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Impact of some heavy metals on bacterial utilization of kerosene in liquid medium

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Potential petroleum degrading bacteria, *Pseudomonas aerogenosa* and *Micrococcus* sp. was isolated from petroleum contaminated soil in petroleum microbiology laboratory, Department of Microbiology University of Nigeria, Nsukka. Hydrocarbon utilization in liquid media was assessed through time – course optical density measurement. Concentrations of the heavy metals ranging from 50 to 200 mg/L were introduced into 100 ml of mineral salt medium containing 10 ml of kerosene inoculated with *P. aeruginosa* or *Micrococcus* sp. and incubated at room temperature (25-30°C) for 384 h. Control samples contained kerosene but no heavy metals. At low concentrations the heavy metals significantly ($p < 0.05$) improved the growth of the isolates as indicated by the highest OD_{600} obtained with 50 mg/L of the metals which was the lowest concentrations. This was confirmed by the bacterial cell number extrapolated from an OD standard curve. At higher concentrations of the heavy metals (150 to 200 mg/L) there was a decline in the growth rates of the isolates. The effects of the heavy metals on the hydrocarbonoclastic efficacy of the isolates were dose-dependent and their growth rates in the presence of the metals were in the following order: Lead > Cadmium > Mercury.

Key words: Heavy metals, kerosene, bioremediation, bacteria.

INTRODUCTION

Hydrocarbon spills in form of petroleum products both on land and water have been a problem since discovery of oil as an energy source (Abioye et al., 2019). Kerosene also known as paraffin or paraffin oil is a clear flammable liquid distilled from petroleum. It is a blend of different hydrocarbons which is less volatile than gasoline (Matveev et al., 2017; Ellison et al., 1999). Kerosene currently has several uses such as aircraft gas turbine and jet fuel for both commercial airlines and military activities, as heating oil and as a spray oil to combat insects on Agricultural plants (Shamiyan et al., 2015). Irwin et al. (1997) stated that because of kerosene availability compared to gasoline during wartime, commercial illuminating kerosene was the fuel chosen for early Jet engines. This resulted to the development of commercial Jet aircraft following World war two, which focused primarily on the use of kerosene type fuels. The blend of kerosene used for Jet fuel consists of 20% aromatic hydrocarbons ranging from $C_9 – C_{16}$ (Ritchie et al., 2003).

According to the US Coast Guard Emergency Response Notification System, kerosene is one of the most commonly spilled petroleum products containing paraffins (alkanes), Cycloparaffins (cycloalkanes),

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aromatics and olefins with carbon numbers (Matveev et al., 2017; Irwin et al., 1997). There is a risk of contamination of ground water whenever hydrocarbons penetrate the top soil into the sub soil (Poi et al., 2018). Spill of kerosene (petroleum hydrocarbons) can occur due to blow outs, leakage from underground storage tanks, sabotage, accidental rupture of pipeline, dumping waste of petroleum products as well as tanker accidents (Aguilera et al., 2010). Environmental pollution from kerosene spill can lead to non-organic carcinogenic and growth inhibiting chemicals present in crude oil and other toxicity to microorganisms and Man. (Atlas and Bartha, 1973; Okpokwasili and Odokuma, 1990). Spill can also result in significant decline in quality of soil and make it unfit for use (Shabir et al., 2008). As well as affects plants and animals (Plohl et al., 2002; Giwa and Ibitoye, 2017). The West Africa sub region, particularly the Niger Delta region of Nigeria, has experienced serious contamination of its rivers, swamps, soils, underground and coastal waters following the ever increasing oil exploration and drilling activities (Iyagba and Offor, 2014).

Heavy metals are major components of inorganic contaminants and cannot be degraded (Krishna et al., 2017). Ekperusi and Aigbodion (2015) reported that heavy metals are non-biodegradable in nature and can remain bound in the soil for a longtime, can bioaccumulate into soil biota, leached into underground water and pose a considerable threat to the environment, biodiversity and public health. Meanwhile, some heavy metals like Zinc (Zn), Magnesium (Mg), Manganese (Mn), Copper (Cu), Nickel (Ni), Chromium (Cr^{3+}), Sodium(Na^{+}) are major elements needed in small quantity for metabolic and Redox functions. Heavy metals like Lead (Pb), Aluminium (Al), Cadmium (Cd), Gold (Au), Mercury (Hg), Silver (Ag) etc play no biological role and are toxic to living organisms (Siddiquee et al., 2015). Physical and other chemical remediation methods for hydrocarbons pollution are very expensive and ecotoxic (Raeds and Shimaa, 2014).

