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CRUDE OIL BIOREMEDIATION BY INDIGENOUS BACTERIA ISOLATED FROM OILY SLUDGE

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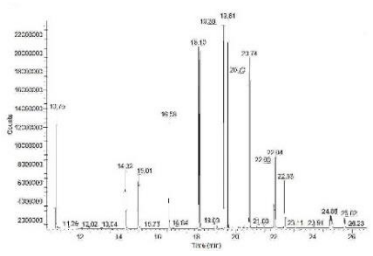
Graphical abstract



Abstract

Enrichment culture technique leads to the discovery of six presumptive TPH-degrading bacteria. Identification and characterization tests using morphological, biochemical and molecular techniques have successfully isolated *Pseudomonas aeruginosa* (UMAS1PF), *Serratia marcescens* (UMAS2SF) and *Klebsiella* spp. (UMAS3KF). All strains were able to use crude oil as sole carbon and energy source for their growth since they were able to survive in Minimal Salt medium supplemented with 1% (v/v) crude oil. Growth study showed that they produced the highest cell counts on the third or fourth day by 10^8 – 10^{11} CFU/ml. Six artificial consortium inoculums have been produced from the growth study. Gas chromatography analysis showed that all isolates had the ability to degrade aliphatic hydrocarbon with 100% degradation of nC_{19} – C_{24}. Among the isolates, UMAS2SF was the best and fastest *n*-alkane degrader with degradation percentage between 55 – 90% of *n*-C_{14} – C_{18} in 14 days. This was followed by UMAS1PF and UMAS3KF with 11 – 82% and 1.3% degradation, respectively. Enhancement study showed that plot with inoculum and NPK addition successfully enhanced *n*-alkane degradation. Plot A2:B3+NPK degraded *n*-alkane the fastest followed by plot treated by C+NPK, A1:B2, B+NPK and A2:B3. Result showed that UMAS1PF was the best PAHs degrader as most of the high molecular weight PAHs was degraded. In the enhancement study, the plot amended with A2:B3 showed the highest PAHs degradation, followed by plots A1:B2, A3:B1:C2 and A1:C3 that was assigned as the third, fourth and fifth best in mineralizing PAHs, respectively.

Keywords: Aliphatic hydrocarbon, consortium, PAHs, n-alkane degrader

1.0 INTRODUCTION

The need in petroleum and by-products to cover for a tremendous energy demand and as a primary raw material for industrialized society has brought an increasingly higher stress on the natural environment [1]. The release of hydrocarbons into environment through industrial operations, accidental spillages and leaks or dumping of wastes are main causes of terrestrial and marine ecosystems pollution [2]. Petroleum refining activities also inescapably generates substantial volumes of oil sludge. This attracts people attention nowadays with concerns of their toxic, mutagenic and carcinogenic properties that may cause progeny's death or mutation [3]. This hazardous compound enters human body usually through the food chain, where the hazardous compound adsorbs to organic-rich soils and sediments, accumulates in fish and other aquatic organisms and transferred to human through seafood consumption [4][5].

A range of cleaning techniques has been developed to curb this problem that is caused by oil pollution. Many approaches have been implemented such as physical and chemical methods that are capable to rapidly remove most of oil on the contaminated area. However, the removals are rarely completely successful and there is evidence that some of these techniques can be harmful to the recovery of certain habitats. In addition, according to Head (2002) [6], mechanical method to reduce hydrocarbon pollution is expensive and time consuming. Thus, new approaches have been focused towards techniques which assist the natural processes to remove oil pollutants. The use of microorganisms for bioremediation of hydrocarbon-contaminated environment is an attractive technology for the restoration of polluted sites. This can be achieved by supplying the indigenous hydrocarbon-degrading microbial community with electron acceptors and nutrients that are not present in sufficient amounts naturally [7]. Hence, the aims of this study were to isolate and characterize the novel indigenous TPH-degrading bacteria that are present in contaminated sludge from an oil refinery in Sungai Bera and to examine the best amendment or formulation that enhances the crude oil bioremediation process.

