Current Topics in
Biotechnology
Vol. 6, 2011

Original Communication

Diesel-oil degrading potentials of bacterial isolates from diesel polluted soil

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ABSTRACT

The study investigated the diesel-oil degrading potentials of single and mixed bacterial cultures from diesel polluted soil. The isolates were purified and identified as members of the genera *Pseudomonas*, *Bacillus*, *Staphylococcus* and *Proteus* using their cultural and biochemical characteristics. They were screened for diesel oil utilization using the vapour phase transfer method and the plates with very high turbidity in modified mineral salts medium containing diesel as the sole source of carbon and energy were selected as pure cultures, and their growth profiles monitored. This was done by monitoring the pH, optical density and total viable count for 14 days at 2 days interval. The effect of diesel oil and glucose on the isolates was monitored. Adherence and emulsification tests were as well performed on the isolates. The results showed changes in pH, optical density and total viable count after the 14 days incubation period. The isolates used adherence and emulsification as mechanisms for oil uptake with the highest percentage obtained in the consortium for both tests. Diesel oil and glucose had little or no effect on the lag phase of the isolates but the exponential phases were stimulated at all concentrations except for *Bacillus* sp. whose exponential phase was stimulated at 0.5% and depressed at 1.0% and 2.0% in diesel oil and depressed at 0.8% and 1.6% in glucose.

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KEYWORDS: diesel oil, bacterial isolates, biodegradation

INTRODUCTION

Biodegradation involves transformation of chemicals mediated by microbes that satisfy nutritional and energy requirements, detoxify the immediate environment or occur fortuitously such that the organism receives no nutritional or energy benefit [1]. Some physical, chemical and biological factors that contribute to the degradation of petroleum and individual hydrocarbons emanating from anthropogenic or natural sources have been studied [1].

Rates of biodegradation depend greatly on the composition, state and concentrations of the hydrocarbons with dispersion and emulsification enhancing rates in aquatic systems and adsorption by soil particulate being the key feature of terrestrial ecosystem [2]. Indigenous soil microbial population, soil structure, temperature, oxygen and nutrient concentrations are important variables in both types of environments. Salinity and pressure may also affect biodegradation rates in some aquatic environments, and moisture and pH may limit biodegradation in soil [3].

Many bacteria and fungi degrade hydrocarbons. Soil microbes degrading hydrocarbons under favorable conditions include: *Pseudomonas, Achromobacter, Micrococcus* and *Acinetobacter*. Hydrocarbons with less than ten carbon atoms, e.g. benzene, toluene, tend to be relatively easy to

degrade as long as the concentration is not too high or toxic to the organisms. Complex molecular structures such as branched paraffin, olefins or cyclic alkanes are much more resistant to biodegradation [4].

At some contaminated sites, as a result of oxygen consumption by aerobic microbes and slow recharge of oxygen, the environment becomes anaerobic and mineralization, transformation, and co-metabolism depend on microbial utilization of electron acceptors other than oxygen. Nitrate, iron, manganese, sulphate and carbon dioxide can act as electron acceptors if the organisms present have the appropriate enzymes [5]. JP-4 jet fuel constituents have been observed to be biodegraded only in the presence of added manganese [6].

Adaptation prior to exposure of microbial communities to hydrocarbon increases hydrocarbon degradation rates. Adaptation is brought about by selective enrichment of hydrocarbon utilizing microorganisms and amplification of the pool of hydrocarbon-catabolizing genes. The latter phenomenon can be monitored through the use of DNA probes. Increase in plasmid frequency may also be associated with genetic adaptation. Seeding of polluted soils with genetically modified organisms accelerates rate of biodegradation and is effective particularly when used under control conditions [7]. In the present study, the diesel oil degrading potential of single and mixed bacterial cultures was examined with the hope of isolating and stocking useful organisms with high potential for diesel oil degradation.

MATERIALS AND METHOD

Screening test for utilization of diesel oil

Diesel oil polluted soil samples were collected in clean sterile glass bottles and analyzed within 1 hour of collection. A ten-fold serial dilution was performed on one gram of the sample in normal saline. A 0.1 ml of the appropriate dilution was spread on nutrient agar in duplicates. Bacterial species growing on the nutrient agar plates were isolated, purified and identified using their cultural and biochemical characteristics. The isolates were screened for diesel oil utilization using the modified mineral salts medium of Okpokwasili and Okorie [8]. Both liquid and solid mineral salts medium, which contained diesel oil at concentrations of 0.05, 0.1, 0.5 and 1.0% were used. The isolates were also screened for diesel oil utilization using the vapor phase transfer method. Inoculated controls were also set up. Inoculated plates were incubated at 37°C for 7 days after which, each plate was scored for colony development.

