academicJournals

Vol. 10(39), pp. 1637-1644, 21 October, 2016
DOI: 10.5897/AJMR2016.8249
Article Number: BFAEEFF61243
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effectiveness of augmented consortia of *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* in the degradation of diesel polluted soil supplemented with pig dung

Fagbemi O. Kehinde[1] and Sanusi A. Isaac[2]*

[1]The Department of Microbiology, University of Lagos, Akoka, Lagos State, Nigeria.
[2]Discipline of Microbiology, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa.

Received 3 August 2016, Accepted 11 October, 2016

Laboratory studies were developed to compare the effectiveness of inoculated bacteria consortia and indigenous microorganisms on diesel-polluted soil for 18 days. Bacteria isolated from the unpolluted soil sample were: *Pseudomonas* spp. (LBI), *Pseudomonas cepacia* (LB5), *Micrococcus luetus* (LB2), *Bacillus subtilis* (LB3) and *Bacillus cereus* (LB4). Their ability to degrade different substrates were first studied by the presence of growth in minimal salt broth. All the isotates were unable to grow in hexane. LB1 and LB2 had a strong growth in n-dodecane and n-hexadecane. Only LB1, LB4 and LB5 were able to grow in paraffin. LB1 and LB5 had poor growth on xylene. LB1 and LB3 had moderate growth in phenol. All the isolates had little growth in kerosene and only LB3 and LB4 grow in diesel. The three most promising of the isolates, with moderate to strong growth (LB2, LB4 and LB5) on crude oil were further used for diesel bioremediation. The bacterial population in the augmented diesel-contaminated soil showed a reduction in the population density from days 15 to 18, an indication of nutrients (diesel oil) exhaustion. While the un-augmented diesel-polluted soil samples showed potential of more days of increased bacterial population after the 18th day of observation, a pointer of more diesel in the soil samples that can be metabolized/utilized by the microorganisms present in soil samples. The consortia of *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* was effective in the removal of 73.8% diesel oil from the diesel-polluted soil sample while natural attenuation resulted in 41.0% diesel oil removal and 35.8% in the control.

Key words: Bioaugumentation, consortia, diesel, effectiveness, pig dung, soil.

INTRODUCTION

Oil producing conutries of the world are, faced with challenges related to rehabilitation of polluted

*Corresponding author. E-mail: Sanusi_isaac@yahoo.com. Tel: +2761618162.

