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Hydrocarbon Biodegrading Potentials of a *Proteus vulgaris* Strain Isolated from Fish Samples

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Abstract: A *Proteus vulgaris* bacterium SR-1 was isolated from a freshly killed fish sample collected close to the point of crude oil spill in the Niger Delta region, Nigeria. **Problem statement:** The application of native bacterial species in bioremediation processes has long been desired, because they would be cost effective and efficient in terms of acclimation time. The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is evidence that these microorganisms are the active degraders of that environment. In this study, we reported the potential of a candidate bacterium- *Proteus vulgaris* SR-1 in the biodegradation of Bonny light crude oil, diesel and kerosene. **Approach:** To screen for oil degrading capability, the bacterium was cultivated in Minimal Salts Medium (MSM) supplemented with 1% (v/v) sterile Bonny Light Crude Oil (BLCO). Oil degradation was monitored by measurement of turbidity using a spectrophotometer and the pH, total viable counts of the culture fluids were determined at time intervals as biodegradation indices. The ability of strain to degrade diesel and kerosene oils was also studied while the level of used hydrocarbon degradation was determined using the gravimetric analysis. The bacterium was screened for presence of Plasmid DNA and implication of plasmid in hydrocarbon degradation was investigated. **Results:** (1) The bacterium utilize hydrocarbons as sole source of carbon and it biodegraded Bonny light crude oil, kerosene and diesel media by as much as 78, 79 and 73.8% respectively, in the presence of 1.0% NaCl (w/v) after 96 h. The total viable count after 96, 120 and 168 h of biodegradation of the test hydrocarbons range between 6.2 and 9.1 $\log_{10}$ c.f.u mL^{-1}, (2) The results showed that increasing NaCl concentration in water had decreasing effect on hydrocarbon degradation. (3) pH of media decreased from 7.0 to between 3.29 and 5.02 during the reaction period while growth increases. (4) Plasmid analysis revealed the presence of a plasmid of approximately 9.1 kb in the bacterial isolate. **Conclusion/Recommendations:** The results of this study showed that *Proteus vulgaris* SR-1 is a highly adapted bacterium with great potential to biodegrade hydrocarbons and the genes responsible for hydrocarbons biodegradation could be located on the (9.1 kb) plasmid it harbors.

Key words: Niger delta, bacteria, hydrocarbons, biodegradation, Plasmid

INTRODUCTION

In Nigeria, the scourge of environmental pollution has reached a frightening scale in recent years especially in the Niger-Delta region, the largest delta in Africa and the third largest in the world where most of the crude oil in the country is found (HRW, 1999). This region encompasses an area of approximately 70,000 km^2 accounting for about 7.5% of the country's total land mass, covering a coastline of 560 km, about two-third of the country's entire coastline. In the Niger Delta, increasing petroleum exploration and transportation has introduced large amounts of hydrocarbons into the area (Odokuma and Dickson, 2003a; 2003b).

Accidental and deliberate crude oil spills have been and still continue to be, a significant source of environmental pollution and poses a serious environmental problem, due to the possibility of air, water and soil contamination (Trindade *et al*., 2005). According to Fasasi (2006), oil spill also destroys the biodiversity of the delicate ecosystem of the Niger Delta. The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves the trio of physical, chemical and biological alternatives (Okoh, 2006).

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Many bacteria and fungi have demonstrated potentials in the biodegradation of hydrocarbon pollution and these organisms are widely distributed in marine, freshwater and soil habitats (Head and Swannell, 1999). The biodegradation of crude oil by microorganisms is one of the primary ways for eliminating crude oil from contaminated sites and appears to be the most environmentally friendly method of removal of oil pollutant (Barathi and Vasudevan, 2001; Balba *et al*., 2002; Urum *et al*., 2003). The ability to isolate high numbers of certain oil degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). The relevance of bacterial isolates in bioremediation of hydrocarbon contaminated systems especially in Nigeria is continually been investigated with the hope of stocking organisms that are useful for the bioremediation of crude oil polluted environments (Okoh *et al*., 2001; Okoh, 2003; Ogbeifun *et al*., 2004; Lu *et al*., 2000; Holt *et al*., 1994; Colle *et al*., 1996). In this study, we report the potential of a candidate bacterium-*Proteus vulgaris* SR-1in the biodegradation of Bonny light crude oil, diesel and kerosene.

