broth was then centrifuged at 5000 rpm, 4°C and the
158 supernatant was analyzed for surface tension measurements. Each treatment was performed in
159 triplicates [16].

160 The residual amount of the total petroleum hydrocarbon (TPH) in the flask and the cotton was
161 recovered by adding 20 mL of petroleum ether thrice. The petroleum ether fraction containing the
162 residual crude oil of each treatment was pooled and dehydrated using anhydrous sodium sulfate
163 for 4 h to remove moisture. This was then vacuum dried using a rotary evaporator at 40ºC. The
164 mass of the residual oil was measured by gravimetric method using a high accuracy electronic
165 balance (Sartorius, Germany). The degrading efficiency of the isolates was calculated as follows
166 [17],

167 $$\text{TPH degrading efficiency} = [1 - (Y + e) / X] * 100 \qquad \textbf{Eq (A.2)}$$

168 Where 'X' is initial crude oil concentration (g)
169 'Y' is residual oil (g)
170 'e' is oil loss (g) due to evaporation, [Oil loss = weight of crude oil in control (uninoculated)
171 flask at zero day of incubation - weight of crude oil in control (uninoculated) flask after seven
172 days of incubation]

173 **Gas Chromatography -FID Analysis**

174 Approximately 0.1g of degraded TPH was dissolved in *n*-pentane to remove all asphaltenes;
175 subsequently, the soluble fraction was air dried and re-dissolved in 1 mL of hexane and loaded on
176 silica gel (60-120 mesh) column. Aliphatic and aromatic fractions of the crude oil were separated
177 by eluting the loaded sample using hexane (150 mL) and toluene (150 mL) respectively [18]. The
178 hexane and toluene extracted fractions were further vacuum dried and analyzed by gas
179 chromatography (GC) (Shimadzu GC-2010 7890A) with flame ionization detector (FID) using
180 capillary Rtx-5MS column (25 m × 0.25 mm × 0.25 um) to examine the degradation profile of
181 crude oil by these eleven isolates. The oven temperature program was: initial temperature: 110ºC

held for 2 min, then heated to 250ºC at 10ºC/min and held for 5 min, then heated to 280ºC at 15ºC/min and held for 17 min. Nitrogen was used as carrier gas (28.4 mL/min).

2.7. Biosurfactant production profile for four best biosurfactant producers

Based on the biosurfactant production and hydrocarbon degradation profile, four organisms were selected to evaluate the biosurfactant production pattern in terms of supplementation of carbon source in MSM. The experiment involved three sets; A: 1% (w/v) dextrose supplemented with 1% (v/v) diesel; B: 1% (w/v) dextrose only and C: 1% (v/v) diesel only.

2.8. Extraction of biosurfactant

The culture filtrate obtained was acidified with 6N HCl to pH 2.0 and kept overnight for precipitation. The precipitate was separated after centrifugation and extracted twice with a mixture of chloroform: methanol (2:1 v/v). The extracts were pooled and were concentrated under vacuum using a rotary evaporator [16].

2.9. Characterization of the partially purified biosurfactant

2.9.1. Thin layer chromatography

The partially purified extract was analyzed by thin layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck Co., Inc., Damstadt, Germany) with a solvent system consisting of chloroform/methanol/water (65:15:2, v/v/v) and visualized with different color developing reagents. Ninhydrin reagent (0.2% ninhydrin in ethanol) was used to detect lipopeptide biosurfactant as red-pink spots, iodine vapour for detecting lipids, orcinol-sulphuric acid reagent (1% in concentrated sulphuric acid) for the presence of glycolipids which shows the appearance of brown spots on heating to 110ºC [16].

2.9.2. Fourier transform infrared (FT-IR) spectroscopy

204 Fourier transform infrared spectroscopy (FT-IR) analysis for molecular composition of
205 biosurfactant was carried out using a Varian-7000 Fourier transform-infrared spectrophotometer
206 by first preparing its pellet in spectral grade KBr applying 5–6 tons /cm^2 of pressure for 10 min
207 using the hydraulic press followed by its scanning in the transmittance mode in the range of 400–
208 4000 cm^{-1} with a resolution of 4 cm^{-1} [12].

