ISSN: 0067-2904
GIF: 0.851

Determination of Oil Biodegradation Activity of *Pseudomonas aeruginosa* Isolated from Soil and Molecular Detection of Responsible Genes

Maysoon Munther Al-Hamando*
Department of Biology, College of Sciences, University of Baghdad, Baghdad, Iraq

Abstract

This study concerns the isolation of oil degraded bacterial samples from oil polluted soil in Al-Dora refinery/ Baghdad – Iraq. Soil samples (15) were on mineral salt agar medium (MSM) used to screen the oil degrading bacteria by forming clear zones around the colonies. To confirm the degradation of oil by these bacteria, the isolates were inoculated in mineral salt broth, 15 isolates of *Pseudomonas spp.* was detected from which two isolates identified as *P. aeruginosa* by morphological, physical and biochemical characteristics that confirmed by using Vitick identification system. Growth was estimated in terms of whole cell by measuring optical density at 620 nm and free extract protein was estimated by protein measurement with Folin phenol reagent method. PCR technique was used to detect the *alkB* genes from the DNA extracted from the two *Pseudomonas aeruginosa* isolates.

Keywords: *Pseudomonas aeruginosa, alkB* genes, oil degradation bacterial.

الكشف عن بكتيريا *Pseudomonas aeruginosa* المحللة للدهون والمعزولة من التربة والتحري الجزيئي عن الجينات المسؤولة عن التحلل

ميسون منذر الحمندو*
قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

في هذه الدراسة تم التحري عن البكتيريا المحللة للدهون والمعزولة من التربة الملوثة في مصفى الدورة/ بغداد العراق حيث تم اخذ 15 عينة من التربة والكشف عن البكتيريا المحللة للدهون باستخدام وسط اكار الاملاح المعدنية من خلال تكون هالات شفافة حول مستعمرات البكتير يا النامية على الوسط المذكور وللتأكد من قابلية هذه البكتيريا المعزولة من تحليل الدهون تم تلقيحها في وسط الاملاح المعدنية السائل وقد تم عزل وتشخيص 15 عزلة بكتيرية عائدة لجنس الزوائف من عينات التربة قيد التجربة وتشخيص عزلتين تعود لنوع الزوائف الزنجارية من خلال الخواص المظهرية والفيزيائية والبايوكيميائية والتأكد من التشخيص بواسطة نظام Vitik. ثم تم قياس النمو البكتيري بواسطة قياس الكثافة الضوئية بطول موجي 620 nm بينما تم قياس مستخلص البروتين من خلايا البكتيريا بواسطة طريقة قياس البروتين باستخدام كاشف Folin phenol، كما استخدامت تقنية تفاعل الكوثرة للكشف عن الجينات المسؤولة عن تحلل الدهون من المادة الوراثية المستخلصة من العزلتين العائدة لبكتيريا الزوائف الزنجارية.

*Email: maysoon_alhamando@yahoo.com

Introduction

Soil contamination or soil pollution is caused by the presence of xenobiotic (human-made) chemicals or other alteration in the natural soil environment. It is typically caused by industrial activity, agricultural chemicals, or improper disposal of waste. The most common chemicals involved are petroleum hydrocarbons, polynuclear aromatic hydrocarbons (such as naphthalene and benzo(a)pyrene), solvents, pesticides, lead, and other heavy metals. Contamination is correlated with the degree of industrialization and intensity of chemical usage [1].

Contaminated or polluted soil directly affects human health through direct contact with soil or via inhalation of soil contaminants which have vaporized; potentially greater threats are posed by the infiltration of soil contamination into groundwater aquifers used for human consumption, sometimes in areas apparently far removed from any apparent source of above ground contamination The most common chemicals involved are petroleum hydrocarbons, solvents, pesticides, lead, and other heavy metals [2].

Health consequences from exposure to soil contamination vary greatly depending on pollutant type, pathway of attack and vulnerability of the exposed population. Chronic exposure to chromium, lead and other metals, petroleum, solvents, and many pesticide and herbicide formulations can be carcinogenic, can cause congenital disorders, or can cause other chronic health conditions. Industrial or man-made concentrations of naturally occurring substances, such as nitrate and ammonia associated with livestock manure from agricultural operations, have also been identified as health hazards in soil and groundwater [3]. The aim of this study is to select the bacteria which can breakdown of crude oil.

