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Biodegradation of Naphthalene using *Pseudomonas putida* and *Bacillus subtilis* Immobilized on Snail Shell

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ABSTRACT: This study investigated snail shell as a carrier to immobilize *Pseudomonas putida* and *Bacillus subtilis* isolated from refinery effluent for the degradation of naphthalene in synthetic wastewater at various process conditions such as initial naphthalene concentration, pH, adsorbent dosage and ambient temperature of 30 ºC in batch mode. The results showed that the adsorption and the biodegradation capacity increased with increase in naphthalene concentration, where 73.11%, 74.46% and 65.20% of the optimum concentration (50 mg/L) were removed by immobilized *Pseudomonas putida*, *Bacillus subtilis,* and snail shell respectively after 72 hours incubation. The optimal degradation occurred at the adsorbent dosage of 2 g at pH 9 and pH 7 for the adsorption and biodegradation respectively. The results were well fitted to both Langmuir and Freundlich models. Therefore, snail shell can be employed as a low-cost adsorbent and solid support matrix for immobilizing microorganisms in remediating hydrocarbon contaminants.

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Petroleum hydrocarbons and their derivatives are the main the energy source for various industrial, automotive and domestic uses. But the deliberate use of these products has been a cause for concern, due to the oil spills as a result of exploration and exploitation (Onyegeme-Okerental *et al.,* 2017). This problem of oil spillage has been a global problem that has occurred since the discovery of crude oil (Kadafa, 2012). Most of the components of petroleum hydrocarbons are toxic, carcinogenic and mutagenic in nature (Das and Chandra, 2011). According to reports by international environmental groups, the Niger Delta in Nigeria has been listed as one of the five most severely oil-damaged ecosystems in the world (Kadafa, 2012). Also, Vidal (2010) reported that due to the anthropogenic activities occurring within this region, their waters are polluted resulting in varying degrees of health problems. Therefore, the effective removal of crude oil and their products is a problem of paramount concern to the world at large.

Several methods have been employed for the remediation of petroleum hydrocarbon, they include physical, chemical and biological techniques (Yadav and Hassanizadeh, 2011). However, these physicochemical methods are acquainted with problems such as incomplete decomposition or breakdown of contaminants, and moreover, the technologies are comparatively expensive when compared to biodegradation (Das and Chandra, 2011Benchiuk and Chibani, 2017). Although, freely suspended cells used in biodegradation have limited capacity, recently the new trend which has gained more attention is cell immobilization on solid support or adsorbent (Sreenivasulu, 2012), and many of these technologies have been used successfully for crude oil bioremediation. Adsorbents such as activated carbon, kaolin, orange peel, groundnut shell, sugarcane bagasse, natural polymeric matrices (agar, alginate, carrageenan, chitosan) and also synthetic matrices such as polyvinyl alcohol (PVA) and polyurethane have been used extensively for hydrocarbons remediation (Agarry and Aremu, 2012; Partovinia and Naeimpoor, 2013). However, snail shell with the incorporation of microorganisms has rarely been used to remediate oil pollutants. Therefore, this study investigated the potential of snail shell for immobilizing *Pseudomonas putida* and *Bacillus subtilis* to remove naphthalene.

MATERIALS AND METHODS

Collection of samples and Reagents: All reagents used were of analytical grade purchased from Sigma Aldrich Chemicals, USA. The refinery effluent used for the isolation was collected from Kaduna Refinery and Petrochemical Company (KRPC) effluent ponds.

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The sample was then transported at ambient temperature to the Department of Microbiology, Ahmadu Bello University, Zaria for the isolation. The snail shell was obtained from a dumpsite located within National Research Institute of Chemical Technology (NARICT) staff quarters, Basawa, Zaria and identified as African giant snail shell (*Achatina achatina*) at the Department of Biological Sciences, Ahmadu Bello University, Zaria.

