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Utilization of Hexadecane and Heptane by *Serratia s*p. under Different Potassium Nitrate (KNO_3) Concentrations

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ABSTRACT

Composition of mineral salts medium vary depending on the scientific protocol used. Potassium nitrate (KNO_3) is one of the components of mineral salts medium (MSM) used in microbiology and other experiment. In this work, the ability of Serratia sp. to degrade hexadecane and heptane at different KNO_3 concentrations was studied. Four experiments were carried out with variations in hexadecane, heptane, salt concentrations and the days of observations. Mineral salts medium composed of 0.125 g/ml of KH_2PO_4, 0.35 g/ml of Na_2HPO_4, 0.05 g/ml of $MgSO_2.7H_2O$, 0.075 g/ml of KNO_3 and 0.25 g/ml of $(NH_4)_2SO_4$ was used. Growth rate using plate count method was assessed during the days of observation. Results showed the ability of Serratia sp. to degrade hexadecane and heptane as the only source of carbon and energy, because the total viable count increased from 79 x 10^2 to 172 x 10^6 cfu/ml for the normal MSN with hexadecane as carbon source. In the set up with normal MSM with heptane, total viable count increased from 22 x 10^2 to 452 x 10^6 cfu/ml. In the set up with hexadecane and 4 times KNO_3 in normal MSM, the total viable count increased from 26 x 10^2 to 33 x 10^6 cfu/ml. In the fourth set up with heptane and 4 times KNO_3 in MSM, the total viable count increased from 117 x 10^2 to 238 x 10^6 cfu/ml. It is concluded that the mixture of the hydrocarbons enhanced the degradation efficiency of Serratia sp.

1. INTRODUCTION

Oil spills have been discovered to affect infant mortality rate in Nigeria (Bruederle and Hodler, 2019). Alkanes are saturated hydrocarbons and are a major fraction (50%) of crude oil depending upon the oil source (Labinger and Bercaw, 2002). Most hydrocarbons can be used as substrates in metabolism by bacteria, archaea, fungi and algae (Abbasian et al., 2015; Rebus *et al.,* 2016). Fungi and algae degrade

hydrocarbons aerobically. Bacteria and archaea are capable of both aerobic and anaerobic degradation (Xue *et al*., 2015).

Hexadecane has been used as a standard to estimate the biodegradation of aliphatic compounds (Dombrowski *et al.,* 2016). Fuels used in most combustion engines are dominated by aliphatic compounds (Chenier *et al*., 2003). Microbial degradation is an alternative that has been applied as a remediation technique in hydrocarbon-polluted sites. The rate of petroleum degradation depends on the microbiological and physicochemical properties of the specific site (Varjani and Upasani, 2017). Microbial degradation of oil has been shown to occur by attack on aliphatic fractions of the oil (Tang *et al*, 2012; Chang *et al*., 2013).

Serratia marcescens has been reported as the most important *Serratia* species due to its ability to produce prodigiosin, serrawettina and also enzymes like as proteases, nucleases, lipases, chitinases, benzonases and cloroperoxidases (Kalivoda *et al.,* 2010). Due to the metabolic capacity of *S. marcesens* strains to degrade petroleum, these strains have been used alone or in microbial consortia, aimed at the recovery of environments contaminated by petroleum spills and derivatives (Ortega-González *et al.,* 2013; Silva *et al.,* 2015). This study was conducted to examine the utilization of hexadecane and heptane by *Serratia s*p under different potassium nitrate ($KNO_{3)}$ concentrations.

2. MATERIALS AND METHODS

2.1. Isolation and Identification of *Serratia* sp.

Serratia sp. was obtained from the stock culture in the Department of Microbiology, University of Ilorin. *Serratia* sp was originally isolated from kerosene contaminated Ilorin alfisol loam using the pour plate technique. Traditional colonial, microscopic and biochemical tests were initially used to identify the organism. The result of the traditional test was confirmed in this work using molecular tools. PCR was performed using 16S Rrna gene primers: Bact 27F (5' AGAGTTTGATCMTGGCTCAG3') and U1492R (5'-TACGGYTACCTTGTTACGACTT-3') as described by Spear *et al*. (2005) for the identification of the isolates. Amplified PCR products were cleaned and sequenced. Amplified 16S rRNA gene sequences were analyzed using the National Center for Biotechnology Information (NCBI) database - BLASTn. The organism was identified based on the percentage similarity of the obtained sequences with those in the NCBI database.

