VOL. 8, NO. 2, FEBRUARY 2013 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science


www.arpnjournals.com

BIODEGRADATION OF OILY WASTEWATER BY PURE CULTURE OF *Bacillus cereus*

Marina Bujang[1], Noor Azlina Ibrahim[1] and Aweng a/l Eh Rak[2]
[1]Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Locked Bag, Jeli, Kelantan, Malaysia
[2]Faculty of Earth Science, Universiti Malaysia Kelantan, Locked Bag, Jeli, Kelantan, Malaysia
E-Mail: aqua_rie169@yahoo.com

ABSTRACT

The discharges of oily wastewater to environment caused serious damages to human, animal and environment and bioremediation are an attractive alternative to chemical method for removal of hydrocarbon from industrial effluents. This study has demonstrated a very good biodegradation capability of oily wastewater by *Bacillus cereus*. In spite of the complex composition of oily wastewater from three different automotive workshops, biodegradation could be accomplished by *Bacillus cereus*. The total hydrocarbon in oily wastewater degraded by the *Bacillus cereus* expressed in percentages showed that the *Bacillus cereus* has the ability to degraded oily wastewater component in range 3% to 91%. From three different oily wastewater samples from different automotive workshop in Kota Bharu, the highest growth and degradability was obtained on oily wastewater from automotive workshop T. After 5 days of incubation, from 5% to 91% of oily wastewater components from automotive workshop T were removed by *Bacillus cereus*. Meanwhile, *Bacillus cereus* degraded only 20% to 62% of hydrocarbon components present in oily wastewater collected from automotive workshop P and 3% to 47% of hydrocarbon component in oily wastewater from automotive workshop K. The present studies suggested that the *Bacillus cereus* grew maximally on the three different oily wastewaters from automotive workshops when supplied as the sole source of carbon and energy and the greatest *Bacillus cereus* growth was detected on oily wastewater from automotive workshop T followed by oily wastewater from automotive workshop K and automotive workshop P. Apart from growth, the changes in pH in MSM medium during oily wastewater degradation by *Bacillus cereus* were also determined and it found that the pH of medium during the degradation of oily wastewater by *Bacillus cereus* was at acidic condition for all flasks. In conclusion, this study demonstrated the hydrocarbon degrading organism can be isolated from hydrocarbon polluted area and the degrading ability by *Bacillus cereus* is a clear indicator that this bacteria can be applied in the bioremediation techniques.

Keyword: *Bacillus cereus*, biodegradation, bioremediation, oily wastewater, hydrocarbon.

INTRODUCTION

Oil contaminated wastewater has been identified as one of the most concerned pollution sources. This kind of wastewater widely produced from a variety of sources such as crude oil production, automotive garage, oil refinery, petrochemical industry, metal processing, lubricant and car washing. These sources serve as the major contributor to the environmental problems especially in soil and water. Both the waste and unused compounds from the prescribed sources are grouped as oily waste which is difficult to be treated or recycled (Tri, 2002). The discharges of oily wastewater to sewer or environment have potential to cause significant environment harm through the release of contaminants to the environment and considered as hazardous industrial wastewater. This is because, this kind of wastewater contain toxic substances such as petroleum hydrocarbons, phenols, polyaromatic hydrocarbon which are inhibitory to animal and plant growth and also are mutagenic and carcinogen to human being.

Oily wastewater from automotive workshops are a combination of water with some surface oil, oil sludge or sediments which containing lubricants, cutting fluids and heavy hydrocarbon such as tars, grease and diesel oil, bacteria and light hydrocarbon at concentration that may vary from a few hundred parts per million to as much as 1 to 10 percent by volume. In addition, the oily wastes may also contain chemically and mechanically emulsified oils, acid/alkaline cleaner, solvents, suspended solids and heavy metals (Kanluen and Amer., 2000).

As concern for effect of hydrocarbon contaminated wastewater continues to grow, so are the different technologies that continue to emerge to remediate contaminated site. One of these technologies is bioremediation. Bioremediation of oily waste water is treatment technology that use of microorganisms or their enzymes to reduce the concentration or toxicity of hydrocarbon contaminants into less toxic forms (Vidali, 2005; Bobeye, *et al*., 2010). The microorganisms may be indigenous to contaminated area or they are isolated from other area and brought to the hydrocarbon contaminated area (Vidali, 2005).

