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Growth kinetics of a diesel-degrading bacterial strain from petroleum-contaminated soil

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Abstract

A diesel-degrading bacterium was isolated from a diesel-contaminated site in Selangor, Malaysia. The isolate was tentatively identified as *Acinetobacter* sp. strain DRY12 based on partial 16S rDNA molecular phylogeny and Biolog® GN microplate panels and Microlog® database. Optimum growth occurred from 3 to 5% diesel and the strain was able to tolerate as high as 8% diesel. The optimal pH that supported growth of the bacterium was between pH 7.5 to 8.0. The isolate exhibited optimal growth in between 30 and 35ºC. The best nitrogen source was potassium nitrate (between 0.6 and 0.9% (w/v)) followed by ammonium chloride, sodium nitrite and ammonium sulphate in descending order. An almost complete removal of diesel components was seen from the reduction in hydrocarbon peaks observed using Solid Phase Microextraction Gas Chromatography analysis after 10 days of incubation. The best growth kinetic model to fit experimental data was the Haldane model of substrate inhibiting growth with a correlation coefficient value of 0.97. The maximum growth rate- μmax was 0.039 hr^{-1} while the saturation constant or half velocity constant Ks and inhibition constant Ki, were 0.387% and 4.46%, respectively. MATH assays showed that 75% of the bacterium was found in the hexadecane phase indicating that the bacterium was hydrophobic. The characteristics of this bacterium make it useful for bioremediation works in the Tropics.

Key words

Isolation, Characterization, Diesel-degrading, *Acinetobacter* sp., Haldane

Introduction

Pollution of diesel and hydrocarbons in Malaysian soils and waters are mostly due to incidents of spills from tankers and storage tanks. The largest crude oil spill was reported in the Straits of Malacca when 44,900 tons of crude oil was spilled by the tanker Tadotsu (Etkin, 1999). There are also cases of soil pollution due to diesel spills. About 15 tons of diesel spilled from an overturned lorry tanker in Seremban, polluting surrounding soils while more than one ton of diesel was spilled into the soils in Gelugor, Penang from a 1,000 kw-mobile generator unit (Shukor *et al.,* 2009a).

Petroleum and xenobiotic pollutants are generally toxic to many organisms (Sarma et al., 2011). However, many bacterial strains are not only resistant towards these pollutants but have the ability to break down or transform the chemical substances to support their life cycle (Salam *et al.,* 2011). There are several genus that have been reported as hydrocarbon utilizers, such as *Pseudomonas*, *Bacillus, Proteus*, *Aeruginosa*, *Klebsiella*, *Aeromonas*, *Micrococcus*, *Serratia*, *Acinetobacter*, *Staphy lococcus*, and *Flavobacterium* (Wrenn *et al.,* 1994; Burkhard *et al.,* 1997; Shukor *et al.,* 2009a,b). There have been many reports on the isolation of diesel-degrading bacteria but the quest for the best degrader means that more bacteria with better properties

must be isolated to enhance diesel remediation especially for local environment. In light of above, the present study reports the isolation of a diesel-degrading bacterium that grew optimally at 5% diesel concentration and could tolerate diesel as high as 8%.

Materials and Methods

Isolation and identification of diesel-degrading bacteria: Soil samples were collected from petroleum-contaminated sites at several locations all over in Malaysia during December 2004. Soils were collected 15-20 cm beneath the surface and were placed in sterile screw-capped vials and placed on ice until returned to Universiti Putra Malaysia, Serdang, Selangor, Malaysia for further examination. Soil samples were resuspended in 10 ml of sterile saline solution (0.9% NaCl) and vigorously shaken for 5 min. The enrichment culture media consisted of a modified basalt salt media (pH 7.0) supplemented with 2% (v/v) diesel (density is 0.85 g ml^{-1}) as carbon source composed of (per liter of distilled water): KH_2PO_4, 1.360 g; Na_2HPO_4, 1.388 g; $MgSO_4$, 0.01 g; $CaCl_2$, 0.01 g; $(NH_4)_2 SO_4$, 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, $CoCl_2.6H_2O$, $Fe_2SO_4.2H_2O$, $CuCl_2.2H_2O$, $NaMoO_4.2H_2O$ (Shukor *et al.,* 2009a). The flasks were incubated at 30°C and 150 rpm (YIH DER, Taiwan) for 6 days. The controls were devoid of inoculums. Isolates exhibiting distinct colonial morphologies were isolated by repeated sub culturing into basal salt medium and solidified basal salt medium until purified strains were obtained. Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies.

Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). The PCR mixture contained 0.5 pM of each primer, 200 mM of each deoxynucleotide triphosphate,1x reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 ml. 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 (Devereux and Wilkinson, 2004). 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 combined 16S RNA gene sequences, and the resultant 1470 bases were compared with the GenBank database using the Blast server at NCBI (Altschul *et al.,* 1990). The partial 16S rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under the following accession number; DQ226213.

Phylogenetic analysis: Nineteen 16S rRNA gene sequences closely matches strain DRY12 were retrieved from GenBank and were aligned using ClustalW (Thompson *et al.,* 1994) with the PHYLIP output option. The alignment was observed for any obvious mis-alignments. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed by using PHYLIP, version 3.573 (Retief, 2000) with *Bacillus subtilis* as the outgroup in the cladogram. Evolutionary distance matrices for the neighbour-joining/ UPGMA methodology were computed using the DNADIST algorithm program. The program reads in nucleotide sequences and writes an output file containing the distance matrix. The model of nucleotide substitution is those of Kimura (1980). Phylogenetic tree was inferred by using the neighbour-joining method of Saitou and Nei (1987). With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps (Felsenstein, 1985) by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the MI methods using the CONSENSE program and the tree was viewed using TreeView (Page, 1996).

Analysis of diesel : An SPME Polydimethyl siloxane, 7 m thickness, Supelco, USA) coated with polydimethy lsiloxane layer was used as a hydrocarbon compounds extraction device. The volatility of diesel fuel is well suited for sampling with SPME fibers (Eriksson *et al.,* 1998). 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 m (Milipore) membrane and stored in 1.5 ml eppendorf tube. For GC analysis, 100 l of the diesel constituents were transferred into 1.5 ml glass vials heated on the hot-plate. An SPME fiber coated with a 7 m polydimethyl siloxane layer (Supelco, USA), was pierced through the Teflon septum, 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 ºC for 10 min and immediately inserted into the injector manually for GC analysis. The fingerprint of the separated diesel residues 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 m) (Varian). The column temperature parameters were set at as an initial temperature of 50ºC for 5 min followed by a 10ºC increment per minute to 300ºC and the isothermal held for 10 min. Carrier gas velocity was 30 ml min^{-1}, and makeup gas velocity, 30 ml min^{-1} with a total run time of 35 min.

Kinetic studies : The profile of various biomass growth rates could be used in obtaining kinetic parameters from batch

experiments. The values of the specific growth rate coefficient m at each initial diesel concentration could be obtained by plotting ln X (bacterial dry weight) vs. time. When these values were plotted against substrate concentrations a nonlinear curve will be obtained and modeling could be done to determine constants such as μmax (maximum growth rate), Ks (half saturation constant) and Ki (inhibition constant).

Microbial adhesion to hydrocarbon (MATH) assay : A modified method of Rosenberg (1984) and Zoueki *et al.* (2010) was used for the MATH assay. The bacterial suspension was adjusted to an absorbance of 1.0 at 600 nm on a spectrophotometer (Shimadzu) with the addition of NaCl to a final concentration of about 0.2 M. About 300 ml of hexadecane was added to 5 ml of bacterial suspension in a clean borosilicate round-bottom glass tube (16 x 150 mm, Pyrex). The tube was vortexed for 2 min and then was set aside resting for 15 min to allow for the phases to separate. About 2 ml of the bacterial suspension was removed carefully using Pasteur pipet and transferred to a quartz cuvette for absorbance measurement at 600 nm. Bacterial adhesion to the hydrocarbons was evaluated using the formula FPC = 1 - Af/Ao where FPC is fraction partitioned to the hydrocarbon phase, Af is final absorbance and Ao is initial absorbance.

Statistical analysis: Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test (Miller and Miller, 2000). $P < 0.05$ was considered statistically significant.

Results and Discussion

Isolation and identification of diesel-degrading bacteria: Five bacterial isolates were successfully isolated from water and soil samples collected from various locations in Malaysia. Most of the samples were collected from oil-contaminated locations. All isolates showed optimal growth on day 4 followed by a general decline in growth afterwards. Strain 12 showed the highest growth implying efficient cellular assimilation of diesel to carbon source for growth and energy. It has been shown that there is a high correlation between cellular growth and diesel assimilation in microbes (Shukor *et al.,* 2009a). The lowest growth was exhibited by isolate M; isolated from uncontaminated soils in the grounds of University Putra Malaysia. The fact that isolate 12 was isolated from soil under a leaking diesel storage tank gave testament to acclimatization advantages of xenobiotics-degrading microbes over non-acclimatized strains. Isolate 12 was chosen for further studies.

