REMOVAL OF NAPHTHALENE FROM SYNTHETIC WASTEWATER BY SIMULTANEOUS ADSORPTION-BIODEGRADATION (SAB) USING BACTERIA IMMOBILIZED SNAIL SHELL

By

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NIGERIA

SEPTEMBER, 2018

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DEPARTMENT OF CHEMISTRY,
FACULTY OF PHYSICAL SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

SEPTEMBER, 2018

DECLARATION

I declare that the work in this Dissertation entitled **"Removal of Naphthalene from Synthetic Wastewater by Simultaneous Adsorption-Biodegradation (SAB) using Bacteria Immobilized Snail Shell"** has been carried out by me in the Department of Chemistry under the supervision of Prof. E. B. Agbaji and Dr. N. C. Nwokem. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or in any other Institution.

AINA Oluwatoyin Emmanuel	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled **"Removal of Naphthalene from Synthetic Wastewater by Simultaneous Adsorption-Biodegradation (SAB) using Bacteria Immobilized Snail Shell"** by AINA Oluwatoyin Emmanuel meets the regulations governing the award of the degree of Master of Science in Analytical Chemistry of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This work is dedicated to Almighty God.

ACKNOWLEDGEMENT

My profound gratitude goes to the Almighty God for His gift of life, grace and strength He bestowed on me during the course of this research. May His name alone be praised.

I wish to express my sincere appreciation to my supervisors: Prof. E. B. Agbaji and Dr. N. C. Nwokem for their assistance, moral guidance, constructive criticism and encouragement expressed towards the success of this work. I am also indebted to the Head and Staff of the Department of Chemistry for creating the best avenue to carry out this research.

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ABSTRACT

This study investigated the use of snail (*Achatina achatina*) shell; an alternative adsorbent to natural matrices (such as polyvinyl alcohol and polyurethane) and agricultural wastes (such as sugarcane bagasse, orange peels, spent tea leaves, corn cobs and cotton seed hull) as a carrier to immobilize *Pseudomonas putida* and *Bacillus subtilis* for the degradation of naphthalene in synthetic wastewater. The hydrocarbon-degrading bacteria (HDB) used for the study were isolated from a refinery effluent and were further cultured in a nutrient medium. Naphthalene concentrations of 10, 20, 30, 40 and 50 mg/dm^3 were studied at constant temperature of 30 ^{o}C, pH of 5, 7 and 9 and adsorbent dosage of 2, 3 and 5 g for 72 hours in batch mode. The naphthalene concentrations were determined using UV-spectrophotometer (Agilent CARY 300, USA) and the products of the degradation were determined using Fourier Transform Infra-red spectroscopy (CARY 630, USA) to assess the adsorption and simultaneous adsorption-biodegradation capacity and equilibrium. The results showed that optimal adsorption and biodegradation occurred at naphthalene concentration of 50 mg/dm^3 and adsorbent dosage of 2 g. For the effect of pH, optimal adsorption took place at basic pH of 9, while the optimal biodegradation occurred at neutral pH of 7. The ranges of the percentage removal for the *Pseudomonas putida* and *Bacillus subtilis* immobilized on treated snail shell were: 73.23 - 82.12 % and 66.33 - 76.79 % respectively while on untreated snail shell were: 63.26 - 73.11 % and 60.17 – 74.46 % respectively. These values were significantly higher ($P<0.05$) than the values obtained from the adsorption of naphthalene which ranges were: 50.62 – 65.20 % and 57.05 – 67.10 % respectively for untreated and treated snail shell. The results of the equilibrium study indicated favourable adsorption and biodegradation, the R^2 values obtained were: 0.9933,

0.9997, 1.0000, 0.9973, 0.9947 and 1.0000. This indicated that the sorbent systems were well fitted to both the Langmuir and the Freundlich models. Therefore, snail shell can be employed as low-cost adsorbent and solid support matrix for the immobilization of microorganisms for the remediation of hydrocarbon contaminants.