Contamination of water hydrocarbon waste stimulates indigenous microbial populations, which are capable utilizing the hydrocarbon substrates as their sole carbon and energy sources thereby degrading the contaminants. Several bacterials species have been identified as having the ability for oil degradation. In general, *Bacillus* sp. has been identified as petroleum hydrocarbon degraders (Ghazali, *et al.,* 2004; Das and Mukherjee, 2007) and is known as naphthalene and pyrene degraders (Ron and Rosenberg, 2001; Zhuang, *et al.,* 2002). According to Sorkhoh, *et al.* (1993), 368 isolates belonging to the genus Bacillus were isolated from desert

samples and two strain of bacillus was able to degraded 80% - 89% of crude oil (5g/L) within five days at 60ºC. Recently, Obuekwe, *et al*., (2009) identified *Bacillus* sp. and *Paenibacillus* sp. as two of the most prominent crude-oil degraders in the Kuwait desert environment. The ability of bacillus species in hydrocarbon degradation has consistently been observed by Antai, (1990) and associated with competent hydrocarbon enzyme system of the organism; its ability to form spores and emulsify hydrocarbon (Joshi and Pandey, 2011). The aim of the present study is to evaluate the biodegradation of hydrocarbon potentials of the *Bacillus cereus* isolated from oily wastewater from automotive workshop.

MATERIALS AND METHODS

Sample collection

In this research, oily wastewater in oil trap was collected from three automotive workshops in Kota Bharu. Oily wastewaters samples were collected in sterile sampling bottles. The samples were labeled and transport to the laboratory and stored in the refrigerator at temperature 4ºC prior to analysis.

Analysis of hydrocarbon content

Hydrocarbon content analysis in oily wastewater was performed by independent lab (UKM UNIPEQ). The hydrocarbon content analyses were carried out according to the Standard Methods for the Examination of Water and Wastewater (2005) 21st Edition, APHA, AWWA, WPCF.5520D and GC-MS/MS, respectively.

Isolations and identification of hydrocarbon degrading bacteria

Microorganisms used in all experiments were isolated from oily wastewater from three different automotive workshops in Kota Bharu. Pure colonies were isolated from spread plate method using mineral salt medium (MSM) containing 1% v/v oily wastewater. Firstly, different types of colonies were selected and taken by using sterile loop and streaked onto mineral salt medium agar plate containing 1% v/v oily wastewater concentration. Then, the plates were incubated overnight at 30°C. The growth of pure colonies was observed after 24 hours.

The isolated bacteria were characterized and identified by their morphological characteristic based on size, shape and colony morphology on nutrient agar plate. Besides that, all isolates were examined by gram staining and 16SrDNA.

Biodegradation and growth studies

Growth and degradation studies over a time course were carried out using untreated oily wastewater from automotive workshop as the sole carbon and energy sources. In this study, the bacteria were inoculated into 75mL of MSM containing 10% of oily wastewater. While, for control preparation, 10 ml of oily wastewater was added into 90 mL MSM medium without inoculum. After that, the culture was incubated in 30°C for 5 days with 150 rpm agitation speed. At 24 hours interval during the incubation, samples were drawn from the flask for pH and optical density at 600nm wavelength measurement.

Degradation of oily wastewater

The media bottle containing cultures of *Bacillus cereus* and oily wastewater were incubated in the same experimental conditions as well as same period as growth studies. In order to determine the extent of biodegradation of oily wastewater by *Bacillus cereus*, the residue of oil in liquid medium at the end of incubation session were extracted with dichloromethane (DCM) using method which was published by Ainon, *et al*., (2009). The bacteria were inoculated into 75mL of MSM containing 10% of oily wastewater. While, for control preparation, 10 ml of oily wastewater was added into 90 mL MSM medium without inoculum. The cultures were incubated at 30°C for 5 days before removed the flask and the medium was centrifuged at 5000 g for 20 minutes to pellet the bacteria. The oil residue in supernatant then was extracted with dichloromethane. The oil was concentrated to 1.0 mL through evaporating the solvent in solvent oil mixtures by rotary evaporator at 37^{o}C. One micro liter of the extracted oil was analyzed on DB-1HT column in capillary gas chromatograph equipment with a flame ionization detector. Temperature was programmed to increase from 50^{o}C to 300^{o}C with 5^{o}C increment per minute. Hydrogen gas with flow rate of 3 mL/min was used as a carrier.

RESULTS AND DISCUSSIONS

Analysis of hydrocarbon content

The components of hydrocarbon in oily wastewater from three automotive workshops in Kota Bharu were identified by using GCMS. Identification of hydrocarbon compounds was performed by comparison of the GC retention time and the mass spectrometric fragmentation with reference substances.

