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Research article

Characterization of a diesel-degrading strain isolated from a local hydrocarbon-contaminated site

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ABSTRACT

A diesel-degrading bacterium has been isolated from a primary school soil. The isolate was tentatively identified as *Serratia* sp. Strain DRY5 based on the carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny. Isolate 5 showed an increase cellular growth with respect to diesel concentrations from 0% until 2% where the optimum growth occurring at 2% (v/v) diesel concentration and was able to tolerate up to 8% (v/v) diesel. Based on the optimization studies using different nitrogen sources, Ammonium chloride proved to be the best nitrogen source. Ammonium chloride was optimum at 7.7 gl^{-1} and concentrations higher than that resulted with inhibiton of the cellular growth. The optimal temperature and the optimal pH that supported the growth of this bacterium was between 23 to 40 ^{o}C and pH 7.5 to pH 8.5 respectively. Diesel components was proven to be completely removed from the reduction in the hydrocarbon peaks monitored by Solid Phase Microextraction Gas Chromatography analysis after 7 days of incubation. All of these characteristics suggest that this bacterium is the right bacterium for bioremediation of diesel spills and pollution in the tropics.

INTRODUCTION

Pollutions of soils and aquatic bodies are usually caused by industrials' wastes. The physical and chemical treatments that exist nowadays are expensive and are not able to remove trace quantities of pollutant. There are a few successful remediation techniques that have been developed and used in petroleum-contaminated sites [26]. Indigenous bioremediation plays an important part in diesel pollution because diesel produces damaging vapors and intolerable smell that have to remediate immediately [9,26]. Other than that, the use of indigenous bioremediation product posses a few advantages such as it is easy to maintain, can be apply to large area, affordable, and completely degrade the contaminant [3].

In malaysia, oil and grease are rank as the highest industrial pollution [12]. Since Malaysia is one of the oil and gas producer in the world, oil pollution could not be avoided especially since Malaysia owns the Straits of Malacca - the busiest waterway in the world. Contamination occurs in Malaysia mostly because of human error. For instance is when two oil tankers collided with each other in the coastal areas of the Straits of Malacca spilling almost 150 ton of diesel making the case to be one of the largest hydrocarbon spills to be reported [4]. Other than that, there was another spilled contaminating the soils in Seremban from an overturned lorry tanker spilling almost 15 tons of diesel [33] and another spilled of one ton of diesel into the soils in Gelugor from a 1,000 kw-mobile generator unit [33]. A locally – isolated bacterial consortium that can effectively degrade diesel has been reported [17]. 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 that could degrade diesel at a broad range of temperature from 23 to 40 ^{o}C and could tolerate up to 8% (v/v)

diesel. Based on the characteristics of this bacterium, it is practical to use as a bioremediation agent in the tropics.

MATERIALS AND METHODOLOGY

Isolation of Diesel-Degrading Bacteria

Soil samples of 10 grams were taken randomly from a depth of 5cm from topsoil using a sterile spatula and stored in a sterile screw-capped polycarbonate tubes. The soil samples were taken in December 2003 near the King Edward VII (2^{nd}) Primary School in the city of Taiping, State of Perak, Malaysia. The soil samples were placed in sterilized plastic bags and were stored on ice during the transferation from 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 with diesel as carbon source was used as the enrichment culture media. A modified basal salt medium [25] 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, $CoCl_2.6H_2O$, $Fe_2SO_4.2H_2O$, $CuCl_2.2H_2O$, $NaMoO_4.2H_2O$. The flasks were then incubated at 30°C and were shaken at 150 rpm (YIH DER, Taiwan) for six days. Culture isolation and enumeration was performed using the spread plate technique. The cultures were then incubated at 30°C. Isolates showing a distinct colonial morphologies were isolated by repeated sub culturing into basal salt medium and solidified basal salt medium to obtaine purified strains. Identification at species level was performed by using Biolog GP MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies.

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 ionization detector (FID) fitted with a Chrompack Capillary Column, WCOT Fused Silica 30 m x 0.39 (film thickness 0.25 µm) (Varian). The column temperature parameters 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.