2.0 METHODOLOGY

2.1 Sampling

Oily sludge samples were obtained from Sungai Bera, Brunei Darussalam. The sludge was kept at 4°C before analysis.

2.2 Isolation of TPH-degrading Bacteria

The bacteria strains were isolated by using enrichment culture technique. Five grams of the sample was inoculated into 100 ml of Minimal Salt Media (MSM) with crude oil (1% v/v) as the carbon source. Minimal Salt Media contained per liter: 0.1 g Na_2CO_3, 0.01 g $CaCl_2.6H_2O$, 0.02 g $MnSO_4.7H_2O$, 0.01 g $FeSO_4$, 1.5 g Na_2HPO_4, 1.0 g KH_2PO_4, 0.02 g $MgSO_4.7H_2O$, 2.0 g NH_4Cl, 1.0 g NaCl. Culture was incubated at room temperature with shaking at 145 rpm for four days. After five cycles (4 days per cycle) of enrichments, a serial dilution was performed and plated on Plate Count Agar (PCA) followed by incubation at 37°C for overnight. Bacteria colonies formed on the agar surface were selected and subsequently subcultured onto MSM agar with 1% (v/v) crude oil. All culture plates were stored at 4°C.

2.3 Morphological and Biochemical Characterization

Morphological and biochemical characterization were carried out using standard gram staining method and conventional biochemical testing, which includes citrate utilization test, SIM test, Methyl Red and Voges Proskauer test (MRVP) and Kligler's Iron Agar test (KIA).

2.4 Molecular Characterization

Molecular characterization was performed using method as described by Ausubel et. al. (1992) [8] with several modifications. 16Sr RNA characterization performed using two universal primers, namely pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') designed by Edwards *et al*. (1989) [9]. Twenty five microliters of the PCR mixture containing 1X PCR buffer (Buffer A Vivantis), 3 mM of $MgCl_2$ (Vivantis), each deoxynucleoside triphosphate at concentration of 600 µM (Vivantis), each primer at final concentration of 500 µM, 3 µl of 1 u *Taq* polymerase (Vivantis) and 3 µl (10 – 15 ng/µl) of DNA template. PCR mixture without DNA template was used as negative control. The PCR parameters consisted of 5 minutes of initial denaturation at 94°C, followed by 30 cycles of denaturation step at 94°C (30 seconds), primers annealing at 55°C (30 seconds) and elongation step at 72°C (30 seconds). The PCR was ended with final extension of PCR product for 7 minutes at 72°C. Finally, 5 µl of the PCR product was examined using agarose gel electrophoresis at 90 volts for one and the half hour.

2.5 Phylogenetic Analysis

Multiple-sequence alignment was performed with ClustalX 1.81 and was subsequently manually aligned. Mega version 3.0 was used to determine pair-wise distance and nucleotide compositions (Percentage of A, C, T and G bases). The phylogenetic tree was constructed by using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods with PAUP software version 4.0b4.

2.6 Growth Determination

Growth study was conducted by inoculating a single colony of each isolate into 10 ml Minimal Salt Media (MSM) broth. The incubation was at 37°C with agitation at 145 rpm for 48 hours. When the OD_{600} of the inoculum was in range of 0.1-0.3, 1 ml was transferred into fresh MSM with 1% (v/v) crude oil to make the final proportion of the inoculum to crude oil to 1:1. This culture was used as seed for a larger volume. Two hundred fifty milliliter Erlenmeyer flask was used to place 100 ml MSM containing 1% (v/v) crude oil and 1% (v/v) bacterial inoculum. The incubation condition of each flask was maintained for 14 days at room temperature with agitation at 145 rpm. A control without the isolate was prepared. At every 24-h intervals, the bacterial cultures were monitored spectrophotometrically at 600 nm wavelength and total viable counts (cfu/ml) was undertaken by spread plate technique using Plate Count Agar (PCA). In order to minimize the statistical error, the experiment was done in triplicate.

