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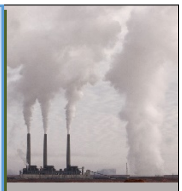

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Allochthonous Diesel Biodegradation by *Bacillus* sp. MO1 Isolated from Diesel-contaminated Soil

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ABSTRACT

Ten bacterial isolates were successfully isolated from pristine and contaminated areas in Malaysia. There was no lag phase observed signifying each of these isolates was able to quickly induce cellular machinery for diesel assimilation. The best isolate was tentatively identified as *Bacillus* sp. strain MO1 based on carbon utilisation profiles using Biolog GP plates and partial 16S rDNA matching. Strain MO1 grew optimally at 37 ºC on 4% (v/v) diesel, pH 7.5 and 0.9% (w/v) KNO_3. Diesel components were proven to be completely eliminated from the reduction in the hydrocarbon peaks monitored by gas chromatographic analysis after 6 days of incubation. The bacterium was found to be a better remediation agent than a commercial bioremediation product. All of these characteristics suggest that this bacterium is the suitable bacterium for bioremediation of diesel spills and pollution in the tropics.

INTRODUCTION

Pollutions of soils and aquatic bodies are usually instigated through the build-up of the industrials' wastes. Currently, the physical and chemical treatments that exist are expensive and are not capable to get rid of trace quantities of pollutant. There are a few successful remediation techniques that have been developed and applied in petroleum-contaminated sites. Indigenous bioremediation plays a critical part in diesel pollution because diesel produces damaging fumes and intolerable smell that has to be remediated immediately [1]. Other than that, the use of indigenous bioremediation product posses a few advantages such as low maintenance, applicable to large area, affordable, and completely degrade the contaminant [2].

In Malaysia, oil and grease rank as the highest industrial pollutants [3]. Since Malaysia is one of the oil and gas producers in the world, oil pollution could not be avoided especially since Malaysia owns the Straits of Malacca - the busiest waterway in the world. The occurrence of diesel contamination in Malaysia is highly associated to anthropogenic error. For instance, it has been reported that the collision between two tankers in the coastal areas of the Straits of Malacca had spilled almost 150 ton of diesel - making the incident to be one of the largest hydrocarbon spills to be reported [4].

Furthermore, there was another oil spill contaminating the soils in Seremban from an overturned lorry tanker which spilled an estimated of 15 tons of diesel [5] and another spillage of one ton of diesel into the soils in Gelugor from a 1,000 kW-mobile generator unit [6]. Even though there are a lot of reports regarding on the isolation of diesel-degrading bacteria, the search for the best degrader is still going at full speed in order to isolate bacteria with better properties to improve diesel remediation. In this work, we report on the isolation of a diesel-degrading bacterium and soil diesel biodegradation studies. Based on the results obtained, the isolate is suitable to be used as a bioremediation agent in the tropics.

MATERIALS AND METHODS

Isolation of diesel-degrading bacteria

Ten grams of soil samples were taken randomly from all over Malaysia from a depth of 5 cm from topsoil using a sterile spatula and stored in sterile screw-capped polycarbonate tubes. The soil samples were placed in sterilised plastic bags and stored on ice during the transfer from the site to the laboratory. The soil samples were resuspended in 10 ml of sterile saline solution (0.9% NaCl) and were vigorously shaken for 5 min.

A basal salt media consist of diesel as the sole carbon source was used as the enrichment culture media. A modified basal salt medium [7], pH 7.5, was composed of (per liter of distilled water): KH_2PO_4, 1.360 g; Na_2HPO_4, 1.388 g; KNO_3, 0.5 g; $MgSO_4$, 0.01 g; $CaCl_2$, 0.01 g; $(NH_4)_2SO_4$, 7.7 g; and 100 ml of a mineral solution containing 0.01 g of $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, H_3BO_4, $CoCl_2.6H_2O$, $Fe_2SO_4.2H_2O$, $CuCl_2.2H_2O$, $NaMoO_4.2H_2O$. Diesel was purchased from an Esso petrol station in Seri Serdang, Selangor. The established culture medium was supplemented with 2.5% (v/v) diesel [7] prepared with the pH adjusted to 7.5 and autoclaved at 121°C for 15 minutes before use.

