

Enhancing Biodegradation of Crude Oil in Soil Using Fertilizer and Empty Fruit Bunch of Oil Palm

(Peningkatan Biodegradasi Minyak Mentah dalam Tanah Menggunakan Baja dan Tandan Kosong Buah daripada Kelapa Sawit)

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ABSTRACT

Bioremediation of crude oil using biostimulation and/or bioaugmentation was done by simulation study in the green house under uncontrolled environment temperature. In this study, the soil with indigenous microbes was spiked with Tapis crude oil at 200 g/kg. The microbial density of the amended soils was augmented by addition of fresh inoculum of microbial consortium which consist of Pseudomonas aeruginosa UKMP-14T, Acinetobacter baumannii UKMP-12T and seed culture two strains of fungi, Trichoderma virens UKMP-1M and Trichoderma virens UKMP-2M at ratio 1:1:1:1 (v/w). The amendment soil was added with 20% (v/w) of standardize consortium inoculum, 20% (w/w) of dried empty fruit bunch (EFB) and the effect of EFB was compared with 0.7% commercial fertilizer (v/w) which contain NPK (8:8:1). Soil with indigenous microbes was used as a control. Results showed total petroleum hydrocarbon (TPH) degradation for treatment added with NPK fertilizer was 70.36%, addition with EFB bulking agent 68.86% and addition of both NPK and EFB was 100% at day 30 of incubation. The control plot, 62% of TPH degradation was achieved after 30 days incubation.

Keywords: Bioaugmentation; biostimulation; microbial consortium

ABSTRAK

Bioremediasi minyak mentah menggunakan biostimulasi dan/atau bioaugmentasi telah dijalankan secara kajian simulasi di dalam rumah tumbuhan dengan suhu persekitaran tidak dikawal. Dalam kajian ini, tanah yang mengandungi mikrob indigenous telah ditambah dengan minyak mentah Tapis pada 200 g/kg. Kepadatan mikrob dalam tanah yang diubahsuai telah ditambah dengan inokulum segar konsortium mikrob yang terdiri daripada Pseudomonas aeruginosa UKMP-14T, Acinetobacter baumannii UKMP-12T dan kultur benih dua strain kulat Trichoderma virens UKMP-1M dan Trichoderma virens UKMP-2M pada nisbah 1:1:1:1 (i/b). Tanah yang diubahsuai ditambah dengan 20% (i/b) inokulum piawai konsortia, 20% (b/b) tandan kosong buah kelapa sawit (EFB) yang telah dikeringkan dan kesan EFB telah dibandingkan dengan 0.7% baja komersil (i/b) yang mengandungi NPK (8:8:1). Tanah dengan mikrob indigenous dijadikan sebagai kawalan. Hasil kajian menunjukkan degradasi TPH bagi rawatan yang ditambah dengan baja NPK adalah 70.36%, penambahan agen pemukul EFB ialah 68.86% dan penambahan kedua-dua NPK dan EFB adalah 100% pada hari ke 30 eraman. Plot kawalan pula menunjukkan 62% degradasi TPH telah dicapai selepas 30 hari pengeraman.

Kata kunci: Bioaugmentasi; biostimulasi; konsortium mikrob

INTRODUCTION

Bioremediation of hydrocarbon using bacteria was mainly studied in the lab (Mirdamadian et al. 2010; Sathishkumar et al. 2008) compared with field study (Chorom et al. 2010). The activities of microbes in the control environment will not be the same as in field study since many uncontrolled environmental factors such as temperature, pH, humidity together with the presence of other microbes will affect their growth.

Nutrient addition has enhanced the biodegradation of petroleum hydrocarbons in contaminated soils due to stimulation of native microorganisms (Cunningham & Philp 2000). Biostimulation requires the evaluation of both the intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the *in situ* process. One of those parameters

is aeration, which can be improved in bioremediation systems by the use of plant crop residues that act as bulking agents (Boodoosingh, 2007; Molina-Barahona et al. 2004). Bioremediation of complex hydrocarbons usually requires the cooperation of more than a single species because the individual microorganism can metabolize only a limited range of hydrocarbon substrates (Shabir et al. 2008). Therefore, assemblages of mixed populations with overall broad enzymatic capabilities are required to bring the rate and extent of petroleum hydrocarbon degradation much faster (Zhong et al. 2007).

In this study, application of NPK inorganic fertilizer and palm oil empty fruit bunch (EFB) were used to stimulate the growth of microbial consortium to degrade hydrocarbon contaminated soil. Since Malaysia is one of the world's largest producers of palm oil, the use of EFB is an effective

and economic way to supply nutrient for microbes. The objective of this study was to compare the efficiency of microbial consortium in stimulating *in situ* bioremediation of crude oil-contaminated soil.

