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Original Article

STUDIES ON METAL- RESISTANT BACTERIA ISOLATED FROM SPENT- ENGINE OIL CONTAMINATED SOIL, IN OGBOMOSO, OYO STATE, NIGERIA.

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Abstract

This study was carried out to establish the relationship between hydrocarbon utilization potential and heavy metal tolerance in bacteria species. Nineteen bacteria species were isolated from soil samples collected from four (4) automobile repair workshops in Ogbomosho, Oyo state Nigeria. The bacteria isolated were identified to be *Klebsiella oxytoca*(5), *Klebsiella edwardsii*(13) and *Shigella alcalescens*(1). Metal tolerance assay revealed that *Klebsiella oxytoca*, *Klebsiella edwardsii* and *Shigella alcalescens* have the ability to grow in the presence of $ZnCl_2$, $PbCl_2$ and $CuSO_4$. The minimum inhibitory concentration (MIC) of the metal salts ranged from 1mM to >16mM for $PbCl_2$, 2mM to >16mM for $ZnCl_2$ and 8mM to >24 for $CuSO_4$. The ability of the bacteria strains to utilize hydrocarbons was also investigated by measuring the optical density of the strains in minimal salt medium supplemented crude oil at 625nm. The results showed that there is relationship between hydrocarbon utilization and heavy metal tolerance among the bacteria.

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Keywords: Tolerance, Degradation, Crude oil, Incubation.

INTRODUCTION

Environmental pollution is a global problem because of its adverse effects on human beings, plants and animals. Heavy metals are increasingly found in our environment due to natural and industrial process. It has been well reported that microbial interactions with metals may have several implications for the environment such that microbes can be used in the biogeochemical cycling of toxic heavy metals as well as in cleaning up or remediating metal contaminated environments (Badar *et al.*, 2000). Salt *et al.*, (1998) also reported that bacteria tolerance to heavy metals can be used to minimize the effects of heavy metals on total biological activity of the ecosystem. Processes involved in reducing metal toxicity are of considerable current interest particularly for highly contaminated heavy soils.

Anthropogenic activities like energy production, construction, waste disposal, coal and fuel combustion are the major sources of soil pollution. The vehicle repairs also release heavy metals to air that ultimately get deposited on the soil. Mokokha *et al.*, (2008) reported that the Fe, Cu, Zn were essential component of alloy, pipe, wire and tyre in motor vehicles and these metals were released into soil by either mechanical abrasion or normal wear and tear.

The pollutants present in soil not only affect the micro flora and fauna of soil ecosystem but also contaminate fruits and vegetables. Last but not the least, they result in formation of leachates that ultimately contaminate the ground water passing through the soil profiles or by directly entering the surface water system through run offs.

The consumption of the contaminated food and water causes severe health problems in human beings. Rapid

motorization has resulted in release of deleterious mutagens/genotoxins/carcinogens to all components of environment. The direct DNA damaging effects of these pollutants further result in long term effects such as carcinogenicity (Watanabe et al., 2005). Some authors documented the adverse effects of toxic metal like lead that is released from vehicular emissions (Flora, 2002; Sharma and Prasad, 2010) while some authors reported the accumulation of other metals in plants (Sajawan et al., 1996) and rivers (Pande and Sharma, 1999). This study was carried out with the aim of isolating and identifying indigenous bacteria having heavy metals and hydrocarbon remediation capabilities.

MATERIALS AND METHODS

Four (4) Sampling sites were selected by observing regular practice of metal contaminated wastes in the vicinity of auto batteries and automobile workshops near a pharmaceutical company in Ogbomoso, Oyo state Nigeria. Automobile workshops that have been in existence for more than five years were selected. Surface soil sample was collected from automobile workshops into sterile polythene bags and transported to the laboratory of the Department of Pure and Applied Biology of Ladoke Akintola University of Technology, Ogbomoso, Oyo State Nigeria.

