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Full Length Research Paper

An isolated bacterial consortium for crude oil biodegradation

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Crude oil utilizing bacteria were isolated from crude-oil contaminated soil (COCS) and tested for their ability to degrade COCS. Fifty strains were isolated, purified and monitored on agar plated for their growth. Twenty strains were selected for the second monitoring in shake flask using minimal salt medium with crude oil as sole carbon source. The growth was monitored by measuring the optical density (OD_{600}) on daily basis. The twelve best growing strains were selected to form four bacterial consortiums. Each consortium contains three strains combined in accordance with their growth. The four consortiums were tested for their growth and the best one (Cons.1) was selected for the kinetics study and the biodegradability test. The first order kinetic study showed that the μ_{max} of Cons.1 was 0.02 h^{-1} while the half saturation constant k_s was 2.2 %v/v TPH. The strains forming IMNC201 belong to *Klebsiella pneumoniae* and *Enterobacteriaceae* species. The bioremediation experiments were designed using DesignExpert 6.0.8 software by optimizing the amount of crude oil, microbial inoculum and sludge which are initially added to the autoclaved soil. Fifteen runs were carried out until no more microbial activity was noticed. The bioremediation results were compared with natural attenuation and biostimulation to determine the feasibility of the bioremediation process. From the optimization of the bioremediation, the optimum degradation obtained was 98.8% within 4 days at 5% v/w crude oil, 5% v/w inoculum and 10% v/w sludge. Biostimulation showed 98.3% TPH removal, while natural attenuation resulted in 97.7% in 13 days. The removal of crude oil by bioremediation was achieved in less time as compared to natural attenuation and biostimulation.

Key words: Crude oil, bacterial consortium, kinetics, bioremediation, biostimulation, natural attenuation.

INTRODUCTION

Environmental pollution, especially with hydrocarbons is a major environmental and health concern. Hydrocarbons such as crude oil are highly toxic and can affect plants, animals and human. This environmental threat is mainly due to the leakage of underground storage tanks and accidental spills and leakage from petroleum pipelines (Yakubu, 2007). The need to remediate the contaminated sites has led to the development of new technologies that emphasize the detoxification and destruction of the contaminants rather than the conventional approach of disposal (Okoh, 2006). Moreover, crude oil and its products are by their nature biodegradable, thus, bioremediation techniques have been developed and improved for cleaning up oil-contaminated soil and has become an alternative to chemical and physical techniques (Evan and Furlong, 2003).

Bioremediation of petroleum contaminated soil is an environmentally sound and cost-effective technique of treating contaminated soils. It can be applied *in situ* or *ex situ* and does not require high skilled technology (Yakubu, 2007).

There are different types of bacteria that are naturally present in the environment and capable of utilizing hydrocarbons as a carbon source. However, the cooperation of more than one microbial species is usually required for an effective biodegradation. This can be due

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Abbreviation: COCS, Crude-oil contaminated soil.

to the synergistic interactions among the microbes (Mukred et al., 2008). Wide varieties of bacterial species, which are capable of the degradation of hydrocarbons, are aerobic bacteria. Some of these bacteria are *Rhodococcus, Pseudomonas, Arthrobacter, Corynobacterium and Bacillus* (Jain, 2005). Moreover, the application of a bacterial consortium that is well-adapted to the environmental conditions of the contaminated site is a main factor for an effective bioremediation process. For example, if bioremediation process is to be applied in Malaysia, a bacterial consortium that is isolated from a location in Malaysia would be a better degrader as it is well adapted to the Malaysian environmental and weather conditions.

This work discusses the isolation and selection of crude oil utilizing bacteria from a crude oil-contaminated soil (COCS) samples and its application in degradation of crude oil in soil.

MATERIALS AND METHODS

Isolation of microorganisms

COCS samples were collected from a location near a storage tank at 5 different depths: 10, 20, 30, 40 and 50 cm. The soil samples were analyzed to determine their physical, biological and chemical characteristics. Enrichment culture was prepared by mixing 2.5 g/L NaCl, 4.74 g/L K_2HPO_4, 0.56 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl.6H_2O$ and 0.5 g/L NH_4NO_3 in dH_2O. 1% v/v crude oil was added as a sole carbon source. One gram soil was added to the enrichment media and incubated at 37°C, 250 rpm for two weeks. One milliliter of the enrichment media was plated on minimal salt medium (MSM)-agar plates and crude oil was added by soaking a filter paper with 0.5 ml sterile crude oil and placing it on the lid of the agar plate. The plates were incubated at 37°C for one week in an inverted position to avoid moisture (Kleyn and Bicknell, 2007).