Solid Phase Microextraction (SPME)

An SPME (PDMS (Polydimethylsiloxane), 7 µm thickness, Supelco, USA) was used as a hydrocarbon compounds extraction device. Since diesel fuel is volatile, it is well suited for sampling with SPME fibers [13]. In order to analyze the aromatic hydrocarbons during the biodegradation process, 1.5 ml of homogenized culture were extracted from the incubated growth medium and filtered through 0.45 µm (Millipore) membrane and stored in 1.5 ml eppendorf tube. For GC analysis, 100 µl of the diesel constituents were transferred into 1.5 ml glass vials heated on the hot-plate. Teflon septum was pierced through by an SPME fiber coated with a 7 µm polydimethylsiloxane layer (Supelco, USA), and was pushed down into the middle of the static headspace by using SPME holder Supelco (Bellefonte, PA, USA). The fiber was then retracted after extraction (headspace) at 110 °C for 10 minutes and immediately inserted manually into the injector for GC analysis.

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 with a final volume of 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'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' corresponding to the forward and reverse primers of 16S rDNA, respectively [11]. 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 BLASTN option with the matrix turned off and default parameters available from the server NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used to analyze the pair-wise comparisons to measure the level of homology between the two nucleotide sequences of the forward and the reverse complement of the Reverse primer sequences. The two sequences were compared and checked for errors and omissions of bases especially at the overlapped region based on the overlapped region between the forward and reverse complement of the reverse 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 RNA gene sequence, and the resultant 1478 bases with the GenBank database was made using the Blast server at NCBI [1].

Phylogenetic Analysis

Based on GenBank, a multiple alignment of 19 16S rRNA gene sequences were closely matches with the strain DRY5 and this were aligned by using clustal_W [34] with the PHYLIP output option. Mis-alignment was being observed and alignment positions with gaps were excluded from the calculations. *Bacillus subtilis* was used as the outgroup in the cladogram to construct a phylogenetic tree by using PHYLIP, version 3.573 [15]. On the other hand, the DNADIST algorithm program was used to compute the evolutionary distance matrices for the neighbour-joining/UPGMA methodology. This program reads the nucleotide sequences and give an output file containing the distance matrix. The model of nucleotide substitution was based on Kimura, 1980 [18]. A method from Saitou and Nei called neighbour-joining method was used to derive a phylogenetic tree [31]. Repetition of the PHYLIP analysis with 1000 bootstraps [15] by the SEQBOOT program in the PHYLIP package was used with each algorithm to check the confidence levels for individual branches within the tree. The finding of the topologies using a family of consensus tree methods called MI method using the CONSENSE program was used to construct the majority rule (50%). The tree was viewed using TreeView [28].

RESULTS AND DISCUSSION

Isolation of Diesel-Degrading Bacteria

There were several successfull isolation of bacterial from king Edward VII (2^{nd}) Primary School soil . Isolate 5 was selected for further investigation since it gave high cellular growth in diesel as a carbon source. There was little growth or no growth observed on the uncontaminated soil samples. There are studies that prove the high correlation between cellular growth and diesel assimilation in mircobes [29]. Generally, diesel that act as the carbon source would be assimilated by the bacterium for growth and energy. The degradation of diesel can be observed by using CFU ml^{-1} and the higher CFU ml^{-1} , the higher the amount of diesel being degraded.

Identification of Isolate 5

The isolate was tentatively identified as *Serratia* sp. Strain DRY5 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny [24]. The phylogenetic tree of isolate 5 is presented elsewhere.

Bacterial growth optimization on diesel

The effect of Carbon source

This experiment was performed in order to study the optimum diesel concentration as a carbon source for strain DRY5. Figure 1 is showing the result of carbon source optimization. Based on figure 1, the optimum carbon source (diesel) concentration for the growth of strain DRY5 was 2% (v/v) and it also showed that strain DRY5 was able to grow on 5% (v/v), 8% (v,v) and 10% (v/v) with a declining bacterial growth. Even though diesel is needed as a carbon source, at a high diesel concentration, diesel would be considered toxic to microorganisms due to the solvent effect of diesel that could destroy bacterial cell membrane. This is why a lot of biodegradation studies on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5% [17,20,21,27,30,35]. Concentration higher than 1 or 1.5% has been proven to cause retardedness in degradation [5,14,20,21]. A degradation at a much higher degradation (6% v/v) is possible but it requires glucose (0.2% w/v) and yeast extract (0.1% w/v) [19]. Strain DRY5 was proven to be able to tolerate higher diesel concentrations thus, it is the best candidate for diesel bioremediation. DRY5 can grow on hexadecane (C_{16}) as the single carbon source contains essentially 100% of its cellular fatty acid as 16-carbon fatty acid [16] because diesel and its medium chain hydrocarbon components are such a highly assimilable carbon source.