The gas chromatography (GCMS) profile of oily wastewater from automotive workshop K showed that, 61.3% of polar hydrocarbon and 38.8% of non polar hydrocarbon was found in oily wastewater from automotive workshop K. The hydrocarbon compounds in oily wastewater K were n- alkenes (Tetradecane, Pentadecane, Hexadecane, Octadecane, Nonadecane, Eicosane, and Hexacosane), cycloalkanes, PAHs (Naphthalene), phytane, and oxygen containing compounds (aldehydes, ketones and ester). In addition, GCMS method also shows the existence of aliphatic and aromatic hydrocarbons in oily wastewater from automotive workshop P. The results showed that, oily wastewater P compounds consist of 72.1% polar hydrocarbon and 27.9% non polar hydrocarbon. Meanwhile, the hydrocarbon components in the oily wastewater from automotive workshop P were n-alkanes (Tetradecane, Hexacosane, Pentadecane, Hexadecane, Heptadecane, Octadecane and Docosane), Cycloalkanes, phytane, aromatic hydrocarbon (Benzene, 1, 3, 5-

trimethyl) and oxygen containing compounds (aldehydes, ester and aromatic acid) and fatty acid. This study also showed that, oily wastewater from automotive T consist of 84.5% of polar hydrocarbon and 15.5% of non polar hydrocarbon compounds. The commonly found hydrocarbon compounds in oily wastewater from automotive workshop T were n-alkanes, Cycloalkanes, PAHs (0-xylene, Benze [e] acephenanthrylene, Benzo [a] pyrene, pristine, phytane and oxygen containing compounds (aldehydes, ester, and aromatic acid) and fatty. The results of this study showed that, oily wastewater from automotive workshop T was polluted with high toxic index aromatic hydrocarbon (PAHs) listed as priority by US environmental protection agency (US-EPA).

Isolations and identification of hydrocarbon degrading bacteria

A total of thirteen bacterial strains were isolated on nutrient agar plates from 3 samples of oily wastewater originated from three different automotive workshops in Kota Bharu. The results of isolation showed that, five strains were isolated from workshop K and four strain from automotive workshop P and T respectively. The result of 16SrDNA revealed that, bacterial strain belongs to the genera *Bacillus*, *Staphylococcus* and *Acinetobacter*. *Bacillus cereus* was chosen for biodegradation test due to its ability to growth well in Mineral salt medium (MSM) with hydrocarbon as sole carbon and energy sources compared to other isolates.

Biodegradation and growth studies

The present studies suggested that, *Bacillus cereus* showed optimum growth on the three different oily wastewaters from automotive workshops when supplied as the sole source of carbon and energy and the highest growth was recorded on oily wastewater from automotive workshop T followed by oily wastewater from automotive workshop K and automotive workshop P. Apart from growth, the changes in pH in MSM medium during oily wastewater degradation by *Bacillus cereus* were also determined and it was found that, the pH of medium during the degradation of oily wastewater by *Bacillus cereus* was at acidic conditions for all flasks.

Degradation of oily wastewater

Furthermore, this study has demonstrated a very good biodegradation capability of oily wastewater by *Bacillus cereus*. In spite of the complex composition of oily wastewater from three different automotive workshops, biodegradation could be accomplished by *Bacillus cereus*. Total hydrocarbon in oily wastewater degraded by *Bacillus cereus* was expressed in percentage where the degradation rate was recorded ranged between 3% to 91% (Table-1). From three different oily wastewater samples from different automotive workshop in Kota Bharu, the present studies revealed that, the best performance in hydrocarbon degradation by *Bacillus cereus* was observed in medium containing oily wastewater from automotive workshop T. The results of chromatography showed that, *Bacillus cereus* able to degrade 5% to 91% of hydrocarbon in oily wastewater collected from automotive workshop T after 5 days of incubation (Figure-3). Meanwhile, *Bacillus cereus* able to degrade only 20% to 62% of hydrocarbon in oily wastewater collected from automotive workshop P (Figure-2). On the other hand, degradation rate for oily wastewater from automotive workshop K was ranged between 3% to 47% (Figure-1). The possible explanation of the difference in hydrocarbon degradation rate in oily wastewater from automotive workshop K, P and T by Bacillus cereus is the differences in waste characteristics where each type of oily wastewater has its own characteristic composition (Ainon, *et al*., 2010). This could be further strengthening by the composition of oily wastewater from automotive workshop T where it is acid and cycloalkanes because according to Zhang, *et al.* (1998), biodiesel is easier to metabolize compared to diesel due to natural product for biodiesel contained pure fatty acid. Hydrocarbon with oxygen atoms attached to the end, which are very biological active, being recognized and attacked by enzymes such as acetyl - CoA dehydrogenase. So this finding might be able to explain the high percentages of oily wastewater degradation in oily wastewater from automotive workshop T compared to other automotive workshops.

