Int.J.Curr.Microbiol.App.Sci (2019) *8*(12): 2612-2622

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 12 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.812.305

Study on Bioremediation and Growth Curve Patterns of Bacteria Cultured in Hydrocarbon Formulated Media at Different Concentrations

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Keywords

Biosorption, Biodegradation, Hydrocarbon, Effluent, Bacteria

Article Info

Accepted:
20 November 2019
Available Online:
10 December 2019

A B S T R A C T

Bioremediation, which employs the biosorption and or biodegradation potentials of organisms or their attributes, is an effective technology that can be used to accomplish both effective detoxification and volume reduction. Research was carried out to further provide experimental evidences that support the use of biological method which is a better, less expensive and safer means of biosorbing hydrocarbon contaminants. Bacteria isolated from the African locust bean (*Parkia biglobosa*) effluent namely; *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and *Acetobacter* spp were used to biodegrade crude-oil products such as petrol, kerosene and diesel oil at varying concentrations in a culture medium. The pattern of microbial growth differs from organism to organism due to several factors such as the incubation period, the nature and composition of the nutrient in which the organism was cultured. Microbial load was between the range of 4.0-5.0 x 10^6 Cfu/ml for *B. cereus*, *B. subtilis*, *P. aeruginosa and Acetobacter spp* when cultured in 0.2-0.4 ml petrol concentration. Also, increase in the concentration of the hydrocarbon in the medium from 0.2ml to 0.6ml led to a declination of cell density in all the four microbes investigated. This work established the fact that *B. cereus* proved to be a better hydrocarbon degrader as compared to the other bacteria isolates used in this study in this order: *B. cereus* > *B. subtilis* > *P. aeruginosa* > *Acetobacter* spp. In conclusion, this work revealed that *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and *Acetobacter* species isolated from effluent collected from production site of fermented African locust beans condiment are able to utilize hydrocarbon compounds as their carbon source, thereby proving their biodegradability potentials.

Introduction

Petroleum has been known for several years even as far back as the pre-Christian times by the Chinese, and over the years, petroleum product have been modified and used for different purposes. The modern petroleum industry had its beginning in Romania and in a well-sunk in Pennsylvania by Colonel E. A. Drake in 1859 (Alloway and Ayres, 1993).

Petroleum can be defined as any mixture of natural gases, and crude oil composed of hydrocarbons consisting almost entirely of the elements Hydrogen and Carbon in the ratio of about 2 to 1. It also contains elements such as Nitrogen, Sulphur and Oxygen, all of which constitute less than 3% (v/v). Phosphorus and heavy metals such as Vanadium and Nickel are also trace constituents comprising less than 1% (v/v) (Atlas, 1981).

Despite the many uses of petroleum product and its benefits to man, petroleum and its derivatives pose a serious threat to the health of man, animals, plants and to the ecosystem as a whole regionally or globally. The threats posed by mismanagement of petroleum product include air pollution, global climate change, and death of aquatic life in oil spills in bodies of water. Exploration for and production of petroleum have caused local detrimental impacts to soils surface and groundwater, hence damaging the ecosystems (Richter and Kreitler, 1993; Kharaka and Hanor, 2003). These impacts arose predominantly from the inappropriate disposal of some of the large volumes of effluent, some of which contained heavy metals, radioactive compounds and toxic organic and inorganic compounds produced with oil and gas, or from accidental hydrocarbon and waste water releases, and from abandoned oil wells (Kharaka *et al.,* 1995; Veil *et al.,* 2004). Impacts and ground-surface disturbances can also arise from related activities such as site clearance, construction of roads, tank batteries, brine pits and pipelines, and other land alterations required for the drilling for exploration and production wells and construction of production facilities. The cumulative impacts from these operations are high.