The flasks were then incubated at 30°C and were shaken at 150 rpm (YIH DER, Taiwan) for six days. The spread plate technique was used for culture isolation and enumeration. The cultures were then incubated at 30°C. Isolates showing a distinctive colonial morphologies were isolated by repeated subculturing into basal salt medium and solidified basal salt medium to obtain pure strains. Identification at species level was performed by using Biolog GP MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and 16s rDNA database search.

Diesel analysis using gas chromatography

The fingerprint of the individual diesel residues and the intermediate products produced in this research were quantified by Varian 2900 (Varian, USA) gas chromatograph equipped with a flame ionisation detector (FID) fitted with a Chrompack capillary column, WCOT fused silica 30 m x 0.39 (film thickness 0.25 µm) (Varian). The parameters for the column temperature were set at an initial temperature of 50°C for 5 minutes followed by a 10°C increment per minute to 300°C and the isothermal held for 10 minutes. Carrier gas velocity was 30 ml/min, and makeup gas velocity, 30 ml/min with a total run time of 35 minutes.

Identification of bacterium

16S rDNA gene sequencing

Alkaline lysis was performed to extract genomic DNA from bacterial colonies. PCR amplification was performed using Biometra T-Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture (50 µl) contained 0.5 pM of each primer, 200 µM of each deoxynucleotide triphosphate,1x reaction buffer, 2.5 U of Taq DNA polymerase (Promega). The 16S rDNA gene from the genomic DNA was amplified by PCR using the following primers; 5'–AGA GTT TGA TCC TGG CTC AG-3' and 5'-AAG GAG GTG ATC CAG CCG CA-3' corresponding to the forward and reverse primers of 16S rDNA, respectively.

PCR was performed under the following conditions: initial denaturation at 94 °C for 3 min; 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer.

Sequence analysis

The BLAST 2 sequences algorithm using the nucleotide blast option with the matrix turned off and default parameters available from the NCBI server (http://www.ncbi.nlm.nih.gov/blast/) was used to evaluate the pair-wise comparisons to quantify the homology level between the sense and antisense (constructed through the reverse complement option) nucleotide sequences. The two sequences were compared and checked for any errors and omissions of bases especially at the overlapped region between the sense and antisense primer sequence using the CHROMAS software version 1.45. The sequences were combined at bases giving the least ambiguous characters and gaps. The comparison of the combined 16S rRNA gene sequence, and the resultant 1281 bases with the GenBank database was made using the Blast server at NCBI [8]. The 16s rRNA ribosomal gene sequence for this isolate has been deposited in GenBank under the accession number **GU085507**.

Biodegradation studies

Biodegradation studies were conducted on soil contaminated with diesel. The soil was obtained from an agriculture plot in Universiti Putra Malaysia (**Table 1**). The collected soil was classified as sandy clay loam in accordance to the match of PSD and the USDA soil textural class.

Table 1. The specifics of agricultural soil obtained from Universiti Putra Malaysia.

Soil characteristic(s)	Value
pH	6.4
Clay content (<2 µm) (%)	31.60
Silt content (2-50 µm) (%)	8.83
Sand content (>50 µm) (%)	58.29
Water content (%)	15
Total N (ppm)	0.14 % ± 0.03
Available P (ppm)	98.78 ± 28
Exchangeable K (ppm)	132.00 ± 30
Ca (ppm)	661.22 ± 86.77
Mg (ppm)	121.89 ± 26.24
Total petroleum hydrocarbon (TPH)	53,000 mg/kg*

*measured using gravimetric analysis for diesel contaminant

The course and other plant materials were removed prior to thorough soil mixing and usage. The degradation of hydrocarbon sludge in soil was examined by using non-sterile soil. Fresh soil was taken in 300 ml beaker glass and arranged in three different sets with 100 g of soil in each beaker. The soils were inoculated with 10 ml of the bacterial culture grown on medium containing diesel to obtain a cell concentration of approximately $1x10^8$ cells per grams of soil.

