http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (17): 2122-2127, 2008
ISSN 1028-8880

Enhancement of Biodegradation of Crude Petroleum-Oil in Contaminated Water by the Addition of Nitrogen Sources

A.M. Mukred, A.A. Hamid, A. Hamzah and W.M. Wan Yusoff
School of Biosciences and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Abstract: Addition of nitrogen sources as supplementary nutrient into MSM medium to enhance biodegradation by stimulating the growth four isolates, *Acinetobacter faecalis*, *Staphylococcus* sp., *Pseudomonas putida* and *Neisseria elongata* isolated from petroleum contaminated groundwater, wastewater aeration pond and biopond at the oil refinery Terengganu Malaysia was investigated. The organic nitrogen sources tested not only supported growth but also enhances biodegradation of 1% Tapis crude oil. All four isolates showed good growth especially when peptone was employed as the organic nitrogen compared to growth in the basal medium. Gas chromatography showed that more then 91, 93, 94 and 95% degradation of total hydrocarbon was observed after 5 days of incubation by isolates *Pseudomonas putida*, *Neisseria elongate*, *Acinetobacter faecalis* and *Staphylococcus* sp., respectively.

Key words: Biodegradation, crude oil, contaminated water

INTRODUCTION

Biodegradation as the core process of bioremediation technologies depends on culture conditions, aeration and nutrients addition to enhance hydrocarbon breakdown. Rahman *et al.* (2002) reported that optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients are present (Atlas and Bartha, 1992). Biodegradation conditions within contaminated aquifers are often found to be limited by the availability of nutrients including nitrogen. Consequently, microorganisms that are capable of degrading contaminants as well as fixing molecular nitrogen as their sole nitrogen could have a growth advantage in fixed-nitrogen-deficient environments would be favorable for promoting *in situ* bioremediation (Westrick *et al.*, 1994). Heterotrophic populations require a group of other nutrients in addition to the organic compounds that serves as a source of carbon (C) and energy and thus in the study of biodegradation, much attention has been given to the roles of nitrogen (N) and phosphorus (P). In any environment, the ratio of C: N: P needs to be maintained at about 120:10:1 to sustain microbial activity (Thomas *et al.*, 1992). Nutrient such as nitrogen from urea, ammonium or nitrate ions and phosphorus from orthophosphate are required to stimulate growth and biodegradation (Atlas, 1981). Biostimulation involves introduction of additional nutrients in organic or inorganic form into a contaminated environment which then increases the population of the indigenous microorganisms (Pankrantz, 2001). Therefore, the goal of the present study is to examine the effect of nitrogen source addition for enhancement of biodegradation of crude petroleum oil of four potential local isolate.

MATERIALS AND METHODS

Source of microorganisms, media and culture condition: Bacterial strains were isolated from samples collected (Jun 2005) from groundwater and wastewater aeration pond and biopond located at the Terengganu oil refinery, Malaysia. Ten milliliter of each sample were washed with 90 mL saline and filtered with membrane. Incubation was carried out for 24 h at 37°C on nutrient agar plate. Mineral Salts Medium (MSM) (Zajic and Supplisson, 1972) was prepared by dissolving 1.8 g K_2HPO_4, 4.0 g NH_4CI, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4.7H_2O$ in 1 L of distilled water. Bacteriological agar was added (15 g L^{-1}) to the solution where solid basal medium was required. The pH was adjusted to 6.90 and the medium was autoclaved at 121°C for 15 min. 1.0% (v/v) Tapis crude oil was added as sole carbon source and complex vitamin solution (1.0 ml L^{-1}) according to manufacturer's suggestion.

Corresponding Author: A.M. Mukred, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Inoculum preparation: Bacterial inoculums were prepared in 50 mL nutrient broth by inoculating a loopful of cells from nutrient agar plate. The cultures were incubated for 24 h at 37°C at agitation of 150 rpm. The cells were then harvested by centrifugation (Appendorf) at 4000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 10 mL saline to give the inoculum suspension at absorbance of 0.5 and wavelength at 550 nm. Unless specified otherwise, bacterial inoculums was added to give a final concentration of 10% (v/v). The cultures were incubated at 37°C for 5 days. Growth was evaluated by the resulting colony forming unit (cfu mL^{-1}).

