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An Iron Determination Method for Azo Dyes-Contaminated Wastewater

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Abstract

We have designed an improved iron determination method based on the ability of ferrous iron to reduce 12-molybdophosphate (12-MP). The method allows for direct determination of ferrous iron in the presence of azo dyes. The iron determination method had a specific extinction coefficient of 0.0514 (mg l^{-})$^{-1}$.cm^{-1} at 865 nm with a correlation coefficient of 0.999. The relative standard deviation for each data point does not exceed 5% (n=3) suggesting precision. In its oxidized state 12-MP is yellowish in color and when reduced it forms a blue solution. The iron determination method is less interfered with azo dyes compared to common iron determination methods such as 1,10-phenanthroline and the commercial FerreMo^{TM}.

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 [26;9]. 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 [37] and another spilled of one ton of diesel into the soils in Gelugor from a 1,000 kw-mobile generator unit [38]. A locally – isolated bacterial consortium that can effectively degrade diesel has been reported [16]. 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 range of temperature from 10 to 40 ºC . 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 15 – 20 cm from topsoil using a sterile spatula and stored in a sterile screw-capped vials. The soil samples were taken in the year 2004 near Bukit Ekspo, (University Putra 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 seven 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 (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. 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.

Identification of bacterium

16S rDNA gene sequencing

Alkaline lysis was performed to extract genomic DNA from bacterial colonies. PCR amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). 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 758 bases were compared with the GenBank database using the Blast server at NCBI [1]. This analysis showed that this sequence is closely related to *rrs* from Gammaproteobacteria.The partial 16S rRNA ribosomal gene sequences for this isolate have been deposited in GenBank under the following accession number **DQ851856**. The Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to compare the resultant 852 bases.

This analysis showed that this sequence is similar to *Burkholderia* species with 99% similarity. The results obtained from Biolog™ Identification system showed that Isolate 27 was similar to *Burkholderia cepacia* with 75 % similarity. Together with the Biolog™ results, for now, isolate 27 is assigned tentatively as *Burkholderia* sp. strain DRY27.

Phylogenetic analysis

Based on GenBank, a multiple alignment of 19 16S rRNA gene sequences were closely matches with the strain DRY27 and this were aligned by using clustal_W [39] 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 [18]. A method from Saitou and Nei [34] called neighbour-joining method was used to derive a phylogenetic tree, (1987). 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 [31].

RESULTS AND DISCUSSION

Isolation of diesel-degrading bacteria

There were several successfull isolation of bacterial from Bukit Ekspo, (University Putra Malaysia) soil . Isolate 27 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 [32]. Generally, diesel that act as the carbon source would be assimilated by the bacterium for growth

and energy [30]. 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 27

Biolog identification system showed that isolate 27 gave the closest ID to *Burkholderia cepacia* with 75 % similarity. Based on these results, isolate 27 is assigned as *Burkholderia* sp. strain DRY27. The phylogenetic tree of isolate 27 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 DRY27. 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 DRY27 was 3% (v/v) and it also showed that strain DRY27 was able to grow on 2% (v/v), 4% (v,v) and 5% (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 effect of solvent in diesel that could damage bacterial cell membrane [29]. This is why a lot of biodegradation studies on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5% [28;21;17;20;40;33]. Concentration higher than 1 or 1.5% has been proven to cause retardedness in degradation [14;5;21;20]. 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]. DRY27 can grow on aliphatic hydrocarbons or diesel oil as the carbon source and display high cell surface hydrophobicity when cultured in these carbon sources[27]. *Burkholderia* sp can also degrades a wide variety of alkanes, toluene, benzoate, *m*-, *p*- and *0*- toluate as sole carbon [22].