environments, leading to the development of a wide range of clean up techniques including physical, chemical and biological methods (Wang et al., 1994, Rui et al., 2012). The first two categories hardly achieve holistic elimination of oil contamination from environments with second method involving application of expensive chemical dispersants introducing even more pollutants (Jain et al., 1992; Esin and Ayten, 2011). This makes biological method indispensible as the most natural method to eliminate the bulk of oil contaminants from the environment. The biological method exploits the diverse degradation abilities of microorganisms to convert the complex chemical components of crude oil to harmelss products by mineralization (Chandran, 2011; Chemlal et al., 2013). The acute need for energy in the world today has resulted in a large output of oil and oil products; thus, much of the hydrocarbon material that is extracted from the earth in various parts of the world is transported to other parts of the world for refining. A measure of the oil, both in crude form and in various refined forms is lost to the environment, particularly accidental spills. Terrestial oil spills arise from production, transportation and storage accidents. From these, pipeline failures are the most likely ones to inundate agricultural or wilderness areas (Roy et al., 2014). Diesel oil, sometimes called fuel oils, is classified as middle distillates of crude oil and consists of hydrocarbons with number of carbons atoms mainly in the range of $C_9 – C_{20}$. Although, the proportion of diesel fuel that maybe subjected to volatilization ($C_2 – C_{10}$) is small (1500 – 6300 µg g^{-1}), some of these volatilized hydrocarbon are toxic and can cause health risk. It is known that oil pollution can occur naturally, like the tar sands in Alberta, where oil has worked its way to the surface (Mielke, 1990; Erin et al., 2010; Roy et al., 2014). However, this sources seems minute as compared to the oil spilled by man into the soil through routine leaks from tanks, from oil rigs and pipelines and the most sensational of all supertanker accidents. Oil spills have many adverse effects on the environment. When oil spills occur, the domestic, agricultural and industrial uses are impaired (Ibrahim et al., 2008; Roy et al., 2014, Abu et al., 2016). The cummulative impact of repeated small oil spills can devastate the environment. Elimination of these small oil spills is essential to ensuring the health and productivity of our agricultural lands, not only for us but for future generations. In Nigeria, it takes a long time for arable land polluted with crude oil to regain its fertility. Thus, the amount of compensation paid to farmers by polluters (mainly oil industries and government agencies) is sometimes grossly under estimated, considering the damage done to the soil fertility and the expected produce (Nwachukwu, 2000). Biological reactions involved in the degradation of petroleum are also important in natural systems. In general, it can be assumed that when crude oil is discharged into a system, those fractions with boiling points less than 370°C will evaporate from the system in a matter of days. This leaves biological and autocatalytic decomposition to operate on the remaining fraction. An important concept to remember at this point is that biological decay usually involves only specific compounds. Crude oil is a complex mixture of hydrocarbons, many of which are toxic to be tolerated and degraded (Bragg, 1994; Abu et al., 2016). Thus, when microorganisms attack crude oil, certain fractions are utilized preferentially and certain fractions remain. Van Hammed et al. (2003) listed the general hierarchy of hydrocarbons with respect to preference for microbial degradation. They suggested that alkanes are more readily degraded than aromatic hydrocarbons and that within the alkane's straight-chain compounds are more susceptible to microbial action than branching chains. Methane, ethane and propane are attacked by only a few highly specialized organisms and the more refractory materials, such as waxes and compounds containing more than 30 carbon atoms are insoluble and therefore highly resistant to degradation (Bossert and Compeau, 1995; Yousseria et al., 2016). Biodegradation is a biologically catalyzed oxidation or reduction reaction involving complex chemical compounds. This process can be based on either growth (organic pollutants are used as the sole source of carbon and energy) or co-metabolism. Co-metabolism is the breaking down of organic compounds in the presence of a growth substrate which is used as the primary carbon and energy source (Das and Chandran, 2011). These microbial activities occur with effective cooperation from the soil (Laleh et al., 2016). Biological degradation of hydrocarbons in the environment is also linked to a number of physical and chemical factors, including the concentration and chemical structure of contaminant, physicochemical properties of soil, the content of biogenic salts, moisture content, oxygen and other terminal electron acceptor availability, organic compounds level, temperature and pH of soil. The rate and efficiency of the purification process of soil depends on the occurrence of adequately numerous and active microflora in the contaminated soil (Sobral et al., 2009; Zanaroli et al., 2010). Bioaugmentation involves the addition of microorganisms, indigenous or exogenous to the contaminated sites (Abu et al., 2016). A limiting factor in the use of microbial cultures in land treatment unit is that non-indigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels; and most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degraders if the land treatment unit is well managed (Silva-Castro et al., 2013; Cerqueira et al., 2014)**.** Soil bioaugmentation is a solid phase process where specific microorganisms are added to the soil in order to enhance its biological activities. The seeded microorganisms are often developed through an enrichment process. This procedure results in the selection of the most efficient microorganisms that possess the necessary metabolic pathway and enzymatic

system for degradation of contaminants (Thompson et al., 2005; Sprocati et al., 2012). Soil bioaugmentation is most effective when the soil is not nutrient deficient, but the indigenous microbial population lacks the required metabolic activity. However, this technology has a limited capacity if the bioavailability of the contaminants, controlled by their desorption from soil, is the rate-limiting step in bioremediation (Laleh et al., 2003). Therefore, bioremediation protocols involving application of exogenous competent organisms as a supplement to those naturally present can improve the rate of recovery of polluted environments. Of course, the inoculation of diesel-contaminated soil with microbial consortia having high metabolic activity is essential in achieving effective bioremediation (Zanaroli et al., 2010). But despite the apparent simplicity of bio-augmentation, there have been many failures (Vogel and Walter, 2001; Wagner, 2003; Liu et al., 2016). Some of these failures have been attributable to harsh environmental conditions, pH and redox factors, the absence of key co-substrates (Thompson et al., 2005; Liu et al., 2016). Co-substrates such as surfactant and organic wastes (such as poultry waste, wheat straw) has shown to improve the bioremediation efficiency of diesel-contaminated soils (Soleimani et al., 2013; Laleh et al., 2016). Consequently, exploring different microbial species, their optimum degrading parameters, co-substrates or nutrient supplements with high efficiency to breakdown/degrade hydrocarbons, is of importance in the bioremediation of crude oil and its products. The objective of this study was to investigate the bioremediation of diesel-polluted soil using augmented bacteria and pig dung as nutrient supplement.

MATERIALS AND METHODS

Source of soil used

Unpolluted soil (7 kg) was collected from the Botanical Garden, University of Lagos, Lagos State, Nigeria.

