Available online at www.elixirpublishers.com (Elixir International Journal)

Chemical Engineering

Elixir Chem. Engg. 80 (2015) 30945-30948

ISSN: 2229-712X

Performance evaluation of oil degrading microbes isolated from crude oil contaminated soil in Niger delta area of Nigeria

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ARTICLE INFO

Article history:

Received: 11 January 2015;

Received in revised form:

25 February 2015;

Accepted: 5 March 2015;

Keywords

Degradation,
Cellulosic carriers,
Bioremediation,
Immobilized bacteria,
Total petroleum hydrocarbon.

ABSTRACT

Artificially contaminated soil was treated with hydrocarbon degrading bacteria previously isolated from soil that was consistently contaminated with petroleum. The consortium of bacteria used consists of *Enterobacter aerogenes*, *Serratia marcescens* and *Proteus myxofaciens.* immobilized in cellulosic materials such as coconut fibre and groundnut husk. The results of the laboratory tests show that the immobilized bacteria have good self life with bacteria load of 3.33 x 10^{22} and 3.20 x 10^{19} on the 1st and 28th day respectively. The immobilized bacteria system shows promise in the degradation of petroleum hydrocarbons. After 21 days of application of the immobilized bacteria system in laboratory scale degradation of Forcados light crude oil, the residual concentration of petroleum hydrocarbons decreased to 14.87% for one of the samples as compared to a residual concentration of 58.97% in the control sample. Therefore the immobilized bacteria system using cellulosic as a carrier can serve as an effective and fast bioremediation tool for cleaning up petroleum contaminated soil at low cost.

Introduction

Crude oil production began on commercial scale in Nigeria in 1958, two years after it was discovered at Oloibiri in the oil-rich Niger Delta region of Nigeria. The oil industry in Nigeria has grown in the last five decades to become the major foreign exchange earner and has replaced agriculture as the main stay of the national economy. There are over 606 on-shore and off-shore oil fields and more than 3,000 kilometers of oil pipeline laid across the Niger Delta (Adebanwi, 2001). One of the greatest challenges facing the Nigerian petroleum industry today is the impact on the environment of the production and refining activities. The production, transportation, and refining of petroleum releases substantial quantities of both refined and crude petroleum to the environment and impacting seriously the air, land and water bodies. Oil spillages still occur through tanker accidents, well blow out, sabotage and accidental rupture of pipelines (Atlas, 1981; Colwell and Walker, 1977).

Oil spills constitute the most significant source of petroleum hydrocarbons in the Nigerian environment especially in the Niger Delta. Oil pollution and the resultant degradation of the environment have negative impacts on the oil producing communities. In Nigeria, statistics of some possible causes of oil spillage shows that fifty percent (50%) of the spillage is due to corrosion, twenty eight percent (28%) to sabotage, twenty one (21%) to oil production operation and one percent (1%) to engineering drills, inability to effectively control oil wells, failure of machines and inadequate care in loading and offloading oil vessels (Nwilo and Badejo, 2005). Spilt oil vaporization takes care of one to two thirds of the oil while what is left must be cleaned by other methods including physical removal and chemical treatments.

According to Onwurah, *et al.*, (2007), bioremediation is a technology that exploits the abilities of microorganisms and other natural habitat of the biosphere to improve environmental quality for all species, including man. Remediation techniques are eventually employed in a bid to restore the polluted area to a status as near as possible to the original. It has been known for many years that the major constituents of most crude oils are biodegradable (Prince, 1997). Most of the petroleum which enters the environment is degraded naturally because many species of bacteria in the environment are endowed with the ability to oxidize petroleum hydrocarbons (Zobell, 1964). However the rate at which natural degradation occurs is slow and does not provide immediate or rapid relief when accidents occur or to the problem of chronic pollution (Atlas and Bartha, 1992). Many reasons account for why degradation is slow in the natural environment, these include: low counts of hydrocarbon-degrading microbes, toxicity of some components, limited oil/water or soil interface, insufficiency of oxygen and lack of essential mineral nutrient (Atlas and Bartha, 1992). Many bacteria are capable of degrading the constituents of oil and the oil degrading bacteria are the most important input in bioremediation technique (Gimsing, *et al.*, 2010). Interest has arisen in the use of immobilized bacteria technology to treat oil pollution to enhance the rate of degradation. Whereas several materials have been used to immobilize bacteria cells for application in crude oil degradation locally available agriculture by products have not been seriously considered.

Seven petroleum hydrocarbon degrading bacteria species, *Micrococcus roseus, Leuconostoc mesenteriodes, Streptococcus thermophilus, Aerococcus viridians, Flavobacterium thalpophilium, Micrococcus varians and Bacillus sp* were isolated from soil samples that were consistently contaminated with petroleum hydrocarbon in our previous work (Mamah, *et al.*, 2013). This involved cultivation in mineral salt medium using the enrichment technique (Schlegel, 1993).