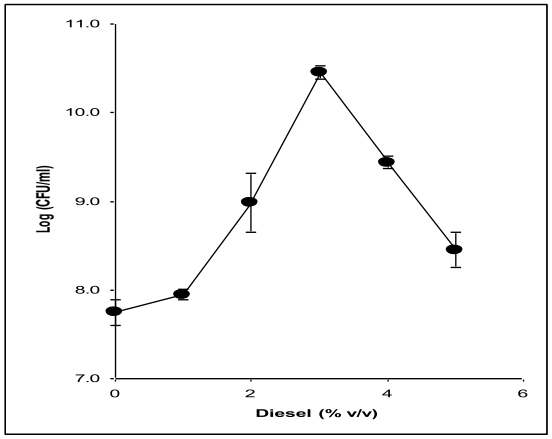

Figure 1. The effect of diesel concentrations on the growth of strain DRY27 grown at room temperature for 3 days on an orbital shaker (150 rpm). Bacterial growth was measured by determination of colony forming unit (Log CFU ml^{-1}). Data represents mean ± SEM, n=3.

The effect of temperature

Based on Figure 2, strain DRY27 grew optimally in a wide range of temperatures ranging from 10 to 40 ºC and it was decreasing dramatically at higher temperatures. The optimum temperature for the growth of strain DRY27 was 30 ºC. Temperature of 30 ºC isreported to be the most optimum temperature for diesel degradation [6;28;17;20;19]. In Ma and Herson, growth for *Burkholderia* sp is reported to be at 37 °C. Growth are also reported in a lower temperature optima in between 10 and 15 °C [29], 10 and 25 °C [23], at 20 °C [8;21;40] at 27 ºC [33] and in between 27 to 37°C [30]. Temperature of 37 °C is reported to be a higher growth optima by [24] at 37 °C for a tropical diesel-degrading bacterium from Mexico. The temperature of 37 °C is 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 DRY27. This is an advantage since Malaysia has a tropical climate where soil temperature can vary from 24 to 35 °C year round [36].

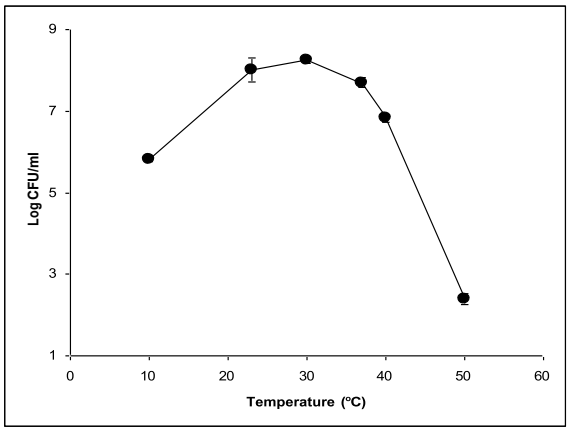

Figure 2. The effect of temperature on the growth of strain DRY27. Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

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 [29]. 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 [10]. The optimal pH that supported growth of the bacterium was between pH 7.5 to pH 8.5 in borate, 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 [14;8;5;23;6;28;17;24;21;20;33;40;19]. However, in Somtrakoon [35], *Burkholderia* sp was grown in 4.9 pH.

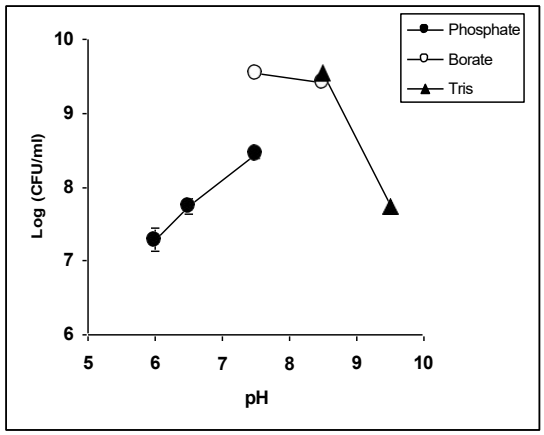

Figure 3. The effect of pH on the growth of strain DRY27 using three overlapping buffers. The buffer system used were phosphate (●), carbonate (○) and tris (▲). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

The effect of Nitrogen Sources

Inorganic nitrogen sources such as $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl and KNO_3 were tested as the nitrogen source at 1 gl^{-1} . Sodium nitrate gave the highest growth on diesel compared to the other carbon sources ($p<0.05$) while growth on ammonium sulphate and ammonium chloride were almost the same ($p<0.05$). Ammonium chloride gave the lowest growth compared to the other nitrogen sources (Figure 4). The optimum concentration was at 7.7% 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 [14;8;5;23;6;28;17;24;21;20;33;40;19]. 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 96.5% after 7 days of incubation (Figure 6). In Mohanty and Mukherji, *B. Cepacia* is able to degrade only 51.37% of diesel for 15 days which is a longer time compared to DRY27.

