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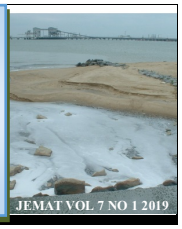

Biodegradation of Hydrocarbon Sludge by *Pseudomonas* sp. Strain UPM-KV

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ABSTRACT

A hydrocarbon-utilizing microorganism isolated locally was characterized and investigated. This study involved standard biochemical tests and investigation of the bacterial growth based on the uptake of carbon and nitrogen source, temperature and optimum pH growth. The bacterium was found to be a Gram-negative rod, non-motile property with unique property to degrade hydrocarbon sludge. By using diesel as sole carbon source the bacterium was found to be an aerobe as further proven by the oxidase testing. The optimum conditions for the growth was found to be at 30 °C and pH 6.8 with optimum diesel concentration, 1% (v/v). The bacterium ideally used ammonium sulfate as source of nitrogen and was identified as *Pseudomonas* sp. strain UPM-KV. The ability of this bacterium to efficiently grow on hydrocarbon sludge makes the bacterium an important tool for bioremediation of this toxic sludge that contains high concentration of heavy metals.

INTRODUCTION

Hydrocarbon sludge is considered as weakly biodegradable compound if compared to many hydrocarbons containing organic molecules that involved in carbon cycle. It leaves small molecular weight residues [1] and no information was stated that the hydrocarbon can be degraded completely. The susceptibility of the hydrocarbon sludge to undergo biotransformation is closely related to its chemical structure and molecular weight. Different types of hydrocarbons can be arranged according to their susceptibility to biodegradation as follows: n-alkanes > branched chain alkanes > branched alkanes > low molecular weight n-alkyl aromatics > monoaromatics > cyclic alkanes > asphaltenes [2]. However, some compounds and their derivatives are refractory to biodegradation such as polycyclic aromatic hydrocarbons (PAHs) especially those with four or more fused aromatic rings [3]. Highly branched compounds are more persistent to microbial degradation as compared to the less complex hydrocarbon [4]. Since n-alkanes are the most easily degradable, an aromatic hydrocarbon like benzenes are more difficult to undergo biodegradation than n-alkanes which can be degraded readily [5]. Hydrocarbon sludge is consisting of thousands of different kind of hydrocarbon compounds. These hydrocarbons have different molecular weight ranging from 16 for methane to high molecular weight hydrocarbons like benzene and cyclic compounds which have weight above 20,000. Most of hydrocarbon sludge are black and some are dark brown in colour. Hydrocarbon is mainly made of alkanes of straight chain, branched chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen [6].

Only a few types of microorganisms can degrade high molecular weight polycyclic aromatics hydrocarbons [7] and until present the biodegradation of hydrocarbon has yet not been extensively explored. To resolve these problems, variety of chemical techniques were developed to eliminate the spilled

hydrocarbon oils from the environmental pollution areas. These techniques include incineration, thermal desorption, soil washing and solvent extraction [8]. However, some of these cleaning techniques are costly and not efficient, and even create other environmental problems [9]. Microbial degradation has been proven to be the most interesting process of bioremediation of oil spill among all the proposed cleaning techniques [10]. Enzymatic is the key reactions of biodegradation that involve oxidation process catalyzed by oxygenase and peroxidase enzymes. It was claimed that the biodegradation mechanism relates to the electron transport system in which oxygen is the final electron acceptor. This is why hydrocarbon is said to be the best to be degraded in aerobic condition while in anaerobic conditions inorganic chemical can act as electron acceptor [11].

Byproducts of metabolic degradation of hydrocarbon are the production of carbon dioxide, water and some intermediate compounds such as non-toxic cellular residues [5], therefore, bioremediation is a method that can be well classified as an environmental friendly process. The exposure to photo oxidation, dilution, volatilization, dispersion and sedimentation can introduce the hydrocarbon to the environmental hazard [8]. Even hydrocarbons of higher molecular weight can undergo volatilization under ultraviolet radiation from the sun [7]. Hydrocarbon sludge is accumulated hydrocarbons at the bottom of storage tanks such as in petroleum and crude oil separation systems with complex chemical properties, therefore, the processes are crucially important in order to avoid pollution to the environment. This study aims at isolating a hyrdrocarbon-sludge utilizing bacterium from local soils.