Source of hydrocarbon used

The type of crude oil used is bony light from Shell Flow Station near Portharcourt (4°49`N 7°2`E), River State. The diesel oil used was bought from Total Filling Station in Ketu, Lagos (6°35`N 3°45`E).

Bioremediation protocols

Two kilograms of the soil contained in open tray, 13.5 x 8.5 x 4 cm (internal dimension) was contaminated with 200 g of diesel oil, to give approximately 10% (v/w) pollution. The contaminated soil in the tray was then inoculated with 200 mL of 8.8 x 10^{-3} cfu/ml of bacteria (*Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria*) capable of degrading hydrocarbon and supplemented with 200 g of pig dung (powder) thoroughly mixed and was subsequently designated A. The second tray contained 2 kg of soil sample contaminated with 200 mL of diesel oil only and labelled as B. The last tray C, which serves as the second control contain 2 kg of sterilized soil and 200 mL of diesel oil covered with foil paper to prevent contamination. To achive sufficient aeration, the content of the trays were mixed thoroughly every 3 days. Immediately after starting the experiment, and at intervals, 1 g of a polluted soil sample of each tray were taken to evaluate the bacterial population of the polluted soil sample. The collected samples were either analysed immediately or stored in a refrigerator at 4°C and later analyzed.

Determination of physiochemical parameters

The pH, moisture content, total phosphate, exchangeable bases (sodium, potassium, magnesium and calcium) and nitrogen were determined using methods described by AOAC, (2012).

Isolation and counting of organism from samples

Measured 9.0 mL of water in McCartney bottles were used as diluents for this purpose. One gram of the soil sample in each tray was weighed using weighing balance into McCartney bottle containing 9.0 ml of sterilized water and shaken vigorously. This was taken as 10^{-1} dilution measured using sterile pipette. An aliquot (1.0 ml) was then taken from this tube into a fresh tube with 9.0 ml sterile water to give 10^{-2} dilution. This process was continued until 10^{-10} dilution of the sample was obtained. Thereafter, 0.1 ml aliquot of 10th dilution was introduced into freshly prepared nutrient agar and spread thoroughly, using a hockey stick until the agar surface becomes dry. Plates were incubated at 30°C for 24 h. Bacteria population was monitored every three days. The resulting colonies were later sub cultured unto fresh plates by streaking along line of inoculation and gradually thinned out to obtain distinct and well separated colonies. The platinum loop was flamed after each streak or transfer.

Isolation of hydrocarbon utilizers

The isolation of hydrocarbon oil degraders was done by seeding the minimal salts agar medium with colonies isolated from the samples. Each was then inverted on to a Petri dish cover containing filter paper soaked with the tested hydrocarbon (soil sample + hydrocarbon). The hydrocarbon served as the major carbon source. Incubation was done at 28°C for 48 to 72 h. Each colony was then picked with a sterile aluminium loop, emulsified in distilled water and 0.1 ml aliquots plated unto the minimal salts agar plates to obtain distinct colonies. The isolated strains were maintained on nutrient agar medium, incubated for 24 h and kept at 4°C. The ability of these organisms to degrade the hydrocarbon was further tested by culturing on minimal salt broth with each hydrocarbon as sole carbon source. Each test-tube was filled with 9.0 ml minimal salt broth, 1% hydrocarbon to be tested, both autoclaved at 121°C for 15 min.

The isolates were then aseptically inoculated into the minimal salt broth and plugged with cotton wool to allow for aeration. These tubes were incubated at 30°C for 7 to 14 days with intermittent shaking to allow contact between the oil phase and the liquid phase, which contain the bacterial isolate. The amount of growth was observed comparatively with the control medium set-up, containing no bacterial inoculum (Amund et al., 1987).

