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

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Abstract: A diesel-degrading bacterium has been isolated from a diesel-polluted site. The isolate was tentatively identified as Staphylococcus aureus strain DRY11 based on partial 16S rDNA molecular phylogeny and Biolog® GP microplate panels and Microlog® database. Isolate 11 showed an almost linear increase in cellular growth with respect to diesel concentrations with optimum growth occurring at 4% (v/v) diesel concentration. Optimization studies using different nitrogen sources showed that the best nitrogen source was potassium nitrite. Sodium nitrite was optimum at 1.2 g l^{-1} and higher concentrations were strongly inhibitory to cellular growth. The optimal pH that supported growth of the bacterium was between 7.5 to 8.0 and the isolate exhibited optimal broad temperature supporting growth on diesel from 27 to 37 °C. An almost complete removal of diesel components was seen from the reduction in hydrocarbon peaks observed using Solid Phase Microextraction Gas Chromatography analysis after 5 days of incubation. The characteristics of this bacterium suggest that it is suitable for bioremediation of diesel spills and pollutions in the tropics.

Key words: Isolation, Characterization, Diesel-degrading, S. aureus

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Introduction

Industrialization has produced large quantity of wastes that end up as pollutants of soil and aquatic bodies. Current physical and chemical treatments of wastes are generally expensive and are not able to remove trace quantities of pollutants. Bioremediation is a cheaper alternative and could remove traces of contaminants. To date numerous bioremediation of industrial wastes have been attempted and several have been successful (Bishnoi *et al*., 2006; Potentini and Rodriguez-Malaver, 2006; Demir *et al*., 2007; Singh *et al*., 2007). The use of local indigenous bioremediation product would help in dealing with accidents and spills before indigenous bacteria at the sites of pollution could adapt and remove residual pollutant. This is important since pollutant such as diesel emits harmful vapors and unbearable smell and must be rapidly remediate and this warrants the use of ready-made bacterial innocula (Mohammed *et al*., 2007; Classens *et al*., 2006).

Out of the many types of industrial pollutants, oil and grease rank as the number one pollutant in Malaysia (DOE, 2007). Oil pollution in Malaysia is contributed by oil and gas activities as an oil and gas producer and also due to its vicinity to the Straits of Malacca-one of the busiest waterways in the world for centuries. There have been several contaminated sites in Malaysia due to human error. For example, one of the largest hydrocarbon spills to be reported occurs when 150 ton of diesel spilled into the coastal areas of the Straits of Malacca as a result of a collision between two oil tankers (Berita Harian Online, 1997). In another incident, about 15 tons of diesel spilled from an overturned lorry tanker in Seremban, polluting surrounding soils (The New Straits Times, 2000) whilst more than one ton of diesel was spilled into the soils in Gelugor, Penang from a 1,000 kw-mobile generator unit (The New Straits Times, 2001). Efficient diesel-degrading capacity of a locally-isolated bacterial consortium has been reported (Ghazali *et al*., 2004). 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. In this work we report on the isolation of a versatile diesel-degrading bacterium that could degrade diesel at a broad range of temperature from 27 to 37°C. The characteristics of this bacterium suggest that it is useful as a bioremediation agent in the tropics.

Materials and Methods

Isolation of diesel-degrading bacteria: Soil samples, each measuring approximately 10 grams were taken randomly to a depth of 5 cm from the topsoil using sterile spatula and stored in sterile screw-capped polycarbonate tubes. The soil samples were taken from a diesel polluted site underneath a 10 m^3 diesel tank in Selangor, Malaysia in December 2004. The soil samples were placed in sterilized plastic bags and stored on ice during transfer from site to the laboratory. Soil samples were resuspended in 10 ml of sterile saline solution (0.9% NaCl) and vigorously shaken for 5 min. The enrichment culture media consists of basal salt media supplemented with diesel as carbon source. A modified basal salt medium (Michaud *et al*., 2004) 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 incubated at 30°C and 150 rpm (YIH DER, Taiwan) for six days.

Spread plate technique was used for culture isolation and enumeration. The cultures were then incubated at 30°C. 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 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 separated diesel residues and 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 min followed by a 10°C increment per minute to 300°C and the isothermal held for 10 minutes. Carrier gas velocity was 30 ml per min, and makeup gas velocity, 30 ml per 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. 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 mm (Milipore) 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. An SPME fiber coated with a 7 µm polydimethylsiloxane 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°C for 10 min and immediately inserted into the injector manually for GC analysis.

