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Bioremediation Experiment Using Hydrocarbon Degrading Bacteria

Percobaan Bioremediasi dengan Menggunakan Bakteri Pengurai Hidrokarbon

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Abstract- A laboratory experiment was set up to demonstrate the capability of microbe to remediate petroleum hydrocarbon contaminated beach sand. Oil contaminated soil was used as a source of inoculum for hydrocarbon degrading bacteria (HDB) while oil contaminated beach sand was used as remediation object. The growth of HDB in the inocula was enriched and stimulated through the addition of nutrient in the form of vitamin and mineral as well the addition of oil waste as a source of carbon. Experiment took place in the course of approximately five weeks. Microscopic observation clearly showed the interaction between microbe and oil contaminant both in enrichment and bioremediation samples. The result of the experiment also suggests that approximately 25% of the petroleum hydrocarbon mass in the contaminated beach sand was biodegraded over the course of one month. Overall, the results of this experiment suggest the potential of bioremediation method to treat petroleum hydrocarbon polluted environment.

Keywords: bacteria, bioremediation, hydrocarbon

Abstrak- *Percobaan laboratorium dilakukan untuk menunjukkan kemampuan mikroba dalam memulihkan kondisi pasir pantai yang terkontaminasi minyak bumi. Sumber inokula untuk bakteri pengurai hidrokarbon (HDB-hydrocarbon degrading bacteria) diambil dari contoh tanah yang telah terkontaminasi miyak bumi sementara pasir pantai yang juga telah terkontaminasi minyak bumi digunakan sebagai objek bioremediasi. Petumbuhan HDB dalam inokula diperkaya dan dirangsang melalui penambahan nutrisi dalam bentuk vitamin dan mineral dan juga penambahan limbah minyak sebagai sumber karbon. Percobaan berlangsung selama kurang lebih lima minggu. Pengamatan mikroskopis jelas menunjukkan interaksi antara mikroba dan kontaminan minyak baik dalam conto inokula maupun dalam conto pasir pantai. Hasil penelitian juga menunjukkan bahwa dalam kurun waktu satu bulan, proses bioremediasi berhasil menguraikan sekitar 25% hidrokarbon yang mencemari pasir pantai. Secara keseluruhan, hasil percobaan ini menunjukkan potensi metode bioremediasi untuk memperbaiki kondisi lingkungan yang tercemar minyak bumi.*

Katakunci*: bakteri, bioremediasi, hidrokarbon*

INTRODUCTION

Petroleum hydrocarbons are an important source of energy for various human activities. However, as a result of accidental spills or improper disposal practices, petroleum hydrocarbons have become ubiquitous contaminants of soils and waters worldwide. Many kinds of toxic compound present in petroleum hydrocarbon, proving to be hazardous to the surroundings natural ecosystem. The polycyclic aromatic hydrocarbons (PAHs) are of most concern owing to their toxicity and tendency to bio accumulate. Bioremediation has been considered as an environmentally friendly and cost effective method for remediation of hydrocarbon contaminated sites (Crawford, 2006; Fukuhara et al., 2013).

Bioremediation uses living organisms (microorganisms, plants or products produced from living organisms) to degrade, detoxify, or sequester toxic chemicals including PAHs present in soil, sediment, sludge or water. The microorganism used the pollutant or contaminant as their sources of carbon and energy for growth. Bioremediation process can be enhanced through bio-stimulation or bio-augmentation. Bio-stimulation is a process that attempt to stimulate growth of indigenous contaminant degrading bacteria through the addition of nutrients and other supplementary growth components while bio-augmentation is a process where selected bacterial strains with specific catabolic activities or genetically modified microorganisms are added to accelerate the removal of undesired compounds in polluted sites (Crawford, 2006).