MATERIALS AND METHODS

Microorganism, identification and maintenance: The hydrocarbon degrading bacterium strain used in this study were isolated from our previous study (Olajide *et al*., 2009) from newly killed fish samples collected close to the point of spill in the Niger Delta region in Nigeria. The purified bacterium strain was characterized for Gram reaction, cell morphology (Olajide *et al*., 2009) and biochemical/enzymatic analysis. The bacterium strain was grown in nutrient broth culture medium with a 2% (v/v) inoculum and incubated 37°C with shaking at 125 rpm (Lab-line No 3590) and regenerated twice before use in the manipulations. The regenerated strain was maintained on nutrient Broth agar (Merck) slants at 4°C and sub-cultured every three months or when necessary.

Inoculum was prepared by growing cells at 37°C for 24 h in Nutrient Broth and stirred in a rotary shaker at 120 rpm. The composition of the mineral salt medium used in this study was described by Tuleva *et al.* (2002).

Chemical reagent and hydrocarbon substrates: The Bonny light crude oil, diesel and kerosene were obtained from the Nigerian National Petroleum Corporation (NNPC) Warri, Delta State, Nigeria and all other chemicals used for this study are of analytical grade from BDH Chemicals Ltd. Poole, England except otherwise stated.

Screening of hydrocarbon degrading bacterium: Fifty milliliter of the mineral salt medium was distributed in a sterile conical flask, 1% of each hydrocarbon was aseptically added and controls also set up. After sterilization, the bacteria isolate *Proteus vulgaris* was inoculated into the flasks. The flasks were incubated at 37°C on a rotary shaker (Lab-line No 3590), with shaking at 120 rpm for one week. Oil degradation was monitored by measurement of turbidity at a wavelength of 540 nm using a spectrophotometer (NOVASPEC II, Pharmacia Biotech). The measurement was taken for one week period to allow appreciable monitoring of bacteria growth under experimental conditions alongside that of sterile control. The pH, Total Viable Counts (TVC) of the culture fluids were determined at time intervals as biodegradation indices (Rahman *et al*., 2002; Emtiazi and Shakarami, 2004).

Biodegradation of hydrocarbons by pure culture: A 50 mL of cooled sterilized mineral salts medium was dispensed into several sterilized 100 mL conical flasks and 0.5 mL of each sterilized hydrocarbon added to make the usual 1% w/v oil. To each of the flasks, a pure culture of isolate *Proteus vulgaris* already grown in nutrient broth and standardized to constant mass was added at about 1.0 mL of culture to a flask to maintain uniformity as much as possible. The flasks were incubated on rotatory shaker with shaking at 120 rpm for one week at room temperature. After incubation, the level of used hydrocarbon degradation was determined using the gravimetric analysis (Chang, 1998; Marquez-Rocha *et al*., 2001). The percentage of hydrocarbon remaining was calculated compared to the control.

The effect of hydrocarbon degradation on pH: To study the effect of pH on hydrocarbon degradation, the pH of the medium was adjusted to the desired pH by adding either 0.1 M HCl or 0.2 M NaOH using glass electrodes. Following inoculation, the flasks were incubated on shaker at 150 rpm for 3 days. The emulsification capacity of the culture broth free of cells was also determined (Yakimov *et al*., 1995).

Investigation of different salt concentrations effect on hydrocarbon degradation: The effect of salinity on hydrocarbon degradation was determined by adding different concentrations (0.0-2%) of NaCl to

the minimal salts medium. The mixtures were incubated at 37°C on shaker at 150 rpm for 3 days (Prommachan *et al*., 2001). The emulsification capacity of the culture broth free of cells was also determined.

Plasmid DNA detection procedure: The presence of plasmid DNA in *Proteus vulgaris* SR-1 was done in accordance with the description of Kado and Liu (1981). Cells were grown in 3 mL of LB broth overnight at 37°C to an optical density at 600 nm of 0.8 and pelleted by centrifugation (5,700 rpm, 4°C, for 7 min). The cell pellet was thoroughly resuspended in 1 mL of TE buffer (40 mM Tris-acetate and 2 mM sodium EDTA). The Tris was adjusted to pH 7.9 with glacial acetic acid. The cells were lysed by adding 2 mL of lysing solution (3% SDS and 50 mM Tris (pH 12.6). The solution was adjusted to pH 12.6 by adding 1.6 mL of 2 N NaOH, which was mixed by brief agitation. The solution was heated at 50-65°C for 20 min in a water bath and 2 volumes of phenol-chloroform solution (1:1 vol/vol) were added. The solution was emulsified by shaking briefly and the emulsion was broken by centrifugation (6,000 rpm, 15 min, 4°C). Avoiding the precipitate at the interface, the upper aqueous phase was transferred to Eppendorf micro centrifuge tubes. Samples were withdrawn directly for electrophoresis immediately or stored at 4°C until tested. As plasmid DNA samples contain RNA, 1 µL of RNase was added to each 35 µL of sample, mixed and incubated for 5 min at 37°C prior to gel loading. This was followed by addition of 7 µL of loading dye. Plasmid DNA was analyzed by 1.0% agarose gel electrophoresis in TAE buffer pH 8.5 for 30 min. Lambda HindIII DNA molecular mass marker (23130-564 bp) was used as a standard for molecular mass determination. The gel was stained with ethidium bromide (0.5 mg mL^{-1}) and observed under UV transilluminator for the presence of plasmid band.
above, were digested an hour at 37 °C with ten units of various restriction enzymes and the fragments thus generated were fractionated by electrophoresis on 1.0 % agarose gel as described.