A moderately high bootstrap value (62.4%) linked strain DRY12 (isolate 12) to *Acinetobacter* sp. indicating that the phylogenetic relationship of the strain to this genera was significant. The closest relationship to a particular species was *A. calcoaceticus* with moderate bootstrap values (65.7%) which also includes an uncultured bacterium. Together with the Biolog identification system which gave the closest ID to the *Acinetobacter* genus, at least for now, isolate 12 is assigned tentatively as *Acinetobacter* sp. strain DRY12. The genus *Acinetobacter* is a well known hydrocarbon degrader with degrading species including *Acinetobacter venetianus* (Baldi *et al.,* 1999), *Acinetobacter calcoaceticus* (Wales and Fewson, 1994a) and several strains under *Acinetobacter* sp. (Wales and Fewson, 1994b; Espeche *et al.,* 1994; Sakai *et al.,* 1996; Tanase *et al.,* 2012). It has been documented that the genus is the most predominant bacteria found on a diesel spillage site and has the ability to accumulate alkane intracellularly without any structural modification of the hydrocarbon. The alkane-degradative genes found in *Acinetobacter* sp. appeared to be located on the chromosome and is known to excrete emulsifying lipopolysaccharide; emulsan when grown on diesel (Baldi *et al.,* 1999). Hence, the genus *Acinetobacter* is expected to have great potential for bioremediation.

Effect of carbon and nitrogen sources : The result of carbon source optimization is shown in Fig. 3. Isolate 12 showed an almost linear increase in cellular growth with respect to diesel concentrations with an optimum growth occurring from 3 to 5% (v/v) diesel concentration and was able to tolerate as high as 8% diesel. Growth inhibited at higher diesel concentrations. Similar range of optimal diesel concentration supporting growth was reported in *Staphylococcus aureus* strain DRY11 and *Pseudomonas* sp. strain DRYJ3 (Shukor *et al.,* 2009a,b).

Diesel is needed as a carbon source but at certain concentrations, diesel can be toxic to microorganisms due to the

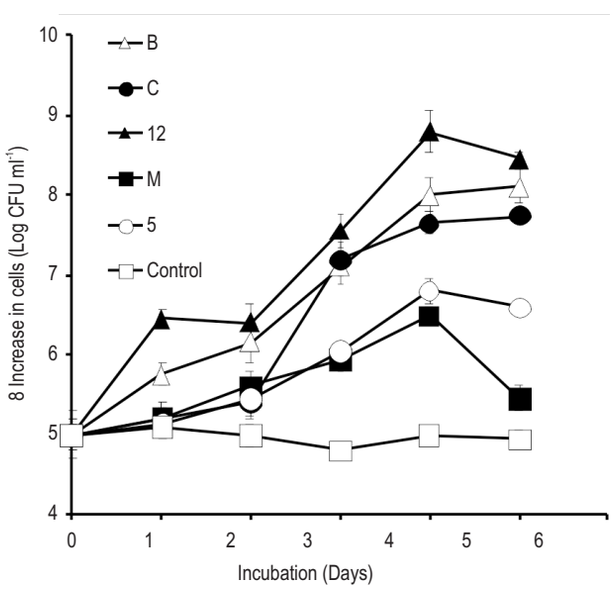

Fig. 1 : Growth curve of diesel-degrading isolates on medium supplied with 2.5% diesel incubated on an orbital shaker (150 rpm) at 30ºC for 5 days. The bacterial growth measurement was based on colony forming unit (Log CFU ml^{-1}). Value are mean of three replicates ± SE