Isolation and identification of microorganisms: All colonies that had grown on crude oil agar were subcultured on Nutrient Agar (NA) and were incubated at 37°C for 24 h. A colony on NA was recultured to obtain pure culture. All the pure strains were conserved on glycerol and stored at -20°C. Screening of the isolates was carried out based on colony morphology, size, shape, color, Gram staining and biochemical tests, methyl red-Voges proskauer (MR-VP) tests, indole, catalase and oxidase. The isolates were identified according to the descriptions in the Bergeys manual of Systematic Bacteriology, vol. 1 (1984), vol. 2 (1986). API 24 E (Commercial Kit) was used for identification of Gram negative bacteria.

Effect of nitrogen source on growth and biodegradation: To determine effect of organic and inorganic nitrogen source on biodegradation, the inorganic nitrogen source of the basal medium were substituted by the addition of the following; peptone, yeast extract and ammonium sulfate at concentrations of 0.30, 0.50, 1 and 1.50% (v/v). Cultivations were carried out after 5 days and the growth was measured as CFU count.

Extraction of residual crude oil for Gas Chromatography (GC) analysis: Crude oil was extracted with a pre-cleaned separating funnel, following the modified methods of Chaillan *et al.* (2004). Fifty milliliter of sample (BM + crude oil + bacteria), then centrifuged at 4000 rpm for 15 min. The separating funnel was stoppered with a glass stopper and it was shaken vigorously. Vapour was carefully vented out through the stopcock. The process of venting and vigorous shaking was repeated for several minutes and the mixture was allowed to separate into two phases. After the phases have been separated in the funnel, chloroform was collected in a 250 mL pre-cleaned bottle. The extraction was repeated with 25 mL chloroform twice, then filtered and dried by passing it through of 3 g pre-combusted Na_2SO_4 (150°C for 3 h in incubation) and collected in a round bottom flask. The extract was then evaporated by using a rotary evaporator. The crude oil extracted washed with 2 mL chloroform. The solvents were removed under a gentle stream (dried in fume chamber) for 7 days. The residue was reconstituted with 1 mL of chloroform and analyzed by GC.

Analysis of extract: A detailed analysis of the hydrocarbon extract was performed by Gas Chromatography (GC). The clarus GC 500 was equipped with a split injector (split ratio 50/1) and a Flame Ionization Detector (FID) both set at 300°C; carrier gas was nitrogen 1.50 mL min^{-1}; the column was fused silica capillary column (30.0 m×0.32 mm, film thickness 0.25 μm); temperature programming was 60-320°C, 5°C min^{-1}, injection volume 1 μL.

RESULTS AND DISCUSSION

Identification of bacteria: Biochemical and growth characteristics of four isolates (WD2, DD3, TDA4.2 and TAM4.4) that exhibited good potential were further investigated. The results showed that two isolates were short rod Gram Negative *Acinetobacter faecalis* from groundwater sample and *Pseudomonas putida* from aeration pond sample. The other two isolates were coccus Gram Positive *Staphylococcus* sp. from groundwater sample and *Neisseria elongata* from biopond sample (Table 1).

Effect of peptone on growth and degradation: The bacterial growths in different percentages of peptone as nitrogen source were studied (Table 2). All four isolate showed good growth at different peptone concentration 0.3, 0.5, 1.0 and 1.50%. Optimum growths were observed at percentages 1% Tapis crude oil on the 5 day of incubation. Among the four isolates, isolate WD2 and DD3 showed a maximum growth with 1% peptone while, isolates TDA4.2 and TAM4.4 showed a maximum growth with 0.5% peptone. From this study, 1% peptone was the best nitrogen source tested for growth compared to the basal medium, yeast extract and ammonium sulfate. However, increasing the concentration of peptone from 1 to 1.50% significantly decreases growth and degradation. This is in agreement with the finding by Tano-Debrah *et al.* (1999) that due to enhanced growth; degradability of oil was better in a medium containing 1% peptone, compared to medium containing either ammonium sulfate or potassium nitrate. Zeng *et al.* (2004) also reported cell growth increase in the medium with 1% peptone for *Pseudomonas fluoresences* FS1.

