BIODEGRADATION OF CRUDE OIL USING HYDROCARBONOCLASTIC BACTERIA ISOLATED FROM GUT OF EARTHWORMS IN CRUDE OIL POLLUTED REGIONS OF NIGER DELTA, NIGERIA

[1*]Gideon I Ogu, [1]Ehiobu M John
Department of Biological Sciences,
Novena University, Ogume,
Delta State, Nigeria
*Email: gideoniogu@gmail.com

[2]Imbolo F Diete-Spiff, [2]Churchill E Ndego
Department of Chemical Sciences,
Novena University, Ogume,
Delta State, Nigeria

ABSTRACT: This work was carried out to investigate the abilities of hydrocarbonoclastic bacterial species isolated from the gut of earthworms, which were collected from crude oil polluted sites in Niger Delta region, Nigeria, to degrade 2.0 % crude oil in mineral salt medium over a 25 day period using standard laboratory techniques. Among the crude-degrading bacterial cultures isolated, *Flavobacterium* sp, *Micrococcus* sp and *Bacillus* sp were selected for the study based on the preliminary efficiency of crude oil utilization. The total viable counts ranged from 8.8 x 10^{11} cfu/ml to 15.7 x 10^{11} after 25 days, with *Flavobacterium* sp. and mixed culture having the lowest and highest counts respectively. A maximum degradation of 92.5 % was obtained with the mixed bacterial consortium, while *Bacillus* sp, *Micrococcus* sp. and *Flavobacterium* sp individually gave 85.7, 74.34 and 71.95 % biodegradation respectively. The optimum temperature and pH range for maximum degradation were found to be 35 – 40 ^{o}C and 6.5-7.4 respectively. Therefore, the use of the aforementioned mixed hydrocarbonoclastic bacterial consortium isolated from gut of earthworm under optimized conditions will be an effective and eco-friendly technology for bioremediation of crude oil polluted environemnts.

Keywords: Eco-friendly, Crude oil, Biodegradation, consortium

1. INTRODUCTION

The complex hydrocarbons resulting from crude oil pollution has remained a major environmental concern in many countries, and over the years, concerted effort had been made in studying the viability of using oil-degrading microorganisms for their bioremediation. Bioremediation have over the years received keen attention because of its obvious merits. It has been established that virtually all types of hydrocarbons are susceptible to microbial degradation and hence the relevance of using the biotechnological approach (microbial capability) for bioremediation of the hazardous waste is justified (Atlas, 1991; Head, 1998; Okoh, 2006). The success of the bioremediation efforts in the clean-up of the oil tanker *Exxon Valdez* oil spill of 1989 in Prince William Sound and the Gulf of Alaska has created tremendous interest in the potential of biodegradation and bioremediation technology (Atlas and Bartha, 1998). Thus, many countries are currently convinced of the full potential of this technology and are, therefore, encouraging research into improving and optimizing the procedures (Okoh, 2006).

Currently, several reports and sound recommendations had been made in utilizing microorganisms isolated from

crude oil polluted environments as candidate in crude oil degradation. However, less attention had been paid to sourcing microbial flora potentials of common perennial soil organism such as earthworm. Earthworms are often exposed to various pesticides and other organic pollutants in the soil and their intestines have excellent detoxification capability with a large number of aerobic and anaerobic bacteria (Karsten and Drake, 1995; Smitha et al., 2014). This study therefore attempt to isolate and characterise potential hydrocarbon degrading bacteria that might possibly be employed as alternative cost-effective, green candidate(s) in bioaugumentative bioremediation of crude oil contaminated environment.

2. MATERIALS AND METHODS

2.1 Source of Crude oil and Mineral salts

Crude oil (dark brown in colour) used in this study was obtained from NPDC, NNPC subsidiary, Warri, Delta State, Nigeria. The mineral salts components were purchased from Chris Colon Scientific Company and Equator Laboratory, Port-Harcourt, Rivers State, Nigeria.