For liquid medium, the method of Okpokwasili and Okorie [8] was adopted. The medium which contained 0.5, 0.5 and 1.0% diesel oil was prepared in screw cap test tubes. Another set of test tubes containing mineral salts medium but no diesel oil was prepared to serve as control. Each set of tubes were inoculated with 0.1 ml of 24 hour broth culture of the corresponding bacterial isolates. All the tubes were incubated at 37°C for 7 days with constant shaking at 24 hours interval. Each tube was scored for turbidity after the incubation period.

Monitoring the growth of the bacterial isolates in diesel oil

Modified mineral salts medium of Okpokwasili and Okorie [8] was prepared and dispensed in 99 ml volumes into 250 ml flasks. To each of the flasks was added 1ml of the diesel oil. Selected isolates which showed the highest turbidity in the screening test were used as pure cultures to inoculate the flasks. A consortium of the isolates was also inoculated to one flask. Each of the flasks was inoculated with 0.2 ml of 24-hour broth cultures of the corresponding isolate. One of the flasks remained uninoculated and they were incubated at room temperature and hand-shaken at intervals for 14 days. The optical density (OD) at 420 nm, Total Viable Count (TVC) and pH of the culture in each flask were monitored at 48 hours interval.

Effect of diesel oil and glucose on bacterial isolates

The marine medium of Okpokwasili and Nnubia [9] that contained sodium glycerophosphate, 0.05 g; glycerol, 0.50 ml; bacteriological peptone, 0.01 g; and yeast extract, 0.01 g in 1 liter of mineral salts solution was prepared and used. About four concentrations of the diesel oil (0.0, 0.5, 1.0 and 2.0% v/v) were prepared in the medium in 100 ml volumes in 250 ml flasks. Also the marine medium was supplemented with 0.4, 0.8 and 1.6% (w/v) glucose as a reference substrate.

Duplicate flasks containing 100 ml of each test substrate concentration (glucose-and diesel oil-amended marine broth) were inoculated with 0.2 ml of each culture. The flasks were incubated at room temperature and hand-shaken at intervals for a period of 24 hours. The absorbance (A_{420nm}) and the total count were taken every 2 hours to follow the growth curve.

Emulsion and adherence profile of the isolates

Each of the isolates and the consortium were grown in diesel oil-glucose-mineral salts broth medium containing 1%(v/v) diesel oil and 1%(w/v) glucose in mineral salts medium of Okpokwasili and Okorie [8] at room temperature for 7 days. Cells were harvested by centrifuging the culture at 30, 000 rpm for 30 minutes. The cells were washed twice with potassium, urea, magnesium (PUM) buffer which contained 16.87 g K_2HPO_4; 7.26 g KH_2PO_4; 0.2 g $MgS0_4$. $7H_20$ and 1.8% filter sterilized urea in 1 liter of distilled water. After washing, the PUM buffer was used to suspend the cells and the optical densities adjusted to approximately equal values.

The cell suspensions were dispensed in 10 ml volumes into duplicate test tubes. The adherence test for the cells were carried out by taking the absorbance (at 420 nm) of the cell suspension (initial absorbance) and then adding 0.1 ml of the diesel oil into the tubes. The tubes were vigorously shaken and allowed to stand for 15 minutes during which time, the oil with the adhering cells separated. The lower portions of the tubes were carefully separated into separate cuvettes and the absorbance measured (final absorbance). Adherence is expressed as percentage decrease in absorbance of the lower aqueous phase after mixing, as compared to that of the cell suspension prior to mixing with oil.

$$\% \text{ Adherence} = \frac{\text{initial absorbance - final absorbance}}{\text{initial absorbance}} \times \frac{100}{1}$$

The emulsification activity of the cell-free supernatants was determined by the method of Marin *et al.* [10]. Equal volumes of the cell-free supernatants and diesel oil were added to duplicate test tubes. The mixtures were shaken vigorously and allowed to stand for 1 hour. Emulsification activity was expressed as the percentage of the total height occupied by the emulsion.

$$\% \text{ Emulsification} = \frac{\text{Height of the emulsion layer} \times 100}{\text{Total height of the mixture}}$$

RESULT

The growth of the bacterial isolates in the diesel oil

The isolates were identified as members of the genera: *Pseudomonas, Bacillus*, *Staphylococcus* and *Proteus*. The growth profile of the bacterial isolates and the consortium determined by monitoring the optical density (OD), total viable counts (TVC) and pH of the cultures utilizing diesel oil as sole source of carbon and energy are presented in Figures 1 to 5. The results showed that the TVC of the pure cultures and the consortium increased with corresponding increase in optical density. The OD and TVC appeared in the following order: consortium > *Pseudomonas* sp. > *Bacillus* sp. > *Staphylococcus* sp. > *Proteus* sp. The highest pH drop was observed in the consortium where the pH dropped from 7.87 to 5.29. This was followed by *Proteus* species (PH 7.25-5.65) and the least was in *Staphylococcus* species which dropped from 7.35 to 6.47. *Pseudomonas* species and *Bacillus* species had the same pH drop with the values of 7.75 to 6.50 and 7.55 to 6.30, respectively. The TVC of the consortium was significantly higher than that of the individual isolates at $P < 0.05$.