Identification of bacterium

16S rDNA gene sequencing: Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture contained 0.5 pM of each primer, 200 µM of each deoxynucleotide triphosphate,1x reaction buffer, 2.5 U of *Taq* DNA polymerase (Promega) to achieve a final volume of 50 µl. 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: 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 was analyzed using the BLAST 2 sequences algorithm using the BLASTN option with the matrix turned off and default parameters available from the server at NCBI (http://www.ncbi.nlm.nih.gov/blast/). Based on the overlapped region between the forward and the reverse complement of the reverse primer sequence, both of the sequences were compared and checked for errors and omissions of bases especially at the overlapped region using the CHROMAS software Version 1.45 and the sequences were combined at bases giving the least ambiguous characters and gaps. The combined 16S rRNA gene sequence, and the resultant 1478 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; DQ226212.

Phylogenetic analysis: A multiple alignment of 19 16S rDNA gene sequences closely matches strain DRY11 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 (Felsenstein, 1985) 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).
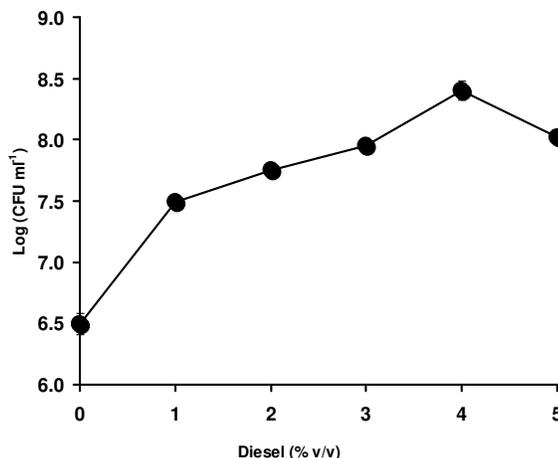
Results and Discussion

Isolation of diesel-degrading bacteria: Several bacterial isolates were successfully isolated from a diesel spillage site from a 10 m^3 diesel tank in Selangor, Malaysia. One isolate named Isolate 11 that gave high cellular growth on diesel as a carbon source was selected for further studies. Other isolates from uncontaminated soil samples
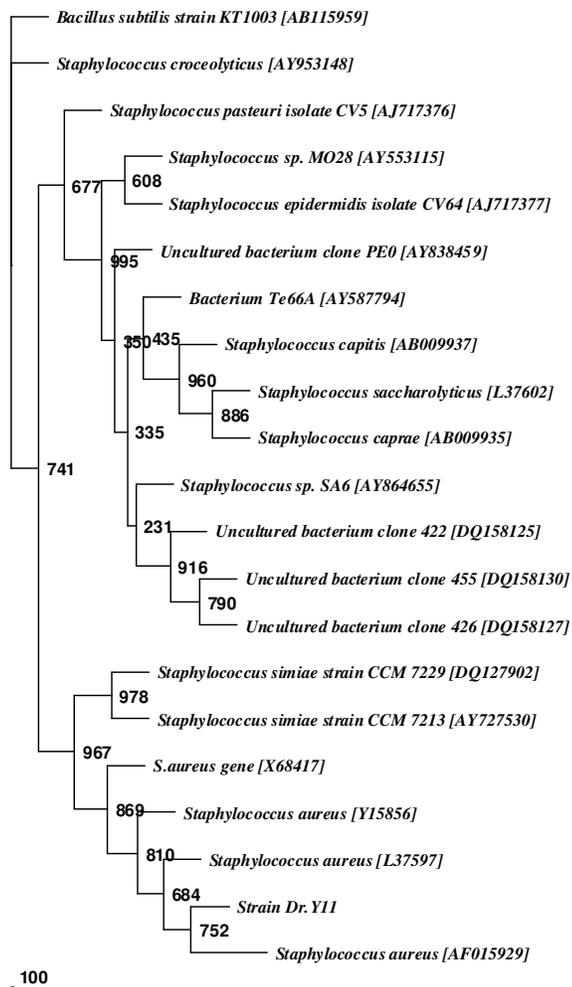
Fig. 1: A phylogram (neighbour-joining method) showing genetic relationship between strain DRY11 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. *Bacillus subtilis* strain KT1003 is the outgroup