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in the environment (Brooijmans *et al.,* 2009). Several bacteria species are known to feed entirely on hydrocarbons. Bossert and Bartha (1984) listed 22 genera of bacteria and 31 genera of fungi. Yakimov *et al.,* (2007) listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. In earlier days, the extent to which bacteria participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but the increase discovery of microorganisms that obtain nutrient from hydrocarbon, initiated the appreciation of biodegradation as a function of the ecosystem and local environmental conditions, thus encouraging the study of their impact in degradation of petroleum hydrocarbon (Das *et al.,* 2007).

Malaieswari *et al.,* (2017) in their work have also reported the isolation of fungal isolates and evaluated their heavy metal utilization and other pollutants degradation potential using atomic absorption spectroscopy.

Several studies have examined the fate of petroleum in various ecosystems (Bruheim and Eimjjelle 1998). The development of petroleum industry into new borders, the apparent inevitable spillages that occur during routine operations, and records of acute accidents during transportation has called for more studies into oil pollution problems (Cooney 1984), which has been recognized as the most significant pollution problem in the world. Also, the extensive use of petroleum products leads to the contamination of almost all part of the environment, and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the remediation of hydrocarbon-polluted environments (Challaina *et al.,* 2004), the mechanism of which has been extensively studied and reviewed (Lindstrom and Braddock, 2002).

Crude oil can be accidentally or deliberately released into the environment leading to serious pollution problems (Thouand *et al.,* 1999). Even small releases of petroleum hydrocarbons into water bodies can lead to increase in the concentrations of dissolved hydrocarbons above the regulatory limits (Spence *et al.,* 2005). These pollution problems often result in huge disturbances of both the biotic and abiotic components of the ecosystems (Mueller *et al.,* 1992), more so that some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organo-pollutants (Hallier-Soulier *et al.,* 1999). It is a common stance that many farmers in the oil exploration areas in developing countries are experiencing tremendous difficulties in restoring the fertility of extremely polluted farmlands due to lack of knowledge on appropriate remediation procedures. The non-chalant attitude to the problem of oil pollution is particularly of serious concern for food safety in such neglected areas such as the Niger delta regions of Nigeria as persistence of the pollution could result in the release of toxic pollutants into the food chain and water products (Bradley *et al.,* 1997).

The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves three major processes which are physical, chemical and biological alternatives. The biodegradation of oil pollutants is not a new concept as it has been intensively studied in controlled conditions (Sugiura *et al.,* 1997; Chaillana *et al.,* 2004) and in open field experiments (Chaıneau *et al.,* 2003; Gogoi *et al.,* 2003), but it has acquired a new significance as an increasingly effective and potentially cost-friendly cleanup technology. Bioremediation, which employs the biodegradative potentials of organisms or their attributes, is an effective technology that can be used to accomplish both effective detoxification and volume reduction (Caplan 1993).

Besides, bioremediation technology is believed to be non-invasive and relatively cost effective (April *et al.,* 2000). In some cases it may not require more than the addition of some degradation enhancers to the polluted system. It could end up being the most reliable and probably least expensive option for exploitation in solving some chemical pollution problems (Mesarch *et al.,* 2000).

Materials and Methods

Collection of effluent sample

The African locust bean effluent used for this study was collected from a locally producing source located at Eyenkorin, via Ilorin, Kwara State, Nigeria. The effluent was collected in sterile sample bottles and stored in refrigerator at 4^{o}C.

Isolation of microbes

1ml of the locust bean effluent was mixed with 9 ml of normal saline as a diluent in a test tube. Subsequently, serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} 10^{-9}, 10^{-10}) were prepared and diluent 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} was used for the isolation. Nutrient agar used for the isolation was prepared according to the manufacturer's instruction. Plates were labeled appropriately, inverted and incubated at 36^{o}C for 24 hours in an incubator.

Characterization and Identification of Isolates

Pure colonies of the different organisms isolated were sub-cultured from mixed cultures onto a fresh nutrient agar plates. Morphological characteristics were performed and cultures were preserved in agar slant at 4^{o}C.