MATERIALS AND METHOD

PREPARATION OF CONTAMINATED SOIL

The soils used in this study were red soil and sand which were confirmed free from any type of hydrocarbon contamination. The soil was air dried and passed through 2 mm sieve to remove stones and gravel. Soil compositions were analysed by ALS Technichem Sdn. Bhd. The sieved soil was spiked with 10% (v/w) of Tapis light crude oil, by repeatedly spraying on the top layer of soil, mixed thoroughly with sterile spatula, until the whole soil homogeneously polluted. The soil samples were kept overnight and then divided into 200 g for each container. The EFB used in this study was obtained from an oil palm mill in Dengkil, Selangor. The EFB was dried in the oven at 100°C, then cut into small pieces using blender about 2-3 mm in size.

EXPERIMENTAL DESIGN AND SOIL TREATMENT

The crude-oil polluted soil was divided into 4 treatment sample-containers, which were 40 cm length, 27 cm width and 10 cm depth and designated as tray A, B, C and D. Tray A which did not receive any treatment, served as a control, to account for the abiotic loss and uptake of crude oil by the indigenous microbes. All the other 3 trays were inoculated with 20% (v/w) bacterial-fungal consortium. Tray B was added with fertilizer with NPK content 8:8:1 (0.7% v/w), tray C with 20% (w/w) EFB and tray D with both NPK fertilizer and EFB (Table 1). Each treatment was done in duplicate and for each tray, the soil samples were taken from three different locations. The treatment soils were kept in the green house with temperature 29-33°C daytime and 23-25°C at night.

The pH of the soil was maintained at pH6.5 – 7.0 by using calcium carbonate powder (Ca₂CO₃) and the soil moisture was adjusted to 40% water holding capacity (WHC) by adding distilled water every two days. The moisture and pH of the soil were observed using moisture meter ProCheck (Decagon Devices, USA) and pH meter (IQ Scientific Instruments, Model IQI50, USA), respectively. The

treatment soils were mixed every 2 days to eliminate the effect lack of oxygen and to homogenies the soil contents.

PREPARATION OF MICROBIAL CONSORTIUM

Two species of bacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* UKM12T and two species of fungi *Trichoderma virens* UKMP-1M and 2M were used in this study which has been isolated previously by Ainon and Raja Farzarul Hanim (2006) and Ainon et al. (2012), respectively.

The standard bacteria inoculum was prepared as described by Ainon et al. (2010a), while fungal spore suspensions was prepared as described by Ainon et al. (2012). The microbial consortium was constructed by mixing each of the bacterial and fungal species in equal ratio of 1:1:1:1 (v/v).

ENUMERATION OF MICROBIAL GROWTH

The enumeration of bacterial population was performed using spread plate method on nutrient agar (NA) for bacteria and potato dextrose agar (PDA) for fungi. Sampling was done on every sixth day by taking 1 g of soil from each treatment, mixed with 9.0 mL sterile normal saline (0.85%) and tenfold serial dilution was performed. The plates for bacteria and fungi growth were incubated at 37 and 30°C, respectively for 24 h. The bacterial colonies were counted and reported as CFU/g of soil.

DETERMINATION OF TOTAL PETROLEUM HYDROCARBON (TPH)

The soil sample was added with chloroform for hydrocarbon extraction and the mixtures was then filtered using phase separators filter paper and the filtrate was evaporated in the fume cupboard until dry. The dried crude oil was dissolved in chloroform and injected into gas chromatography with flame ionization detector (GC-FID) using Clarus GC 500 (Perkin Elmer-Auto System). The biodegradation of crude oil was determined as total petroleum hydrocarbons (TPH) and percentage of TPH degradation as described by Ainon et al. (2010a).

DATA ANALYSIS

Data obtained in the study were analyzed using statistical analysis of one way ANOVA and post hoc Tukey's test with SPSS Version 17.0 and considered significance if $p < 0.05$.

