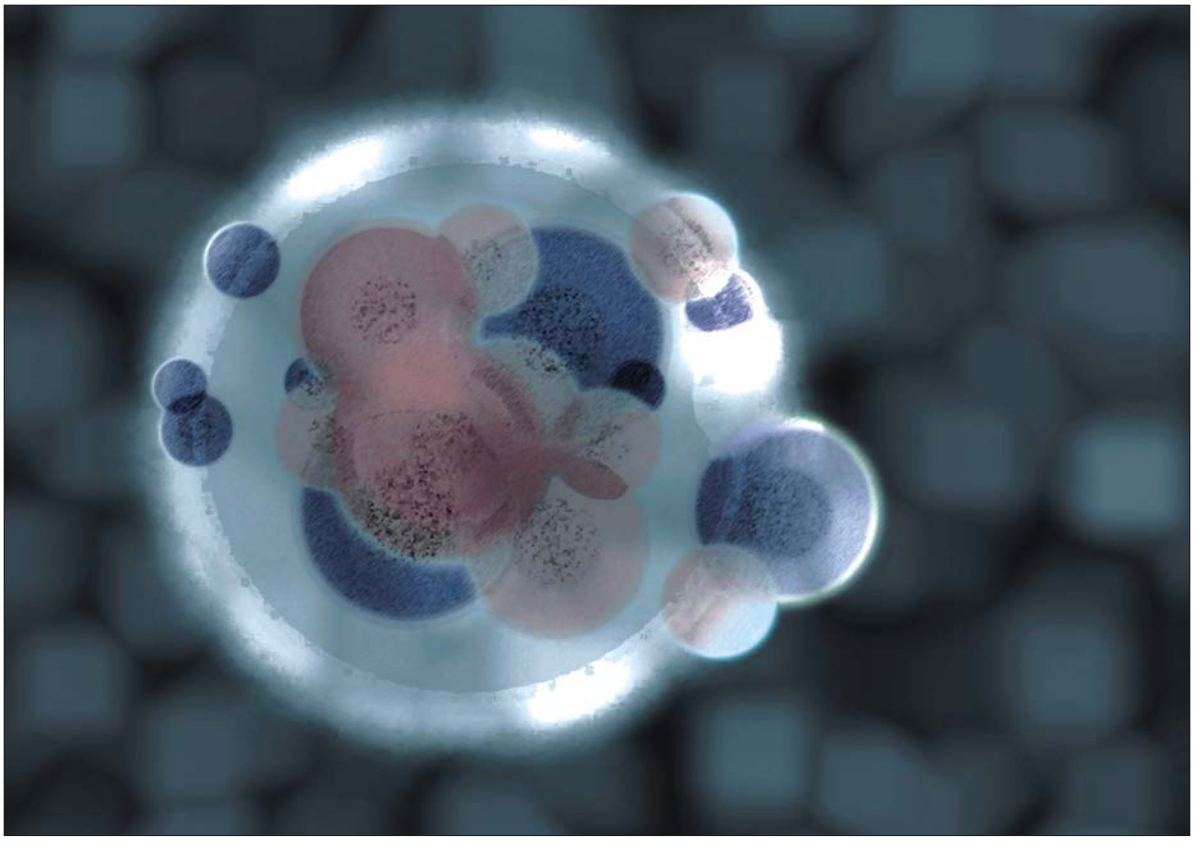

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 15 (1): 16-24, 2015
ISSN 1727-3048

RESEARCH ARTICLE OPEN ACCESS

DOI: 10.3923/jbs.2015.16.24

Effect of Different Organic and Inorganic Nitrogen Sources on the Kinetics of the Breakdown of Crude Oil Using Pseudomonas

[1]A. Oje Obinna, [2]N.E. Onwurah Ikechukwu, [3]S. Ubani Chibuike and [3]Nweke Alexandra
[1]Department of Chemistry, Biochemistry and Molecular Biology, Faculty of Science and Technology, Federal University Ndufu Alike Ikwo (FUNAI), Ikwo Ebonyi State, 400002, Nigeria
[2]Biotechnology and Pollution Control Unit, Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka Enugu State, Nigeria
[3]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka Enugu State, Nigeria

ARTICLE INFO

Article History:
Received: January 12, 2015
Accepted: August 19, 2015

Corresponding Author:
A. Oje Obinna
Department of Chemistry, Biochemistry and Molecular Biology, Faculty of Science and Technology, Federal University Ndufu Alike Ikwo (FUNAI), Ikwo Ebonyi State, 400002, Nigeria
Tel: 08036431336