Bioremediation has been found to be a cost effective and less harmful means of converting these toxic pollutants into a less harmful state. Among several clean up strategies available, bioremediation technology is gaining prominence due to it's simplicity, environmental friendliness, higher efficiency and cost effectiveness in comparison to other technologies (Azubuike et al., 2017). The direct use of microorganisms with distinctive features of catabolic potential and/or their products such as enzymes and biosurfactant is a novel approach to enhance and boost their remediation efficacy (Le et al., 2017). One of the major problems of microbial remediation of petroleum polluted environment is the lingering rate at which biodegradation occurs when several biodegradation traits is required, as in the case of co-contaminated site. Presence of toxic metals above trace amounts has been noted to impact on the bioremediating capabilities of the microorganisms like mercury, lead and cadmium (Ekpenyong and Antai, 2007; Aniruddha and Sarma, 2010). Because of the importance of hydrocarbonoclastic microorganisms in restoration of petroleum polluted environment, this work has been designed to investigate the effects of some heavy metals (Lead, Cadmium and Mercury) on the bacterial utilization of kerosene.

MATERIALS AND METHODS

Collection of samples

Petroleum degrading bacteria isolates (*Pseudomonas aerogenosa* and *Micrococcus* sp) were collected from Petroleum Microbiology laboratory, Microbiology department, University of Nigeria, Nsukka. The salts of heavy metals cadmium chloride ($CdCl_2$), lead chloride (PbCl) and mercury chloride ($HgCl_2$) were purchased from Jeochem Laboratories, Enugu Road, Nsukka. Kerosene were purchased from M.R.S. filling station, Ogurugu Road, Nsukka while Bonny light crude oil was obtained from Warri Refinery, Delta State.

Media for bacterial screening

According to Okpokwasili and Okorie (1988) with slight modification, two mineral salts media designated A and B were formulated for screening and bioutilization of petroleum hydrocarbon degrading bacteria. Medium A which is used for isolation was also used in screening the isolates for the ability to degrade hydrocarbons as well as for bioutilization studies. Medium A consisted (g/L) of NaCl, 10; $MgSO_4$.$7H_2O$, 4.2; KCl, 2.9; $KHPO_4$, 8.3; Na_2HPO_4, 12.8; $NaNO_3$, 2.8; Distilled water 100ml. Medium B consisted (g/l) of all the ingredients in medium A and 15.0g of agar. Medium A was mineral salt broth (MSB) while medium B was mineral salt agar (MSA).

Bacterial screening and enhancement for degradation of kerosene

Four hundred milliliters of mineral salt (MS) broth was prepared as described by Okpokwasili and Okorie (1988) used for the isolation of petroleum hydrocarbon degrading bacteria. The broth medium was dispensed in 200 ml volume into 500 ml conical flask and sterilized at 121°C for 15 min. After cooling, the medium in each flask was enriched with 1.0 ml of filter-sterilized kerosene, after which 10 g sterilized crude oil contaminated soil were measured into each flask to supply nutrients such as Nitrogen and Sulphur apart from carbon and energy source. The flasks were incubated for seven days at room temperature (25-30°C) to improve the multiplication of hydrocarbonoclastic bacteria in the media. Thereafter 0.1ml of the turbid suspension was inoculated on to mineral salt agar. Whatman filter paper impregnated with kerosene was placed inside the plate cover to supply the organisms carbon by the vapour phase transfer method as described by Ekpenyong and Antai (2007). The plates were incubated for 3 days in an inverted position. The resulting organisms were sub cultured on nutrient agar and stored in nutrient agar slants.