Sample Preparation and Characterization: The snail shells were washed thoroughly with detergent and rinsed with tap water and distilled water, to ensure complete removal of all adhering impurities. The washed snail shells were then sun-dried for 48 hours, pulverized using a laboratory mill (Thomas Wiley model 4, USA) and then oven dried at 100 ^{o}C for 12 hours. The dried powdered sample were screened with a sieve of particle size 0.125mm and finally subjected for characterization using an X-ray Fluorescence spectroscopy (X-Supreme 8000, UK).

Preparation of Reagents: A 0.1 g/L naphthalene stock solution was prepared by dissolving the compound in acetone-water 30:70% v/v, standard concentrations ranging from 10 - 50 mg/L were then prepared by serial dilutions of the stock solution in distilled water.

Microorganisms' strains and inoculum preparation for biodegradation: The relevant microorganism strains were isolated using the method described by Naga et al., (2016) with a slight modification. The isolates were identified with a combination of microscopic and biochemical techniques using microgen kits, which identified the most likely species as *Pseudomonas putida* and *Bacillus subtilis* with 90.50 % and 99.68 % similarity. The strains were stored on a nutrient agar slant medium at 4 ^{o}C for further studies. Primary and secondary cultures were prepared respectively as described by Agarry and Aremu (2012). The secondary culture was then used as the inoculum for the degradation studies as this ensures full acclimatization of the organisms to grow on the naphthalene as the sole source of carbon and energy.

Batch Experiment: The degradation study was carried out by adding 100 cm^3 each of the prepared inoculums of the *Pseudomonas putida* and *Bacillus subtilis* separately into different glass bottles containing a known amount of snail shell (2 - 5 g). Exactly 150 cm^3 of synthetic wastewater (K_2HPO_4, 1.0 g; KH_2PO_4, 0.5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.5 g; $CaCl_2$, 0.02 g, $MnSO_4$, 0.02 g and Molybdenum powder, 0.02 in 1000 cm^3 of deionized water) with known amount of naphthalene concentration (10 – 50 mg/L) was added to the respective glass bottles, the wastewater was maintained at a known pH (5 - 9). The glass bottles were placed in a rotary shaker for 72 hours at a speed of 180 rpm and temperature of 30 ^{o}C so as to reach equilibrium. A similar process was repeated for the control (Agarry and Aremu, 2012). The naphthalene concentrations were determined using a UV-VIS spectrophotometer (Agilent Cary 300, USA).

The percentage naphthalene removal was also calculated using equation 1;

$$\text{Removal efficiency } (\%) = \frac{C_o - C_e}{C_o} * 100 \quad (1)$$

The amount of the naphthalene adsorbed, q_e, was also calculated from equation 2;

$$q_e = \frac{V(C_o - C_e)}{m} \quad (2)$$

Where, qe is the amount of adsorbate naphthalene adsorbed in milligram per gram of the adsorbent; C_o is the initial concentration of the naphthalene before adsorption process; C_e is the equilibrium concentration of the naphthalene in the aqueous medium after adsorption process; m is the mass in gram of the adsorbent; V is the volume of the solution in Litre.

RESULTS AND DISCUSSION

The X-ray fluorescence (XRF) analysis of the snail shell for the major and minor components are given in Table 1. The snail shell was substantial with CaO, this showed calcium as the major element making up to 96.13%. This major element present in the shell aids microorganism in producing enzymes needed for the breakdown of contaminants, although, excess nutrient contents can also inhibit biodegradation of hydrocarbons (Chaineau *et al.,* 2005); in this study, moderate elemental values were obtained except for calcium. Therefore, the composition of the snail shell makes it a potential adsorbent material for contaminant adsorption.