Meanwhile, the chromatogram of oily wastewater from automotive workshop K showed similar trend with the chromatogram of oily wastewater from automotive workshop P. It is probably the oily wastewater from automotive workshop K has similar characteristics with oily wastewater from automotive workshop P.

Table-1. Degradation of oily wastewater by *bacillus cereus.*

Oily wastewater (carbon sources)	**Maximum growth** (OD600nm)	**Changes of pH in culture medium** (D0 to D5)	**Percentage of degradation range** (%)
Automotive workshop K	0.979±0.02	6.78±0.16 to 5.93±0.08	3%-47%
Automotive workshop P	0.969±0.00	6.51±0.23, to 5.05±0.05	20%-62%
Automotive workshop T	0.980±0.00	6.27±0.04 to 5.40±0.11	5%-91%

Legend: The percentages of degradation range of oily wastewater by *Bacillus cereus* in Mineral salt medium after 5 days of incubation at 30ºC

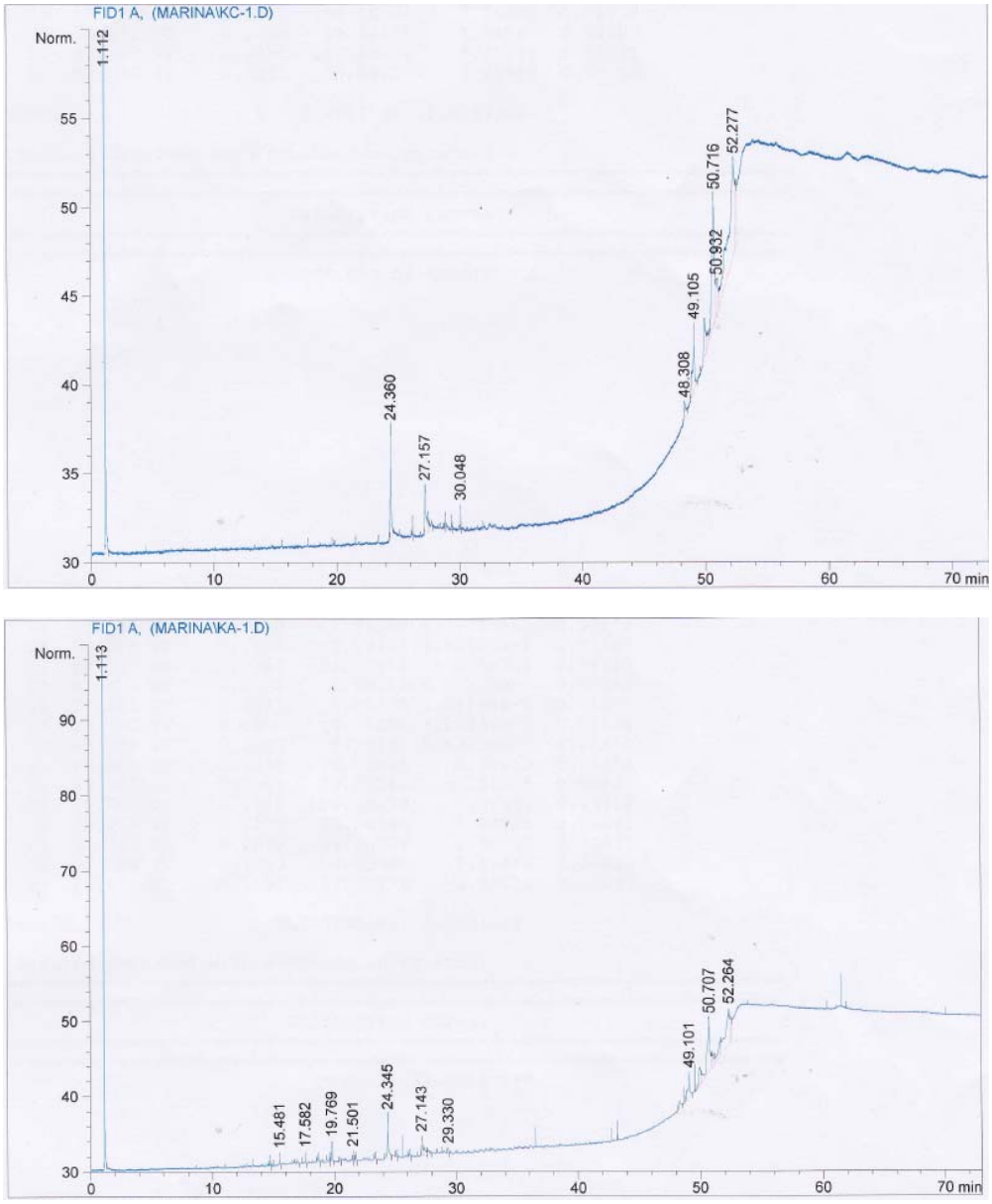

Figure-1. GC chromatograms of oily wastewater from workshop K extracted by dichloromethane after inoculation with KC (No bacterial cultures) and KA (*Bacillus cereus*) after 5 days of incubation at 30ºC.

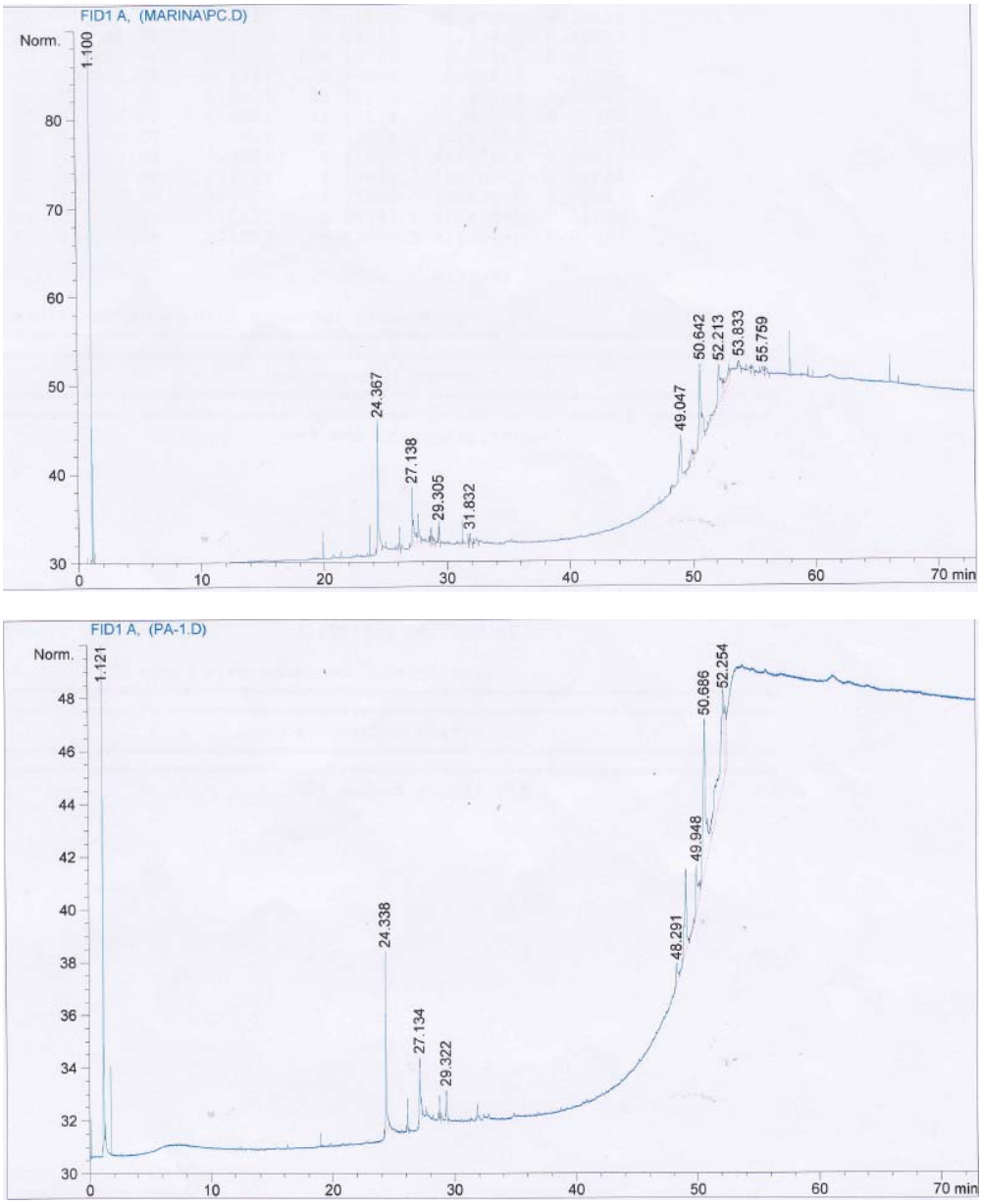

Figure-2. GC chromatograms of oily wastewater from workshop P extracted by dichloromethane after inoculation with PC (No bacterial cultures) and PA (*Bacillus cereus*) after 5 days of incubation at 30ºC.

