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USE OF CELLULOLYTIC BACTERIA FOR PETROLEUM HYDROCARBON BIODEGRADATION

Lovet. T. Kigigha and Ebimoboere E. Odayibo
Department of Biological Sciences, Niger Delta University Wilberforce Island, PMB 71, Yenagoa, Bayelsa State, Nigeria

ABSTRACT

Cellulolytic bacteria were isolated from decaying leaves of fruit trees viz. Mango (MN), Guava (GU) and Bush mango (BM). Ten isolates were randomly isolated from each type of the leaf sample and identified. The cellulolytic bacteria that were tentatively identified were: *Staphylococcus* sp 3(6.7)*, *Bacillus* and other Gram positive rods 5(16.7), *Enterococcus* sp 3(10.0), *Serratia* sp 1(3.3), *Escherichia* sp 3(10.0), *Pseudomonas* sp 7(23.3), *Flavobacteria / Xanthomonas* spp 1(3.3), *Proteus* sp 3(10.0), *Alcaligenes* sp 2(6.7) and *Enterobacter* sp 2(6.7); (the number in parenthesis (*) represent percentages of occurrence). The bacteria species which occurred in all the leave samples were: *Staphylococcus sp*, *Bacillus* and other Gram positive rods, *Escherichia* sp, *Pseudomonas* sp, and the *Proteus* sp. The TCFU g $^{-1}$ of the leaf samples indicated that guava leaf was significantly higher than those of mango and bush mango (P = 0.007) ranging from 1.20E+05 to 4.30E+05. The tentatively identified hydrocarbonoclasic species were: *Bacillus* and other Gram positive rods, *Escherichia* sp, *Pseudomonas* sp, *Proteus* sp, *Alcaligenes* sp and the *Enterobacter* sp. The objective of the paper was the characterization of the cellulolytic bacteria from decaying leaf samples and the testing of these isolates for their ability to degrade petroleum hydrocarbons.

KEYWORDS: Cellulose, petroleum hydrocarbon, bacteria, biodegradation, bioremediation.

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Corresponding Author: *lovet_k@yahoo.com*

INTRODUCTION

Petroleum hydrocarbon is a major source of energy for industrial and for domestic purposes. As the demand for crude oil is ever increasing, leaks and accidental spills occur regularly during the exploration, production, refining, transportation and storage of petroleum. This has constituted a major source of contamination of both land and marine ecosystems. The addition of hydrocarbons to an ecosystem results in the selective increase in microorganisms that are capable of utilising the hydrocarbons.

The technology commonly used for the remediation of petroleum hydrocarbon contaminants from an ecosystem include mechanical collection using floating booms, burying in landfills, evaporation of volatile fractions, and washing. Chemical methods employ the use of dispersants to aid sedimentation and the application of adsorbents. However, these technologies are expensive and could lead to further contamination of the environment. Biological methods are considered to be more eco-friendly as they offer *in situ* biodegradation of oil fractions by microorganisms.

Earlier studies by John and Okpokwasili (2012), on crude oil degradability and plasmid profile of autotrophic nitrifying bacteria, Nitrosomonas and Nitrobacter species, isolated from mangrove sediment in the Niger Delta of Nigeria, indicated that nitrifying bacteria could utilize kerosene, diesel oil, jet fuel and engine oil as carbon sources. Also, Latha and Kalaivani, 2012, investigated the isolation of bacteria from crude oil contaminated site using

gravimetric analysis of degradation. Indication was that increase in oil degradation was correlated to an increase in cell number which implied that the bacterial isolates were responsible for the oil degradation. The present study involves the characterization of the cellulolytic bacteria from decaying leaf samples and the testing of these isolates for their ability to degrade petroleum hydrocarbons.

Source of leaf samples

Mango (*Mangifera indica)*

Mangifera indica (Mango) peel and pulp contain compounds, such as the pigments carotenoids and polyphenols, and omega-3 and -6 poly-unsaturated fatty acids. Photochemical and nutrient content appears to vary according to the species. Up to 25 different carotenoids have been isolated from mango pulp. Peel and leaves also have significant polyphenolic content, including xanthonoids, mangiferin and garlic acid. In Nigeria, different parts of mango are commonly used as herbal preparations in the treatment of tooth ache, gastrointestinal disorders, dysentery, diarrhoea gastrointestinal tract infections, respiratory and urinary tract infections. Phytochemicals screening showed the presence of active pharmacological components such as tannins, saponins, sterols, cardiac glycoside, flavonoid and alkaloids. These components are known to be biologically active because they protect the plant against infections and predations by animals. Phytochemicals generally exert their antimicrobial activities through different mechanisms to that of synthetic drugs (Bala, 2006).