Materials and Methods

Sample Collection

From polluted soil in Al-Dora refinery in Baghdad /Iraq, 15 samples were collected to isolate oil degradation bacteria. Samples were collected at a depth of 5cm from the surface of the soil in a sterile polythene bags and tightly packed then carefully transferred to the laboratory for the analysis and stored at 4°C aseptically before processing [4]**.**

Screening of oil degrading Bacteria

A volume of 5 gm of soil sample was inoculated in mineral salt medium broth (MSM) and was incubated at 37°C for 2 days. After incubation 0.1ml of the broth culture, that matched the turbidity of the 0.5 McFarland turbidity standards (1.5 x 10^8 CFU/ml), was plated in mineral salt agar medium using spread plate technique. An ethereal solution of crude oil (10% w/v) was uniformly sprayed over the surface of the agar plate. The ether immediately vaporized and thin layer of oil remained on the entire surface. The plates were incubated at 25°C for 2 days. The organisms that formed clear zones around the colonies were considered as crude oil degraders [5].

Oil Degradation

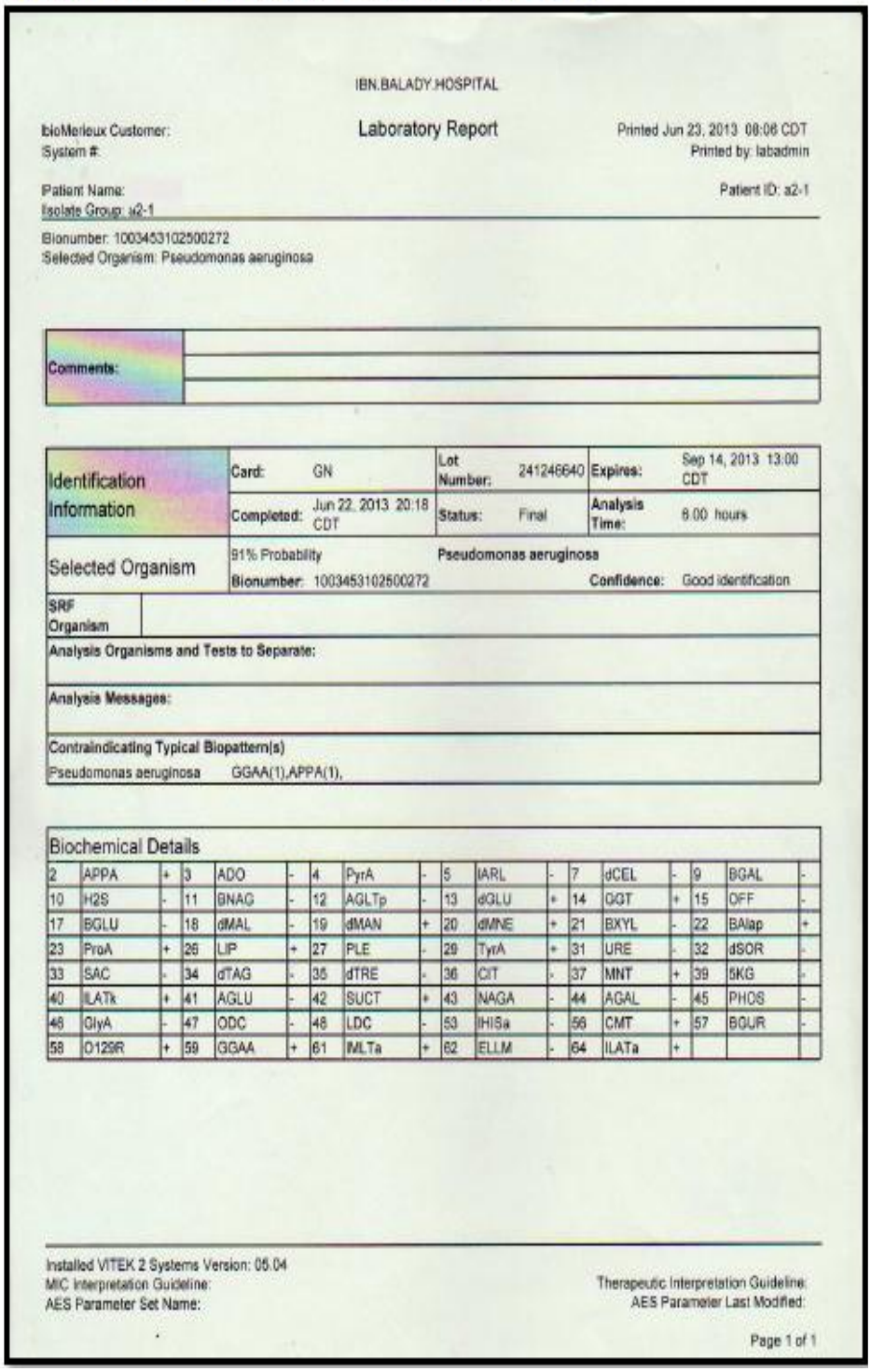
To confirm the degradation of oil, mineral salt medium broth (MSM) supplemented with 2 ml of oil was used. About 100 ml medium was dispensed in 250ml conical flasks. The medium was inoculated with 2 ml of oil degrading bacteria, that matched the turbidity of the 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/ml), (bacteria obtained by screening of crude oil degrading bacteria) and incubated at 28°C for 10 days on a rotary shaker at 175rpm [6].