An analysis of hydrocarbon degrading bacteria (HDB) distributions in the soil environment including enumeration of hydrocarbon degraders as well as bacterial structural characterization may significantly important to improve the efficiency of bioremediation. Light microscopy and scanning electron microscopy have been used to observed interaction between bacterial and oil in the contaminated sites (Horowitz et al., 1975; Southam et al., 2001). The Most Probable Number (MPN) procedures have been used to estimate the population density and composition of hydrocarbon-degrading microbial populations (Southam et al., 2001; Wrenn and Venosa, 1996). The MPN enumeration involves the dilution method for estimating, without any direct count, the density of organisms in a liquid as non-hydrocarbon degrading bacteria can grow on impurities present even in highly purified agars, the MPN procedures is considered as the most reliable method for enumeration of hydrocarbon degraders (Mills et al., 1978; Randall and Hemmingsen, 1994; Wrenn and Venosa,1996).

This laboratory experiment aims to demonstrate the potential of bioremediation process on the treatment of petroleum-hydrocarbon contaminated soils. In this experiment the ability of microorganisms present in the soil sample to degrade oil waste has been investigated so that contaminated site can be treated using those microbes.

MATERIAL AND METHOD

Soil Sampling

Soil sample as a source of inoculum for HDB was collected from Soccer Field near garden shed in University of Queensland St Lucia at September 2013 (Figure 1). The site experienced occasional hydrocarbon contamination as a result of oil spill from garden equipment stored in the shed. Soil sampled stored in sterile 15 ml falcon tube and immediately taken to the laboratory for enrichment and inoculation. A flow chart describing method used in this bioremediation experiment appears in Figure 2.

Figure 1. Sampling location of HDB used in this experiment, UQ St Lucia

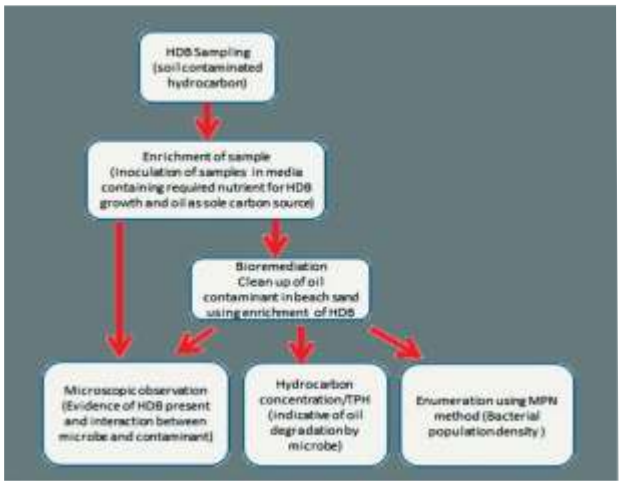

Figure 2. Flow chart of the method used in thisbioremediation experiment.

Enrichment of HDB

Enrichment was carried out by inoculating 1 gram of soil samples to 100 ml of sterile medium containing (per 100ml dH_2O); 0.02 g $MgSO_4.{}_7H_2O$, 0.002 g $CaCl_2$, 0.1g K_2HPO_4, 0.1 g NH_4NO_3 and approximately 1 ml of oil waste as a sole carbon source. Soil slurry (Figure 3) was then incubated at room temperature for two weeks on shaker table.

Bioremediation

In this experiment, 100 gram of beach sand was artificially contaminated with 3ml of waste oil. The contaminated sand was placed in a 250 cc beaker and subjected for bioremediation process (Figure 3). At week 2, 3ml of HDB soil slurry was transferred into oil contaminated beach sand along with 1 ml of oil waste and 15 ml of fresh new medium (recipe as for enrichment). The contaminated sand was then incubated in the dark at room temperature. Enrichment of HDB in oil contaminated beach was maintained in week 3 through the addition of more HDB soil slurry (6ml) and fresh medium (2ml).

Microscopic examination and Total Petroleum Hydrocarbon (TPH) count

In order to examine the enrichment cultures as well as to determine the location of hydrocarbon degrading bacteria (i.e., free living vs. oil associated bacteria), samples from soil slurry and samples from contaminated beach were observed under phase contrast light microscopy in week 2 and week 4 of experiment.

