Bio-Enrichment of Waste Crude Oil Polluted Soil: Amended with *Bacillus 139SI* and Organic Waste

Arezoo Dadrasnia and Salmah Binti Ismail

***Abstract*—Biodegradation of waste crude oil contaminated soil amended by *Bacillus 139SI* and used tea leaf amendments was investigated to determine the rate of hydrocarbon remediation. Previously, *Bacillus 139SI* was isolated from an agricultural soil in the Serdang agricultural center, Malaysia. Within 60 days, 14% oil loss was recorded in unamended polluted autoclaved soil, while waste crude oil disappeared more rapidly in the soil amendment with both strain and organic waste, recorded above 89%. Utilizing bacteria counts were significantly higher in all amended treatments comparing to control soil. Dehydrogenase activity in soil was markedly enhanced by the application of amendments. Waste crude oil composition monitored by GC/FID indicated complete degradation of n-C_9–C_{25}. First-order kinetic model revealed that organic waste and strain were the best of treatments, with biodegradation rate constant of 0.17day^{-1}and half life of 4 days. The results showed there is potential for tea leaf and *Bacillus 139SI* to enhance biodegradation of waste crude oil contaminated soil.**

***Index Terms*—Bioaugmentation, biostimulation, microbial consortium, organic waste, waste crude oil.**

I. Introduction

Modern industrial society is built and ruled by petroleum hydrocarbons. Petroleum is essential to the current global networked economy, without it, our economic order would cease to function, bringing disaster to many populations. The unintended release of hydrocarbons into the environment can negatively impact human and animal health, and change the characteristics of soils impacting the plant populations they can support [1]. Sonawdekar [2] reported that the amount of natural crude oil spill is estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. The rapid transition from an agricultural economy to an industrial economy in some developing countries like Malaysia makes it probable that hydrocarbon environmental pollution is a significant issue. As long as oil is stored, used and transport, there is a potential threat of oil spillage in environment Crude oil, as a result of PAHs content, interrupts the survival, reproduction, development and growth of organisms. This may increase the risk of mortality from infectious diseases [3]. A diversity of bioremediation techniques has been developed to increase the degradation rate of contaminated sites [4], [5]. More than 200 species of bacteria, yeasts, and fungi have been identified, which are capable of degrading hydrocarbons. In addition, many studies have proven the positive effects of biostimulation in the restoration of total petroleum hydrocarbon contaminated sites [6], [7].

The seriousness of waste crude oil pollution in our environment and the possibility of bioremediation by biostimulation and bioaugmentation are the driving force behind this study. The aim of this research was to explore the feasibility of using biosurfactant (*Bacillus 139SI*) and organic waste (used tea leaf) to remedy soil, which has been contaminated by waste crude oil. A series of microcosm study was conducted in a greenhouse condition for evaluation of oil-degrading microorganisms in the bioremediation process.

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II. Material and Method

A. Collection of Samples

Organic waste (tea leaf) and soil were collected from the Science Canteen and the garden section University of Malaya, respectively. Waste crude oil collected from a refinery station in Malaysia and its profile analyzed using GC. Physicochemical properties such as moisture content, pH, total nitrogen, organic carbon and phosphorus were determined using the standard methods.

B. Bacterial Culture and Inoculum Preparation

Bacillus 139SI was originally obtained from an agricultural soil in the Serdang agricultural center, Malaysia, which reported earlier [8]. Cultivation was carried out at 37^0C in brain-heart infusion (BHI) agar including 5% sheep blood [8]. This soil was suspended in 3mL of sterile distilled water; the suspension was streaked on BHI for a period of 24h at 37^0C. Colonies obtained from the plates were sub-cultured on BHI every month to maintain its survival. In order to obtain a standard inoculum, *Bacillus 139SI* was grown in BHI broth in an orbital shaker at 200 rpm to yield an absorbance reading (OD = 1) at 600 nm. The cells were harvested in mineral salt media (MSM).