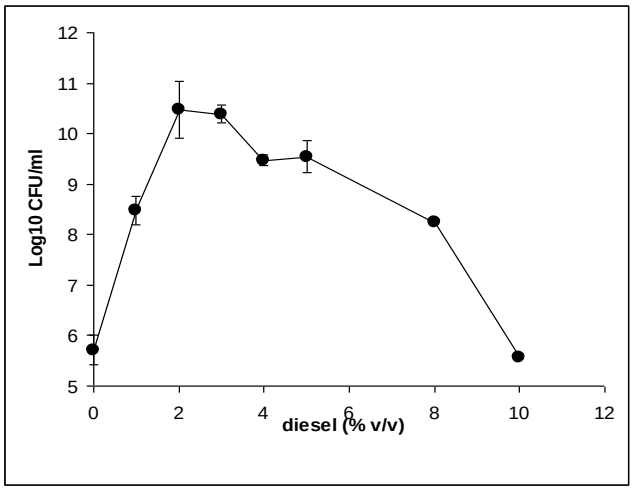


Figure 1. The effect of diesel concentrations on the growth of strain DRY5 grown at room temperature for 3 days on an orbital shaker (150 rpm). Bacterial growth was measured by determination of colony forming unit (Log CFU ml^{-1}). Data represents mean ± SEM, n=3.

The effect of Temperature

Based on Figure 2, strain DRY5 grew optimally in a wide range of temperatures ranging from 23 to 40 ^{o}C and it was decreasing dramatically at higher temperatures. Temperature of 30 ^{o}C was reported to be the most optimum temperature for diesel degradation [6,16,17,20,27]. Growth were reported in a lower temperature optima in between 10 and 25 °C [22], at 20 °C [8, 21,35] and at 27^{o}C [30]. Temperature of 37 °C was reported to be a higher growth optima by Márquez-Rocha and co-workers, 2005 [23] at 37°C for a tropical diesel-degrading bacterium from Mexico. The temperature of 37°C was also reported to be able to grow *Rhodococcus ruber* and *Rhodococcus erythropolis* [5]. Even though these bacteria were reported to grow well at 37 °C, they were not able to grow in a wide temperature range as strain DRY5. This is an advantage since Malaysia has a tropical climate where soil temperature can vary from 24 to 35 °C year round [32].

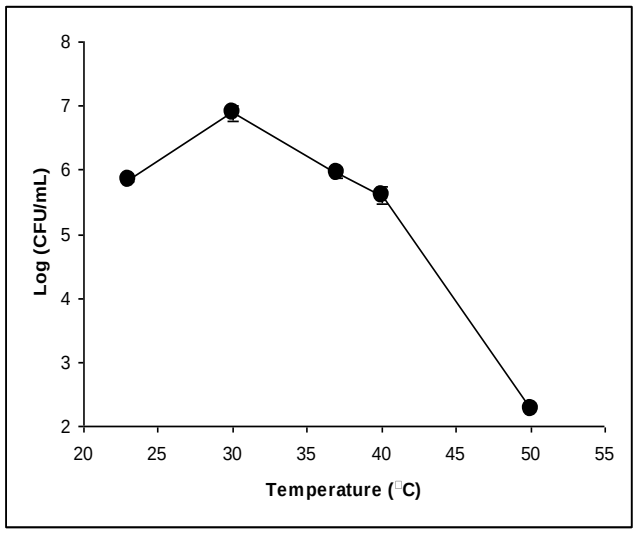
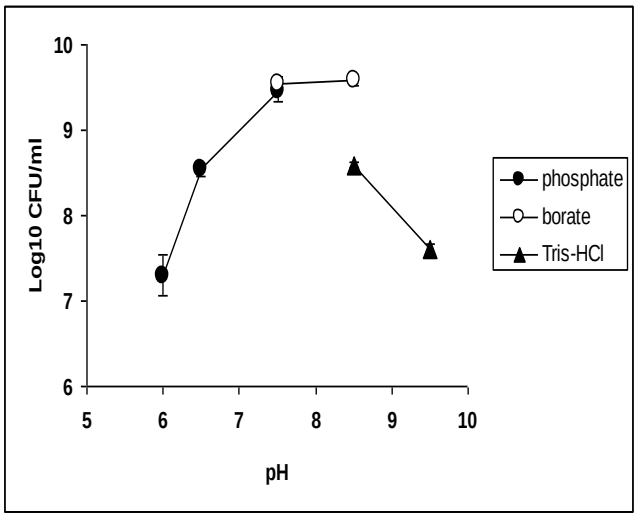