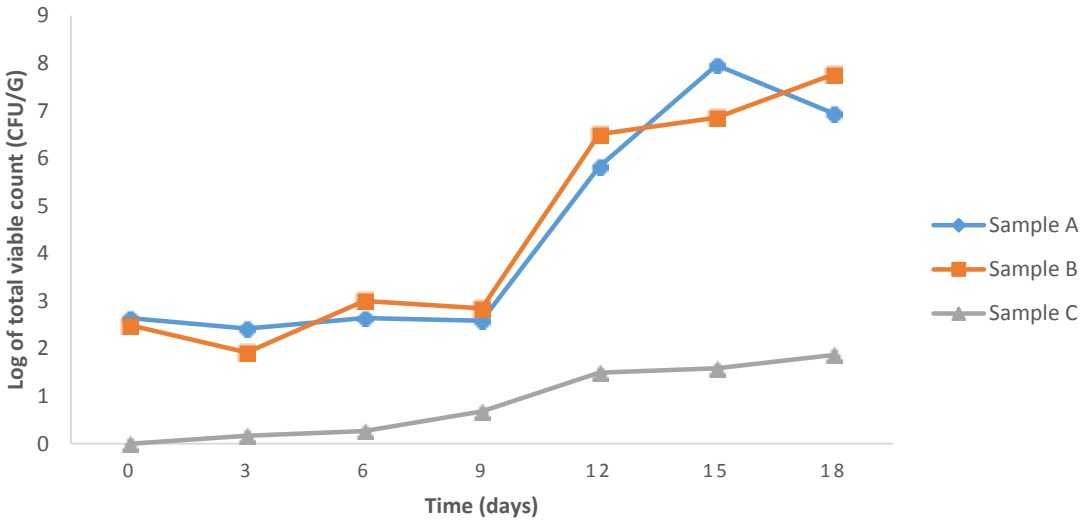

Figure 1. Growth profile of bacteria present in Trays A, B and C polluted with diesel oil.

Identification of the bacteria isolates

Bacterial isolates were prepared on agar plates and the characteristics of the colonies of the pure cultures were observed, recorded and used for their probable identification. Subsequently, biochemical tests were carried out on the bacterial isolates. These include: Gram's staining, motility, catalase test, oxidase test, gelatine hydrolysis, citrate utilization, indole production, methyl red test, acetylmeyhyl carbinol production (Voges Proskauer test), carbohydrate metabolism (hush and leifson's test), starch hydrolysis, acid-fast test, urease activity, nitrate test, acid and gas production from sugars.

Determination of residual oil concentration

The residual diesel concentration in the soils samples were determined according to Vallejo et al. (2001), 8 g of the soil were extracted using 20 ml hexane in a flask tilted with a cap. The residual diesel was analysed by 8200 auto sampler gas chromatography equipped with a 50 m fused silica open tube capillary column internal coated with crosslinked methyl silicon and flame ionization detection. The degradation percentage was determined using the following formula according to Bento et al. (2005).

Percentage degradation of diesel = {(Total diesel in Tray "C" – Total diesel in Tray "A/B")/Total diesel in Tray "C"}*100.

Tray A = soil + diesel + organisms + pig dung; Tray B = soil + diesel; Tray C = sterile soil + diesel.

RESULTS

The pH value of the soil sample was 6.9, while that of pig dung was 5.5. The moisture content of the soil was 12.9% while that of pig dung was 9.9%. The nutrient present in soil sample was lower as compared to that of the pig dung.

Substrate specificity test

The bacteria isolated from the soil sample were: *Pseudomonas* spp., *Pseudomonas cepacia, Micrococcus luetus, Bacillus subtilis* and *Bacillus cereus.* The ability of the bacteria to degrade hydrocarbons varies depending on the type of substrates available. Therefore, their ability to degrade the different substrates were observed by the presence of growth in the broth (Minimal Salt Broth + hydrocarbon been tested) (Table 2). All the isotate were unable to grow on hexane. LB1 and LB2 had a strong growth on n-dodecane and n-hexadecane. Only LB1, LB4 and LB5 were able to grow on paraffin. LB1 and LB5 had poor growth on xylene. LB1 and LB3 had moderate growth in phenol. All the isolates had little growth in kerosene and only LB3 and LB4 had poor growth in diesel.

Bacterial population

Figure 1 illustrates the different growth phases of the bacteria in each of the trays. Bacteria population in tray "A" reduces from days 15 to 18, an indication of nutrient exhaustion, while tray "B" and "C" still show potential to increase after day 18. This can be attributed to the presence of some residual diesel in the contaminated soil sample that can still be used by the bacteria in those trays.

Residual oil concentration

Tray "A" had a degradation of 73.82%, tray "B" had 40.95% and tray "C" had a degradation of 35.76%. Tray "A" shows the highest percentage followed by tray "B"

Table 1. Physiochemical parameters of soil and pig samples prior to bioremediation.

Parameter	Levels detected	
	Soil	Pig dung
pH	6.9	5.5
Moisture (%)	12.9	9.9
Sodium (Na) (%)	0.005	0.4
Potassium (K) (%)	0.002	0.06
Magnesium (Mg) (%)	0.04	0.6
Calcium (Ca) (%)	0.18	0.9
Nitrogen (N) (%)	0.02	1.2
Phosphorus (P) (%)	0.001	0.002

Table 2. Substrate specificity test of isolates on different carbon sources.