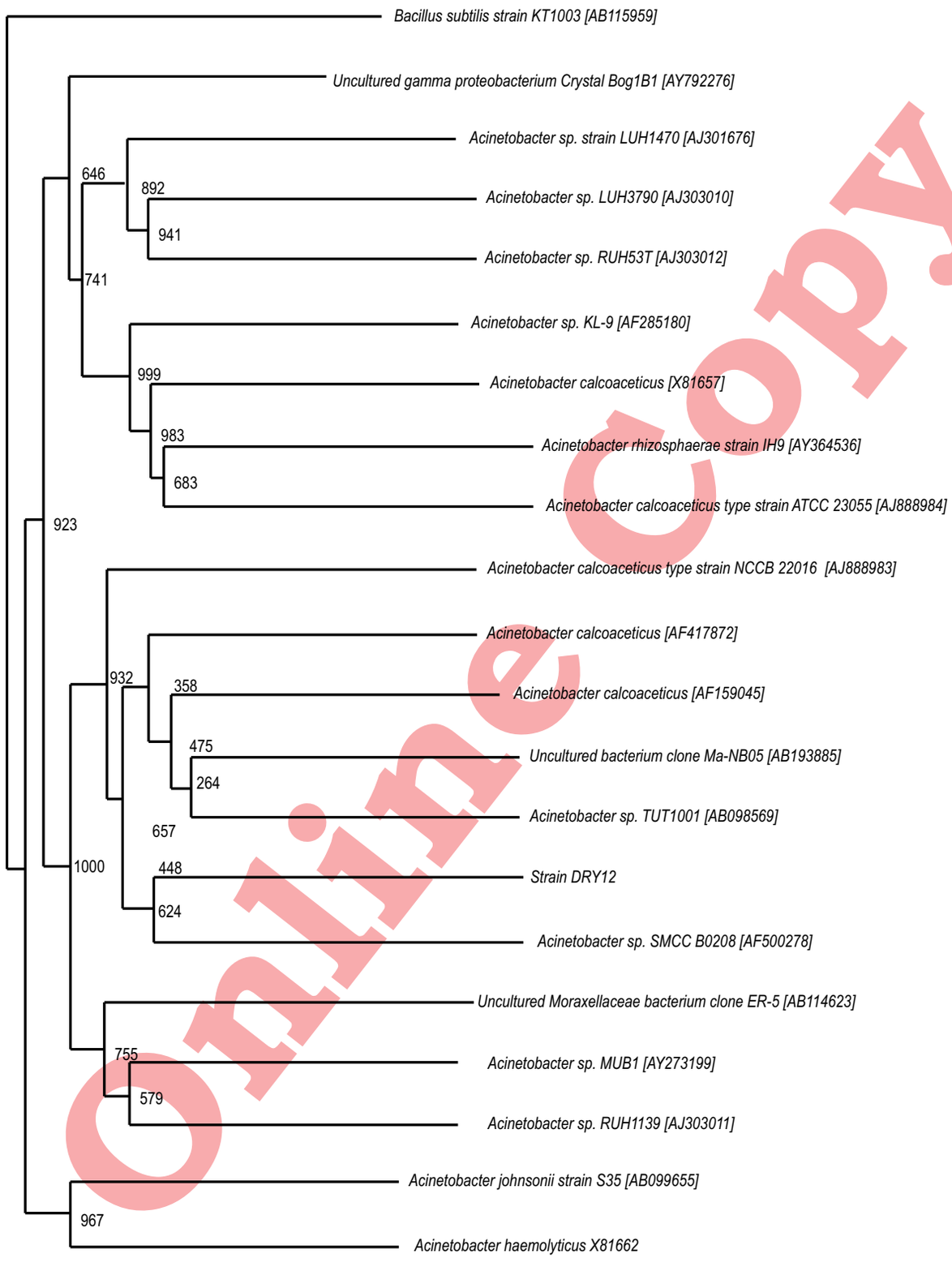

Fig. 2 : Neighbour-joining method cladogram showing phylogenetic relationship between strain DRY12 (isolate 12) and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rDNA sequences. The numbers at branching points refer to bootstrap values, based on 1000 re-samplings. The branch lengths in the cladogram are not to scale. Bacillus subtilis strain KT1003 is the outgroup

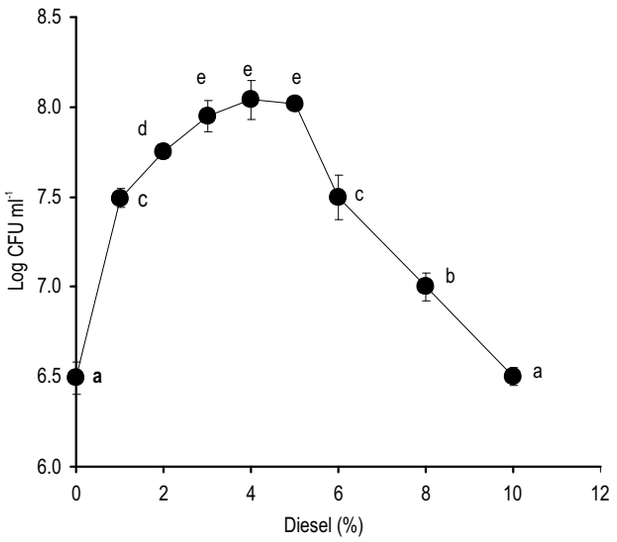

Fig. 3 : The effect of diesel (sole carbon source) concentrations on the growth of strain DRY12. Values are mean of three replicates ± SE; Values with the same letter are not significantly different (p>0.05)