METHODS

Soil sample collection

The soil samples (3 portions) were taken from the top (15 cm) layer at TPU (Taman Pertanian Universiti), Universiti Putra Malaysia. The soil had a pH 6.4, a percentage of clay (<2 µm), silt content (2-50 µm) and a sand (>50µm) content are 31.60%, 8.83% and 58.29%, respectively. According to the match of PSD and the USAD soil textural class, all points are considered as Sandy clay loam. The chemical content of soil sample are total N of 0.14 % ± 0.03m, available P is 98.78 ± 28 ppm, exchangeable K is 132.00 ± 30 ppm, Ca is 661.22 ± 86.77 ppm, Mg 121.89 ± 26.24 ppm and water content of 15%. The soil was mixed thoroughly then removed coarse and other plant materials before used.

The degradation of hydrocarbon sludge in soil was examined by using non sterile soil. Fresh soil was taken in 300 ml beaker glass and arranged in three different sets with each with 100 g of soil. To each set, sterilized hydrocarbon sludge (1000 mg hydrocarbon sludge/kg soil) was added and mixed aseptically. The soils were inoculated with 10 mL of bacteria culture grown on medium containing diesel as carbon source. Basal mineral salt media was used for enrichment. This experiment has three controls, which are non-sterile soil containing hydrocarbon sludge without bacteria that were sampled simultaneously to study biological breakdown of the monomer.

Media preparation

The inorganic basal media used in the present research consisted of: 0.7 g of potassium hydrogen phosphate (KH_2PO_4), 1.5 g of ammonium nitrate (NH_4NO_3), 0.5 g of calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4.7H_2O$) which were dissolved in 1 liter distilled water [12] [13]. The pH of the media was adjusted to pH 7.2 using 0.1 M sodium hydroxide (NaOH) and was autoclaved at 121 °C for 30 minutes.

Media for isolation

Two different media for isolation were prepared. The method of preparation is as follows:

Inorganic basal media with diesel (1% v/v)

To prepare the inorganic basal media with 1% (v/v) diesel, 100 µl of sterilized diesel was first added into 10 ml of basal media in a universal bottle. The pH was then adjusted to pH 7.2. Finally, the media was autoclaved for sterilization purposes.

Inorganic basal agar with diesel (1% v/v)

The inorganic basal agar with diesel was prepared by dissolving 18 g of agar powder I 1 liter of inorganic basal media. Similarly, the pH of the mixture was adjusted to pH 7.2. The sterilized diesel (1% v/v) was added before the agar solution was autoclaved. The mixture of agar and diesel was then homogenized and poured onto petri dishes.

Isolation

The sample taken from the top (15 cm) layer at TPU (Taman Pertanian Universiti), Universiti Putra Malaysia was added to the inorganic basal media and was incubated for 24 hours at ambient temperature with constant stirring. After the incubation, 100 µl of bacteria culture was taken out and a serial of dilution was performed. The aliquot was diluted to 10^8 times before spreading onto nutrient agar. Colonies of hydrocarbon degrading bacteria with different morphologies were then isolated into a new diesel broth.

Bacterial stock preparation

The isolate of hydrocarbon degrading bacteria were maintained as bacteria stock in broth media with 1% (v/v) diesel. Every week the bacteria cultures were maintained by sub culturing.

Gram staining

Bacteria smear was prepared by using a sterilization technique. The smear was air-the smear was flooded with gram's iodine mordant and let for 1 minute. The smear was then discolored by 95% ethyl alcohol before being counterstain with safranin for 45 seconds. Finally, the slide was bottled with bibulous paper and was examined under oil immersion.

