1 ***Original Research Article***

2 Investigating the effectiveness between using 3 *Pseudomonas fluorescens* and its 4 biosurfactant in bioremediation of petroleum 5 hydrocarbon contaminated soil

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10 ABSTRACT

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Aim: To investigate the effectiveness of using *Pseudomonas fluorescens* and its biosurfactant in bioremediation of petroleum hydrocarbon contaminated soil.
Study design: (1) Culturing *Pseudomonas fluorescens* for biosurfactant production using an optimized glycerol-mineral salt medium. (2) Separately using the biosurfactant and the bacterium to remediate hydrocarbon contaminated soil, (3) Determining the extent of hydrocarbon removal between the use of the bacterium and its biosurfactant.
Place and Duration of Study: Department of Microbiology, University of Port Harcourt, Nigeria; Between February 2017 and July 2017.
Methodology: *Pseudomonas fluorescens* was cultured for biosurfactant production using a glycerol-mineral salt medium with optimized parameters deciphered from a previous study. About 100 ml of the biosurfactant produced was added to petroleum hydrocarbon contaminated soil in a bioremediation setup. Also, 100 ml broth culture of the bacterium was added to hydrocarbon contaminated soil in another bioremediation setup. A control was also set up. The bioremediation and control setups were monitored for Total heterotrophic bacterial population, Hydrocarbon utilizing bacterial (HUB) population, pH, and total hydrocarbon concentration.
Results: Biosurfactant production was indicated by a reduction of the surface tension of the culture broth from 60.04 $mN.m^{-1}$ to 30.64 $mN.m^{-1}$. Addition of the biosurfactant to petroleum hydrocarbon contaminated soil resulted in about 69 % decrease in hydrocarbon concentration. On the other hand, the addition of the bacterium resulted in about 66 % decrease in hydrocarbon concentration. There was about 50 % decrease in hydrocarbon concentration in the control setup. The HUB population in the bioremediation setup in which biosurfactant was added ranged from 1.70×10^4 - 4.80×10^6 $cfu.g^{-1}$, while the HUB population in the setup in which the bacterium was added ranged from 2.17×10^4 - 1.35×10^6 $cfu.g^{-1}$. The HUB population in the control setup ranged from 6.33×10^3 - 9.15×10^4 $cfu.g^{-1}$.
Conclusion: Though the extent of hydrocarbon attenuation via the use of biosurfactant was higher than that using the bacterium, analysis of variance of the results showed that there is no significant difference between the use of the biosurfactant producing bacterium and its biosurfactant in bioremediation of petroleum hydrocarbon polluted the soil.

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13 *Keywords: Pseudomonas fluorescens; biosurfactant; petroleum hydrocarbon; hydrocarbon utilizing*
14 *bacterial population; bioremediation*

15

16 1. INTRODUCTION

17

18 Bioremediation of petroleum hydrocarbon contaminated environment is usually achieved by initiating
19 or enhancing natural biological processes that will lead to degradation of the hydrocarbons. Chemical-
20 surfactants and biosurfactants have been suggested for use in bioremediation of petroleum
21 hydrocarbon contaminated environment [1, 2]; biosurfactant been preferred over chemical-surfactants
22 due to their biodegradable and low toxic nature. Surfactants aid indigenous hydrocarbon utilizing
23 bacteria and fungi in the petroleum hydrocarbon contaminated environment to degrade hydrocarbons
24 by enhancing the apparent solubility of the hydrocarbons [3], and enhancing the bioavailability of
25 hydrocarbons through adsorption and emulsification [4, 5].

26

27 Bacteria found in the petroleum hydrocarbon contaminated environment that have been shown to
28 degrade hydrocarbons include *Pseudomonas, Arthrobacter, Micrococcus, Vibrio, Acinetobacter,*

Corynebacterium, Flavobacterium, etc [6, 7, 8]. In petroleum hydrocarbon contaminated environments where the microbial load is low or stressed, or there is a huge quantity of toxic or recalcitrant fractions of the hydrocarbons, there may need to add any of these bacteria. Some of the hydrocarbon-degrading bacteria mentioned above also produce biosurfactants, e.g. *Pseudomonas aeruginosa* [4, 9]. The use of biosurfactants and biosurfactant-producing microorganisms in the bioremediation of contaminated environments has been investigated by several researchers [10, 11, 12, 13, 14, 15]. Contaminated environments which have been studied include those contaminated with heavy metals, pesticide, chlorinated aromatics, naphthalene, petroleum hydrocarbons, and polycyclic aromatic hydrocarbons.