2.7 Soil Physiochemical Properties Analysis

Soil physiochemical properties were determined prior bioremediation to be conducted. pH was determined according to method described by Tan (1996) [10] with several modifications. The pH of the soil extract [soil:water (1:10)] was estimated using pH meter. Soil dry matter content, moisture content, water holding capacity and organic matter content were carried out according to method by Rothamsted (2004) [11]. Soil texture test was done according to method by Brown (2003) [12].

2.8 Bioremediation Experimental Design

Bioremediation test was designed according to Adoki and Orugbani (2007) [13] with several modifications. Treatments were set up in sets of plastic container (21.5 cm x 21.5 cm). Six hundred grams of oil-contaminated soil constituted of 150 g of oily sludge, 450 g of uncontaminated composite soil and 100 ml of inoculum [UMAS1PF (1.91 ± 0.76 x 10^9 CFU/ml), UMAS2SF (2.30 ± 0.3 x 10^8 CFU/ml) and UMAS3KF (2.61 ± 0.9 x 10^8 CFU/ml)] was prepared per treatment. Control that contained oily sludge and soil mixture without inoculum was set up together with the experiment. Soil moisture was maintained at 15%. All treatments were incubated at room temperature for 14 days and tilled twice a day. Day 0 and day 14 control samples together with the day 14 of treated samples were analyzed to examine the bacterial counts, the percentage of TPH degradation, soil organic matter and the pH variation.

2.9 Bioremediation Enhancement of Petroleum Hydrocarbon-contaminated Soil through Biostimulation and Bioaugmentation Process

Bioremediation plot was prepared based on the ratios of the bacteria consortia which were selected depending on their kinetic growth in the medium containing crude oil as sole carbon source. Control was set up with no amendment (without NPK and bacterial inoculum). Other amendment containing bacteria consortia and/or NPK were also set up to test the effects of these additives on biodegradation (Control, Control+NPK, A+NPK, B+NPK, C+NPK, A1:B2, A1:B2+NPK, A1:C3, A1:C3+NPK, A2:B3, A2:B3+NPK, A:B:C, A:B:C+NPK, A3:B1:C2, A3:B1:C2+NPK, A3:B2:C1 and A3:B2:C1+NPK).

2.10 Hydrocarbon Analysis

Extraction of remaining crude oil present in the soil after 14 days of incubation was performed by Soxhlet extractor [14]. Asphaltane was removed by mixing 15 ml of *n*-pentane with TEL. The mixture was then evaporated to dryness.

Column was prepared a day before used. Glass column (50 ml) was packed with slurry activated (130°C, overnight) silica gel (7.5 g). Sample was introduced into the silica gel chromatographic column. Total aliphatic hydrocarbon (F1) fraction was obtained by eluting the column with 30 ml of hexane. PAH fraction (F2) was eluted with 30 ml of hexane:DCM (1:3 v/v) mixture. Eluants were collected and then rotary evaporated. The concentrated fraction was diluted with 1 ml DCM and was sonicated prior to being transferred into a Teflon-caplined vial. Solvent was evaporated to the point of dryness under a gentle stream of purified N_2.

2.11 Gas Chromatography-Flame Ionization Detector (GC-FID) Analysis

The quantitative and qualitative analysis of aliphatic hydrocarbons was performed on computerized capillary gas chromatography Shimadzu GC-17A 5890 Series III with standard FID. One microliter sample was injected in splitless injector (280°C) and detector (300°C) and separated on a DB-5 fused silica capillary column with 25 m long x 0.22 mm internal diameter with 0.25 µm film thickness. Temperature in the oven was maintained at 50°C for 2 minutes before increased to 300°C at temperature program rate of 6.5°C/min. Dichloromethane was used to dilute F1 fraction prior to GC-FID analysis. *n*-alkane mix (50 ppm) with octadecene and was injected as the *n*-alkane standard.