2.2. Estimation of Hydrocarbon Degradation

Mineral salts medium (20 ml) containing 0.125 g/ml of KH_2PO_4, 0.35 g/ml of Na_2HPO_4, 0.05 g/ml of $MgSO_2.7H_2O$, 0.075 g/ml of KNO_3 and 0.25 g/ml of $(NH_4)_2SO_4$ was used for the induction experiment. All media were autoclaved at 121 °C for 15 minutes. *Serratia* sp. was obtained from a previously prepared stock culture in the laboratory which was maintained on nutrient agar slant, subcultured periodically aseptically in nutrient broth and stored at room temperature for 24 hours before inoculating serially (100 µl) into the MSM. The hydrocarbons (hexadecane and heptane) were used as carbon source for the biodegradation.

Four experiments were performed. In the first experiment consisting of 24 reaction tubes contained the MSM (20 ml), hexadecane (26 µl) and *Serratia* sp. (serial dilution at 10^5 of 100 µl) were labelled in triplicates with controls but with an absence of the hydrocarbon. After sterilization of the reaction tubes and tips, 0.9 µl of sterile distilled water was pipetted into the reaction tubes using micropipette and labelled serially. Exactly 200 µl of the MSM, hydrocarbon and organism in the reaction tube was pipetted into the reaction tubes which were serially diluted and then plated using nutrient agar and incubated at room temperature. After 24 hours, growth was then counted and recorded. *Serratia* sp. was examined on the days 0, 3, 6, 13, 33 and 36 by growth using plate count method.

In the second experiment, the same procedure was conducted with heptane and growth was examined on days 0, 3, 6, 9, 12 and 15 on nutrient medium. The third and fourth experiments were similar to the second, only that there was an increment in the concentration of KNO_3 to 0.3 g/ml. Also, only hexadecane was used in the third experiment while hexadecane and heptane were used in the fourth experiment.

2.3. Bacteria Growth Determination

The initial and final growth was determined by pour plate method using nutrient agar. The plates were incubated at 37 °C for 24 hours. After the incubation period, colonies were counted and total microbial count/ml was calculated.

3. RESULTS AND DISCUSSION

In Figure 1, the growth of *Serratia* sp. was examined on day 0, 3, 6, 13, 33 and 36. It was found to increase gradually from day 0 up to day 6. A decline occurred at day 13 before an exponential increase that continued up till day 36. It was observed that in the first experiment (Figure 1), the growth of *Serratia* in the presence of hexadecane and with normal concentrations of the salts had a bit of a fall in the graph at the log phase and towards the end of the growth curve. The growth increased gradually from day 0 to day 3, which then grew rapidly to day 6, and later back on the gradual process to day 9 till the last day 12 (Figure 2). In the second experiment (Figure 2), in the presence of hexadecane and heptane and with normal salt concentration, there was a gradual increase in the growth profile until the last days. This adds to the fact that the bacteria degraded and utilized better in both hydrocarbons. In Figure 3, the growth was examined on day 0, 3, 6, 9 and 12. It started at a point of declining from day 0 to day 3 which then had a full increase sporadically to day 6. The growth then gradually continued up to day 9 till the last day of 12. In Figure 4, the growth rate was observed on the days; 0, 3, 6, 9 and 12. At day 0, the growth started by declining to day 3 which then gave a sporadic increase up till day 6. Gradually, it then increased at day 9 and up till day 12. It was observed that *Serratia* sp. grew sporadically and was able to degrade in the presence of both hydrocarbons at increased KNO_3 concentration (Figure 4). Finally, the fourth experiment which had both hydrocarbons and increased concentration of KNO_3, also had a decline at the lag phase of growth before gradual increase in the bacteria growth. Adetitun *et al.* (2014) had previously reported the growth of *Serratia* on kerosene as the sole energy and carbon source at different extents when assessed for hydrocarbon consumption.

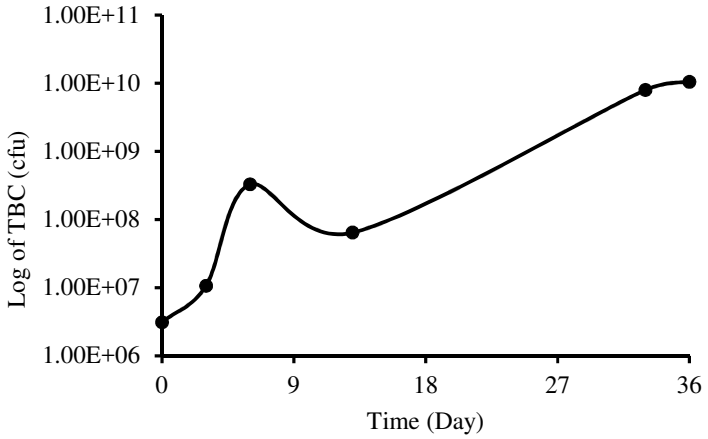

Figure 1: Growth of *Serratia* sp. in the presence of hexadecane (TBC= total bacterial count)