Pseudomonas comprises a group of aerobic, Gram-negative, rod-shaped bacteria that can degrade an exceptionally wide variety of organic compounds [16]. The principal species of *Pseudomonas* that are easily noticed on isolation from environmental media due to their ability to produce greenish pigments that fluoresce under ultraviolet (UV) light include *P. aeruginosa*, *P. fluorescens*, *P. putida,* and *P. syringae* [17]. *P. fluorescens* is able to degrade various pollutants such as herbicides, hydrocarbons, and phenol [18, 19, 20, 21]. The degrading ability of the bacterium and its ability to produce biosurfactants [22, 23] make it a potential candidate in the bioremediation of polluted environment. *P. fluorescens* is not generally considered a bacterial pathogen of humans, and its virulence to humans is significantly low especially when compared to the virulence of *P. aeruginosa* [24]. The bacterium can thus be used in environmental applications without much concerned about an ensuing health hazard.

The aim of this research is to investigate the effectiveness of using *Pseudomonas fluorescens* and its biosurfactant in the bioremediation of petroleum hydrocarbon contaminated soil. Results generated from the study will aid in making a choice between the use of a bio-agent capable of producing biosurfactant and biosurfactant in the bioremediation of crude oil or petroleum hydrocarbon polluted environments.

2. MATERIAL AND METHODS

2.1 Source of *Pseudomonas fluorescens*

P. fluorescens bv. 3 (EU543578.1) isolated from river water in a previous study [25] was used for this study.

2.2 Biosurfactant production

Glycerol-Mineral salt medium with optimized parameters deciphered in a previous study [26] was used in culturing the bacterium for biosurfactant production. The optimized parameters of the medium were pH = 5.5, C:N = 20, and C:P =16. The constituent of the medium is outlined in Table 1 and Table 2.

About 200 ml of the glycerol-mineral salt broth was placed in 250 ml capacity conical flasks and sterilized in an Autoclave. After sterilization and cooling, 20 ml of a 48 h old broth culture of *P. fluorescens* was transferred into the content of the flask. The culture flask was incubated at ambient temperature (27 ^{0}C – 31 ^{0}C) for seven days on a PSU-20i Multi-functional Orbital Shaker (Keison Products, UK) operated at 150 rpm for 8 hrs per day. At the end of the incubation period, the pH of the broth was determined and screened for biosurfactant activity.

88 **Table 1**: Composition of the glycerol-mineral salt medium used in culturing the bacterium for
89 biosurfactant production
90

Composition	Concentration
Glycerol (% v/v)	3
KH_2PO_4 (G.L^{-1})	4.03
$MgSO_4.7H_2O$ (g.L^{-1})	0.4
NaCl (g.L^{-1})	1.0
$CaCl_2.2H_2O$ (g.L^{-1})	0.1
$NaNO_3$ (g.L^{-1})	4.46
TES* (% v/v)	0.1

91 **TES - Trace elements solution*
92
93 **Table 2**: Composition of TES
94

Trace element salts	g.L^{-1}
$MnSO_4.H_2O$	1.5
$FeSO_4.7H_2O$	0.5
$CuSO_4.5H_2O$	0.2
$Na_2MoO_4.2H_2O$	0.1
$ZnSO_4.7H_2O$	1.5
H_3BO_3	0.3

