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BIODEGRADATIVE ACTIVITIES OF SOME GRAM- NEGATIVE BACILLI ISOLATED FROM KEROSENE TREATED SOIL GROWN WITH COWPEA(Vigna unguiculata)

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ABSTRACT

The biodegradative activities of three Gram negative bacilli (Aeromonas hydrophila, Vibro parahaemolyticus and Actinobacillus sp) isolated from soil contaminated with kerosene and planted with cowpea was investigated. The isolates were identified using MicrobactTM ID 24E system for the identification of Enterobacteriaceae and common miscellaneous Gram negative bacilli (MGNB). 2kg of soil collected from University of Ilorin was placed inside transparent, drilled buckets. Physicochemical properties of the soil were recorded. The soil was contaminated with kerosene at different concentrations of 0ml, 7ml, 14ml, 21ml, 56ml, 112ml, 168ml and 224ml. The setup was laid out in a randomized complete block design with three replicates. Cowpea seeds of the variety Samaru-40 were cultivated and observed for eight weeks. The results indicated that kerosene contamination of soils significantly affected the growth parameters: germination percentage, time of germination, rate of germination, plant height, number of leaves, leaf area and root nodules. A negative interaction existed between the level of the contaminant and the growth characteristics measured. Their biodegradative activities were studied and confirmed by the change in the Total Petroleum Hydrocarbon (TPH) using gravimetric method. The biodegradative abilities of the isolates were compared by measuring the optical densities, total viable count, pH and emulsification activity. The results showed that the organisms did better as a consortium rather than singly. Aeromonas hydrophila had the highest biodegradative activity followed by Vibro parahaemolyticus and then Actinobacillus sp. The study recommends the use of Aeromonas hydrophila and consortium for more effective biodegradation.

Keywords: Bacterial Biodegradation, Soil, Cowpea, Kerosene, Total Petroleum Hydrocarbon.

INTRODUCTION

The frequency and risk of oil pollution has led to extensive research. Approximately five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995). A wide range of studies have dealt with biotransformation, biodegradation, and bioremediation of petroleum hydrocarbons and interest in exploiting crude oil-degrading organisms for environmental clean-up has become central to petroleum microbiology (Head and Swannell, 1999), but scientists have reported that indigenous and adapted micro-organisms are more efficient for biodegradation of oil pollutant. (Bharathi and Vasudevan, 2001; Seklemova et al., 2001).

Oil-contaminated soils are of environmental concern because they are unsuitable for agricultural and recreational uses and are potential sources of surface and ground water contamination (Ikhajiagbe et al., 2013).Oil pollution of soil leads to build up of essential organic carbon (C), phosphorus (P), calcium (Ca), and non-essential (magnesium (Mg), lead (Pb), zinc (Zn), iron (Fe), copper (Cu) elements in soil and the eventual translocation in plant tissues (Vwioko et al., 2006). This therefore, poses high toxicity and public health challenges as plant consumers are exposed to dangerous heavy metals.

Cowpea is an essential legume in Nigeria (Abayomi and Adeyini, 2005). The current world research efforts now supports the development of plant products with proven crop protection potentials compared to the use of chemicals which may be toxic to both the plants and environment (Aliyu et al., 2011).

Bacteria play the central role in hydrocarbon degradation. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs (Trindade et al., 2005). In many ecosystems, there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favourable for oil-degrading metabolic activity (Capelli et al., 2001). This work focuses on the potential of 3 Gram negative bacilli isolated from kerosene to degrade hydrocarbon.

MATERIALS AND METHODS

Soil Analysis

The soil analysed was collected from fallow land close to the University of Ilorin Dam area. It was dark in colour and loose alfisol loam according to USDA classification. The soil was collected in sterile polythene bags using hand trowel. The experimental arrangement comprised eight (8) treatment options including the control. Each option had three replicates. 3000g of soil was poured into twenty four (24) transparent plastic planting pots. Soil physicochemical parameters were measured using appropriate testing tools. Each planting pot was treated with eight (8) different concentrations of kerosene; 0 (control), 7, 14, 21, 56, 112, 168 and 224ml according to the method of (Adetitun et al., 2014; Ekpo and Thomas, 2007). Three healthy cowpea seeds were planted in each pot and thinned to two to allow vigorous growth after germination. The cowpea was sourced from the National Seed Council, Kwara State. The cowpea seeds were identified as Samaru-40 type. The arrangement of the pots was randomized according to standard methods. The plants were watered daily by using a graduated cylinder, since the experiment is a controlled one. The planting pots were perforated at the sides and bottom to allow for drainage of excess water and aeration.

Determination of Microbial Load

The pour plate method of Zajic and Suplison (1972) was used in determining the microbial load of the treated soil in the bottles with an interval of 7 days for 8 weeks. From each bucket, 0.5g of soil

was taken and serial dilution ranging from 10-6 to 10-8 was prepared. Three plates from each dilution was inoculated and incubated at 37oC for 24 hours. Bacterial counts were done using the colony counter.

Characterization and Identification of Isolates
Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and some biochemical characteristics according to the scheme of Cowan and Steel (Barrow and Feltham, 1995). This organisms were further characterized and identified with the aid of MicrobactTM ID 24E system for the identification ofEnterobacteriaceae and common miscellaneous Gram negative bacilli (MGNB). The MicrobactTM ID 24E kit was used according to manufacturer's specifications (Oxoid Ltd., Basingstoke, Hants, UK).

Determination of Total Petroleum Hydrocarbon (TPH)
The gravimetric method described by Matthew (2009) was used for calculating the TPH. This was done at the start and at the end of the experiment.

Biodegradation Studies
The method described by Adetitun et al. (2014) was used for the biodegradation studies. The isolates used include Vibrio parahaemolyticus, Aeromonas hydrophila, Actinobacillus sp, Vibrio parahaemolyticus + Aeromonas hydrophila, Aeromonas hydrophila + Actinobacillus sp, Vibrio parahaemolyticus + Aeromonas hydrophila + Actinobacillus sp. They were used singly and as a consortium and also with controls.