Table 1: Chemical Composition of the Snail Shell

Component	Weight composition (%)
MgO	0.05
Al_2O_3	0.65
SiO_2	1.97
P_2O_5	0.15
SO_3	0.18
Cl	0.07
K_2O	0.14
CaO	96.13
TiO_2	0.01
Fe_2O_3	0. 10
SrO	0.56

Figure 1 is an illustration of the effect of pH on the adsorption and biodegradation of naphthalene at constant naphthalene concentration of 50 mg/L and optimum dosage value (2 g). The optimum removal rate occurred at pH 9 for the adsorption of naphthalene. It was clear that the adsorption of naphthalene varies with pH, that is, as the pH increased from 5 – 9, there was a corresponding increase in the percentage removal. However, it was a contrasting observation for the biodegradation of naphthalene with *Pseudomonas putida* and *Bacillus subtilis* incorporated on the snail shell with the optimum removal achieved at pH 7. This shows that the bacteria were more active at a neutral pH. This observation is in agreement with the literatures, that microorganisms achieve better efficiency at pH 6 – 8, with the optimum obtainable at pH 7 (Agarry and Aremu, 2012; Darsa et al., 2014;).

Figure 2 shows the percentage of naphthalene adsorbed and biodegraded at varying adsorbent doses set at 2, 3 and 5 g respectively with a fixed naphthalene concentration of 50 mg/L and a pH of 7. It was found that the percentage naphthalene removal of both adsorption and biodegradation decreases with increasing amounts for adsorbent from 2 g to 5 g. An initial increase in adsorption capacity was observed with an increase in the adsorbent dose, since the number of particles increased and, therefore, more surface was available for hydrocarbon binding. But a further increase in the adsorbent dosage beyond the maximum adsorption capacity, however, resulted in a decrease in capacity as shown in Figure 2. This capacity reduction could be due to the overlap of adsorption as a result of overcrowding of adsorbent particles beyond the optimal dose (Oladunni *et al.,* 2012). However, for the biodegradation, a high substrate content may inhibit the activity of microorganisms (Lin *et al.,* 2015), as excess nutrient aid microbial growth but does not necessarily improve the rate of biodegradation (Chaineau *et al.,* 2005). Thus, the results show that the adsorbent dose of 2 g has the highest removal efficiency for this study.

Figure 3 shows the effect of concentration on the adsorption and biodegradation of naphthalene. This was investigated over a concentration of 10 – 50 mg/L at constant pH and adsorbent dosage of 7 and 2 g respectively. The results obtained in both the adsorption and biodegradation study indicated that as the initial concentration increased, there was a corresponding increase in the percentage naphthalene removal. This could be attributed to the availability of active sites for naphthalene removal. However, as the concentration increases, the percent removal was higher in the biodegradation process across all the concentration range than the adsorption.

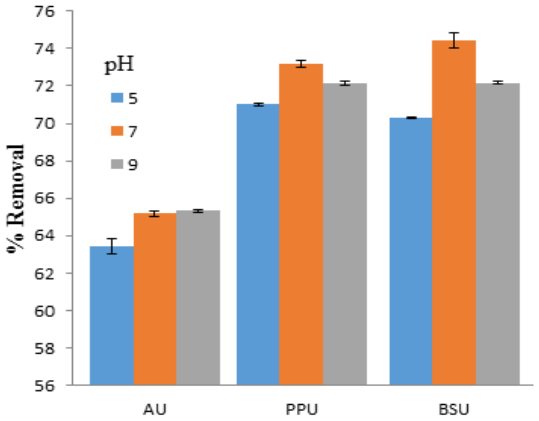

Fig 1: Effect of pH on the Adsorption/Biodegradation of Naphthalene: **Key:** AU = snail shell; PPU = *Pseudomonas putida* immobilized on snail shell; BSU = *Bacillus subtilis* immobilized on snail shell