209 **2.9.3. Gas chromatography–mass spectrometry (GC–MS) of fatty acids**

210 The partially purified biosurfactant (10 mg) was dissolved and derivatized in 1 mL of 1M HCl–
211 methanol using a closed screw-cap tube for 4 h at 100ºC. The product containing the fatty acid
212 methyl esters (FAMEs) was partitioned by adding double distilled H_2O (1 mL) and the organic
213 phase was extracted over anhydrous sodium sulfate for moisture removal. The samples were
214 analyzed by GC-MS: 1 µl was injected into a Shimadzu GC–MS (QP2010 ultra) equipped with a
215 RTX-5MS fused silica capillary column (0.25 mm film thickness, 0.25 mm internal diameter, 30
216 m in length). Helium at 1.5 mL/min flow rate was used as the carrier gas and the operating
217 temperature of the column and injector were 140ºC and 260ºC respectively, the electron impact
218 ion source was maintained at 230ºC. Electron impact mass spectra were recorded at 70 keV. The
219 mass spectrum of each fatty acid methyl ester was matched to the "National Institute of Standards
220 and Technology" (NIST) mass spectral library database to determine probable fatty acids
221 composition of the biosurfactant.

222 **3. Results and Discussion**

223 **3.1. Screening and identification of biosurfactant producing isolates**

224 Amongst forty-seven total isolates, thirty-seven isolates showed positive results for DCM and
225 OSM respectively in primary screening (Table 1). Of these thirty-seven primary screened colonies,
226 eleven colonies were finally selected based on secondary screening results which involved

227 lowering of surface tension in the range between 28 to 42 mN/m with the maximum reduction
228 observed in *Bacillus* sp. (SB2), *Achromobacter* sp. (PS1), *Bacillus* sp. (SLDB1) and
229 *Ochrobactrum* sp. (GREW1) with values of 28.16 ± 0.19 mN/m, 30.43 ± 0.44 mN/m, 31.10 ± 0.71
230 mN/m and 31.14 ± 0.68 mN/m respectively as against the un-inoculated (control) medium with
231 a surface tension of 69.84 ± 2.2 mN/m and deionized water of 70.31 ± 1.84 mN/m (Table 2).
232 Cooper [19] reported that an isolate can be a promising biosurfactant producer if it reduces the
233 surface tension of a liquid medium to 40 mN/m or less. The highest emulsification index (E_{24}) of
234 69.90 ± 0.97%, 65.23 ± 1.48%, 64.22 ± 1.44% and 61.35 ± 1.15% was observed in isolates
235 *Achromobacter* sp. (PS1), *Bacillus* sp. (SLDB1), *Pseudomonas* sp. (MRBSIT1), and *Bacillus* sp.
236 (SB2) respectively with stable emulsions even after one week. The similar rating was observed for
237 emulsification activity with highest in *Achromobacter* sp. (PS1) (97.05 ± 1.62 EU/mL). These
238 colonies also showed mucoid colony morphology on BH agar plates supplemented with diesel
239 indicating the production of exopolysaccharides as reported by Subudhi et al. [20].
240 Among the eleven isolates, five isolates were found to be Gram negative and six were Gram
241 positive. The sequence and the phylogenetic analysis are shown in Fig. 1. The 16S-rRNA gene
242 revealed the presence of six different genera *Achromobacter, Bacillus*, *Citrobacter, Lysinibacillus*,
243 *Ochrobactrum* and *Pseudomonas*. These genera belonged to either Proteobacteria or Firmicutes
244 phylum (Table 3). The 16S-rRNA gene sequence analysis showed 100% sequence similarity for
245 *Pseudomonas* sp. (MRBSIT1) and *Ochrobactrum* sp. (GREW1), whereas 99% for all the other
246 isolates. Occurrence of *Pseudomonas* as the dominant genera for biosurfactant production in
247 hydrocarbon contaminated environments has also been reported by Chirwa and Bezza [21]. Saisa-
248 Ard et al. [22] reported species belonging to *Acinetobacter*, *Bacillus*, *Corynebacterium*,
249 *Pseudomonas*, *Rhodococcus* and *Serratia* as the most widely reported genera for biosurfactant-

250 producing bacteria from hydrocarbon contaminated terrestrial or marine sites, however only very
251 few reports have been highlighted related to biosurfactant production from genera *Achromobacter*
252 [23], *Citrobacter* [24], *Lysinibacillus* [25] and *Ochrobactrum* [26].