2.2 Collection and Processing of Earthworms

Adult African earthworms were sourced from crude oil contaminated sites (Eleme and Tai LGA) in Ogoni-land, Niger Delta region of Nigeria. they were transported in ice-packed bag to the laboratory for further processing. The earthworm samples were washed with sterile tap water and placed on a petri plate moistened with filter paper and disinfected with 70 % ethanol. The gut content was then dissected out, homogenized in sterile 0.85 % NaCl Solution for 5-10 min (Maheswari and Sudha, 2013).

2.3 Isolation of crude degrading Bacterial from Earthworm gut

The gut bacteria able to degrade crude oil were isolated using crude oil amended mineral salts medium (MSM) by enrichment method as described by Odjadjare et al. (2008). Ten grams (10 g) of the gut samples was added separately to 100ml sterile defined growth Mineral Salt Medium (MSM) (pH 7.0) in 250 ml conical flask containing the desired petroleum as the carbon and energy source to a concentration of 1 % (v/v). The cultures were then incubated at ambient temperature (28 ± 2 ^{o}C) on an orbital shaker at 100 rmp. After seven (10) days incubation, samples were then serially diluted and 1ml portions were plated following standard pour plate techniques onto sterile MSM agar containing 1 % petroleum. Isolates were purified and transferred to agar slants (stored at 4 ^{o}C) as working stock cultures for further use and strain characterization. Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 2002).

2.4 Determination of Crude oil utilizers

The ability of the isolates to utilize crude oil was confirmed by inoculating each isolate into separate cotton plugged 250 ml Erlenmeyer flasks containing sterile Mineral Salts Medium (MSM). The MSM broth and crude oil were autoclaved separately at 121 °C for 15 min. Sterile crude oil which served as source of carbon and energy was added at 1 % (v/v) to make up a final volume of 100 ml sterile liquid MSM. Each isolate was subsequently inoculated into separate flask of the medium. Control flask containing the MSM and 1 % (w/v) of crude oil but without organism was also prepared. The flasks were monitored and agitated daily for a period of 21 days. Utilization of crude oil was assessed by monitoring the cell density

at 600 nm wavelength with spectrophotometer after 21 days period (Khan and Shukla, 2011).

2.4 Biodegradation study

The method of Sathishkumar et al. (2008) was adopted with sight modifications. The individual and mixed gut bacterial consortium from overnight culture at the log phase of growth were adjusted with sterile distilled water to give a bacterial cell count of 1.0 x 10^7 cfu/g and transferred to three 250 ml conical flasks each containing 100 ml of sterile-defined mineral salts medium with 2 % of crude oil. Un-inoculated flask containing 100 ml of sterile-defined mineral salts medium with with 2 % of crude oil served as control. All the flasks were replicated in triplicates. The flasks were then incubated on orbital shakers set as 100 rpm for 25 days at room temperature. At every seven days intervals, duplicate flask per organisms and control set up were removed from the incubator and the residual crude oil were determined spectrophotometrically (Odu et al., 1985). Samples (5 ml) from different treatments were mixed with equal volume of toluene to extract hydrocarbons from the sample. The extracts were detected spectrophotometrically at 420 nm. A standard curve prepared using known concentrations of crude oil was used to estimate the amount of hydrocarbons in the sample. Degradation was estimated as the difference between the initial and final concentrations of total hydrocarbons expressed in percentage.

2.5 Statistical Analysis

Calculation of means and standard deviations, using Microsoft Excel office 2007 version. test of significance were performed using SPSS 16.0 version for Windows program (SPSS, Inc.)

3. RESULTS AND DISCUSSION

Among the crude-degrading bacterial cultures isolated from the gut, *Flavobacterium* sp, *Micrococcus* sp and *Bacillus* sp were characterized and selected for the biodegradation study based on the efficiency of preliminary crude oil utilization (Table 1). These isolates were combined appropriately as the mixed culture. These gut bacterial isolates had been reported in the past to possess enzymatic machinery needed for break down petroleum complex hydrocarbons in the environment. *Bacillus*, *Micrococcus*, and *Flavobacterium* isolated from crude oil contaminated sites had been reported to possess versatile crude oil degradaing properties (Atlas, 1992; Okoh and Trejo-Hernandez, 2006; Odjadjare et al., 2008). It was also reported elsewhere that *Bacillus* sp. and *Micrococcus* sp. isolated from oil contaminated site possess significant biodegradation capacities (Sebiomo et al., 2010; Afuwale and Modi, 2012). Therefore, thess bacteria isolated from the gut of earthworms in this study are members of groups that have been well reported in the literature as petroleum hydrocarbon degraders