Bacteria growth curve study

Bacteria culture (100 µl) was taken and a serial of dilution was performed until a dilution factor of 10^8 was attained. The diluted sample was then spread onto the nutrient agar by using a spreading plate technique. The single colonies formed were then counted periodically, i.e. at day 1, 2, 3, 4, 5, 6, 7 and 8 until a decreasing trend was observed [13]. The study was carried out in triplicate to assess the reproducibility of the results.

Effects of various factors on the growth of bacterial isolate

Effect of pH

Buffers such as acetate buffer, phosphate buffer and Tris buffer were used in this study to adjust the pH of the inorganic basal broth to pH 4.0, 4.5, 5.0, 6.0, 7.0, 8.0 and 9.0. The bacterial cultures were transferred onto the media of different pH in triplicate and were then incubated for 72 hours at ambient temperature in a constant stirring condition. The single colonies formed were then enumerated to determine the optimum pH.

Effect of temperature
To study the effects of temperature, the bacterial cultures were incubated at 10, 20, 30, 37, 40 and 50°C. Each temperature media was prepared in triplicate. After 72 hours, the sample was diluted to 10^8 times and was then spread onto nutrient agar. The single colonies formed were counted.

Effect of carbon concentration
To prepare the media with 1, 2, 3, 4 and 5% of diesel, different volumes of diesel were added into 10 ml of inorganic basal broth. After 72 hours of incubation in ambient temperature, the procedure for single colonies enumerating was carried out. This experiment was undertaken in triplicate [13].

Effect of nitrogen sources
Various sources of nitrogen (1.5 g/l) which included ammonium sulfate [$(NH_4)_2SO_4$], potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), sodium nitrate ($NaNO_2$), and aluminum ferric sulfate ($Al^{3+}Fe^{3+})_2(SO_4)_3$ were added into each basal media, respectively. The media was prepared in triplicate. Samples were then incubated for 72 hours at ambient temperature in a constant stirring condition. The growth of the isolate was observed by counting the formation of single colonies.

Effect of nitrogen concentration
Basal media with various concentration of ammonium sulfate were prepared. The concentration included 0.01, 0.05, 1, 1.5, 2.0 and 2.5%. As usual, the samples were incubated for 72 hours at room temperature in a constant stirring condition. A volume of 100 µl of bacteria was then taken from the media and a serial dilution was performed. The aliquots were then spread onto nutrient agar. Single colonies formed were then enumerated.

Identification using Biolog

Gram Stain (differentiation) procedure.
The same procedure as mentioned before was carried out for differentiating between two principal groups of bacteria, which is gram-positive and gram-negative.

Oxidase test on gram-negative bacteria
A separated and well-grown colony from a culture medium was taken with an inoculating loop. After that, the colony was applied to a reaction zone of oxidase strip and spread. After a few seconds, the color change was compared with a color scale.

Preparation of Test inoculums and Inoculation into ID Microplate
Bacteria colonies from a pure culture plate were taken out by using a sterile cotton swab. The bacteria were dissolved in inoculation fluid to prepare the bacteria suspension. The bacteria suspension was adjusted to 52% ± 2. Once the required turbidity was attained, the suspension was poured onto a sterile petri dish. The culture was then dispended into the wells of ID Microplates by using the 8-channel micropipette and was incubated for 4, 5 and 24 hours at 30 °C. At each incubation time, the sample was identified by using Microstation Reader.

Identification of bacterium
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. A pure culture of a bacterium was grown on a Biolog Universal Growth agar plate. The bacteria were swabbed from the surface of the agar plate and suspended to a specified density in GN Inoculating Fluid. A 150 µl of a bacterial suspension was pipetted into each well of the MicroPlate. The MicroPlate was incubated at 30 or 35 °C depending upon the nature of the organism for 4-24 hours according to manufacturer's specification. The MicroPlate were read with the Biolog MicroStationTM System and compared to database [13,14].