A commercial and proprietary blend of wetting and nutritional agents with mixture of safe and non-pathogenic *Bacillus* strains (Microblaze® Microbial Products, Gift from Corro-Shield Sdn Bhd.) which commonly used for hydrocarbon-based contaminants was also tested using the same number of cells. The soils were wetted and mixed with 10 ml of Basal mineral salt media containing the optimized conditions every three days including control (without bacterial inoculation) to maintain the moisture of between 10 to 15%. The soils were incubated at room temperature and sampled periodically.

RESULTS AND DISCUSSION

Screening and isolation of diesel-degrading bacteria

Ten bacterial isolates were successfully isolated from different places in Malaysia (**Table 2**). Each isolate was evaluated upon their growth potential on 2.5% (v/v) diesel over the course of six days. Isolate 4 was chosen for further studies based on the growth profile (**Fig. 1**). There was no lag phase observed demonstrating each of these isolates' capability to quickly induce cellular machinery for diesel assimilation.

Table 2. Locations of soils from which diesel-degrading bacteria were obtained.

Isolate	GPS location	Location	Sample type
1	N 02°31.746' E 101°49.24'	Port Dickson, N.Sembilan	soil
2	N 02°31.756' E 101°49.221'	Port Dickson, N.Sembilan	water
3	N 02°18.102' E 102°07.837'	Sg. Udang Recreational Park, Melaka	water
4	N 02°59.900' E 101°39.500'	Seri Kembangan, Selangor	soil
5	N 02°14.955' E 102°08.220'	Tangga Batu Industrial Area, Melaka	water
6	N 01°26.855' E 103°54.719'	Pasir Gudang Industrial Area, Johor	water
7	N 02°26.084' E 103°50.337'	Mersing Jetty, Johor	water
8	N 02°33.197' E 103°45.340	Gunung Arong Forest Reserve, Johor	water
9	N 03°04.624' E 103°04.272'	Bdr. Muadzam Shah, Pahang	soil
10	N 03°04.547' E 103°04.783'	Bdr. Muadzam Shah, Pahang	water

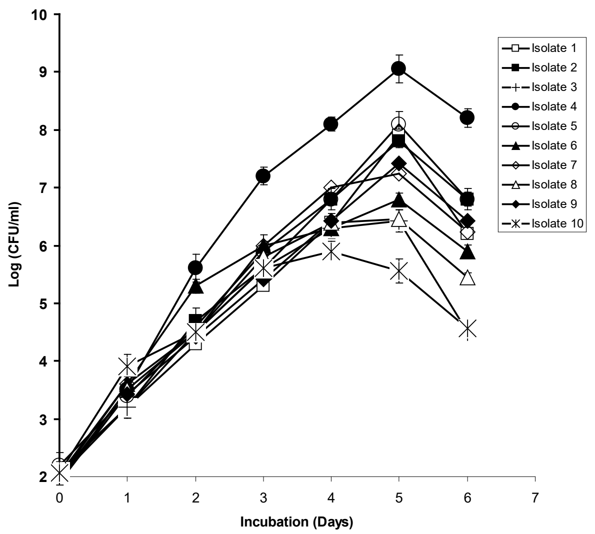

Fig. 1. Growth curve of 10 diesel-degrading isolates on liquid medium supplied with 2.5% (v/v) commercial diesel incubated on an orbital shaker (150 rpm) at pH 7.5 and 30 °C for 6 days. The bacterial growth measurement was based on the colony-forming unit (Log CFU/ml).

