Original Research Article

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

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ABSTRACT

Aim: To investigate the effectiveness between 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 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 setup. 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, 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 soil.

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

1. INTRODUCTION

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

Bacteria found in petroleum hydrocarbon contaminated environment that have been shown to 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 be 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 of 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 between 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.

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

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

**TES - Trace elements solution*

Table 2: Composition of TES

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

2.3 Screening the culture broth for biosurfactant activity

Biosurfactant activity was screened via measurement of surface tension, oil-spread diameter, and determination of drop-collapse activity.

The capillary rise method was used in determination of the surface tensions of the culture broth with the aid of the equation $\gamma = ½.rhdg$ [27]. Where 'r' is the radius (cm) of the capillary tube; 'h' is the rise in height (cm) of the liquid; 'd' is the broth density ($g.cm^{-3}$); and 'g' is the acceleration due to gravity (980 $cm.s^{-2}$).

The oil spread diameter was determined as follows: About 40 ml of water was poured into Petri dishes and oil films generated on the surface of the water by applying several drops of diesel oil. A drop of broth culture was placed in the centre of the oil films, and the diameter of the ensuing zone of clearance was measured.

The drop collapse activity was determined as follows: Each well in a ceramic well plate were coated with a drop of used engine oil. The well plate was then incubated at 37 ^{0}C for about 1 hr. After incubation, two drops of the culture broth were transferred into the oil-coated wells. After 1 minute, the shapes of the drops were observed.

2.4 Bioremediation setup

The bioremediation setup consisted of three tanks labelled CT (control tank), BS, and PA. The tanks have a dimension of 30 cm x 30 cm x 40 cm (L x W x H), and were fabricated from rectangular amber coloured glass pane. About 5 Kg of soil was placed in each tank. The soils in the tanks were contaminated with about 500 ml of 1:1 diesel oil and used engine oil mixture. The resulting contaminated soils in the tanks were analyzed for pH and total hydrocarbon concentration (THC). Contaminated soils in the tanks were treated in the following manner: Tank BS - about 100 ml of crude biosurfactant solution was added, Tank PA - about 100 ml 24 h old broth culture of *P. fluorescens* of known population was added. The moisture content of the soils in all the tanks were adjusted to about 10 % using sterile warm (35 – 40 ^{0}C) distilled water, and was checked weekly and adjusted to a value between 10 – 15 % where the need arose. Also, the soils in all the tanks were tilled twice weekly with the aid of a disinfected hand trowel.

2.5 Monitoring of bioremediation

Soil samples were collected from the tanks at weekly intervals. The samples were collected with the aid of a disinfected hand trowel, and sterile small size wide-mouth bottles of about 50 ml capacities. The samples were analysed for, Total heterotrophic bacterial (THB) population, Hydrocarbon utilizing bacterial (HUB) population, pH, and THC.

THB and HUB were enumerated using the standard plate count method. In this method, nutrient agar (NA) plates were used for THB, while mineral salt agar (MSA) containing fluconazole were used for HUB. Due to the insolubility of Fluconazole in water based medium, the content of a 50 mg Fluconazole capsule was used for an MSA medium volume of 300 ml so as to achieve an optimum distribution of the particles of Fluconazole in MSA plates. Petroleum hydrocarbons were supplied into inoculated MSA plates using the vapour phase transfer method, and the plates were incubated at ambient temperature for 5 – 7 days. Inoculated NA plates were incubated at 37 ^{0}C for 24 h.

2.6 Quantification of THC in soil samples

The THC of the contaminated soils in the tanks were determined via spectrophotometric method. About 10 g of the soil samples were placed, separately, in a 150 ml capacity beaker, followed by the addition of 20 ml Xylene. The mixtures were agitated for about 5 minutes, and then filtered using a Whatman No. 1 filter paper. The extracts from the filtration were subjected to absorbance measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set at 420 nm. Absorbance readings of the extracts, with the aid of the equation of the straight line of the calibration graph previously obtained, were then used to calculate the THCs.

2.7 Statistical analysis

The analysis of variance (ANOVA) was used to determine if there was any significant difference between the extents of hydrocarbon degradation in the different bioremediation setup tanks.