Turbidity standard equivalent to McFarland 0.5

According to Eze et al. (2019), this is a barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula gave

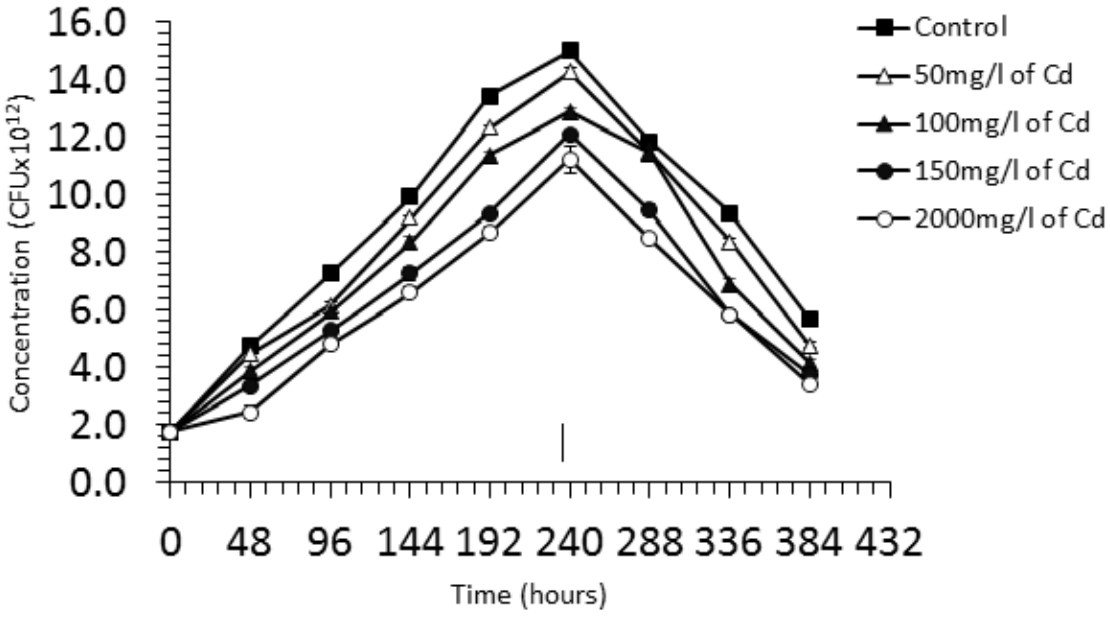

Figure 1. The effects of cadmium on the growth rate of *P. aeruginosa* in kerosene.

confluent or almost confluent growth. The standard was shaken immediately before use and was stored in a well-sealed container in the dark at room temperature (20-28°C).

Inoculum preparation

The organisms were subcultured into sterilized nutrient broth and allowed to grow for 48 h as described by Eze et al. (2019). Thereafter the cells were washed in normal saline. Sterile centrifuge tubes were used in separating the cells from the liquid broth at 10,000 rotations per minute for 15 min.

Experimental set up

According to Ekpenyong and Antai (2007) with slight modification, determination of the effects of heavy metals on the hydrocarbonoclastic capacities of the organisms were carried out using four different concentrations (50, 100, 150 and 200 mg/L) in a liquid medium containing 10 ml of kerosene each. The control is kerosene in liquid medium without heavy metals. The pH of the medium was adjusted to 7.0 before sterilization. The time course growth optical density to determine the rate of petroleum utilization in the presence and absence of heavy metals was determined every 48 h using optical density (OD) measured at 600 nm. The experiment lasted for 384 days.

RESULTS

Figures 1 to 3, show the effects of heavy metals (Cd, Ld and Hg) on the growth rate of *P. aeruginosa* in kerosene. According to the figures optimum growth of the microbes, indicated by the highest OD_{600} were obtained with 50 mg/L (low concentrations) of heavy metals which was confirmed by the bacterial cell number extrapolated from an OD standard curve. Each of the flasks for the experiment contained an initial cell concentration of 2×10^{12}cfu/ml at 0hr. 50 mg/L concentrations of heavy metals significantly ($p < 0.05$) improved the growth of *Pseudomonas aeruginosa* in kerosene. Their cell concentrations (cfu/ml) at 240h (exponential growth phase) include 14.997×10^{12}, 14.245×10^{12}, 14.262×10^{12} and 13.115×10^{12} for control, Cd, Pb and Hg at the same time above. In 100 mg/L growth in kerosene generated 12.905×10^{12}, 13.870×10^{12} and $11.934\text{x}10^{12}$cfu/ml for cadmium, lead and mercury respectively. 150 mg/L produced reduced cell concentrations of 12.092×10^{12}, 12.986×10^{12} and 11.670×10^{12} cfu/ml. A decline in the growth rates were observed in 200 mg/L of Cd, Pb and Hg with cell concentrations 11.217×10^{12}, 11.946×10^{12} and 10.314×10^{12}cfu/ml.

