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ENHANCED REMOVAL OF CRUDE OIL IN SOIL BY CO-CULTURE OF *Bacillus subtilis* AND *Pseudomonas aeruginosa* ISOLATED FROM CONTAMINATED SOIL IN KANO STATE, NIGERIA

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ABSTRACT
There is increasing concern over the issues of environmental pollution especially one caused by hydrocarbon. Soil samples were obtained from oil contaminated fields in Kano and screened for crude oil utilizing bacterial populations. The composition of the bacterial isolates recovered from the contaminated soil include Bacillus subtilis 3 (37.5%), Bacillus cerrus 2 (25%), Pseudomonas aeruginosa 2 (25%) and Staphylococcus aureus 1 (12.5%). All the isolates were able to utilized Bonny light crude oil as a source of carbon and energy to varying extent. B. subtilis and P. aeruginosa, with very high ability in utilizing the crude oil, were used as co-culture for this bioremediation studies, and were subjected to 5% of crude oil for 56 days at room temperature. The isolates, used singly and co-culture, exhibited strong ability to grow in crude oil within the incubation period with co-culture significantly (P<0.05) had the highest microbial counts of 1.05±1.04 x 10^6 cfu/ml on 42 days of the experiment while B. subtilis had the least count on the zero day of the experiment with mean value of 6.90±1.15 x 10^4 cfu/ml. The results of the study indicated the potentials of Co-culture of B. subtilis and P. aeruginosa in treating oil spills contaminated soil.
Keywords: Biodegradation, Hydrocarbon, Crude oil, Bacteria, Coculture, Soil, Kano

INTRODUCTION

Soil pollution due to crude oil is becoming a widespread environmental problem of major concern. Oil spills due to pipeline rupture, tank failures, various production and storage problems and transportation accidents are the major causes. Crude oil is a complex mixture of hydrocarbons, mainly composed of aliphatic, aromatic and asphaltene fractions along with nitrogen, sulfur and oxygen containing compounds (Kulkarni, 2014).

Crude oil changes the physicochemical characteristics of the land, contaminating it to the detriment of living organisms. Wildlife, vegetation, crops and farmland are adversely affected (Okecha, 2000). Biodegradation as a means of remediation of contaminated site has drawn positive attention because of its economic viability and environmental friendliness (Dinkla *et al.*, 2001). In view of the above, the present study aimed at determining the potential of a co-culture of *Bacillus subtilis* and *Pseudomonas aeruginosa*, without the addition of nutrients to speed up oil biodegradation in soil.

MATERIALS AND METHODS

Sample Collection

Nigerian crude oil (Bonny Light Oil) was collected from Kaduna Refinery Petroleum Corporation (KRPC), Kaduna state, Nigeria. Soil samples from oil contaminated area of Nigerian National Petroleum Corporation (NNPC) Deport, Hotoro, Kano state, north-western Nigeria were aseptically collected at different points using ditch auger. In the same way, non-oil contaminated soil samples were collected from Botanic Garden of the Biological Sciences, Bayero University, Kano, Nigeria and transported to Microbiology Laboratory, Department of Microbiology of the same institution for further analysis in accordance with the method of Romanus *et al.*, (2015).

Isolation and Identification of Bacteria

This was carried out using the method of Hemalatha and Veeramanikandan (2011). Hydrocarbon degrading bacteria were isolated from soil samples using Bushnell Hass (BH) medium containing Bonny light crude oil as a sole source of carbon. 1.0gram of soil was introduced in 250ml Erlenmeyer flask containing 100ml of BH broth and 100µl of crude oil and incubated at 37°C for 7 days on an orbital shaker operated at 200 rpm.

After one week, similar process was repeated. At the end of the second week, samples were serially diluted up to 10^{-6} dilutions. Then 1.0ml from 10^{-6} and 10^{-5} dilutions were spread on BH agar and incubated under the same condition.

Mixed colonies were obtained and each of the selected colonies was grown on BH agar plate in the presence of Bonny light crude oil and stored at 4°C for further studies. Cultural, morphological and biochemical characterization of the isolates were carried out using standard microbiological methods (Chessbrough, 2006).

Standardization of the Inoculums

McFarland turbidity standard solution used for standardization of bacterial inocula was prepared as described by Andrew (2009) and Cheesbrough (2006).