Guava (Psidum guajava L)

Extracts of the leaves and stem barks of *Psidum guajava* were studied by Elekwa et al, 2009, for their phytochemical constituents. Results revealed the presence of alkaloids, saponins, cardenolides with steroided rings, and cardenolides with deoxy - sugar. Aqueous extracts were inhibitory to *Bacillus subtilis* and *Fusarium* spp. The presence of these constituents appear to support the uses of guava in traditional medicine.

Bush mango (Irvingia gabonensis)

Irvingia gabonensis (Aubry-Lecomte ex Ororke) Baillon. Family: *Irvingiaceae* (African mango , dika, odika , ogbono, bush mango or iba-tree). African mango leaves and root extracts have been documented to have inhibitory activity against several bacteria and fungi diseases. Potential mechanisms of action include membrane disruption by terpenoids and inactivation of microbial adhesion, enzymes, and cell envelope transport proteins by allergic acid-like compounds (Kuete et al, 2007; Fadare and Ajaiyeoba, 2008).

Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)n$, a polysaccharide consisting of a linear chain of several hundred to over ten thousand β (1→4) linked D-glucose units. Cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulose is one of the most abundant organic polymers on Earth (Klemm et al. 2005). Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cello-dextrins or completely into glucose units; this is a hydrolysis reaction. Most mammals have only very limited ability to digest dietary fibres such as cellulose. Some ruminants like cows and sheep contain certain symbiotic anaerobic bacteria (like *Cellulomonas*) in the flora of the rumen, and these bacteria produce enzymes called cellulases that help the microorganism to break down cellulose. The breakdown products are then used by the ruminants and the symbiotic bacteria as nutrient (Brás, 2008).

Petroleum hydrocarbons (Crude oil)

Petroleum hydrocarbons (PHC) naturally occur as organic compounds found in the anaerobic decay of plants and/or animal remains under diagenic conditions beneath geological formations. Petroleum hydrocarbons are composed of various proportions of hydrogen and carbon containing compounds such as the alkanes, aromatics and polycyclic aromatic hydrocarbon (PAHs) components. Petroleum hydrocarbons are classified into two broad groups viz. aliphatic (open chain) compounds and the cyclic (closed chain) compounds.

Distribution of Hydrocarbonoclasic Microorganisms

Kigigha and Underwood (2013a) reviewed the action of indigenous microorganisms on petroleum hydrocarbon and indicated that many indigenous microbes have the ability to utilise hydrocarbons as their major source of energy and carbon, and that such microbes were widely distributed in nature. Once a site is contaminated, the microbial community composition will be greatly changed both in quantity and composition. Populations of hydrocarbon-degraders normally constitute less than 1% of the total microbial communities, but when oil pollutants are present these hydrocarbon-degrading populations increase. Certain hydrocarbon-degrading populations become dominant in oil impacted environments because of natural selection resulting from the presence of oil contaminants.

MATERIALS AND METHODS

Samples of leaves

Decaying leave samples were collected from beneath three fruit trees within Niger Delta University campus. Any other decaying source of cellulose material from different plants could be used as a source of cellulose (Klemm et al. 2005). The samples were from Mango (MN), Guava (GU) and Bush Mango (BM). They were collected separately into pre-sterilised beakers.

Crude oil

Qua Iboe light crude was kindly provided for by the Nigeria National Petroleum Cooperation (NNPC) in stoppered pre-sterilised glass conical flasks and stored in a refrigerator to prevent evaporation of volatile fractions.

Sterilisation of Materials

All the glass-wares used for the purpose of culturing were washed and thoroughly rinsed for three times with distilled water and autoclaved for 15 minutes at 121^{o}C. All reagents were prepared according to manufacturers specifications.

Enumeration of microbial load of leaf samples.