Emulsification Test (E24)
This test was done as described by Cooper and Goldenberg (1987); Dhail and Jasuja (2012).
Plant Studies in Relation to Pollution Effects.
The plant's height, leaf length and breadth were measured weekly for 4 weeks. Also at the end of the whole planting process, the cowpea plant was uprooted and the nodules counted.

Statistical Analyses
One-way analysis of variance (ANOVA) test was used to determine whether the measured parameters differed significantly. P value less than 0.05 was considered to indicate statistical significance.

RESULTS

Table 1: Physicochemical Properties of the Unpolluted Soil

Soil Properties	Values
Temperature	35.0oC
pH	7.06
Moisture Content	57.6%
Water Holding Capacity	0.59 ml/g

Table 2: Total Petroleum Hydrocarbon at the Start and End of the Experiment

Treatment (ml)	TPH (mg/kg) at week 1	TPH (mg/kg) at week 8
0	0	0
7	28.4	18.4
14	32.4	22.1
21	38.9	27.8
56	78.4	33.4
112	109.2	102.3
168	118.8	108.6
224	210.9	132.4

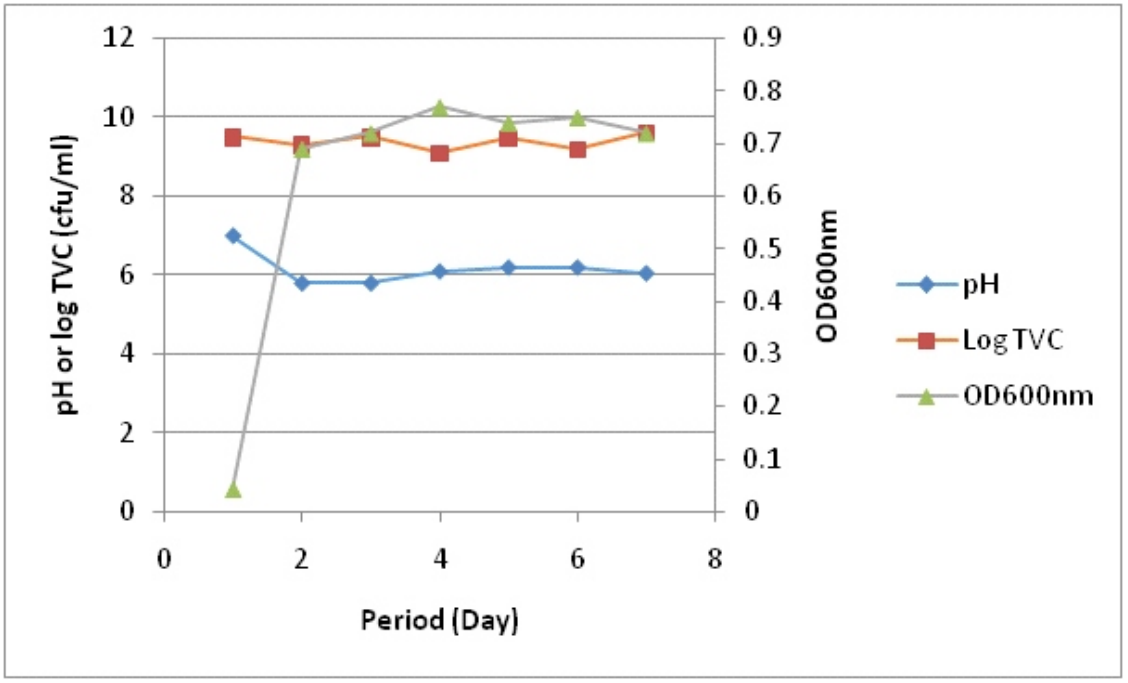
Table 3: Identity of the Gram Negative Bacterial Isolates from the Contaminated Soil Grown with Cowpea.

Isolate	Identity
A	Vibrio parahaemolyticus
C	Aeromonas hydrophila
F	Actinobacillus sp

Table 4: Occurrence of Bacteria in the Contaminated Soil during the Period of Experimentation.

Organism	Treatment Concentration (ml)	Period (weeks)				Percentage frequency (%)
		1	2	3	4	
Vibrio parahaemolyticus	0	-	+	+	+	75
	7	+	-	-	+	50
	14	-	-	+	+	50
	21	-	-	-	+	25
	56	-	-	-	+	25
	112	-	-	+	+	50
	168	+	+	+	+	100
	224	+	+	+	+	100
Aeromonas hydrophila	0	-	+	+	+	75
	7	+	+	+	+	100
	14	-	+	+	+	75
	21	-	+	+	+	75
	56	-	+	+	+	75
	112	-	+	+	+	75
	168	-	+	+	+	75
	224	+	+	+	+	100
Actinobacillus sp	0	-	+	+	-	50
	7	+	+	+	+	100
	14	+	-	+	-	50
	21	-	+	+	-	50
	56	-	-	+	+	50
	112	-	+	+	+	75
	168	-	-	+	+	50
	224	-	+	+	+	75

Legend: + = Present, - = Absent.