The growth profile of the hydrocarbon utilizing bacteria in crude oil-MSM further confirmed that these bacteria could utilized petroleum hydrocarbons. The total viable count (TVC) varied significantly ($P \leq 0.05$) with sampling days. Although, there was an initial gradual rise in TVC of the bacteria, the highest TVC recorded by *Bacillus* sp. increased sharply from 3.7 x 10^8 cfu/ml after day 5 to 7.9 x 10^9 cfu/ml at day 10 and increased steadily to 11.9 x 10^{11} after 25 days of incubation (Fig 1.). A similar situation was observed among the *Flavobacterium* and *Micrococcus* sp., though with relatively lower TVCs of 8.8 x 10^{11} cfu/ml and 10.1 x10^{11}cfu/ml respectively after 25 days. The growth pattern of the

combined culture was however in zig-zag form. The initial consortia bacterial culture count rose insignificantly until day 5, where a sudden sharp rise was observed till day 10, followed by a steady decline in the growth rate till after day 20, where a sudden rise was recorded again till day 25. This observed growth profiles revealed that there were little or no periods of lag phase before active metabolic activities suggesting the constitutive expression of hydrocarbon catalyzing enzymes or physiological activities owing to previous exposure to exogenous crude oil hydrocarbons residues in the gut system of the earthworms dwelling in the polluted soils. The observation is in consonance with previous reporters (Olivera *et al.,* 1997; Okeretungba and Ezeronye, 2003; Oboh *et al.,* 2006; Sebiomo *et al*., 2010), who opined that prior exposure to hydrocarbon contamination confers acclimatization or adaptation to the bacterial species or populations.

The results of biodegradation of the crude oil revealed that there was a direct relationship between the growth rate and loss of crude oil by the axenic and mixed bacteria culture (Table 2). The loss of the crude oil in the medium containing the mixed bacterial culture was the highest (92.5 %), followed by *Bacillus* sp (85.7 %), *Micrococcus* sp (74.5%) and *Flavobacterium* sp (71.9 %) after 25 days of incubation. The significant percentage crude oil degradation observed among these gut bacteria shows that they possess efficient enzymatic machinery probably due to their previous exposure to crude oil residues in the gut of earthworms. Earthworms had been reported to feed voraciously on diverse organic compounds with efficient enzymes that could breakdown the ingested compounds. Also, it was reported that most of the microbiota that make up that make up flora of earthworms are same as those in the soil environment (Smitha et al., 2014).

Table 1: Morphological and Biochemical characterization of crude oil degrading gut bacterial isolates

Isolate code	Shape	Gram Stain	Motility test	Catalase test	Niterate test	Oxidase test	Indole test	Citrate test	O/F test	Sugar fermentation test					Presumptive Identity
										Glucos	Lactose	Sucros	Fructos	Mannit	
CDGB 1	Rod	+	+	+	+	+	-	+	F	A	-	A	A	A	*Bacillus* sp.
CDGB 2	Rod	-	-	+	+	+	-	+	F	A	-	AG	A	-	*Flavobacetrium* sp.
CDGB 3	Coccus	+	-	+	+	+	-	-	O	-	-	-	-	-	*Micrococcus* sp.

CDGB = Crude oil-degrading gut bacterial isolate; F = Fermentation; O = Oxidation; AG = Acid and Gas; - = Negative; + = Positive