Ten grams of each of the decaying leaf samples after drying to constant weight in desiccators, were separately ground into fine powder. One gram of each were subjected to ten-fold serial dilution using normal saline. Spread plates in triplicates were made on Nutrient Agar using 0.1 ml from the dilutions from the 4th ten-fold serial dilution tubes and colonies counted after incubated at 37^{o}C for 24 hours. The TCFU g^{-1} of the leaf samples were determined in triplicates.

Screen Test for Cellulose Utilisation by Isolates

Cellulose degrading bacteria were isolated from decaying leaf samples using serial dilution and pour plate method. Bacteria were further identified by morphological and biochemical test (Muhammad et al, 2012). Three grams each of the powdery decaying leaf samples, serving as sole carbon source were added to 250 ml of molten Agar-Agar preparation respectively. The mixture was rocked gently and thoroughly to aid mixing; this was sterilized in the autoclave and then poured into plates. The content was allowed to solidify at room temperature and surface dried in an incubator at 40^{o}C. A 1.0 ml 24h old filtered broth culture of the ten-fold serial diluted leaf samples (in normal saline) at 10^{-6} dilution, was each separately spread on the Agar-agar plates and then incubated at 37^{o}C for 48-72hrs and the TCFU ml^{-1} was counted.

Enumeration of total hydrocarbon utilizing bacteria

The vapour phrase transfer method (Okpokwasili and Amanchukwu, 1988) was adopted. Agar-agar (21g/litre) was dissolved in distilled water and sterilised by autoclaving. This was allowed to cool and then poured into plates and allowed to solidify at room temperature. Crude oil (1.0 ml) was then used to impregnate sterile filter papers to serve as sole carbon source and sterilized by autoclaving. The filter papers were placed on the cover of the plates. The agar-Agar was spot-inoculated with a loop-full of day-old isolates in broth medium; the plates were then inoculated

at 37^0C for 72 h and observed for growth. An un-inoculated plate was also observed as control. The bacterial isolates which degraded cellulose and could grow on petroleum hydrocarbon were counted.

RESULTS

Using the spread plate method, 1ml filtrate of each of three decaying leaf samples (mango, guava and bush-mango) were tested for their TCFU ml-^{1}as shown in Fig 1. Ten isolates based on their morphological differences were randomly selected from each type of the leaf samples and identified (as shown in Table 1). The cellulolytic bacteria that were tentatively identified were: *Staphylococcus* sp 3(6.7)*, *Bacillus* and other Gram positive rods 5(16.7), *Enterococcus* sp 3(10.0), *Serratia* sp 1(3.3), *Escherichia* sp 3(10.0), *Pseudomonas* sp 7(23.3), *Flavobacteria / Xanthomonas* spp 1(3.3), *Proteus* sp 3(10.0), *Alcaligenes* sp 2(6.7) and *Enterobacter* sp 2(6.7); (the number in parenthesis (*) represent percentages of occurrence). In Fig 1, the TCFU g $^{-1}$ of the leaf samples was shown in which GU was significantly higher than those of mango and bush-mango (P = 0.007); ranging from 1.20E+05 to 4.30E+05. Fig 2, shows the distribution of the cellulolytic bacteria while Fig 3, shows the cumulative frequency of isolation of cellulolytic and PHC degrading bacteria from the leaf samples. The tentatively identified hydrocarbonoclasic bacteria species were: *Bacillus* and other Gram positive rods, *Escherichia* sp, *Pseudomonas* sp, *Proteus* sp, *Alcaligenes* sp and *Enterobacter* sp.

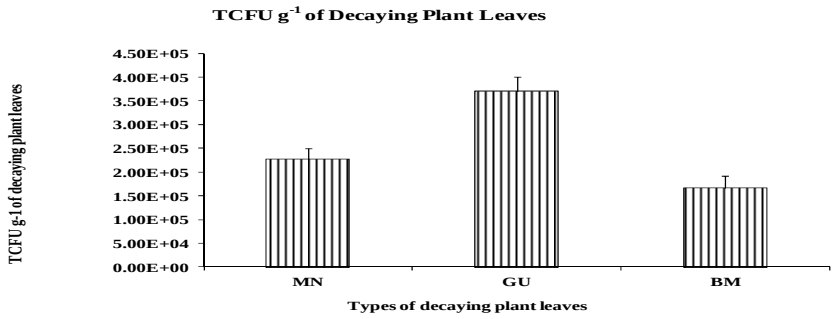

Fig 1: Microbial load of bacteria, TCFU g^{-1} of decaying leaves. The mean values were greater than would be expected by chance, there was a statistically significant difference P= 0.007. MN = Decaying leaf sample mango; GU = Decaying leaf sample of guava and BM = Decaying leaf sample of bush mango. Data = mean (± S. E.; n=3).