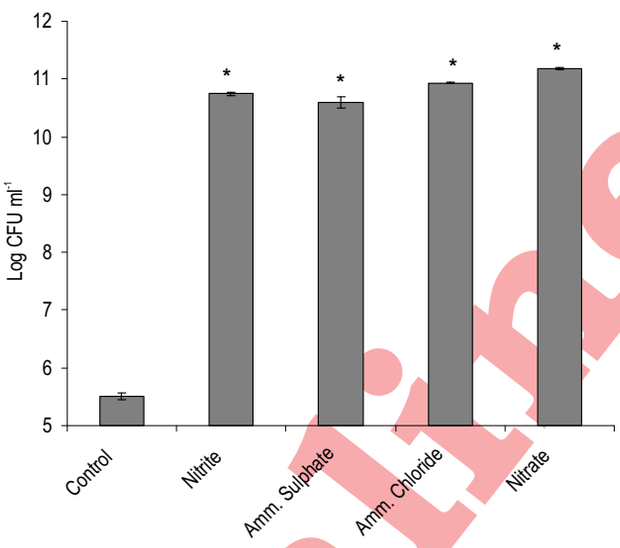

Fig. 4 : The effect of different nitrogen sources (0.77%, w/v) on the growth of strain DRY12. Values are mean of three replicates ± SE; Values significantly different from those obtained from control are marked by stars (p<0.05)

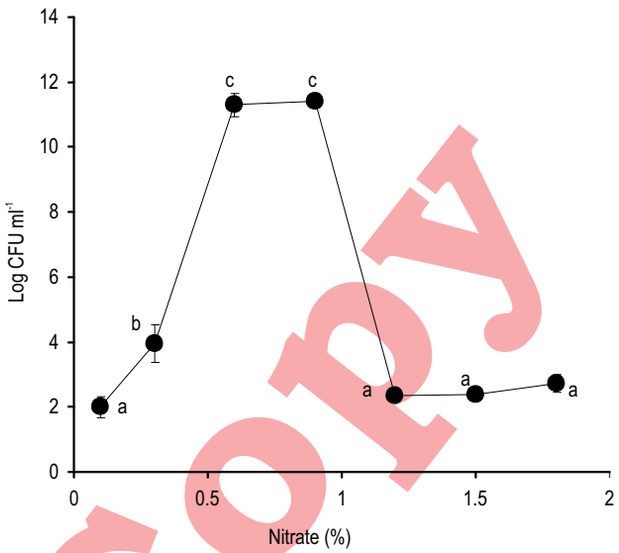

Fig. 5 : The effect of nitrate concentration as the sole nitrogen source on the growth of strain DRY12. Values are mean of three replicates ± SE; Values with the same letter are not significantly different (p>0.05)

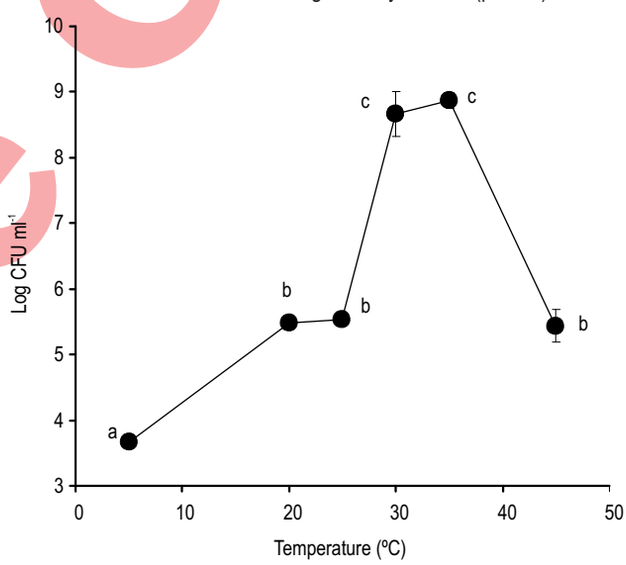

Fig. 6 : The effect of temperature on the growth of strain DRY12. Values are mean of three replicates ± SE; Values with the same letter are not significantly different (p>0.05)

solvent effect of diesel which destroy bacterial cell membrane. A much lower optimal diesel concentration at 1% supporting optimal growth was reported for both *Rhodococcus ruber* and *Rhodococcus erythropolis* (Bicca *et al.,* 1999). Strain DRY12 was able to tolerate higher diesel concentration suggesting that strain DRY12 is a good candidate for diesel bioremediation. This high tolerance is not surprising since the soil has been contaminated with diesel for several years and acclimatization process favouring high diesel tolerance strains had occurred. The great majorities of soil microorganisms are heterotrophic and use available organic materials for energy. Soil microbiologists have observed that whenever available energy material is abundant in soil, microbes capable of using that material are usually abundant (Onuoho *et al*., 2011; Dharni *et al.,* 2012). Diesel concentration may also affect biodegradation itself. Excessive quantities of diesel may reduce biodegradation rate due to toxic effect. Conversely, very low concentrations of diesel may also reduce overall degradation rates because contact between the diesel and the microorganisms is limited (Shukor *et al.,* 2009a).