Micrococcus sp also utilized kerosene in the presence of heavy metals (Cd, Ld and Hg) with good growth. They followed the same pattern of growth (Figures 4 to 6) as *P. aeruginosa.* At 240h (exponential growth phase) with 50 mg/l concentrations of the metals, it's corresponding cell concentrations during utilization of kerosene were 14.139×10^{12}, 12.980×10^{12}, 13.857×10^{12} and 11.702×10^{12}cfu/ml for control Cadmium, Lead and Mercury. While in 100 ml of kerosene generated 12.745×10^{12}, 12.980×10^{12} and 11.018×10^{12}cfu/ml (Cd, Ld and Hg). At 150 mg/L in kerosene $11.018\times10^{12}11.563\times10^{12}$ and 8.411×10^{12}cfu/ml were obtained. The least growth was generated by 200 mg/L, $8.454\times10^{12}10$, $829\times10^{12}$and 6.129×10^{12}cfu/ml. The microbes exhibited a steady decline in their growth from

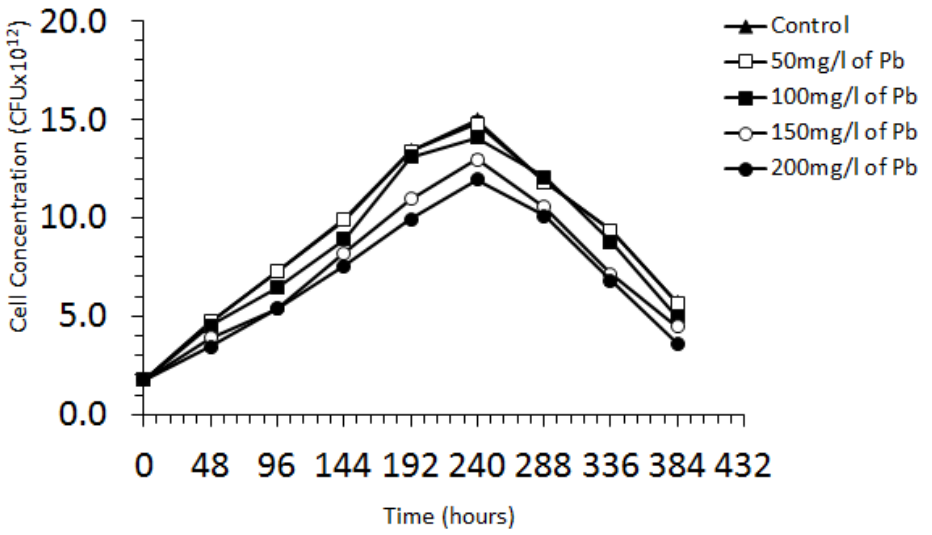

Figure 2. The effects of lead on the growth rate of *P. aeruginosa* in kerosene.