When the colonies appear on agar plates, each one was subcultured on a new agar plate until pure strains were obtained. More than 50 pure strains were obtained. The strains were monitored on MSM-agar plates and the first 20 to appear on agar were selected for the next screening. The pure colonies were inoculated into 250 ml shake flasks containing 100 ml of MSM and 1 ml crude oil as a carbon source. The flasks were incubated in shaker for one week at 37°C and 250 rpm. The growth of the bacteria was monitored by measuring the OD_{600} using spectrophotometer on daily basis and the best 12 strains were selected. Four bacterial consortiums were formed with three bacterial strains per consortium. The consortiums were again incubated for 1 week and the growth was monitored to select the best growing consortium.

Consortium preparation

For the preparation of the consortiums, colonies were plated on MSM agar and incubated overnight at 37°C. After 12 h, the strains at log phase were inoculated onto 250 ml shake flasks containing 100 ml nutrient broth and incubated at 37°C and 200 rpm for 12 h. A sample of 5 ml from each broth was added to a sterile falcon tube and centrifuged at 5000 rpm for 15 min. The supernatant was discarded and normal saline solution was added to the pellets and vortexed well. A volume of 0.1 ml of each colony was added to nutrient broth and the broth was incubated overnight. The consortium in nutrient broth was used to inoculate flasks for kinetic study and biodegradability test (Rahman, 2002).

Kinetic study

The selected consortium (Cons.1) was characterized based on colony characteristics, gram staining, catalase test and lactose fermentation (Mudili, 2007). To establish the kinetics of Cons.1, the consortium was grown at different TPH concentrations such as: 0.1, 0.5, 1, 1.5 and 2% v/v. The growth and degradation of TPH were monitored on daily basis for one week.

Sludge collection and characterization

Domestic wastewater sludge was collected from Indah Water Consortium (IWK) treatment plant in Gombak, Malaysia. The sludge was collected from the sludge holding tank before the addition of polymer which is usually applied in the sludge treatment in the mechanical thickener. The sludge pH and nutrient content were analyzed using standard methods (MS, 1980; IH-MS, 1980 and APHA, 1992).

Bioremediation runs

Bioremediation optimization runs were designed using the software DesignExpert 6.0.8 using half factorial design with three replica and three centre points. The optimized parameters were the percentage of crude oil, inoculum and sludge that were initially added to the soil. The design resulted in fifteen runs with the residual TPH as the response.

Contaminated soil was dried, sieved and autoclaved at 121°C for 20 min. The soil was placed in microcosms made of aluminum with 100 g soil/microcosm. The values for oil were adjusted according to the design and the inoculum (about 10^6 CFU/ml) and sludge were added and mixed well with the soil. The soil microcosms were moisturized by spraying with sterile dH_2O and mixed with the soil. A control microcosm was prepared by putting 100 g of autoclaved and moisturized soil in a microcosm without the addition of inoculum. Another two microcosms were prepared for natural attenuation and bioaugmentation. For the natural attenuation, 100 g of non-autoclaved soil was placed in a microcosm without any additions, while for the bioaugmentation, 100 g of non-autoclaved soil was mixed with 50% v/w autoclaved sludge to provide nutrient to the indigenous microbes. The soil microcosms were kept in the dark to avoid the algal growth.

The bioremediation microcosms were monitored by taking samples every three days for TPH and MPN measurements. Moreover, the soil pH and soil moisture were measured regularly to ensure that the process conditions are within the optimum range.

RESULTS AND DISCUSSION

The characterization of the soil showed that the pH is near neutrality (6.9), which is favorable for the biodegradation by microbes. Moreover, the moisture content, which is an important limiting factor in biodegradation, is 14.83% and is sufficient to maintain the soil pH in the neutral range. However, the moisture content needs to be increased to fit within the optimum range for growth and degradation of the bacteria which is 30 to 90%. Moreover, the soil texture with low clay or silt (8%) is required for optimum growth and degradation (Vidali, 2001). The soil analysis results are shown in Table 1.

Table 1. Characteristics of the contaminated soil samples.