Effect of diesel oil and glucose on bacterial isolates

The effect of diesel oil on the optical densities of the pure isolates and the consortium are shown in Figures 6 to 10. Generally the diesel oil had little or no effect on the lag phases. The exponential phases were stimulated at all concentrations of the diesel oil in all the isolates except in *Bacillus* species where the exponential growth phase was stimulated at 0.5% and depressed at 1.0% and 2.0%.

Statistical analysis (ANOVA) at $P < 0.05$ revealed that there were significant differences among the optical densities of the isolates and the consortium. Again, there were significant differences among the optical densities obtained at various times of the analysis and among various concentration of the diesel oil.

The effects of varying concentrations of glucose on the growth of pure isolates and the consortium

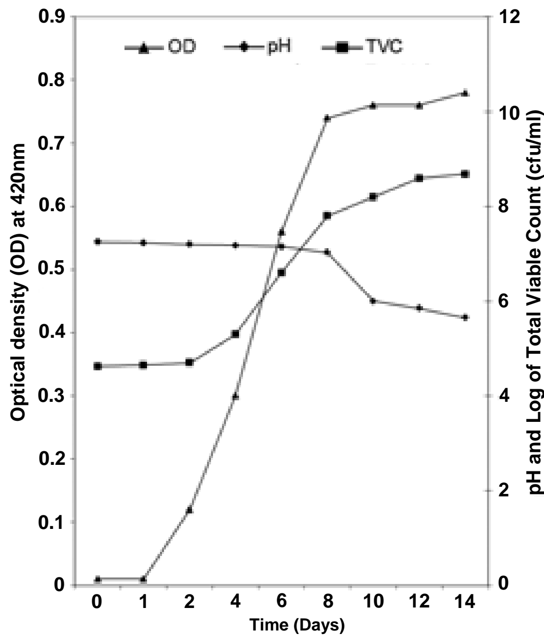

Figure 1. Growth profile of a culture of *Proteus* sp. in liquid mineral salts medium containing diesel oil as the sole source of carbon and energy.

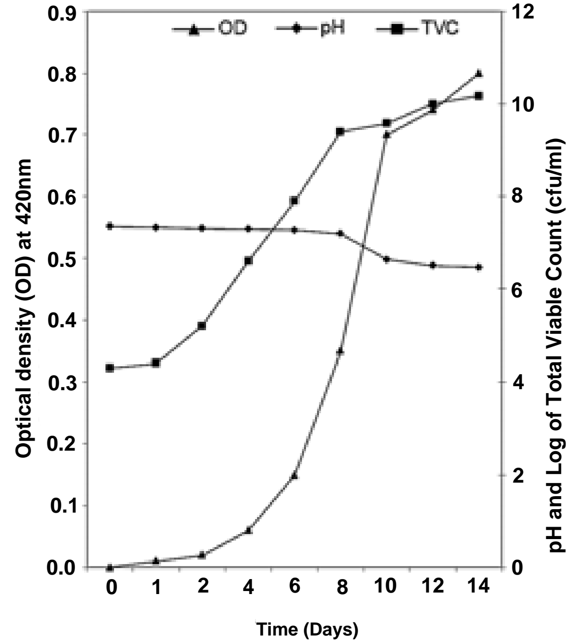

Figure 3. Growth profile of a culture of *Staphyloccocus aureus* in liquid mineral salts medium containing diesel oil as the sole source of carbon and energy.

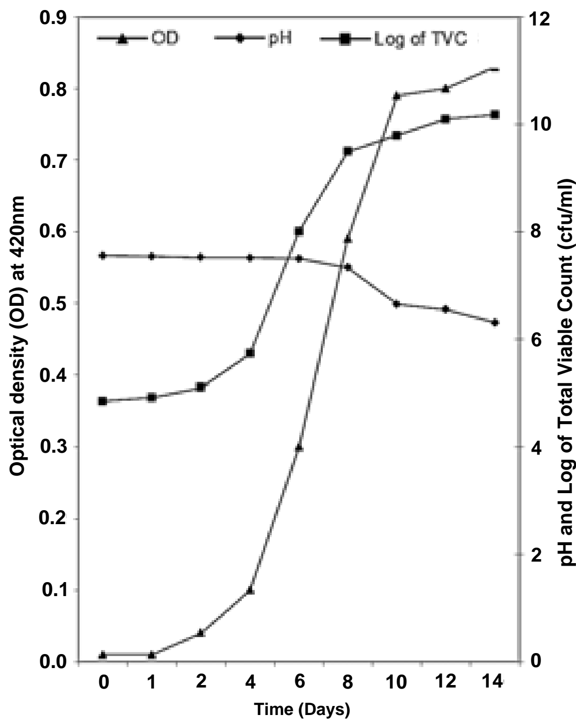

Figure 2. Growth profile of a culture of *Bacillus* sp. in liquid mineral salts medium containing diesel oil as the sole source of carbon and energy.