ABSTRACT

The option of using microbes in the remediation of polluted sited have received wide acceptance due to its sustainability and environmental friendliness. This study is aimed at using the kinetic parameters (V_{max} and K_m) to assess the effect of organic and inorganic nitrogen sources on the performance of *Pseudomonas* sp., during the remediation of crude oil polluted sites. The rate of crude oil breakdown was assessed by determining the total petroleum hydrocarbon in the soil (after contaminating the soil with different percentages of crude oil) daily for 14 days. The experiment was repeated for different nitrogen sources (*Azotobacter vinelandii*, $(NH_4)_2SO_4$, $NaNO_2$ and $NaNO_3$). The results of the experiments showed that the rate of breakdown of crude oil increases with increase in crude oil contamination in the different nitrogen sources except for the nitrate and the nitrite groups, where the rate reduced at 10% crude oil contamination. It was also observed that the introduction of various nitrogen sources decreased the V_{max} and the K_m. *Pseudomonas* sp., during the breakdown of crude oil showed a decrease in V_{max} from 0.236±0.018-0.090±0.009, 0.137±0.005, 0.106±0.011 and 0.116±0.010 for the introduction of *Azotobacter vinelandii*, $(NH_4)_2SO_4$, $NaNO_2$ and $NaNO_3$, respectively, 0.521±0.194. This shows that the introduction of the nitrogen source increase the affinity of the organism for crude oil.

Key words: Bioremediation, crude oil, ammonium, nitrite, nitrate, *Pseudomonas* sp., *Azotobacter vinelandii*

INTRODUCTION

Pollution is the contamination of the environment with substances and in doses that are harmful to the body. Environmental pollution caused by crude oil spills is a major problem in oil producing country as Nigeria (Agbogidi and Eshegbeyi, 2006). Petroleum is the world's leading energy fuel but the enormous scale of the petroleum industry's operation has inevitably created a new set of difficult environmental problems as being experienced today in the Niger Delta region of Nigeria (Ekanem *et al*., 2010; Ugbomeh and Atubi, 2010; Eneh, 2011; Ojimba and Iyagba, 2012). Oil spilliage which is caused by exploration, exploitation, storage and transportation even vandalization and bunkering has lead to the pollution of the environment (Nicolotti and Egli, 1998). The polluted environment is therefore, not useful for agricultural purposed both farming and fishing as, the case may be. This also affect the microbial flora thereby reducing microbial population (Baker, 1970; Mackay, 1991; Siddiqui and Adams, 2002; Lundstedt, 2003).

Microorganism plays some catalytic roles in the breaking down crude oil (Das and Chandran, 2011). This is because the

produce a lot of enzymes that play active part in the breaking down processes. Just like the enzymes, the kinetic of the enzymes in an enzyme catalyzed reaction helps on to determine the rate of product formation or substrated depliction, the kinetics of the whole organism can be used to obtain such information. It lows concentration of substrate, the rate of degradation by microorganism increases tending to a first order rate chemical reaction but as the substrated increases that it overwhelm the organisms, the rate of breakdown tend to a zero order rate of reaction. In other words, the organisms can be seen to obey the Michealis-Mentens assumptions and equation:

$$V_0 = \frac{V_{max} \times [5]}{K_m + [5]}$$

Where:

Vo = Initial velocity (initial rate of reaction)
V_{max} = Maximum velocity
K_m = Michealis-Mentens constant
[S] = Substrate concentration

Pseudomonas sp. are hydrocarbonoclastic bacteria that are naturally endowned with the ability to breakdown compounds with carbon backbone chain (Mansur *et al*., 2014).

Because of this ability, *Pseudonmonas* sp. have been implicated in many disease conditions (Musefiu *et al*., 2014), *Pseudomonas* sp. also possess the ability to solubilize phosphorus from the soil (Yasser *et al*., 2014; Sarker *et al*., 2014) and therefore, utilize it for cellular activity and also make it available for plants. It has been reported also that some species of *Pseudomonas* sp. posses that ability to denitrify nitrate in the soil (Jangiam *et al*., 2013) and release them as free nitrogen into the atmosphere. Many bioremediation studies have proven that *Pseudomonas* sp. can breakdown straight chain hydrocarbons (Fathepure, 2014; Sharma *et al*., 2014), which is a major component of crude oil but finds it fairly difficult to breakdown Poly Aromatic Hydrocarbons (PAHs) which are recalcitrants. For *Pseudomonas* sp. to carry out their functions, they need to be a supply of carbon sources (in most cases the pollutants serves this purpose), phosphorus source (which the organism can get by phosphate solubilization of soil) and Nitrogen. Although some *Pseudonmonas* sp. are known to contain *nif* gene for nitrogen fixation (Yan *et al*., 2008) , most a times, these genes are not expressed in the organisms. Therefore, for *Pseudomonas* to work efficiently in the environment, nitrogen must be supply to the organism. This study is therefore, aimed at determine the best form in which nitrogen should be supplied (both organic and inorganic forms) for optimum activity organism using the V_{max}, K_m and the V_{max}: K_m ratio.

MATERIALS AND METHODS

Crude oil: The Crude oil used was gotten from the Directorate of Petroleum Resources Port-Harcourt, Rivers State Nigeria.

Soil: The soil samples that was used in this study were obtained from the Agric Farm, Department of Agriculture, University of Nigeria, Near Green House.

Microorganism: Two microorganisms were used in the course of the research. The *Pseudomonas* species was gotten from the culture collection Center Department of Microbiology, University of Nigeria, Nsukka while the *Azotobacter vinelandii* was isolated from the soil around the postgraduate laboratory, Department of Biochemistry, University of Nigeria, Nsukka using *Azotobacter vinelandii* specific media.