TABLE 1. Experimental design for the treatments

Plot	Treatment	Amendments
A	Control	Soil + crude oil
B	Bioaugmentation + Biostimulation	Soil + crude oil + Consortium 20% (v/w) + NPK 0.7% (v/w)
C	Bioaugmentation + Biostimulation	Soil + crude oil + Consortium 20% (v/w) + EFB 20% (w/w)
D	Bioaugmentation + Biostimulation	Soil + crude oil + Consortium 20% (v/w) + NPK 0.7% (v/w) + EFB 20% (w/w)

RESULTS

SOIL CHARACTERISATION

The main physico-chemical and microbiological characteristics of the contaminated soil were evaluated before assays. The soil was acidic at pH 4.4 and the moisture content was low and could influence biodegradability negatively, as good soil aeration would be difficult to attain. In order to achieve suitable condition for microbial growth the soil was amended by adding water to 50% water-holding capacity and Ca_2CO_3 for pH maintaining at 6.5-7.0 (Table 2). The nutrient content in the soil was low in carbon 0.2-1.0% but high in phosphorus and potassium 128 and 599 mg/kg with low bacterial population 7.8×10^4 cfu/gm soil.

BIODEGRADATION OF HYDROCARBONS IN SOIL BY MICROBIAL CONSORTIUM

Biodegradation of crude oil was analyzed every 6 days to determine the amount of crude oil that has been degraded. In general, all the treatments including the control showed an increment of TPH degradation from day 6 to day 30. The

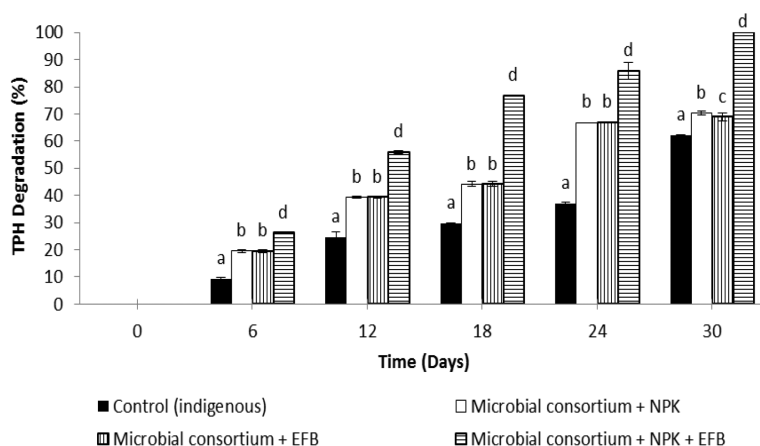
biodegradation of crude oil in soil added with microbes and fertilizer (Treatment B) showed no significant difference in percentage of TPH degradation with soil added with EFB (treatment C) from day 6 to day 24. This microbial consortium together with fertilizer and EFB degraded 100% of 50000 ppm of Tapis crude oil in 30 days compared to 38% with the control (Figure 1). The chromatogram of TPH degradation showed all carbons peaks including pristane (C19) and phytane (C20) in the crude oil were degraded in 30 days (Figure 2). Meanwhile, the TPH degradation for treatment using NPK fertilizer and EFB showed 72% and 68% degradation, respectively.

MICROBIAL GROWTH

The number of bacteria in all treatments (A, B, C, D) showed an increment in growth with significant difference from day 0 to day 12 (Figure 3). From day 18 to day 30, there is no significant difference between treatment with addition of EFB or NPK with EFB. The number of bacterial colonies was stabilized for tray with addition of EFB and NPK with EFB from day 24 to day 30. At the end of day 30 incubation, addition of NPK, EFB and both NPK and EFB, the

TABLE 2. Physicochemical characteristics of the soil used in this study

Parameters	Value	Unit	Methods
Total organic carbon (TOC)	0.2 -1	%	Oxidization/titration
Nitrogen	20	mg/kg	APHA 4500
Phosphorus	128	mg/kg	Acid Digest/APHA 3120 B
Potassium	599	mg/kg	Acid Digest/APHA 3120 B
Viable cell count	7.8×10^4	CFU/g soil	Spread plate method
Moisture	4.4	% (WHC)	ProCheck Moisture Meter (Decagon Devices, USA)
Sand	25	%	APHA method
pH	4.4		pH meter
Temperature	22.6	°C	ProCheck Moisture Meter (Decagon Devices, USA)



Different letters indicate significantly different at $p < 0.05$

FIGURE 1. The effect of bioaugmentation and biostimulation on biodegradation of crude oil in soil