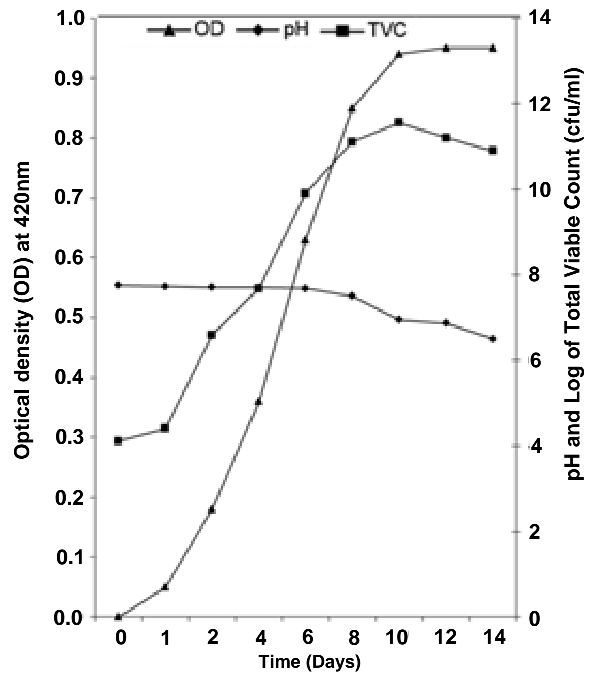

Figure 4. Growth profile of a culture of *Pseudomonas* sp. in liquid mineral salts medium containing diesel oil as the sole source of carbon and energy.

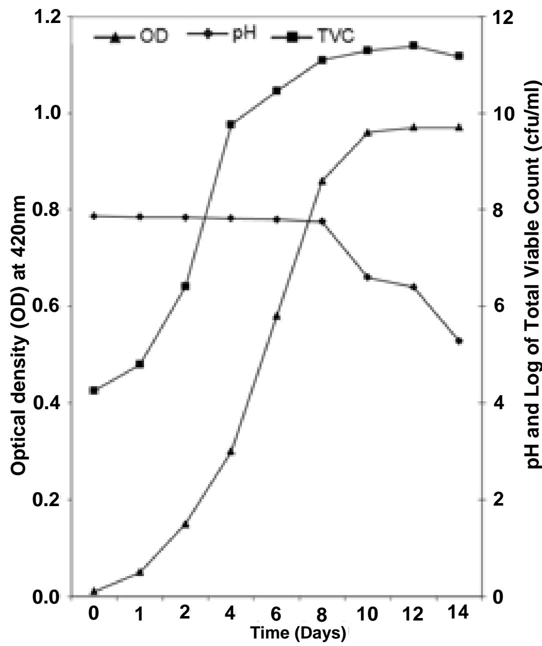

Figure 5. Growth profile of a culture of consortium in liquid mineral salts medium containing diesel oil as the sole source of carbon and energy.

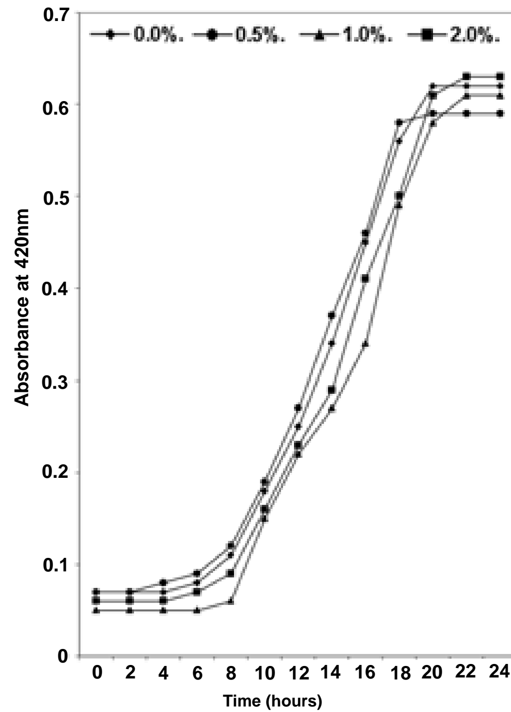

Figure 7. Effects of diesel oil on the growth of *Bacillus* species.