Estimation of Growth

Growth was estimated in terms of whole cell. 5ml of MSM medium inoculated with activated bacteria suspension was centrifuged at 3000rpm for 10min. The pellet was washed twice with normal saline and resuspended in 1.0ml of 4.6M NaOH at boiling temperature for 10 min. to obtain cell free extract protein concentration in cell free extracts was estimated by protein measurement with the folin phenol reagent method. Growth was also monitored by measuring optical density at 620nm [5, 6].

Identification of the isolates:

The colony characteristics, the morphology of the isolates, pigmentation, staining reactions, physiological and biochemical characteristics were examined by standard methods and the isolates were identified and confirmed the results by vitik identification system (Figure-1).



IBN.BALADY.HOSPITAL

bioMerieux Customer:
System #:

Laboratory Report

Printed Jun 23, 2013 08:06 CDT
Printed by: labadmin

Patient Name:
Isolate Group: a2-1

Patient ID: a2-1

Bionumber: 1003453102500272
Selected Organism: Pseudomonas aeruginosa

Comments:	

Identification Information	Card: GN	Lot Number: 241246640	Expires: Sep 14, 2013 13:00 CDT
	Completed: Jun 22, 2013 20:18 CDT	Status: Final	Analysis Time: 8.00 hours
Selected Organism	91% Probability	Pseudomonas aeruginosa	
	Bionumber: 1003453102500272		Confidence: Good identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			
Pseudomonas aeruginosa	GGAA(1),APPA(1),		

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	+	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Installed VITEK 2 Systems Version: 06.04
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

Figure 1- The identification of *Pseudomonads aeuroginosa* by Vitik identification system

Detection of *alkB and alkB1* genes

Extraction of genomic DNA

DNA were extracted from 2 isolates by using a commercial purification kit (Presto Mini Genomic DNA Kit, (Bioneer, Korea), which is used for extraction of DNA from Gram negative and Gram positive bacteria. The Gram negative bacteria extraction protocol was used and the extracted DNA was detected in 0.8% agarose gel by gel electrophoresis. The extracted DNA concentration and purity was detected by Nano-drop system.

Amplification of *alkB and alkB1* genes by Polymerase Chain Reaction

Two isolates were screened by PCR for the detection of genes involved in the biodegradation of n-alkanes as described by [7]. Primers used are shown in Table-1.

Table 1- Primers used in this study

Gene	Primer sequences	Fragment size
alkB	Fw(5- TGGCCGGCTACTCCGATGATCGGAATCTGG -3) Rv (5- CGCGTGGTGATCCGAGTGCCGCTGAAGGTG -3)	870 bp
alkB1	Fw(5- CGGGGTTCAAGGTCGAGCAT -3) Rv (5- CAGGACCAGGTTGGTGAAGA -3)	434 bp

PCR reactants were prepared in final volume of 50 µl, as follows in (Table-2) and tab:

Table 2- PCR reactants in final volume of 50 µl

PCR reactants	Volume
Template genomic DNA < 250 ng	5 µl
Forward and reverse primer 10 pmol/ µl	5 µl
Go Taq green Master mix 2X	25 µl
Deionized D.W	10 µl

While the PCR reaction was performed according to the following conditions in (Table-3), then PCR products were detected on 1.5% agarose gel by gel electrophoresis after staining with ethidium bromide and visualized by UV illumination photographed.