The concentration of oil in the sample and control beach was determined using an InfraCal TOG (total oil and grease)/TPH (total petroleum hydrocarbon) Analyser (WilksIR) as per manufacturer's instructions.

Enumeration using MPN method

Oil contaminated samples was obtained for enumeration using MPN method at week 4 of incubation. 1 gr of oil contaminated sand slurry was diluted in 9 ml of HDB media (for media recipe see Southam et al., 2001) and subjected to tenfold serial dilutions (10-1 to 10-5). Five tubes per dilution, each contained 4.5 ml of media are also prepared for inoculation. Samples from each dilution were inoculated by adding 0.5 ml of each dilution fold to those 4.5 ml serial tubes. The 10-5 dilution was inoculated into row 5; the 10-4 dilution was inoculated into row 4 and so on. All tubes then incubated in the dark for one week. In week 5, tubes were scored for positive growths and the HDB population density was determined based on the MPN method (Tabel 1)

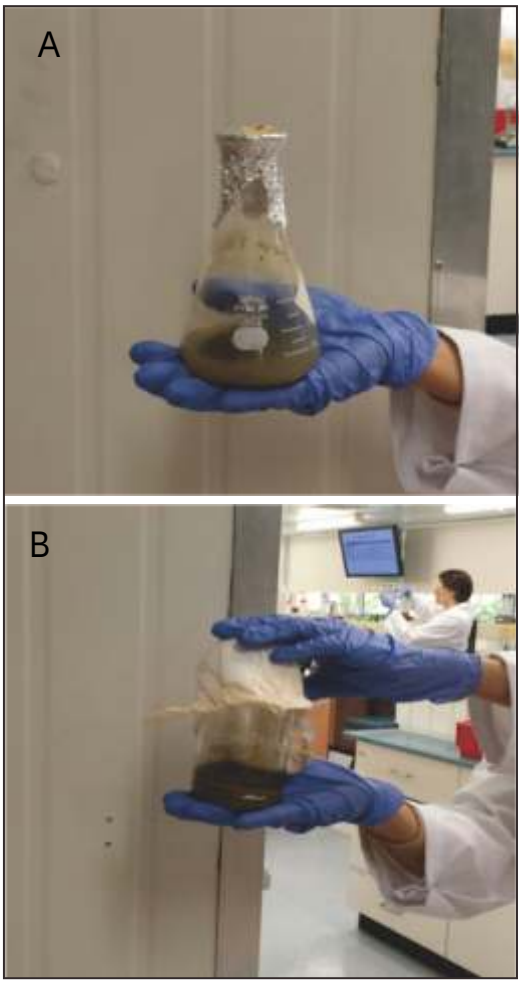

Figure 3. A. Culture of UQ soil enrichment as a HDB source of inoculum, B. Artificial contaminated beach sand as an object of lab scale bioremediation project.

Table 1. MPN table of most probable numbers for use with ten-fold dilutions and 5 tubes per dilution (Cochran, 1950)

p_1	p_2	0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.09
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.081	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.10	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.40
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16	-

RESULTS

Phase contrast microscopy

After two weeks of incubation, microscope observation of our enrichment sample gave evidence for the present of bacteria and interaction between bacteria and the oil contaminant (Figure 4).Further microscope observation on our bioremediation sample, conducted after 3 weeks of the remediation process, shows the breakdown of the oil and bacterial cell attachment to the oil surface (Figures 4B and C).

Amount of oil degraded

The concentration of oil in the beach was measured using an InfraCal TOG/TPH Analyzer (WilksIR) using the manufacturer's instructions. Standard curve for TPH machine was created with waste oil (Figure 5). This standard was used to quantify the concentration of hydrocarbon in this experiment bioremediation sample.