The objective of the work is to immobilize hydrocarbon degrading bacteria in cellulosic materials such as: coconut fibre and ground husk. Second, to treat the petroleum contaminated soil with the immobilized bacteria. Third, to determine the self life and evaluate the effectiveness of the immobilized bacteria in the bioremediation of petroleum contaminated soil.

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Materials and Methods

Cellulosic Materials

Coconut fibre was obtained from the fibrous husk (mesocarp) of the coconut (*Cocos nucifera*) fruit obtained from Eku, Delta State. The individual fibre was pulled out from the husk and reduced in size by cutting. The cuttings were then sun dried for several days and ground in a grinding mill. The brown coloured powder was sieved through 0.48mm and stored in a plastic container at room temperature in the laboratory.

Groundnut husk was collected from groundnut shelling mills in Eku, Delta State. The husk collected was crushed by a grinding mill, sun dried for ten days and finally ground to powdery form. The light brown coloured meal was sieved through 0.48mm and stored in plastic container in the laboratory at room temperature.

Immobilization of Bacteria Isolates in Cellulosic Carriers and Check for Shelf life

Petroleum hydrocarbon degrading bacteria isolates in stock was obtained from previous work (Mamah, *et. al.,* 2013). Fresh cultures of bacteria isolates code named C, F, and G were prepared according to the method described by (Mishra, *et. al.,* 2001). Mineral Salt Media (MSM) solution prepared according to the composition of Mills *et al.,* (1978) as modified by Okpakwasili and Okorie (1988). The trace element solution was sterilized in separate containers using autoclave at 0.14 Mpa for 15 minutes. Trace element solution (2ml) was added to the MSM solution (1 litre) and the MSM preparation adjusted to pH 7.3 before it was used for cultivating the petroleum degrading bacteria. 1ml of the MSM broth culture was transferred from each of the growth flask into separate fresh 100ml MSM (with 1 ml of crude-oil, (Escravos light, sterilized by means of membrane filter 0.45 µm) added in sterile conical flasks. These were again incubated for 10 days at 30^{o}C to obtain bacteria acclimatized to utilizing crude oil as carbon source for metabolism. All flasks incubated showed growth at the end of the 10 days incubation period. The immobilized systems were prepared by immobilizing each of the bacteria isolates in samples of the coconut fiber and groundnut husk at a ratio of 1ml of bacteria broth to 1g of cellulosic material (Mishra, *et. al.,* 2001). Seeded carriers were stored in a refrigerator at 4^{o}C and shelf life checked after 120 days for the following parameter, Survival of isolates in each carrier and level of deterioration e.g. fungal growth in the carrier. The quantification of the hydrocarbonoclastic bacteria population was done by the most probable number technique (MPN) (Oblinger and Koburger, 1975), using the liquid mineral medium by Vecchioli, *et. al.,* (1990).

Laboratory Scale Test for Immobilized Bacteria Abilities to Degrade Contaminated Soil

Sterile sharp sand contaminated with sterile crude oil (10% v/w) was inoculated with the bacteria isolates immobilized in coconut fiber and groundnut husk. NPK 15-15-15 fertilizer and MSM were applied as amendments. The remediation of the soil was monitored by the variation in the concentration of petroleum hydrocarbons with time over a period of 28 days using gas chromatography.

Gas Chromatography Analysis

The analysis of whole oil, aliphatic and aromatic fractions was performed on HP 6890 gas chromatograph using FID detector. The Conditions for aliphatic hydrocarbon and aromatic hydrocarbon analysis are: Column, HP-5 (30 m × 0.25 mm); Initial temperature, 60^{o}C; final temperature, 300^{o}C; ramp rate, 8^{o}C per min; carrier gas, helium (30cm/sec); injection temperature, 250^{o}C; detector temperature, 300^{o}C and Column, HP-5 (30 m × 0.25 mm); Initial temperature, 100^{o}C; final temperature, 310^{o}C; ramp rate, 4^{o}C per min; carrier gas, helium (30cm/sec); injection temperature, 250^{o}C; detector temperature, 300^{o}C respectively.

Results and Discussion

Evaluation of potential of bacteria isolates to degrade petroleum hydrocarbon

In a previous work (Mamah, *et. al.,* 2013), the potential of the seven bacteria isolates to degrade petroleum hydrocarbons has been evaluated by a procedure which measures the ability of the isolates to grow in MSM broth with crude oil as carbon source as reflected in turbidity readings using a Turbid meter (Vasileva - Tonkova and Gesheva, 2004). The utilization of crude oil as carbon source results in increasing turbidity of the MSM broth, with the highest turbidity produced by the most active crude oil degrading bacteria isolate.