RESULTS AND DISCUSSIONS

Bacterial growth curve
From the mixed cultures of hydrocarbon degrading bacteria, a certain species of bacterial was isolated for further investigation. In this growth curve study (**Fig. 1**), it was found that the lag phase happened at the first day where the bacterium was adapting itself by synthesizing the inducible enzyme required for crude-oil degradation [15]. The number of bacteria growth is lower compare to mixed population. This further support the theory that each member in a microbial community has significant roles and may need to depend on the present of other species or strains in order to be able to survive when the readily available carbon source is limited and confined to complex carbon [16]. Logarithmic phase occurred at day four followed by rapid decreasing which may due to continuing depletion and buildup of toxic metabolic waste [15].

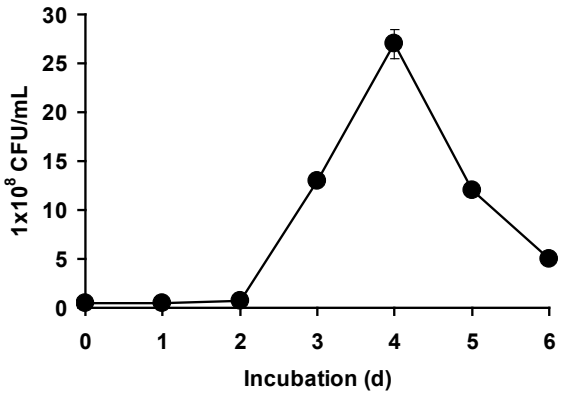
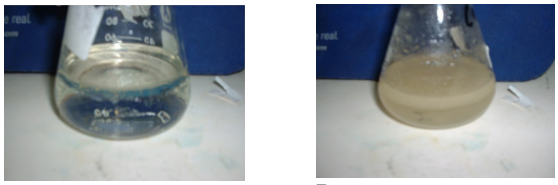


Fig. 1. Growth curve of isolated bacterial strain.

Characterization of isolated bacterial

Morphological test indicated that this isolated bacterial was gram-negative, rod-shaped with smooth and convex colony. Based on oxidase test, it was positive and non-motile similar to the *Pseudomonas aeruginosa* recorded by Nordin et al., [13] which was isolated from several environmental samples contaminated with oil.



A B

Fig 2. The initial media at 0 (A) and 4 (B) days after incubation.

Media after four days incubation shows well emulsification of oil in water due to the bacterial activity for oil consumption during their growth process (**Fig. 2**).

Effect of pH

The result (**Fig. 3**) shows the isolated bacterial was well grown at pH 7, however, it was decline at extreme alkaline and acidic pH (pH 4 and 9) which same to the work reported by Dibble and Bartha, [17]. In their study, they found that the optimum pH for hydrocarbon bioremediation activity can be observed at the pH ranges from 5.0-7.8. Extreme pH was observed to have negative effect on the ability of bacteria to degrade hydrocarbon [18].

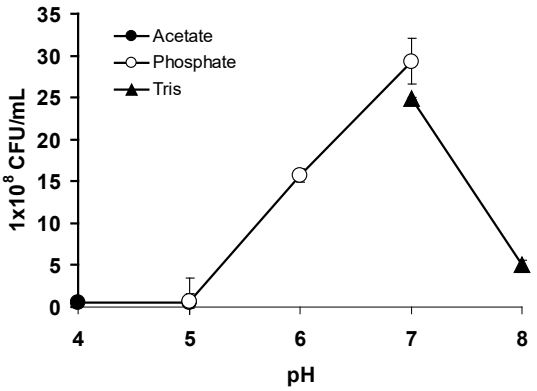


Fig. 3. Effect on pH on the growth of *Pseudomonas* sp. strain UPM-KV. Different (3) types of buffer were required in preparing the media indicating to the different range of pH value. The growth rate was determined by enumerating the growth rate of a single colony.

Effect of temperature

The effect of temperature on the growth of isolated bacterial was studied at different temperatures ranging from 10 °C to 50 °C (**Fig. 4**). At 30 °C, the isolate showed the highest growth rate. There has no growth at 10 and 50 °C indicating that the isolated bacterial is a mesophilic type. According to Venosa and Zhu, [19] hydrocarbon degradation can occur over a wide range of temperature but generally decreases with decreasing temperature. Usually temperature is not a limiting factor except if it is related to other factor such as physical state of available hydrocarbon [1].