Preparation of media

Preparation of Broth for the isolation *Azotobacter vinelandii* using specific media: The media for the isolation of *Azotobacter vinelandii* media was prepared as follows; Sodium benzoate (1.0 g), Di-Potassium hydrogen phosphate (K_2HPO_4) (0.5 g) and Mannitol (0.5 g) were put in a 1 L conical flask and small quantity of distilled water was added and the volume was brought to 999 mL the mixture was thoroughly mixed and autoclaved for 15 min at 15 psi pressure-121°C. Cool to 45-50°C after that, ethanol (1 mL) was aseptically added and then aseptically distributed into sterile tubes or flasks.

Preparation of *Pseudomonas* maintenance media: The *Pseudomonas* maintenance media was prepared as follows:

- Di-Potassium hydrogen phosphate (K_2HPO_4) (12.5 g), Potassium dihydrogen phosphate (KH_2PO_4) (3.8 g), Ammonium sulphate ($(NH_4)_2SO_4$) (1.0 g), Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) (0.1 g), Carbon source (0.8 M solution) (100.0 mL) (which was prepared by adding 14.4 g of glucose in 100 mL of distilled water) and Trace elements solution (5.0 mL) were put in a 1 L conical flask, small quantity of water was added to dissolve the salts and then made up to make using distilled water. The pH of the solution was adjusted to 7.2 using (KH_2PO_4) and (K_2HPO_4) as, acid and base, respectively. All the components, except carbon source, were added to the conical flask and brought to the volume to 900.0 mL. It was mixed thoroughly and gently heated to boiling. The mixture was autoclave for 15 min at 15 psi pressure-121°C, after cooling to about 45-50°C. The carbon source was aseptically added and mixed thoroughly. It was then aseptically distributed into 250 mL conical flask

Preparation of trace element solution: The trace element was prepared by adding Boric acid (H_3BO_3) 0.232 g, Zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) 0.174 g, Ferrous Ammonium sulphate ($FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$) 0.116 g, Cobalt (II) Sulphate heptahydrate ($CoSO_4 \cdot 7H_2O$) 0.096 g, Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) 0.022 g Copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) 8.0 mg and $MnSO_4 \cdot 4H_2O$ 8.0 mg were added to a 1 L conical flask and made up to make with distilled water.

Determination of the remaining Total Petroleum Hydrocarbon (TPH): One gram of soil was put in a test-tube and ten milliliter (10 mL) of Chloroform/Ethanol mixture (1:1) was added. The mixture was agitated for 5 min and then allowed to stand for 10 min. The sample was then filtered and the absorbance of the filtrate was taken at 520 nm using chloroform/Ethanol mixture (1:1) as a blank. The quantity of crude oil was estimated using a crude oil standard curve.

Determination of degraded TPH will be done by calculation: The amount of crude oil degraded was estimated by simple calculation. This was done by subtracting the initial concentration of crude oil from the final concentration crude oil.

Amount of TPH degraded = Initial concentration-
Final concentration

Experimental design: The experiment was setup in 6 groups each group contain various concentrations of crude oil (0.1, 0.5, 1.0, 5.0 and 10%). Each group contain either *Pseudomonas* sp or *Azotobacter* sp. with amendment. The groups are as follows:

- **Group 1:** *Pseudomonas* sp alone
- **Group 2:** *Azotobacter vinelandii* alone (as an organic source of nitrogen since the organism has the ability to fix atmospheric nitrogen into the soil)
- **Group 3:** *Pseudomonas* sp and *Azotobacter vinelandii*
- **Group 4:** *Pseudomonas* sp and Ammonium sulphate ($(NH_4)_2SO_4$)
- **Group 5:** *Pseudomonas* sp and Sodium nitrite ($NaNO_2$)
- **Group 6:** *Pseudomonas* sp and Sodium nitrate ($NaNO_3$)

Two hundred grams of soil was put in a conical flask and groups 4-5 was impregnated with 10 mL of 0.1 M of their salt solution, the soil was mixed very well to ensure proper spreading of the salts. It was then autoclaved to kill the organisms present in the soil and allowed to cool to room temperature. After that 1 mL of the microbial broth containing *Pseudomonas* sp. Except for the group 3 that contain 0.5 mL of each organism. After stirring, the experiment was monitored for two weeks.

Statistical analysis: The results of the experiments were analyzes statistically and are presented as Mean±SEM, the linear regression model was used to analyze the rate of breakdown, while the non-linear regression model was used to determine the V_{max} and the K_m the level of significant was determined at $p<0.05$. The graphpad prism v 6 was used for the analysis.