One gram of soil sample was suspended in 9ml sterile distilled water and was diluted serially up to 10^{-10} . 0.2ml (aliquot) of the suspension was inoculated separately in to Mueller- Hinton agar supplemented with 2.0mM, 2.0mM and 4.0 mM of $PbCl_2$, $ZnCl_2$ and $CuSO_4$ respectively. The plates were incubated at $35^\circ C$ for 24-72 hours. Distinct colonies growing on each metal supplemented plate were selected, subcultured and stored in glycerol broth at freezing temperatures. The bacteria isolates were inoculated into MHA slant bottles and sent for identification.

All the isolates were tested for tolerance to three metals by determining the minimum inhibitory concentration of the metals to inhibit the growth of the organisms on MHA plates. Mueller-Hinton agar supplemented with $PbCl_2$, $ZnCl_2$ and $CuSO_4$ metal salts was prepared. The following ranges of concentration were used: 1- 28mM for $CuSO_4$ and 0.25-16mM for $PbCl_2$ and $ZnCl_2$. The metal tolerance assay was carried out by inoculating each isolate on the plates containing different metal salts ($PbCl_2$, $ZnCl_2$ and $CuSO_4$) at different concentrations.

The ability of all the metal resistant bacteria isolated to utilize hydrocarbon (crude oil) was investigated. In order to quantify the growth of the bacteria isolates, the optical density of the culture of each isolate growing on the crude oil was measured at 625nm using a Gold spectrum 53 UV-Visible spectrophotometer.

RESULTS AND DISCUSSION

DISCUSSION

In this study out of nineteen (19) bacteria that were isolated, thirteen (13) were identified to be *Kl. edwardsii* (i.e P1, P4, P8, P9, P10, C2, C3, C4, C5, C6, C7, C8, and C9), five (5) were identified to be *Kl. oxytoca* (i.e. P3, P5, P7, P11 and P12) and One (1) was identified to be *S. alcalescens* (i.e. P7) the identification result conformed with the assertion that majority of soil microbes at the

surface are Gram – negative bacteria (Fierer et al., 2003). Duxbury and Bicknell (1993) also reported higher frequency of heavy metal tolerance in Gram – negative metal bacteria over Gram – positive bacteria. All the bacteria isolates used in this study were Gram – negative bacteria as revealed by the result of gram – staining (Table 1).

Since *S. alcalescens*, *Kl. oxytoca* and *Kl. edwardsii* are all enterobacteriaceae, the results obtained in metal tolerance assay correlated with the reports of Frank and Henrik (2003) in which *E. faecium*, *Salmonella* isolates had MIC of 20 – 28mM of Copper sulphate and for Zinc chloride the MIC for *Salmonella* was reported to be 2mM. Willam et al., (1995) also identified copper – resistant *E. coli* isolate that were able to grow on agar containing 18mM copper sulphate. Mergeay et al., (1995) reported that the MIC of lead for *E. coli* is only 5mM. There is no molecular information about uptake of lead, although many lead tolerant bacteria have been isolated, precipitation of lead phosphate within the cell of these bacteria has been reported (Levinson and Mahler, 1998).

One of the best characterized determinant of Copper resistance in prokaryotes reported in *Pseudomonas spp* is the copABCD operon system that reside on a 35kb plasmid of the Copper- resistant strain *Pseudomonas syringae* of Pv. Tomato PT 23.3 isolated from California tomato fields (Mellano and Cooksey, 1988, Cooksey, 1994). Copper resistance by the cop determinant has been reported to contain six genes, cop ABCDRS, arranged in a single operon and homologous to the equivalent Pco genes (Cooksey, 1993). The pco and cop determinants are regulated by a plasmid – encoded and a chromosomally encoded two- component system (Munson et al., 2000).

Atlas and Bartha (1973) reported that among the different technologies used during oil spill response, a widely preferred and promising technology is bioremediation i.e the use of highly adapted or indigenous microbial cultures to degrade toxic petroleum hydrocarbons into harmless products (ultimately to remove the contaminants from the environment). Based on this assertion, the ability of the metal-resistant bacteria to bioremediate ecosystems contaminated by hydrocarbon (crude oil) was investigated in this study. The results obtained showed that some of the bacteria isolates have innate potential to utilize crude oil (fig.1 to fig.19). This may be due to their exposure to the toxic effect of used engine oil in the automobile repair workshop where the organisms were isolated.