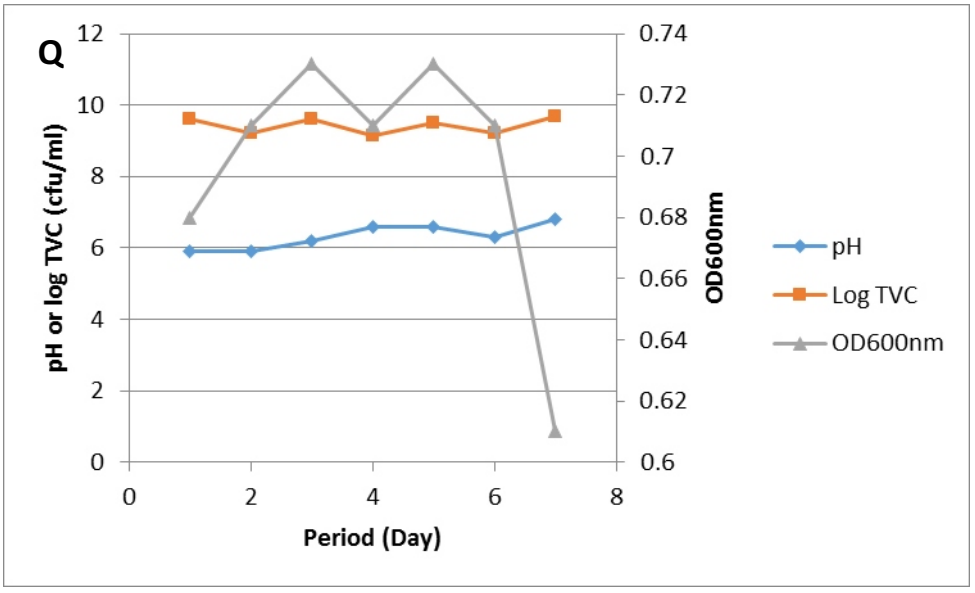
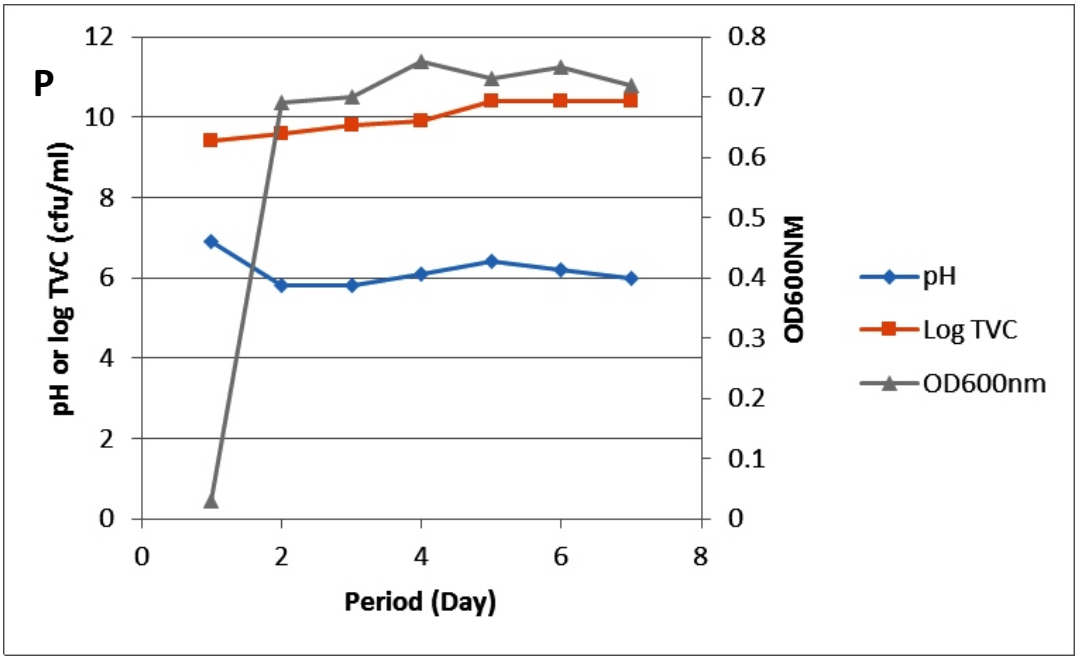


Figure 1: Growth property of Vibrio parahaemolyticus on Kerosene. (P) with kerosene; (Q) without kerosene



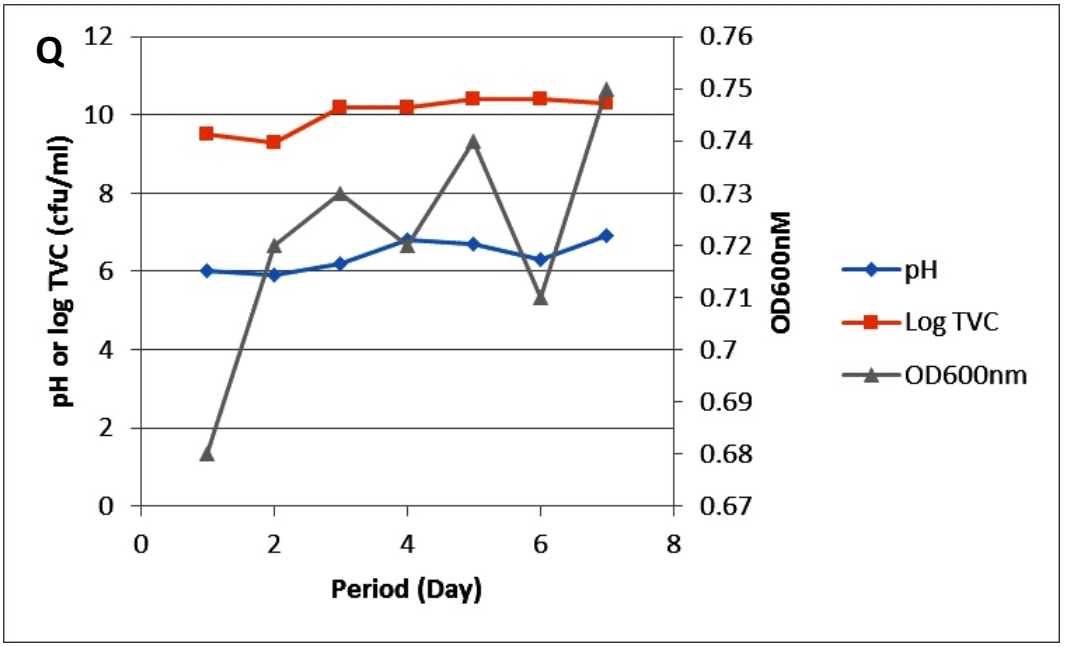


Figure 2: Growth property of Aeromonas hydrophila on Kerosene. (P) with kerosene; (Q) without kerosene.