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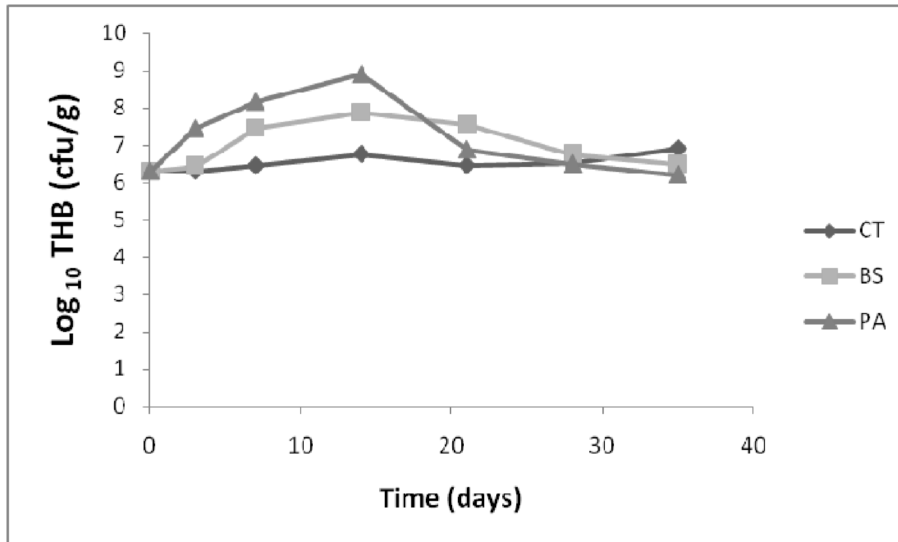
96
97 **2.3 Screening the culture broth for biosurfactant activity**
98
99 Biosurfactant activity was screened via measurement of surface tension, oil-spread diameter, and
100 determination of drop-collapse activity.
101 The capillary rise method was used in the determination of the surface tensions of the culture broth
102 with the aid of the equation $\gamma = ½.rhdg$ [27]. Where 'r' is the radius (cm) of the capillary tube; 'h' is the
103 rise in height (cm) of the liquid; 'd' is the broth density (g.cm^{-3}), and 'g' is the acceleration due to
104 gravity (980 cm.s^{-2}).
105
106 The oil spread diameter was determined as follows: About 40 ml of water was poured into Petri dishes
107 and oil films generated on the surface of the water by applying several drops of diesel oil. A drop of
108 broth culture was placed in the centre of the oil films, and the diameter of the ensuing zone of
109 clearance was measured.
110
111 The drop collapse activity was determined as follows: Each well in a ceramic well plate was coated
112 with a drop of used engine oil. The well plate was then incubated at 37 ^{0}C for about 1 hr. After
113 incubation, two drops of the culture broth were transferred into the oil-coated wells. After 1 minute, the
114 shapes of the drops were observed.
115
116 **2.4 Bioremediation setup**
117
118 The bioremediation setup consisted of three tanks labelled CT (control tank), BS, and PA. The tanks
119 have a dimension of 30 cm x 30 cm x 40 cm (L x W x H), and were fabricated from rectangular amber
120 coloured glass pane. About 5 Kg of soil was placed in each tank. The soils in the tanks were
121 contaminated with about 500 ml of 1:1 diesel oil and used engine oil mixture. The resulting
122 contaminated soils in the tanks were analyzed for pH and total hydrocarbon concentration (THC).
123 Contaminated soils in the tanks were treated in the following manner: Tank BS - about 100 ml of
124 crude biosurfactant solution was added, Tank PA - about 100 ml 24 h old broth culture of *P.*
125 *fluorescens* of the known population was added. The moisture content of the soils in all the tanks was
126 adjusted to about 10 % using sterile warm (35 – 40 ^{0}C) distilled water and was checked weekly and
127 adjusted to a value between 10 – 15 % where the need arose. Also, the soils in all the tanks were
128 tilled twice weekly with the aid of a disinfected hand trowel.