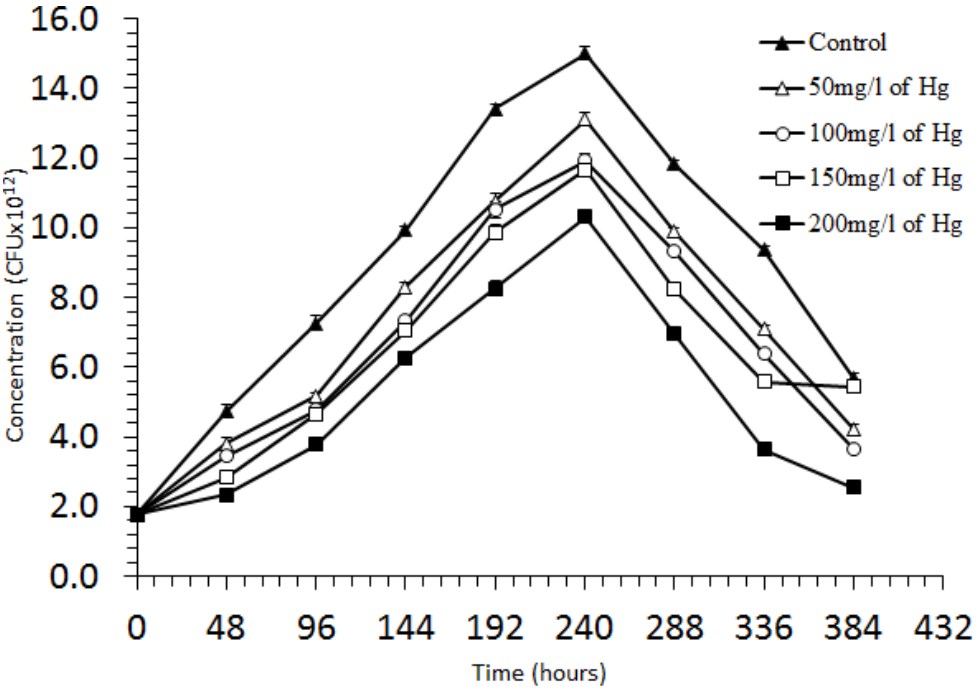

Figure 3. The effects of mercury on the growth rate of *P. aeruginosa* in kerosene.

288- 384 h except for medium containing 200 mg of mercury per litre with *Micrococcus* sp. in kerosene which started declining at 192 h.

DISCUSSION

The bacteria growth in liquid medium containing kerosene as carbon and energy source with or without heavy metals shown in Figures 1 to 6. According to the figures optimum growth of the microbes, indicated by the highest OD_{600} were obtained with 50 mg/L (low concentrations) of heavy metals. Hence, 50 mg/L concentrations of heavy metals significantly ($p < 0.05$) enhanced the growth of *P. aeruginosa* and *Micrococcus* sp. in kerosene. This is in agreement with earlier

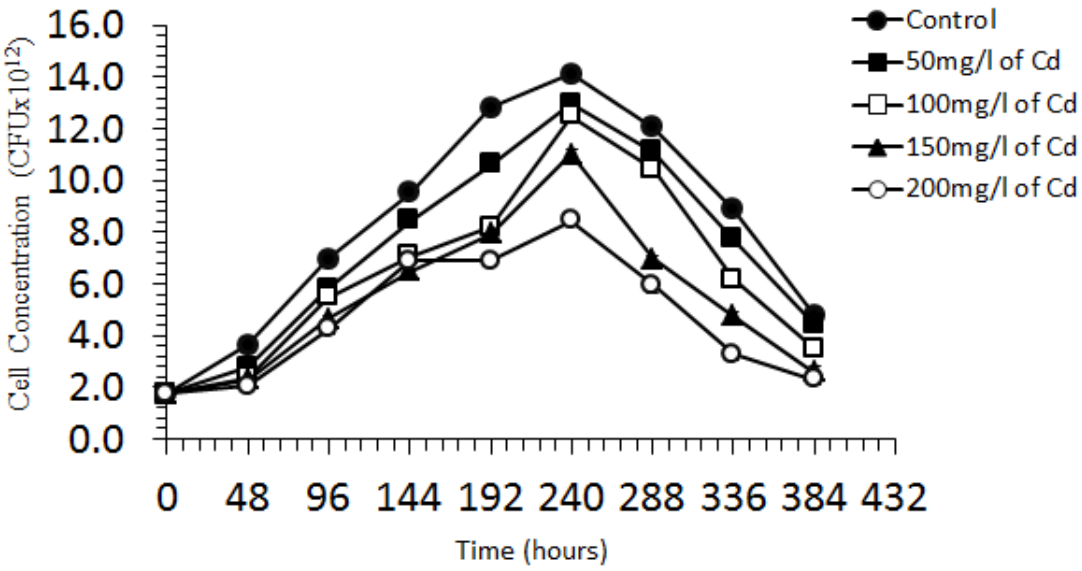

Figure 4. The effects of cadmium on the growth rate of Miocrococcus sp in kerosene.