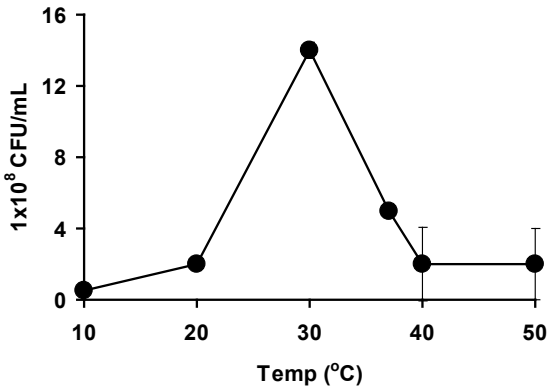


Fig. 4. Effect of temperature (10-50 °C) on bacterial growth.

Effect of carbon source concentration

The result shows bacterial growth increased from 0.1% (v/v) and reached optimum level at 1% (v/v) of diesel (**Fig. 5**). According to Singh and Ward [9], the hydrocarbon degradation proceeds at optimal rates as long as the contaminant levels in the soil are below 5%. The biodegradation slowed considerably at sludge loading rates from 10% to 15% [17]. This may due to nutrient or oxygen limitations or toxic effects of byproducts saturation [19]. The addition of large quantities of hydrocarbon may also results in a high C:N ratio that was unfavorable to microbial activities [20].

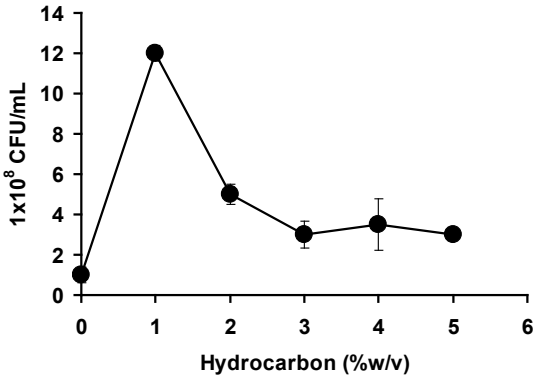


Fig. 5. Growth of isolated bacterial at various crude-oil concentrations (n=3).

Effect of nitrogen sources

Nitrogen sources such as aluminum ferric sulfate, ammonium sulfate, ammonium chloride, potassium nitrate and sodium nitrate are used to study the effect of nitrogen sources. All of the nitrogen sources support the bacterial growth with ammonium sulfate was the most dominant source (**Fig. 6**).

The result was accurate with the media suggested by Ermolenko et al., [12] where ammonium sulfate is used in a study related to the hydrocarbon degrading bacteria. According to Singh et. al., [9], the addition of inorganic nutrient may speed up the process of biodegradation and bioremediation. Jackson and Pardue [21] found that addition of ammonia appeared to be more effective than nitrate in stimulating degradation of hydrocarbon in salt marsh soils. It was attributed to the fact that ammonia is less likely to be loss from the system due to its higher adsorptive capacity to organic matter.

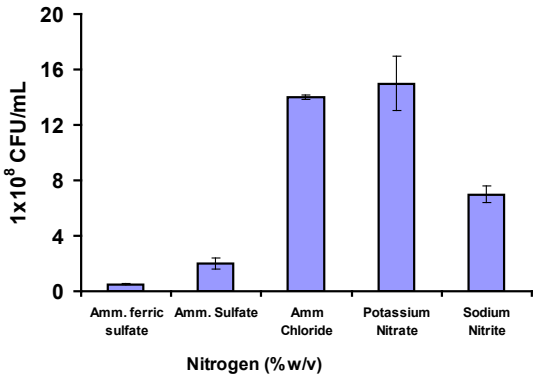


Fig. 6. The effects of various nitrogen sources on the growth of isolated bacterial. The isolate was grown in basal media and was incubated at ambient temperature in a shaking condition. The experiment was carried out in triplicate.