129
130 ## 2.5 Monitoring of bioremediation
131
132 Soil samples were collected from the tanks at weekly intervals. The samples were collected with the
133 aid of a disinfected hand trowel, and sterile small size wide-mouth bottles of about 50 ml capacities.
134 The samples were analysed for, Total heterotrophic bacterial (THB) population, Hydrocarbon utilizing
135 bacterial (HUB) population, pH, and THC.
136
137 THB and HUB were enumerated using the standard plate count method. In this method, nutrient agar
138 (NA) plates were used for THB, while mineral salt agar (MSA) containing fluconazole were used for
139 HUB. Due to the insolubility of Fluconazole in water-based medium, the content of a 50 mg
140 Fluconazole capsule was used for an MSA medium volume of 300 ml so as to achieve an optimum
141 distribution of the particles of Fluconazole in MSA plates. Petroleum hydrocarbons were supplied into
142 inoculated MSA plates using the vapour phase transfer method, and the plates were incubated at
143 ambient temperature for 5 – 7 days. Inoculated NA plates were incubated at 37 ^{0}C for 24 h.
144
145 ## 2.6 Quantification of THC in soil samples
146
147 The THC of the contaminated soils in the tanks were determined via the spectrophotometric method.
148 About 10 g of the soil samples were placed, separately, in a 150 ml capacity beaker, followed by the
149 addition of 20 ml Xylene. The mixtures were agitated for about 5 minutes and then filtered using a
150 Whatman No. 1 filter paper. The extracts from the filtration were subjected to absorbance
151 measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set
152 at 420 nm. Absorbance readings of the extracts, with the aid of the equation of the straight line of the
153 calibration graph previously obtained, were then used to calculate the THCs.
154
155 ## 2.7 Statistical analysis
156
157 The analysis of variance (ANOVA) was used to determine if there was any significant difference
158 between the extents of hydrocarbon degradation in the different bioremediation setup tanks.
159
160 # 3. RESULTS
161
162 At the end of the incubation period, biosurfactant production by the bacterium was indicated by a
163 reduction of the surface tension of the culture broth from 60.04 mN.m^{-1} to 30.64 mN.m^{-1}. The pH
164 increased from 5.5 to 8.3, oil spread diameter ranged from 30 to 40 mm, and the drop-collapse activity
165 was positive. The extent of reduction in surface tension, relatively wide oil spread diameter, and the
166 positive drop-collapse test indicates surfactant activity.
167
168 Average bacterial population in the 24 h old broth culture of *P. fluorescens* added to contaminated soil
169 in Tank PA, as determined via the standard plate count using the spread plate technique, was found
170 to be 5.04×10^{8} cfu.ml^{-1}.
171
172 The THB population in tank CT ranged from 2.01×10^{6} cfu.g^{-1} to 8.03×10^{6} cfu.g^{-1}; in tank BS ranged
173 from 2.73×10^{6} cfu.g^{-1} to 7.70×10^{7} cfu.g^{-1}; and in tank PA ranged from 1.63×10^{6} cfu.g^{-1} to 8.07×10^{8}
174 cfu.g^{-1}. In Fig. 1 it can be seen that tank PA had the highest THB population for the first half of
175 bioremediation period, while tank CT had the least.
176
177 The HUB population in tank CT ranged from 6.33×10^{3} cfu.g^{-1} to 9.15×10^{4} cfu.g^{-1}; in tank BS ranged
178 from 1.70×10^{4} cfu.g^{-1} to 4.80×10^{6} cfu.g^{-1}; and in tank PA ranged from 2.17×10^{4} cfu.g^{-1} to 1.35×10^{6}
179 cfu.g^{-1}. In Fig. 2 it can be deduced that tank BS and PA had higher HUB population than tank CT.
180
181 The pH and THC of the soil samples from the different bioremediation tanks at weekly intervals is
182 presented in Fig. 3 and Fig. 4 respectively. Figure 3 shows that in the course of the bioremediation
183 the pH of the contaminated soil in the different tanks increased form acidic values to values close to
184 neutral pH. Figure 4 shows a general decrease in the THC with tank BS almost taking the lead.
185
186 The result of the analysis of variance (ANOVA) in determining if there is a significant difference
187 between the extents of hydrocarbon degradation in the different bioremediation setups is presented in
188 Table 3 and 4. In Table 3 it can be seen that the $F_{calculated}$ is greater than the $F_{tabulated}$, while in Table 4

189 F $_{calculated}$ is lesser than F $_{tabulated}$. There is thus a significant difference between the extents of
190 hydrocarbon degradation in any of the two bioremediation setups (BS and PA) and the control setup
191 (CT), but no significant difference between the extents of hydrocarbon degradation in the two
192 bioremediation setups.
193