Screening of hydrocarbon degrading bacteria for potential to Utilize Bonny Light Crude oil

The hydrocarbon degrading ability of the isolates was measured using turbidity method as described by Okpokwasili (1988) and the extent of bacterial growth was represented as maximum growth (+++), moderate growth (++), minimal growth (+) and no growth (-).

Biodegradation of Crude oil using individual and Co-bacterial Culture

Plastic buckets containing 5% crude oil and 1000 grams of sterilized soil were inoculated with standardized inoculums of the bacterial isolates. *B. subtilis, Pseudomonas aeruginosa* and co-culture of (*B. subtilis* and *P. aeruginosa*) were inoculated into plastic buckets labeled A – C respectively. A quantity of (20ml) of the standardized inoculum was used in all the inoculations and the entire tests were performed in triplicates with control having no bacterial isolates. These were incubated for 56 days at room temperature (Chinenye, *et al.*, 2014). At every 2 weeks interval, crude oil degrading bacteria were enumerated and at the end of incubation time, residual crude oil was extracted with n-hexane using Soxhlet extraction method and analyzed by Gas Chromatography Mass spectrophotometery. (Romanus *et al.*, 2015; Riskuwa and Udeme, 2016).

RESULTS AND DISCUSSION

The increase in the exploration and usage of petroleum products have resulted in wide spread contamination of the environment. This has led to the concerted efforts in studying the feasibility of detoxifying oil contaminants using bioremediation technique. Analysis of soil samples from oil contaminated soil of National Petroleum corporation deport, Hotoro, Kano State, Nigeria revealed eight (8) bacterial isolates belonging to three genera: *Bacillus* (5 isolates), *Pseudomonas* (2 isolates), and *Staphylococcus* (1 isolate). Two isolates (25%), *Bacillus subtilis* and *Pseudomonas aeruginosa* utilized the crude oil at a maximum rate and were able to grow in the oil medium after 7 days of incubation. Similar organisms were also isolated by other investigators (Deepika and Rajalakshmi 2013, Anupa and Padma, 2009).

Table 1 showed the variation in bacterial counts with time in soil polluted with 5% Bonny light crude oil. The results indicated that the individual isolates gave higher bacterial counts at 42 days of incubation whereas the co-culture gave the highest counts with the mean value of $1.05\pm1.04 \times 10^6$cfu/ml under the same conditions. This was significantly ($P<0.05$) higher than the count obtained at 0, 14, 28 and 56 days for both the isolates. However, the lower bacterial counts initially observed, may be due to the lag phase as the cells are synthesizing essential enzymes and nutrients for growth. Similar results were obtained by Romanus *et al.*, (2015).

Table 1: Total bacterial counts (cfu/ ml) in the presence of 5% Bonny light crude oil

	Time (Days)				
Bacterial Isolates	**0**	**14**	**28**	**42**	**56**
Bacillus subtliis	6.90×10^4 ±1.15j	1.38×10^5 ±1.15h	2.78×10^5 ±1.15f	5.74×10^5 ±1.12c	3.25×10^5 ±1.41e
Pseudomonas aeruginosa	1.10×10^5 ±1.10i	2.20×10^5 ±1.10g	4.40×10^5 ±1.10d	8.40×10^5 ±1.10b	5.40×10^5 ±1.10c
Co-culture	1.15×10^5 ±1.20i	2.69×10^5 ±1.21f	5.67×10^5 ±1.13c	1.05×10^6 ±1.04a	7.64×10^5 ±1.09b

Figure 1 below showed the overall main effect (growth) performance of *B. subtlis, P. aeruginosa,* and Co-culture in 56 days. The results indicated that when overall growth (degradation ability) was compared, the co-culture performed significantly ($P<0.05$) higher than the individual isolates. On the other hand, when individual isolates were considered, *P. aeruginosa* was significantly higher than the *B. subtilis*. These findings agree with the work of Mandri and Lin (2007) who reported that high degradation of crude oil observed by the co-culture of *P. aeruginosa* and *Flavobacterium species* showed significant reduction in petroleum hydrocarbon as compared to the individual isolates.