Identification of strain MO1

The best isolate (no. 9) was tentatively identified as *Bacillus* sp. strain MO1 based on carbon utilisation profiles using Biolog GP plates and partial 16S rDNA matching. The isolate was obtained from a diesel-contaminated site in the state of Pahang. The optimal carbon sources (diesel), pH and temperature for the growth of strain MO1 is at 4% (v/v), between 20 to 40 °C and between pH 7.5 and 8.0, respectively.

The effect of nitrogen sources

Various inorganic nitrogen sources were used to evaluate the best nitrogen sources for strain MO1 which includes $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl and KNO_3. From the results obtained shown in **Fig. 2**, KNO_3 appeared to be the best nitrogen supplier for the diesel-degrading bacteria. Similar findings have been reported by Hunkeler *et al.* [9]. The effect of concentration of nitrogen sources was studied at concentrations ranging from 0.1% to 1.8%. The optimum concentration of KNO_3 was 0.9% (w/v).

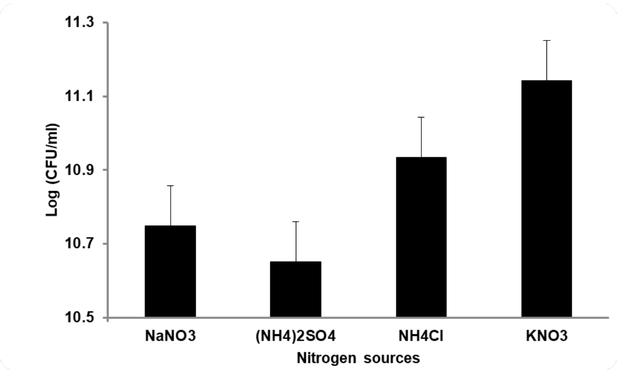

Fig. 2. The effect of different nitrogen sources (0.77 %, v/v) on the growth of Strain MO1. Bacterial growth was measured by colony-forming unit (Log CFU/ml). Growth was carried out at 37 °C on 4% (v/v) diesel for 6 days on an orbital shaker (150 rpm).

Gas chromatographic analysis

The GC profile of diesel degradation is shown in **Fig. 3**. From the profile, the optimal condition for strain MO1 to grow demonstrates a promising degradation of diesel as more than half of the original concentration of diesel in control (without inoculation of MO1) have been degraded.

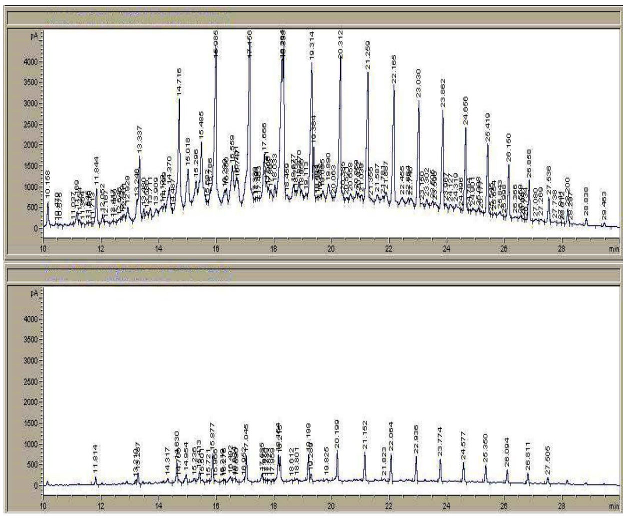

Fig. 3. GC profiles of diesel oil extracted from the aqueous phase of the medium at 37 °C on 4% (v/v) diesel, pH 7.5 and 0.9% (w/v) KNO_3 for 6 days on an orbital shaker (150 rpm) with and without inoculation with strain MO1. (Upper panel) Abiotic control (uninoculated); (Lower panel) diesel media inoculated with bacterium. The internal standard was *n*-hexane.

Comparison between allochthonous bacterium MO1, Microblaze® and native microbial flora in biodegradation of diesel

The results showed that allochthonous bioremediation agents either locally isolated or commercial (Microblaze®) are better at reducing TPH levels in soil especially in the first five days of incubation where the locally-isolated allochthonous agent (strain MO1) showed the highest degradation compared to later incubation days.