Hydrocarbon utilization test

Hydrocarbon utilization test was carried out by inoculating an actively growing bacteria into nutrient broth media containing different sources of hydrocarbon product such as kerosene, diesel, petrol at varying concentrations of 0.2 ml, 0.4 ml, 0.6 ml and 0.8 ml. The culture in test tubes were incubated at 36^{o}C and their corresponding absorbance measured at a two days interval at 540 nm using UV- Spectrophotometer. The procedure was monitored for 14 days.

Results and Discussion

Using cultural characteristics, cellular morphology and biochemical, two *Bacillus* species namely; *B. subtilis* and *Bacillus cereus,* species of *Acetobacter,* and *Pseudomonas aerouginosa* were isolated from locust bean effluent from a local factory. Atlas (1981), Okoh and Trejo-Hernandez (2006) had earlier reported that *Pseudomonas, Bacillus, Proteus, Salmonella and Streptococcus* as bacteria isolated from oil contaminated soil samples. Two of these bacteria (*Pseudomonas* and *Bacillus*) have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites. Parte *et al.,* (2017) also reviewed that major bacterial genera such as *Bacillus, Pseudomonas, Flavobacterium, Moraxalla, Acinetobacter, Arthrobacter, Paracoccus, Aerobacter, Alkaligens, Burkholderia* and *Sphingomonas* have been found to help remove or detoxify chlorinated pesticides; polychlorinated diphenyl, polycyclic aromatic hydrocarbons and organo-phosphorus. Kharlifa, (2017) also isolated a new strain of *Kocuria sediminis* DDK6 which was confirmed to have metabolic versatility to degrade diesel oil, thereby providing ecological and environmental merits for its application in bioremediation of hydrocarbon pollutants. Three petroleum products namely; petrol, kerosene and diesel were used as sources of hydrocarbon to be utilize and biodegrade as carbon source and energy by the tested bacteria. The petroleum hydrocarbon used as carbon source are those with carbon chain ranging from 4 to 21 carbons, it can be postulated that the isolates should be able to degrade any hydrocarbon within that range. Among the four bacteria isolates, there were variations in the growth pattern and microbial cell density at different crude-oil concentrations of 0.2ml, 0.4ml and 0.6 ml. Figures 1-3 shows varying concentrations of Petrol as the carbon source to utilize at concentrations of 0.2-0.6ml. Considering Figure 1, microbial load was between the range of 4.0-5.0 x 10^{6} cfu/ml for *B. cereus, B. subtilis, P. aerouginosa and Acetobacter* when cultured in 0.2-0.4ml petrol concentration. At day 4, *B. cereus* showed a double cell density which indicated a faster biodegradation rate of the hydrocarbon as compared with the other three isolates. Continuous increase was recorded at the exponential phase in the four isolates till day 14. when the death phase emerged (Figure 1). Wang *et al.,* (2017) also reported microbial degradation of biomass using a novel anaerobic, thermophilic, and cellulolytic bacterium (strain CSK1) isolated from MC1. The cellulase activity of the CSK1 bacterium used in their work reached the highest level on culturing day 8; the results which also correlates with our present study.

The pattern of microbial growth differs from organism to organism due to several factors such as the incubation period, the nature and composition of the nutrient in which the organism was cultured. It was noted that increase in the concentration of the hydrocarbon in the medium from 0.2ml to 0.6ml led to a declination of cell density in all the four microbes investigated as shown in Figures 1-3. Moreover, it was observed that each of the microbes have different rates at which they utilize and degrade hydrocarbons as carbon and energy source.