Table 1: Evaluation of bacteria isolates' potential to degrade crude oil using turbidity readings

ISOLATES	DAY O	DAY 7	DAY 14	DAY 21	DAY28
A	5.70	56.0	43.50	52.50	87.50
B	5.65	59.60	63.50	66.75	74.20
C	5.92	224.0	257.50	288.0	297.0
D	5.80	174.0	123.0	101.70	97.60
E	23.76	110.0	123.0	133.0	161.0
F	12.76	112.50	167.0	242.0	393.0
G	25.50	113.50	130.0	197.0	277.0

Key: A = *Micrococcus roseus*, **B** = *Leuconostoc mesenteriodes*, **C** = *Enterobacter aerogenes* **D** = *Aerococcus viridians*, **E** = *Flavobacterium thalpophilium*, **F** = *Serratia marcescens,* **G** = *Proteus myxofaciens*

The results of the evaluation show that three of the seven bacteria isolates, *Enterobacter aerogenes*, *Serratia marcescens* and *Proteus myxofaciens*. with the highest turbidity readings of 297.0, 393.0 and 277.0 respectively possess the best potential to degrade petroleum hydrocarbons after 28 days. The turbidity generally increased with time for all the isolates but the most active bacteria isolates to degrade the crude oil carbon source produced the highest turbidity values.

Table 2: Shelf Life of Immobilized Bacteria

Immobilized system	Bacteria laod (cfu/ml)	
	Day 1	Day 120
gA	1.54×10^{34}	3.19×10^{31}
gB	1.32×10^{35}	5.90×10^{31}
gC	1.02×10^{30}	6.63×10^{31}
gE	8.12×10^{33}	1.70×10^{12}
gF	3.17×10^{35}	8.20×10^{21}
gG	1.56×10^{32}	2.30×10^{22}
kA	7.27×10^{31}	1.60×10^{11}
kB	6.66×10^{31}	5.12×10^{21}
kC	1.23×10^{34}	6.90×10^{33}
kE	6.14×10^{33}	5.74×10^{33}
kF	7.36×10^{34}	4.20×10^{23}
kG	3.28×10^{31}	1.04×10^{21}

Key to the codes: **gA** – groundnut husk + bacteria isolate A; **gB** – groundnut husk + bacteria isolate B; **gC** – groundnut husk + bacteria isolate C; **gE** – groundnut husk + bacteria isolate E

gF – groundnut husk + bacteria isolate F; **gG** – groundnut husk + bacteria isolate G

kA – coconut fiber + bacteria isolate A; **kB** – coconut fiber + bacteria isolate B

kC – coconut fiber + bacteria isolate C; **kE** – coconut fiber + bacteria isolate E

kF – coconut fiber + bacteria isolate F; **kG** – coconut fiber + bacteria isolate G

Shelf life

The three bacteria isolates, *Enterobacter aerogenes*, *Serratia marcescens* and *Proteus myxofaciens.* that demonstrated the best potentials to degrade petroleum hydrocarbons (Table 1), were each immobilized in the two cellulosic carriers: coconut fiber and groundnut husk. The bacteria load of the immobilized material was enumerated and results showed that the bacteria isolates survived very well in the coconut fiber and groundnut husk. The enumeration of bacteria load was performed to determine the viability of the immobilized bacteria isolates after some period of storage. The population of the bacteria isolates immobilized in the coconut fiber and groundnut husk only decreased slightly over 120 days of storage (Table 2). The results therefore demonstrate that the cellulosic materials did not pose adverse conditions to the viability of bacteria isolates, hence their suitability to immobilize the bacteria isolates.

The bacteria counts of the three most active petroleum hydrocarbon degrading bacteria isolates: *Enterobacter aerogenes, Serratia marcescens and Proteus myxofaciens.* codes (C,F,G) respectively immobilized in ground nut husk and coconut fiber (Table 3) shows a high load of bacteria to effect rapid degradation of crude oil.

Table 3. Bacterial Count of Laboratory Bioremediation Experiment

Immobilized system	Days Bacteria laod (cfu/ml)			
	1	7	21	28
gC	3.33×10^{22}	2.05×10^{21}	5.77×10^{19}	3.20×10^{19}
gF	3.12×10^{22}	3.07×10^{22}	6.40×10^{18}	5.12×10^{22}
gG	4.04×10^{22}	2.05×10^{21}	3.20×10^{19}	7.04×10^{19}
kC	3.17×10^{22}	6.02×10^{17}	5.76×10^{19}	9.28×10^{19}
kF	3.17×10^{22}	3.20×10^{16}	3.20×10^{19}	6.40×10^{18}
kG	1.54×10^{21}	2.56×10^{17}	4.50×10^{13}	3.20×10^{16}