Table 3- PCR amplification program

No.	Stages	Temperature & Time		
1	Initial denaturation	94 °C	5 min.	
2	Denaturation	94 °C	30 sec.	35 cycles
3	Annealing	59 °C	30 sec.	
4	Extension	72 °C	45 sec.	
5	Final extension	72 °C	5 min.	

Results and Discussion

Pseudomonas was identified by using morphological, physiological and biochemical characteristics in addition to Gram stain reaction from all 15 isolates of soil. Out of 15, two isolates were identified as *P. aeruginosa* by using Vitick identification system.

In MSM agar medium, isolates of *P. aeruginosa* which showed maximum clearing zone of oil in the medium comparing with the control (MSM agar medium without bacterial growth) (Figure-2), indicates the presence of oil degradation bacteria, may be due to production of emulsifiers, surfactants etc.

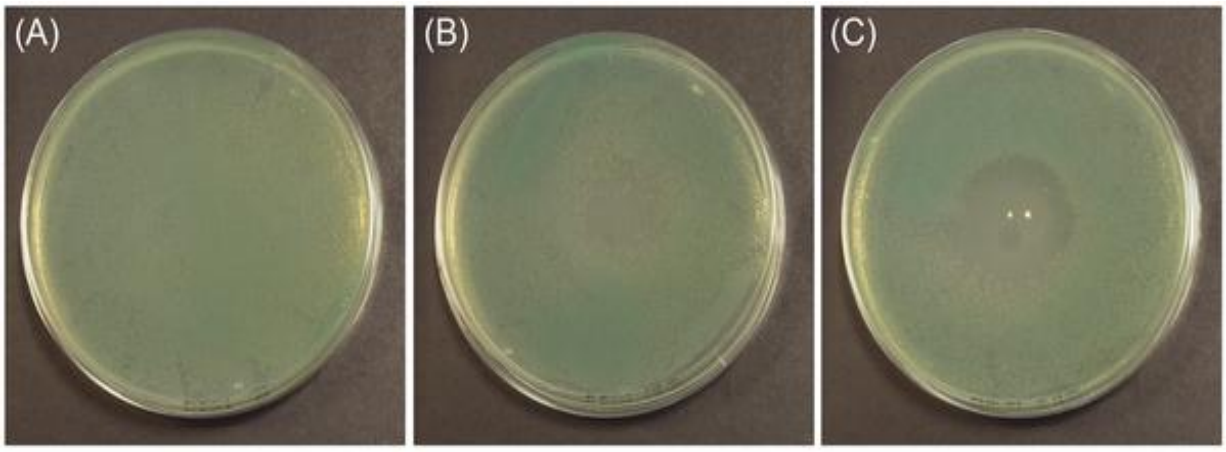


Figure 2- The clear zone of *P. aeruginosa* isolates (A: control, B & C: tested bactria)

Efficiency of *P. aeruginosa* isolated from polluted soil for oil degradation was investigated by screening of oil degradation using MSM broth, Isolates of *P. aeruginosa* were able to utilize oil as a sole carbon and energy source and to degrade it metabolically after 10 days (Figure-3).