Various inorganic nitrogen sources such as $NaNO_2$, $(NH_4)_2SO_4$, NH_4Cl and KNO_3 were tested as the nitrogen source for the diesel degrading bacteria. From the results obtained, KNO_3 was the best nitrogen source (Fig 4). Although the

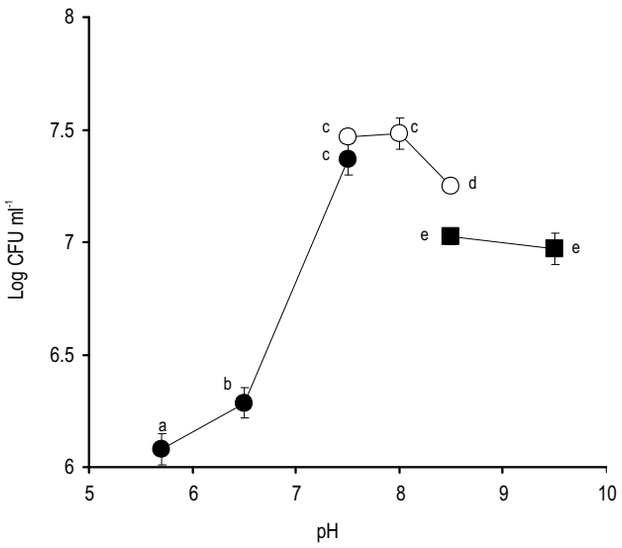

Fig. 7: The effect of pH on the growth of strain DRY12 using three overlapping buffers. The buffer system used are phosphate (●), carbonate (○) and borate (■). Data represents mean SEM, n=3. Values with the same letter are not significantly different (p>0.05)

elemental nitrogen content of $(NH_4)_2SO_4$ was much higher than KNO_3 at 0.77% (w/v), nitrogen in the latter is the better bioavailable form. In contrast, *Pseudomonas* sp. strain DRYJ3 requires ammonium sulphate for optimal growth (Shukor *et al.,* 2009a) while *Staphylococcus aureus* strain DRY11 grows optimally on nitrite (Shukor *et al.,* 2009b).

The optimum KNO_3 concentration was between 0.6 and 0.9% (w/v) bracketing the original 0.77 % (w/v) used throughout the characterisation studies (Fig. 5). Higher nitrate concentrations showed strong inhibitory effect on the growth of the bacterium suggesting monitoring of nitrate concentration during bioremediation is very important in future works. This study is important because low levels of fixed forms of nitrogen in the bacterial environment limits the rate of hydrocarbon degradation (Wrenn *et al.,* 1994; Onuoha *et al.*, 2011). Nitrogen is the nutrient most likely to be limiting. It is usually added as a nitrogen source for cellular growth, but it can also serve as an electron acceptor (Shukor *et al.,* 2009a).