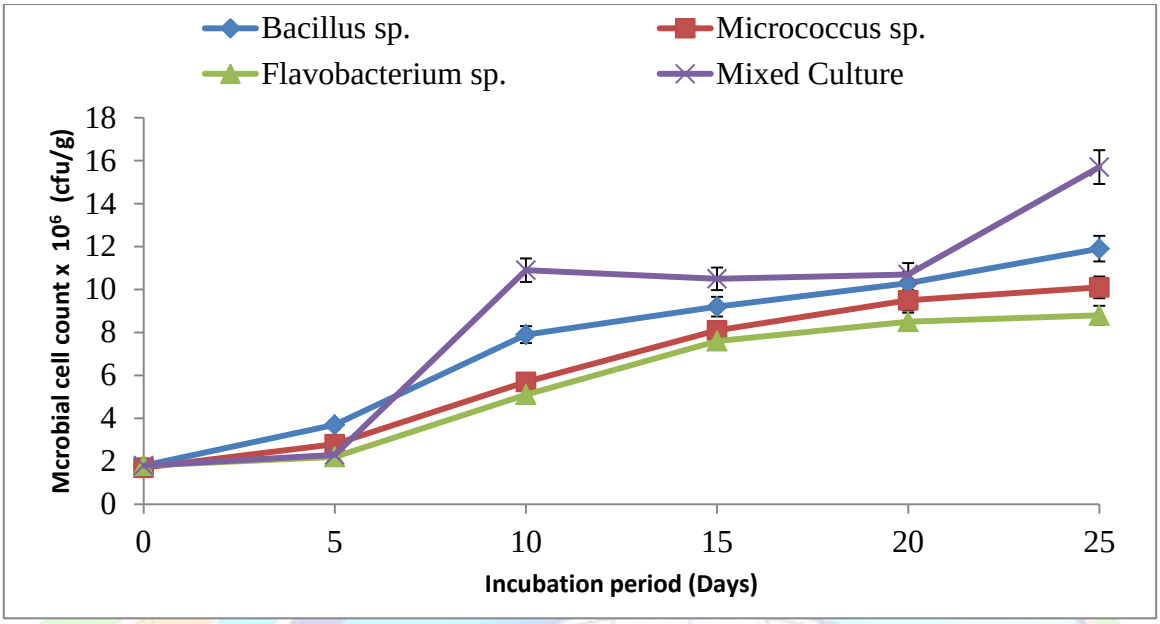

Fig. 1: Growth profile of bacteria isolates in crude oil-MSM

Table 2: Rate of biodegradation of crude oil in MSM by single and mixed gut bacterial isolates after 25-day treatment.

Gut Bacterial Isolates	Residual oil (g)	Undegraded oil (%)	Degraded oil (%)
Flavobacterium species	0.0734	28.1	71.9
Micrococcus species	0.0618	25.5	74.5
Bacillus species	0.0206	14.3	85.7
Mixed culture	0.0098	07.5	92.5