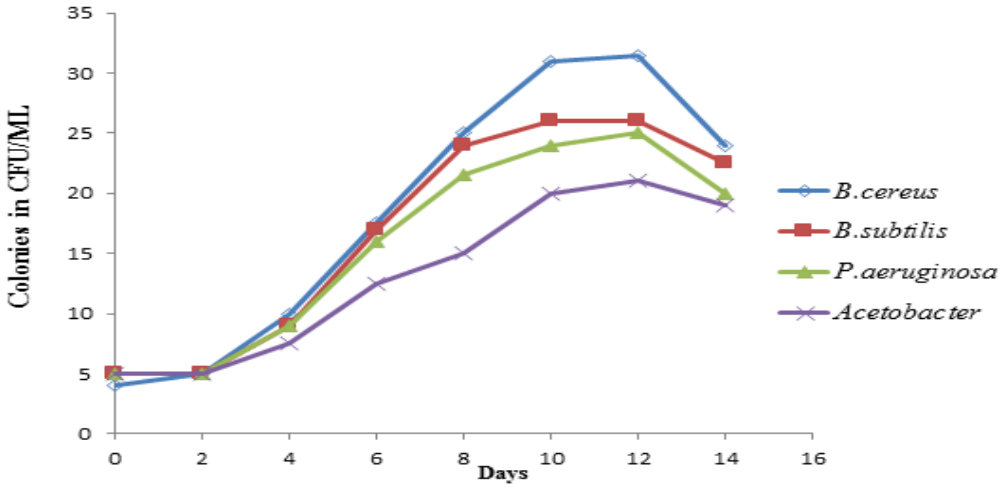

Figure 1 Growth curve pattern of *B.cereus, B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.2ml of petrol