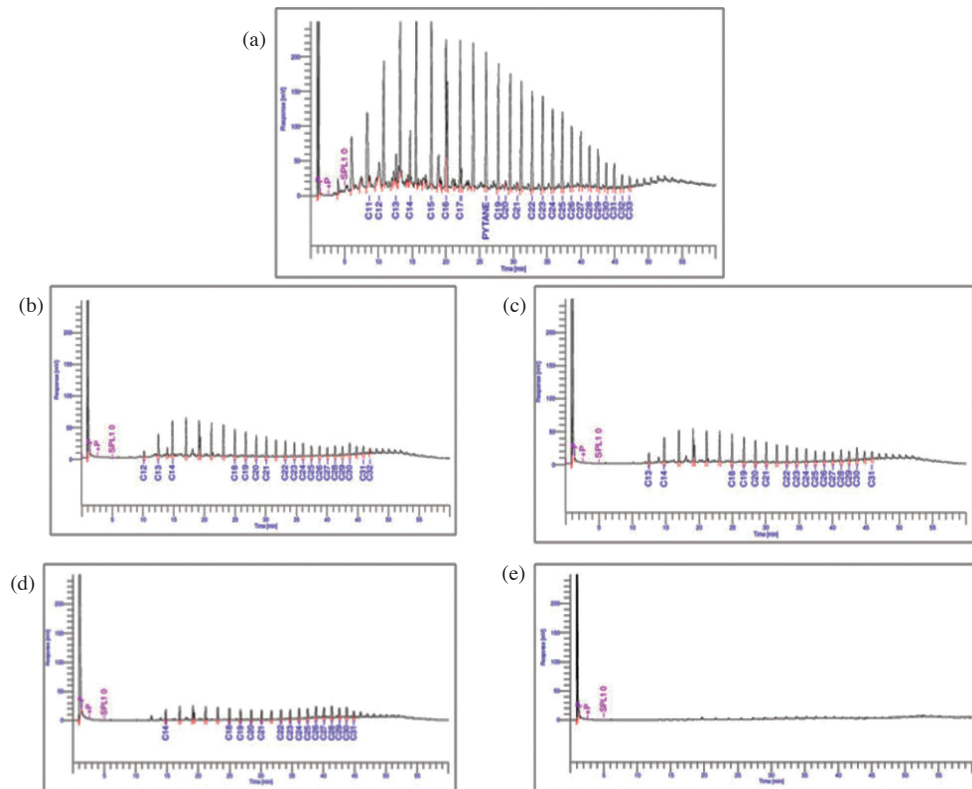
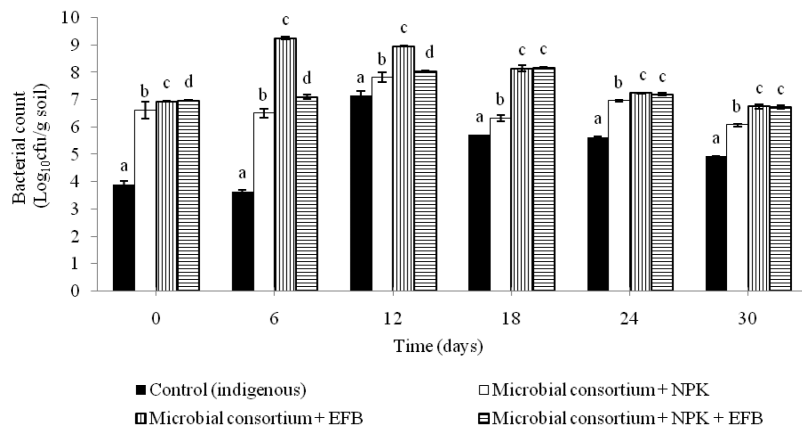


FIGURE 2. Chromatogram profile of the residual crude oil (a) Control D0, (b) control (indigenous) (c) addition of NPK (d) addition of EFB and (e) addition of NPK and EFB, after 30 days incubation



Different letters indicate significantly different at value $p < 0.05$

FIGURE 3. The number of bacteria during biodegradation process in different soil treatments.

number of bacterial colonies were 1.195×10^6 , 5.807×10^6 and 5.388×10^6 CFU/g soil, respectively.

In this study, the number of fungi was not enumerated. However, the presence of inoculated *Trichoderma virens* was confirmed by observation on PDA plate. The growth of the fungi was still observed until day 30 of incubation. The fungi growth was compared to the pure culture and the physical characteristics were confirmed as *T. virens*.

DISCUSSION

Microbes used in this study have been isolated from oil contaminated site in Malaysia (Ainon et al. 2012, Ainon & Raja Farzarul Hanim 2006). Formulation of this consortium has been tested in laboratory scale study (Ainon et al. 2010b). Using microbial consortium rather than a pure culture for bioremediation is more advantageous because it offers more metabolic diversity for co-metabolism of

hydrocarbon complexes for field application. This study showed bioaugmentation with microbial consortium significantly increased the biodegradation of Tapis crude oil compared to indigenous microbes. Alisi et al. (2009) also obtained complete degradation of diesel oil and phenanthrene and 75% of TPH in 42 days using microbial formula with selective native strains.