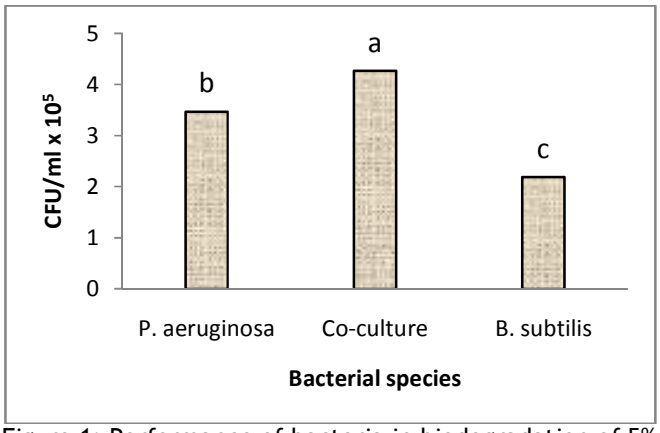

Figure 1: Performance of bacteria in biodegradation of 5% Bonny light crude oil

Figure 2 showed the effect of incubation time on bacterial biodegradation of Bonny light crude oil. The results revealed that crude degrading bacterial counts for single and co-culture increased from day zero to 42 days and was the best incubation time. However, when the organisms were allowed to stay up to 56 days, degradation ability significantly ($P<0.05$) decreased except for the control organism. The gradual decline in colony forming units indicated that the cells entered a stationary phase as it was also observed by Nikhil *et al.*(2013; Romanus *et al.*, 2015). On the contrary, these outcomes disagree with the results obtained by Chinenye *et al.*, (2014), who recorded highest number of hydrocarbon degrading bacteria on 84 days of the experiment.

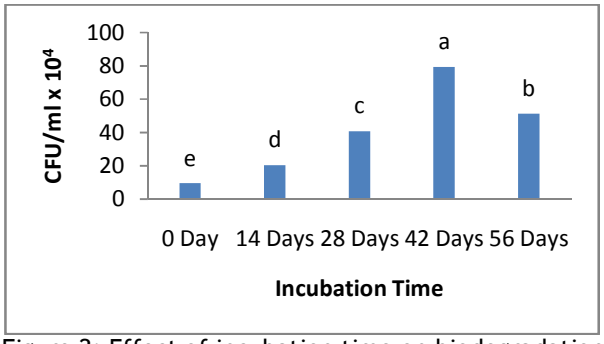

Figure 2: Effect of incubation time on biodegradation of 5% Bonny light crude oil

Figure 3-6 showed Gas Chromatography results and these indicated that all the isolates had degraded the crude oil. This is because the untreated crude oil showed 28 components (number of peaks) while the components degraded gave less than that. This means that some components had degraded to volatile compounds that might have escaped. This agrees with the work of Thenmozhi *et al.*, (2011) who observed significant reductions of the peaks after 30 days of the experiment.

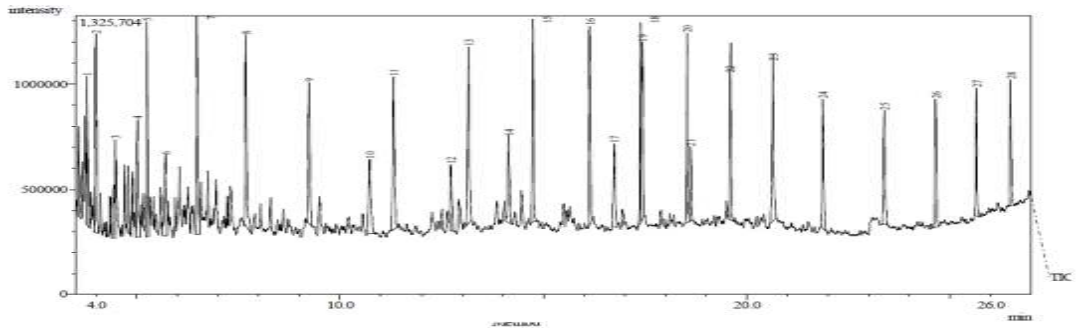

Figure 3: Chromatogram of Bonny light crude (control) after 56 days of incubation