RESULTS

The result in Fig. 1-6 show the graphs for the breakdown of crude oil using different nitrogen sources. All the graphs show decreases in crude oil (TPH) as time increases in other words, it was observed that TPH decreased with time. The curves in Fig. 2 were observed to tilt more to the horizontal signifying very little decrease as the time increases. The linear regression was used to determine the slope of the graphs, which represent the rate of degradation of crude oil

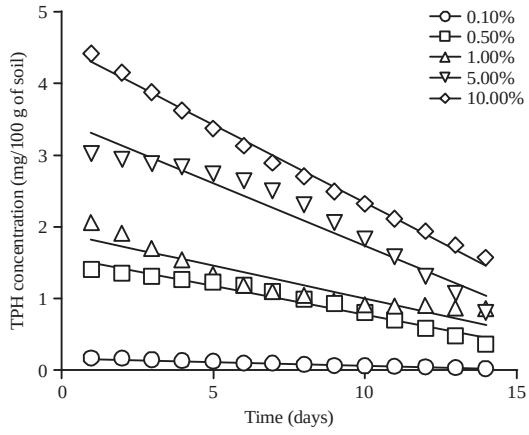

Fig. 1: Degradation of various concentration of TPH using *Pseudomonas* sp. alone

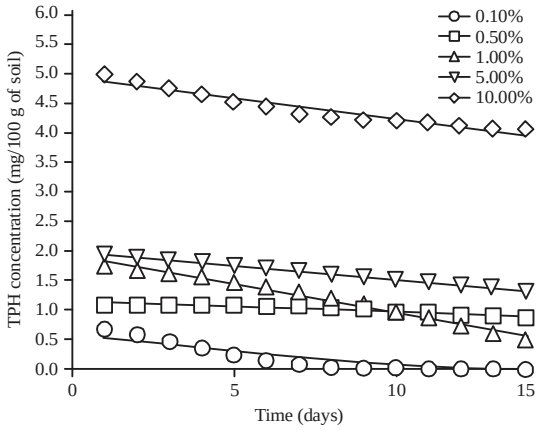

Fig. 2: Degradation of various concentration of TPH using *Azotobacter vinelandii* alone

will the R-square value was used to identify how accurate (perfect) the curve is the data was presented in Table 1.

The result in table 1 shows the rate of degradation crude oil as obtained from Fig. 1-6. The equation of the line of best fit was determined and the slope which is the rate of degradation. The R-square values were also seen to be more than 0.7 or one could say that the curves were more than 70% perfect. The negative values of the slope simple shows the rate of reduction. It was also observed that the value of the slope increases with increase in the percentage of crude oil with except in the group containing *Azotobacter vinelandii* which fluctuate as the percentage of crude oil increases. Also the group containing Nitrite and nitrate also showed a decrease in the slope after 5.0% crude oil contamination.

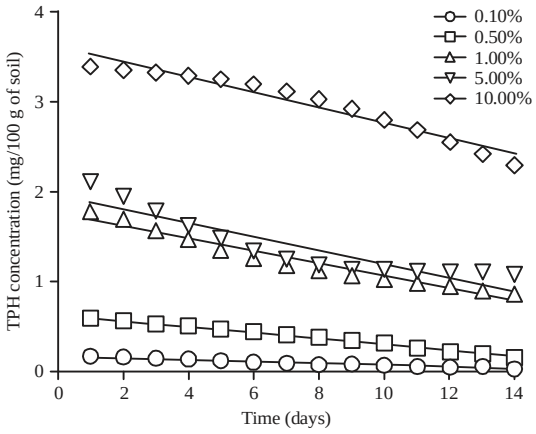

Fig. 3: Degradation of various concentration of TPH using a consortium of *Pseudomonas* sp and *Azotobacter vinelandii*

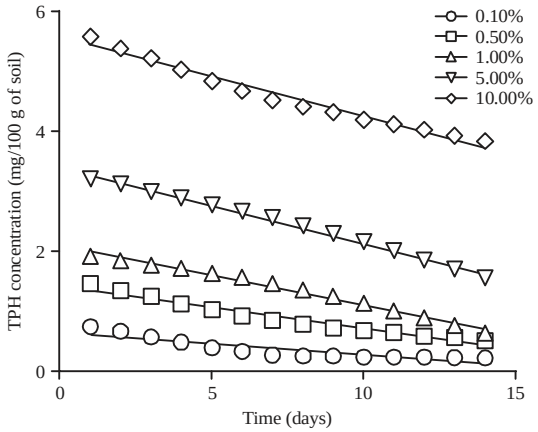

Fig. 4: Degradation of various concentration of TPH using *Pseudomonas* sp and Ammonium salt