253 **3.2. Evaluation of the hydrocarbon degrading potential of biosurfactant producers**

254 Evaluation of qualitative hydrocarbon degradation using DCPIP by the eleven isolates of genera
255 *Achromobacter*, *Bacillus*, *Citrobacter*, *Lysinibacillus*, *Ochrobactrum,* and *Pseudomonas* showed
256 positive results for 1% (v/v) crude oil and diesel (Table 4). Of all the genera, only *Pseudomonas*
257 (MRBSIT1) and two isolates of *Bacillus* (TC2 and SB2) were found to degrade hexane. For
258 benzene, *Pseudomonas* (MRBSIT1) and *Citrobacter* (BRRO1) showed positive results. This
259 limitation in degradation behavior with respect to hexane and benzene hydrocarbons may be
260 explained on the basis of log K_{ow} (octanol/water partition coefficient), which proves to be a useful
261 parameter to predict the suitability of the solvent for bioconversion [27]. It is well established that
262 the solubility of hydrocarbons in aqueous system decrease while their respective log K_{ow} values
263 increase with the corresponding increase in the molecular weight of hydrocarbons. This allows the
264 diffusion of low molecular weight hydrocarbons hexane and benzene having low log K_{ow} values
265 of 2.3 and 3.5 respectively from the surrounding aqueous system to the cells thereby destabilizing
266 the cell membrane integrity and thus resulting in the limitation of hydrocarbon degradation. On
267 the other hand, the higher molecular weight hydrocarbons (decane and diesel) with low solubility
268 in aqueous system and corresponding higher K_{ow} values of 5.6 and 7.7 respectively form a biphasic
269 system with the W medium used in DCPIP method. In the presence of these less soluble
270 hydrocarbons, the logarithmic phase cells with cell density of 1.0 at OD_{600} nm, shows an adaptation
271 by producing biosurfactant. These amphiphilic biosurfactant molecules partition themselves at the
272 hydrocarbon water interphase and acts as a mediator by increasing the mass transfer rates of

273 hydrophobic contaminants (hydrocarbon based substances) into the aqueous phase through
274 specific interaction resulting in mobilization and solubilization. Mobilization involves the
275 reduction of surface and interfacial tension while solubilization results in the dramatic increase in
276 the apparent solubility of hydrocarbon due to its aggregation within the surfactant micelles [28].
277 Similarly, Kubota et al. [15] reported degradation of decane using DCPIP method by one *Bacillus*
278 (ODMI57) and two *Pseudomonas* sp. (F721 & F722). While none of the isolates amongst the
279 thirty-six bacterial colonies belonging to different genera *Acinetobacter*, *Rhodococcus*, *Gordonia*,
280 *Pseudomonas*, *Ralstonia*, *Bacillus* and *Alcaligens* were able to degrade benzene.
281 The gravimetric results of the residual total petroleum hydrocarbon (TPH) showed 46.32%
282 degradation of 2% (w/v) crude oil by *Achromobacter* sp. (PS1) with surface tension value of 32.43
283 $\pm$ 0.83 mN/m, followed by 38.93% degradation by *Ochrobactrum* sp. (GREW1) with surface
284 tension value of 33.14 $\pm$ 0.54 mN/m respectively as against the un-inoculated control (59.27 $\pm$ 1.16
285 mN/m). The GC-FID results of the percentage degradation of the aliphatic fraction by
286 *Achromobacter* sp. (PS1) and *Ochrobactrum* sp. (GREW1) was observed as 70.77% and 79.24%
287 respectively and that of aromatic fraction as 77.17% and 72.13% respectively (Table 4). Mnif et
288 al. [29] also reported degradation of aliphatic fractions of 1% crude oil from micro-organisms of
289 different genera with maximum degradation of 96.20% and 93.30% by *P. aeruginosa* and *H. lutea*
290 in twenty days. Microbial degradation of crude oil has been shown to be fast in aerobic conditions
291 as compared to anaerobic and occurs by the action of oxygenases on the aliphatic and aromatic
292 fractions. Aerobic degradation of aliphatic hydrocarbons results in the formation of fatty acids.
293 The carboxylic acid groups in the fatty acids are then further metabolized *via* the β-oxidation
294 pathway to form acetyl CoA or propionyl CoA depending on the number of carbon atoms (even
295 or odd) in the *n*-alkane. These compounds are then subsequently metabolized *via* the tricarboxylic