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197 **Fig. 1:** Total heterotrophic bacterial population (THB) of the hydrocarbon contaminated soil in the
198 bioremediation setups.
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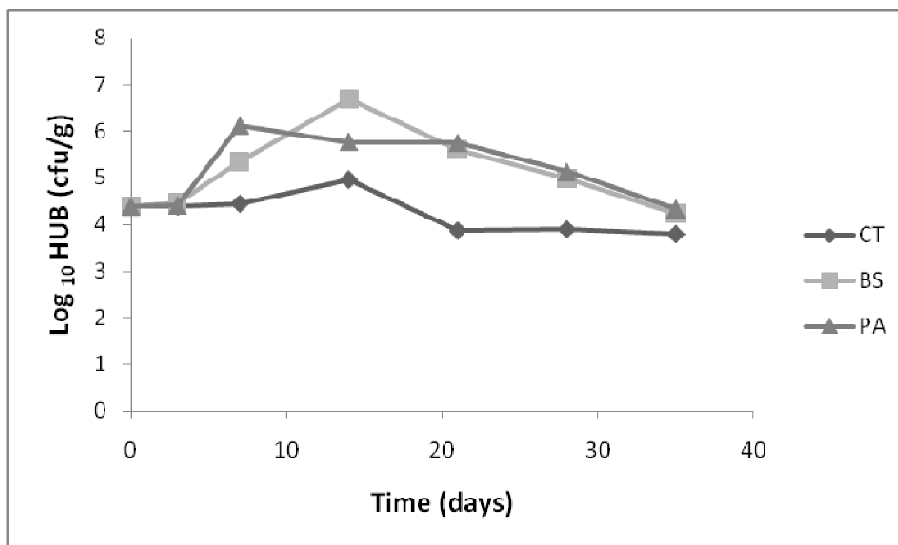

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204 **Fig. 2:** Hydrocarbon utilizing bacterial population (HUB) of the hydrocarbon contaminated soil in the
205 bioremediation setups.
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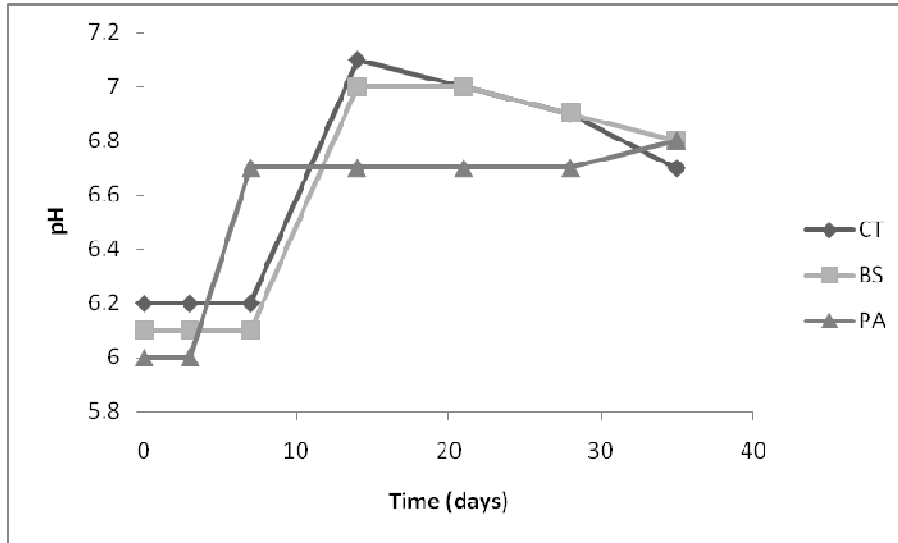

210 **Fig.3:** pH of the hydrocarbon contaminated soil in the bioremediation setups.

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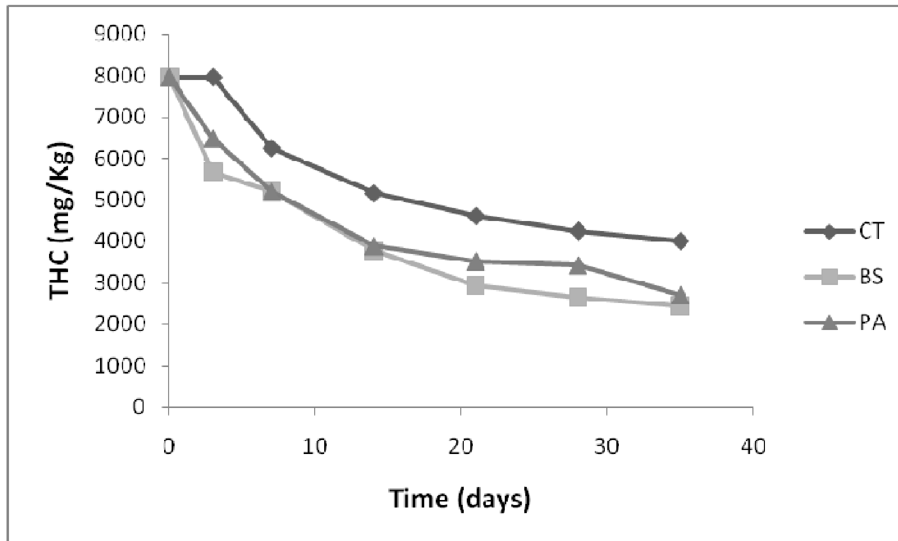