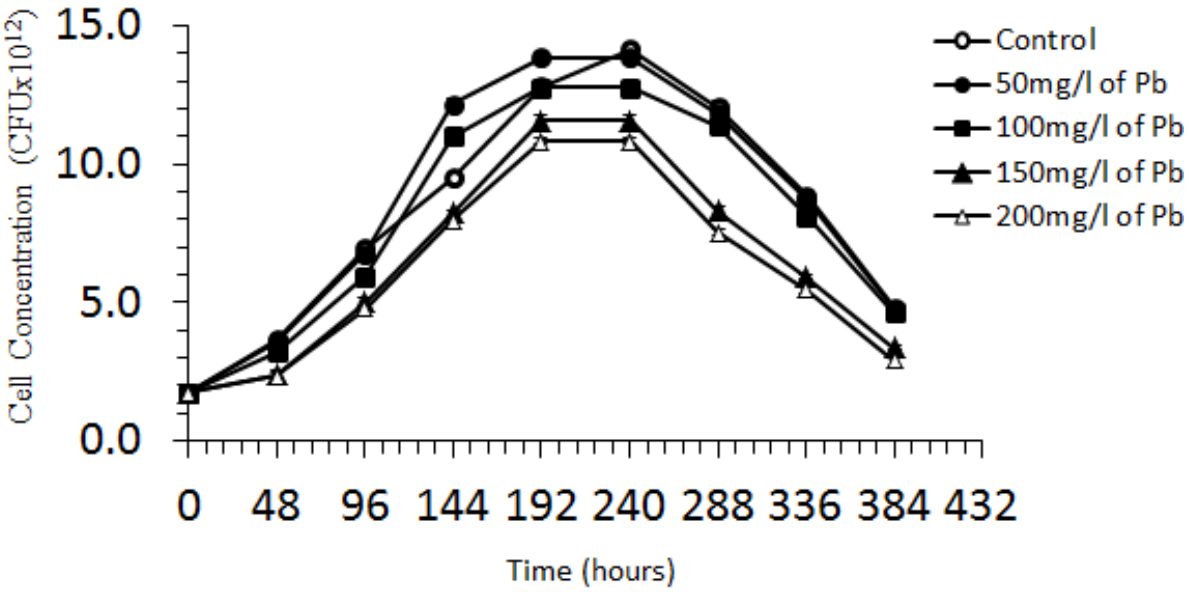

Figure 5. The effects of lead on the growth rate of *Miocrococcus* sp. in kerosene.

literature reports which showed slightly enhanced enzyme production at lower concentrations of heavy metals and subsequent reduction in hydrocarbon content (Owabor et al., 2011). A decline in the growth rates were observed in 200 mg/l of Lead, Cadmium and Mercury (Figures 1 to 6). The decline observed in growth at higher concentrations of the heavy metals is also in total agreement with the reports of Mona et al. (2014) who stated that excessive levels of heavy metals can be damaging to the organisms.

There is a significant ($p < 0.05$) decrease in growth among all concentrations of heavy metals in comparison to their control (Figures 1 to 6). Meanwhile, *P. aeruginosa* proved a better bioremediation agent and showed better heavy metal tolerance when compared to *Micrococcus* sp. This is related with Vinothini et al. (2015) who reported that microorganisms possess mechanisms by which they degrade crude oil compounds by utlilizing them as carbon and nitrogen sources. Panet and Marchal (2006) reported that the pattern of degradation varies for