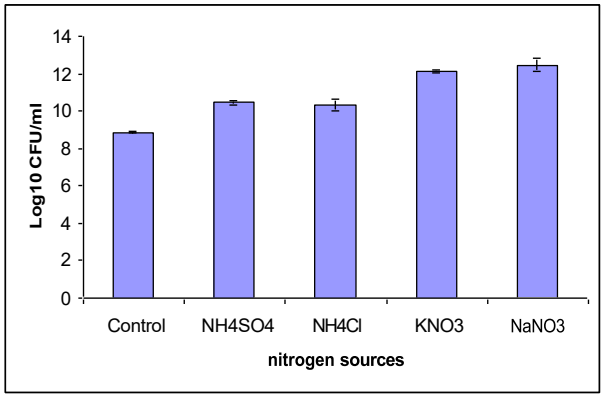

Figure 4. The effect of different nitrogen sources on the growth of strain DRY27. Bacterial growth was measured by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3.

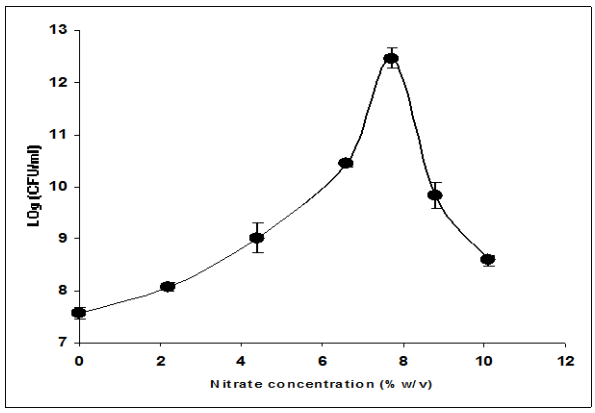

Figure 5. The effect of nitrite as a nitrogen source on the growth of Isolate 27. Bacterial growth was determined by colony forming unit (Log CFU ml^{-1}). Growth was carried out at room temperature for 3 days on an orbital shaker (150 rpm). Data represents mean ± SEM, n=3**.**

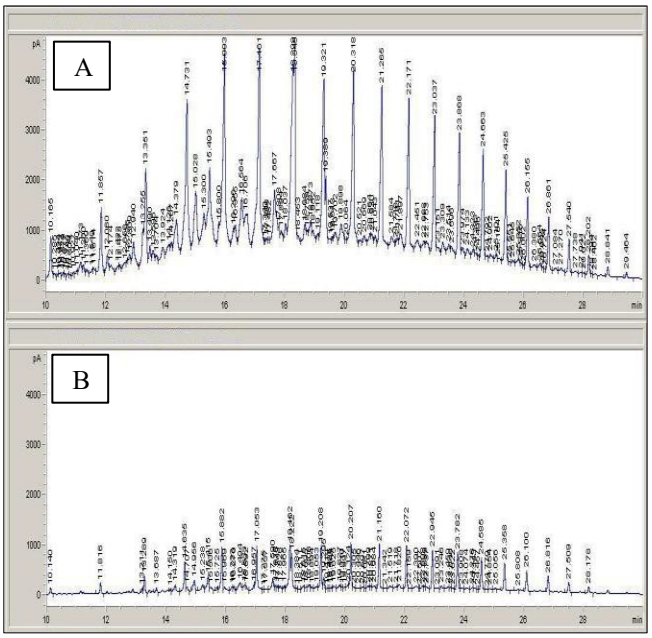

Figure 6. GC profiles of diesel oil extracted from the aqueous phase of the medium after 7days of incubation with 3 % diesel (v/v) with and without inoculation with strain DRY27. (A) Abiotic control (uninoculated); (B) inoculated with diesel. The internal standard was n-hexane

CONCLUSION

This work is served as an introductory study for the actual bioremediation works on the polluted site using the autochthonous strain DRY27. 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 DRY27 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.

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