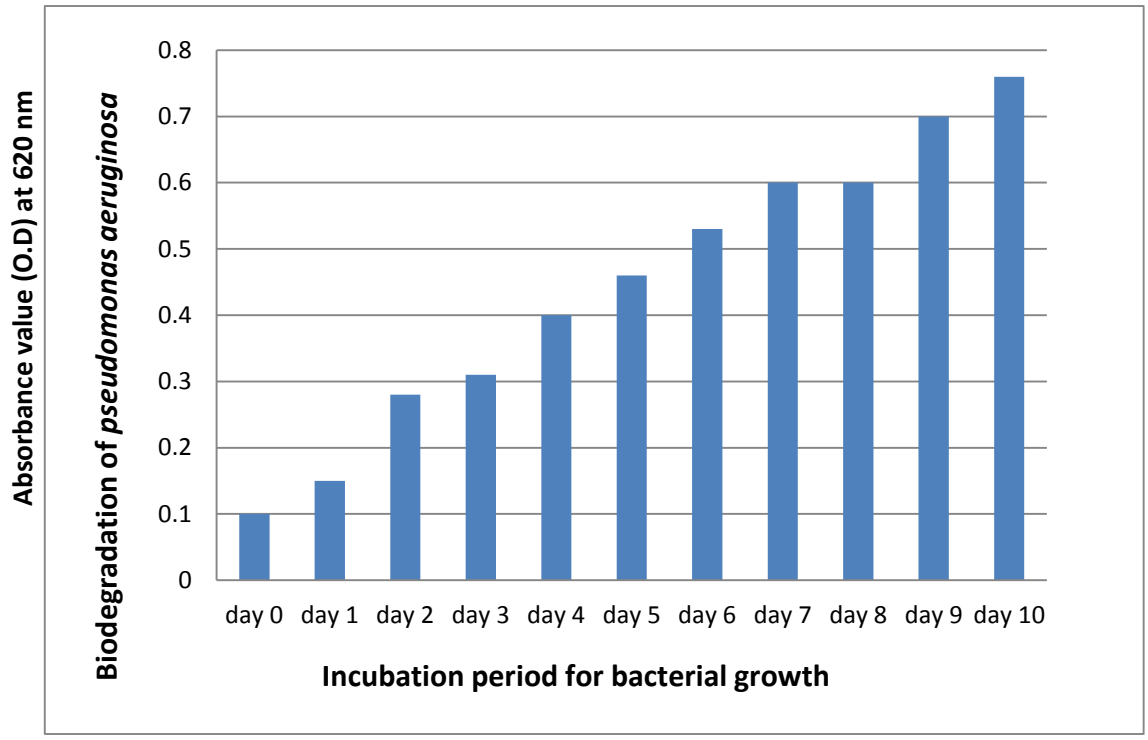


Figure 3- Biodegradation of oil by *P. aeruginosa*

The results in this study agreed with [8, 9] who mentioned that the maximum of clearing zone formed around bacterial isolates grown on mineral salt agar medium covered with an ethereal solution of crude oil at 25˚C for 2 days was observed, indicated their ability of crude oil degradation and they can be considered as crude oil degraders. The growth results were clarified by growth estimation in a minimal salt broth medium supplied with 100 ml oil and the absorbance was measured at 620 nm (Table-4).

Table 4- Optical density of tested bacterial isolate

Days no.	O.D at 260 nm wave length
day 0	0.1
day 1	0.15
day 2	0.28
day 3	0.31
day 4	0.4
day 5	0.46
day 6	0.53
day 7	0.6
day 8	0.6
day 9	0.7
day 10	0.76

The results showed that the biodegradative activity increases with time. The consumption of crude oil was observed after different incubation periods that the greater level of degradation takes place in the first 5 days of culturing, which means that the component gradient was degraded. The yield biomass for bioremediation purpose is very convenient with a little biomass production and a high utilization of the carbon source in order to not altering the ecosystem [10].

In conclusion *P. aeruginosa* is capable of assimilating crude oil. The presence of different pathways is favorable for contaminated soil bioremediation [11]. In order to detect the *alkB genes*, two DNA of *P. aeruginosa* isolates were extracted, their concentration and purity was listed in (Table-5).

Table 5- The concentration and purity of extracted DNA

Isolates	Concentration	Purity
Ps1	167.4 ng/µl	1.85
Ps2	208.8 ng/µl	1.85

Multiplex PCR technique was carried on to detect *alkB* and *alkB1* genes in two isolates of *P. aeruginosa* that are responsible of oil biodegradation activity. In this assay, specific primers were used and the results showed a band with 434 bp in the tested isolates after being confirmed by gel electrophoresis technique with molecular weight of *alkB1* gene by using 100bp DNA ladder (Figure-4).