From the results obtained it was noticed that some of the bacteria isolates were able to utilize crude oil up to the 96hours of incubation before they entered into their stationary phase, these isolates include *Kl. edwardsii* (P1, P9, C3, C4, C5, C6, C7, C8 and C9), *Kl. oxytoca* (P3, P5 and P7²) and *S. alcalescens* (P7). The growth of these bacteria isolates is an indication that they were utilizing the crude oil (Fig.1 to Fig.19). Similarly, three (3) out of the bacteria isolates stopped growing after 48hours of incubation, the bacteria isolates stopped growing are *Kl. edwardsii* (P10) and *Kl. oxytoca* (P11 and P12). This is probably an indication of their inability to utilize

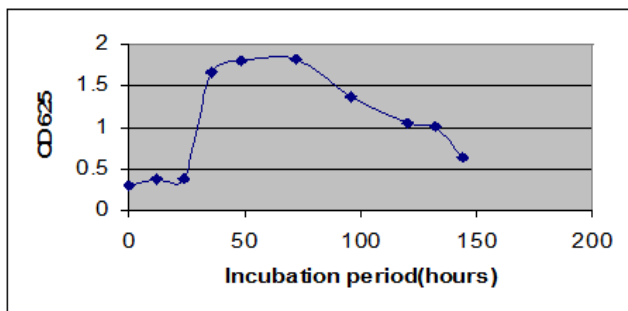


Fig.1: Growth curve of *K. edwardsii* (P1) on crude oil

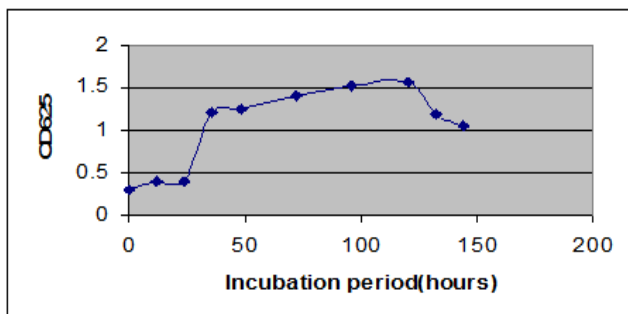


Fig.2: Growth curve of *Klebsiella oxytoca* (P3) on crude oil

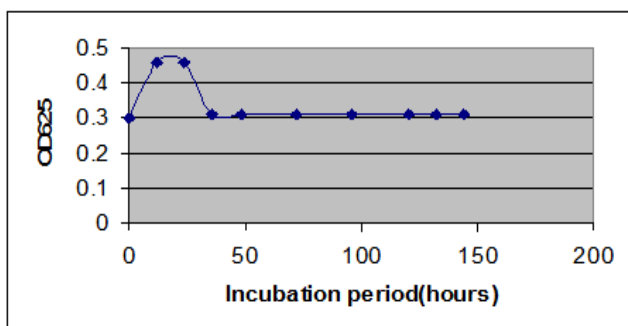


Fig.3: Growth curve of *K. edwardsii* (P4) on crude oil