The TPH measurement result was appeared in Table 2. This experiment TPH reading was 53 which correspond to 2946.65 ppm of oil while the initial contaminated beach sand sample (control beach sample) that show 71 in TPH reading correspond to 3970.32 ppm. Table 2 also showed the result of the same experiment performed by other student in Geomicrobiology class.

Microbial counts using MPN method

Positive results were found in all five tubes of each dilution series. Positive tubes show brownish cloudy colour, which more prominent to be seen if the tubes were shake. Having all tubes positive, our MPN counts fall outside the ranges provided in the Cochran table (Table 1). Within this condition, we can only concluded that our bacterial population is >105 MPN per gram of sample.

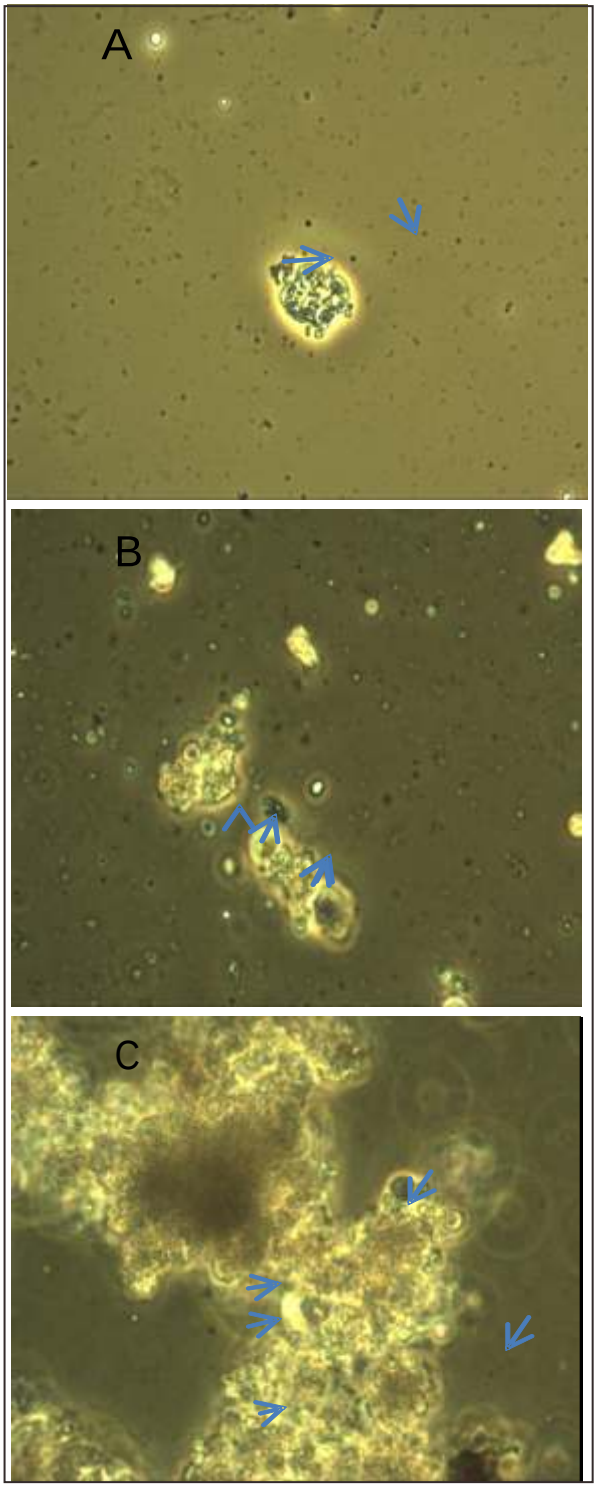

Figure 4. Contrast light micrograph: A. Picture was taken from HDB enrichment sample at week 2; B and C. Picture were taken from bioremediation sample at week 4. Blue arrow pointed to bacterial cell. Magnification: x40 (Field width 250 µm).