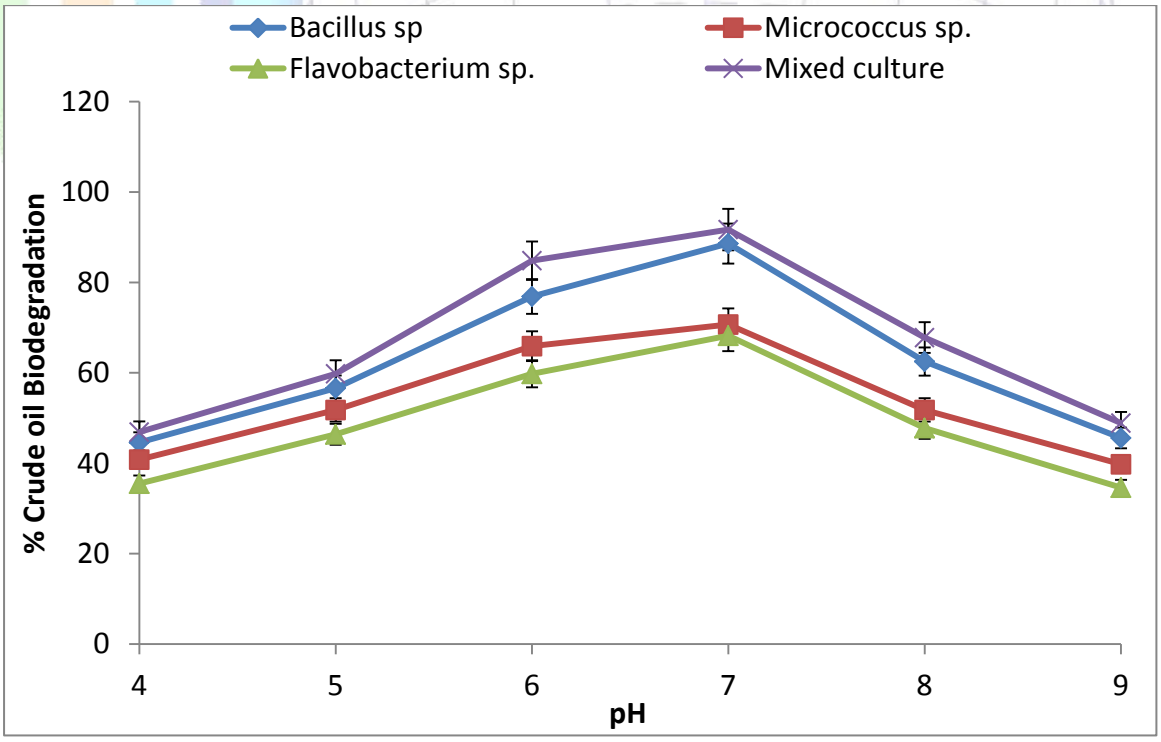

Fig. 2: Effect of pH on the rate of crude oil biodegradation

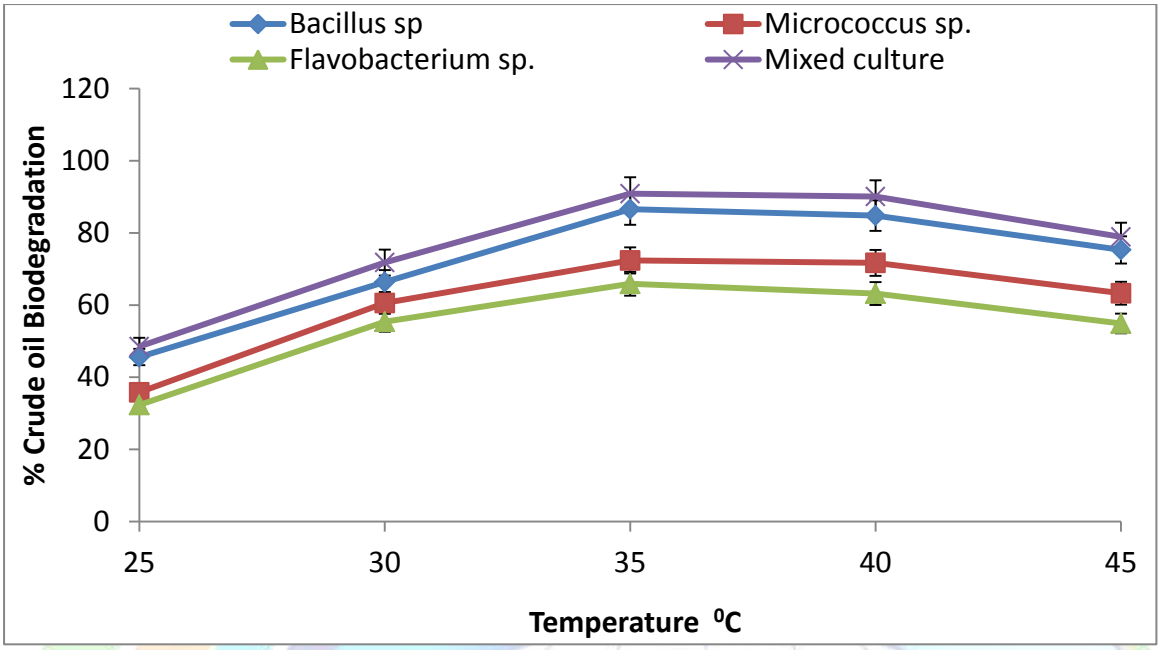

Fig. 3: Effect of Temperature on the rate of crude oil biodegradation

Thus, these bacterial isolates were probably native soil petroleum degraders that might have been taken in by the earthworm while feeding on the hydrocarbon organic polluted soil. The extent of crude oil biodegradation (71.9% - 92.5%) observed in this study is consistent with the findings of others workers who studied oil degradation potentials of same bacterial species isolated from crude oil polluted soils (Okoh, 2003; Odjadjare et al., 2008).