2.12 Gas Chromatography-Mass Spectrophotometer (GC-MS) Analysis

Gas chromatography mass spectrophotometry analysis was performed to analyze PAHs. The GC oven was programmed from 50 – 300°C at 4°C min^{-1} with the transfer line held at 290°C. Samples for analysis were dissolved in dichloromethane and injected on-column using HP 7673 auto sampler at an oven temperature of 50°C. Helium was used as the carrier gas at a linear velocity of 23 cm s^{-1}. Typical mass spectrometer source conditions were ionization energy of 70 eV and temperature of 200°C. PAH mix (50 ppm) with d-10 anthracene was injected for PAH standard.

2.13 Data Analysis

Data was subjected to analysis of variance using SPSS 16.0. Tukey HSD with a significant level of 0.05 was applied to the results in order to determine the statistical significance.

3.0 RESULTS AND DISCUSSION

3.1 Isolation and Characterization of TPH-degrading Bacteria

Isolation of bacteria from the contaminated soil produced three isolates that belongs to genera of *Pseudomonas*, *Serratia* and *Klebsiella*. These bacteria were gram negative and rod-shaped. According to results obtained, isolate G was characterized as

Pseudomonas aeruginosa. Morphological characteristic demonstrates that isolate G secretes blue-green pigment that was apparent when it was incubated on NA and PCA and the color becomes weak when streaked on MSM agar with 1% (v/v) crude oil. *Pseudomonas aeruginosa* was also reported by Adoki and Orugbani (2007) [13] to be predominant in petroleum hydrocarbon contaminated soils and the isolated species were assumed to be able to utilize petroleum hydrocarbon as sole carbon source.

Isolate R on the other hand, was identified as *Serratia marcescens*. This isolate produced red pigmentation which was produced during incubation on PCA and MSM agar with 1% (v/v) crude oil. Nevertheless, no significant pigment formation was observed during incubation on hydrocarbon containing medium in conical flask. Therefore, it is assumed that the pigment formation is elucidated at certain condition as the pigment production is highly variable among species and is dependent on many factors such as species type and incubation time.

Isolate BC that exhibited drop-like characteristic and milky on NA, PCA and MSM agar with 1% (v/v) crude oil has been identified to be *Klebsiella* spp. Citrate utilization test, indole test, MR and VP test for this species were negative as well as H_2S production and for glucose. Although morphological and biochemical characteristics has been determined, sometimes the genus of the isolates however could not be determined as description of reactions provided in Bergey's manual varies among species. Thus, molecular characteristic need to be performed in order to precisely determined the species of each isolate.

Phylogenetic tree constructed by the NJ, MP and ML methods in this study illustrates a constant relationships pattern among isolates (Figure 1). This study showed that *Serratia marcescens* and *Klebsiella* spp. was in a sister-group relationship. Although morphological features of these isolates did not elucidate the close relationship among them, 16S rRNA gene sequence did discriminate the evolutionary relationships. Meanwhile, *Pseudomonas aeruginosa* was grouped independently.

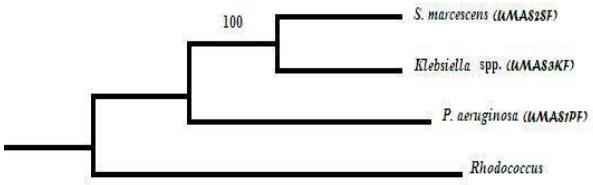


Figure 1 Rooted Neighbor-joining tree. Calculated by performing 1000 neighbor-joining bootstrap replicates.

3.2 Bioremediation Experimental Design

Bioremediation trial was conducted to verify the capability of individual isolate in degrading hydrocarbon on soil that was intentionally contaminated. Each isolate was inoculated independently for 14 days and monitored for growth and hydrocarbon losses. Other parameters for instance soil texture, pH, moisture content, organic matter content, water holding capacity are among the characteristics that were also considered in this experiment. By monitoring the bioavailability of *n*-alkane and PAHs fractions in soil sample at the end of the study, the work has shown that the indigenous microorganisms could degrade and transform hydrocarbons in two weeks.