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Abbreviations

AOAC:	Association of Official Agricultural Chemists
AT:	Treated snail shell
AU:	Untreated snail shell
BST:	*Bacillus subtilis* incorporated on treated snail shell
BSU:	*Bacillus subtilis* incorporated on untreated snail shell
DOE:	Design of Experiment
FTIR:	Fourier Transform Infra-red spectroscopy
HDB:	Hydrocarbon-Degrading Bacteria
MSM:	Mineral salt medium
PAC:	Powered activated carbon
PAHs:	Polycyclic Aromatic Hydrocarbons
PCBs:	Polychlorinated Biphenyls
PPT:	*Pseudomonas putida* incorporated on treated snail shell
PPU:	*Pseudomonas putida* incorporated on untreated snail shell
SAB:	Simultaneous Adsorption-Biodegradation
Sp. Gr.:	Specific gravity
UV-VIS:	Ultra violet-Visible spectroscopy
XRF:	X-ray Fluorescence spectroscopy

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Petroleum hydrocarbons and their other derivatives are majorly the energy source of various industrial, automobiles and domestic use. Petroleum (crude oil) is a complex mixture of several organic compounds such as aromatic cyclic hydrocarbons, complex branched aliphatic and cyclic alkanes as well as residual substances. Some of these do not degrade easily, and as such are termed recalcitrant (Nilanjana and Preethy, 2011). The increasing soil, surface and groundwater pollution through the release of petroleum hydrocarbons has been seen as one of the major issues in environmental pollution (Shah and Bhatt, 2006). Petroleum products have a long-term impact on the aquatic, soil, natural resources, and human health. Most of these contaminants have been found to be carcinogenic and toxic, and they easily find their way into the food chain (Farrington and McDowell, 2004).

Hydrocarbons produced by natural activities are usually present at trace levels, whereas hydrocarbons originating from different anthropogenic activities are present at higher levels, especially in industrial and transportation associated areas (Okedeyi *et al.*, 2013).

In most cases, the pollutant released through industrial emissions are deliberate and well regulated, while cases such as chemical spills are mostly accidental and largely unavoidable (Akpofure *et al.*, 2000). Also, anthropogenic activities from several auto mechanic workshops increasingly contaminate the environment with various petroleum products and motor spent oil such as diesel, engine oil and petrol (Yahaya, 2016).

There are several methods for removing petroleum hydrocarbons such as physical and chemical methods, but these methods have limitations and are very expensive. However, biodegradation as a method involves the decomposition of organic pollutants by microorganisms into products that are non-toxic and environmentally friendly, which can enter into trophic levels of food chain without posing any challenges to live. This is an adequate method for remediation (Plaza *et al.,* 2005), and it is considered as a more sustainable method than excavation (Penn *et al.,* 2002; Plaza *et al.,* 2005). The biodegradation of petroleum hydrocarbons in the environment is found to be a slow complex process that depends mainly on the nature and amount of the hydrocarbons present in the contaminants. It is also influenced by factors such as degrading microbial community, temperature, and nutrients (Ekpo and Udofia, 2008). The addition of nutrients like nitrogen and phosphorus are essential for the enhancement of biodegradation process (Wang, 2011). The success of this technology depends on the ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the polluted environment (Nilanjana and Preethy, 2011).

1.2 Statement of Problem

Petroleum hydrocarbons are recalcitrant pollutants that do not degrade easily and are deleterious to human health, animals, and plants due to their possible carcinogenic and mutagenic actions. The release of oil or hydrocarbons into the environment either by accident or through various anthropogenic activities is a major cause of water and soil pollution (Nilanjana and Preethy, 2011). In most mechanic workshops and automobiles, several liters of waste oil are generated daily and discharged carelessly into the environment (Adegoroye, 1997; Fagboya, 1997; Adelowo *et al.*, 2006). Oil spillage during

exploration and pipelines vandalization without effective clean-up is capable of destroying the natural habitat of an oil-rich region(United States Environmental Protection Agency,1996), especially as had been witnessed in the Niger Delta region of Nigeria (Oluyege and Oluyemi, 2005). Adelowo *et al*. (2006) reported that one litre of petroleum hydrocarbon is capable of contaminating a million litres of fresh water.