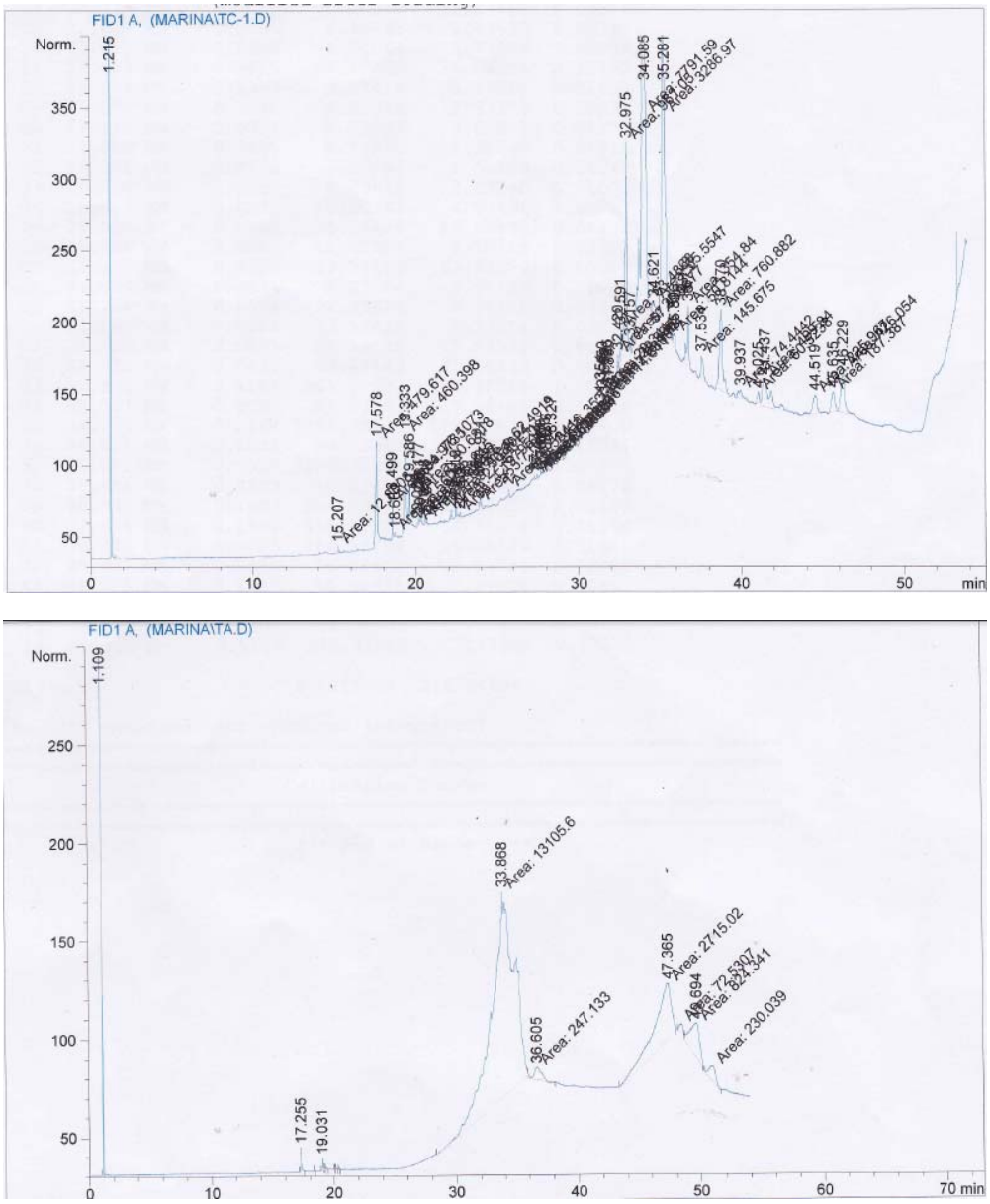

Figure-3. GC chromatograms of oily wastewater from workshop T extracted by dichloromethane after inoculation with TC (No bacterial culture) and TA (*Bacillus cereus*) after 5 days of incubation at 30ºC.