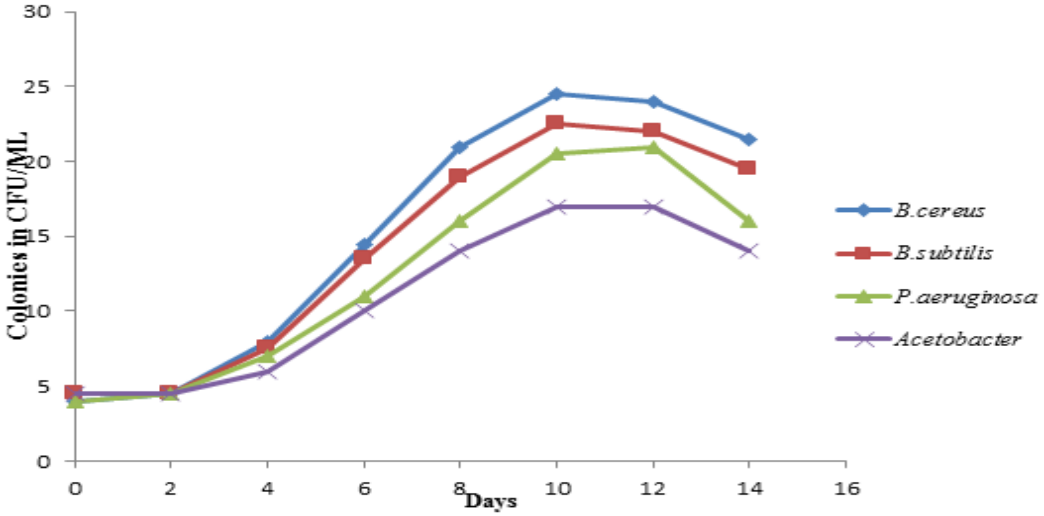

Figure 2 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.4ml of petrol

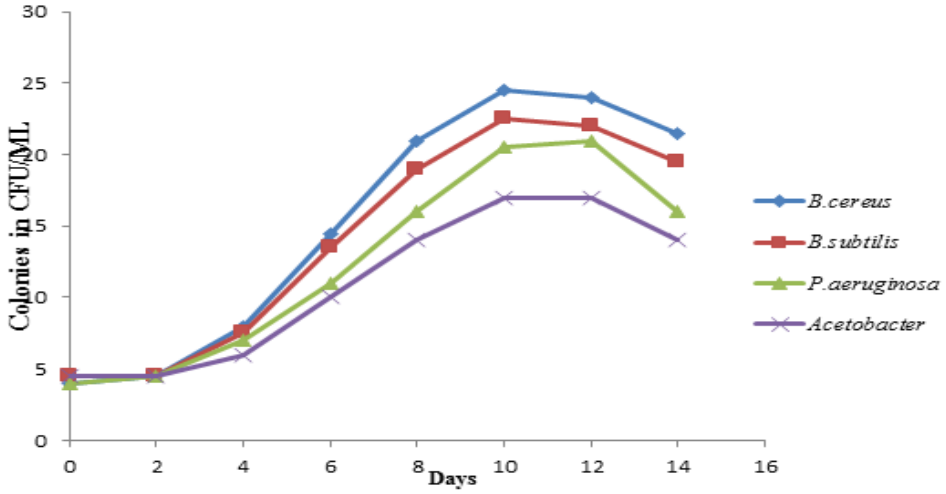

Figure 2 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.4ml of petrol

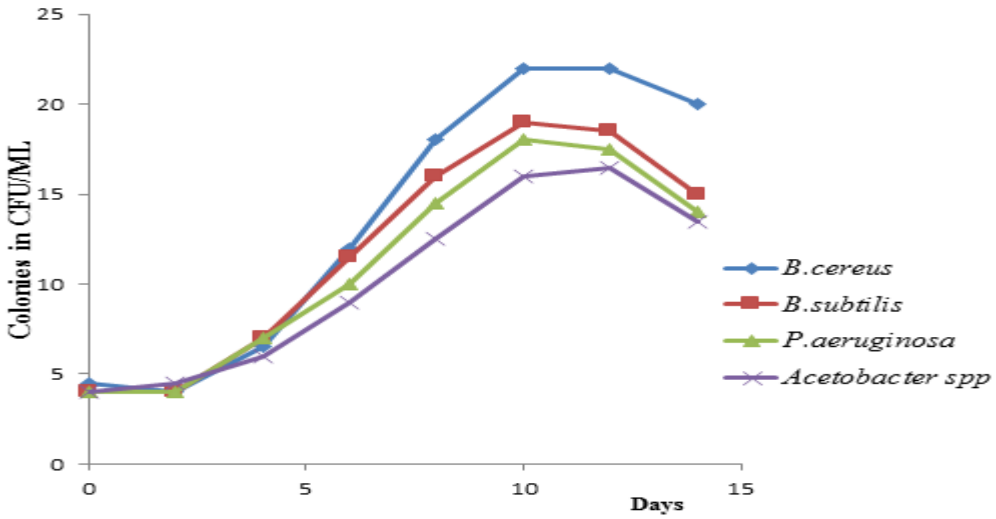

Figure 3 Growth curve pattern of *B.cereus, B.subtilis, P. aeruginosa* and *Acetobacter spp.* in growth broth containing 0.6ml of petrol