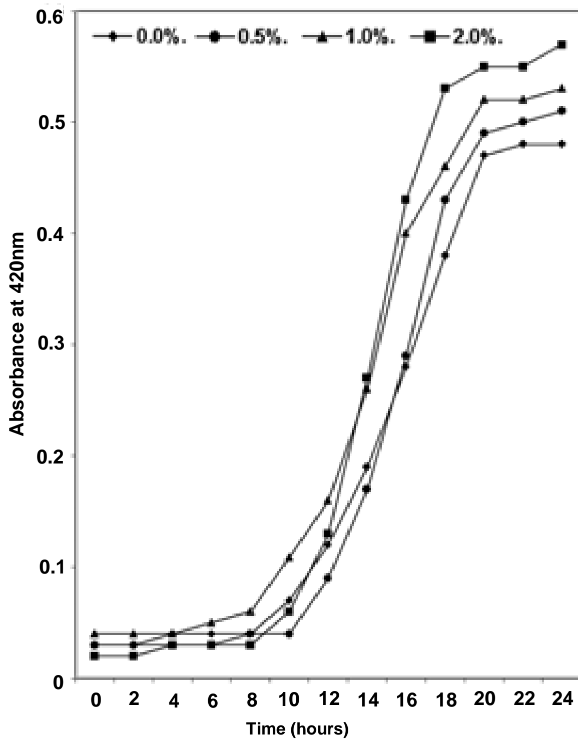

Figure 6. Effects of diesel oil on the growth of *Proteus* species.

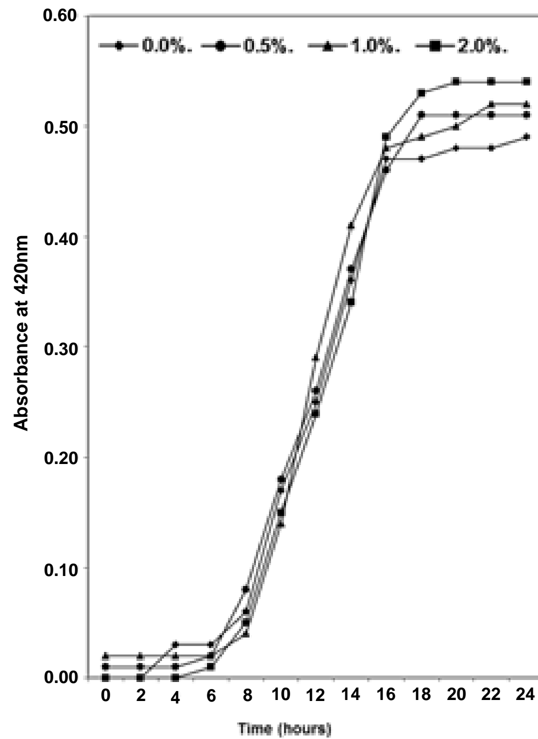

Figure 8. Effects of diesel oil on the growth of *Staphylococcus* species.

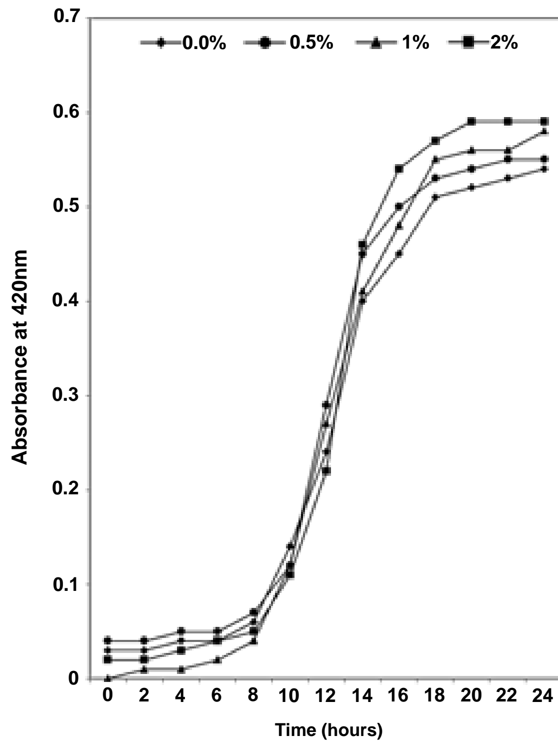

Figure 9. Effect of diesel oil on the growth of *Pseudomonas* species.

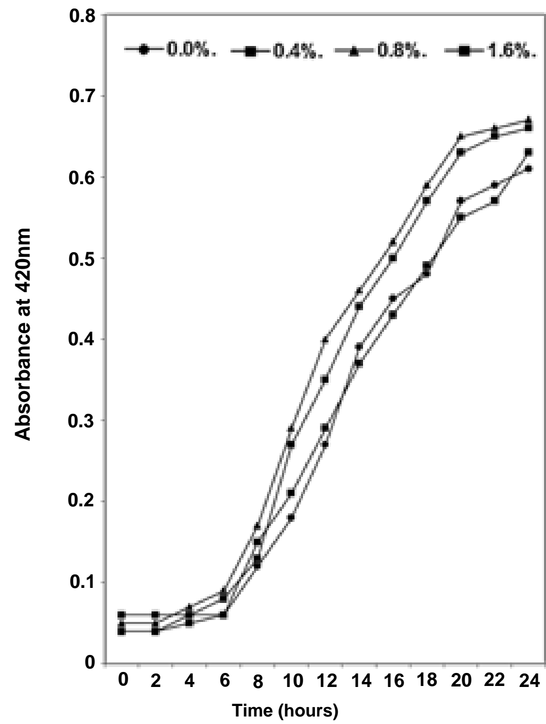

Figure 11. Effects of glucose on the growth of *Proteus* species.