Table 1: Biochemical and growth characteristics of isolated bacterial cultures

	Isolates			
Characteristics	WD2	DD3	TAM4.4	TDA4.2
Gram stain	-	+	-	+
Cell morphology	Rod	Cocci	Rod	Cocci
Cell size	3 µm	5 µm	2 µm	2 µm
MacConkey	+	-	-	+
Indole test	-	-	-	-
MR test	-	-	-	-
V-P test	-	-	-	-
Citrate test	+	-	+	-
TSI test	K/K	A/A	K/K	K/K
Oxidation/fermentation	O	F	O	F
Motility	+	-	+	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Growth at room temperature at 29°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 40°C	+	+	+	+
Colony colour	Translucent	White	Creamy	White
Spore forming	+	-	+	-
Genus	*Acinetobacter*	*Staphylococcus*	*Pseudomonas*	*Nesseria*
Species	*faecalis*	sp.	*putida*	*elongata*

-: Negative, +: Positive, K/K: Alkaline no fermentation, A/A: Acid glucose, lactose and/or sucrose fermentation, O: Oxidative, F: Fermentive

Table 2: Growth (cfu mL^{-1}) of isolates WD2, DD3, TDA4.2 and TAM4.4 with different concentration of nitrogen sources in the MSM medium with 1% Tapis crude oil at pH 7.0 after 5 days

		Concentration of nitrogen sources										
		Peptone (%)				Yeast extracts (%)				Ammonium sulfate (%)		
Bacteria	Control	0.3	0.5	1.0	1.50	0.3	0.5	1.0	1.50	0.3	0.5	1.0
Acinetobacter faecalis WD2	14.77	22.24	23.69	27.37	25.14	22.26	22.69	23.40	22.87	18.20	18.93	18.34
Staphylococcus sp. DD3	14.38	23.85	23.36	24.30	22.89	22.41	22.60	23.10	22.80	19.14	18.88	18.40
Neisseria elongate TDA 4.2	14.27	23.43	24.51	23.55	17.68	23.66	23.19	22.92	-----	18.78	19.20	18.50
Pseudomonas putida TAM 4.4	14.10	23.41	24.20	23.60	18.23	23.12	23.35	22.51	-----	18.10	18.25	17.92

Control: MSM medium without nitrogen sources

Effect of yeast extract on growth and degradation: Medium containing different concentrations of yeast extract as nitrogen source promotes cell growth and hydrocarbon degradation (Table 2). Good growth was observed with all the four isolates when yeast extract were present in the medium. However the four isolates isolate WD2 and DD3 showed high growth with 1% yeast extract while, isolates TDA4.2 and TAM4.4 showed high growth with 0.5% yeast extract. However, increasing the concentration of yeast extract from 0.5 to 1% significantly lowered the amount of growth hydrocarbon degradation. Khleifat (2006) reported that the nitrogen sources supplied, except yeast extract and casein, led to the enhancement of the phenol biodegradation of *Ewingella americana*. In another report, Okeke and Frankenberger (2003) demonstrated that good growth and degradation of crude oil in medium containing 1% yeast extract. Optimal amount of yeast extract that should be supplemented for phenol biodegradation was reported by Loh and Tan (2000).