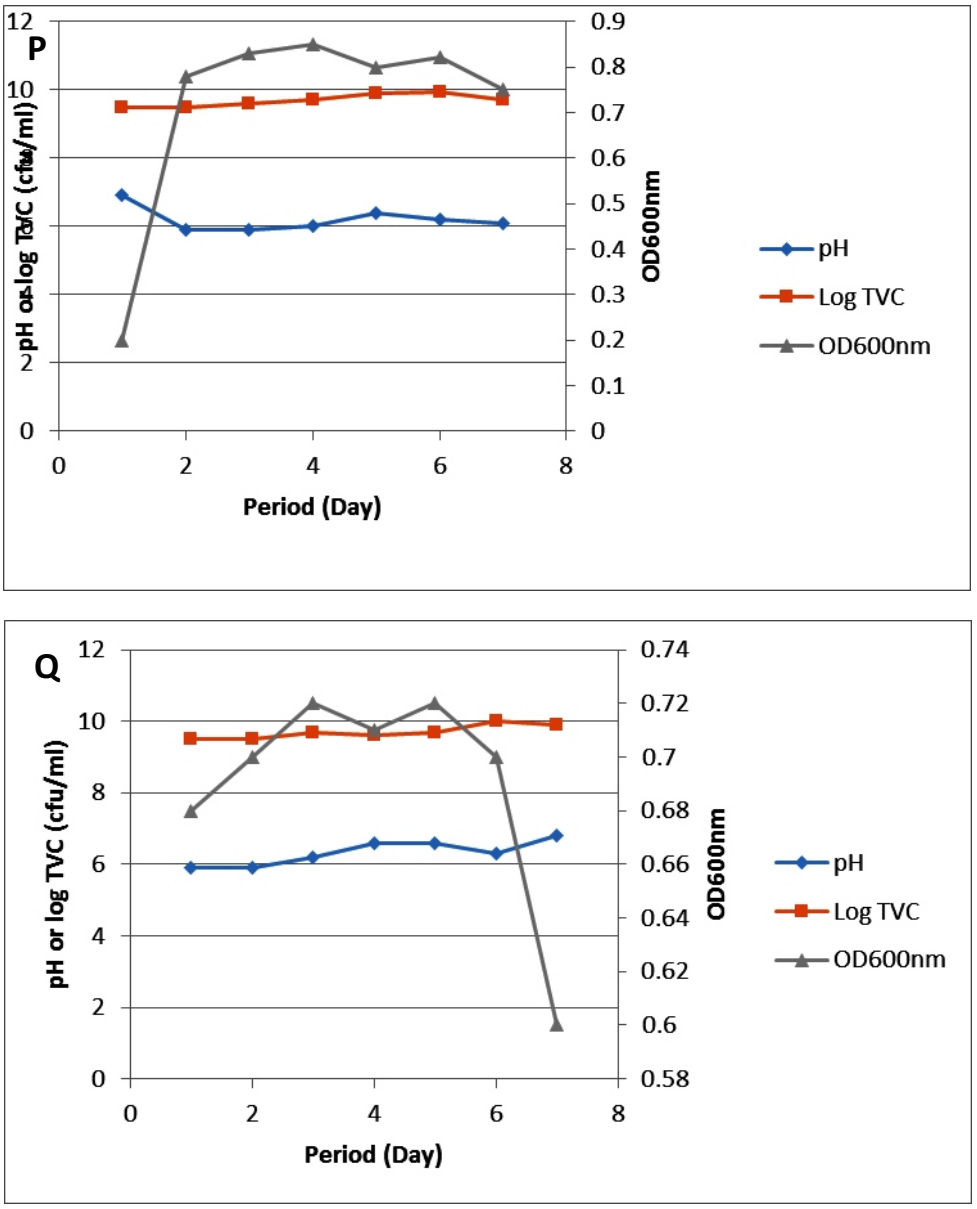
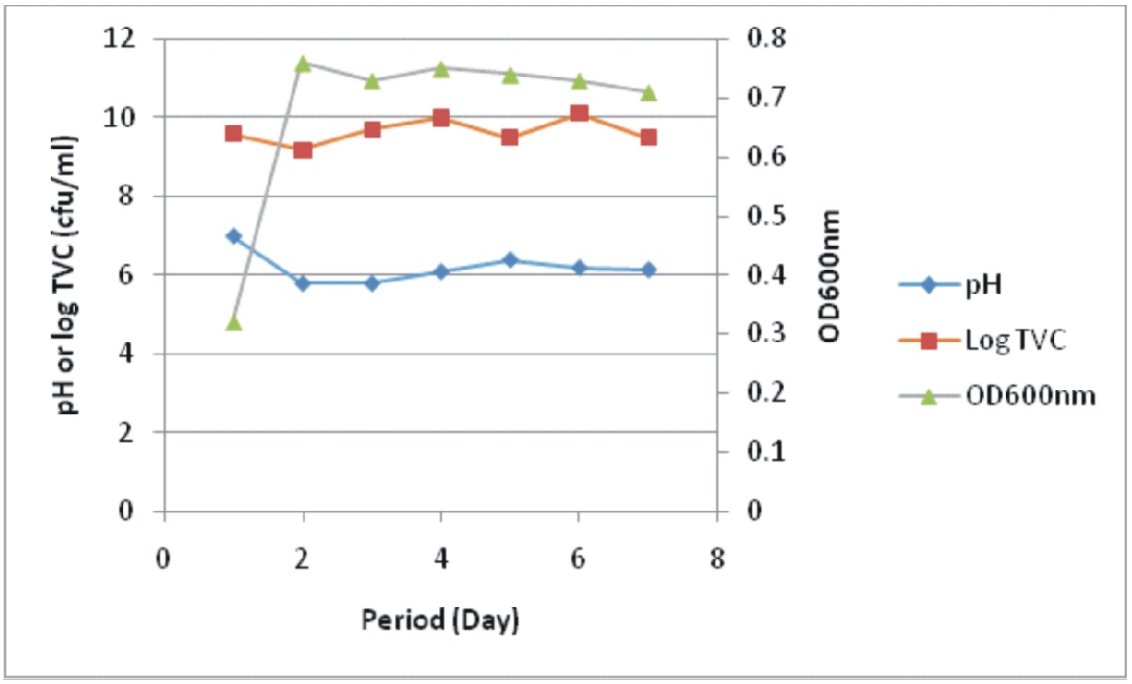


Figure 3: Growth property of Actinobacillus sp on Kerosene. (P) with kerosene; (Q) without kerosene.