RESULTS

Isolation and identification of bacterial strains: The test bacteria is a small, motile, Gram-negative, single rods, indole positive, oxidase negative, facultative anaerobe, while the results of biochemical and enzymatic assay correct identification rate without additional tests assay was 88.9% at the species level and 98.4% at the genus level. The overall rate of correct species identification was about 90% consistency index of *Proteus vulgaris.*

Hydrocarbon biodegradation: The utilization of hydrocarbons (Bonny light crude oil, Diesel and kerosene) as a substrates by isolate *Proteus vulgaris* SR-1 is evident by the increase in cell density. The results showed maximal increase in optical densities and total viable count concomitant with decrease in pH on fourth, fifth and eight day of reactions (Fig. 2-4). The maximum cell densities were 6.2 log_{10} c.f.u mL^{-1} and over a 96-120 h of growth in 1% (v/v) Bonny light crude oil and diesel respectively. The maximum cell density of 9.1 log_{10} c.f.u mL^{-1} was obtained on 168 h during growth in the presence of 1% (v/v) kerosene (Fig. 4).

Effect of Hydrocarbon Degradation on pH: The initial pH of medium was 7.0 in this experiment. Generally, a decreasing trend of pH was observed in the experimental flasks within the incubation period as growth increases in the presence of the three different hydrocarbons investigated (Fig. 1-3).

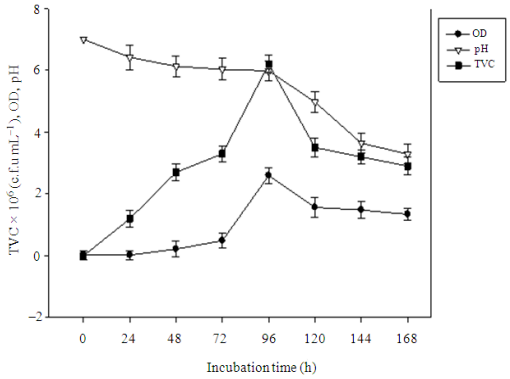

Fig. 1: Growth profile of *Proteus* species in mineral salt medium containing bonny light crude oil as sole carbon and energy source

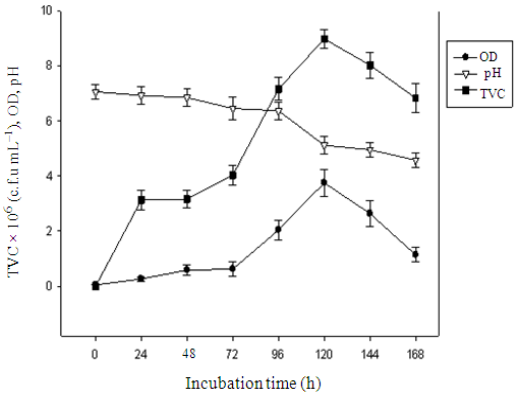

Fig. 2: Growth profile of *Proteus* species in mineral salt medium containing diesel oil as sole carbon and energy source

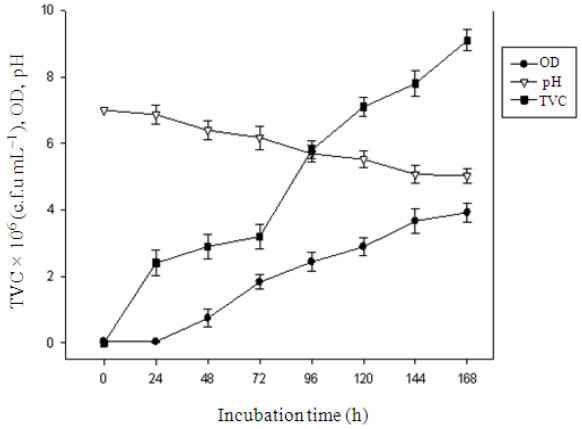

Fig. 3: Growth profile of *Proteus* species in mineral salt medium containing kerosene oil as sole carbon and energy source