Figure 2. The effect of temperature on the growth of strain DRY5. Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

The effect of pHs

pH plays an important role in bacterial growth. pH in a media can be changed simply by the production and accumulation of bacterial waste products. This is why it is very important to optimize the environmental conditions for the enhancement of bacterial growth. In order to design an effective bioremediation strategy, identification of the pH optima is important. The optimal pH that supported growth of the bacterium was between pH 7.5 to pH 8.5 in borate, phosphate buffer and Tris-HCL buffer (Figure 3). There are a lot of other bacterial strains that need the neutral or near neutrality for the optimal growth of bacteria on diesel [5,6,8,14,16,17,20,21,22,23,27,30].



.**Figure 3.** The effect of pH on the growth of strain DRY5 using three overlapping buffers. The buffer system used were phosphate (●), carbonate (○) and borate (■). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

The effect of Nitrogen Sources

Inorganic nitrogen sources such as $NaNO_2$, $(NH_4)_2SO_4$, NH_4Cl and KNO_2 were tested as the nitrogen source at 1 gl^{-1}. Ammonium chloride gave the highest growth on diesel compared to the other carbon sources ($p<0.05$) while growth on nitrite and nitrate were almost the same ($p<0.05$). Ammonium sulphate gave the lowest growth compared to the other nitrogen sources except for the control while the control gave the lowest growth compared to the others (Figure 4). The optimum concentration was at 7.7 gl^{-1} and higher concentrations resulted with the inhibition of cellular growth (Figure 5). It was stated that the best nitrogen source in diesel biodegradation works is mostly either ammonium or nitrate salts [5,6,8,14,16,17,20,21,22,23,27,30,35]. The use of nitrite in bioremediation have to be handled carefully since it is known to inhibit cellular growth during hydrocarbon biodegradation [7]. The study of nitrogen sources is important as decreasion of it in the bacterial environment will results with limitation of the rates of hydrocarbon degradation [2]. After achieveing all of the optimization results on strain DRY27, biodegradation of diesel was performed. The reduction in hydrocarbon peaks was shown to be 95.1% after 7 days of incubation (Figure 6).

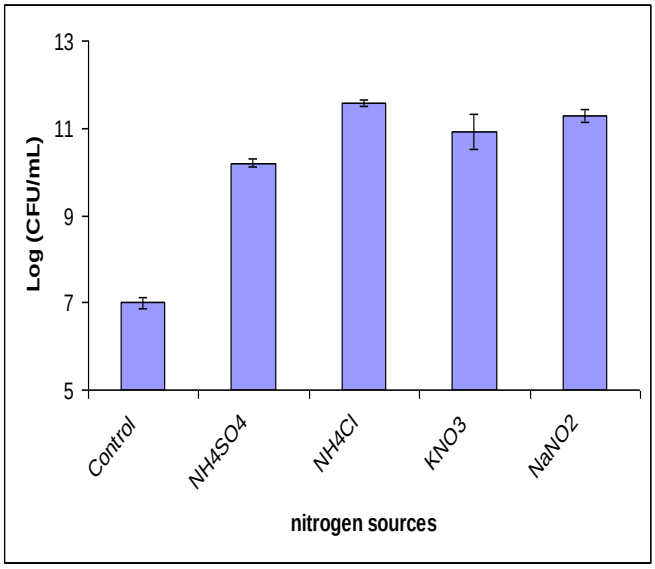


Figure 4. The effect of different nitrogen sources on the growth of strain DRY5. Bacterial growth was measured by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