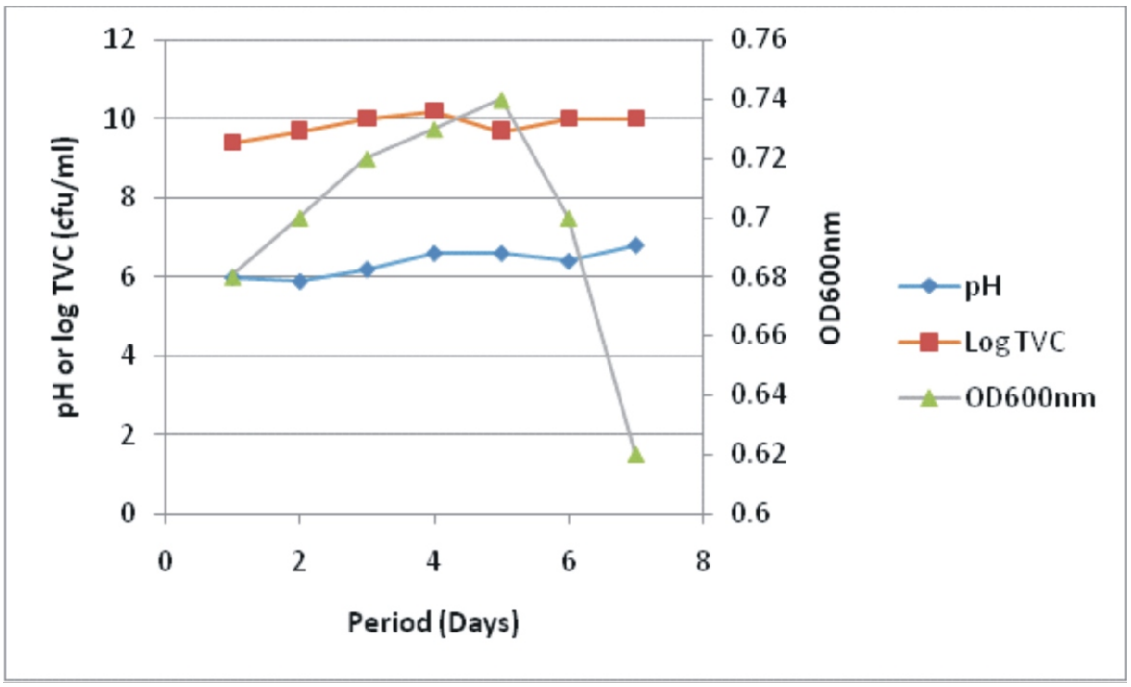
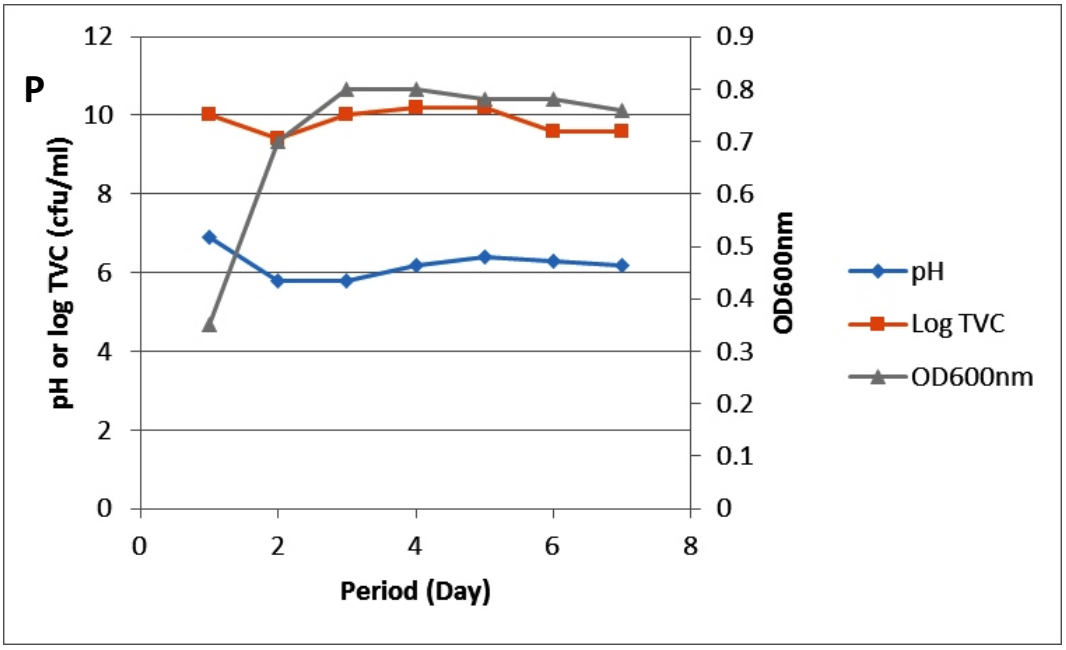


Figure 4: Growth property of Vibrio parahaemolyticus and Aeromonas hydrophila on Kerosene. (P) with kerosene; (Q) without kerosene.