Effects of temperature and pH: Strain DRY12 exhibited

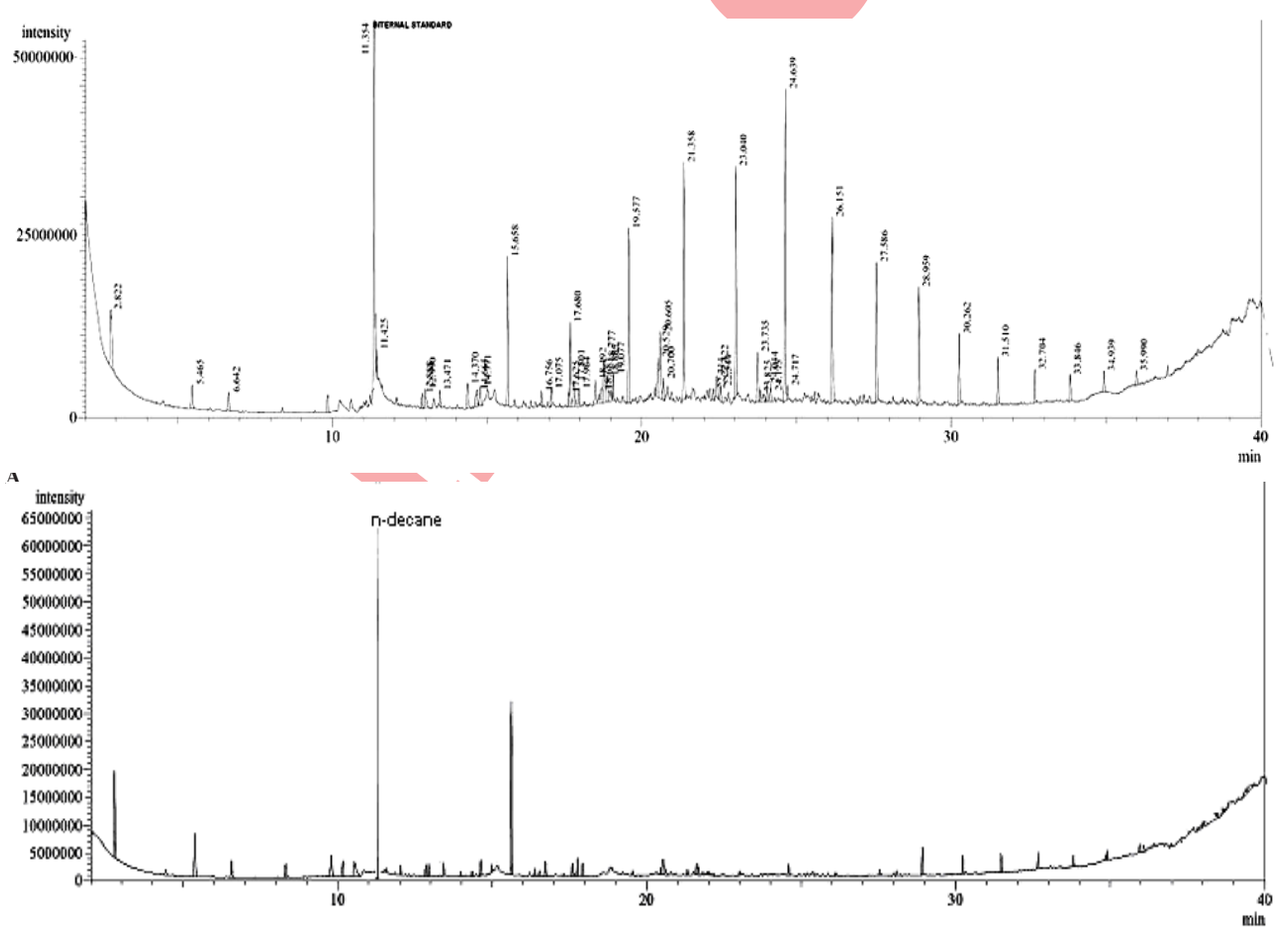

Fig. 8 : GC profiles of diesel oil extracted from the aqueous phase of the medium after 10 days of incubation at 30 °C with 3 % diesel (v/v) using optimized conditions with and without bacterial inoculation. (A) Abiotic control (uninoculated); (B) inoculated with diesel. The internal standard was n-decane

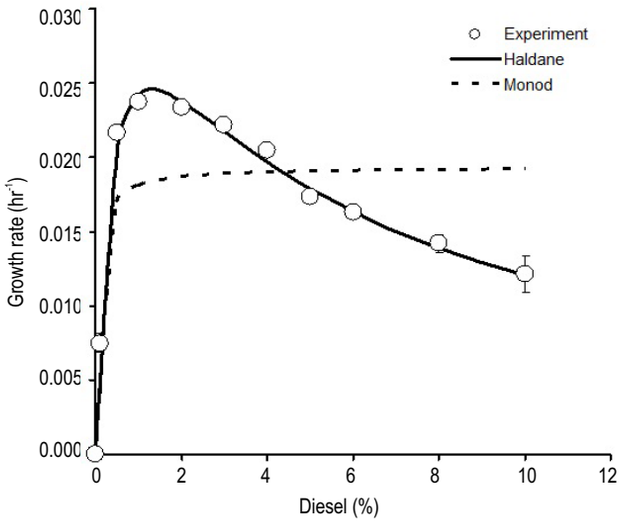

Fig. 9 : Growth kinetics of strain DRY12 on diesel. Values are mean of 3 replicates ± SE

optimum temperature in between 30 and 35ºC (Fig. 6). Growth decreased dramatically at higher temperatures. The geographical origin of diesel-degrading isolates is reflected strongly by their optimal temperature requirement. For example, a local diesel-degrading *Staphylococcus aureus* strain DRY11 grows optimally in between 27 and 37ºC (Shukor *et al.,* 2009b) and other tropical diesel-degrading bacteria require similar temperature for optimal growth (Bicca *et al.,* 1999; Márquez-Rocha *et al.,* 2005) while diesel-degrading bacteria isolated from Polar regions often have optimal growth temperatures in the range of 10 to 25ºC (Margesin and Schinner, 1999; Shukor *et al.,* 2009a).