3. RESULTS

At the end of the incubation period, biosurfactant production by the bacterium was indicated by a reduction of the surface tension of the culture broth from 60.04 $mN.m^{-1}$ to 30.64 $mN.m^{-1}$. The pH increased from 5.5 to 8.3, oil spread diameter ranged from 30 to 40 mm, and the drop-collapse activity was positive. The extent of reduction in surface tension, relatively wide oil spread diameter, and the positive drop-collapse test indicates surfactant activity.

Average bacterial population in the 24 h old broth culture of *P. fluorescens* added to contaminated soil in Tank PA, as determined via the standard plate count using the spread plate technique, was found to be 5.04×10^{8} $cfu.ml^{-1}$.

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 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} $cfu.g^{-1}$. In Fig. 1 it can be seen that tank PA had the highest THB population for the first half of bioremediation period, while tank CT had the least.

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 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} $cfu.g^{-1}$. In Fig. 2 it can be deduced that tank BS and PA had higher HUB population than tank CT.

The pH and THC of the soil samples from the different bioremediation tanks at weekly intervals is presented in Fig. 3 and Fig. 4 respectively. Figure 3 shows that in the course of the bioremediation the pH of the contaminated soil in the different tanks increased form acidic values to values close to neutral pH. Figure 4 shows a general decrease in the THC with tank BS almost taking the lead.

The result of the analysis of variance (ANOVA) in determining if there is a significant difference between the extents of hydrocarbon degradation in the different bioremediation setups is presented in 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

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

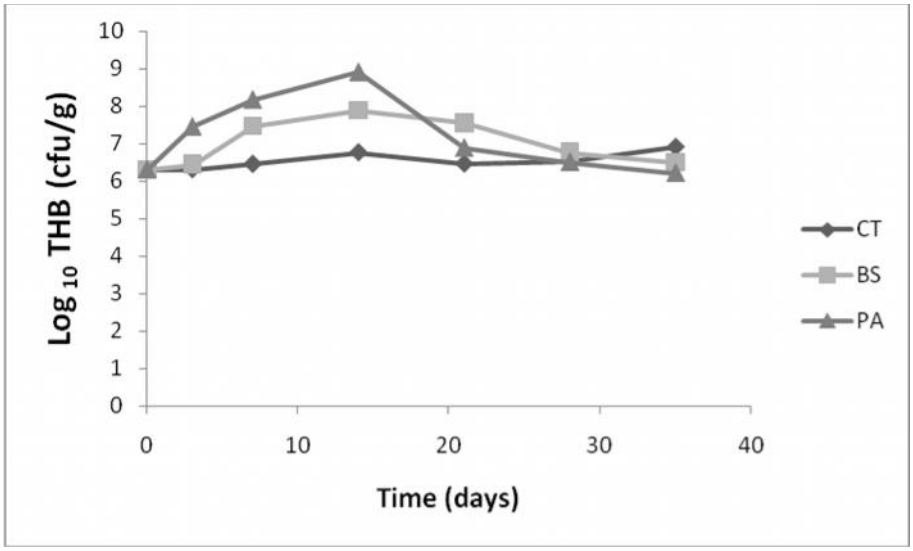

Fig. 1: Total heterotrophic bacterial population (THB) of the hydrocarbon contaminated soil in the bioremediation setups.