Fig. 2: The effect of diesel concentrations on the growth of strain DRY11 grown at room temperature for 5 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
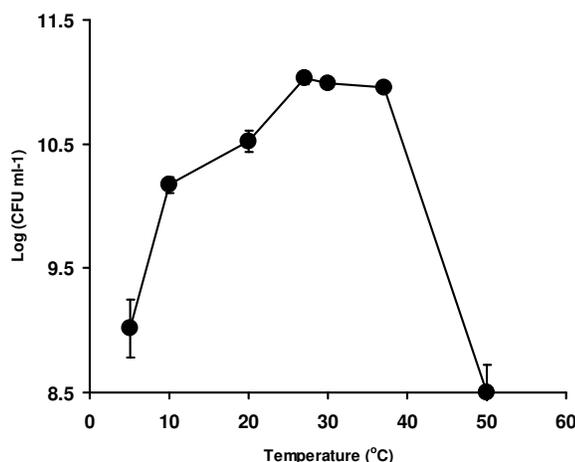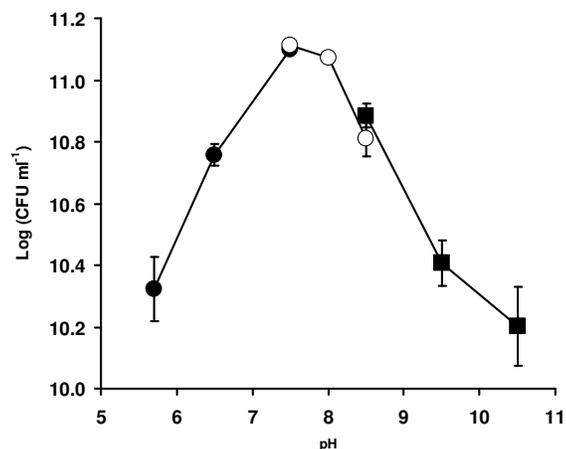

Fig. 3: The effect of temperature on the growth of strain DRY11. Growth was carried out at room temperature for 5 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3

gave little to no growth on diesel. It has been shown that there is a high correlation between cellular growth and diesel assimilation in microbes (Petrikevich *et al.*, 2003). Generally, the entire carbon source is assimilated by the bacterium for growth and energy and an increase of CFU ml^{-1} is regarded as an indicator of degradation with higher CFU ml^{-1} correlating with higher amount of diesel being degraded. The fact that Isolate 11 was isolated from soil under a leaking diesel storage tank gave testament to acclimatization advantages of xenobiotics-degrading microbes over non-acclimatized strains.

Identification of isolate 11: A high bootstrap value (75.2%) linked strain DRY11 (Isolate 11) to *Staphylococcus aureus* indicating that
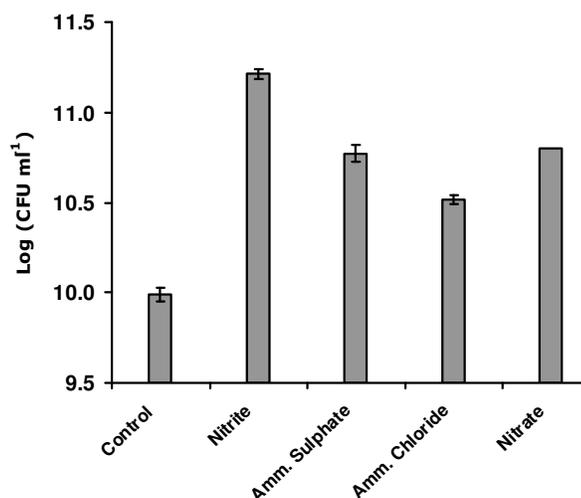
the phylogenetic relationship of isolate 11 to this species is strong (Fig. 1) since bootstrap values has been known to be underestimated and a value over 70% is approximately over 95% in accuracy (Alfaro *et al.*, 2003). Together with the Biolog identification system which gave the closest ID to *S. aureus* with high probability (99%), isolate 11 is assigned as *S. aureus* strain DRY11. The genus *Staphylococcus* has been identified to be a key hydrocarbon-degrading bacteria found in many hydrocarbon-polluted sites (DeRito *et al.*, 2005). However there is limited report on the isolation of diesel-degrading *S. aureus*.