Effect of ammonium sulfate on growth and degradation: Different inorganic nitrogen concentration sources can be used by the microorganisms, the effect of ammonium sulfate for the study of the influence of inorganic nitrogen source on growth hydrocarbon biodegradation of the four strains (Table 2). The addition of ammonium sulfate to the medium of all the four isolates showed good growth but isolate DD3 showed high growth with 0.3% while, isolates WD2, TDA4.2 and TAM4.4 showed high growth with 0.5%. The cell growth values obtained with inorganic nitrogen sources as ammonium sulfate were considerably lower than when peptone and yeast extract were used as organic nitrogen sources. However, increasing the concentration of ammonium sulfate from 0.5 to 1% significantly decrease hydrocarbon degradation because the addition of ammonium sulfate greater than the optimal amount would result in slow growth and consequently biodegradation by Lee *et al*. (2003) reported that the growth kinetics of *Pseudomonas putida* G7 on pyruvate was determined from a chemostat culture of *Pseudomonas putida* G7 using pyruvate as carbon and ammonium source.

Biodegradation of crude oil with MSM medium containing peptone: The biodegradation in MSM containing peptone was studied using four bacteria *Staphylococcus* sp., *Acinetobacter faecalis*, *Neisseria*

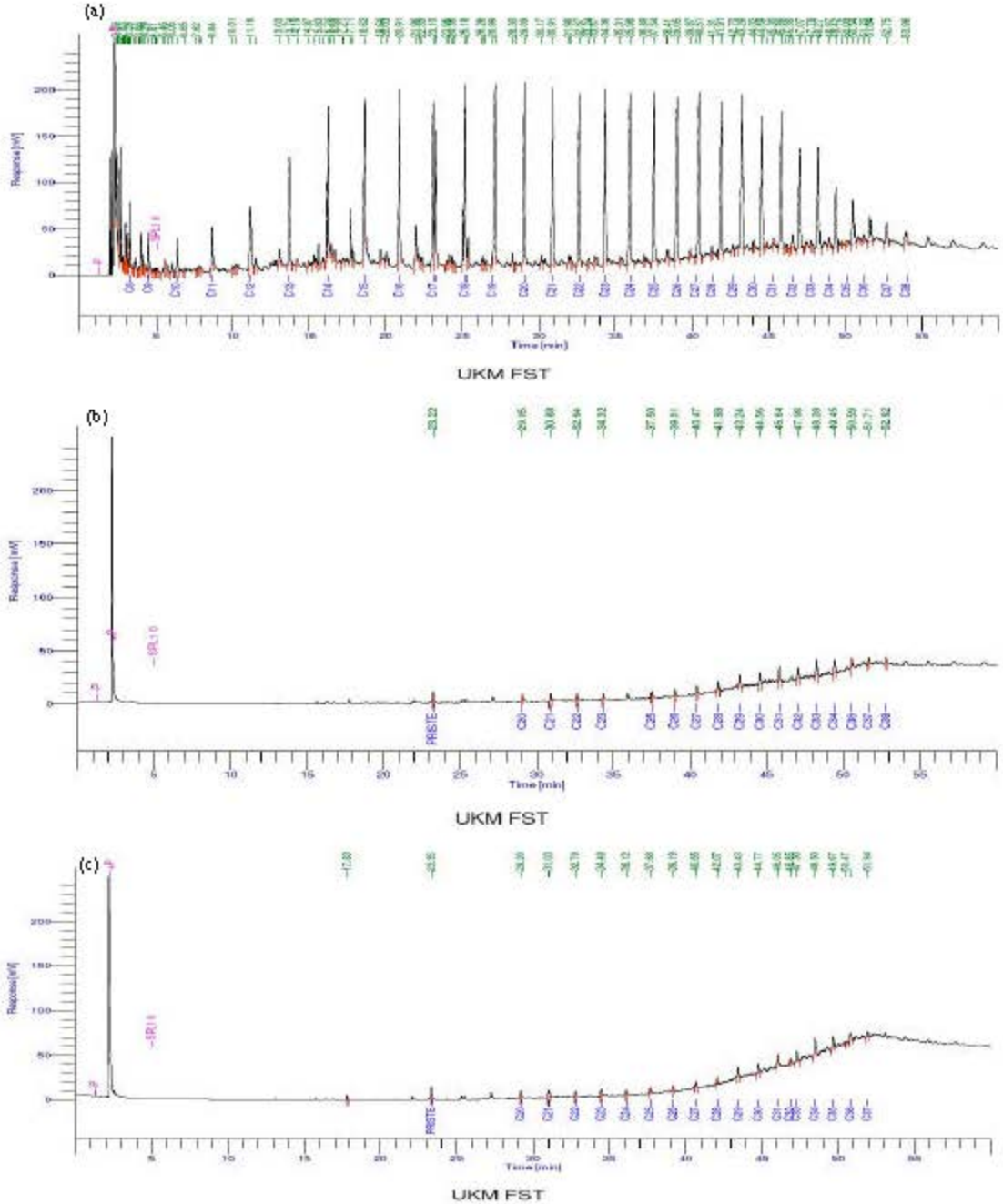

Fig. 1: (a) Chromatograph of Tapis crude oil before microbial degradation (b) Chromatograph of Tapis crude oil after microbial degradation using *Staphylococcus* sp. and (c) using *Acinetobacter faecalis* after 5 days incubation

elongata and *Pseudomonas putida* following enhanced biodegradation of 1% Tapis crude oil polluted water after 5 day of incubation. GC results showed 95, 94, 93 and 91% degradation of Tapis crude oil by *Staphylococcus* sp., *Acinetobacter faecalis*, *Neisseria elongata* and *Pseudomonas putida*, respectively. Maximum degradation was achieved after 5 days of incubation of *Staphylococcus* sp. Aliphatic compounds in the range of C_8-C_{19} was degraded completely followed by C_{20}-C_{38} with percentage degradations of 100, 96 and 97%, respectively. Compared to control (Fig. 1), between 3 and 2% of C_{20}-C_{38} remained after 5 days. The medium-chain aliphatic and