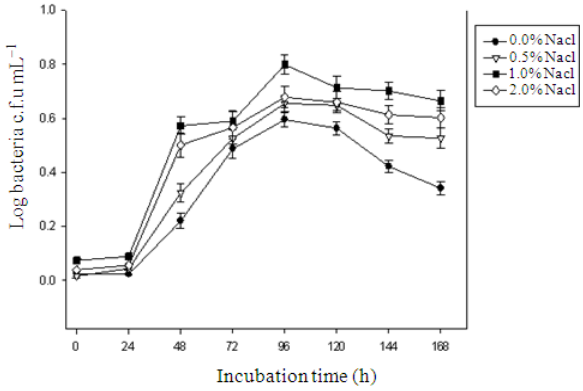

Fig. 4: Population growth of bacterial cells of *Proteus vulgaris* in crude oil medium containing different concentrations of NaCl (0.0-2.0%)

Effect of salinity on hydrocarbon degradation: The effect of different NaCl concentrations (0-2%) on hydrocarbons degradation from the crude oil-contaminated water was studied. The results showed that increasing NaCl concentration in water had decreasing effect on hydrocarbon degradation. The amount of oil degraded by *Proteus vulgaris* SR-1 strain increased initially to a maximum level at 1.0% w/v NaCl, but thereafter decreased with increasing salt concentration and the patterns were similar for the three different test hydrocarbon substrates (Fig. 4-6). In all the various concentrations of NaCl used, salt concentrations did not affect the viable cell count during biodegradation. The results obtained were almost the same for the different hydrocarbons, though the number of total viable counts varied in each oil as shown in Fig. 4-6.

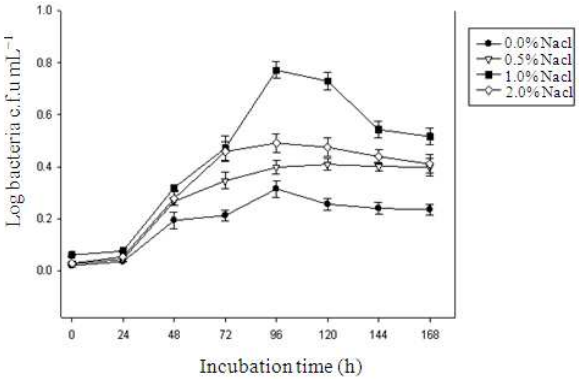

Fig. 5: Population growth of bacterial cells of *Proteus vulgaris* in kerosene oil medium containing different concentration of NaCl (0.0-2.0%)

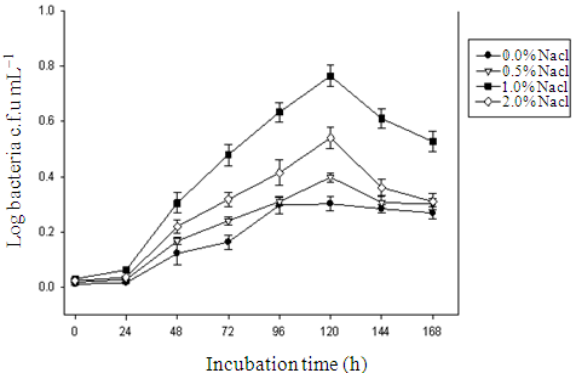

Fig. 6: Population growth of bacterial cells of *Proteus vulgaris* in diesel oil

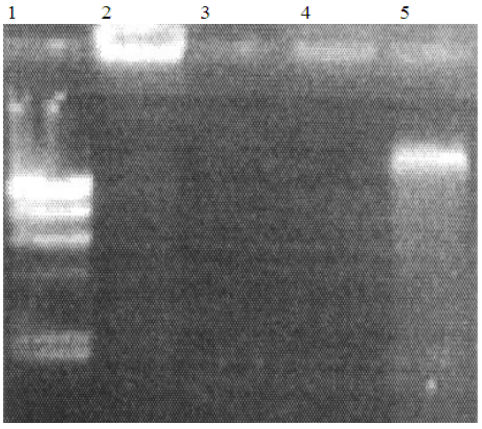

Fig. 7: Agarose gel electrophoresis of plasmid DNA from *Proteus vulgaris* (SR-1). Lane 1 is Hind111 marker and lane 5 contains the plasmid DNA from SR1