Table 1: Rate of degradation of crude oil, which was obtained from Fig. 1-6

Organism and amendment	Concentration of crude oil in soil (%)	Equation	Slope	R-square
Pseudomonas sp. alone	0.10	Y = -0.0108×X+0.1733	-0.0108±0.0005	0.9715
	0.50	Y = -0.0806×X+1.5810	-0.0806±0.0045	0.9646
	1.00	Y = -0.0906×X+1.9150	-0.0906±0.0102	0.8678
	5.00	Y = -0.1745×X+3.4860	-0.1745±0.0121	0.9452
	10.00	Y = -0.2170×X+4.5140	-0.2170±0.0051	0.9937
Azotobacter vinelandii alone	0.10	Y = -0.0515×X+0.5878	-0.0515±0.0071	0.8152
	0.50	Y = -0.0166×X+1.1480	-0.0166±0.0022	0.8220
	1.00	Y = -0.0966×X+1.9320	-0.0966±0.0035	0.9840
	5.00	Y = -0.0472×X+1.9870	-0.0472±0.0002	0.9997
	10.00	Y = -0.0696×X+4.9310	-0.0696±0.0055	0.9299
Pseudomonas sp.+*Azotobacter vinelandii*	0.10	Y = -0.0094×X+0.1628	-0.0094±0.0005	0.9652
	0.50	Y = -0.0328×X+0.6225	-0.0328±0.0007	0.9946
	1.00	Y = -0.0702×X+1.7550	-0.0702±0.0043	0.9567
	5.00	Y = -0.0765×X+1.9560	-0.0765±0.0094	0.8455
	10.00	Y = -0.0855×X+3.6210	-0.0855±0.0058	0.9480
Pseudomonas+Ammonium salt	0.10	Y = -0.0382×X+0.6477	-0.0382±0.0056	0.7940
	0.50	Y = -0.0720×X+1.4310	-0.0720±0.0046	0.9541
	1.00	Y = -0.1005×X+2.1070	-0.1005±0.0037	0.9841
	5.00	Y = -0.1274×X+3.4040	-0.1274±0.0026	0.9949
	10.00	Y = -0.1333×X+5.6070	-0.1333±0.0062	0.9745
Pseudomonas+Nitrite salt	0.10	Y = -0.0443×X+0.7007	-0.0443±0.0017	0.9817
	0.50	Y = -0.0661×X+1.4610	-0.0661±0.0062	0.9031
	1.00	Y = -0.1119×X+2.5560	-0.1119±0.0023	0.9950
	5.00	Y = -0.1059×X+3.1530	-0.1059±0.0120	0.8664
	10.00	Y = -0.0947×X+4.0950	-0.0947±0.0007	0.9994
Pseudomonas+Nitrate salt	0.10	Y = -0.0138×X+0.4749	-0.0138±0.0007	0.9727
	0.50	Y = -0.0522×X+1.6560	-0.0522±0.0007	0.9978
	1.00	Y = -0.0823×X+1.8890	-0.0823±0.0066	0.9280
	5.00	Y = -0.1173×X+3.0760	-0.1173±0.0097	0.9246
	10.00	Y = -0.0976×X+4.0250	-0.0976±0.0079	0.9271

Table 2: V_{max} and K_m of *Pseudomonas* with various nitrogen sources

Parameters	Ps	Az	Ps+Az	$Ps+NO_3$	$Ps+NO_2$	$Ps+NH_4$
V_{max}	0.236±0.018	Not converging	0.090±0.009	0.116±0.010	0.106±0.011	0.137±0.005
K_m	1.370±0.368	Not converging	0.584±0.239	0.521±0.194	0.151±0.089	0.366±0.067
R square	0.976	0.039	0.932	0.941	0.775	0.980
Vmax/Km	0.1725	Not determined	0.155	0.223	0.702	0.373

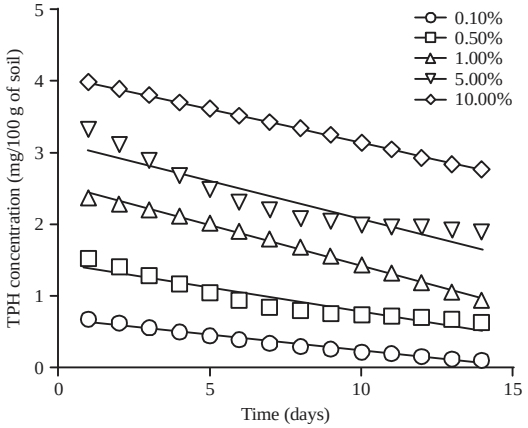

Fig. 5: Degradation of various concentration of TPH using *Pseudomonas* sp and Nitrite salt

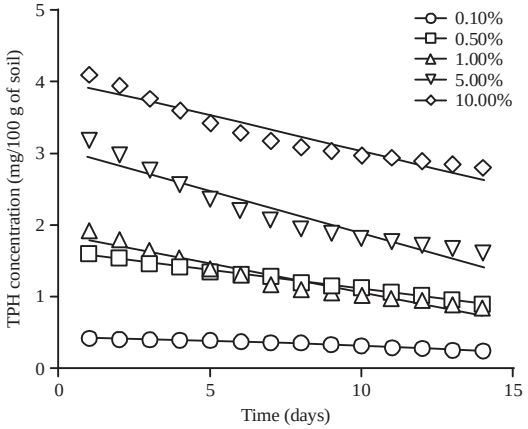

Fig. 6: Degradation of various concentration of TPH using *Pseudomonas* sp and Nitrate salt

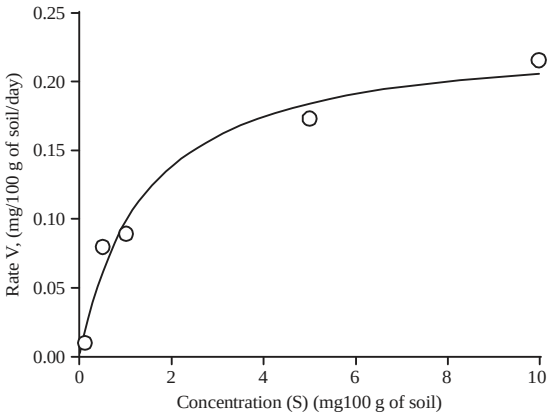

Fig. 7: Graph of the rate of breakdown of TPH using *Pseudomonas* sp. alone against substrate concentration