296 acid (TCA) cycle to CO_2 and H_2O, together with the production of electrons in the electron
297 transport chain. This chain is repeated, further degrading the aliphatic hydrocarbons. In aromatic
298 hydrocarbons, under aerobic conditions arenes in PAH rings are oxidized leading to formation of
299 intermediates such as protocatechuate and catechols. The catechols are further cleaved to
300 metabolites such as acetate, succinate, pyruvate or acetaldehyde, which subsequently enter the
301 TCA cycle and are thus available as energy and carbon sources to the cell [30]. On the other hand,
302 *Bacillus* sp. (SB2) though showed maximum reduction in surface tension of 28.16 mN/m in 2%
303 crude oil, yet the degradation percentage of TPH was found to be 20.91%. This may be explained
304 on the basis that the biosurfactant produced by *Bacillus* sp. (SB2) was not that efficient to emulsify
305 and utilize the extremely complex high molecular weight recalcitrant carbon structures of
306 asphaltenes present in crude oil due to its relatively lower emulsification index (E_{24}) of 61.35%.
307 Also, it has been reported that asphaltenes are recalcitrant to degradation [31] while NSO (nitrogen,
308 sulphur, and oxygen) compounds show an inhibitory effect on the degradation of many creosote
309 compounds comprising of six major classes – aromatic hydrocarbons (polyaromatic hydrocarbons
310 and alkylated polyaromatic hydrocarbons), tar acids/phenolics, tar bases/nitrogen containing
311 heterocycles, aromatic amines, sulphur containing heterocycles and oxygen containing
312 heterocycles [32]. *Pseudomonas* sp. (MRBSIT1) showed the preferential percentage degradation
313 of aliphatic fraction of crude oil to 93.86% though with a low TPH degradation of 25.19%. These
314 differences in the ability of the bacteria to degrade hydrocarbons may also be linked to several
315 other inherent factors such as cell surface hydrophobicity, catabolic enzyme activity, gene
316 arrangement besides the nature of the biosurfactant production [33]. Thus, this study gives a clear
317 association between the synthesis of emulsifying agent and concomitant hydrocarbon degradation

318 with solubilization of hydrocarbons rendering them more accessible for their breakdown and
319 uptake.

320 **3.3. Biosurfactant production profile of best biosurfactant producing and concomitant**
321 **hydrocarbon degrading isolates**

322 The biosurfactant production profile of the isolates *Achromobacter* sp. (PS1) and *Bacillus* sp.
323 (SLDB1) showed low surface tension values of 32.12 ± 0.78 mN/m and 30.54 ± 0.70 mN/m
324 respectively in set B comprising of only glucose against the set A comprising of glucose and diesel
325 (Fig. 2). *Bacillus* sp. (SB2) showed almost same surface tension values (28.41 ± 0.41 and 28.52 ±
326 0.26 mN/m) in sets A and B. While *Ochrobactrum* sp. (GREW1) showed a different pattern of
327 biosurfactant production with low surface tension values of (31.06 ± 0.28 and 31.38 ± 1.36 mN/m)
328 in set A and C comprising of glucose with 1% (v/v) diesel and only 1% (v/v) diesel respectively
329 as against the set B (only glucose) with surface tension of 56.75 ± 0.38 mN/m. This difference in
330 production pattern may be attributed owing to the involvement of complex synthetic machinery
331 governed by several intrinsic and extrinsic interacting parameters and also on the substrate
332 composition.

333 **3.4. Characterization of the biosurfactant**

334 **3.4.1. Thin layer chromatography (TLC)**

335 The TLC result of the extracted biosurfactant from *Achromobacter* sp. (PS1) and *Bacillus* sp.
336 (SLDB1) suggested a glycolipid nature of the biosurfactant with two prominent spots at R_f of 0.34
337 and 0.72 relating to di-rhamnolipid and mono-rhamnolipid moieties similar to Jeneil JBR 215
338 rhamnolipid standard when developed with orcinol reagent (Fig. 3A). Similar results were reported
339 by Bhat et al. [34] for Jeneil standard rhamnolipid as well as rhamnolipid produced from
340 *Pseudomonas aeruginosa* with the following R_f 0.35 and 0.73 values. Till date glycolipid

341 production has not been reported from *Achromobacter* sp. The genus *Bacillus*, has been most
342 commonly reported for the production of lipopeptide type of biosurfactants [12]. Our results are
343 however contrary to earlier reports as production of glycolipid type of biosurfactant was observed
344 from *Bacillus sp.* (SLDB1) using 1% glucose but are in accordance with the reports of
345 Chandankere et al. [35] wherein the production of glycolipid biosurfactant from *Bacillus*
346 *methylotrophicus* USTBa has been reported using 2% crude oil. The TLC results for extracted
347 biosurfactant from *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2) showed positive spots
348 when developed with ninhydrin reagent indicating the presence of peptide moieties in the molecule
349 confirming the lipopeptide nature of the biosurfactant. The peptide moiety was observed as a single
350 spot (R_f 0.55) in *Ochrobactrum* sp. (GREW1) and two spots (R_f 0.72 and 0.55) in case of *Bacillus*
351 sp. (SB2) as shown in Fig. 3B. Similar TLC pattern was reported by Qiao and Shao [36] for
352 lipopeptide type biosurfactant.