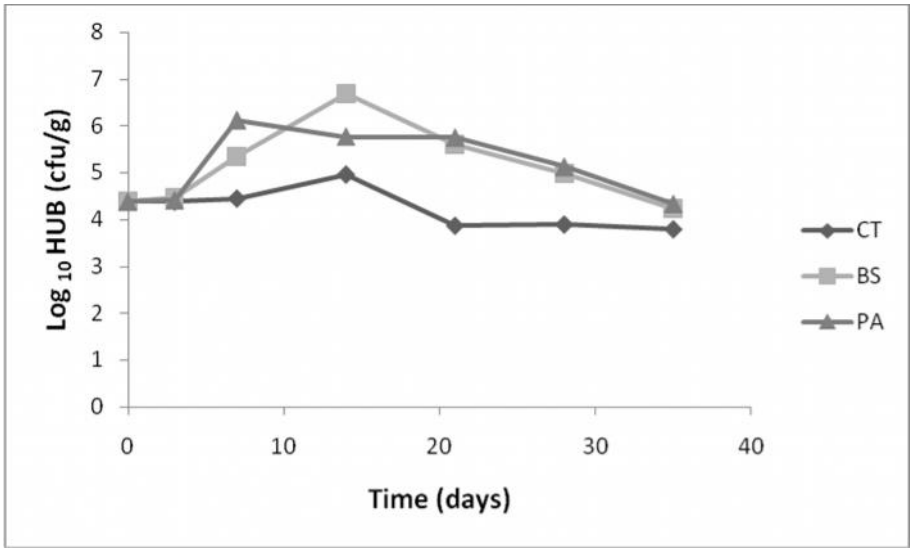

Fig. 2: Hydrocarbon utilizing bacterial population (HUB) of the hydrocarbon contaminated soil in the bioremediation setups.

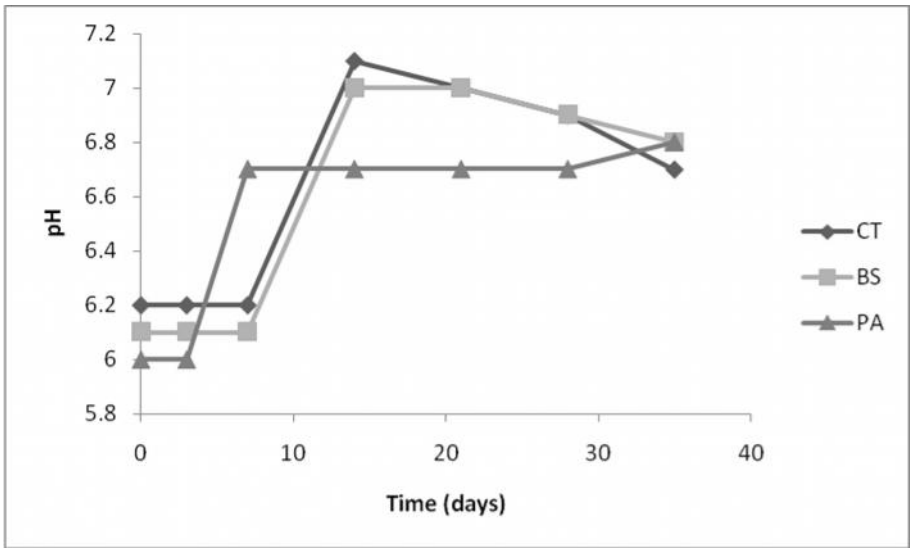

Fig.3: pH of the hydrocarbon contaminated soil in the bioremediation setups.

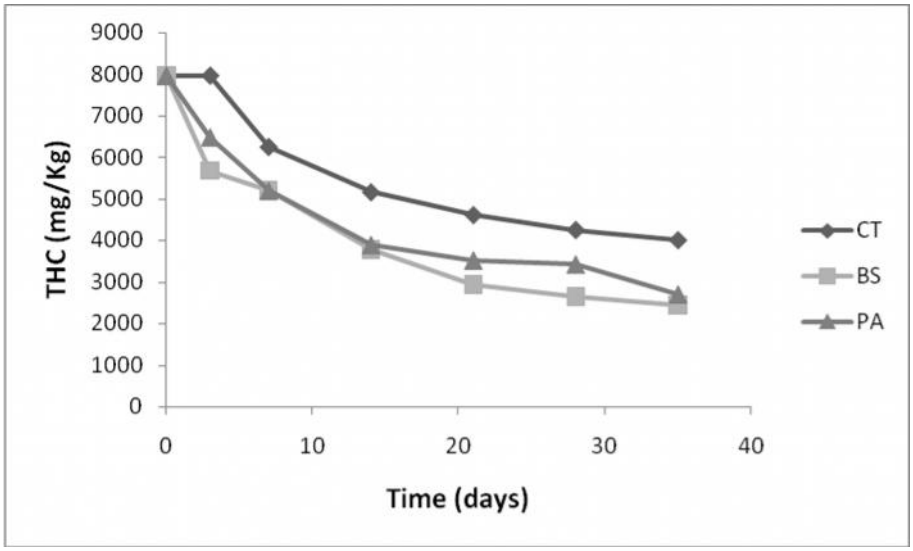

Fig. 4: Reduction in the Total Hydrocarbon Concentration (THC) of the contaminated soil in the bioremediation setups.