Several technologies like mechanical and physicochemical processes such as evaporation, burying, dispersion, and washing are the commonly employed remediation for an oil spill contamination. However, the problem of incomplete decomposition or breakdown of contaminants can occur, and moreover the technologies are comparatively expensive when compared to biodegradation (Nilanjana and Preethy, 2011). Biodegradation is the most efficient method for hydrocarbon degradation but the process is very slow. Combining biodegradation with adsorption makes degradation to be completed within a short period of time. Several studies on adsorption-biodegradation method have been reported, and these have drawn great attention (Agarry and Aremu, 2012; Lin *et al.,* 2015). Solid media such as activated carbon, kaolin, periwinkle shell, orange peel, groundnut shell and sugarcane bagasse have been extensively used for mineralizing hydrocarbons (Agarry and Aremu, 2012; Lin *et al.*, 2015), but so far there is little or no report on snail shell as solid media (adsorbent) for immobilizing microorganisms for degradation of hydrocarbons. Thus, this study sought to evaluate the removal of mineralization of petroleum hydrocarbon by simultaneous adsorption-biodegradation using snail shell incorporated bacteria to achieve synergistic effects.

1.3 Justification

Biodegradation is an eco-friendly and cost-effective alternative to all other methods of degradation. The conventional physicochemical remedies have high expenses and can generate products or deposits that are toxic to the biota (Bidoia *et al.,* 2010). However, biodegradation is non-invasive as it involves complete transformation of the pollutants into carbon dioxide, inorganic or simpler inorganic compounds (Nilanjana and Preethy, 2011). Also, combining high effectiveness and ease; bioremediation processes represent a highly important way of recovering polluted areas among a few other cleaning-up techniques (Bidoia *et al.,* 2010). Moreover, the remediation technique adopted in this study is simple and cheap, as the snail shell to be used is available locally and in abundance.

1.4 Aim

This research aimed at investigating the removal of naphthalene by adsorption and simultaneous adsorption-biodegradation using snail shell.

1.5 Objectives

The aim of this research will be achieved through the following objectives:

i. Determination of the physicochemical properties of the snail shell;
ii. Composition of the snail shell using x-ray fluorescence (XRF);
iii. Isolation and identification of bacteria capable of utilizing hydrocarbon from petroleum contaminated wastewater;
iv. Optimization of process parameters (initial hydrocarbon concentration, adsorbent dosage, and pH) for the mineralization of hydrocarbon;

v. Comparison of adsorption of naphthalene on snail shell and its biodegradation using snail shell incorporated with bacteria; and

vi. Evaluation of the performance of the snail shell using some selected models (Langmuir and Freundlich isotherms).

CHAPTER TWO

2.0 REVIEW OF RELATED LITERATURE

2.1 Environmental Pollution

The pollution of soils and aquifers by oil spills is a common and persistent problem destroying almost all levels of the environment, and thus imposing severe health implications and ecological problems (Bundy *et al.,* 2002; Okoh, 2006). Generally, poor waste management has been identified as the major causes of pollution and degradation in most developing countries (Adedosu *et al.,* 2015).

The increasing environmental degradation which occurs as a result of an increase in population, industrialization, urbanization, agriculture, mining and transportation activities are global challenges facing the world (Yashim, 2015). Petroleum hydrocarbons are persistent pollutants in the environment, owing to their recalcitrant nature to biodegradation, bioaccumulation and the extent of health effects associated with their exposure (Allen *et al.,* 1999). Furthermore, the functioning ecosystems such as respiration and the nitrogen cycle are disrupted by petroleum hydrocarbons (Schafer *et al.,* 2009).

Apart from the major oil spills, a small number of petroleum hydrocarbons are being released into the environment during transport, distribution process or through other anthropogenic activities (Kumar *et al.,* 2011). Oil spills on the ecological balance of aquatic ecosystems can have intense consequences. Exxon Valdez, Amoco Cadiz and Prestige oil spills as some of the most prominent examples of oil spillage have been observed within their marine environments (Atlas and Bartha, 1998). High mortality rates in algae, invertebrates, sea mammals and birds occurred as a result of direct physical contact or exposure to toxic compounds released from the spills (Peterson *et al.,* 2003).

2.2 Crude Oil/Petroleum

Petroleum as a naturally occurring oil consists mainly of hydrocarbons with some other elements such as sulphur, oxygen and nitrogen. Petroleum is a natural product which emerged from the anaerobic conversion of matter of biological origin under high temperature and pressure (Balba *et al.,* 1998). Crude oil is a complex mixture of aliphatic, aromatic asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolones, carbazoles, sulfoxides and amides) (Colwell and Walker, 1997; Jain *et al.,* 2011). Other elements found in trace amount include iron, silicon, aluminium and nichrome (Harayama *et al*., 2004; Ionescu *et al.,* 2013). Petroleum is a fossil fuel formed from prehistoric fossilised organic materials such as zooplankton and algae which have settled down over time under anaerobic conditions at the bottom of a sea or lake (Kvenvolden, 2006). The formation of this petroleum occurs from the pyrolysis of hydrocarbon which is exothermic in reactions at high temperature or pressure. Over time, the biological matter mixes with mud and then buried under heavy layers of sediment resulting in high heat and pressure, thus making the organic matter to change, first into kerogen (a waxy material found in various oil shale around the globe) and then change into liquid and gaseous hydrocarbons with the addition of more heat through catagenesis (Braun and Burnham, 1993).