353 **3.4.2. Fourier transform infrared (FT-IR) spectroscopy**

354 Characteristic absorption bands corresponding to specific functional groups present in each of the
355 four biosurfactants were determined and confirmed to characterize the type of biosurfactant. In
356 *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1), a significant broad band at 3423 cm^{-1} and
357 3416 cm^{-1} respectively corresponds to O–H stretching vibrations of free hydroxyl groups and the
358 stretching bands around 1080 cm^{-1} indicates the presence of polysaccharide or polysaccharide-like
359 substances revealing the glycolipid nature of biosurfactants. Similar absorption bands were
360 reported by Singh and Tiwary [37].

361 On the other hand, respective characteristic absorbance bands at 3275 cm^{-1} and 3277 cm^{-1}, resulting
362 from the N-H stretching mode and the absorption bands corresponding to C–O stretching and N–
363 H bending of amide groups around 1536 cm^{-1} and 1658 cm^{-1} in *Ochrobactrum* sp. (GREW1) and

364 *Bacillus* sp. (SB2) signifies the presence of peptide bonds and lipopeptide nature of the
365 biosurfactant. Ramani et al. [38] also reported that the characteristic stretching frequency of amides
366 which lie in the region 3250-3300 cm^{-1} and 1500-1650 cm^{-1} is specific for lipopeptide type of
367 biosurfactant and generally not observed in glycolipid biosurfactants.

368 **3.4.3. GCMS of Fatty acids**

369 The fatty acid compositions of the biosurfactant produced from *Achromobacter* sp. (PS1) and
370 *Bacillus* sp. (SLDB1), showed the presence of 3-hydroxydecanoic acid ($C_{10:0}$) as the most
371 abundant fatty acid with 88.27 ± 0.07 and 90.11 ± 0.07 relative percentage respectively. Other
372 fatty acids were also found however in minor amounts and were identified as 3-hydroxydodecanoic
373 ($C_{12:0}$), 3-hydroxytetradecanoic ($C_{14:0}$), 3-hydroxyhexadecanoic ($C_{16:0}$), 3-hydroxyoctadecanoic
374 ($C_{18:0}$), and 3-hydroxyheneicosanoic ($C_{21:0}$). A similar pattern was observed in the fatty acid
375 composition of rhamnolipid standard (Jeneil biosurfactant) with 3-hydroxy decanoic fatty acid as
376 the major (91.45 ± 0.04%) component (Table 5). This result of our finding is in accordance with
377 the reported literature for glycolipids where decanoic acid is the most commonly reported fatty
378 acid [39].

379 In case of *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2), the fatty acid content of the
380 lipopeptides was diverse with several longer chain fatty acids. Major fatty acid composition peaks
381 of 3-hydroxyoctadecanoic acid (33.03 ± 0.09%) and 3-hydroxyheneicosanoic acid (32.82 ± 0.03%)
382 in *Ochrobactrum* sp. (GREW1) and 3-hydroxyhexadecanoic acid (56.89 ± 0.01%) and
383 hydroxyheneicosanoic (39.02 ± 0.03%) in *Bacillus* sp. (SB2) were observed. This diversity
384 observed in lipopeptide production may be a consequence of differences the fatty acid components
385 which not only depends on the producing bacteria but also on the culture conditions and substrates
386 used. A mixture of β-hydroxy fatty acids of dodecanoic, tetradecanoic, pentadecanoic,

387 hexadecanoic, octadecanoic, (9)-octadecenoic and (9,12)-octadecadienoic in the lipoprotein
388 biosurfactant have also been reported by Qiao and Shao [36], Ramani et al. [38] and Leon et al.
389 [40].

390 **4. Conclusions**

391 The potent biosurfactant producers and concomitant hydrocarbon degraders obtained from the
392 present investigation provides an insight for the productive competence of different types of
393 biosurfactants with *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1) producing glycolipids and
394 *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2) producing lipopeptides as confirmed by TLC,
395 FT-IR and GC-MS results. All the biosurfactants exhibited high emulsification activity with low
396 surface tension values and efficiently degraded crude oil revealing their promising applicability in
397 bioremediation processes. Moreover, the utilization of cheap glucose as the carbon source in the
398 case of *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1 and SB2) further directs towards the
399 use of agro-waste residues for sustainable cost effective biosurfactant production with high yields.

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