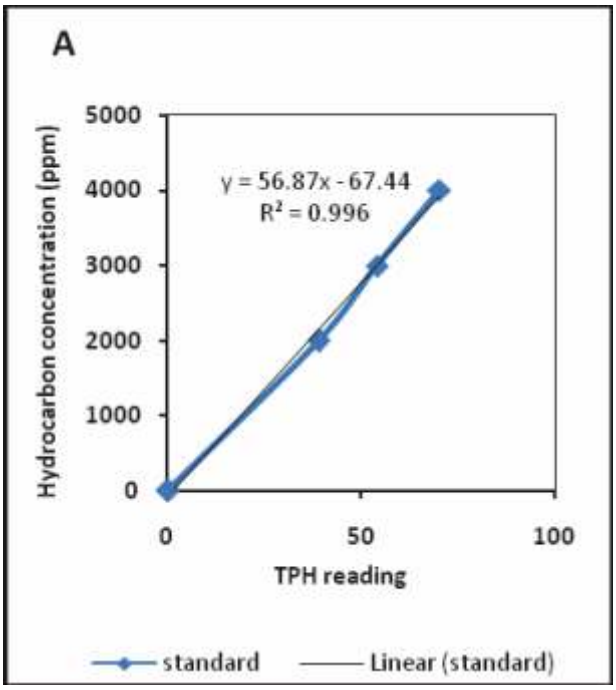

Figure 5. Standard curve to quantify hydrocarbon content in this experiment.

Table 2. Hydrocarbon content in the bioremediation samples (Calculated based on the equation provided by standard curve)

Sample	Reading at 10x dilute sample	Hydrocarbon content (ppm) y=56.87x - 67.4446	% of oil degraded**)
A	53	2,946.66	25.78
B	51	2,832.92	28.65
C	44	2,434.83	38.67
D	45	2,491.70	37.24
E	62	3,458.49	12.89
F	42	2,321.09	41.54
G	68	3,799.71	4.30
H	59	3,287.88	17.19
I	64	3,572.23	10.03
J	53	2,946.66	25.78
K	58	3,231.01	18.62
L	53	2,946.66	25.78
Control beach	71	3,970.32	na

DISCUSSION

Experimental design is crucial for the success of bioremediation system. At such, this experiment was designed to include the entire necessary step that ensures the success of the bioremediation process. First, in order to get naturally viable HDB that capable in biodegrading the oil contaminant, sampling area was carefully chosen when oil contamination has occurred. Naturally occurring microbial activities are and have been the starting point for various biotechnological applications including bioremediation process. Secondly, the growth of HDB in the soil sample was stimulated through the addition of nutrition and the addition of oil as HDB sole source of carbon, ensuring that an adequate number of viable HDB to be present in the sample and ready to be introduced to the bioremediation object (contaminated beach sand). Activation of HDB bacteria will contribute to efficient biostimulation in addition to bioaugmentation (Fukuhara et al., 2013). The nutrient contain inorganic salt as well as nitrogen and phosphorous. Inorganic salts commonly used to grow and maintain the indigenous HDB in various biostimulation system (Aislabie et al., 2006; Fukuhara et al., 2013; Southam et al., 2001; Subathra et al., 2013). Inorganic nitrogen (N) and phosphorous (P) were used for the adjustment of the C/N/P ratio against hydrocarbon (Fukuhara et al., 2013; Leys et al., 2005).

A phase contrast microscopy may assist in characterising the HDB present in the sample. The binding of HDB to hydrocarbon can be observed using this type of microscope. The study has revealed that there are two types of hydrocarbon interactions during biodegradation which are adhesion to oil and a hypothesized pseudosolubilization in which the HDB assimilate small droplets of emulsified oil. Our microscope observation suggested that culture was in several different phase of growth. Oil droplet with bacterial cell seen in the media and in the oil (Figure 4A) suggests that culture observed was in the stationary phase (Horowitz et al., 1975). It has been observed that in stationary phase, most cells were not attached (Horowitz et al., 1975). This study observation is also document bacterial cell that was attached to large oil droplets (Figure 4C). This condition indicates that culture was in exponential growth (Horowitz et al., 1975).