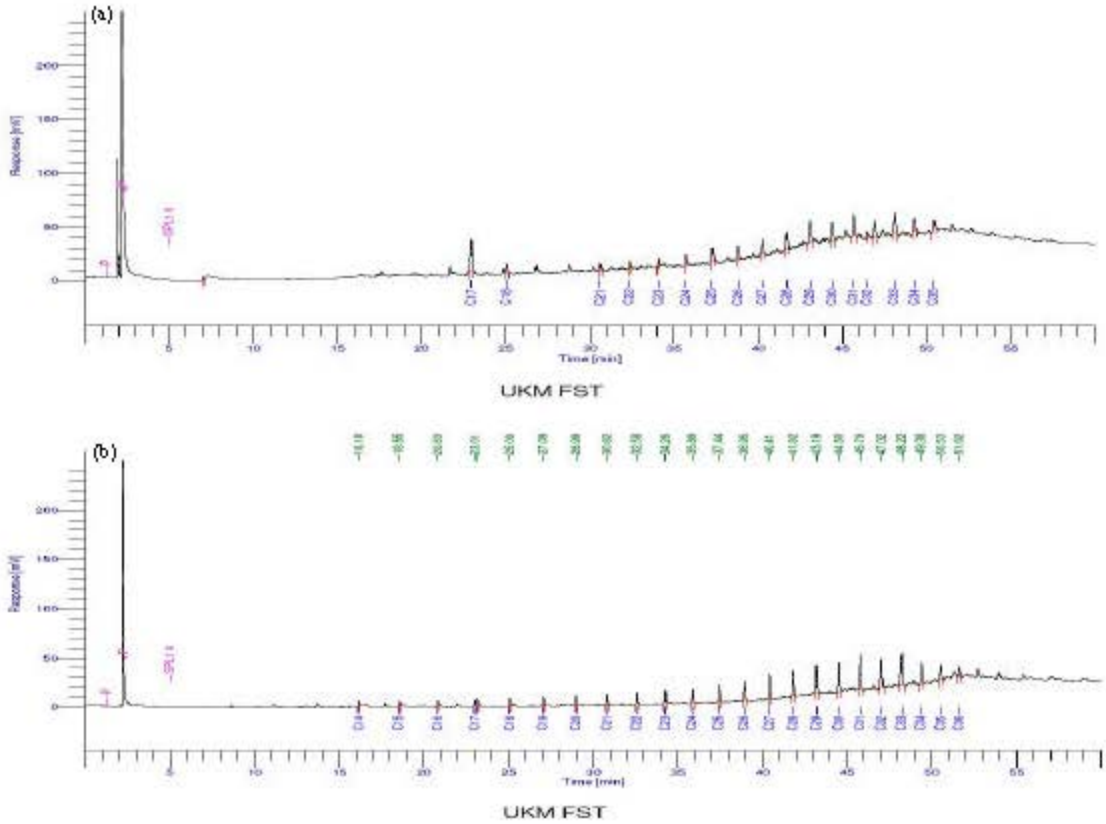

Fig. 2: (a) Chromatograph of Tapis crude oil before microbial degradation (a) Chromatograph of Tapis crude oil after microbial degradation using *Neisseria elongate* and (b) using *Pseudomonas putida* after 5 days incubation

aromatic compounds C_8-C_{19} were almost fully degradation where less than 5% of these compounds remained. More then 94% of these aliphatic compounds have been removed; a percentage of hydrocarbon degradation of approximately 100% C_8-C_{37} and between 95 and 97% of C_{20}-C_{37} was degraded by *Acinetobacter faecalis* (Fig. 1) after 5 days of incubation compared to control, with 4 and 2% of C_{20}-C_{37} remained. Short-chain and medium-chain aliphatic and aromatic compounds C_8-C_{20} were degraded up to 94% where less than 6% of these compounds remained. Gas chromatographic analysis showed degradation of all aliphatic in the range of C_8-C_{38}. Degradation of approximately 100% of C_8-C_{16}, and between 80 to 96% of C_{17}-C_{26} and between 95 and 87% of C_{27}-C_{38} was recorded by *Neisseria elongata* after 5 days of incubation (Fig. 2). However less than 7% of short-chain and medium-chain aliphatic and aromatic compounds C_8-C_{20} remained. *Pseudomonas putida* was able to degrade alkanes in the range of C_8-C_{13} completely followed by C_{14}-C_{29} and C_{30}-C_{38} with percentage degradation of 100, 90 and 91%, respectively after 5 days of incubation with only 9 and 8% of these compounds remained (Fig. 2). Rahman *et al*. (2003) reported that the percentage of hydrocarbon degradation of approximately 100% of (C_8-C_{11}), 83-98% of (C_{12}-C_{21}), (80-85%) of (C_{22}-C_{31}) and 57-73% of (C_{32}-C_{40}) using bacterial consortium. In other report by Verma *et al*. (2006), the chromatograms of oily sludge inoculated with three isolates for 5 days, indicate that the *Acinetobacter* sp., SV9 degrades the aliphatic fraction to a greater extent than the aromatic fraction, *Bacillus* sp., SV9 consumed almost all aliphatic and aromatic, while the *Pseudomonas* sp., SV9 degraded 60% of both fractions of oily sludge. Among 368 isolates belonging to the genus *Bacillus* isolated by Sorkhoh *et al*. (1993) from desert soils, two *Bacillus stearothermophilus* strains degraded 80-89% of crude oil within 5 days at 60°C. Ijah (1998) reported crude oil components of chain length C_{12}-C_{32} were extensively degraded by *Serratia marcescens* OCS-21 after 16 days of incubation while *Acinetobacter calcoaceticus* COU-27 was able to degrade C_{22}-C_{30} components of the crude oil. All four isolates showed maximum removal of short-chain

and medium-chain aliphatic compounds compared to longer-chain aliphatic compounds. This seems logical as short-chain and medium-chain alkanes are generally more easily degraded due to their lower hydrophobicity.

CONCLUSION

The best nitrogen source was peptone. Two isolates (*Acinetobacter faecalis* WD2 and *Staphylococcus* sp. DD3) showed maximum growth in 1% peptone and two isolates (*Neisseria elongate* TDA4.2 and *Pseudomonas putida* TAM4.4) showed maximum growth in 0.5% peptone. Degradation of total hydrocarbon of 91, 93, 94 and 95% in Tapis crude oil after 5 days of incubation with MSM + peptone was achieved by isolates *Pseudomonas putida* TAM4.4, *Neisseria elongate* TDA2.4, *Acinetobacter faecalis* WD2 and *Staphylococcus* sp. DD3, respectively.