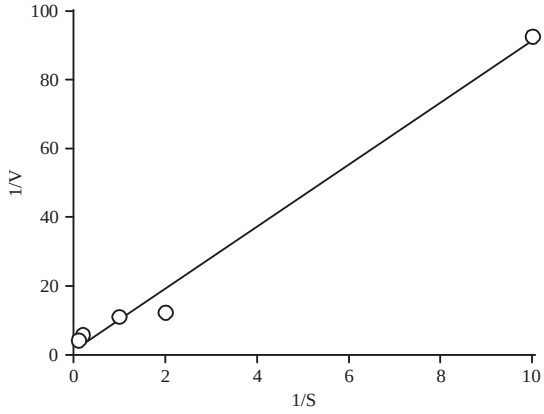

Fig. 8: Graph of the inverse of the rate of breakdown of TPH using *Pseudomonas* sp alone against the inverse of the substrate concentration

To determine the Vmax and Km, the graph of rate of degradation (slope) against substrate concentration were plotted and represented in Fig. 7, 9, 11, 13, 15 and 17. Figures were observed to be sigmoid in shape according to the Michealis -Mentens pattern, Fig. 9 falling out of place. due to the fact that the points in the graph could not converge. In order to linearize the curves, a plot of the inverse of the rate of degradation against the inverse of the substrate concentration was done and represented in Fig. 8, 10, 12, 14, 16 and 18. The values of V_{max} were obtained as the inverse of the value of the rate when substrate concentration is zero, while the K_m were obtained as the negative of the inverse of the value of substrate when the rate is zero. The values of V_{max} and K_m were represented in Table 2.

The Table 2 shows the result of the V_{max} and K^m of *Pseudonmonas* sp. and the various amendment. The result reveals that the introduction of nitrogen sources reduced both the V_{max} and K_m. *Azotobacter vinelandii*'s V_{max} and K_m were not determined because the point were not converging and the line of best fit gave an R-square value of 0.039, which means that the curve is about 3.9% perfect. The consortium of *Pseudomonas* sp. and *Azotobacter vinelandii* showed lowest V_{max} value (0.090±0.009) and highest K_m value (0.584±0.239) in the groups containing nitrogen sources. The V_{max}: K_m ratio showed that $Ps+NO_2$>$Ps+NH_4$>$Ps+NO_3$>Ps>Ps+Az.

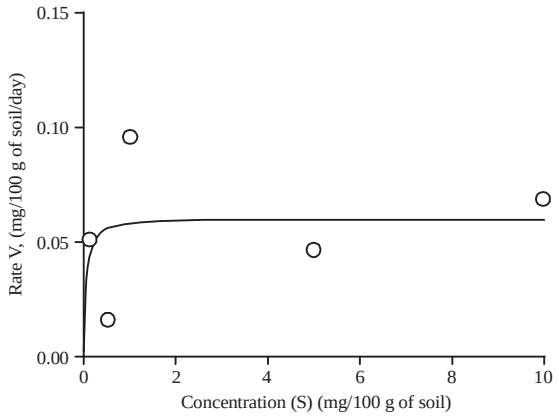

Fig. 9: Graph of the rate of breakdown of TPH using *Azotobacter vinelandii* alone against substrate concentration

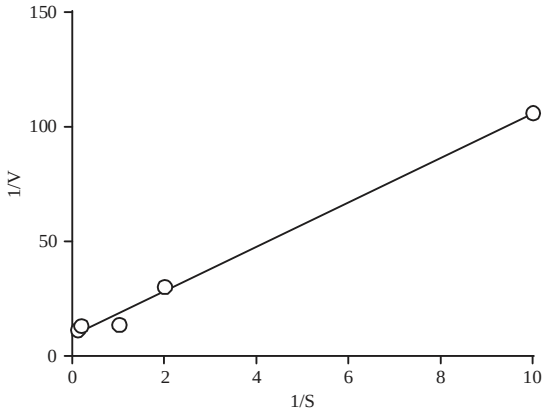

Fig. 12: Graph of the inverse of the rate of breakdown of TPH using *Azotobacter vinelandii* and *Pseudomonas* sp. against the inverse of the substrate concentration

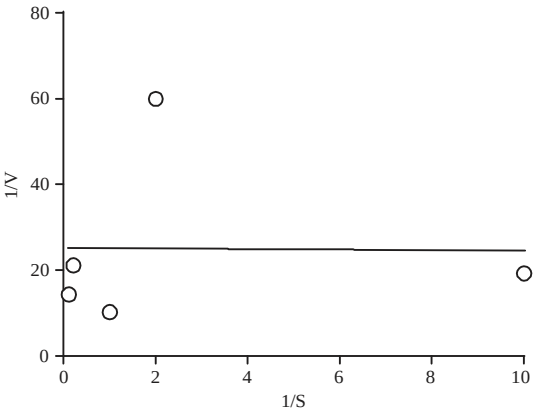

Fig. 10: Graph of the inverse of the rate of breakdown of TPH using *Azotobacter vinelandii* alone against the inverse of the substrate concentration