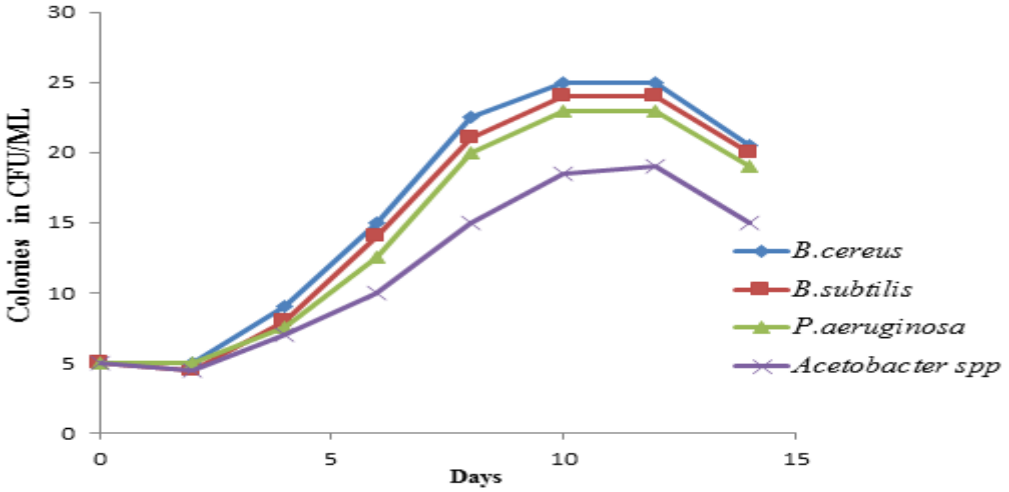

Figure 4 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.2ml of kerosene

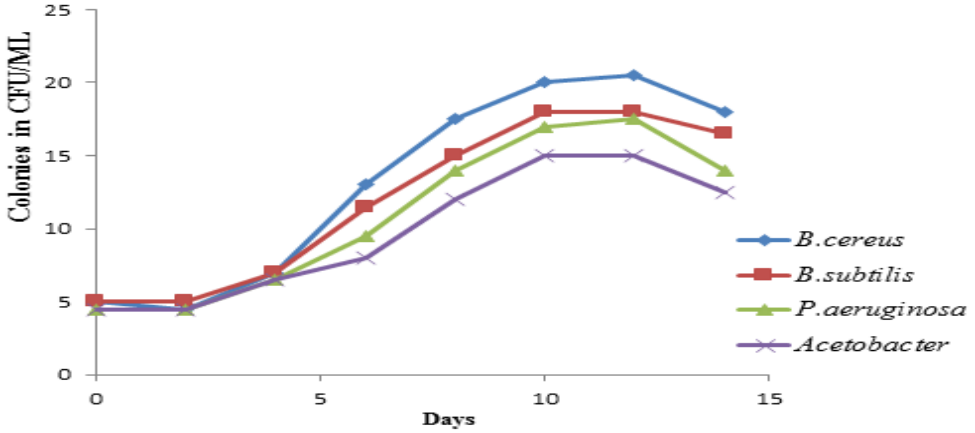

Figure 5 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.4ml of kerosene

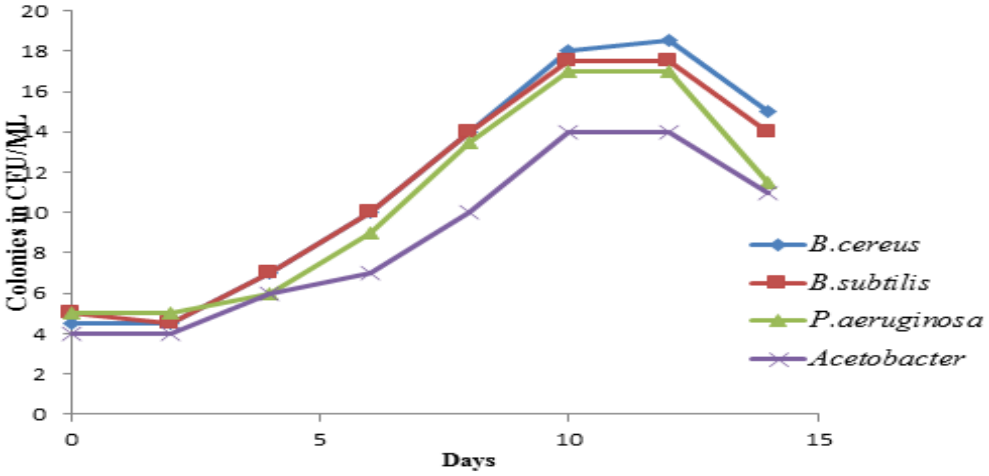

Figure 6 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.6ml of kerosene