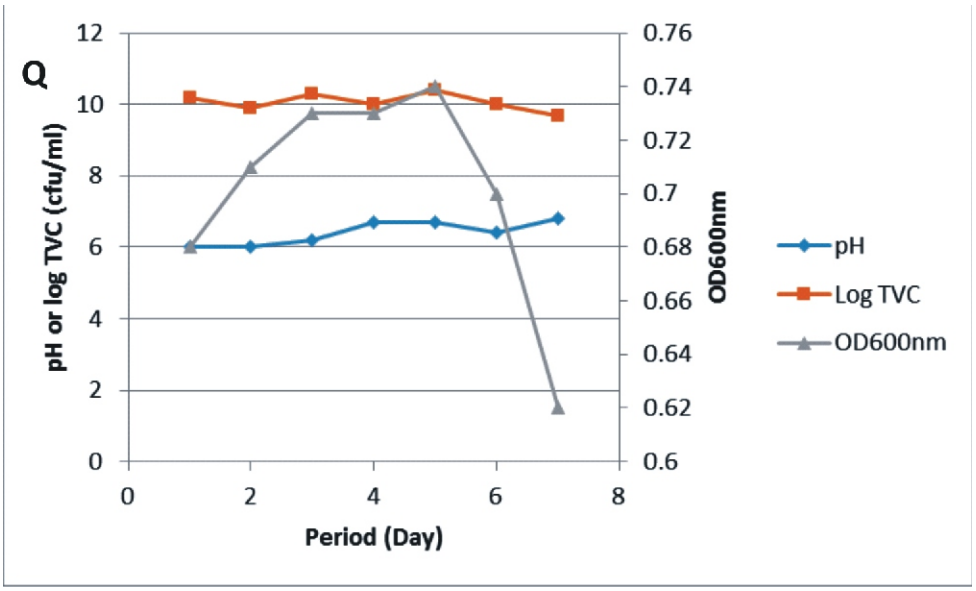
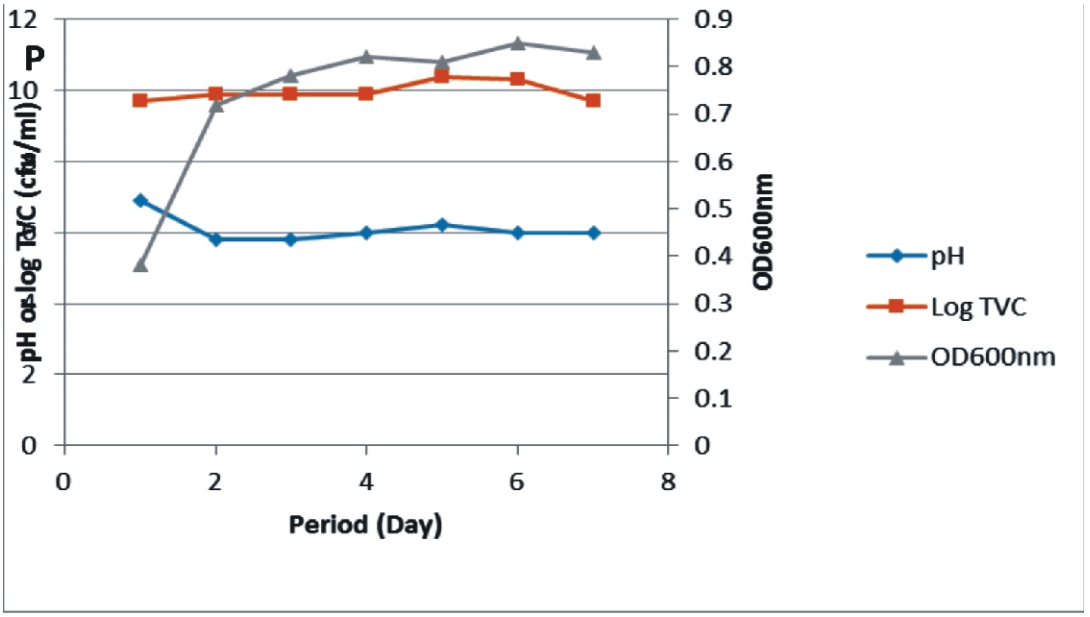


Figure 5: Growth property of Aeromonas hydrophila and Actinobacillus sp on Kerosene. (P) with kerosene; (Q) without kerosene.



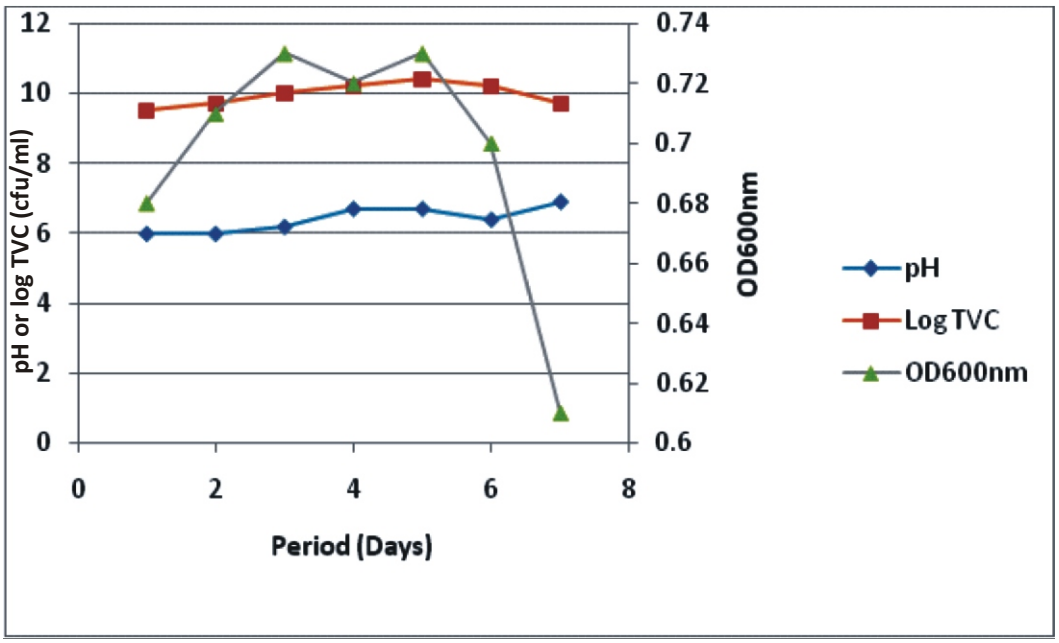


Figure 6: Growth property of Vibrio parahaemolyticus, Aeromonas hydrophila and Actinobacillus sp on Kerosene. (P) with kerosene; (Q) without kerosene.