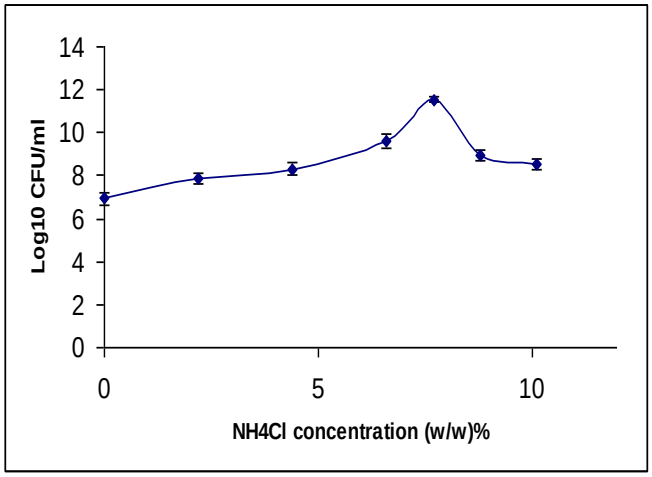
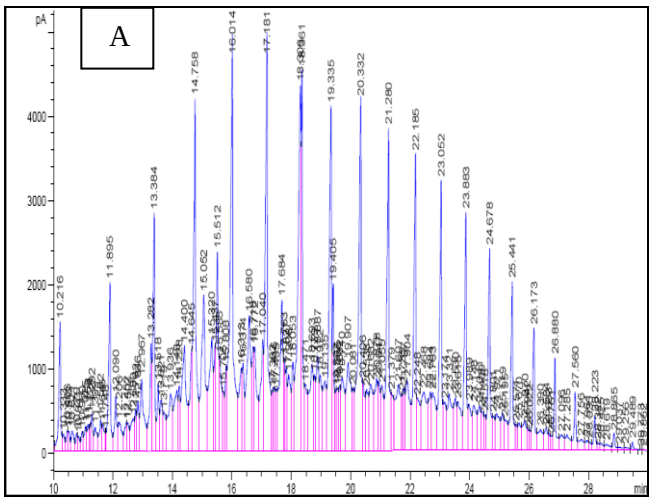


Figure 5. The effect of nitrite as a nitrogen source on the growth of Isolate 5. Bacterial growth was determined by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.



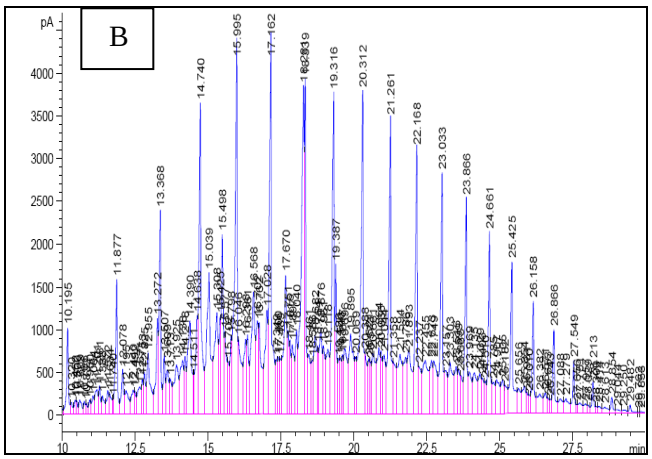


Figure 6. GC profiles of diesel oil extracted from the aqueous phase of the medium after 7days of incubation with 2 % diesel (v/v) with and without inoculation with strain DRY27. (A) Abiotic control (uninoculated); (B) inoculated with diesel. The internal standard was n-hexane.

CONCLUSION

This work is served as an introductory study for the actual bioremediation works on the polluted site using the autochthonous strain DRY5. Crude and processed hydrocarbons is known to contain significant amount of heavy metals that could inhibit bioremediation of the diesel polluted site. This is why a study of resistant of strain DRY5 to heavy metal is being performed. Work is also underway to characterize the enzymes and genes involved in diesel degradation. We are also working on bioaugmentation studies using this strain to remediate hydrocarbon sludge from a petroleum-processing plant as part of a bioremediation study using allochthonous bacterium.