ACKNOWLEDGMENT

The authors would like to thank UKM Zamalah (S 1.14/32/3 Jilid XI) and PETRONAS (No. CRO50833), for the financial support provided to carry out this study.

REFERENCES

Atlas, R.M., 1981. Microbial degradation of petroleum hydrocarbons an environmental perspective. Microbiol. Rev., 45: 180-209.

Atlas, R.M. and R. Bartha, 1992. Hydrocarbon biodegradation and oil-spill bioremediation. Adv. Microbial. Econ., 12: 287-338.

Chaillan, F., L.A. Fleche, E. Bury, Phantavong, Y. Hui, P. Grimont, A. Saliot and J. Oudot, 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Res. Microbiol., 155: 587-595.

Ijah, U.J.J., 1998. Studies on relative capabilities of bacterial and yeast isolates from tropical soil in degrading crud oil. Waste Manage., 18: 293-299.

Khleifat, K.M., 2006. Biodegradation of phenol by *Ewingella americana*: Effect of carbon starvation and some growth conditions. Process Biochem., 41: 2010-2016.

Lee, K., J.W. Park and I.S. Ahn, 2003. Effect of additional carbon source on naphthalene biodegradation by *Pseudomonas putida* G7. J. Hazard Mater. B, 105: 157-167.

Loh, K.C. and C.P.P. Tan, 2000. Effect of additional carbon sources on biodegradation of phenol. Bull. Environ. Contamin. Toxicol., 64: 756-763.

Okeke, B.C. and W.T. Frankenbergerjr, 2003. Biodegradation of methyl tertiary butyl ether (MTBE) by a bacteria and its monoculture isolates. Microbiology, 158: 99-106.

Pankrantz, T.M., 2001. Environmental Engineering Dictionary and Directory. 1st Edn., CRC Press, Boca Raton.

Rahman, K.S.M., T.J. Rahman, P. Lakshmanaperumalsamy and I.M. Banat, 2002. Occurrence of crude oil degrading bacteria in gasoline and diesel station soils. J. Basic. Microbiol., 42: 284-291.

Rahman, K.S.M., T.J. Rahman, Y. Kourkoutas, I. Petsas and I.M. Banat *et al.*, 2003. Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol., 90: 159-168.

Sorkhoh, N.A., A.S. Ibrahim, M.A. Ghannoum and S.S. Radwan, 1993. High temperature hydrocarbon degradation by *Bavillus stearrothermophilus* from oil polluted Kuwait desert. Applied Microbiol. Biotechnol., 39: 123-126.

Tano-Debrah, K., S. Fukuyama, N. Otonari, F. Taniguchi and M. Ogura, 1999. An inoculum for the aerobic treatment of wastewaters with high concentration of fats and oils. Bioresour. Technol., 69: 133-139.

Thomas, J.M., C.H. Ward, R.L. Raymond, J.T. Wilson and R.C. Loehr, 1992. Subsurface microbial ecology and Bioremediation. J. Hazardous Mater., 32: 369-385.

Verma, S., R. Bhargava and V. Pruthi, 2006. Oily sludge degradation by bacteria from Ankleshwar, India. Int. Biodet. Biodeg., 57: 207-213.

Westrick, J.J., J.W. Mello and R.F. Thomas, 1994. The groundwater supply survey. JAWW, pp: 52-59.

Zajic, E. and B. Supplisson, 1972. Emulsification and degradation of bunker C; fuel oil by microorganisms. Biotechnol. Bioeng., 14: 331-343.

Zeng, F., K. Cui, X. Li, J. Fu and G. Sheng, 2004. Biodegradation kinetics of phthalateesters by *Pseudomonas fluoresences* FS1. Process Biochem., 39: 1125-1129.