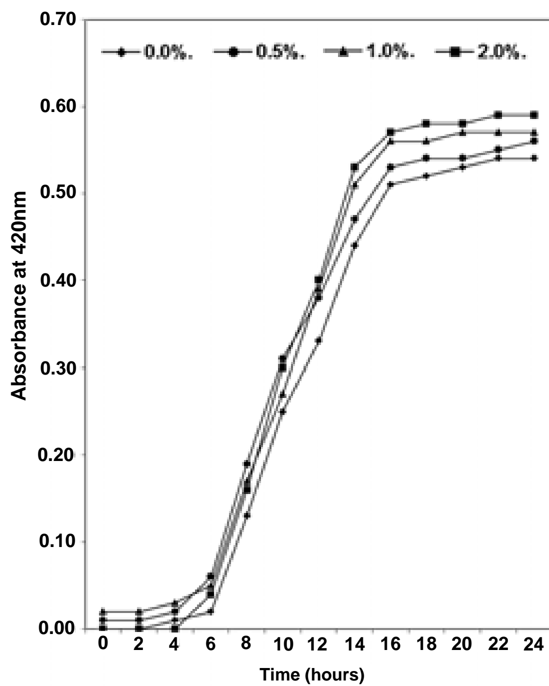

Figure 10. Effects of diesel oil on the growth of consortium.

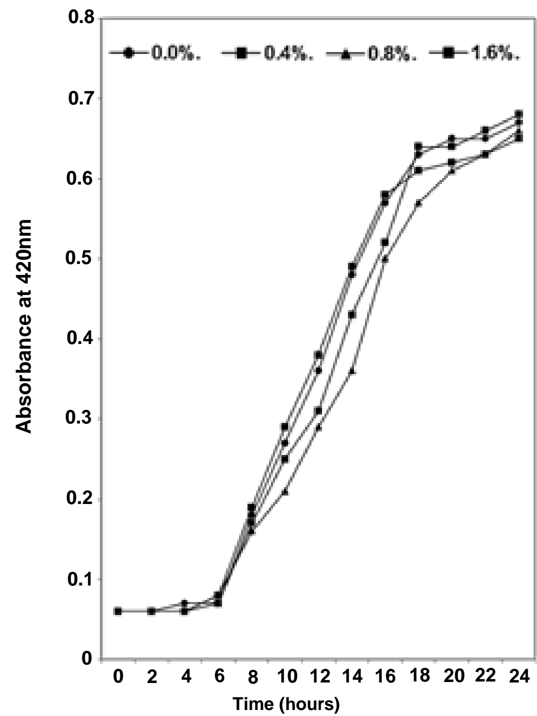

Figure 12. Effects of glucose on the growth of *Bacillus* species.

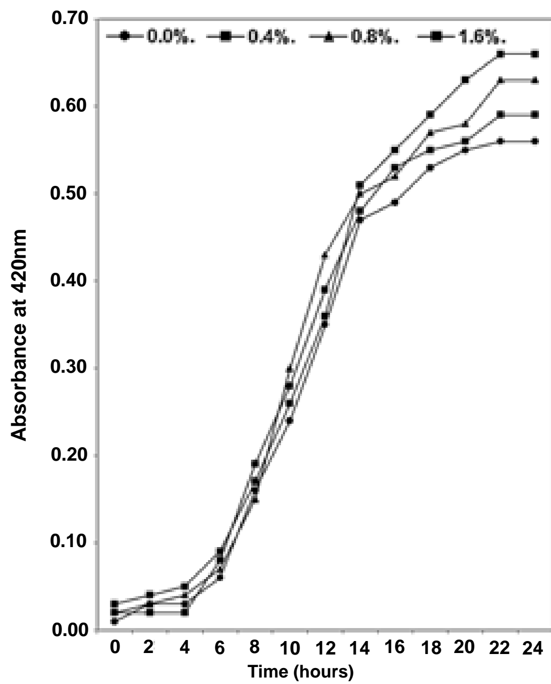

Figure 13. Effects of glucose on the growth of *Staphylococcus* species.