Substrates	LB1	LB2	LB3	LB4	LB5
Crude oil	+	++	-	+++	++
Xylene	+	-	-	-	+
Phenol	++	-	++	-	-
Engine oil	+	-	-	+	-
Diesel	-	-	+	+	-
Kerosene	+	+	+	+	+
Benzene	+	+	-	+	+
Cyclohexane	-	+	+	+	+
Paraffin	+	-	-	++	+
n- Decane	++	-	-	-	+
n-dodecane	+++	++	+	+	-
n-hexadecane	+++	+++	-	-	++
Hexane	-	-	-	-	-

No growth, + Poor growth, ++ Moderate growth, +++ Strong growth, LB1- *Pseudomonas* spp, LB2- *Micrococcus luetus,* LB3- *Bacillus subtilis,* LB4- *Bacillus cereus,* LB5- *Pseudomonas cepacia*

and this was due to bio augmentation and natural attenuation respectively of the polluted soil samples. The degradation obtained in Tray “C” can be attributed to volatilization of the diesel oil and probably later by invading bacteria.

DISCUSSION

Bioremediation of diesel-contaminated soil which involves the use of augmented bacteria and pig dung to reduce high levels of diesel to levels that can be harmless/safe and as a result, will minimize the subsequent damages caused to the environment. Bacteria either pure or mixed cultures used for the bioremediation of diesel-contaminated soils require nutrients, carbon and energy sources to grow and proliferate in harsh polluted environments (Lahel et al., 2016). Also, of importance is that, biodegradation/bioremediation rates of these microbes depend on the hydrocarbon composition and environmental conditions such as temperature, pH, moisture content, bioavailability of the pollutant, contamination levels and the presence of additional nutrients (such as pig dung in this research). Competition between indigenous and exogenous microorganisms for limited carbon sources, as well as antagonistic interactions and predation by protozoa and bacteriophage determines the final outcome of the bioremediation process (Franco et al., 2014; Fernandez et al., 2016; Lahel et al., 2016). The soil sample (soil sample before pollution) in this study was nutrient deficient (Table 1). The moisture content was also low and this is an important factor that can have an adverse effect on the metabolic activities of microorganisms during biodegradation of hydrocarbon (Van hammed et al., 2003; Thompson et al., 2005; Abu et al., 2016). The

Table 3. Residual oil concentration and % degradation of each sample after 18 days.

Tray	Oil concentration at day 0 (mg/g)	Oil concentration at day 18 (mg/g)	Degradation (%)
Tray A	246097.02	64439.55	73.82
Tray B	246097.02	145323.59	40.95
Tray C	246097.02	158083.07	35.76

Tray A = soil + diesel + organisms + pig dung, Tray B = soil + diesel, Tray C = sterile soil + diesel (control).