C. Bio-Enhanced of Waste Crude Oil

0.5 kg of fresh soil was placed in the plastic poly bags labeled A to H and polluted with 3% (w/w) waste crude oil. After 2 days, 5% of the tea leaf (TL) added into each of the oil polluted soil, labeled A, B and C, respectively. The soils were mixed daily to provide sufficient aeration. After 3 days of stabilization, 10% (v/w) of *Bacillus 139SI*. As a biosurfactant (BF) with inoculum $\times 10^8$ colony forming units (CFU/g) added to contaminated soil. Soil moistened by the addition of water every other day to adjust the water holding capacity to

maintain at 60% throughout the experimental period. In addition, the control with only soil and oil and an additional control treatment, autoclaved twice (within the same day at 121 ^{0}C and 15 psi for 1 h) and then 0.5 % (w/w) NaN_3 was added, to determine the non-biological loss of oil from the soil. All the treatments were set-up in triplicates. The contaminated soils were sampled every 10 days for a period of 60 days. Experiments were conducted with the following combinations:

A: Soil + 3% waste crude oil + 5% TL + BF
B: Soil + 3% waste crude oil + BF
C: Soil + 3% waste crude oil + 5% TL
D: Soil + 3% waste crude oil
E: Autoclaved Soil + 3% waste crude oil + 5% TL + BF
F: Autoclaved Soil + 3% waste crude oil + BF
G: Autoclaved Soil + 3% waste crude oil + 5% TL
H: Autoclaved Soil + 3% waste crude oil

D. Determination of Oil Content in Soil

The concentration of oil in soils was determined gravimetrically by ultrasonic extraction. 10 g of soil sample placed in 250 ml conical flask with n-hexane: acetone (ratio 1:1) for 30 min, using an ultrasonic instrument (121OE-MTH, USA) at 25 ^{0}C. After sonication, the extract was separated from the sample by centrifugation at 5000 rpm for 5 min. The above procedures were repeated twice and the extracts were decanted off after each extraction. Solvent was separated from the extract by distillation and drying. Percentage of degradation of oil calculated using the following formula [6]:

$$\text{Biodegradation}(\%)=\text{TC-TT/TC}\times 100 \quad (1)$$

where TPH is total petroleum hydrocarbon. TC: TPH in control, TT: TPH in treatment. The analysis of the residual hydrocarbon in the soil will determine using GC coupled to a flame ionization detector (FID). Helium carrier gas flow will be 1.27 ml min-1. The column oven was initially held at 50 ^{0}C for 2 min, increased to 300 ^{0}C at a rate of 6 ^{0}C min^{-1}, then to 300 ^{0}C (held for 16 min).

E. Dehydrogenase Activity

Dehydrogenase activity was determined by monitoring the rate of reduction of 2, 3, 5-triphenyltetrazolium chloride (INT) as a substrate [9].

F. Kinetics of Oil Removal and Half Life

First-order kinetics model used is expressed by the following equation [10],

$$C_t = C_i \exp(-kt) \quad (2)$$

where C_t (mg/g), is the oil concentration in soil at instant t, C_i (mg/g) is the initial concentration of soil, k is the rate constants of the first order expressed in (day^{-1}), and t is the time. The model estimated the biodegradation rate and half - life of hydrocarbons in soil relative to treatments applied.

$$\text{Half-life}=\text{Ln}2/k \quad (3)$$

To indicate the proportion of the variation explained by the model, the coefficient of multiple determinations (R^2) was calculated;

$$R^2=1-\text{RSS/CTSS} \quad (4)$$

where RSS was the residual sum of squares, and CTSS was the corrected total sum of squares. The data will be analyzed for significant differences ($p < 0.05$) between treatments using one-way analyses of variance with SPSS 18.

III. Results and Discussion

The physicochemical properties of the investigated soil (Sandy loam) and biowaste used in this biodegradation study are presented in Table I. It is clear that the soil had a low N (0.04%) and P (0.6%) content compared to TL. Hence, it is needed addition of organic waste as a source of nutrient. TL had the highest N and P content compare to soil used; this is one of the most important limiting nutrients for effective bioremediation [11].