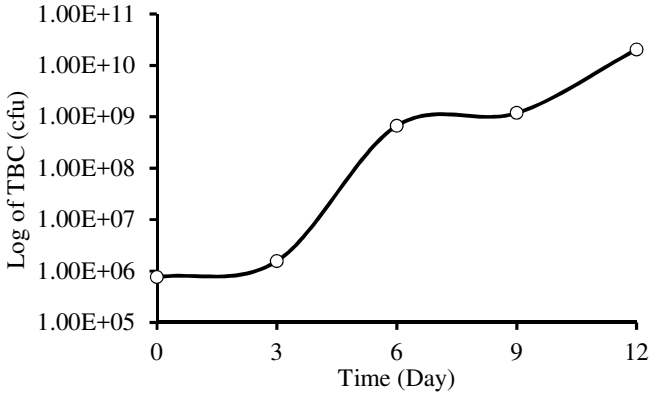

Figure 2: Growth rate curve and biodegradation by *Serratia* sp.

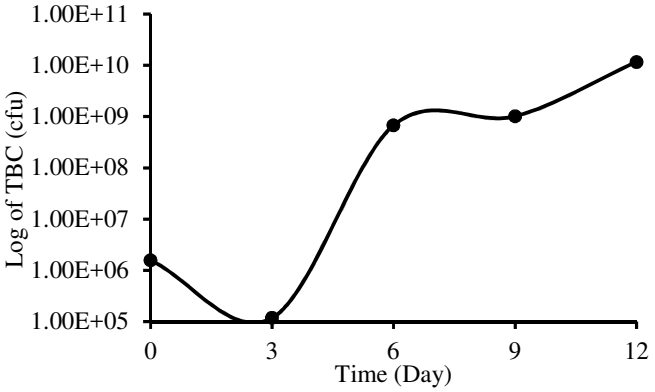

Figure 3: Growth rate curve of *Serratia* sp. with increased concentration of KNO_3 (biodegradation of hexadecane)

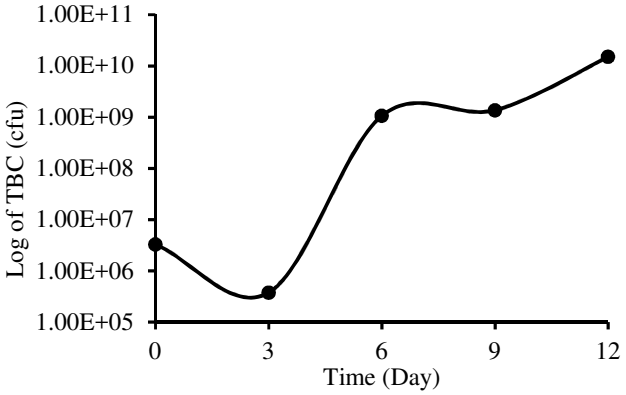

Figure 4: Growth rate curve of *Serratia* sp. (biodegradation of hexadecane and heptane)

Obire and Nwaebuta, (2002) also reported that *Serratia marcesens* persisted in kerosene contaminated soils from two weeks of contamination. It was further reported by De Souza *et al.* (2016) that *S. marcescens* can grow on diesel fuel. The species *S. marcesens* has been described as having the capability to degrade the most diverse types of petroleum hydrocarbons and derivatives (Jaysree *et al.,* 2015). Due to the metabolic

capacity of *S. marcesens* strains to degrade petroleum, these strains have been used alone or in microbial consortia, aiming at the recovery of environments impacted by petroleum spills and derivatives (Ortega-González *et al.,* 2013; Silva *et al.,* 2015).

In this study, the experiment was performed to enable a comparison with biodegradation of model hydrocarbons at different salt concentrations. Pour plate method was used to evaluate the cell growth. However, results showed differences in their growth and the hydrocarbon degradation rates in MSM. As indicated by the data, the degradation of the hydrocarbons, hexadecane and heptane in increased salt concentration proceeded by *Serratia* sp. was more effective in the comparison with the degradation of hexadecane and heptane and also in hexadecane alone. The use of autochthonous microorganisms inhabiting hydrocarbon polluted niches for biodegradation and Bioremediation has been widely accepted as a formidable approach due to the avalanche of successes recorded by various researchers. Adaptation of autochthonous microbial communities, which implies series of physiological and genetic changes that confer degradative ability on the autochthonous organisms have been regarded as a key factor. However, increasing evidences have demonstrated the propensity for hydrocarbon degradation by microorganisms isolated from non-hydrocarbon polluted sources.