Bacterial growth optimization on diesel

The effect of carbon source: This study was carried out to study the optimum diesel concentration as a carbon source for strain DRY11. The result of carbon source optimization is shown in Fig. 2. The
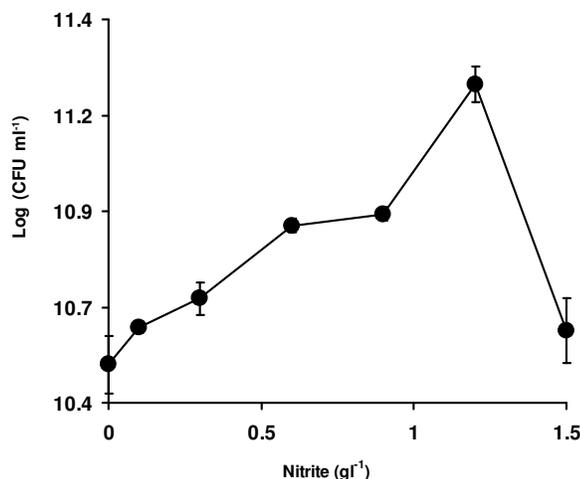
Fig. 4: The effect of pH on the growth of strain DRY11 using three overlapping buffers. The buffer system used were phosphate (●), carbonate (○) and borate (■). Growth was carried out at room temperature for 5 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3


Fig. 6: The effect of nitrite as a nitrogen source on the growth of Isolate 11. Bacterial growth was determined by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 5 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3

Fig. 5: The effect of different nitrogen sources on the growth of strain DRY11. Bacterial growth was measured by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 5 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3

results showed that the optimum carbon source (diesel) concentration for the growth of strain DRY11 was 4% (v/v). Although bacterial growth started to decline above this, the bacterium was still able to grow on 5% (v/v) diesel. 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 destroy bacterial cell membrane. Thus many biodegradation studies on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5% (Mukherji *et al.*, 2004; Lee *et al.*, 2005,2006; Hong *et al.*, 2005; Ueno *et al.*, 2007; Rajasekar *et al.*, 2007). It has been found that degradation is generally retarded at concentrations higher than 1 or 1.5% (Espeche *et al.* 1994; Bicca *et al.*, 1999; Lee *et al.*, 2005, 2006). Degradation at a much higher concentration (6% v/v diesel) has been reported

but degradation requires glucose (0.2% w/v) and yeast extract (0.1% w/v) (Kwapisz *et al.*, 2008). Since strain DRY11 was able to tolerate higher diesel concentrations, this suggests that strain DRY11 is a good candidate for diesel bioremediation due to its relatively high tolerance to diesel. Diesel and its medium chain hydrocarbon components are such a highly assimilable carbon sources that *Acinetobacter* sp. H01-N growing on hexadecane (C_{16}) as the sole carbon source contains essentially 100 % of its cellular fatty acid as 16-carbon fatty acid (Finnerty, 1977).

The effect of temperature: Strain DRY11 grew optimally in a wide range of temperatures ranging from 27 to 37°C (Fig. 3). Growth decreased dramatically at higher temperatures. One of the most reported temperature optima supporting diesel degradation is at 30°C (Cavalca *et al.*, 2000; Mukherji *et al.*, 2004; Hong *et al.*, 2005; Lee *et al.*, 2006; Kwapisz *et al.*, 2008). Lower temperature optima have been reported such as between 10 and 25°C (Margesin, 2000), at 20°C (Chapman and Shelton, 1995; Lee *et al.*, 2005 and Ueno *et al.*, 2007) and at 27°C (Rajasekar *et al.*, 2007). Higher growth optima were reported by Marquez-Rocha *et al.* (2005) at 37°C for a tropical diesel-degrading bacterium from Mexico. Similarly, Bicca *et al.* (1999) noted that *Rhodococcus ruber* and *Rhodococcus erythropolis* grew well at 37°C. However, these bacteria were not reported to grow optimally at such a wide temperature range as strain DRY11. The ability to grow at a wide temperature range is a significant advantage especially in a tropical climate such as Malaysia where soil temperatures can vary from 24 to 35°C year round (Sinnakkannu *et al.,* 2004).