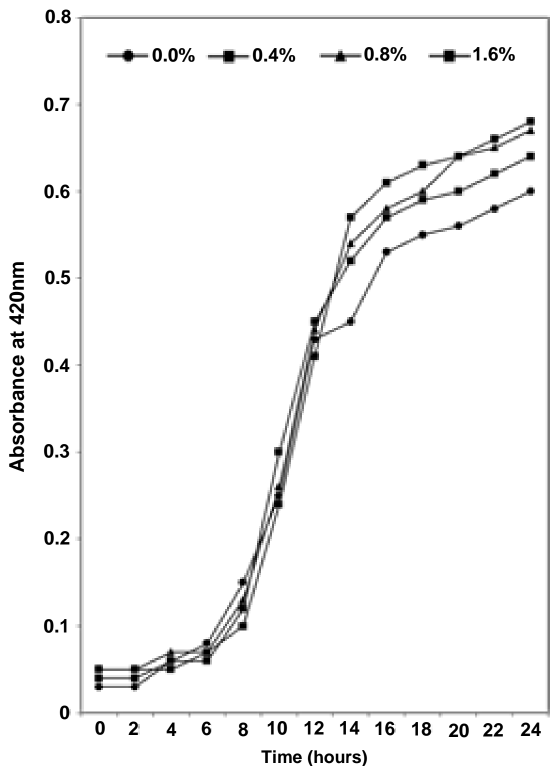

Figure 14. Effects of glucose on the growth of *Pseudomonas* species.

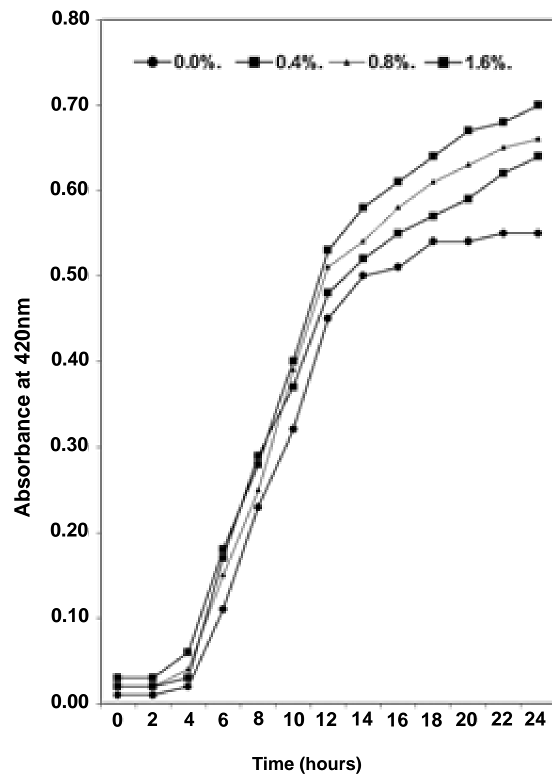

Figure 15. Effects of glucose on the growth of consortium.

are shown in Figures 11 to 15. Glucose had little or no effect on the lag phases but affected the exponential phases of the growth of the organisms. The exponential phases of all the isolates and the consortium were stimulated at various concentrations of glucose except in *Bacillus* species that had its exponential phase depressed at 0.8 and 1.6%. There were high significant differences ($P < 0.05$) among the optical densities of the pure isolates and the consortium and also high significant differences ($P < 0.05$) among the optical densities obtained at various times and concentrations of the glucose.

Emulsification and adherence profiles

The result of the emulsification and adherence tests performed on the pure isolates and the consortium are shown in Figure 16. The consortium had the highest percentage of adherence (42.14%) followed by *Pseudomonas* species (14.29%) and the least percentage of adherence was obtained in the *Proteus* species (10.03%). In the emulsification test, the consortium had the highest percentage