The optimal pH that supported growth of the bacterium was between pH 7.5 to 8.0 in phosphate or borate buffer (Fig. 7). The requirement of neutral or near neutrality for optimal growth of bacteria on diesel is also exhibited by other bacterial strains such as *Rhodococcus ruber* (Bicca *et al.,* 1999), *Acinetobacter* sp. strain B2.2 (Espeche *et al.,* 1994), *Pseudomonas* sp. strain DRYJ3 (Shukor *et al.,* 2009a) and *Staphylococcus aureus* strain DRY11 (Shukor *et al.,* 2009b). Available data on optimum pH for biodegradation of hydrocarbon in soil bioremediation studies (Wrenn *et al.,* 1994; Ruberto *et al.,* 2005) show that the pH of soil where various hydrocarbon-degrading microbes have been isolated were between 4.8 and 9.2. Although the favourable pH range for bioremediation is between 6 and 8 and the most ideal pH was around 7 (Cookson, 1995; Margesin *et al.,* 2003). However, most soil with active diesel metabolizing activity usually exhibited a low pH due to several factors including carbon dioxide production leading to the formation of carbonic acid (Wrenn *et al.,* 1994). Thus, a cheap source of pH controlling chemical such as calcium carbonate can be added to soil during bioremediation to achieve near neutrality in order to optimize remediation (Shukor *et al.,* 2009a). Using all of the optimised conditions an almost complete removal of diesel components as seen from the reduction in hydrocarbon peaks after 10 days of incubation were achieved (Fig. 8).

Growth kinetic studies: Data from experimental value was fitted to two kinetic models of growth *i.e.* Monod and Haldane using CurveExpert Professional software (Version 1.6) custom equation algorithm that minimizes sums of square of residuals. The correlation coefficient value for the substrate inhibition model of Haldane was 0.97 indicating good fitting while the Monod model gave a correlation coefficient value of 0.76 indicating poor fitting (Fig. 9). The value of specific growth rate μ tends to increase as the substrate concentration is increased and rises to a peak value and finally decreases. The maximum growth rate- μmax was 0.039 hr^{-1} while the saturation constant or half velocity constant Ks and inhibition constant Ki, were 0.387% or 3289 mg l^{-1} and 4.46% or 37,910 mg l^{-1} diesel, respectively. There are few data available on diesel-degradation and utilization kinetics. The μmax value was higher than values reported for alkane-degrading *P. frederiksbergensis* and *R. erythropolis* at 0.0154 and 0.0125 (hr^{-1}), respectively (Abdel Megeed and Mueller, 2009), and lower than *Rhodococcus rubber* and *Rhodococcus erythropolis* grown on diesel in another study that showed maximum growth rates of 0.086 and 0.123 hr^{-1}, repectively (Zhukov *et al.,* 2007). A lower value of Ks indicates a high affinity of biomass and degradative enzymes to substrate while a high Ki value indicate good tolerance towards high diesel concentrations.

MATH assay: After extraction with hexadecane 75% of the bacterium was found in the hexadecane phase indicating that the bacterium was hydrophobic. This value is closed to MATH values of between 70 and 80% found in several *Acinetobacter*-degrading strains (Mara *et al.,* 2012). The vortexing part produces fine hydrocarbon droplets that the bacteria can adhere to. The inclusion of high salt concentration prevents attraction due to charge surfaces and adhesion is only due to hydrophobic interaction- the more hydrophobic the bacterial surface the more likely for binding and biodegradation.

In conclusion, a diesel-degrading bacterium that was able to tolerate as high as 8% diesel was isolated. The isolate exhibited relatively broad optimum temperature and was able to completely remove diesel components after a 10-days incubation period. These good characteristics world be useful as an autochthonous organism in bioremediating diesel-contaminated sites in the tropic. Future works includs to study the heavy metal tolerance of this strain since it is well known that crude and processed hydrocarbons contain significant amount of heavy metals which could inhibit bioremediation. Current work includes the characterization of enzymes and genes involved in diesel degradation and at the same time working on bioaugmentation studies using this bacterium to remediate hydrocarbon sludge from a petroleum-processing plant.

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