TABLE I: Physicochemical Properties of Soil and Organic Waste Used for Bioremediation

Parameters	Soil	TL
Nitrogen (%)	0.04	0.1
Phosphorus (ppm)	60	134
Potassium (ppm)	197	236
Moisture content (%)	9.6	41.5
Organic C (%)	4.25	5.34
pH	7.2	6.8
Texture	Sandy loam	-

A. Biodegradation of Waste Crude Oil

Initial of concentration of oil in the soil was 30000 ppm of soil (3% w/w). Fig. 1 shows the profile of residual waste crude oil in different treatments during the period of 60 days. 3120 ppm (10.4%) oil was remained in treatment A compare to 17100 ppm (57%) in treatment D. The reduction of residual hydrocarbons in the soil was observed in all the treatments. Treatments E, F, G and H with autoclaved soil recorded 48%, 46%, 46% and 14% biodegradation, respectively. It is approve the effect of microorganism to enhance the rate of biodegradation. Chang *et al.* [12] illustrated the rate of degradation was enhanced by using microbial inoculants in bioremediation process. However, other environmental factors such as pH, temperature, nutrients and moisture are important key to influence the microbial growth and biodegradation rate which has been reported in many studies. Chang *et al.* [13] reported the increase rate of oil removal by using *A. baumannii* T30C in a period of 35 days; the soil was tilled to improve the chemical and physical properties. On the other hand, temperature also played the main role in the metabolism of microbial hydrocarbon which is attributed to the breakdown of hydrocarbon compounds. Room temperature of soil treatments was conducted was 33 $\pm$ 2 ^{0}C over the 60 days of study in the greenhouse. Zekri and Chaalal [14] reported that increasing the temperature led to increase the rate of hydrocarbon degradation by thermophilic bacteria of *Bacillus sp*. Biological activity also is widely affected by

availability of nutrients and tolerance of microorganisms to pH variation. The present study demonstrated that the rate of degradation in unamended control soil and autoclaved soil was 43 and 14%, respectively, which was significantly lower than soil amended with *Bacillus 139SI* and TL. Similar results were obtained by Van Gestel *et al.* [15] who reported 85% diesel oil reduction in contaminated soil amended with different composts (vegetable, fruit and garden waste) at a ratio of 1:10 (oil/compost) over a period of 12 weeks.

Comparison of means revealed that there was a significant difference ($p < 0.05$) between the unamended soil (control) with treatments amended with BF and TL, which proves the positive effect of biosurfactant during the biodegradation of waste crude oil in the soil. The lowest rate of degradation was recorded is soil amended with TL (treatment C and G) with 72.5% and 46%, respectively compare to those treatments amended with biosurfactant (Bacillus 139SI) only (treatments B and F) with 76.2% and 46% degradation, respectively in polluted soil with crude oil. This is in contrast with results of Chang *et al.* [12] who reported treatments amended with nutrients only had the highest rate of degradation compare to treatments amended with bacterial isolate. The reason could due to limitation of nutrients in the soils and also might be the differences in microbial ecology of the soil used for these two experiments.

Schaefer and Juliane [16] evaluated the effect of different additives such as brewery and horticultural wastes on total petroleum hydrocarbon (TPH) degradation at 5000 mg/Kg concentration of crude oil and indicated that the application of these wastes as treatment amended did not enhance the degradation of oil. They assumed that micro-organisms preferred the additives as nutrient sources over the less easily degradable, nitrogen deficient, long-chain crude oil [16].

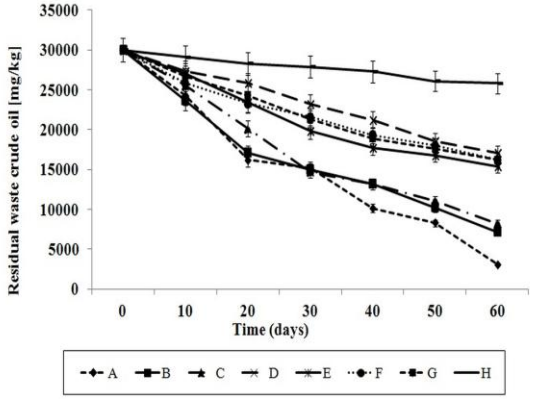

Fig. 1. Residual hydrocarbons in different treatments of waste crude oil contaminated soil.