REFERENCES

[1]Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman: Basic local alignment search tool. *J. Mol. Biol*., **215(3)**, 403-410 (1990).

[2]Atlas R.M. and C.E. Cerniglia: Bioremediation of petroleum pollutants. *Bioscience*, **45(5)**,1-10 (1995).

[3]Bento, F.M., Camargo, F.A.O, Okeke, B.C., and Frankenberger, W.T.: 2005, 'Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation,' *Bioresour. Technol.* 96, 1049 – 1055.

[4]Berita Harian Online: 150 tonnes of diesel spilled in the Straits of Malacca. 6 August (1997).

[5]Bicca F.C, L.C. Fleck, M. Antonio and Z. Ayub: Production of biosurfactant by hydrocarbon degrading *Rhodococcus ruber* and *Rhodococcus erythropolis*. *Rev. Microbiol*., **30**, 231-236 **(**1999).

[6]Cavalca L., P.D. Gennaro, M. Colombo, V. Andreoni, S. Bernasconi, I. Ronco and G. Bestetti: Distribution of catabolic pathways in some hydrocarbon-degrading bacteria from a subsurface polluted soil. *Resour. Microbiol.*, **151**, 877-887 (2000).

[7]Chayabutra, C. and L.K. Ju: Degradation of n-hexadecane and its metabolites by *Pseudomonas aeruginosa* under microaerobic and anaerobic denitrifying conditions. *Appl. Environ. Microbiol.*, **66(2)**, 493-498 (2000).

[8]Chapman, P.J. and M. Shelton: Fossil fuel biodegradation, laboratory studies. *Environ. Health Perspect. Suppl.,* **1035**, 1-7 (1995).

[9]Claassens, S., L. Van Rensburg, K.J. Riedel, J.J. Bezuidenhout and P.J.J. Van Rensburg: Evaluation of the efficiency of various commercial products for the bioremediation of hydrocarbon contaminated soil. *Environmentalist*, **26(1)**, 51-62 (2006).

[10]Davey, K.R.: Review paper, modelling the combined effect of temperature and pH on the rate coefficient for bacterial growth. *Int. J. Food Microbiol.*, **23**, 295-303 (1994).

[11]Devereux, R., and S.S. Wilkinson: Amplification of ribosomal RNA sequences. *In*: Molecular microbial ecology manual, 2nd ed. (*Eds.:* G.A. Kowalchuk, F.J. de Brujin, I.M. Head, A.D.L. Akkermans and J.D. van Elsas. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 509-522 (2004).

[12]DOE: Malaysia Environmental Quality Report 2006, Department of Environment, Ministry of Natural Resources and Environment, Malaysia. ISSN 0127-6433 (2007).

[13]Eriksson, M., A. Swartling and G. Dalhammar: Biological degradation of diesel fuel in water and soil monitored with solid-phase microextraction and GC-MS. *Appl. Microbiol. Biotechnol.*, **50**,129-134 (1998).

[14]Espeche, M.E., W.P. MacCormack and E.R. Fraile: Factors affecting growth of an n-hexadecane degrader *Acinetobacter* species isolated from a highly polluted urban river. *Int. Biodeterior. Biodegrad*., **33(2)**, 187-196 (1994).

[15]Felsenstein, J.: Confidence limits on phylogenies, an approach using the bootstrap. *Evolution.,* **39**, 783–791 (1985).

[16]Finnerty, W.R.: The biochemistry of microbial alkane oxidation, new insights and perspectives. *Trends Biochem. Sci.,* **2**, 73-75 (1977).

[17]Ghazali, F.M., R.N.Z.A. Rahman, A.B. Salleh and M. Basri: Biodegradation of hydrocarbons in soil by microbial consortium. *Int. Biodeter. Biodegrad*., **54**, 61-67 (2004).

[17]Hong, J.H., J. Kim, O.K. Choi, K.S. Cho and H.W. Ryu: Characterization of a diesel-degrading bacterium, *Pseudomonas aeruginosa* IU5, isolated from oil-contaminated soil in Korea. *World J. Microbiol. Biotechnol*., **21(3)**, 381-384 (2005).

[18]Kimura, M.A.: Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* **16**, 111–120 (1980).