Effect of nitrogen concentration

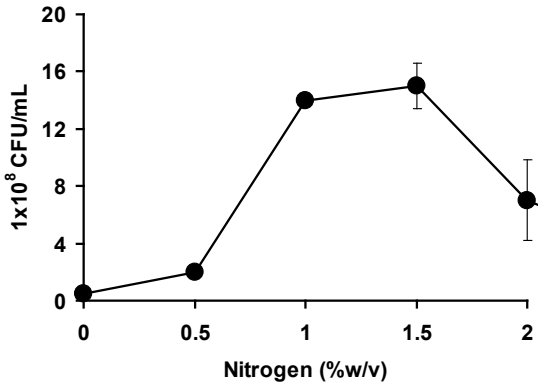


Fig. 7. The effect of nitrogen concentration on the growth of isolated bacterial. The experiment was carried out after ammonium sulfate has been determined as the most dominant nitrogen source. Error bars represent the standard error of the mean between three determinations.

Fig. 7 shows the effect of nitrogen concentration on the growth of isolated bacterial. The procedure was carried out by enumerating the number of viable cells. Based on the previous result, ammonium sulfate ga ve the best growth performance; therefore, it was selected for further investigation. The nitrogen concentration which included 1.0, 1.5, 2.0 and 2.5% were prepared to study the effect of nitrogen concentration. The growth was optimum at 1.5% of ammonium sulfate. According to Venosa and Zhu [19] the nutrient concentration should be maintained at high level to facilitate bacterial growth. However, excessive concentrations of nutrients, such as ammonia, will induce toxic responses due to the acid production [22].

Identification using Biolog

In Biolog identification, the bacteria were identified using the MicroLog™ database software (**Fig. 8**). From the result attained, there were 9 possibilities with all bacteria from genus *Pseudomanas*. The isolate was identified to be *Pseudomonas aeruginosa* with the probability of 99% and similarity of 0.716. This bacterium is a gram negative and non-enteric species. The identifications performed by Biolog GN gave a moderate probability identification to *Pseudomonas* sp. At this juncture, isolate is assigned tentatively as *Pseudomonas* sp. strain UPM-KV.

NAME	PROB	SIM	DIST	TYPE
Pseudomonas aeruginosa	99%	0.716	4.29	GN-NENT OXI+
Pseudomonas fulva	0%	0.001	6.43	GN-NENT OXI+
Pseudomonas citronellolis	0%	0.000	7.34	GN-NENT OXI+
Pseudomonas viridiflivida	0%	0.000	7.76	GN-NENT OXI-
Pseudomonas mendocina	0%	0.000	8.50	GN-NENT OXI+
Pseudomonas maculicola	0%	0.000	8.96	GN-NENT OXI+
Pseudomonas putida	0%	0.000	9.10	GN-NENT OXI+
Pseudomonas putida biotype B	0%	0.000	11.09	GN-NENT OXI+
Ralstonia paucula	0%	0.000	11.44	GN-NENT OXI+
Pseudomonas fuscovaginae	0%	0.000	11.53	GN-NENT OXI+

Fig. 8. Bacterial identifications using Biolog GN.

CONCLUSION

In conclusion, new bacterium was isolated from TPU (Taman Pertanian Universiti), Universiti Putra Malaysia from the soil with pH 6.4, the percentage of clay (<2 µm), silt used inorganic basal media containing 1% (v/v) diesel as sole carbon and energy source. The different percentage of hydrocarbon sludge was able to be degraded by the isolated bacterium. The optimum growth was achieved at day 4 while the optimized condition for this bacterium to grow were at pH 7.0, temperature 30 °C, optimum diesel concentration at 1% (v/v) and ammonium sulfate as the best nitrogen source at concentration of 1.5%. The bacterium was successfully identified through Biolog identification method with 99% of probability as *Pseudomonas aeruginosa* and was named as *Pseudomonas* sp. strain UPM-KV.

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