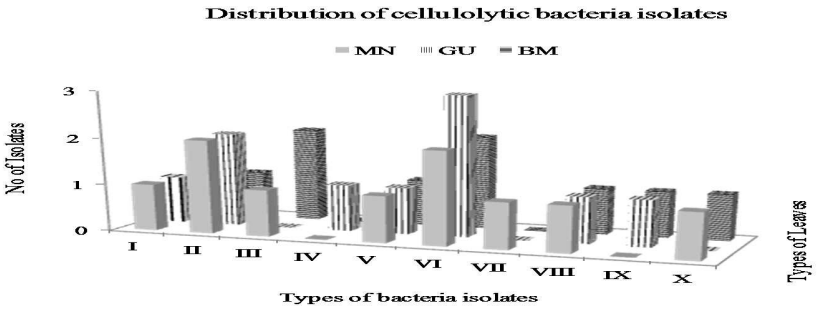

Fig 2: The distribution of cellulolytic bacteria from decaying leaf samples.
Bacteria isolates: I = *Staphylococcus* sp; II = *Bacillus* and other Gram positive rods; III = *Enterococcus* sp; IV = *Serratia species*; V = *Escherichia* sp; VI = *Pseudomonas* sp; VII = *Flavobacteria/Xanthomonas* spp; VIII = *Proteus* IX = *Alcaligenes* sp and X = *Enterobacter* sp

Table1: Characterisation of cellulolytic bacteria isolates from leaf samples

BACTERIA ISOLATES / TESTS	I	II	III	IV	V	VI	VII	VIII	IX	X
Colonial morphology	Golden/ entire	Whitish/dry entire	Creamy / entire	Reddish/ entire	Mucoid/ entire	Mucoid/ entire	Yellow/red /entire	Mucoid/ entire	Mucoid/ entire	Creamy/entire
Gram's reaction	+ve cocci in Clusters	+ve rods in chains	+ve cocci in chains	-ve rods	-ve rods	-ve rods	-ve coco-bacillary	-ve rods	-ve rods	-ve rods
Growth on MSA	+ve	NT	-ve	NT	NT	NT	NT	NT	NT	NT
Tube Coag.	+ve	NT	-ve	NT	NT	NT	NT	NT	NT	NT
Slide Coag.	+ve	NT	-ve	NT	NT	NT	NT	NT	NT	NT
Litmus test	-ve	NT	+ve	NT	NT	NT	NT	NT	NT	NT
Oxidase test	NT	NT	-ve	-ve	-ve		+ve	-ve		-ve
Citrate	NT	NT	NT	+ve	-ve		-ve	+ve		+ve
Sugar Use:	NT	NT	NT							
Lactose Mannitol Glucose Sucrose				-ve +ve +ve -ve	+ve +ve +ve +ve	-ve +ve +ve +ve	- ve - ve - ve - ve	- ve - ve + ve +ve	+ve +ve + ve +ve	+ve +ve + ve +ve
Growth in KIA:	NT	NT	NT							
-Acid/Gas				+ve	+ve	-ve	-ve	-ve	+ve	+ve
-H2S				-ve	-ve	-ve	-ve	-ve	-ve	-ve
-Butt /Slope				R/Y	Y/Y	R/R	Y/Y	R/Y	R/Y	Y/Y
Tentative identification	*Staph*. sp	*Bacil* & other G +ve rods	*Enteroc*. sp	*Serratia sp*	*E. coli*	*Pseudo.* sp	*Flav/Xan*. sp	*Proteus* sp	*Alcaligen . sp*	*Enterobacter. sp*
Cellulolytic bacteria CFU g^{-1} from Leaves:										
MN	1	2	1	0	1	2	1	1	0	1
GU	1	2	0	1	1	3	0	1	1	0
BM	1	1	1	0	1	2	0	1	1	1