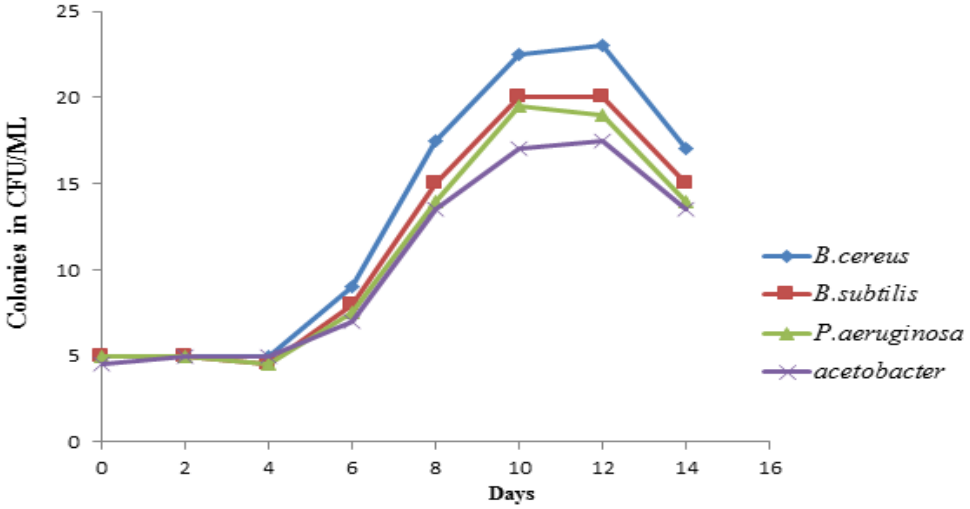

Figure 7 Growth curve pattern of *B.cereus,B.subtilis, P.aeruginosa* and *Acetobacter spp.* in broth containing 0.2ml of Diesel

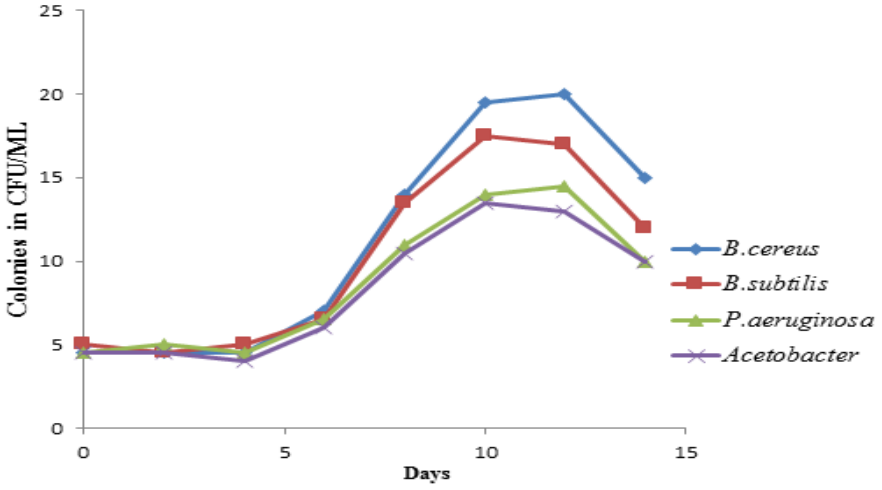

Figure 8 Growth curve pattern of *B.cereus, B.subtilis, P. aeruginosa* and *Acetobacter spp.* in broth containing 0.4ml of Diesel