Table 3: Analysis of variance of the final THC of the contaminated soil in the control and 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				

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				

4. DISCUSSION

Biosurfactants and hydrocarbon degraders have been researched in the bioremediation of petroleum hydrocarbon contaminated environments. In some cases the hydrocarbon degrader could also be biosurfactant producer. A choice between the use of the hydrocarbon degrader or its biosurfactant could thus arise due to the potential pathogenic nature of the organism or the cost of biosurfactant production using the organism.

Biosurfactant production by *P. fluorescens* was indicated by a reduction of the surface tension of the 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 reported by Persson *et al*. [22] and Abouseoud *et al*. [23] respectively for biosurfactant production by *P. fluorescens*. There is thus some level of agreement with the surface tension result obtained in this study and that obtained by Persson *et al*. [22] and Abouseoud *et al*. [23]. The relatively wide oil spread diameter of the culture broth along with the positive drop-collapse test is also indicative of biosurfactant production.

It has been cited that for a bacterium to be added to a hydrocarbon contaminated environment for bioremediation purposes, it should be propagated to a minimum of 2×10^8 $cfu.ml^{-1}$ [28]. The quantity (100 ml) of 24 h old broth culture of *P. fluorescens* added to the petroleum hydrocarbon contaminated soil in tank PA had a population of 5.04×10^8 $cfu.ml^{-1}$. The population size of the bacterium was thus adequate for the bioremediation study.

On scrutinizing Fig. 4 it can be deduced that addition of the biosurfactant to petroleum hydrocarbon contaminated soil in bioremediation setup BS resulted in about 69 % decrease in hydrocarbon concentration; addition of the bacterium (*P. fluorescens*) to petroleum hydrocarbon contaminated soil in bioremediation setup PA resulted in about 66 % decrease in hydrocarbon concentration; while the control setup (tank CT) had about 50 % reduction in hydrocarbon concentration. The higher reduction of hydrocarbon concentration in bioremediation setup BS is supported by the relatively high population of hydrocarbon utilizing bacteria in the setup (Fig. 2). The use of biosurfactant is thus preferable for use in bioremediation.

Application of biosurfactant produced by two *Pseudomonas* species in the bioremediation of a hydrocarbon polluted swamp has been shown to result in about 84 % reduction of the total petroleum hydrocarbons [29]. In another related study, addition of biosurfactant produced by *P. aeruginosa* to a hydrocarbon-contaminated soil resulted in reduction of the total petroleum hydrocarbons from 6 % to 1.3 % [30]. Thus, a reduction of about 78 % (i.e., $\frac{6-1.3}{6} \times 100$) of the total petroleum hydrocarbons was attained. The deviation in the extent of hydrocarbon reduction obtained using biosurfactant from *P. fluorescens* in this study from what was obtained in the works of Okoro [29] and Pradeep *et al*. [30] is

quite narrow. It can be implied thus that there is some level of agreement between the extents of hydrocarbon reduction obtained in this study and that obtained in the other studies.

Addition of a co-culture of a biosurfactant producing *P. aeruginosa* and hydrocarbon degrading *P. putida* to soil matrix polluted with diesel oil have been shown to result in about 80 % degradation of the hydrocarbons [31]. Also, results generated from another study indicated that maximum biodegradation of petroleum hydrocarbons was achieved with isolates, which included *P. aeruginosa*, having the ability to produce biosurfactants [32]. The maximum biodegradation was achieved with the biosurfactant producing isolates, both singly and in consortium, compared to non-biosurfactant producing isolates. *P. fluorescens* have been shown to degrade petroleum-hydrocarbon compounds such as hexadecane, phenol, and naphthalene [19, 21, 33]. In this study, the addition of biosurfactant producing *P. fluorescens* to petroleum-hydrocarbon polluted soil could have aided the indigenous microorganisms in the soil in degrading the petroleum hydrocarbons. This is obvious when comparing the extent of hydrocarbon reduction in tank PA and the control setup which of course also had indigenous microorganisms capable of degrading the hydrocarbons. The presence of indigenous microorganisms capable of degrading the hydrocarbons is confirmed by the presence of substantial quantity of hydrocarbon utilizing bacteria in the control (Fig. 4).