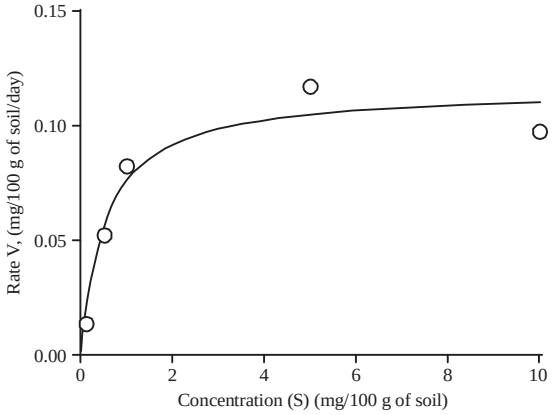

Fig. 13: Graph of the rate of breakdown of TPH using *Pseudomonas* sp. and nitrate salt against substrate concentration

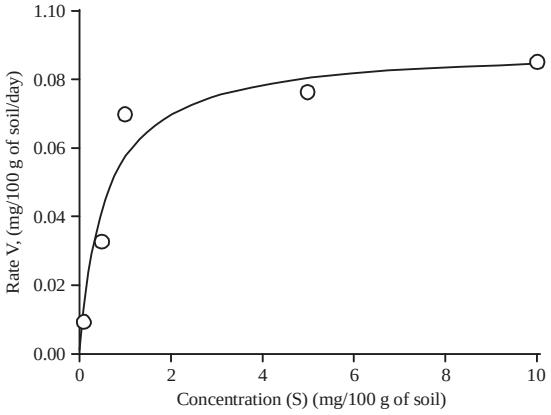

Fig. 11: Graph of the rate of breakdown of TPH using a consortium of *Azotobacter vinelandii* and *Pseudomonas* sp against substrate concentration

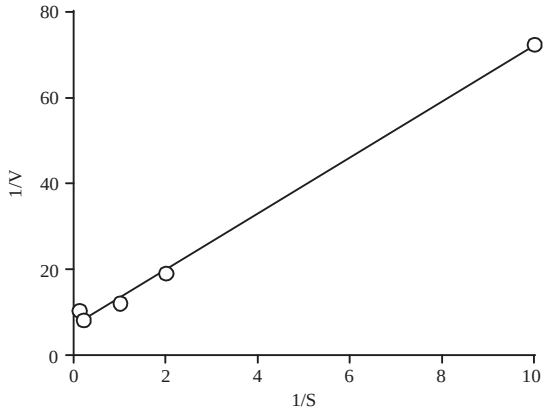

Fig. 14: Graph of the inverse of the rate of breakdown of TPH using *Pseudomonas* sp. and nitrate salt against the inverse of the substrate concentration

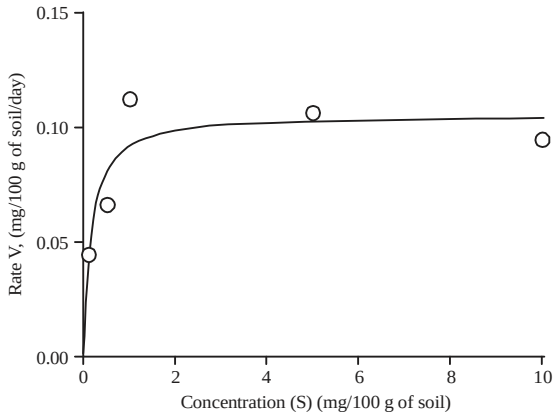

Fig. 15: Graph of the rate of breakdown of TPH using *Pseudomonas* sp. and nitrite salt against substrate concentration

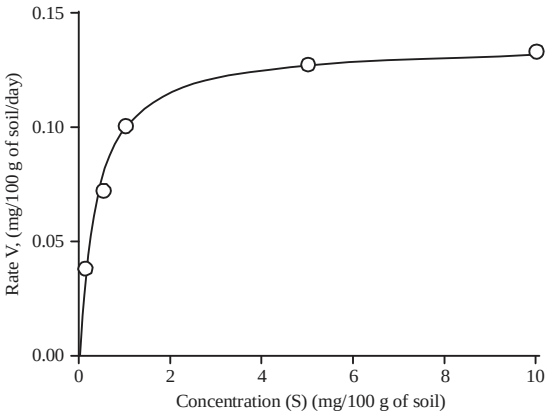

Fig. 17: Graph of the rate of breakdown of TPH using *Pseudomonas* sp. and ammonium salt against substrate concentration