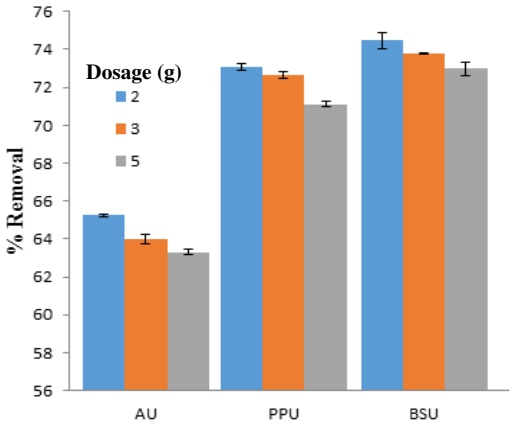

Figure 2: Effect of Dosage on the Adsorption/Biodegradation of Naphthalene

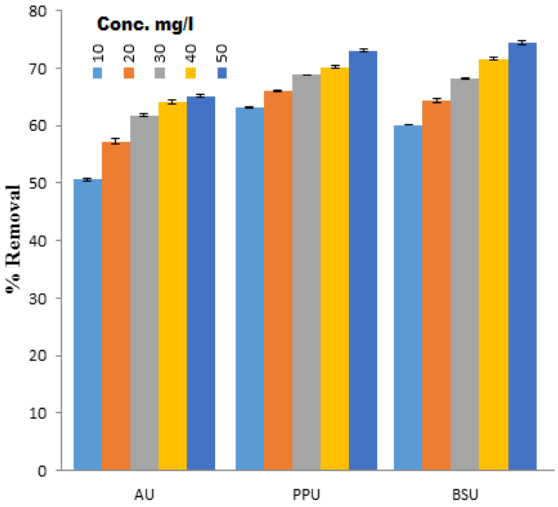

Fig 3: Effect of Initial Concentration on the Adsorption/Biodegradation of Naphthalene

This showed that biodegradation predominates after adsorption has reached equilibrium, thus revealing the synergistic effect of simultaneous adsorption-biodegradation on the removal of naphthalene from wastewater (Agarry and Aremu, 2012). Furthermore, the advantage could also be attributed to the fact that the mechanism works partly due to the fact that naphthalene can be adsorbed onto the snail shell and further degraded by the two immobilized bacteria (Lin *et al.,* 2015).

Adsorption Isotherms: Langmuir model assumes that monolayer adsorption takes place on a surface containing a finite number of adsorption sites. It is defined in equation 1:

$$q_e = \frac{q_{max} b C_e}{1 + bC_e} \qquad (3)$$

The linearized Langmuir model equation is expressed as below:

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \frac{1}{bq_{max}C_e} \qquad (4)$$

Where, q_e is the amount of adsorbate adsorbed per gram of dried adsorbent at equilibrium (mg adsorbate/g of dried adsorbent), q_{max} is the maximum monolayer coverage capacity (mg/g), b is Langmuir constant or adsorption coefficient or the adsorption affinity (L/mg) for binding of adsorbate on the adsorbent sites and C_e is equilibrium (residual) adsorbate concentration in solution after adsorption (mg/L). The values of q_{max} and b can be calculated from the intercept $\left(\frac{1}{q_{max}}\right)$ and slope $\left(\frac{1}{q_{max}b}\right)$ of the plot $\frac{1}{q_e}$ against $\frac{1}{C_e}$.

The essential characteristics of Langmuir model can be described by dimensionless separation factor, R_L, given as:

$$R_L = \frac{1}{1+bC_o} \qquad (5)$$

Where C_o is the highest initial solute concentration. R_L Values indicate whether the adsorption is unfavourable ($R_L > 1$), linear ($R_L = 1$), favourable ($0 < R_L < 1$), or irreversible ($R_L = 0$).

A linear adsorption isotherms were presented in Figure 4-6. The respective q_{max} and b of linear expressions of Langmuir adsorption isotherm were calculated from the slopes and intercepts of the linear plots of 1/qe versus 1/Ce. The values obtained for q_{max}, b, R^2 and R_L were listed on Table 2. It was observed that the linearized form of the Langmuir isotherm was linear over the concentration range studied and the result fitted well with the model with respect to R^2 values obtained. The high degree of correlation for the linearized Langmuir relationship observed suggest monolayer adsorption on specific sites or single surface reaction.