Test		Value
pH		6.9
Moisture content		14.84%
Porosity		57%
Bulk density		1.14076 g/cm^3
Soil texture[a]	Gravel (2 mm)	1%
	Sand (0.06-2 mm)	91%
	Clay /silt (0.06 mm)	8%
Nutrient	Total organic carbon (TOC)[b]	3.8 % w/w
	Total kjaedahl nitrogen (TKN)[c]	182 mg/Kg
	Total phosphorus[d]	22 mg/Kg
Oil composition[e]	C6-C9	2 mg/Kg
	C10-C14	230.6 mg/Kg
	C15-C28	169.5 mg/Kg
	C29-C36	66.8 mg/Kg
Total hetrotrophic bacteria		2 x 10^6 CFU/ml
Crude oil utilizing bacteria		1.48 x 10^6 CFU/mL

Methods applied are: (a) BS, 1990; (b) MS, 1980; (c) IH-MS, 1980 and APHA, 1992; (d) MS, 1980 and (e) USEPA 8015.

The isolation of bacteria resulted in more than 50 strains. The fastest growing 20 strains appearing on MSM agar, within 3 to 4 days were selected for initial screening. The rest of the 50 strains appearing within 5 to 8 days were excluded. Among the tested strains, 12 showed good growth within one week as compared to the rest when grown on MSM flasks containing oil as sole carbon source. The 12 strains were used to construct four consortiums, each containing three strains combined according to their growth during the initial monitoring (the first 3 fastest combined together followed by the next 3 and so on).

The consortiums were monitored for their growth and the results showed that consortium number 1 (Cons.1) which was made from the three highest growing colonies has the highest growth rate.

The kinetics parameters were calculated based on the observations and Michaelis-Menten equation (Equation 1). First, the readings were plotted on the logarithm scale, then the maximum growth rate: μ_{max}= 0.02 h^{-1} and the Half saturation constant: k_s = 2.2% v/v TPH were determined from the slope and x-axis intercept, respectively.

$$1/\mu = k / \mu_{max}C + 1 / \mu_{max} \quad (1)$$

The percentage removal of the TPH at the end of the kinetic study is shown. The results show that at 1% v/v, the highest degradation occurs, while the minimum is the very low TPH percentage of 0.1% v/v due to the limited amount of nutrient available for the bacteria at this percentage. Moreover, the strains belonging to this consortium were identified to be from the species *Klebsiella pneumoniae* and *Enterobacteriaceae,* which are usually present in the soil they have been isolated from. Strains belonging to these species have been widely used for hydrocarbon degradation (Rodrigues et al., 2009; Obuekwe et al., 2009; Sarma, 2004; Sorkhoh et al., 1990).

The sludge was analyzed for pH, moisture and nutrient content. As shown in Table 2, the pH of the collected sludge is neutral which is very good for the soil used in the bioremediation as the addition of the sludge will not cause decrease in the soil pH. Moreover, the moisture content of the sludge is 93.2% which is high and could increase the soil moisture content and thus result in more favorable conditions for the growth of the microorganisms. Moreover, the N/P ratio is almost equal to 10 which is the optimum value required for the degradation of TPH.

The bioremediation runs were kept for thirty days with the sampling done at three days interval. The moisture level was dropped to levels lower than 30% after two days of adjusting the soil moisture, thus moisturizing was done every two days to maintain the moisture level above 30%. Moreover, it was noticed that the soil pH tend to drop during the bioremediation to pH 5, accordingly, lime

Table 2. The sludge analysis results.

Characteristic	Value
pH	7.17
Moisture	93.2%
C (mg/L)	2686
N (mg/L)	354
N/P	9.7
P (mg/L)	36.4

was added every time sample was collected in order to fix the pH within the range of 6.5 to 8. The reason why the pH was reducing could be due to the byproducts produced by the microbes while degrading the crude oil (Mariano et al., 2007). The reduction in the moisture level could be another reason for the drop in pH.

It was noticed that the bacterial growth and degradation activity stopped after the day thirteen and the residual TPH remained constant following that period. This is due to the depletion in the nutrient as it reached a very small concentration at day 13. Thus, the data for 13 days were used for the analysis. The reduction in the TPH in the soil was noticed by the change in the color of the soil from blackish brown, at the beginning of the runs, to reddish brown, after two days, and finally light brown at day 13.

The first group of the runs which contained 3% oil, 10% inoculum and 30% sludge has shown degradation of TPH up to 86.8% at day 13. It was also noticed that the bacterial growth was high at the first seven days due to the availability of the nutrients: Oil and sludge. Moreover, the runs containing 5% oil, 15% inoculum and 50% sludge showed a very fast drop in the TPH concentration in the first four days which is a relatively short time period. This can be related to the high percentage of the oil, inoculum and sludge that were initially added to the soil. The results of this group also indicate that if these conditions are to be applied in large scale, 50% v/w of water sludge is to be mixed with the contaminated soil. This is a large amount that is to be transferred from the wastewater treatment plant and thus incurring more cost than the smaller sludge requirement.