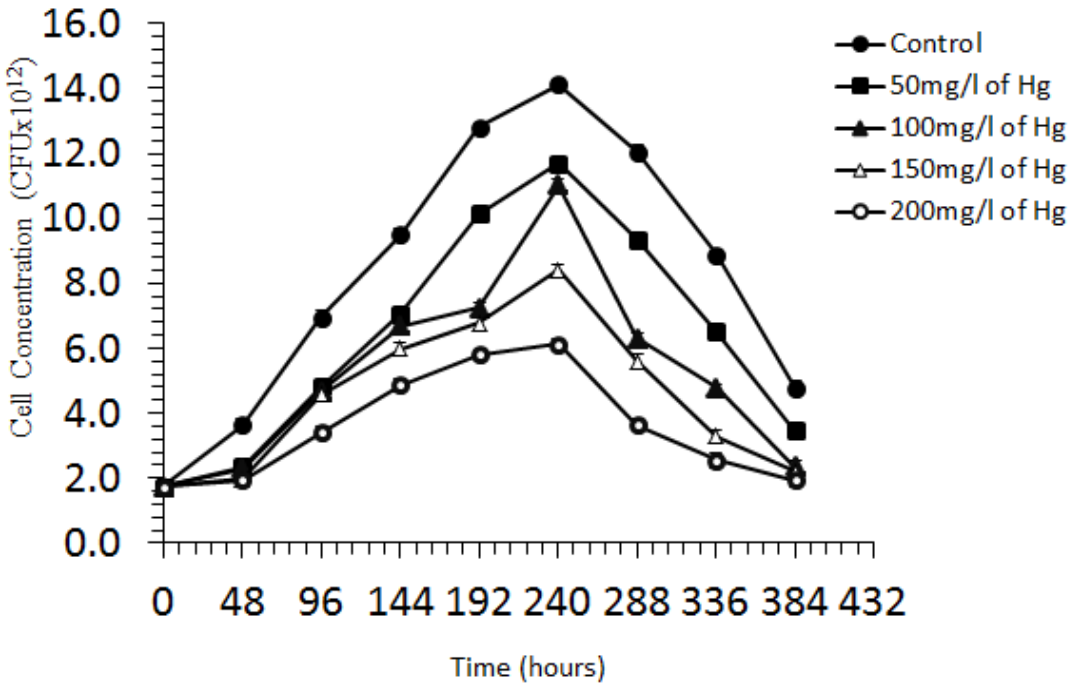

Figure 6. The effects of mercury on the growth rate of *Micrococcus* sp. in kerosene.

different degrading microorganisms because different microorganisms possess different catabolizing enzymes. Crude oil provided a better medium for the growth of the microbes. This agree with the report of Vinothini et al. (2015) who stated that refined petroleum supply only carbon and energy source to resident microbes while crude oil supplies in addition to carbon and energy, mineral nutrients such as nitrogen, sulphur. This proved why crude oil was used to enhance the multiplication of the organisms. Their growth rates in the presence of heavy metals were in the following order: Lead > Cadmium> Mercury. The inhibitory effects shown by Lead, Cadmium and Mercury which were more significant at high concentrations might be as a result of inhibition of enzymatic activities. It is likely that enzymes might have been denatured at higher concentrations of heavy metals leading invariably to retardation of growth with speed, as can be observed from 288- to 384 h. The findings associated with mercury in this study shows that mercury was the most toxic to microbes. This might be connected to the fact that mercury by virtue of it's affinity for thiol-groups in protein acts as an inducer of oxidative stress. The result is the inactivation of enzymes and ultimately the death of the microbes (Owabor et al., 2011). Meanwhile, the reason for the highest growth found in lead may be attributed to the fact that lead belongs to group 4 elements and period 6, does not possess the 3d electrons and this makes the attraction of the S-electrons and nucleus of its atom possible. The weakness of the bond between the nucleus and the outer electrons is responsible for the susceptibility of lead to complexation with other interfering chemicals such as ethylene diameintetracetic acid (Greenwood and Earnshaw, 1997). This interaction ultimately results to its reduced toxicity to the microbes.

Conclusion

(i) Bioremediation provides a safe and healthy environment therefore, the need for its application in eliminating hazardous substances from the environment.
(ii) There is a significant difference between the responses of *P. aeruginosa* and *Micrococcus* sp to the presence of heavy metals in kerosene.
(iii) The effects of heavy metals on bacterial utilization of kerosene was dose dependent.
(iv) The results of the research showed *P. aeruginosa* a better hydrocarbon remediation agent than *Micrococcus* sp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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