Our study further revealed that the mixed gut bacterial cultures displayed better degradation than the individual cultures. Similar findings had been reported by earlier works (Rahman et al., 2002; Bagherzadeh-Namazi et al., 2008; Al-Wasify and Hamed, 2014). However, a contrary observation had been reported by previous researchers (Okerentugba and Ezeronye, 2003; Johnsen et al., 2005; Odjadjare et al., 2014) who opined that single bacterial culture had better biodegradation potentials than mixed microbial communities. The higher oil degradation observed with the mixed culture could be attributed to a combined enzymatic activities expressed by the mixed culture that must have probably enhanced the degradation of the complex mixtures of crude oil compounds. Additionally, the better biodegradation potential observed by the mixed bacterial culture is an indication that there was minimal antagonism or predation by the individual bacteria that make up the mixed culture. Antagonism, ammensalism and predation had been reported as the commonest experience of mixed microbial cultures (Thouand et al., 1999; Johnsen et al., 2005; Odjadjare et al., 2008). Therefore, a consortia culture containing these three gut bacteria would be a veritable potential crude oil bioremediation agent

Studies on the effect of pH on the rate of crude oil biodegradation shows that maximum degradation occurred at pH of 7.0 (Fig. 2), with a range of between 6.5 and 7.4 for both the single and mixed gut bacterial cultures. This finding further buttress the earlier assertion that the mixture of the three gut bacteria culture would be an efficient bioremediation agent since most crude oil polluted sites are relatively acidic in nature, which is the favoured condition for best metabolic

activities of these bacteria. This finding is in consonance with report of (Sathishkumar et al., 2008), who observed a similar pH range for maximum biodegradation activities.

Furthermore, our result also revealed that maximum biodegradation was noted at relatively high temperature range of 35 - 40°C by the mixed and individual cultures (Fig. 3). This is an indication that the enzymatic machinery of these gut bacteria isolates are relatively themostable. Additionally, such physical condition is quite obtainable in most crude oil polluted soils in Nigeria, unlike other temperate countries. Hence, these bacteria culture isolated from gut of earthworm could be applied as potential bioagumentative agent for efficient crude oil bioremediation in the Nigeria Delta environment known for its long history of crude oil pollution.

Conclusion

The results from this study have shown that earthworms inhabiting crude oil polluted environments could harbour potential crude oil degrading bacteria in their guts. Although, significant crude oil hydrocarbon degradation was observed by each bacteria culture, a better result was achieved when the individual bacteria were mined together as consortia culture. Hence, the use of the aforementioned mixed bacterial consortium isolated from gut of earthworm under optimized conditions will be an effective and eco-friendly technology for the degradation of crude oil pollution. Further study is recommended on the bio-augmentative potentials of the isolates in crude oil contaminated systems.

REFERENCES

1. C. Afuwale and H. A. Modi Study of bacterial diversity of crude oil degrading bacteria isolated from crude oil contaminated sites. Life Sci. Leaflets. 6:13-23, 2012.
2. R. S. Al-Wasify and S. R. Hamed Biodegradation of Used Engine Oil Using Mixed and Isolated Cultures. Int. J. Bacteriol. 3,1-8, 2014
3. R. M. Atlas. Microbial hydrocarbon degradation-biodegradation of oil spills. *J.* Chem. Technol. Biotechnol. 52, 149-156, 1991.
4. R. M. Atlas. *Petroleum Microbiology*. In: J Lederberg (Ed) Encyclopedia of Microbiology. Academic Press, Baltimore. 363-369, 1992..
5. R. M. Atlas and R. Bartha. Fundamentals and Applications. In: Microbial Ecology. 4th Ed. Benjamin/Cummings Publishing Company, Inc. California, USA, 523-530, 1998
6. A. Bagherzadeh-Namazi, S. A. Shojaosadati and S. Hashemi-Najafabadi. Biodegradation of Used Engine Oil Using Mixed and Isolated Cultures. Int. J. Environ. Res. 2(4) 431-440, 2008
7. I. M. Head. Bioremediation: towards a credible technology. Microbiol. 144 (3), 599-609, 1998.
8. J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams. Bergey's Manual of Determinative Bacteriology, 9th edition. Lippincot: Williams and Wilkins. Philadelphia, USA. 131-542, 2002
9. A. R. Johnsen, L. Y. Wick, and H. Harms. Principles of microbial AH-degradation in Soil. Environ. Pollut., 133, 71-84, 2005
10. G. R. Karsten and H. L. Drake. Comparative assessment of the aerobic and anaerobic microfloras of Earthworm Guts and Forest Soils. Appl. Environ. Microbiol. 61, 1039-1044, 1995.
11. A. J. Khan and R. U. Shukla. Application of Oil Degrading