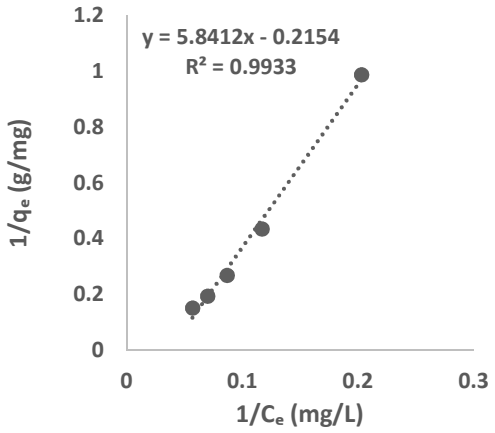

Fig 4: Langmuir Isotherm plot for naphthalene adsorption on snail shell

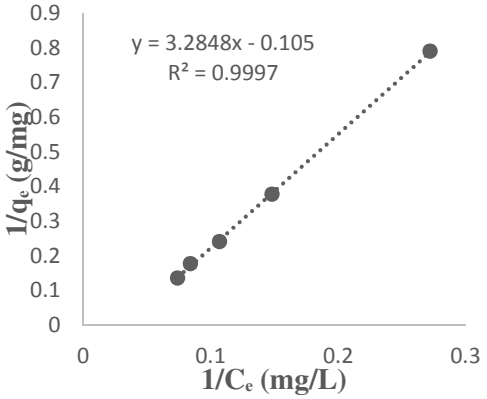

Fig 5: Langmuir Isotherm plot for naphthalene biodegradation with Pseudomonas *putida* immobilized on snail shell

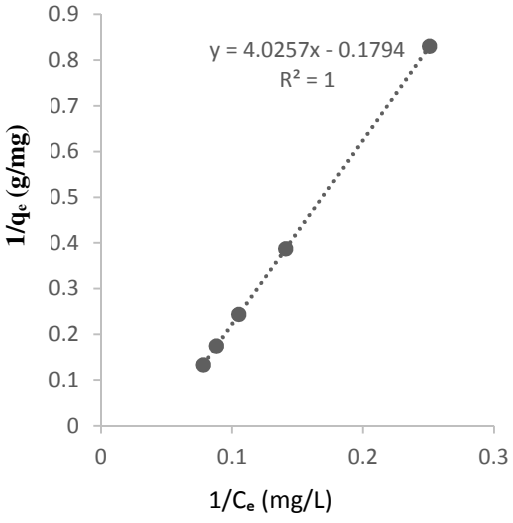

Fig 6: Langmuir Isotherm plot for naphthalene biodegradation with Bacillus subtilis immobilized on snail shell

The Freundlich model, on the other hand, assumes adsorption on a heterogeneous surface. The empirical equation proposed by Freundlich is given by equation 4:

$$q_e = K_f C_e^{1/n} \quad (6)$$

The linearized Freundlich model equation is given as equation 7.0:

$$\log q_e = \log K_f + \frac{1}{n}\log C_e \quad (7)$$

Where K_f is Freundlich Constant measuring adsorption capacity (L/mg), and n is constant related to adsorption efficiency and energy of adsorption or adsorption intensity of the adsorbent. Generally, n > 1 suggests favourable adsorption. It has also been used to evaluate whether the adsorption process is physical (n > 1), chemical (n < 1) or linear (n = 1) (Oladunni et al., 2012). The slope and the intercept correspond to $\frac{1}{n}$ and K_f respectively, which are obtained from the straight line plot of $\log q_e$ against $\log C_e$.

Figure 7-9 are the Freundlich isotherm plots of log qe against log Ce which all gave straight lines. The slopes and the intercepts correspond to $(\frac{1}{n})$ and K_f respectively. The results are presented in Table 2. The obtained n values are all below one, which is an indication of a cooperative adsorption in sites with different binding energies and indicative of favourable adsorption process (Abechi *et al*., 2013).
The correlation coefficient (R^2) values obtained from the adsorption and biodegradation studies showed that the isotherms also fitted well with the Freundlich model.