Key: gC – groundnut husk + bacteria isolate C; **gF** – groundnut husk + bacteria isolate F

gG – groundnut husk + bacteria isolate G; **kC** – coconut fiber + bacteria isolate C

kF – coconut fiber + bacteria isolate F; **kG** – coconut fiber + bacteria isolate G

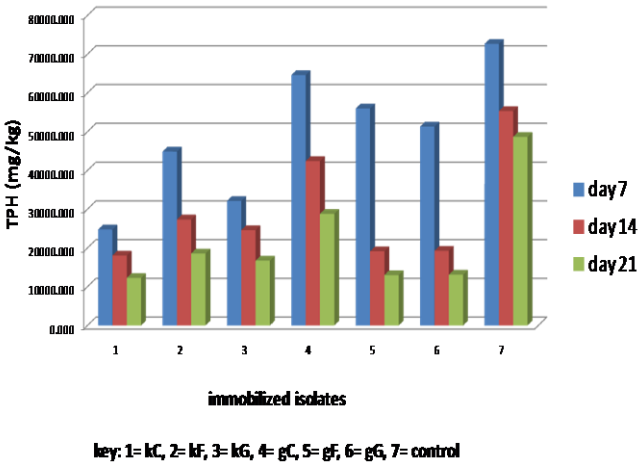

Fig 1. Lab scale monitoring of oil (Escravos light) degradation using immobilized isolates

The results of the treatment of petroleum contaminated soil with the immobilized bacteria isolates C, F, and G in the carrier: coconut fiber and groundnut husk is presented in Fig 1. There is a decrease in the concentration of Total Petroleum Hydrocarbon (TPH) with time. The results show that all of the immobilized isolates were effective in degrading petroleum hydrocarbons when compared to the control experiment. In addition, the bacteria isolate that was immobilized in coconut fiber: kC, kF and kG performed better than the respective bacteria immobilized in groundnut husk. This suggests that the bacteria isolates are more viable in coconut fiber than in groundnut husk. Among the isolates C, F and G, isolates C performed best when immobilized in coconut fiber.

The result of the effectiveness test of the bacteria isolates immobilized in groundnut husk is as shown in Fig. 2. It is evident that isolate F performed best, followed by G and isolate C has the least performance. This reveals that individual bacteria isolates thrives best in different immobilization materials as bacteria will be expected to have different affinities for various cellulosic materials.

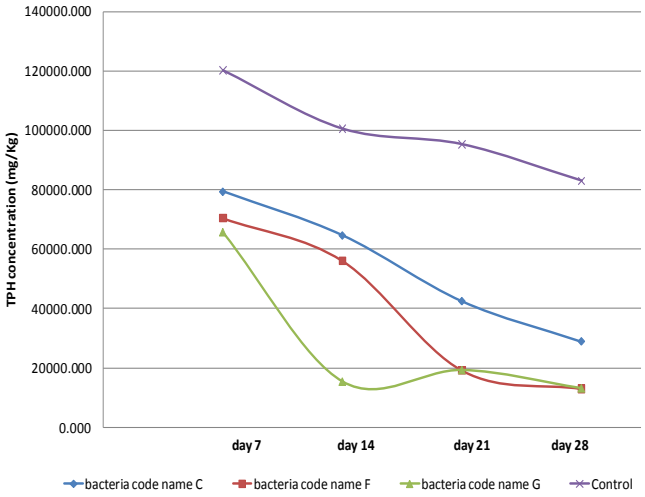

Fig 2. Laboratory scale degradation of oil using different bacteria in Ground Nut Husk

Table 4. Percentage Residual Petroleum Hydrocarbons (TPH) Per Week

Immobilized system	Residual TPH, %		
	Day 7	Day 14	Day 21
Control	88.07	67.05	58.97
kC	30.00	21.88	14.87
Kg	38.95	29.82	20.26
kF	54.37	33.14	22.52
gG	62.21	23.34	15.86
gF	67.87	23.18	15.75
gC	78.32	51.41	34.93

The laboratory scale test on the degradation of petroleum hydrocarbon shows that there was a gradual decrease in the residual concentration of petroleum hydrocarbon. A noticeable decrease was observed in the residual concentration of Total Petroleum Hydrocarbon (TPH) for all immobilized bacteria (Table 4). This is compared to the residual concentration in the control. All the immobilized bacteria proved effective in the removal of hydrocarbon from soil, and kC has the best performance.

Conclusion

The research study has succeeded in developing a bacterial product that possesses certain distinct advantages over the common methods of bioremediation. The product developed could be rapidly deployed for bioremediation of oil polluted soil being in pellet form, and, the application of the product has shown that it is more effective in the remediation of petroleum hydrocarbon contaminate soil having removed as much as 93% content of the original petroleum pollution compared to removal of between 65-85% of original petroleum pollution by the free bacteria cells The products developed have great potential for use as product of choice to remediate oil polluted soils.

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