As it has been mentioned in the previous section, bacterial counts were conducted to assess the population density of HDB bacteria in the contaminated sand. The results of these bacterial enumerations would provide general indication as to the likelihood of success of the bioremediation project. This experiment reveals positive results in all five tubes of each dilution series and the MPN count falls outside the ranges provided in the Cochran table (Table 1). At such, in order to get a reliable count using MPN method, our sample needs more dilution (10-6 to 10-10). The dilution ratio, the number of dilution and the actual sample volume in each dilution as well as the number of samples to be used at each dilution are several factors that need to be considered for accurate count using MPN method. It has been suggested, to use HDB enrichment from the terminal dilution tube of the highest count for further bioremediation application (Southam et al., 2001).

Furthermore, it is also important to have background populations of heterothropic bacteria and HDB of the sampling site (Southam et al., 2001). In this experiment, the initial bacterial population in the original soil sample was not counted. The increase of bacterial population in the contaminated sand compare to background soil may suggest that contaminated sand is amenable to bioremediation, while the higher bacterial counts may indicate that site conditions are not inhibitory to microbial growth and that contaminant levels are sufficient to support a viable population of HDB. In contrast, the decrease in the populations might be caused by unfavourable environment condition for bacterial growth. Laboratory studies have demonstrated the influence of temperature to the rate and the extent of hydrocarbon degradation (Aislabie et al., 2006). Such HDB also need certain range of pH for their optimum growth (Aislabie et al., 2006). Depletion of nutrient or crucial elements (C,N,P) required by HDB to grow may also cause low population density (Leys et al., 2005). For instance, the high concentration of hydrocarbon in the contaminated sample may deplete available nitrogen and phosphorus when they are assimilated during biodegradation, the condition which further may inhibit the growth of bacteria (Aislabie et al., 2006; Leys et al., 2005).

There are many ways to measure the biodegradation of hydrocarbons. Such measurements have focused on the disappearance of particular oil constituents, the appearance of carbon dioxide and the depletion of oxygen (Fukuhara et al., 2013; Subathra et al., 2013; Wrenn and Venosa, 1996). Declining of nutrient level and a decline in the amount of specific oil components are also indicative of biodegradation (Aislabie et al., 2006; Leys et al., 2005; Southam et al., 2001). Additional measurements include detailed chemical analyses of petroleum components, assessments of toxicity levels, and detection of increases in specific oil-degrading microbial populations. In this experiment, the amount of hydrocarbon degraded was evaluated by comparing the TPH of the bioremediation sample with the TPH of control beach. During one month of bioremediation course the TPH concentration in the contaminated beach decreased from 3970.32 to 2946.66 ppm. This data suggests that approximately 25% of the petroleum hydrocarbon mass was biodegraded over the course of one month bioremediation, which further confirms the success of this bioremediation experiment. Overall class data also reveals the decreased concentration of hydrocarbon in the contaminated beach after bioremediation process performed, with the amount of oil degraded ranges from 4% to 38% (Table 2).

CONCLUSIONS

Our experiment indicates the present of viable HDB in the University of Queensland soil sample. Microscopic observation clearly shows the interaction between microbe and oil contaminant both in enrichment and bioremediation sample. Total petroleum hydrocarbon measurement suggests that the HDB in the University of Queensland sample was able to clean up some oil contaminant in the artificially polluted beach sand. Although the MPN count in this experiment did not provide reliable number, overall data suggest the success of this bioremediation experiment. To further maintain the good condition of the bioremediation system, culture should be monitored regularly. Culture maintenance can be done through transferring it to fresh media regularly. Additional fresh nutrient to the contaminated site may also be important.

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