The third group of the runs which started with a very low TPH concentration (0.8834 g/100 g soil) showed up to 98.5% removal in the first four days; however the concentration of TPH did not change significantly after day 4. This is due to the very low concentration of the initial TPH added to the soil. Moreover, the inoculum added was 5% which is also low as compared to the first and second groups, thus the activity of the bacteria reduced significantly at early stages.

The most significant decrease in TPH concentration was attained by the fourth group in which TPH fell by 99.3% in the first two days, although, the initial amount of inoculum and sludge added was 5 and 10%, respectively. However, the amount of the oil available for the microorganisms was 5%. This indicates that when the consortium is used in a highly contaminated soil, the degradation would be very significant in the first few days, while the amount of sludge needed is only 10%. Finally, the last group which started with a low TPH concentration has gone through a similar scenario of the third group since the degradation was high in the first four days, while it showed a very small change in the following period.

To compare the effect of the bioremediation to biostimulation and natural attenuation, two controls were also tested. In biostimulation, the soil was not sterilized in order to see the performance of the indigenous bacteria, from which the consortium was initially isolated, as compared to the performance of the consortium alone. Moreover, 50% sludge was added to the soil in order to stimulate the growth and degradation activity of the microbes. The results obtained showed a great drop in the TPH concentration in the first four days. However, at the end of day 13, 98.3% removal was attained which is considerably high. However, as compared to the fourth group, in which 98.8% removal was attained in the first four days, bioremediation gave a higher removal than biostimulation in a shorter time. Moreover, larger amount of sludge was used in the biostimulation run which would add two disadvantages to the biostimulation. First, the large amount of sludge that is needed to be transferred to the contaminated site; Second, the longer time period which is needed to reach a high percentage of removal relative to the fourth group of bioremediation runs. On the other hand, the natural attenuation showed a less percentage removal of TPH which spanned the thirteen days time period. This indicates that the indigenous microbes are well-adapted to the contaminant and the removal can be achieved without any addition if the process conditions; moisture, pH and aeration are well-controlled. However, if shorter treatment period is needed, addition of nutrient and inoculum would be the best option.

The results of the residual TPH were analyzed by ANOVA using DesignExpert software. The model chosen was linear model. The parameters with $P>0.05$ were excluded from the model and the effect of the other factors on residual TPH was analyzed.

The R-squared of the model is 67.7% which is considered low. This can be due to the complexity of bioremediation process. Moreover, several factors affect the consistency of the degradation such as the instability of the temperature, moisture and pH which are difficult to be controlled in the soil in a narrow range. The genetic alteration that the microbes might go through in the contaminated soil is also a contributing factor. However, there is a good agreement between the adjusted and predicted R^2 which are 58 and 60.1%, respectively.

The final step in the design analysis is the point prediction where the software DesignExpert 6.0.8 predicted the value of the residual TPH to be 0.146 g/100 g soil at the concluded optimum conditions. This data is verified by comparison with the results obtained from the validation runs. The confidence interval (CI) shows that

there is 95% possibility that the results will fall within the range of 0.067 to 0.22, while the prediction interval PI shows a wider range since more scatter is expected in individual values than the averages which are used in the confidence interval. Three solutions were applied in order to verify the model. The conditions of the three solutions were applied to the validation microcosms and the runs were kept for thirteen days. For more reliability, the value of the residual TPH was evaluated at day thirteen by calculating the average of three samples taken from each microcosm.

There is a good agreement of the two data sets, the validation and model calculated results, with R^2 = 99.2% which is a high value. Thus, the optimum conditions of the bioremediation process are concluded to be 5% oil, 5% inoculum and 10% sludge. These conditions are the best because they involve less amount of inoculum and sludge that are needed to be applied to the site. Smaller amount of inoculum is preferred because the culturing and transfer of the microbes is relatively costly. Moreover, if larger amount of sludge is needed, more cost would be incurred on the transportation of the sludge from the wastewater treatment plants to the contaminated site.

Conclusion

Three bacterial strains were isolated and used for constructing the consortium. The consortium showed maximum growth rate on MSM containing crude oil as sole carbon source. The consortium showed a good degradation rate of TPH which suggests the potential application of the consortium for soil bioremediation. The consortium has the advantages of being well-adapted to the contaminated soil environment and the efficient degradation of the crude oil.

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