Cellulolytic bacteria Cumulative CFU g^{-1} from Leaves	3 (10.)*	5 (16.7)	3 (10.0)	1 (3.3)	3 (10.0)	7 (23.3)	1 (3.3)	3 (10.0)	2 (6.7)	2 (6.7)
PHC bacteria biodegraders Cumulative CFU g^{-1} from Leaves	Nil	3	nil	nil	1	4	nil	2	2	1

SPC were averages of three plate counts; * Numbers in parenthesis represent percentages; NT: Not tested.
The bacteria isolates: I = *Staphylococcus* sp; II = *Bacillus* and other Gram positive rods; III = *Enterococcus* sp; IV = *Serratia species*; V = *Escherichia* sp; VI = *Pseudomonas* sp; VII = *Flavobacteria/Xanthomonas* spp; VIII = *Proteus* IX = *Alcaligenes* sp and X = *Enterobacter* sp. PHC = Petroleum hydrocarbon.

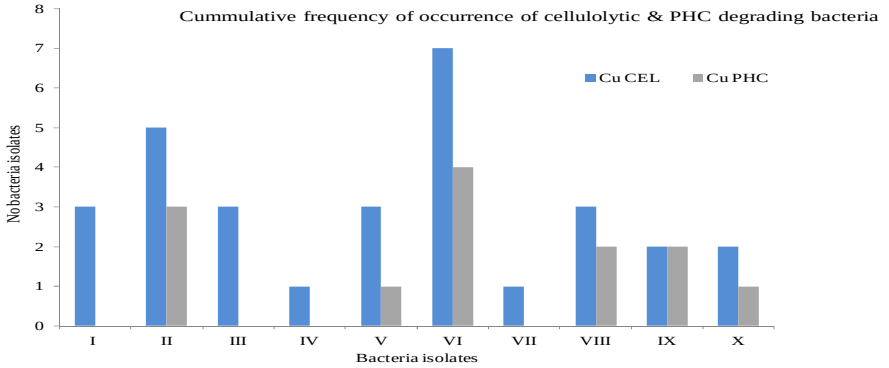

Fig 3. Cumulative frequency of isolation of cellulolytic (Cu CEL) & PHC (Cu PHC) bacteria. Bacteria isolates: I = *Staphylococcus* sp; II = *Bacillus* and other Gram positive rods; III = *Enterococcus* sp; IV = *Serratia species*; V = *Escherichia* sp; VI = *Pseudomonas* sp; VII = *Flavobacteria/Xanthomonas* spp; VIII = *Proteus* IX = *Alcaligenes* sp and X = *Enterobacter* sp. PHC = Petroleum hydrocarbon.

DISCUSSION

The biodegradation of cellulose is a wide spread process common to many bacteria species. All the leaf samples exhibited the presence of cellulolytic bacteria. The guava leaf sample was significantly more colonised, followed by mango leaf sample while the bush mango sample was least colonised. The reason for the observed variation in TCFU ml^{-1} of the decaying leaf samples was not clear; this would require further study to confirm. Indication of greater cellulolytic activity was observed in the isolates from Guava in which three of the ten isolates that were randomly selected were *Pseudomonas* sp and two of *Bacillus* and other Gram positive rods and one each of *Staphylococcus* sp, *Serratia* sp, *Escherichia* sp, *Proteus* sp and *Alcaligenes* sp. In the case of the isolates from the leaf sample of Mango, two each of the isolates, *Pseudomonas* sp, *Bacillus* and other Gram positive rods and one each of *Staphylococcus* sp, *Enterococcus* sp, *Escherichia* sp, Flavobacteria/Xanthomonas, *Proteus* sp and *Enterobacter* sp exhibited cellulolytic activity; while for the bush-mango leaf sample, two each of the isolates; *Enterococcus* sp and *Pseudomonas* sp and one each of *Bacillus* and other Gram positive rods *Escherichia* sp, *Proteus* sp, *Alcaligenes* sp and *Enterobacter* sp were cellulolytic. It was not clear what factors that accounted for the differences in the distribution of cellulolytic bacteria in the leaf samples nevertheless it appears sufficient selective pressure could be derived from the chemical nature of the leaf samples. Bush mango has been known to exhibit potential mechanisms that could disrupt terpenoids and inactivate microbial adhesion, enzymes, and cell envelope transport proteins through exudation of allergic acid-like compounds. Increase in phytotoxic substances appear to have decreased TCFU $ml^{-1}$and cellulolytic activity especially in the bush-mango leaf sample (Kuete et al, 2007; Fadare and Ajaiyeoba, 2008). The ability to degrade petroleum hydrocarbon has been demonstrated to depend on the nature of bacterial biodegraders present and also on the nature of the ecological status of the immediate environment of bacteria (Kigigha and Underwood, 2010). It was not clear if the cellulolytic bacteria from the leave samples were primed by the various phytochemicals found in the leaves and probably environmental condition in which the decaying leaves were found to be able to degrade petroleum hydrocarbon. The tentatively identified hydrocarbonoclasic bacteria species were: *Bacillus* and other Gram positive rods, *Escherichia* sp, *Pseudomonas* sp, *Proteus* sp, *Alcaligenes* sp and *Enterobacter* sp. These isolates have been variously identified by previous researchers to degrade petroleum hydrocarbon (Panda *et al*., 2013). All of these isolates (except the *Alcaligenes* sp) were also implicated in bacteria ureolytic activity studies on nitrogenous waste dump sites by Kigigha et al, 2012, thus indicating their ability to survive in hardy environments.