On comparing the total heterotrophic and hydrocarbon utilizing bacterial population of the bioremediation and control setups (Fig. 1 and 2), it can be deduced that the total heterotrophic bacterial population in all the setups were greater than the hydrocarbon utilizing bacteria population. However, the increase in the hydrocarbon utilizing bacterial population in the two bioremediation setups was higher than that in the control. The trend observed in Fig. 2 partially supports the extent of hydrocarbon reduction in the bioremediation setups (Fig. 4). On comparing the extent of hydrocarbon reduction in the control setup and the two bioremediation setups using ANOVA (Table 3 and Table 4), it can be seen that though there is a significant difference between the extents of hydrocarbon reduction in any of the bioremediation setups and the control setup, there is no significant difference between the extents of hydrocarbon reduction in the two bioremediation setups. The THC results however imply that the use of biosurfactants is a bit better than the use of biosurfactant producing bacteria in the bioremediation of petroleum-hydrocarbon polluted environment.

5. CONCLUSION

Addition of biosurfactant or a foreign biosurfactant producing microorganism to a petroleum-hydrocarbon polluted environment can result in enhanced attenuation of the hydrocarbons. However, due to the relatively high production cost of biosurfactants and the pathogenic nature of some biosurfactant producers, e.g. *P. aeruginosa*, a need arises to weigh the decision between the use of a bio-product or a bio-agent in the bioremediation of polluted environments. In this study, the use of biosurfactant produced by *P. fluorescens* resulted in higher reduction of hydrocarbon concentration. There was however no significant difference between the extents of hydrocarbon attenuation achieved via this means and that achieved using the bacterium, *P. fluorescens*.

REFERENCES

1. Kosaric N. Biosurfactants in industry. Pure Appl. Chem. 1992; 64 (11): 1731-1737.
2. Wong JW, Fang M, Zhao Z, Xing B. Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions. J. Environ. Qual. 2004; 33 (6): 2015-2025.
3. Ron EZ, Rosenberg E. Natural roles of biosurfactants. Environ. Microbiol. 2001; 3 (4): 229-236.
4. Bustamante M, Durán N, Diez MC. Biosurfactants are useful tools for the bioremediation of contaminated soil: A review. J. Soil Sci. Plant Nutrition, 2012; 12 (4): 667- 687.
5. Deziel E, Paquette G, Villemur R, Lepine F, Bisaillon J. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 1996; 62 (6): 1908-1912.
6. Atlas RM. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 1981; 45 (1): 180-209.
7. Das N, Chandran P. Microbial degradation of petroleum hydrocarbon contaminants: An overview. Biotechnol. Res. Int. 2011; Volume 2011, Article ID 941810. http://dx.doi.org/10.4061/2011/941810