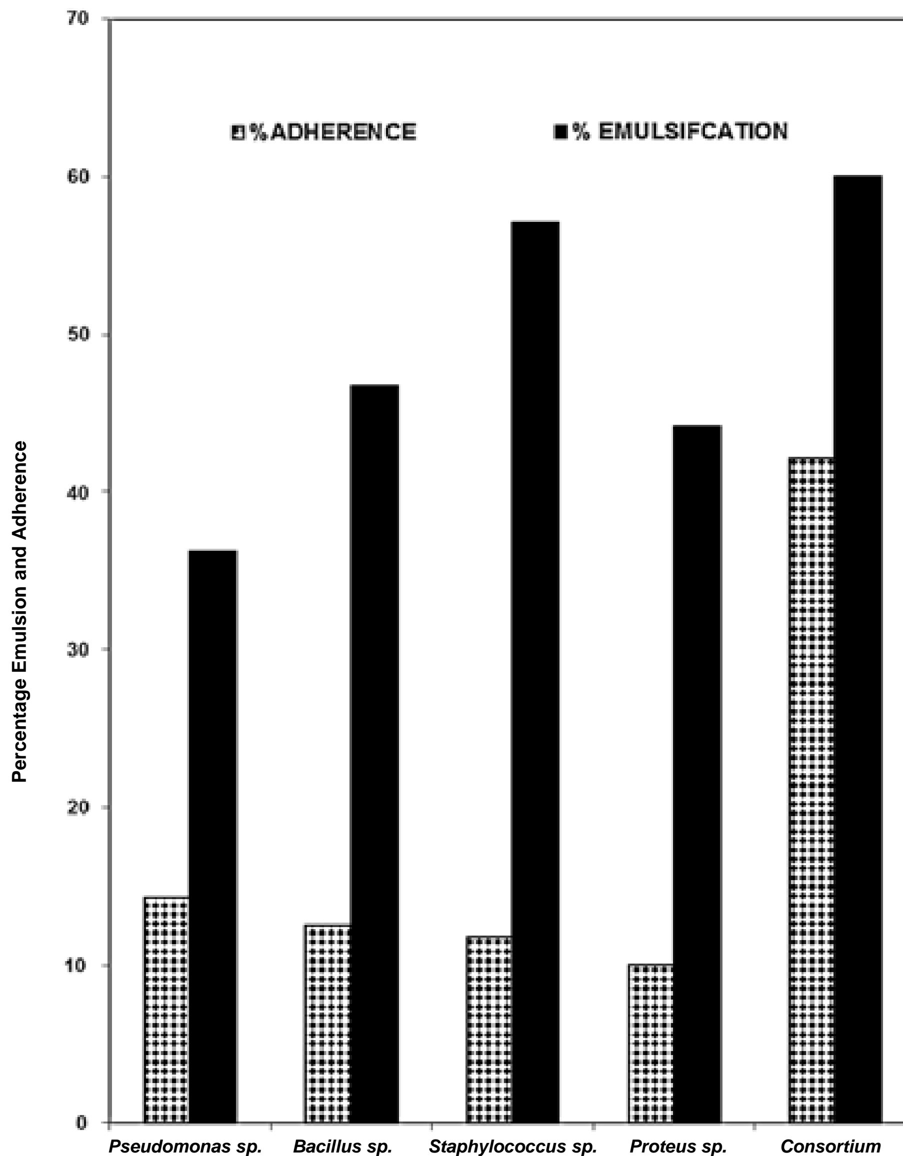

Figure 16. Emulsion and adherence profile of the isolates.

of 60.01% followed by *Staphylococcus* species (51.11%) and the least was *Pseudomonas* species (36.16%).

DISCUSSION

The growth profile of the bacterial isolates and the consortium showed that, as the total viable count increased, there were corresponding increase in the optical density and decrease in pH of the cultures. The utilization of the diesel oil as sole source of carbon and energy by these microorganisms resulted in an increase in their growth with concomitant production of acidic metabolites which might account for the decrease in pH of the culture. The pH drop was within the range of 7.87 to 5.29 in the various organisms and consortium tested. This showed that the consortium, *Proteus, Pseudomonas* and *Bacillus* had greater microbial activities. The total viable counts of these isolates also supported this statement although the *Proteus* species had the least viable count. These isolates also had the highest optical densities showing that they have the potential for application in the bioremediation of diesel-oil

contaminated environment. The consortium of the isolates seems to perform better than the pure isolates in the diesel oil degradation. This is because no organism has been established to degrade both aromatic and aliphatic hydrocarbons completely.

The emulsification and adherence to oil by the bacterial isolates showed that they are able to produce biosurfactants and that the microorganisms could act as emulsifiers by adhering to the oil droplets. This further showed that the organisms have potential to be used in the bioremediation of oil-polluted environment.

Emulsification and adherence have been identified as the major mechanisms for the uptake of hydrocarbons by microorganisms. They increase the bioavailability of non-aqueous phase liquids. Biosurfactants have been applied in the biodegradation of hydrocarbon in polluted soils [11]. This is because biosurfactants enhance the mobility of bacteria and oil in the soil. Lee *et al.* [12] have shown that rhamnolipid biosurfactant enhanced the transport of *Pseudomonas aeruginosa* cells through a sandy soil by decreasing the sorption of cells.

In monitoring the effect of diesel oil and glucose on the growth of isolates, it was observed that diesel oil and glucose had little or no adverse effect on the exponential phases of the microbial growth. The implication is that 2% (v/v) diesel oil is below toxic concentration for the organisms and that there would be no glucose poisoning or toxicity at glucose concentrations up to 1.6% (v/v). This is in accordance with the work of Nweke and Okpokwasili [13] in which glucose toxicity was not discovered in *Staphylococcus aureus*. In the soil, therefore, it could be reasoned that at these concentrations of diesel oil and glucose, the growth rates of these bacterial species will not be reduced and bioremediation involving these organisms will not be slowed down.

The fact that these organisms grew well with glucose showed that glucose and, of course, other carbohydrates could serve as alternative carbon sources. Biodegradation of diesel oil in contaminated soil, therefore, would be slowed down in the presence of alternative carbon sources. It is important to mention here that the organisms do not produce exopolysaccharides with glucose suggesting that glucose molecules are broken down. The fact that these microorganisms fermented glucose, confirmed this statement.

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