B. Dehydrogenase Activity (DHA)

Biological oxidation activity was investigated based on DHA enzyme to evaluate the efficiency of the microbial community to utilize organic compounds (Fig. 2). DHA was significantly increased with time. The highest microbial activity was recorded by soil amended with TL and BF (310 µINTF/g dw) at 60 days, which is 4.4 fold higher than unamended control soil in the same time. These results agree with the findings of Aparna *et al.*, [17] who reported that in the biostimulation process with nutrient addition there was increased dehydrogenase activity from 5.8 µg INTF/g dw to 95.6 µg INTF/g dw in the period of 36 days. At the end of two months, DHA recorded in treatments B, C and D were 256, 121 and 60µg INTF/g dw. The result contrasts with those of Lee *et al.* [18] which demonstrated a significant decrease in DHA during the bioaugmentation and biostimulation process on the 23rd day of study and increased gradually until the end of 40 days due to low water content.

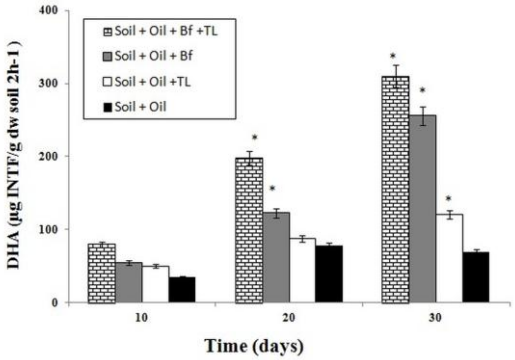

Fig. 2. Dehydrogenase activity in soil polluted.

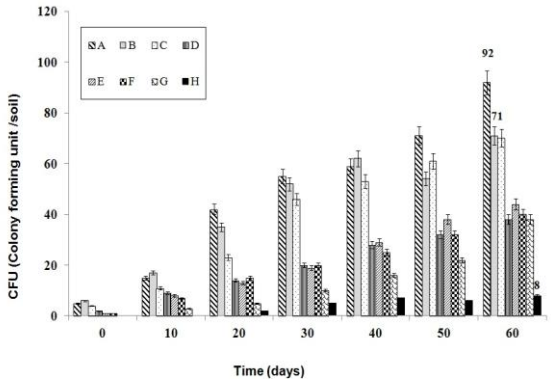

Fig. 3. Total CFU of aerobic heterotrophic bacterial (AHB) in polluted oil. Bars indicate standard error ($n = 3$).

C. The Microbial Population of Soil Polluted with Waste Crude Oil

No significant difference was recorded in aerobic heterotrophic bacterial (AHB) counts among polluted soils with different amendments (Fig. 3). At the end of 60 days, 92 $\times 10^5$ CFU/g of soil was recorded in treatment A which recorded a higher number of AHB. In treatment B refers to the amended with BF only, the number of CFU was increased until 40 days and then decreased to 54 $\times 10^5$ CFU/g of soil followed by increase at 60 days to 71 $\times 10^5$ CFU/g of soil. Thereby, it was approved that *Bacillus 139SI* was played an important role in the reduction of residual hydrocarbon. The result agreed with Padayachee and Lin [19] who reported an increase in bacterial population from 2.0 $\times 10^6$ to 3.2 $\times 10^6$ CFU/ml in soil polluted with diesel oil during the 1st week of all supplemented microcosms amended with fertilizers. This was supported by the results of treatment B in which only bacteria isolated was augmented without the addition of nutrients. However, results indicate that autoclaved soils amended shows lower counts than those treatments with normal soil. In treatments C, the rate of degradation was 72.5%, which was approximately 21750 mg/kg reduction in residual oil; however, the cell count increased from 4 $\times 10^5$ to 7 $\times 10^6$ CFU/g of soil. The difference in the microbial population in other studies compared with this study might be

due to the different type of oil and the microbial ecology of the soil which was used in those studies. This finding is similar to Bento *et al*. [20] who recorded a higher count of AHB in soil polluted with diesel fuel and amended with crop residues.