Several research have suggested that, the extent of oil and total petroleum hydrocarbon (TPH) biodegradation is closely linked to the type of oil and its molecular composition and that the alkanes are more susceptible to biodegradation (Huesemann, 1995; Sugiura, *et al*., 1997). Another researcher, Atlas, (1981) stated that, the microbial utilization of hydrocarbon were highly dependent on the chemical composition of the components in the petroleum mixture and environment determinants. In addition, Ijah, (1998) revealed that, bacteria and yeast isolates from tropical soils capable of degrading up to 52% and 69% of crude oil in 16 days, respectively. Furthermore, Das and Chandran, (2011) stated that, specific enzyme system play an important role in the microbial degradation of oil. All of the literatures finding might be able to explain the differences in hydrocarbon degradation in oily wastewater from automotive workshop K, P and T by *Bacillus cereus*. This is because enzymes system is depending on types and the chain length of hydrocarbon in order to introduce oxygen in the substrates to initiate biodegradation (Das and Chandran, 2011). In addition, the specification of chosen microorganisms and their intrinsic capability of degradation of hydrocarbon also can affect the degradation of hydrocarbon in oily wastewater (Junior, *et al.*, 2009).

Basically, the results of this study (3% to 91%) was in agreement with the finding from other researches where they reported that the efficiency of hydrocarbon biodegradation by bacteria, yeast and fungi in environment was ranged from 6% (Jones, *et al*., 1970) to 82% (Pinholt, *et al*., 1979) for soil fungi, 0.13% (Jones, *et al.*, 1970) to 50% (Pinholt, *et al*., 1979) for soil bacteria, and 0.003% (Hollaway, *et al*., 1980) to 100% (Mulkins and Phillips., 1974) for marine bacteria. The hydrocarbon degradation experiment demonstrated that *Bacillus cereus* is useful to assess the potential for natural attenuation of hydrocarbon contaminated environment. Interestingly, *Bacillus* sp. is the only organisms that been implicated in hydrocarbon degradation (Amund and Adebiyi, 1991; Atlas, 1992; Nwachuku and Ugoji, 1995; Nwachukwu, 2001). *Bacillus* sp. also often been reported as an effective agent for hydrocarbon degradation (Benkacaker and Ekundato, 1997; Diaz, *et al.*, 2000). Another researcher, Ghazali, *et al*. (2004) also stated that, isolated *Bacillus* sp. from hydrocarbon contaminated soils has a potential to biodegrade benzene, crude oil, decanol, ethyl-benzene, n-tetradecanol, octanol and 0-xylene.

In conclusion, this study demonstrated that, hydrocarbon degrading organism could be isolated from hydrocarbon polluted area and *Bacillus cereus* found to be the highest performance among the bacterial isolated. This could be suggested that, *Bacillus cereus* has a potential to be used as hydrocarbon degrading organism in bioremediation for oil contaminated areas.

ACKNOWLEDGEMENT

This research was financially supported by Short Term Grant no R/SGJP/A03.00/00148A/002/2010/000026 Universiti Malaysia Kelantan. We would like to thank all the automotive workshops in Kota Bharu, Kelantan which supported us in terms of providing sampling venue for this project.

REFERENCES

Ainon Hamzah, Amir Rabu, Raja Farzarul Hanim, Raja Azmy and Noor Ainni Yussof. 2010. Isolation and Characterization of bacteria degrading Sumandak and south angsi oils. Sains Malaysiana. 39(2): 161-168.

Amund O.O. and Adebiyi A.G. 1991. Effect of viscosity on the biodegradability of automotive lubricating oils. Tribology International. 24: 235-237.

Antai S P. 1990. Biodegradation of Bonny light crude oil by Bacillus sp and Pseudomonas sp. Waste Management. 10: 61-64.

Atlas R.M. 1981. Microbial Degradation of petroleum hydrocarbons: an experimental perspective. Microbiology Revision. 45(1): 180-209.

Atlas R.M. 1992. Petroleum microbiology. Encycl. Microbiol. Academic Press, Baltimore, Md, USA. pp. 363-369.

Benkacaker M.O. and Ekundayo J.A. 1997. Applicability of evaluating the ability of microbes isolated from an oil spill site to degrade oil. Environmental Monitoring Assessment. 45: 259-272.

Boboye B., Olukunle O. F. and Adetuyi F. C. 2010. Degradative activity of bacteria isolated from hydrocarbon polluted site in llaje, Ondo State Nigeria. African Journal of Microbiology Research. 4(23): 2484-2491.