Conclusion: This study indicated the effectiveness of snail shell for the removal of naphthalene by adsorption and biodegradation from synthetic wastewater. The study was carried out under different conditions in batch and equilibrium modes. The snail shell exhibited competitive properties both for an enhanced adsorption process and bacteria biomass immobilization. The experimental data obtained from the study of both Langmuir and Freundlich Isotherm models could be used to describe the naphthalene sorption equilibrium as all the sorbent systems gave a better fit. The experimental conditions investigated showed that snail shell immobilized *Pseudomonas putida* and *Bacillus subtilis* (adsorption-biodegradation) were very efficient due to the combination of two process. Thus, *Pseudomonas putida* and *Bacillus subtilis* immobilized on snail shell is a promising alternative to bioremediation of polluted sites.

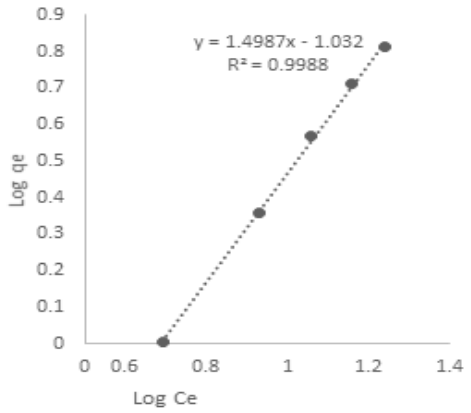

Fig 7: Freundlich Isotherm plot for naphthalene adsorption on snail shell

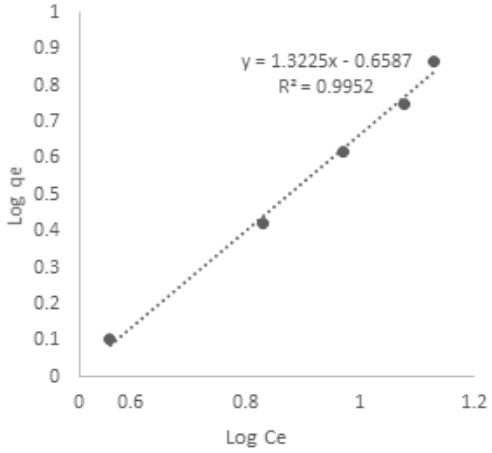

Fig 8: Freundlich Isotherm plot for naphthalene Biodegradation with Pseudomonas *putida* immobilized on snail shell

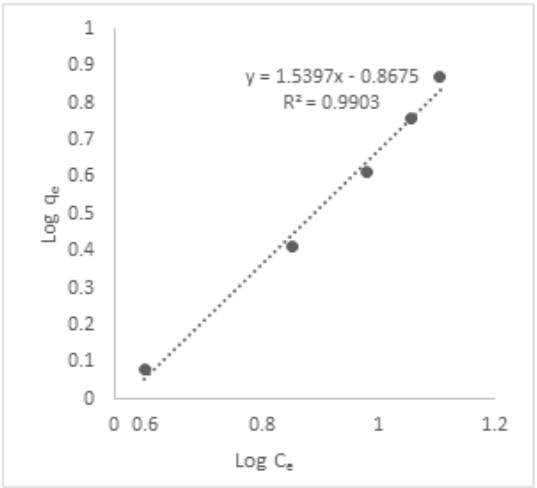

Fig 9: Freundlich Isotherm plot for naphthalene Biodegradation with Bacillus *subtilis* immobilized on snail shell

Table 2: Correlation parameters of Langmuir and Freundlich Isotherms

	Langmuir				Freundlich		
Treatment on Hydrocarbon	q_{max} (mg/g)	b (L/mg)	R_L	R^2	n	K_f (L/mg)	R^2
AU	-4.64	-0.0369	-1.1834	0.9933	0.67	0.09	0.9988
PPU	-9.52	-0.0320	-1.6667	0.9997	0.76	0.22	0.9952
BSU	-5.57	-0.0446	-0.8130	1.0000	0.65	0.14	0.9903

Key: R^2 is the correlation coefficient; q_{max} is the maximum hydrocarbon uptake (mg/g); b, is Langmuir constant (L/mg); K_f, is Freundlich Constant measuring adsorption capacity (L/mg) and n, is constant related to adsorption efficiency and energy of adsorption or adsorption intensity of the adsorbent.

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