216 **Fig. 4:** Reduction in the Total Hydrocarbon Concentration (THC) of the contaminated soil in the
217 bioremediation setups.

218
219

220 **Table 3:** Analysis of variance of the final THC of the contaminated soil in the control and
221 bioremediation setups

Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
CT	3	12023.57	4007.857	139591.8
BS	3	7302.143	2434.048	37261.9
PA	3	8102.143	2700.714	116530.6

Source of Variation	**SS**	**Df**	**MS**	**F calculated**	***P-value***	**F tabulated**

Between Groups	4256610	2	2128305	21.76297	.002	5.14325
Within Groups	586768.7	6	97794.78			
Total	4843379	8				

222
223
224 **Table 4:** Analysis of variance of the final THC of the contaminated soil in the bioremediation setup

Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
BS	3	7302.143	2434.048	37261.9
PA	3	8102.143	2700.714	116530.6

Source of Variation	**SS**	**Df**	**MS**	**F calculated**	***P-value***	**F tabulated**
Between Groups	106666.7	1	106666.7	1.38715	.3	7.70865
Within Groups	307585	4	76896.26			
Total	414251.7	5				

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226
227
4. DISCUSSION

228
229 Biosurfactants and hydrocarbon degraders have been researched in the bioremediation of petroleum
230 hydrocarbon contaminated environments. In some cases, the hydrocarbon degrader could also be
231 biosurfactant producer. A choice between the use of the hydrocarbon degrader or its biosurfactant
232 could thus arise due to the potential pathogenic nature of the organism or the cost of biosurfactant
233 production using the organism.
234
235 Biosurfactant production by *P. fluorescens* was indicated by a reduction of the surface tension of the
236 culture broth to 30.64 $mN.m^{-1}$. A surface tension value of 27 $mN.m^{-1}$ and 33.5 $mN.m^{-1}$ has been
237 reported by Persson *et al*. [22] and Abouseoud *et al*. [23] respectively for biosurfactant production by
238 *P. fluorescens*. There is thus some level of agreement with the surface tension result obtained in this
239 study and that obtained by Persson *et al*. [22] and Abouseoud *et al*. [23]. The relatively wide oil
240 spread diameter of the culture broth along with the positive drop-collapse test is also indicative of
241 biosurfactant production.
242
243 It has been cited that for a bacterium to be added to a hydrocarbon contaminated environment for
244 bioremediation purposes, it should be propagated to a minimum of 2×10^8 $cfu.ml^{-1}$ [28]. The quantity
245 (100 ml) of 24 h old broth culture of *P. fluorescens* added to the petroleum hydrocarbon contaminated
246 soil in tank PA had a population of 5.04×10^8 $cfu.ml^{-1}$. The population size of the bacterium was thus
247 adequate for the bioremediation study.
248
249 On scrutinizing Fig. 4 it can be deduced that addition of the biosurfactant to petroleum hydrocarbon
250 contaminated soil in bioremediation setup BS resulted in about 69 % decrease in hydrocarbon
251 concentration; addition of the bacterium (*P. fluorescens*) to petroleum hydrocarbon contaminated soil
252 in bioremediation setup PA resulted in about 66 % decrease in hydrocarbon concentration; while the
253 control setup (tank CT) had about 50 % reduction in hydrocarbon concentration. The higher reduction
254 of hydrocarbon concentration in bioremediation setup BS is supported by the relatively high
255 population of hydrocarbon utilizing bacteria in the setup (Fig. 2). The use of biosurfactant is thus
256 preferable for use in bioremediation.
257
258 Application of biosurfactant produced by two *Pseudomonas* species in the bioremediation of a
259 hydrocarbon polluted swamp has been shown to result in about 84 % reduction of the total petroleum
260 hydrocarbons [29]. In another related study, the addition of biosurfactant produced by *P. aeruginosa*
261 to a hydrocarbon-contaminated soil resulted in a reduction of the total petroleum hydrocarbons from 6
262 % to 1.3 % [30]. Thus, a reduction of about 78 % (i.e., $\frac{6 - 1.3}{6} \times 100$) of the total petroleum
263 hydrocarbons was attained. The deviation in the extent of hydrocarbon reduction obtained using