Bacterial Isolates for Remediation of oil Contaminated Soil. J. Pharm. Biomed. Sci. 12 (12), 1-4, 2011.

12. N. U. Maheswari and S. Sudha. Enumeration and detection of phosphate solubilising bacteria from the gut of earthworm varieties. J. Chem. Pharm. Res., 5 (4), 264-267, 2013.

13. B. O. Oboh, O. Ilori, O. Akinyemi and S. O. Adebusoye. Hydrocarbon Degrading Potentials of Bacteria Isolated from a Nigerian Bitumen (Tarsand) Deposit. Nat. Sci. (4), 3-10, 2006.

14. A. I. Okoh. Biodegradation of Bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pit in Nigeria. Afr. J. Biotechnol., 2(5), 104-108, 2003.

15. A. I. Okoh. Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnol. Mol. Biol. Rev. 1 (2), 38-50, 2006.

16. A. I. Okoh and M. R. Trejo-Hernandez. Remediation of petroleum hydrocarbon polluted systems: exploiting the bioremediation strategies. 5, 2520-2525, 2006.

17. E. E. O. Odjadjare, S. O. Ajisebutu, E. O. Igbinosa, O. A. Aiyegoro, M. R. Trejo-Hernandez. and A. I. Okoh. Escravos light crude oil degrading potentials of axenic and mixed bacterial cultures. J. Gen. Appl. Microbiol. 54, 277-284, 2008.

18. C. T. Odu, L. C. Nwoboshi and O. F. Esuruoso, Environmental Study ofthe Nigerian Agip oil Company Operation Areas. in Proceedings of the International Seminar on the Petroleum Industry and the Nigerian Environment, Kaduna, Nigeria. 274-283, 1985.

19. A. O. Ojo. Petroleum hydrocarbon utilization by native bacterial population from waste water canal Southwest Nigeria. Afr. J. Biotechnol. 5, 333-337, 2006.

20. P. O. Okerentugba and O. U. Ezeronye. Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. Afr. J. Biotechnol. 2 (9), 288-292, 2003.

21. N. L. Olivera, J. L. Esterics and M. G. Commendatore. Alkane biodegradation by microbial community from contaminated sediments in Pantagonia, Argentina. Int. Biodeterio. Biodeg. 40, 75-79, 1997.

22. K. S. M. Rahman, T. Rahman, P. Lakshmanaperumalsamy and I. M. Banat. Occurrence of Crude Oil Degrading Bacteria in Gasoline and Diesel Station Soils. J. Basic Microbiol. 42 (4), 284-91, 2002

23. M. Sathishkumar, A. R. Binupriya, S. Baik and S. Yun. Biodegradation of Crude Oil by Individual Bacterial Strains and a Mixed Bacterial Consortium Isolated from Hydrocarbon Contaminated Areas. Clean. 36 (1), 92-96, 2008.

24. A. Sebiomo, S. A. Bankole and A. O. Awosanya. Determination of the ability of microorganisms isolated from mechanic soil to utilize lubricating oil as carbon source. Afr. J. Microbiol. Res. 4 (21), 2257-2264,

25. P. Smitha, T. R. Srinivasan and N. Kannan. Isolation and characterization of Endosulfan-degrading bacteria from gut of earthworm Canad. J. Clin. Nutr. 2 (1), 46-57, 2014.

26. G. Thousand, P. Bauda, J. Oudot, G. Kirsch, C. Sutton, and J. F. Vidalie.

Laboratory evaluation of crude oil biodegradation with commercial and natural microbial inocula. Can. J. Microbiol., 45 (2), 106-115, 1999.