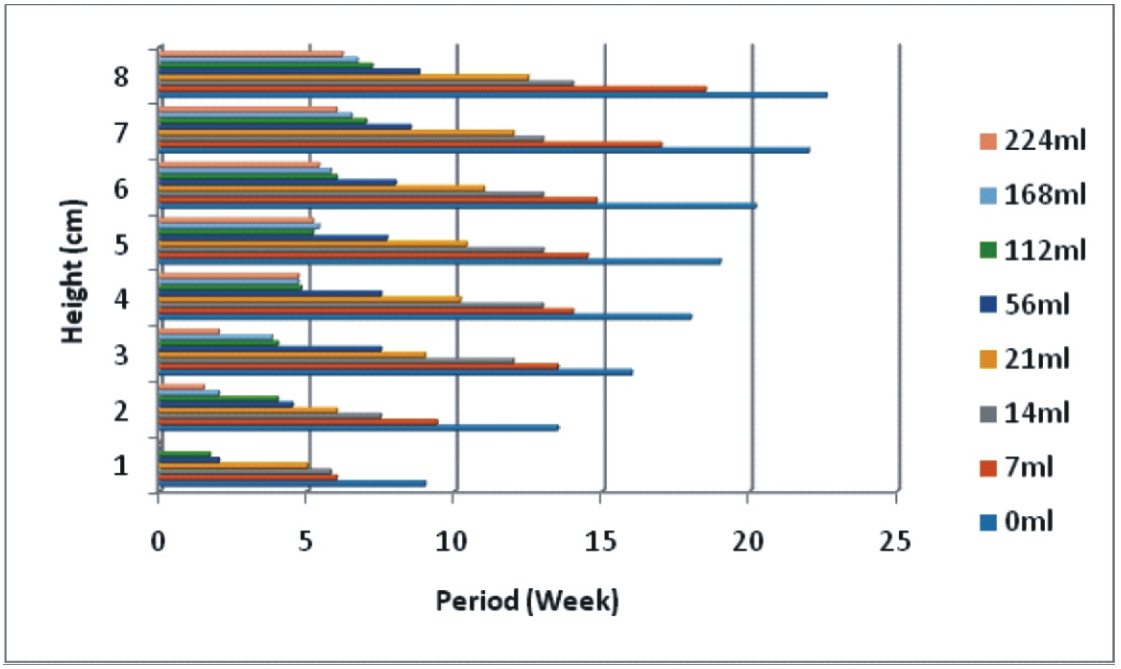


Figure 7: Height of Cowpea (Vigna unguilata) During the Period of Experimentation.

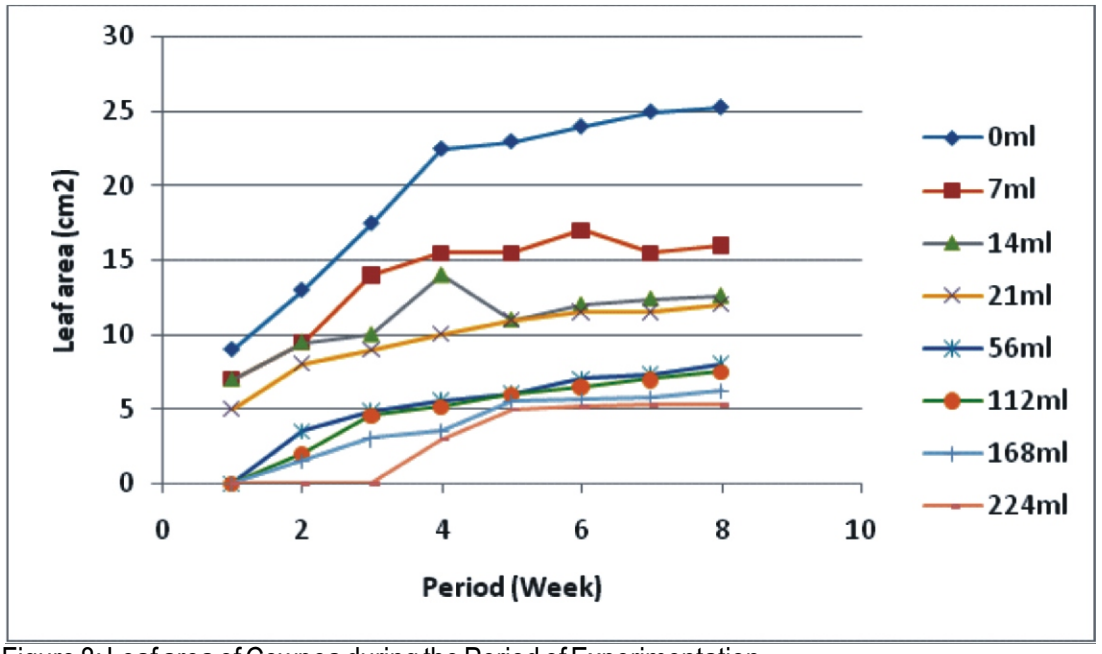


Figure 8: Leaf area of Cowpea during the Period of Experimentation.

Table 5: Emulsification activity of the bacterial isolates individually and as a consortia.

Organism/ Consortium	Emulsification index (%)
Vibrio parahaemolyticus (A)	15.69
Aeromonas hydrophila (C)	57.63
Actinobacillus sp (F)	7.27
Vibrio parahaemolyticus and Aeromonas hydrophila (AC)	11.77
Aeromonas hydrophila and Actinobacillus sp (CF)	55.93
Vibrio parahaemolyticus, Aeromonas hydrophila and Actinobacillus sp (ACF)	7.27