264 biosurfactant from *P. fluorescens* in this study from what was obtained in the works of Okoro [29] and
265 Pradeep *et al*. [30] are quite narrow. It can be implied thus that there is some level of agreement
266 between the extents of hydrocarbon reduction obtained in this study and that obtained in the other
267 studies.
268
269 Addition of a co-culture of a biosurfactant producing *P. aeruginosa* and hydrocarbon degrading *P.*
270 *putida* to soil matrix polluted with diesel oil has been shown to result in about 80 % degradation of the
271 hydrocarbons [31]. Also, results generated from another study indicated that maximum
272 biodegradation of petroleum hydrocarbons was achieved with isolates, which included *P. aeruginosa*,
273 having the ability to produce biosurfactants [32]. The maximum biodegradation was achieved with the
274 biosurfactant producing isolates, both singly and in the consortium, compared to non-biosurfactant
275 producing isolates. *P. fluorescens* has been shown to degrade petroleum-hydrocarbon compounds
276 such as hexadecane, phenol, and naphthalene [19, 21, 33]. In this study, the addition of biosurfactant
277 producing *P. fluorescens* to petroleum-hydrocarbon polluted soil could have aided the indigenous
278 microorganisms in the soil in degrading the petroleum hydrocarbons. This is obvious when comparing
279 the extent of hydrocarbon reduction in tank PA and the control setup which of course also had
280 indigenous microorganisms capable of degrading the hydrocarbons. The presence of indigenous
281 microorganisms capable of degrading the hydrocarbons is confirmed by the presence of a substantial
282 quantity of hydrocarbon utilizing bacteria in the control (Fig. 4).
283
284 On comparing the total heterotrophic and hydrocarbon utilizing a bacterial population of the
285 bioremediation and control setups (Fig. 1 and 2), it can be deduced that the total heterotrophic
286 bacterial population in all the setups were greater than the hydrocarbon utilizing bacteria population.
287 However, the increase in the hydrocarbon utilizing bacterial population in the two bioremediation
288 setups was higher than that in the control. The trend observed in Fig. 2 partially supports the extent of
289 hydrocarbon reduction in the bioremediation setups (Fig. 4). On comparing the extent of hydrocarbon
290 reduction in the control setup and the two bioremediation setups using ANOVA (Table 3 and Table 4),
291 it can be seen that though there is a significant difference between the extents of hydrocarbon
292 reduction in any of the bioremediation setups and the control setup, there is no significant difference
293 between the extents of hydrocarbon reduction in the two bioremediation setups. The THC results,
294 however, imply that the use of biosurfactants is a bit better than the use of biosurfactant producing
295 bacteria in the bioremediation of petroleum-hydrocarbon polluted environment.
296
297 **5. CONCLUSION**
298
299 Addition of biosurfactant or a foreign biosurfactant producing microorganism to a petroleum-
300 hydrocarbon polluted environment can result in enhanced attenuation of the hydrocarbons. However,
301 due to the relatively high production cost of biosurfactants and the pathogenic nature of some
302 biosurfactant producers, e.g. *P. aeruginosa*, a need arises to weigh the decision between the use of a
303 bio-product or a bio-agent in the bioremediation of polluted environments. In this study, the use of
304 biosurfactant produced by *P. fluorescens* resulted in a higher reduction of hydrocarbon concentration.
305 There was, however, no significant difference between the extents of hydrocarbon attenuation
306 achieved via this means and that achieved using the bacterium, *P. fluorescens*.
307
308 **Ethical approval and consent is not applicable.**
309
310
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