pH of the soil was 6.9, which was within the optimum range for microbial activities. It has been reported that such soil is ideal for hydrocarbon degrading microorganisms to be adapted in a bioaugmentation process (Lahel et al., 2016). The pig dung contains more nutrients (such as sodium, magnesium, calcium and nitrogen) as compared to that of the unpolluted soil sample. This is an additional nutrient source for the indigenous microorganisms and the inoculated bacteria. The presence of diesel in the soil samples had an adverse effect on the initial bacterial populations (Figure 1). However, some of the indigenous and the augmented bacteria that can be described as hydrocarbon utilizers (Table 2), soon adapted to the diesel-contaminated environment and utilized the diesel as a substrates for growth and this is the reason for increased bacterial population from the 9th day (Yakimov et al., 2007; Maduka and Okpokwasili, 2016). The bacterial activities in Tray “A” must have been boosted by additional nutrient from the pig dung and complimented by the inoculated crude oil degrading bacteria. These resulted in the rapid utilization of the diesel substrate (Figure 1). The bacterial population in tray “A” shows an initial decrease from day 0 to day 3, which is due to the toxicity of the diesel oil and a steady adapting lag phase from day 3 till day 9. The exponential phase is observed from day 9 to 15, having day 15 as peak growth. The observed reduction in the population density from days 15 to 18 is due to the exhaustion of nutrients (diesel oil depletion). The initial reduction in the population density from day 0 to day 3 in tray “B” can also be attributed to the toxic effect of diesel on the microorganisms present in the soil sample. The relative increase from days 3 to 9, then steady continuous increase in growth showing availability of more nutrient that can be utilized by the microorganisms’ presence. Due to the use of sterile soil in Tray “C”, the bacterial population was low at the early days and later increases steadily from day 6 till day 18 (still lower bacterial population as compared to those of Trays “A” and “B”). Table 3 shows the extent of diesel degradation/mineralization during the bioremediation of diesel-contaminated soil by bioaugmentation process supplemented with pig dung. Mineralization of diesel was found to be highest (73.82%) in tray “A” and lowest in tray “C” (35.76%). Bioaugmentation using the consortia of *B. coagulans, C. koseri* and *S. ficaria* supplemented with pig dung was effective in degradation of diesel-contaminated soil made up of 200 mL of diesel in 2 kg of soil approximately 10% (v/w) contamination. Nevertheless, this was not a complete removal of the diesel contaminant. Complete removal or higher degradation percentage will be preferred because the residue might be recalcitrant by-products from the initial diesel biodegradation which could lead to bioconcentration, bioaccumulation and biomagnification in a real life scenario. The by-products could also have toxic effect on soil organisms (Agnieszka and Zofia, 2010; Hou et al., 2013; Wu et al., 2016; Lahel et al., 2016). Microbial species capable of degrading all the constituents of crude oil/crude oil products are limited in number. Probably, because the inherent ability of each strain/species to degrade one or more hydrocarbon compound is individually confined to them. Hence, efficient degradation of hydrocarbon requires a consortium composed of various microbial strains (Yousseria et al., 2016). The consortium approach improves biodegradation ability. Microbial mixed cultures or consortia have a higher ability to adapt to stress conditions and therefore show increased microbial survival. In addition, they can increase the number of catabolic pathways available for diesel biodegradation and can easily prevent or reduce the accumulation of recalcitrant/toxic compounds from microbial degradation (Briceno et al., 2016). Therefore, increase in the concentration/dose of augmented bacteria could improve the amount of diesel mineralized. Higher microbial dose is believed to boost the adaptability and assimilation capacity of microbial population to the newly introduced soil, which in turn results in higher mineralization efficiency (Trindade et al., 2002; Lahel et al., 2016). The soil sample in Tray “B”, which underwent natural attenuation, resulted in residual diesel of 59% meaning that 41% of the diesel was lost through the activities of indigenous organisms who had utilized the diesel (Bento et al., 2005; Silva et al., 2015). *Pseudomonas* spp., and *Bacillus* spp. which were some of the indigenous bacteria present in the soil sample, have actually been implicated many times in hydrocarbon degradation (Perfumo et al., 2007; Alfreda and Ekene, 2012; Collado et al., 2013). The indigenous organisms utilizing the diesel could take longer time. This will not be in the interest of maintaining healthy environment without harm to the organisms in that environment; hence, the need for effective method such as bioaugmentation and biostimulation. The impact

of hydrocarbons on microorganisms may not be directly related to their toxicity. Several researchers have reported destruction of inorganic nutrient sources that are essential for microbial growth and catabolic activities due to the ability of hydrocarbons to react and form complexes with nitrates, sulphates and phosphates, thus making them unavailable to oil degrading organisms (Andrew and Jackson, 1996; Abu et al., 2016). The activities in tray “C”, was also probably influenced by the soil sorption property. Judging from the conditions in the tray and the bacterial load, it can be said that apart from the microbial activities, some other factors (soil sorption, adsorption, desorption and volatilization) could have influenced diesel concentration in soil sample in Tray “C”. The contaminated soil samples in the other two trays (tray “A” and ” B”) could also have been influenced by same factors. Soil potential to sorb a certain amount of diesel has been documented, especially the clay and humus compounds present in the soil (Lahel et al., 2016). Falciglia et al. (2011), also noted that adsorption and desorption efficiency of diesel is usually affected by the type of soil texture.

Conclusion

The effect of augmented *B. coagulans*, *C. koseri* and *S. ficaria* in biodegradation of diesel-contaminated soil was more effective than natural attenuation in this present study. Their effectiveness can be improved by optimizing bioremediation parameters such as pH, temperature, substrate concentration and moisture content. Cell immobilization technique could as well enhance the degradation activities of these bacteria consortia. Also, the bacterial species isolated in this work had potential for hydrocarbon degradation, with further research, they can be used in the degradation of hydrocarbons. Since they do not occur as pure cultures in nature, the consortia of these organisms could show greater biodegrading abilities.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This material is based on work financially supported by Costech Canada Inc.

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