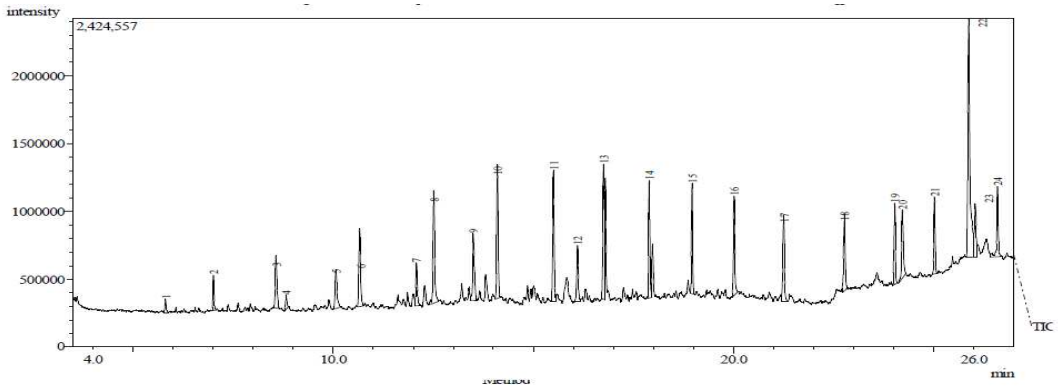

Figure 4: Chromatogram of Bonny light crude oil degraded by *Bacillus subtilis* after 56 days of incubation

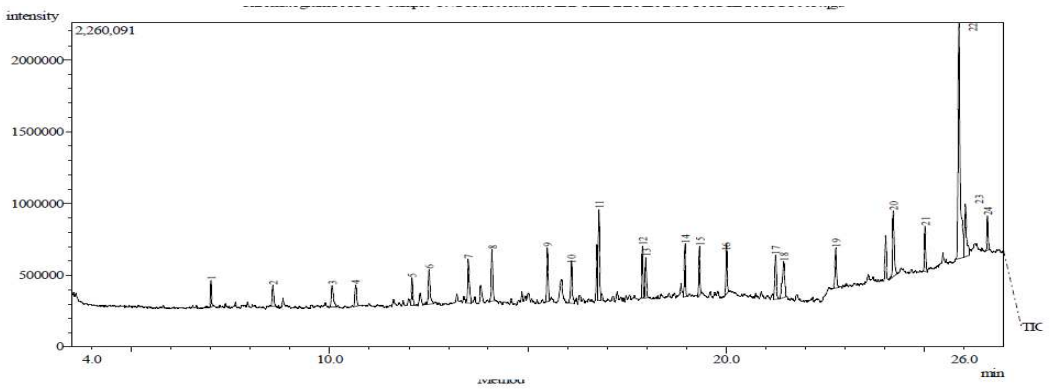

Figure 5: Chromatogram of 5% Bonny light crude oil degraded by *Pseudomonas aeruginosa* after 56 days of incubation.

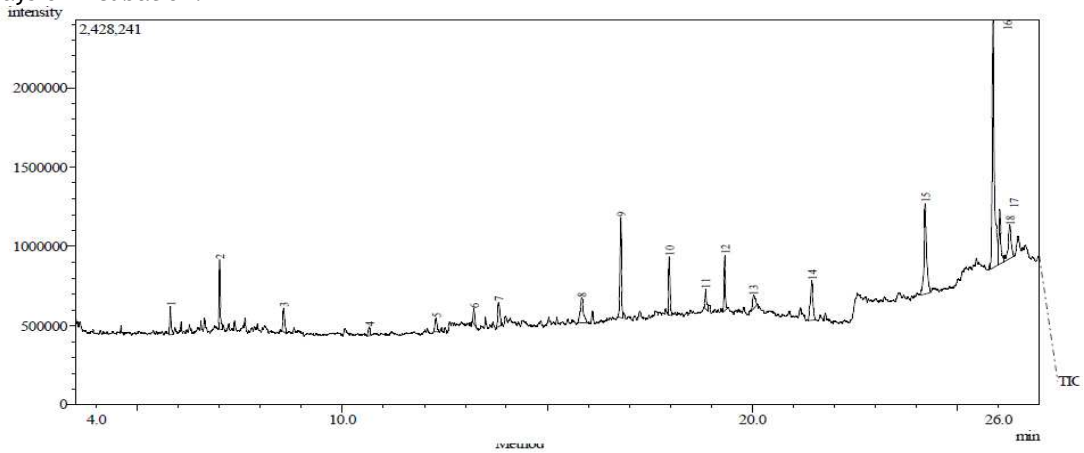

Figure 6: Chromatogram of Bonny li1ght crude oil degraded by co-culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* after 56 days

CONCLUSION

In conclusion, co-bacterial culture (*B. subtilis* and *P. aeruginosa*) has clearly demonstrated high ability to degrade 5% Bonny light crude oil over a period of 56 days of incubation. Therefore, *B. subtilis* and *P. aeruginosa* can be use for bioremediation of soil contaminated with oil.

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