Das K. and Mukherjee A.K. 2007. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from petroleum oil contaminated soil from North-East India. Bioresource Technology. 98: 1339-1345.

Das N. and Chandran P. 2010. Review Article Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. SAGE-Hindawi Access to Research Biotechnology Research International. 2011(ID 941810): 13.

Diaz M.P., Grigson S.J.W., Peppiatt C.J. and Burgess J.G. 2000. Isolation and characterization of novel hydrocarbon-degrading euryhaline consortia from crude oil and mangrove sediments. Marine Biotechnology. 2: 522-532.

Ghazali F.M., Abdul R.N.Z., Salleh A.B. and Basri M. 2004. Biodegradation of hydrocarbons in soil by microbial consortium. International Biodetermination Biodegradation. 54: 61-67.

Hollaway S.L., Faw G.M. and Sizemore R.K. 1980. The bacterial community composition of an active oil field in the Northwestern Gulf of Mexico. Marine Pollution Bulletin. 11: 153-156.

Huesemann M.H. 1995. Predictive model for estimating the extent of petroleum hydrocarbon degradation in contaminated soils. Environmental Science Technology. 29: 7-18.

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

Jones J., Knight M. and Byron J.A. 1970. Effect of gross population by kerosene hydrocarbons on the microflora of a moorland soil. Nature. 227: 1166.

Junior J.S., Mariano A.P. and Angelis D.F. 2009. Biodegradation of biodiesel/diesel blends by Candida viswanathii. African Journal of Biotechnology. 8(12): 2774-2778.

Joshi P. A. and Pandey G. B. 2011. Screening of Petroleum Degrading Bacteria from Cow Dung. Research Journal of Agricultural Sciences. 2(1): 69-71.

Kanluen R. and Amer S.I. 2000. A new treatment successfully removes contaminants from oily wastewater generated by aircraft maintenance operations. Environmental Protection. Aquachem Incorporation.

Mulkins G.J. and Phillips J.E. 1974. Stewart Distribution of hydrocarbon utilizing bacteria in northwestern Atlantic waters and coastal sediments. Canada Journal of Microbiology. 20: 955-962.

Nwachukwu S.U. 2001. Bioremediation of sterile agricultural soils polluted with crude petroleum by application of the soil bacterium, Pseudomonas putida, with organic nutrient supplementations. Current Microbiology. 42(2): 231-236.

Nwachukwu S.U. and Ugoji E.O. 1995. Impacts of crude petroleum spills on microbial communities of tropical soil. International Journal of Ecology Environmental Science. 21: 169-176.

Obuekwe C.O., Al-Jadi Z.K. and Al-Saleh E.S. 2009. Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. International Biodeterioration and Biodegradation. 63: 273-279.

Pinholt Y., Struwe S. and Kjoller A. 1979. Microbial changes during oil decomposition in soil. Holarctic Ecology. 2: 195-200.

Ron E.Z. and Rosenberg E. 2001. Natural roles of biosurfactants. Environmental Microbiology. 3: 22D236.

Sorkhoh N.A., Ibrahim A.S., Ghannoum M.A. and Radwan S.S. 1993. High-Temperature Hydrocarbon Degradation by Bacillus stearothermophilus from oil-polluted Kuwaiti dessert. Applied Microbiology and Biotechnology. 39: 123-126.

Sugiura K., Ishihara M., Shimauchi T. and Harayama S. 1997. Physicochemical properties and biodegradability of crude oil. Environmental Science and Technology. 31: 45-51.

Tri P.T. 2002. Oily wastewater treatment by membrane bioreactor process coupled with biological activated carbon process. Degree of Master of Engineering. Ho Chi Minh City University of Technology.

U.S. Environmental Protection Agency. 1999. Diesel fuel quality, advanced notice of proposed rulemaking. EPA 420-F-99-011, Office of Mobile Sources, U.S. Environmental Protection Agency, Washington, D.C., USA.

Vidali M. 2005. Bioremediation: An overview. Pure Applied Chemical. 7: 1162-1172.

Zhang X., Peterson C.L., Reece D., Haws R. and Moller G. 1998. Biodegradability of biodiesel in the aquatic environment. Trans. ASAE. 41: 1423-1430.

Zhuang W.Q., Tay J.H., Maszenan A.M. and Tay S.T.L. 2002. Bacillus naphthovorovans sp. Nov. from oil contaminated tropical marine sediments and its role in naphtalene biodegradation. Applied. Microbiology Biotechnology. 58: 547-553.