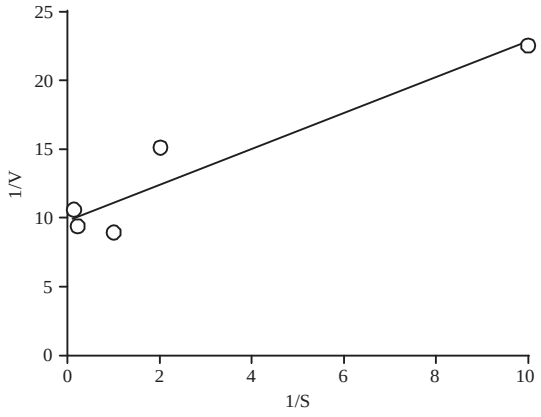

Fig. 16: Graph of the inverse of the rate of breakdown of TPH using *Pseudomonas* sp and nitrite salt against the inverse of the substrate concentration

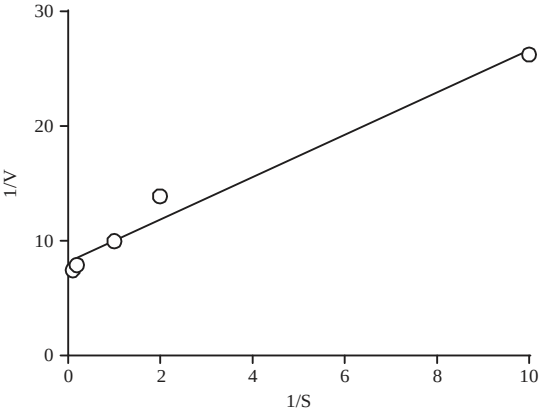

Fig. 18: Graph of the inverse of the rate of breakdown of TPH using *Pseudomonas* sp. and ammonium salt against the inverse of the substrate concentration

DISCUSSION

Pseudomonas sp. has been employed in the bioremediation of crude oil polluted environments (Onwurah *et al*., 2007). One of the challenges facing microorganisms during bioremediation is the availability of macro-nutrients, such as; nitrogen and phosphorus (Bahadur *et al*., 2014) *Pseudomonas* sp has the ability to solubilize phosphorus in the soil (Delgado *et al*., 2014) but its major challenge is the availability of nitrogen. *Pseudomonas* sp. has also the ability to depend on crude oil for energy and biomass production (Goteti *et al*., 2014). When, nitrogen is not available in the soil, the organism begins to decrease in population, because of the inability to synthesize nucleotides and amino acids of which nitrogen is a major component. Therefore to sustain the survival of the *Pseudomonas* in polluted sites, nitrogen sources need to be introduced, since the pollutants (crude oil) acts as a carbon source (Palanisamy *et al*., 2014). This present study sets out to find the effect of different nitrogen sources on the biodegradation ability of *Pseudomonas* sp. Different nitrogen sources both organic and inorganic was used for this purpose. The graphs of the concentration of crude oil against time which was used to determine the rate of degradation showed that *Azotobacter vinelandii* is not a good degrader of crude oil when used alone. In Fig. 2, the curves in the graph were tending towards the horizontal, when compared with the others. It was generally observed that the rate of breakdown of crude oil increases with increase in the concentration of crude oil, with exception of *Azotobacter vinelandii* alone, which did not show any pattern. Also the presence of nitrate and nitrite showed a decrease in the rate of degradation from 5.0% crude oil contamination.

On determining the V_{max} and K_m of the individual groups, the group containing *Azotobacter* could not converge so it was

practically impossible to determine the V_{max} and the K_m for the organism alone. In addition, the line of best fit in figure 10 had an R^2 value of 0.039, which could be interpreted as 3.9% accurate. This also support the claim that *Azotobacter* even though it breaks down the crude oil to some extent, is not an effective degrader.

The results showed that the introduction of nitrogen sources cause a reduction in V_{max} and K_m of the organisms in all the groups. This showed that to some extent, the introduction of nitrogen causes some level of inhibition although, it does not terminate the organisms. The decrease in K_m, when nitrogen source was introduced showed that nitrogen on the other hand increases the affinity of the organism for the substrate (crude oil).

From the result, *Pseudomonas* with nitrite showed the lowerest V_{max} value (0.106±0.011) and K_m value (0.151±0.089). Since, the decrease affects both V_{max} and K_m by different degrees, it becomes necessary to use another parameter that will cushion the effect and give a better comparable parameter. In this regard, the V_{max}: K_m ratio was determined. This ratio shows the level of dilution per quantity of substrate that will produce maximum or optimum activity. In other words, the lower values of this ratio showed that optimum activity could be obtained even at higher concentration of the substrate (crude oil) and this means that the organism with low V_{max}: K_m ratio can also do better at high concentration of crude oil. In this experiment, the order of the ratio is Ps+Az>Ps>Ps+NO_3>Ps+NH_4>Ps+NO_2, making the consortium of Pseudomonas sp and *Azotobacter vinelandii* a better option rather than the use of in organism sources of nitrogen.

CONCLUSION

The argumentation of the breakdown of crude oil by *Pseudomonas* with an organic nitrogen source, such as; *Azotobacter vinelandii* was shown to produce better results. In addition, the sustainability of the consortium and its environmentally friendliness have made the consortium a better choice over the inorganic nitrogen sources that have the ability to increase cost and introduce other pollutant into the environment.

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