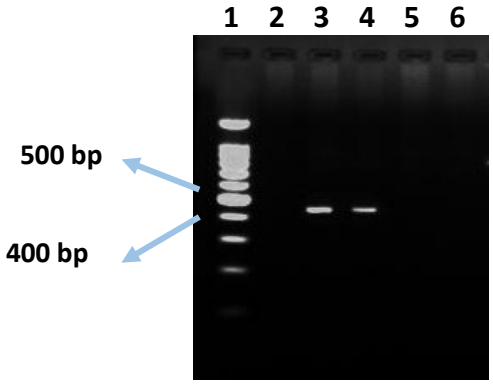


Figure 4- Agarose gel electrophoresis of PCR product for Pseudomonas isolates.

Lane 1: M (DNA marker 100bp ladder)
Lanes 2: Distilled water as control
Lanes (3-4): Detection of *alkB1* in P1, P2 isolates
Lanes (5-6): Detection of *alkB* in P1, P2 isolates
Bands were fractionated by electrophoresis on a 1.5% agarose gel at 5 volt/cm for 2 hrs. visualized under UV light source after staining with ethidium bromide.

While DNA bands that represented the *alkB* gene was absent in the same isolates, which confirm that *P. aeruoginosa* degrading the n-alkanes had the gene encoding for *alkB1* that enable them of degradation of long n-alkenes chains only, while these bacteria have no ability to degrade short chains [12,13].

References

1. Panagos, P., Van Liedekerke, M., Yigini, Y., Montanarella, L. **2013**. Risk Assessment Guidance for Superfund, Human Health Evaluation Manual, Office of Emergency and Remedial Response, U.S. Environmental Protection Agency, Washington D.C. 20450.
2. Snyder, C. **2005**. The dirty work of promoting "recycling" of America's sewage sludge". *Int J Occup Environ Health*, 11(4): 415–27.
3. Olawoyin, S. Oyewole, A. Grayson, L. R. **2012**. Potential risk effect from elevated levels of soil heavy metals on human health in the Niger delta, *Ecotoxicol. Environ. Saf*., 85(1):120–130
4. Afuwale , C. and Modi, H. A. **2012**. Study of bacterial diversity of crude oil degrading bacteria isolated from crude oil contaminated sites. *Life sciences Leaflets*, 6: 13-23.
5. Gupta, S.K. Kincaid, C.T. Mayer, P. R. Newbill, C.A. and Cole C.R**. 1982**. A multidimensional finite element code for the analysis of coupled fluid, energy and solute transport. *Journal of Environmental and Public Health.* 10 (13): 6311.

6. John,R.C. and Okpokwasili, G.C. **2012**. Crude oil-degradation and plasmid profile of nitrifying bacteria isolated from oil-impacted mangrove sediment in the Niger delta of Nigeria. *Bull. Environ.Contam.Toxicol*., 88:1020-1026.

7. Aboulkacem, A., A. Barguigua, I. Maroui, M. Timinouni and A. Belhaj. **2014.** Genetic characterization of environmental and clinical *Pseudomonas aeruginosa* strains egrading the n-alkanes. *International Journal of Scientific and Research Publications,* (10): 2250-3153.

8. Latha, R. and R. Kalaivani, **2012**.Bacterial degradation of crude oil by gravimetric analysis. *Advances in Applied Science Research*, 3: 2789-2795.

9. Vyas, T.K. and Dave, B.P. **2007**. Effect of crude oil concentration, temperature and pH on growth and degradation of crude oil by marine bacteria .2007.*Indian Journal of Marine Sciences*, 36(1): 76 - 85.

10. Ábalos, A., Viñas, M., Sabaté, J., Manresa, M. A. and Solanas, AM. **2004**. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10, *Biodegradation*, 15:249–260.

11. Abed, R. M. M, Safi, N. M. D, Koster, J, Beer, D, El-Nahhal, Y. Rullkotter, J and Garcia-Pichel, F. **2001**. Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. *Applied and Environmental Microbiology*, 68: 1674–1683.

12. Kloos K., J. C. Munch, M. Schloter.**2006**. A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. *Journal of Microbiological Methods*. 66: 486–496.

13. Mathew, S. and Y. H. Hobani.**2015**. A new method for detection of oil degrading genes in *Pseudomonas aeruginosa* based on transformation and PCR Hybridization. *International Journal for Biotechnology and Molecular Biology Research*, 6 (1): 1-6.