2.3 Hydrocarbons

The term hydrocarbon means the compound of carbon and hydrogen only which are the ubiquitous class of natural compounds that are a major constituent of petroleum. Hydrocarbons in petroleum are classified on the basis of molecular composition or

structures as alkanes (normal or iso-alkanes), naphthenes, aromatics and alkenes (Scholz *et al.,* 1999).

The commonly used hydrocarbons such as diesel, petrol and some other petroleum distillates as primary energy and fuel resources are due to the energy produced by them during combustion (Watanabe, 2001). Oil spills are inevitable as large quantities of fuel are required daily to power industry, automobiles and its various uses in homes (Surridge, 2007).

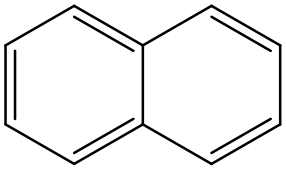
Hydrocarbon contamination is said to be carcinogenic, mutagenic and toxic to the immune system, thus posing a serious threat to human, animal and plant health (Vasudevan and Rajaran, 2001).

2.3.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are a group of organic compounds that contain two or more fused aromatic rings (that is; the organic rings with delocalized electrons). This group of hydrocarbons do not have heteroatom or branching substituent on their ring structures. The example s include; naphthalene, anthracene, phenantrene, flourene, pyrene and so forth. They are recalcitrant and ubiquitous organic pollutants. Hydrocarbons with natural origin occur at trace levels but hydrocarbons emanating from different anthropogenic activities are found at higher levels, mainly in areas known for industrial and transportation activities. Among the list of hydrocarbons, PAHs must be given special attention due to their acute and chronic toxicity, as well as their stability, which delays their degradation (Malawska and Wilkomirski, 2000). Soils contain a large quantity of PAHs and they pose threats to human health, due to their carcinogenic and mutagenic effects (Yang *et al.*,

2005). Also, they are lipophilic in nature and relatively insoluble in water and as a result persist as a potential toxic contaminant (Hafez *et al.,* 2008; Johnsen *et al.,* 2005).

2.3.2 Naphthalene



Molecular formular: $C_{10}H_8$

Molar mass: 128.17 $gmol^{-1}$

Figure 2.1: Naphthalene structure

Naphthalene is the simplest polycyclic aromatic hydrocarbons with two fused benzene rings. It is a white crystalline solid with a characteristic odour. Naphthalene is produced commercially from coal tar or crude oil and it is also used for the production of mothballs, pesticides, plastics, resins and dyes. Exposures to a large amount of naphthalene have been reported to cause Anaemia, damage to red blood cells, and it has been listed as Group C-possible human carcinogen (Agency for Toxic Substances and Disease Registry, 2015).

2.4 Biodegradation

Biodegradation is the decomposition of organic pollutant by microorganisms into products that are non-toxic and environmental friendly and can enter into trophic levels of food chain without posing any challenges to life. It is a treatment process whereby pollutants are metabolised into non- toxic or less toxic compounds by natural microorganisms in the environment (Antwi-Akomeah, 2011). Diaz (2008), reported that biodegradation is enhanced by biosurfactant which is an extracellular surfactant secreted by microorganisms. Viramontes *et al.* (2010) also reported that biodegradation is one of the primary ways to remove petroleum and other hydrocarbon contaminants from the environment, but the

process is slow. However, effective biodegradation is linked to certain factors such as temperature and pH. Biodegradation as a metabolic or enzymatic process, allow microorganisms to break down or mineralize organic pollutant into substances that are innocuous and non-hazardous.