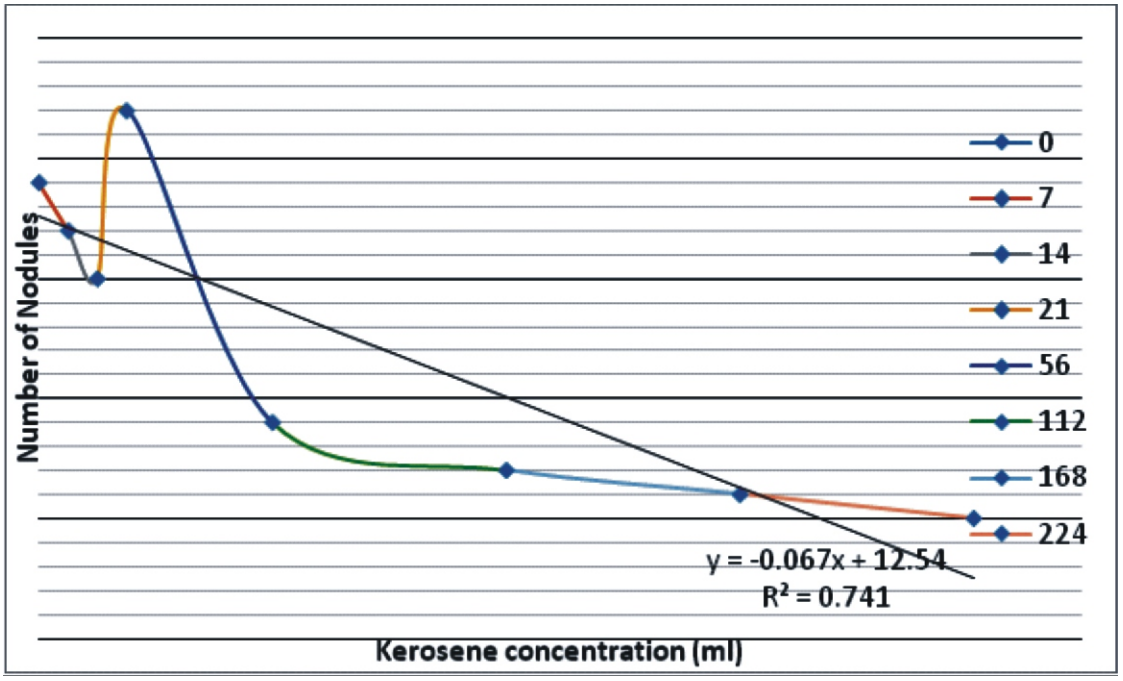


Figure 9: Number of cowpea root nodules at maturity.

DISCUSSION

The total petroleum hydrocarbon reduced at the end of the experiment compared to that at the start. For 0ml it remained 0. For 7ml it changed from 28.4 to 18.4. For 14ml it moved from 32.4 to 22.1. For 21ml it moved from 38.9 to 27.8. For 56ml, it reduced from 78.4 to 33.4. For 112ml, it changed from 109.2 to 103.2, for 168ml it changed from 118.8 to 108.6 while for 224ml it moved from 210.9 to

132.4 (All units in mg/kg). This indicates that some biodegradation activities had occurred. This is in agreement with a study performed by some scientists on petroleum contaminated soil sample in North East India (ONGC). In the Indian study, the results showed reduction of TPH level from 84-21 g/kg soil after treatment for 120 days (Das and Mukherjee, 2007).
The bacterial load revealed an increase in the soil with no contamination but in the polluted soils during the second and third week of isolation, the population reduced but came up again at the fourth week. This may be associated with the lag, log, stationary and death phase of growth in the presence of kerosene. Specifically, there was a gradual increase in the total heterotrophic bacterial count from the third to the fourth week in most of the treatments. This increase could be as a result of the utilization of kerosene by indigenous hydrocarbon degrading bacteria. Similar findings have been reported by (Ekpo et al., 2007) who discovered an increase in the microbial population in crude oil contaminated soils.
The hydrocarbon degraders were found to be persistent during this experimentation. The growth dynamics of the organisms reflected the ability of Aeromonas hydrophila, Vibro parahaemolyticus and Actinobacillus sp to degrade and utilize kerosene as a source of carbon and energy. Data from control experiments in which the isolates were grown on kerosene free mineral salts medium showed that the isolates did not perform well in the absence of kerosene. Similar observation was made in a study to show the ability of bacteria to utilize crude oil (Emtiazi and Shakarami, 2004).

The observable effects that kerosene pollution can have on the crops cultivated in such soils are clearly seen with respect to the increasing concentration of kerosene. The plant's height and leaf area were negatively affected. For instance, pollution level of 56ml significantly delayed emergence while higher kerosene pollution levels, 168ml and 224ml subdued the germination of cowpea seeds. This could be attributed to the fact that kerosene impaired free flow of oxygen in the soil. The effect could also be as a result of formation of polar compounds dissolved in the water that could penetrate the seed coat, exerting polar necrosis (Adam and Duncan, 2002). Nevertheless, plants in the uncontaminated soil thrived normally. This observation is supported by the research of Adesina and Adelasoye (2014). They reported that Maize and Cowpea performed poorly in polluted soil when compared with unpolluted soil.

It can be generally deduced that an increase in the concentration led to decrease in the number of root nodules formed. At 0ml, the root nodules were higher but their numbers reduced at 7ml and 14ml. 21ml had the highest number of nodules and this can be traced to the increase in microbial load at this concentration which may enhance the root-bacteria interaction. Furthermore, kerosene contamination interfered with nitrification because nitrifying bacteria were observed to reduce with increased concentration of kerosene confirmed by the nodules formed. This finding agrees with the report made on diesel contaminated soils (Ekpo and Nkanang, 2010) that it could be due to the fact that under the kerosene oil environment, the nitrifying bacteria could not effectively compete with other organisms that multiplied rapidly, resulting in the exhaustion of the available inorganic nitrogen. Muratova and others (2003) reported that soil polluted with bitumen recorded reduced count of

nitrifying, nitrogen fixing, denitrifying and amonifying bacteria in the rhizosphere of both alfalfa and reed.

Emulsification values increased with increasing cell growth. Emulsification activity of Aeromonas hydrophila (57.63%) was better than Actinobacillus sp and Vibrio parahaemolyticus. The emulsification activity also suggested that the application of bacterial consortiums containing combinations of either the two or the three isolates did not enhance degradation. Rosenberg et al., (1979) reported the ability of the extracellular emulsifying agent of Arthrobacter sp, Aeromonas and Bacillus sp. to emulsify crude oil and fractions of crude oil. This is confirmed by the total bacterial count in this study in which Aeromonas hydrophila recorded the highest at 292*108 cfu/ml on the 5th day due to enhanced metabolism of the kerosene. It can be traced to the production of biosurfactants in agreement with Priya's experiment that confirmed that biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous hydrocarbon interface. This increases the rate of hydrocarbon dissolution and thereby utilization by microorganisms (Priya, 2013). It has been known for many years that Vibrios metabolize crude oil in vitro (Berardesco et al., 1998).

In conclusion, this study has shown clearly the harmful effects of kerosene pollution on Cowpea. It is clear from this investigation that Aeromonas hydrophila can degrade kerosene more efficiently. Further understanding of the metabolic process of this organism on kerosene will increase possibilities of developing models and strategies for removing petroleum and its products from oil-polluted environments.

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