Gas chromatography analyses exhibited that all isolates have the ability to degrade aliphatic hydrocarbon as 100% degradation of $nC_{19} - C_{27}$ and $C_{29} - C_{32}$ were recorded (Table 1). Degradation ability of these isolates was also shown by the studies of a number of researchers [15] [16] [17]. Amongst the isolates, UMAS2SF was the best and fastest *n*-alkane degrader as they degraded $nC_{14} - C_{18}$ at 55% – 90% in 14 days. This was followed by UMAS1PF and UMAS3KF with 11 – 82% and 1.3% degradation of the same carbon number, respectively.

Table 1 Concentration (ng/mg) dry weight of *n*-alkanes and isoprenoids for control and treated sample with UMAS2SF after 14 days of incubation. n.d- Not determined; C- Control; D- Day.

Compounds	CD0	CD14	UMAS2SFD14
C_{12}	7.9	n.d	n.d
C_{13}	24.2	n.d	n.d
C_{14}	36.0	39.2	58.1
C_{15}	38.1	12.3	14.1
C_{16}	27.0	31.9	14.2
C_{17}	17.9	20.6	55.1
Pristane	91.9	15. 3	544.6
C_{18}	13.9	117.2	11.6
Phytane	2.0	13.3	106.0
C_{19}	14.7	31.3	n.d
C_{20}	13.1	17.2	n.d
C_{21}	14.3	18.3	n.d
C_{22}	18.1	21.7	n.d
C_{23}	13.1	16.1	n.d
C_{24}	12.1	15.3	n.d
C_{25}	8.7	n.d	n.d
C_{26}	8.6	n.d	n.d
C_{27}	7.1	n.d	n.d
C_{28}	6.0	29.2	49.7
C_{29}	5.4	n.d	n.d
C_{30}	4.0	n.d	n.d
C_{31}	6.8	n.d	n.d
C_{32}	15.8	n.d	n.d

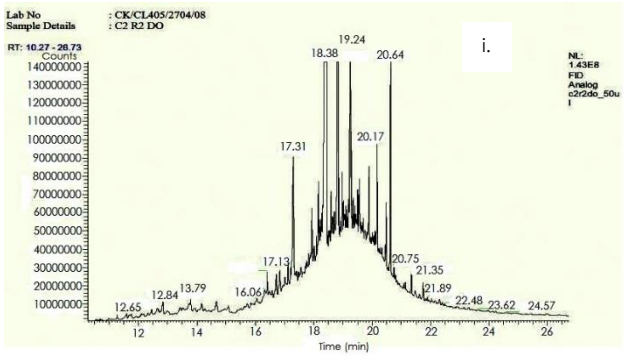
3.3 Bioremediation Enhancement of Petroleum Hydrocarbon-contaminated Soil Through Biostimulation and Bioaugmentation Process

Enhancement study was focused on bioremediation of oil-contaminated soil by amendment of inoculum and/or NPK fertilizer in 19 plots. The study took 14 days to bioremediate 313 ng of hydrocarbon mg dry weight of soil^{-1}. Six bacteria inocula were used in bioaugmentation. The study revealed that A2:B3+NPK, C+NPK, A1:B2, B+NPK and A2:B3 plots were among the

best amendment in degrading *n*-alkanes with 100% degradation of the *n*-alkane nC_{21}, C_{22}, C_{23}, C_{24} and C_{28}. Plot A2:B3+NPK is the best amendment that promotes the highest degradation on *n*-alkane (Table 2), followed by plot C+NPK, A1:B2, B+NPK and A2:B3. This study also showed that two combinations of bacterial strains achieved better degradation than a combination of three bacterial strains. The combination of bioaugmentation and biostimulation in certain plots however degraded *n*-alkane better than in unfertilized plot. However, in PAHs mineralization the addition of consortium solely in plot showed profound effect on PAHs degradation (Figure 2) (Table 3). Plot amended with A2:B3 showed the highest PAHs degradation then followed by plot A1:B2, A3:B1:C2 and A1:C3 that assigned as the third, fourth and fifth best in mineralizing PAHs, respectively.