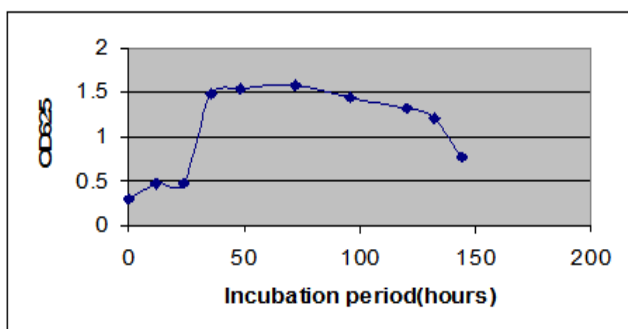


Fig.4: Growth curve of *K oxytoca* (P5) on crude oil

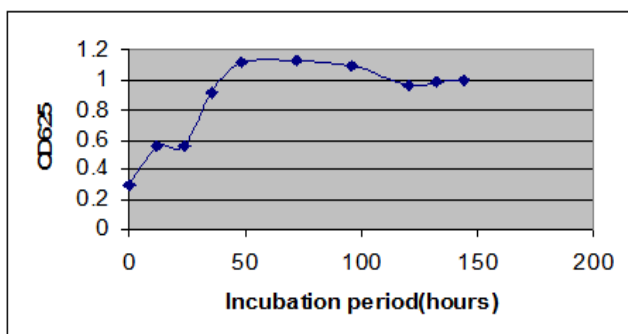


Fig.5: Growth curve of *S. alkalescens* (P7) on crude oil

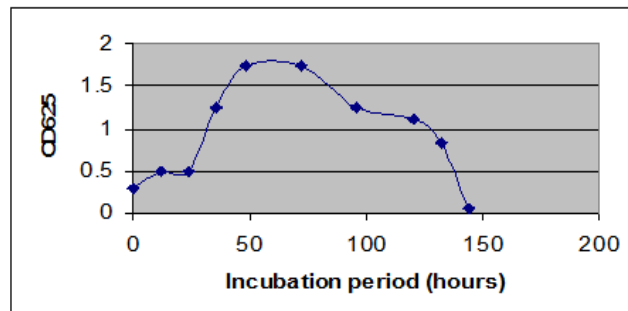


Fig.6: Growth curve of *K. oxytoca* (P7²) on crude oil

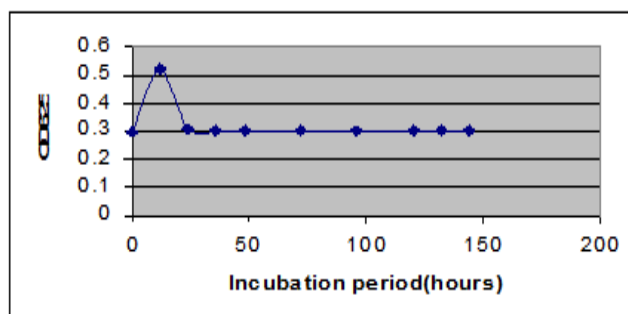


Fig.7: Growth curve of *Klebsiella edwardsii* (P8) on crude oil

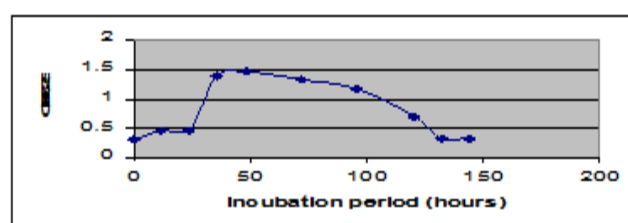


Fig.8: Growth curve of *K. edwardsii* (P9) on crude oil

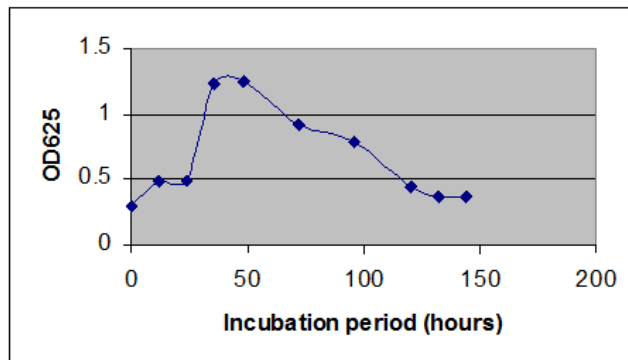


Fig.9: Growth curve of *Klebsiella edwardsii* (P10) *oxytoca* (P11) on crude oil

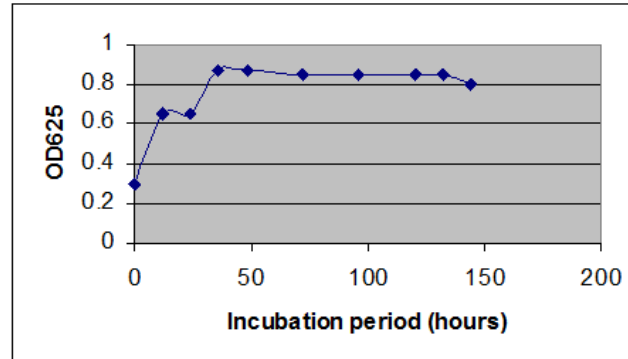


Fig.10: Growth curve of *Klebsiella oxytoca* (P11) on crude oil

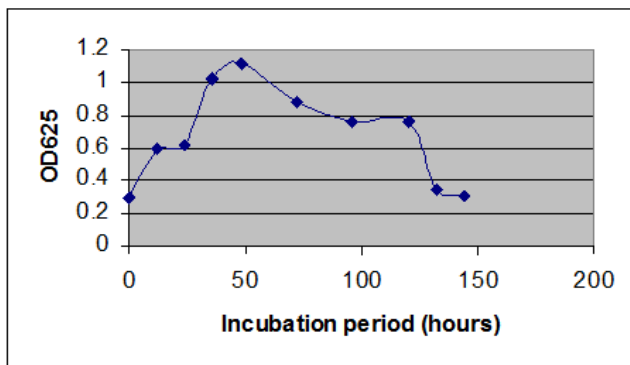


Fig.11: Growth curve of *Klebsiella oxytoca* (P12)

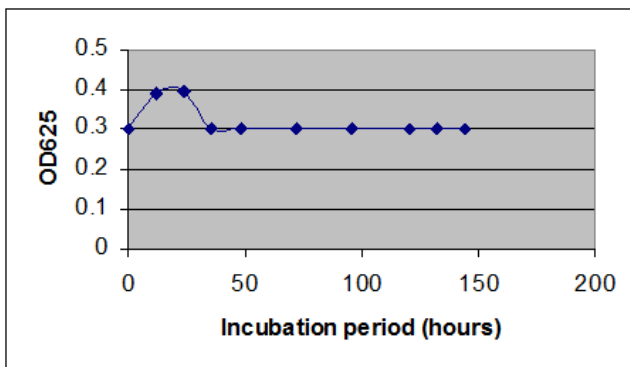


Fig.12: Growth curve of *Klebsiella edwardsiella*

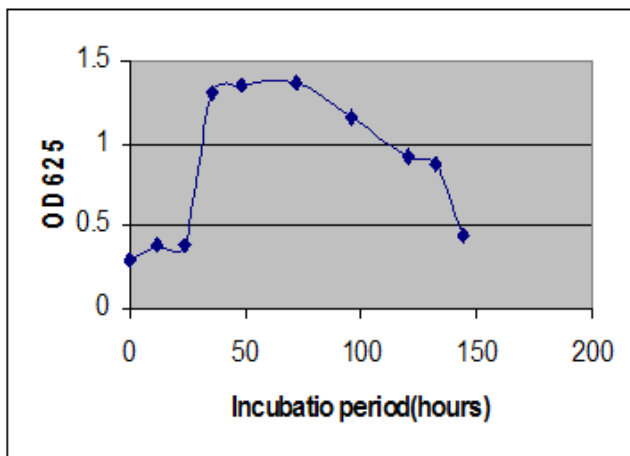


Fig.13: Growth curve of *Klebsiella edwardsii* (C3)