The effect of pH: Maintenance of pH in bacterial medium is important since pH strongly affects bacterial growth. The change in pH of the medium is largely due to the production and accumulation of bacterial waste products. Therefore, the optimization of environmental conditions
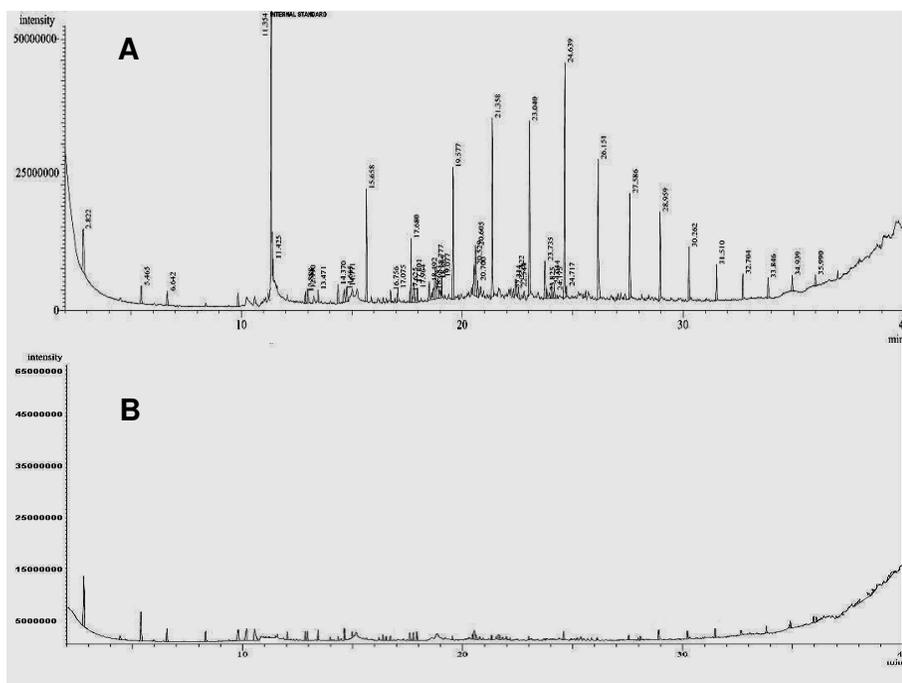
Fig. 7: GC profiles of diesel oil extracted from the aqueous phase of the medium after 10 days of incubation with 0.5 % diesel (v/v) with and without inoculation with strain DRY11. (A) Abiotic control (uninoculated); (B) inoculated with diesel. The internal standard was n-decane

is very important for the enhancement of bacterial growth. Identification of the pH optima for growth would help in designing effective bioremediation strategy (Davey, 1994). The optimal pH that supported growth of the bacterium was between pH 7.5 to pH 8.0 in phosphate or carborate buffer (Fig. 4). The requirement of neutral or near neutrality for optimal growth of bacteria on diesel is also exhibited by many other bacterial strains (Espeche *et al*., 1994; Chapman and Shelton, 1995; Bicca *et al*., 1999; Margesin, 2000; Cavalca *et al*., 2000; Mukherji *et al*., 2004; Hong *et al*., 2005; Marquez-Rocha *et al*., 2005; Lee *et al*., 2005, 2006; Rajasekar *et al*., 2007; Ueno *et al*., 2007; Kwapisz *et al*., 2008).

The effect of nitrogen sources: Various 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}. Nitrite gave the highest growth on diesel compared to other carbon sources (p<0.05) while there was no significant difference in terms of growth on nitrate or on ammonium sulphate (p>0.05). Ammonium chloride gave the lowest growth compared to control (Fig. 5). The optimum concentration was at 1.2 gl^{-1} and higher concentrations were strongly inhibitory to cellular growth (Fig. 6). Nitrite is known to inhibit cellular growth during hydrocarbon biodegradation and the use of nitrite in bioremediation works must proceed with cautions (Chayabutra and Ju, 2000). This study is important because low levels of fixed forms of nitrogen in the bacterial environment limit the rates of hydrocarbon degradation (Atlas and Cerniglia, 1995). Optimum concentration of nitrogen is important as it is the nutrient most likely to be limiting. In many other

diesel biodegradation works, the best nitrogen source is either ammonium or nitrate salts (Espeche *et al*., 1994; Chapman and Shelton, 1995; Bicca *et al*., 1999; Margesin, 2000; Cavalca *et al*., 2000; Mukherji *et al*., 2004; Hong *et al*., 2005; Marquez-Rocha *et al*., 2005; Lee *et al*., 2005,2006; Rajasekar *et al*., 2007; Ueno *et al*., 2007; Kwapisz *et al*., 2008). Thus, the ability to use nitrite as a nitrogen source is an advantage. It is usually added as a nitrogen source for cellular growth, but it can also serve as an electron acceptor (Leeson and Hinchee, 1997). Using all of the optimised conditions an almost complete removal of diesel components was achieved as seen from the reduction in hydrocarbon peaks after 5 days of incubation (Fig. 7).

This work is a preliminary study carried out before actual bioremediation works on the polluted site using the autochthonous strain DRY11 would be carried out. Work is underway to study heavy metals resistant of the strain since it is well known that crude and processed hydrocarbons contained significant amount of heavy metals which could inhibit bioremediation of the polluted site. We are also in the process of characterizing the 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 as part of a bioremediation study using allochthonous bacterium.

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