Al-Thani *et al.* (2009) and Christova, (2004) reported that bacteria and fungi are the principal agents of petroleum biodegradation in soil. Yakimov *et al.* (2007) reported that several bacteria are known to feed exclusively on hydrocarbons. Degradation of these pollutants by microorganisms has been assessed by a variety of strategies including the seeding of the environment with cocktails of oil-utilizing bacteria. Many alkane-degrading bacteria have been isolated and the enzyme systems that oxidize n-alkanes up to C16 have been characterized (Wentzel *et al*., 2007; Rojo, 2009). Long-chain n-alkanes are more persistent in the environment than the shorter but few data are available in particular concerning the metabolism of these compounds in Gram-positive bacteria (Whyte *et al*., 2002; Smiths *et al*., 2002; Lo Piccolo *et al*., 2011).

Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye *et al.* (2007). Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade many different contaminants (Wackett, 2003). Nseabasi and Antai, (2012) reported that bacterial specie of *Bacillus, Pseudomonas, Serratia* and *Micrococcus* had higher counts than that of *Salmonella, Escherichia coli, Streptococcus* and *Staphylococcus aureus* in the degradation of kerosene. Peixoto *et al.* (2017) reported that *S. marcescens* AMS212 strain, in the quantitative analysis, decreased concentration of DCPIP, confirming the metabolic capability of this strain to biodegrade the petroleum. The evaluation of biodegradability using DCPIP molecule has proved to be an effective method in the search for new bacterial strains with potential to degrade petroleum. The species *S. marcesens* has been described as degrading the most diverse types of petroleum hydrocarbons and derivatives (Jaysree *et al.,* 2015). To investigate the stability and antimicrobial activity of biosurfactant, it was reported that *Serratia rubidaea* SNAU02 was employed.

The oil-degrading populations are widely distributed in the lands and water bodies. Microorganisms (bacteria and fungi) have different rates at which they utilize and degrade hydrocarbons in the soil or water. This rate is reflected in the multiplication and colony forming units (cfu) for the isolated organisms. The use of microorganisms to degrade petroleum hydrocarbon resulting from oil spillage has been a subject of extensive research since the first publication of bacterial growth on petroleum hydrocarbons. Several petroleum hydrocarbon-degrading microorganisms have been isolated from both soil and marine sources, which are the two major environments affected by petroleum hydrocarbon pollution. Microorganisms are equipped with metabolic machinery to use petroleum products as a carbon and energy source. *Acinetobacter* sp. was found to be capable of utilizing n-alkanes of chain length C10–C40 as a sole source of carbon (Throne-Holst *et al*., 2007).

Other bacterial genera, namely, *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia* and *Mycobacterium* isolated from petroleum contaminated soil were proven to be potential degraders of hydrocarbons (Chilean *et al*., 2004). Hexadecane degradation was observed by the bacteria, such as *Pseudomonas putida, Rhodococcus erythroplotis* and *Bacillus thermoleovorans* (Abdel-Megeed *et al*., 2010). It was clear that high efficiency of growth rate (equivalent to degradability) was exhibited by *Serratia* sp. Mixed hydrocarbons at normal salt concentrations (Figure 2) > singularity of each hydrocarbon (Figure 1) > hydrocarbons with increased salt concentrations (Figure 3) and (Figure 4). Moreover, if a comparative study was held between the single hydrocarbon (Figure 1), mixed hydrocarbons (Figure 2), single hydrocarbon with increased salt concentration (Figure 3) and mixed hydrocarbon with increased salt concentration (Figure 4). Growth rate increased sporadically after day 3. The third experiment involved hexadecane in increased concentration of KNO_3. It resulted in a decline in the lag phase before a gradual process of increase in the growth. This may be due to the singularity of the hydrocarbon.

Inorganic salt is an indispensable element for organism growth. It could adjust the osmotic pressure of the cell's membrane, maintain enzyme activity and has other important functions. Many studies have demonstrated that salt concentration is a key factor which has a prominent influence on microbial growth. High salt concentration (23.4 g/l NaCl) will inhibit over 90% of microorganisms in the soil (Rousk *et al*., 2011). De Carvalho and Fonseca, (2005) found that higher salinity could prolong the lag phase of *R. erythropolis* strain DCL14. Similar result was also reported by other researchers. Wu *et al.* (2012) used crude oil as a carbon source to detect *Serratia* sp. BF40 strain reproductive capacity and found that the strain grew well at low NaCl concentration (<40 g/l); but when the salt concentration was up to 60 g/l, both the reproduction time and growth plateau decreased. However, bacterial growth was completely inhibited at a salt concentration above 80 g/l. Dastgheib *et al.* (2012) evaluated the effect of different NaCl concentrations on the growth of a bacterial consortium (mixed culture) called Qphe with phenanthrene as sole carbon source.

4. CONCLUSION

The results of this work have revealed that increasing the concentration of KNO_3 has positive effects on the utilization *of Serratia* sp. It is also concluded that the mixture of the hexadecane and heptane enhanced the degradation efficiency of *Serratia* sp.

5. ACKNOWLEDGMENT

The molecular tests were carried out at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria.

6. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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