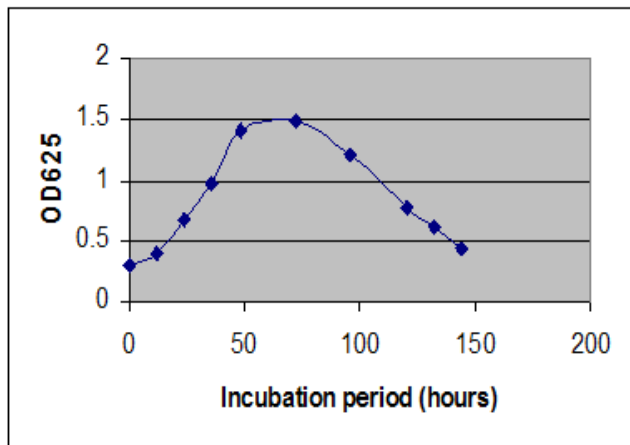


Fig.14: Growth curve of *Klebsiella edwardsii* (C4) on crude oil

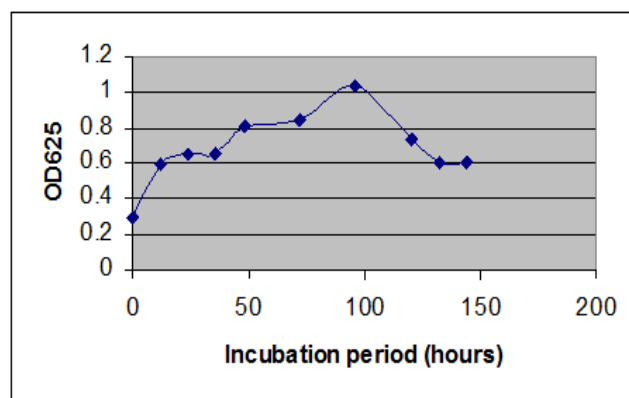


Fig.15: Growth curve of *Klebsiella edwardsii* (C5)