[19]Kwapisz, E., J. Wszelaka, O. Marchut and S. Bielecki: The effect of nitrate and ammonium ions on kinetics of diesel oil degradation by *Gordonia alkanivorans* S7. *Int. Biodeterior. Biodegrad*., **61**, 214–222 (2008).

[20]Lee, M., M.K. Kim, I. Singleton, M. Goodfellow and S.-T. Lee. Enhanced biodegradation of diesel oil by a newly identified *Rhodococcus baikonurensis* EN3 in the presence of mycolic acid. *J. Appl. Microbiol*., **100**, 325–333 (2006).

[21]Lee M., M.K. Kim, M.J. Kwon, B.D. Park, M.H. Kim, M. Goodfellow and S.T. Lee: Effect of the synthesized mycolic acid on the biodegradation of diesel oil by *Gordonia nitida* strain LE31. *J. Biosci. Bioeng*., **100(4)**, 429-436 (2005).

[22]Margesin, R.: Potential of cold-adapted microorganisms for bioremediation of oil-polluted Alpine soils. *Int. Biodeterior. Biodegrad., **46***, 3-10 (2000).

[23]Márquez-Rocha, F.J., J. Olmos-Soto, M. Concepción Rosano-Hernández and M. Muriel-García: Determination of the hydrocarbon-degrading metabolic capabilities of tropical bacterial isolates. *Int. Biodeterior. Biodegrad.,* **55**, 17-23 (2005).

[24] Rahman M.F.A., Shukor, M.Y**.**, Z. Suhaili, S. Mustafa, N.A. Shamaan and M. A. Syed. 2009. Reduction of Mo(VI) by the bacterium *Serratia* sp. strain DRY5. *Journal of Environmental Biology*. **30(1).**In Press**.**

[25]Michaud, L., F. Di Cello, M. Brilli, R. Fani, A.L. Giudice and V. Bruni: Biodiversity of cultivable Antarctic psychrotrophic marine bacteria isolated from Terra Nova Bay (Ross Sea). *FEMS Microbiol. Lett.,* **230**, 63-71 (2004).

[26]Mohammed, D., A. Ramsubhag and D.M. Beckles: An assessment of the biodegradation of petroleum hydrocarbons in contaminated soil using non-indigenous, commercial microbes. *Water, Air, Soil Poll*., **182(1-4)**, 349-356 (2007).

[27]Mukherji S., S. Jagadevan, G. Mohapatra and A. Vijay: Biodegradation of diesel oil by an Arabian Sea sediment culture

isolated from the vicinity of an oil field. *Bioresource Technol*., **95**, 281–286 (2004).

[28]Page, R.D.M.: TREEVIEW, an application to display phylogenetic trees on personal computers. *Computer. Appl. Biosci.*, **12**, 357–358 (1996).

[29]Petrikevich, S.B., E.N. Kobzev and A.N. Shkidchenko: Estimation of the hydrocarbon-oxidizing activity of microorganisms. *Appl. Biochem. Microbiol*., **301**, 19-23 (2003).

[30]Rajasekar A., T.G. Babu, S.T. Pandian, S. Maruthamuthu, N. Palaniswamy and A. Rajendran: Role of *Serratia marcescens* ACE2 on diesel degradation and its influence on corrosion. *J. Ind. Microbiol. Biotechnol*., **34(9)**, 589-598 (2007).

[31]Saitou, N. and M. Nei: The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4**, 406-425 (1987).

[32]Sinnakkannu, S., A.R. Abdullah, N.M. Tahir and M.R. Abas: Degradation of metsulfuron methyl in selected Malaysian agricultural soils. *Fresenius Environ. Bull.*, **13(3)**, 258-261 (2004). The New Straits Times: Oil Spill closes road for six hours. 3 February (2000).

[33]The new Straits Times: Tenaga Nasional Moves Quickly to Avert Disaster after Diesel Spill. 23rd May (2001).

[34]Thompson, J.D., D.G. Higgins and T.J. Gibson: CLUSTAL W, Improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**, 4673–4680 (1994).

[35]Ueno A., Y. Ito, I. Yumoto and H. Okuyama: Isolation and characterization of bacteria from soil contaminated with diesel oil and the possible use of these in autochthonous bioaugmentation. *World J. Microbiol. Biotechnol*., **23**, 1739–1745(2007).