D. Kinetics Model and Half- Life of Biodegradation

Table II shows the biodegradations constant rate in the different treatments. Half-life indicates the length of time it takes to degrade half of the hydrocarbon. The coefficient of determination (R^2) indicates that the model fits well with all the treatments. The kinetics parameter shows the highest rate of degradation in soil polluted with 3% waste crude oil accrued in soil amended with *Bacillus 139SI* and TL (k = 0.17/day and half-life of 4 days). It illustrates that the combination of the TL and biosurfactant is the most effective treatment in stimulating the degradation of soil polluted with waste crude oil throughout the study period. While, the lowest rate of degradation was recorded in unamended control autoclaved soil with 0.04/day. The reason which could be attributed to the reduction of the population of microorganism and enzyme activity in different oil polluted soil. In addition, the reason for the higher rate of biodegradation in soil amended with TL might be the buffering effects of TL and bacterial inoculation and presenting higher quantities of N and P compared to control treatments which attributed to its C: N ratio. The result is similar to that of Medjor *et al.* [21] who reported that at the end of 1200 hours of bioremediation of groundwater polluted with diesel oil, first order reaction showed the constant rate of 0.002 hour-1and half-life (t1/2) of 346.5 hours. Dadrasnia and Agamuthu [7] also have indicated that soil treated with soycake (SC) with a higher amount of N and P recorded a high biodegradation rate at the end of three months of study. They reported low half-life and high biodegradation rate constant in diesel contaminated soil with biowastes amendment compared with unamended control soil.

TABLE II: KINETIC MODEL, AND HALF-LIFE OF WASTE CRUDE OIL DEGRADATION

Treatment	Biodegradation constant (k) day-1	Half- life (days)	R^2
A	0.173	4	0.95
B	0.140	5	0.87
C	0.120	5.8	0.93
D	0.052	13.3	0.94
E	0.107	6.5	0.81
F	0.091	7.6	0.92
G	0.082	8.4	0.80
H	0.040	17.3	0.93

* Shows significant difference at the $p < 0.05$ level.

Representatives of GC/MS chromatograms showing the total petroleum hydrocarbon patterns at 0 day and at the end of 60th days of treatment amended with TL and biosurfactant is illustrated in Fig. 4. Comparison of the chromatograms before and after the biodegradation process demonstrated that the most hydrocarbon fractions had been removed at the end of study compared with unamneded controlled soil. At the end of study, the hydrocarbon fraction in the range of C_{19} to C_{25} in soil higher degradation compared with the start of the experiment. These results agree with Xu and Lu [22] who reported the removal of C_{12} to C_{29} compounds from crude oil polluted soil, at the end of the incubation period. Nevertheless, in this study result of GC revealed the most of the aliphatic hydrocarbons extracted from polluted soil have been utilized by Bacillus 139SI together with indigenous microorganisms.

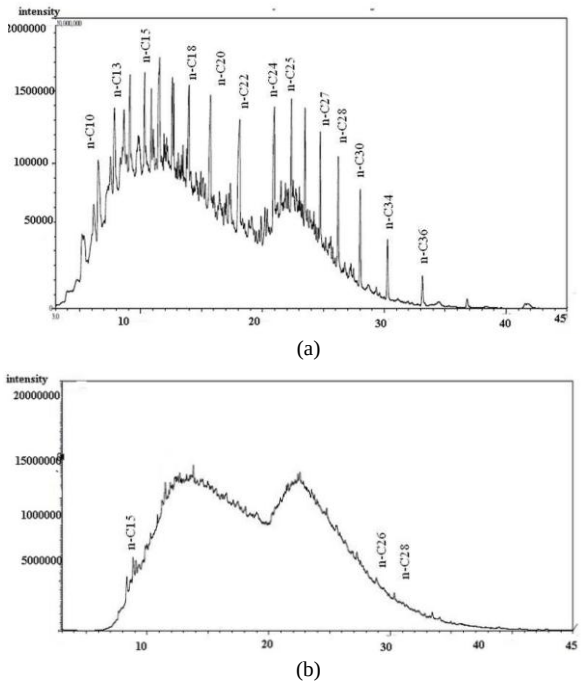

Fig. 4. Chromatogram of residual crude oil in contaminated soil amended with TL and Bacillus 139SI. a) GC profile at 0 day, b) GC profile at 60 days.

IV. CONCLUSION

The mixture of *Bacillus 139SI* and waste tea leaf enhanced the degradation efficiency of waste crude oil contaminated soil. Indeed, multifunction of bio surfactants is known with different chemical structures and surface properties which have important roles in the uptake and mechanisms of mineralization in hydrocarbon polluted soil. However, biosurfactant shows high potential to remediate of hydrocarbons from contaminated soil.

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