8. Salleh AB, Ghazali FM, Zaliha RN, Rahman A, Basri M. Bioremediation of petroleum hydrocarbon pollution. Indian J. Biotechnol. 2003; 2: 411-425.
9. Mukherjee AK, Das K. Microbial surfactants and their potential applications: An overview. In: Sen R, editor. Biosurfactants. New York: Springer Science & Business Media; 2010.
10. Kosaric N. Biosurfactants and their application for soil bioremediation. Food Technol. Biotechnol. 2001; 39 (4): 295-304.
11. Makkar RS, Rockne KJ. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 2003; 22 (10): 2280-2292.
12. Pacheco GJ, Ciapina EMP, Gomes EB, Nei PJ. Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. Brazilian J. Microbiol. 2010; 41: 685-693.
13. Patil SN, Aglave BA, Pethkar AV, Gaikwad VB. *Stenotrophomonas koreensis* a novel biosurfactant producer for abatement of heavy metals from the environment. Afr. J. Microbiol. Res. 2012; 6 (24): 5173-5178.
14. Roy S, Chandni S, Das I, Karthik L, Kumar G, Rao KVB. Aquatic model for engine oil degradation by rhamnolipid producing *Nocardiopsis* VITSISB. 3 Biotech, 2015; 5: 153-164.
15. Singh MJ, Sedhuraman P. Biosurfactant, polythene, plastic, and diesel biodegradation activity of endophytic *Nocardiopsis* sp. mrinalini9 isolated from *Hibiscus rosasinensis* leaves. Bioresour. Bioprocess. 2015; 2:2. Accessed 3 March 2018. Available: http://link.springer.com/article/10.1186%2Fs40643-014-0034-4
16. Prescott LM, Harley JP, Klein DA. Microbiology, 4th ed. New York: The McGraw-Hill companies Inc.; 1999.
17. Stanier RY, Adelberg EA, Ingraham JL. General Microbiology, 4th ed. London: The Macmillan press Ltd.; 1977.
18. Garbi C, Casasús L, Martinez-Álvarez R, Robla JI, Martín M. Biodegradation of oxadiazon by a soil isolated *Pseudomonas fluorescens* strain CG5: Implementation in an herbicide removal reactor and modelling. Water Res. 2006; 40 (6): 1217-1223. http://dx.doi.org/10.1016/j.watres.2006.01.010
19. Vasudevan N, Bharathi S, Arulazhagan P. Role of plasmid in the degradation of petroleum hydrocarbon by *Pseudomonas fluorescens* NS1. J. Environ. Sci. Health. Part A, Toxic/Hazardous Substances & Environ. Eng. 2007; 42 (8): 1141-1146.
20. Moneke AN, Okpala GN, Anyanwu CU. Biodegradation of glyphosate herbicide *in vitro* using bacterial isolates from four rice fields. Afr. J. Biotechnol. 2010; 9 (26): 4067-4074.
21. Mahiuddin M, Fakhruddin ANM, Al-Mahin A. Degradation of phenol via meta cleavage pathway by *Pseudomonas fluorescens* PU1. ISRN Microbiol. 2012; Volume 2012, Article ID 741820. http://dx.doi.org/10.5402/2012/741820
22. Persson A, Österberg E, Dostalek M. Biosurfactant production by *Pseudomonas fluorescens*378: growth and product characteristics. Appl. Microbiol. Biotechnol. 1988; 29 (1): 1-4. http://dx.doi.org/10.1007/BF00258342
23. Abouseoud M, Maachi R, Amrane A. Biosurfactant production from olive oil by *Pseudomonas fluorescens.* In: Méndez-Vilas A, editor. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Formatex, 2007. Accessed 3 March 2018. Available: http://www.formatex.org/microbio/pdf/Pages340-347.pdf
24. Scales BS, Dickson RP, LiPuma JJ, Huffnagle GB. Microbiology, Genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clin. Microbiol. Rev. 2014; 27 (4): 927-948.
25. Peekate PL, Abu GO. Use of Chloramphenicol in the differential enumeration of greenish pigment producing *Pseudomonas*. Basic Res. J. Microbiol. 2017; 4(4): 33-41.
26. Peekate PL, Abu GO. Optimizing C:N ratio, C:P ratio, and pH for biosurfactant production by *Pseudomonas fluorescens*. J. Adv. Microbiol. 2017; 7(2): 1-14. http://dx.doi.org/10.9734/JAMB/2017/38199
27. Negi AS, Anand SC. A Textbook of Physical Chemistry. New Delhi: New Age International; 1985.
28. Thapa B, Kumar KCA, Ghimire A. A review on bioremediation of petroleum hydrocarbon contaminants in soil. Kathmandu University J. Sci., Eng. Technol. 2012; 8 (1): 164-170.
29. Okoro CC. Biosurfactant-enhanced remediation of hydrocarbon contaminated mangrove swamp. Int. J. Biol. Chem. Sci. 2009; 3 (1): 63-74.
30. Pradeep NV, Anupama S, Anitha G, Renukamma AS, Afreen SS. Bioremediation of oil contaminated soil using biosurfactant produced by *Pseudomonas aeruginosa.* J. Res. Biol. 2012; 2 (4): 281-286.

384 31. Kumar M, Leon V, Sisto-Materano A, Ilzins OA. Enhancement of oil degradation by co-culture of
385 hydrocarbon degrading and biosurfactant producing bacteria. Pol. J. Microbiol. 2006; 55 (2):
386 139-146.

387 32. Barin R, Talebi M, Biria D, Beheshti M. Fast bioremediation of petroleum-contaminated soils by
388 a consortium of biosurfactant/bioemulsifier producing bacteria. Int. J. Environ. Sci. Technol.
389 2014; 11: 1701-1710. http://dx.doi.org/10.1007/s13762-014-0593-0
390 33. Sihag S, Pathak H, Jaroli DP. Factors affecting the rate of biodegradation of polyaromatic
391 hydrocarbons. Int. J. Pure Appl. Biosci. 2014; 2 (3): 185-202.