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The results also demonstrate that locally isolated and augmented bacterium is better in degrading contaminant from local soil compared to imported microbes due to geographical conditions advantages. Both allochthonous agents showed little to no lag phase in degrading diesel while bacteria from contaminated soil shows slow adaptivity with a lag period of five days (**Fig. 4**). This is due to the higher diesel-degrading cells that were added to the soil. Native (autochthonous) diesel-degrading bacterial cells are usually slow to grow due to diesel shock. The high concentration of diesel can kill native bacterial cells and the few remaining and surviving diesel-tolerant cells would eventually starts to multiply in a slow rate [10]. Furthermore, the biodegradation profile seen is also related to sample age. An aged diesel-contaminated site generally harbours higher level of diesel-degrading microflora and is more efficient in removing diesel contaminant when compared to the allochthonous system (bioaugmentation) whereas a newly contaminated soil would have few diesel-degrading microflora species and numbers [11].

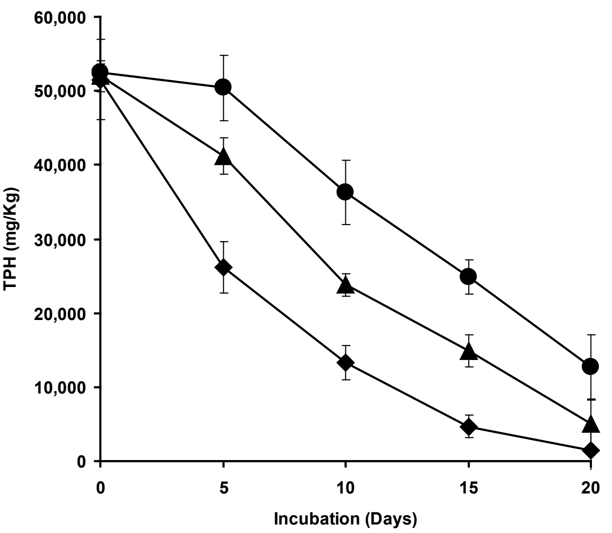

Fig. 4. Comparison between locally-isolated allochthonous bacterium (MO1) (◆) and commercially available allochthonous bacterial consortia (Microblaze®) (▲) to native microbial flora (●) biodegradation of a diesel-contaminated soil

During the last day for the incubation period (day 20), there is no significant difference in terms of diesel degraded in all treatment which indicates that the native cells have progressively catch up with the allochthonous population. Although a mixed report has been documented on the merit of allochthonous bioremediation [10,12,13], it must be noted that allochthonous bioremediation is very crucial in the early part of a spill or contamination. Allochthonous bacteria generally do not survive for a long period under different optimal settings impose by non-native soils, but they help to reduce the contaminant to a certain level until autochthonous or native population that is more suited to the particular soils coup and remove all traces of the pollutant.

All of the diesel-degrading isolates obtained in this work were sampled from contaminated and pristine areas. The ability of bacteria from pristine areas to degrade diesel is anticipated since diesel-degrading bacteria has been shown to be ubiquitous [14,15]. There was no lag phase observed for the isolated bacteria indicating each of these isolates is able to quickly induce cellular machinery for diesel assimilation. The growth pattern can be considered as quite rapid when compared to *Rhodococcus rubber*, which has an optimum growth of 7 days [16]. The high year-round temperatures of Malaysia [17] ensure that bacterial growth on any xenobiotics is rapid. Generally, the entire carbon source is assimilated by the bacterium for growth and energy and an increase of CFU/ml is regarded as an indicator of degradation with higher CFU/ml correlating with higher amount of diesel being degraded. Since isolate 4 has the highest bacterial counts than other isolates, it was chosen for further studies in this research.