REFERENCES

BALA, SA. (2006) Euphorbia hirta linn. In some ethnomedicinal plants of the of the savanna regions of west Africa: Description and phytochemicals. Bala SA(ed.). 1st edn. *The Triump Publishing Company Ltd*, Gidan Saadu Zungur, Kano, Nigeria p.1925.

BRÁS, NATÉRCIA; N. M. F. S. A. CERQUEIRA, P. A. FERNANDES, M. J. RAMOS (2008). "Carbohydrate Binding Modules from family 11: Understanding the binding mode of polysaccharides". *International Journal of Quantum Chemistry* 108 (11): 2030–2040.

ELEKWA, S. C. OKEREKE AND B. O. EKPO (2009). Preliminary phytochemical and antimicrobial investigations of the stem bark and leaves of *Psidum guajava* l,. *J Med Plts Res* Vol. 3(1), pp. 045-048.

FADARE DA, AJAIYEOBA EO(2008). Phytochemical and antimicrobial activities of the wild mango *Irvingia gabonensis* extracts and fractions. *Afr J Med Med Sci* . 37(2):119-124.

JOHN, R. C AND G. C. OKPOKWASILI (2012). Crude Oil-Degradation and Plasmid Profile of Nitrifying Bacteria Isolated from Oil-Impacted Mangrove Sediment in the NigerDelta of Nigeria. *Bull Environ Contam Toxicol.* 88:1020–1026.

KIGIGHA, L.T AND G. J. C. UNDERWOOD (2013 a). Effect of Petroleum hydrocarbon on estuarine microalgal biofilms in the wetland: general introduction, literature review, and methods. *American Journal of Research Communication*, 1(6): pp136-173.

KIGIGHA, L.T; DANIEL SILAS-OLU, D AND D. FINEBONE (2012). Characterization of ureolytic bacteria from nitrogenous waste dumps in Port Harcourt Nigeria. *Cont. J. Microbiol*. Vol 6, (No 1) 9 – 13.

KLEMM, DIETER; HEUBLEIN, BRIGITTE; FINK, HANS-PETER; BOHN, ANDREAS (2005). "Cellulose: Fascinating Biopolymer and Sustainable Raw Material". *Chem. Inform* 36 (36).

KUETE V, WABO GF, NGAMENI B (2007). Antimicrobial activity of the methanolic extract, fractions and compounds from the stem bark of *Irvingia gabonensis* (Ixonanthaceae). *J Ethnopharmacol* . 114(1):54-60.

LATHA, R AND R. KALAIVANI 2012. Bacterial Degradation of Crude Oil by Gravimetric Analysis. *Advances in Applied Science Research*, 3 (5): p2789-2795.

MUHAMMAD IRFAN; ASMA SAFDAR; QURATULAIN SYED AND MUHAMMAD NADEEM (2012). Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity *Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem]*. Vol. 37 (3) ; 287–293.

PANDA S. K, KAR R. N AND C. R PANDA (2013). Isolation and identification of petroleum hydrocarbon degrading microorganisms from oil contaminated environment. *Inter. J. Environ. Sc*. Vol 3, No 5, 2013.