Table 1: Gravimetric analysis of oil degraded at 37°C after incubation for 5 days^{-1}

		Hydrocarbons	
Media (% NaCl)	Bonny light crude oil	Kerosene	Diesel
0.0	73.98	72.73	67.41
0.5	75.86	76.25	70.12
1.0	78.10	79.92	73.80
2.0	60.64	69.56	67.41

Values are means of at least two replicates

Gravimetric analysis of oil degraded: The NaCl concentration for optimal growth was 1.0% (w/v) as observed in Table 1 The biodegradation of hydrocarbons were higher in 1.0% NaCl and was 78.1, 79.9 and 73.8% for Bonny light crude oil, kerosene and diesel respectively.

Evidence of plasmid DNA in isolate: Plasmid analysis revealed the presence of a plasmid of approximately 9.1 kb in the bacterial isolate *Proteus vulgaris* SR-1 (Fig. 7).

DISCUSSION

The isolate, *Proteus vulgaris* SR-1 was able to grow on crude petroleum as the sole source of carbon and energy when screened for hydrocarbon utilization. Interestingly, this same organism has been implicated in hydrocarbon degradation (Kayode-Isola *et al*., 2008).

The growth profiles of the bacterium were monitored by the optical densities, total viable count and the pH of the culture media. The results were shown in Fig. 1-3 and reflect that the isolates grew maximally on the three different hydrocarbon substrates when supplied as the sole source of carbon and energy. This technique was used in several studies to show the ability of bacteria utilizing crude oil (Emtiazi and Shakarami, 2004). In a similar investigation by Rahman *et al.* (2002) the total viable count method was used to confirm the potential of different kind of bacteria utilizing hydrocarbon. Thus bacterium growth reached the stationary phase and moved into the death phase in almost all the cases with the exception of kerosene. This is probably due to the chemistry of the hydrocarbon and the order of hydrocarbon degradation was; Kerosene>Bonny light crude oil>diesel.

The utilization of the petroleum hydrocarbons as sole carbon and energy source by the isolate resulted in the growth with a resultant production of acid. This is probably as a result of chemical change of the crude oil hydrocarbons and production of by products and ability of isolated *Proteus vulgaris* to use crude oil and generate organic acids. Thus reducing the pH as has been reported elsewhere (Matthew, 2006). The initial pH of the medium was 7.0 and this decreased steadily as growth increases in the presence of the three different hydrocarbons as presented in Fig. 4-6. This finding is in agreement with the study of Sepahi *et al*. (2008) who reported that microbial degradation of hydrocarbons often leads to production of organic acids, thus the organic acids probably caused the reduction in pH.

It is evident from this study that isolated *Proteus vulgaris* did not exhibit any lag phase in the culture media (Fig. 1-3). The result can be attributed to genetic makeup due to the constitutive expression of hydrocarbon catalyzing enzymes. This finding is in agreement with the study of Okerentugba and Ezeronye (2003) who reported that microorganisms growing on crude oil hydrocarbon did not exhibit any lag phase.

The tolerance of bacteria to salinity gradients could play a major role in its preferential use in the degradation of oil in marine environment. In this study, the amount of oil recovered from the sterile oil controls increased with increasing concentration of NaCl. The quantity of oil degraded increased with increasing NaCl concentration in the experimental. The results obtained in (Table 1) showed that *Proteus vulgaris* did not tolerate high concentrations of NaCl. It would therefore be unexpected that the isolate would thrive in marine system. Hence, it will be necessary to determine optimum salinity for every studied system.

The implication of plasmids in the degradation of petroleum hydrocarbons has also been a subject of investigation. Several methods have been reported for the isolation of plasmid DNA from Gram-negative bacteria (Davies and Normark, 1980; Anderson and McKay, 1983; Owen and Hernandez, 1990; Mottaleb *et al*., 2003). The application of these methods failed to demonstrate plasmids in *Proteus vulgaris.* The most satisfactory being the procedures described by Kado and Liu (1981). By this procedure, it was possible to identify one plasmid of molecular weight 9.1 kb from *Proteus vulgaris* (Fig. 7). SDS-curing of the isolates leads to complete loss of plasmid and hydrocarbon degradation activity.

CONCLUSION

The results of this study showed that *Proteus vulgaris* is a highly adapted bacterium with great potential to biodegrade hydrocarbons and the genes responsible for hydrocarbons biodegradation could be located on the (9.1 kb) plasmid it harbors.

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