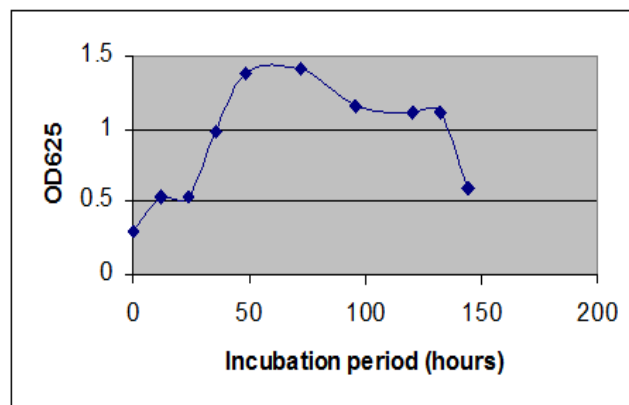


Fig.16: Growth curve of *Klebsiella Edwardsii* (C6) on crude oil

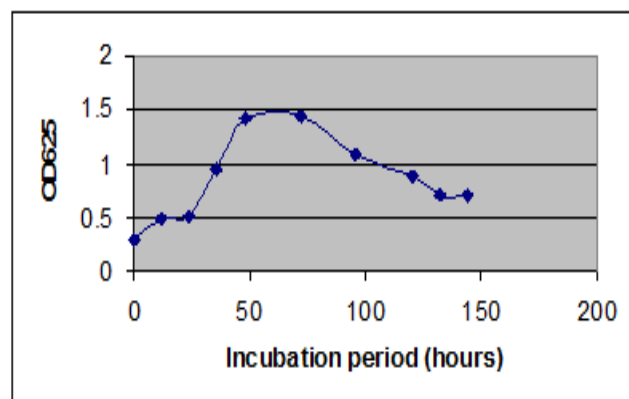


Fig.17: Growth curve of *Klebsiella edwardsii* (C7) on crude oil

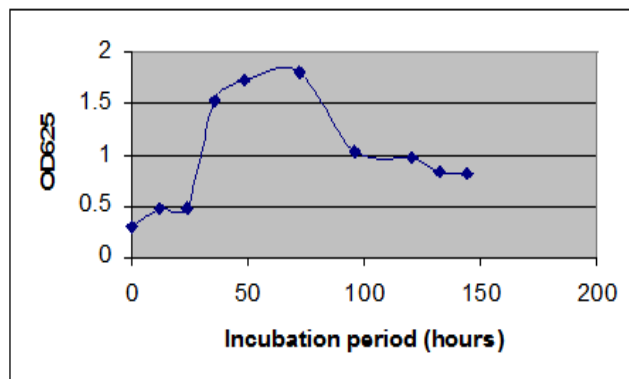


Fig.18: Growth curve of *Klebsiella edwardsii* (C8) on crude oil

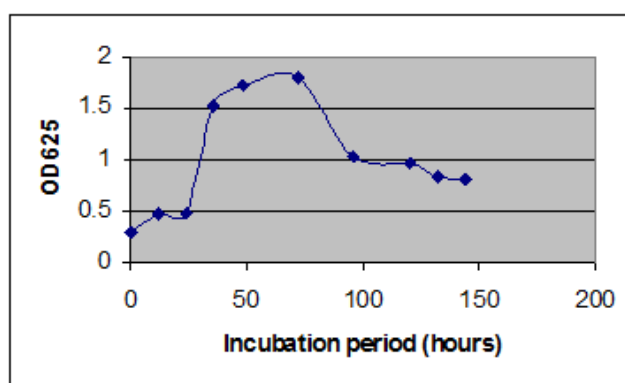


Fig.19: Growth curve of *Klebsiella*

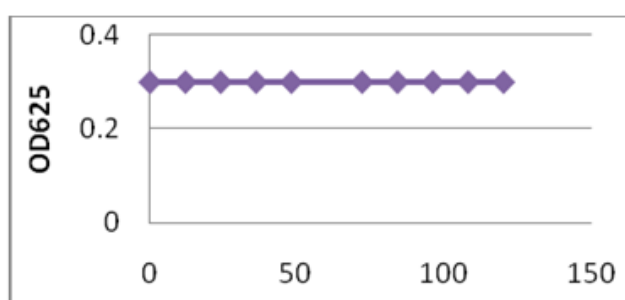


Fig.20: control

Table 1: Minimum inhibitory concentration of tested metals against isolated bacteria strains.

Isolates	Zncl ₂ (mM) ^(a)	Pbcl ₂ (mM) ^(b)	CuSo ₄ (mM) ^(c)
C2 <i>Klebsiella edwardsii</i>	>16	>16	>24
C3 <i>Klebsiella edwardsii</i>	>16	16	>24
C4 <i>Klebsiella edwardsii</i>	>16	>16	>24
C5 <i>Klebsiella edwardsii</i>	>16	>16	>24
C6 <i>Klebsiella edwardsii</i>	>16	>16	>24
C7 <i>Klebsiella edwardsii</i>	16	16	>24
C8 <i>Klebsiella edwardsii</i>	12	16	>24
C9 <i>Klebsiella edwardsii</i>	8	12	>24
P1 <i>Klebsiella edwardsii</i>	12	>16	>24
P3 <i>Klebsiella oxytoca</i>	12	16	8
P4 <i>Klebsiella edwardsii</i>	8	8	8
P5 <i>Klebsiella oxytoca</i>	2	4	12
P7 <i>Shigella alcalescens</i>	8	1	>24
P7 ² <i>Klebsiella oxytoca</i>	4	12	>24
P8 <i>Klebsiella edwardsii</i>	>16	12	>24
P9 <i>Klebsiella edwardsii</i>	>16	>16	>24
P10 <i>Klebsiella edwardsii</i>	>16	>16	>24
P11 <i>Klebsiella oxytoca</i>	>16	16	>24
P12 <i>Klebsiella oxytoca</i>	>16	>16	>24

a: test concentration for Zncl₂ = 0.25-16mM, b: test concentration for Pbcl₂ = 0.25-16mM, c: test concentration for CuSo₄ = 1 – 24mM