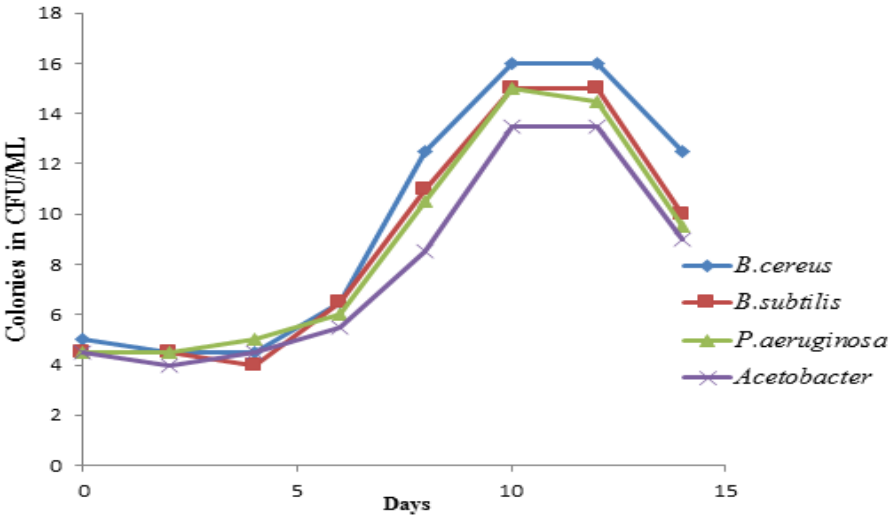

Figure 9 Growth curve pattern of *B.cereus, B. subtilis, P. aeruginosa* and *Acetobacter spp.* in broth containing 0.6ml of Diesel

Plate.1 Biodegradative activities of *P. aeruginosa* in diesel supplemented broth

This rate is mirrored in the degree at which the broth became turbid. This is in correlation with Stanbury and Whitaker (1989) when they highlighted that different organisms have different incubation periods, which range from minutes to several hours. Figures 4-6 shows the growth curve pattern of the four bacteria isolates when cultured in broth containing kerosene as the hydrocarbon source to utilize. The degradation rate of kerosene is quite lower as compared to that of petrol as the hydrocarbon source. This could be as result of increase length of carbon chains in kerosene than petrol. This agrees with Okoh, (2002) who reported that heavier crude oils are generally much more difficult to biodegrade than lighter ones. Figures 7-9 shows the growth curve pattern of the isolates in medium containing diesel as carbon and energy source. Unlike what was noticeable in

Figs. 1-6, in figure 7 growth of the bacteria started on the sixth day in all the four bacteria isolates tested. *B. cereus* showed cell density of 9.0 x 10^6 cfu/ml, *B. subtilis*, 8.0 x 10^6 cfu/ml, *P. aeruginosa* 7.5 x 10^6 (Plate 1), cfu/ml and *Acetobacter* spp. 7.0 x 10^6Cfu/ml at 0.2 ml diesel concentration in the growth medium. All the bacteria isolates showed a declination in cell growth as from day 14 irrespective of the diesel concentration used (Figs. 7-9). Thus, this work established the fact that *B. cereus* proved to be a better hydrocarbon degrader as compared to the other bacteria isolates used in this study in the order: *B. cereus* >*B. subtilis* >*P. aeruginosa* > *Acetobacter* spp. In summary, this work revealed that *Bacillus cereus, B. subtilis, Pseudomonas aeruginosa,* and *Acetobacter species* isolated from effluent collected from production site of fermented African locust beans condiment are able to utilized hydrocarbon compounds as their carbon source, showing that they have the ability to degrade hydrocarbon compounds.

In conclusion, this work revealed that *Bacillus cereus, B. subtilis, Pseudomonas aeruginosa,* and *Acetobacter species* isolated from effluent collected from production site of fermented African locust beans condiment are able to utilize hydrocarbon compounds as their carbon source, thereby proving their biodegradability potentials.

Acknowledgement

The authors are grateful to the Department of Pure and Applied Biology and management of Ladoke Akintola University of Technology (LAUTECH) Ogbomoso, for providing facilities and support for this research project.

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How to cite this article:

Majolagbe O. N., M. O. Olabemiwo, A. Ayandele, I. O. Omomowo and Aina, D. A. 2019. Study on Bioremediation and Growth Curve Patterns of Bacteria Cultured in Hydrocarbon Formulated Media at Different Concentrations. *Int.J.Curr.Microbiol.App.Sci.* 8(12): 2612-2622. doi: https://doi.org/10.20546/ijcmas.2019.812.305