Table 2 Concentration (ng/mg) dry weight of *n*-alkanes for control and treated sample (A2:B3+NPK) after 14 days of incubation. CD14-Control day 14; A-UMAS1PF; B-UMAS2SF; NPK-Nitrogen, Phosphorus, potassium; n.d- Not determined

Compounds	CD14	A2:B3+NPK
C_{14}	39.21	35.23
C_{15}	12.26	17.12
C_{16}	31.93	16.23
C_{17}	20.64	51.42
C_{18}	117.20	94.84
C_{19}	31.33	n.d
C_{20}	17.21	n.d
C_{21}	18.31	n.d
C_{22}	21.73	n.d
C_{23}	16.12	n.d
C_{24}	15.31	n.d
C_{25}	n.d	n.d
C_{26}	n.d	n.d
C_{27}	n.d	n.d
C_{28}	29.24	n.d



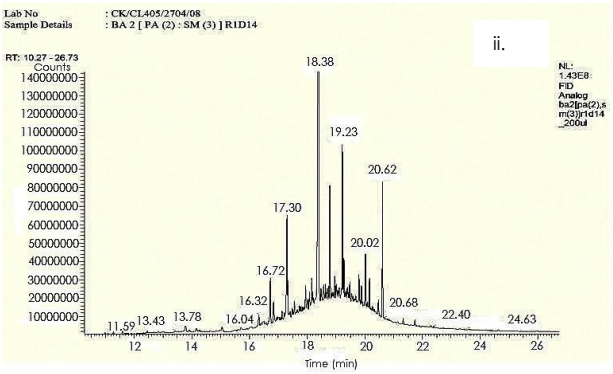


Figure 2 Chromatogram of PAHs in i) Control ii) A2:B3 treated plot; A-UMAS1PF; B-UMAS2SF. ISTD-Internal standard

Table 3 Concentration (ng/mg) dry weight of PAHs in control and in A2:B3 treatment after 14 days of incubation. C-Control; D-Day; A-UMAS1PF; B-UMAS2SF

Compounds	CD14	A2:B3
Naphthalene	20.4	2.3
Acenahthylene	13.9	7.0
Acenapthene	25.1	2.3
Fluorene	6.1	10.8
Phenanthrene	20.0	37.3
Anthracene	78.6	0.7
Fluoranthene	42.9	40.2
Pyrene	14.2	16.7
B(a)Anthracene	9.9	2.7
Chrysene	9.3	3.8
B(b)Fluoranthene	6.3	9.3
B(k)Fluoranthene	14.9	3.8
B(a)Pyrene	14.2	3.5
Ind(1,2,3-cd)Pyrene	4.2	20.8
DiB(a,h)Anthracene	5.6	3.9
B(g,h,i)Perylene	2.2	10.8

4.0 CONCLUSION

Bacteria consortia chosen from previous growth study and 15:15:15 fertilizer were used as the factors in enhancement of a bioremediation study. Fourteen days of incubation revealed that inoculum and NPK addition has successfully enhanced *n*-alkane degradation. Plot A2:B3+NPK degraded *n*-alkane the fastest and followed by C+NPK, A1:B2, B+NPK and A2:B3 plots.

Mineralization of PAHs showed that all of the consortia were able to degrade at least five high molecular weight PAHs tested. Naphthalene, benzo(a)anthracene, chrysene and benzo(a)pyrene were found oxidized in every plot. Besides, the consortia tested was also able to mineralize acenaphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene, b(b)fluoranthene, b(k)fluranthene, dib(a,h)anthracene and b(g,h,i)prylene.

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