Tables 1, showed the results of minimum inhibitory concentration of the tested metals. The minimum inhibitory concentration (MIC) of the metal salts ranged from 1mM to >16mM for Pbcl₂, 2mM to >16mM for Zncl₂ and 8mM to >24 for CuSo₄.

hydrocarbons as source of carbon and energy for growth. Also, bacteria isolates that entered into their decline phase after 24 hours of incubation were also recorded and these are *Kl. edwardsii* (P4 and C2).

The differences that was observed in the growth pattern of all the bacteria isolates is in conformity with the theory of survival and fittest i.e elimination of the fittest and survival of the fittest. Those bacteria isolates that could not cope with the toxic effect of crude oil are those that

entered into their decline phase before 24 hours of incubation. The results obtained in the hydrocarbon utilization assay is in consonance with the reports of Dott *et al.*, (1989); Venosa *et al.*, (1996); and Cerniglia (1996), in which some indigenous bacteria isolates were biostimulated to utilize crude oil and these bacteria isolates utilized the hydrocarbons effectively. Many laboratory and field studies have shown that bioremediation works when properly applied under a given condition.

CONCLUSION

The results obtained from this study showed that there is link between metal tolerance and hydrocarbon utilization among the bacteria isolates. Based on the results obtained in this study, it is therefore recommended that, further scientific investigation should be carried out on the organisms for the optimization of their potential for use in ex-situ bioremediation of heavy metals and hydrocarbons contaminated ecosystems.

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