Diesel is needed as a carbon source but at certain concentrations, diesel can be toxic to microorganisms due to the solvent effect of diesel which destroys the bacterial cell membrane thus devastating the hydrocarbon degrading ability of the cell and reducing the degrading rate of diesel [14]. In a previous study, Bicca *et al*., (1999) [16] have determined that 1% (v/v) of carbon source was suitable for *Rhodococcus ruber* and *Rhodococcus erythropolis*. Several researches on hydrocarbon degradation also chose to carry out the studies using lower diesel concentrations ranging from 0.5 to 1.5% [18–20]. Concentration higher than 1 or 1.5% has been proven to cause retardedness in degradation [16,18,19,21]. A degradation at a much higher degradation (6% v/v) is possible but it requires the addition of glucose (0.2% w/v) and yeast extract (0.1% w/v) [22]. Strain MO1 was proven to be able to tolerate higher diesel concentrations thus, it is the best candidate for diesel bioremediation. The ability of the strain to tolerate high levels of hydrocarbons is probably due to *Bacillus* spp. contain resistant endospores [23].

Márquez-Rocha *et al*., (2005) [24] reported an optimum temperature of 37°C for a tropical diesel-degrading bacterium from Mexico. Similarly, Bicca *et al*., (1999) [16] noted that *Rhodococcus ruber* and *Rhodococcus erythropolis* also grew well at 37°C. The temperature has an emphatic influence on the rate of bioremediation. Generally, as temperatures fall below optimum, the metabolism rate will fall below a satisfactory level. Rise of 10°C in temperature will approximately double the speed of reaction [25]. Growth was reported in a lower temperature optima in between 10 and 15 °C [14], 10 and 25 °C [26], at 20 °C [19,27] and in between 27 to 37°C [15].

pH plays an important role in bacterial growth. pH in a media can be altered simply by the production and accumulation of bacterial waste products [14]. Sepahi *et al*., (2008) [28] proved that the growth of *Bacillus* spp would decrease the pH of the medium from pH 6.8 to 6.2. In general, the favourable pH range for bioremediation is between 6 and 8, with the neutral pH (pH 7) suggested as the most effective one [15]. Most bacteria function best at neutral to slightly alkaline pH in a range between 5 and 9 with the optimum being slightly above 7. Growth is poor at pH 5 or lower [29].

Nitrogen is essential to microbial metabolism and it is necessary to biosynthesise amino acids, proteins and also nucleic acids [30,31]. This property gave a reputation for nitrogen as an essential nutrient for soil bioremediation through microbial biomass. This study highlights the importance in determining the nitrogen uptake because the low level of nitrogen and phosphate will limit the degradation of hydrocarbon [32]. Nitrogen is used by bacteria for growth and also can be used as electron acceptor [33].

The most successful application of nitrogen fertilisers was on the Nakhodka tanker oil spill tragedy off Oki Island in the Japan Sea which had clearly increased the biodegradation rate of more than 5000 tons of heavy fuel oil [34]. This result differs than result from Hunkeler [9] which show that the optimum KNO_3 is 6.8%. Low level of low inorganic nutrient (N) in the soil will decrease and limit the degradation of hydrocarbon in soil. However, the use of nitrite in bioremediation have to be handled carefully since it is known to inhibit cellular growth during hydrocarbon biodegradation [35].

Besides, the study of nitrogen sources is important as reduction of nitrogen element in the bacterial environment will result in the limitation of the rates of hydrocarbon degradation [1].

CONCLUSION

In conclusion, the bacterium which was isolated from soil under the leakage of a diesel storage tank showed the promising result as a bioremediation agent. The bacterium grew optimally in basal media containing 4% (v/v) diesel as the sole carbon source at 37 °C and between pH 7.5 and 8.0. Potassium nitrate was found to be the best nitrogen source for this bacterium at 0.9 % (w/v). The locally isolated bacterium was found to be a better bioremediatory for diesel in soil compared to a commercial preparation. Further study is being carried out to determine the role of surfactant in enhancing the degradation of diesel in soil and soil biodegradation studies.

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