The amount of biomass used as inoculum or bioaugmentation has been optimized in previous study (Siti Nursyazana et al. 2011). The introduced populations to the soil undergo several abiotic and biotic stresses when in contact with complexity of the soil (Tyagi et al. 2011). The bacterial consortium used in this study took about 6 days to tolerate with the new environment before it can start dividing and increased in growth. The stresses that hamper the microbial growth are fluctuation and extreme temperature in the glass house together with decreased in water content, pH and depletion in nutrients. By maintaining the water content, pH and biostimulation with NPK and EFB, the growth of microbes were maintained to 10^6 cfu/g soil till day 30. The best approach for bioremediation of oil contaminated sites was bioaugmentation by inoculating pre-selected microorganisms from the same contaminates sites because they are more likely to survive and propagate as compared to transient or alien strains to the habitat (Bento et al. 2005; Tyagi et al. 2011).

Contaminated soil is always poor in organic matter and thus has low microbial activity. Addition of NPK fertilizer and EFB as inorganic and organic nutrient enhanced the microbial growth and thus concurrently increased the degradation of TPH. Since availability of nutrients, especially nitrogen and phosphorus appears to be one of the most common limiting factors for bacterial growth in soil, therefore addition of these nutrients enhanced hydrocarbon biodegradation to 100% of TPH at day 30 of incubation. Study by Sarkar et al. (2005) also showed the biodegradation of petroleum hydrocarbon increased up to 96% after addition of biosolids and inorganic fertilizer (N and P) to diesel contaminated soils. Similar result was also obtain for kerosene remediation by biostimulation using NPK fertilizer which showed higher degradation of kerosene in 6 weeks incubation (Shabir et al. 2008). Chorom et al. (2010) also found application of NPK agricultural fertilizer at 2 ton/ha in oil-contaminated soil lead to greater rates of biodegradation after 5 weeks incubation. They also found that application of fertilizer enhanced growth of hydrocarbon degrading microorganisms and heterotrophic microorganism in soil contaminated with 1% (w/w) of Maroon oil field crude oil.

Bulking agents such as rice husk, coconut shells and saw dust have been used in biodegradation of hydrocarbon. The efficiency of hydrocarbon degradation by addition of bulking agent from EFB and fertilizer NPK was about the same with no significant in short period of incubation (up to 24 day). Boodosingh et al. (2007) showed by using sawdust exhibiting consistent

removal oil Gator till day 60. In another study by Pala et al. (2005) showed coconut shell always led to higher removals of TPH than rice husks especially in the tests with higher diesel percentage (9%). When organic and inorganic nutrient source were added together, a complete degradation was achieved after 30 days incubation. The efficiency of the degradation may be attributed to the specific bulking agent composition, content and the fiber structure. Organic bulking agent also has the ability to adsorb oil is an advantage to bioremediation.

The EFB is high in organic carbon (54.765%), low in nitrogen (0.86%), phosphorus (0.18%), potassium (2.4%) and magnesium (0.23%) (Yahya et al. 2010). By adding NPK fertilizer this may provide suitable nutrients and conditions for both indigenous and exogenous microbes. EFB can improve the soil structure by adding porosity. This is because the availability of oxygen in soil is important for the microbial enzymes to degrade the hydrocarbon (Rhykerd et al. 1998). Trejo-Hernandez et al. (2006) showed addition of bulking agent, 180.7 mg/kg sugarcane bagasse to the soil contaminated with 10000 mg/g Maya heavy crude oil, achieved 40% TPH degradation in 15 days of treatment.

Addition of either organic or inorganic nutrient source had a significant increased in bacterial count than the control. The microbes have the ability to use hydrocarbon as carbon source and other supplied nutrients to continue growth and sustain higher biomass. As a result the higher number of organism present, hence greater in biodegradation of the hydrocarbon. The initial increase in indigenous and hydrocarbon-degrading microorganisms probably resulted from a selection of those populations which tolerate to petroleum hydrocarbon as carbon source (Molina-Barahona et al. 2004). Beside supplying nutrient to the microbes, addition of bulking agent can also help adaptation process for microbes by absorbing access of hydrocarbon in the soil when the concentration of crude oil is too high (Embar et al. 2006).

CONCLUSION

This study showed addition of NPK fertilizer and EFB had significantly enhanced the growth of microbial consortium, leading to the highest total petroleum hydrocarbon removal of 100% after 30 days incubation. Assemblages of mixed population with overall broad enzymatic capacities are required to increase the rate and extent of TPH biodegradation. Therefore, bioaugmentation and biostimulation has a great effect in enhancing biodegradation of crude oil in real environmental condition.

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