The rate of the degradation of petroleum hydrocarbon is dependent to a large extent on its concentration, and the rate by which bacteria degrade hydrocarbons depends upon the chemical structure and concentration of that hydrocarbon (Abioye, 2012; Matvyeyeva *et al.,* 2014). It has also been reported that high degradation occurred in soil contaminated with 10 % and 20 % crude oil compared to those contaminated with 30 % and 40 % crude oil which showed partial degradation within a 12 months period (Ijah and Antai, 2003).

Organic materials can undergo either aerobic (with oxygen) or anaerobic (without oxygen) degradation. Hydrocarbon-degrading bacteria, yeast and fungi are largely found in habitat such as marine, freshwater and soil (Diaz, 2008).

2.5 Factors Affecting Biodegradation

2.5.1 Temperature

Temperature plays a major role among other factors in biodegradation of hydrocarbons by directly affecting the chemistry of the contaminants. It was reported that at low temperatures, oil viscosity increases, while the volatility of the toxic low molecular weight hydrocarbons reduces thus delaying the process of biodegradation (Sawadogo *et al.,* 2014). The solubility of PAHs increases with temperature, thus increasing the bioavailability of the hydrocarbons. In addition, the oxygen solubility decreases with increasing temperature, which reduces the metabolic activity of aerobic microorganisms (Shallu *et al.*, 2014).

2.5.2 Nutrients

Nutrients are the basic building blocks of life and allow microbes to produce the enzymes necessary for the breaking down of contaminants (Vidali, 2001). Nitrogen and phosphorus are very important nutrients for successful biodegradation of hydrocarbon pollutants (Nkereuwem *et al.,* 2010). Some of these nutrients could become limiting factors thus affecting the biodegradation processes. When a major oil spill occurred in marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for oil degradation (Atlas, 1992; Chaillan *et al.,* 2006).

Mandri and Lin (2007) studied the efficiency of microorganisms to degrade engine oil. It was noted that *Flavobacterium spp,* utilized NH_4NO_3 (46 %) more efficiently than $NaNO_3$ in engine oil degradation metabolic mechanism. The addition of urea or nitrite reduced the cell growth and the degradation rate significantly (6-7 %). *A. calcoaceticum* and *P. aeruginosa*, in contrast, were capable of utilizing different nitrogen sources including nitrite.

2.5.3 Oxygen

Microorganisms use petroleum hydrocarbons as a carbon and energy source, and the metabolic pathways that hydrocarbon-degrading microorganisms use can be either aerobic or anaerobic. Reports have shown that aerobic degradation usually proceeds at a faster rate and is more effective than anaerobic degradation. Aerobic processes require less free energy for initiation of degradation and more energy are produced per reaction (Abdulsalam *et al.,* 2011). Yudono *et al.,* (2011) studied the kinetics approach of biodegradation of petroleum contaminated soil by using indigenous isolated bacteria, and it

was observed that a high number of bacteria degrading organisms grew aerobically in the surface of the soil. This was attributed to the fact that the initial intracellular attack of organic pollutants is an oxidative process.

2.5.4 pH

pH affects both the degree of ionization of the adsorbate (that is, the pollutant) and the surface charge of the adsorbent during the adsorption and biodegradation process. The pH of either the waste water or medium controls the electrostatic interactions between the adsorbent and the adsorbate. The adsorbing nature of an adsorbent depends not only on the surface functional groups, but also on its isoelectric point (Agarry and Aremu, 2012). The pH of the solution affects the extent of adsorption due to the composition of raw materials and the technique of activation, thus varying the extent of adsorption according to the adsorbate functional groups (Gao and Pedersen, 2005; Yoon *et al*., 2005; Putra *et al*., 2009). Mentzer and Ebere (1996) reported that optimum pH for the biodegradation of hydrocarbons occurs within range 6 – 8. pH is an important factor which influences the metabolic activity and growth of microorganisms, different organisms have their tolerance level and as such can grow over a wide range of pH (Darsa *et al.,* 2014). However, microorganisms tend to prefer a neutral pH to a larger extent (Boszczyk-Maleszak *et al.,* 2006).

2.5.5 Concentration of petroleum hydrocarbon

The concentration of petroleum hydrocarbons affects the level of tolerance in the environment. At low concentrations, all fractions are likely to be attacked. However, at high concentrations, only those fractions most susceptible to degradation will be broken down. Chaineau *et al.* (2005) reported that higher concentrations of gasoline in contaminated water were related to higher counts of microorganisms. It was observed that

petroleum biodegradation rates in the soil are concentration dependent. Petroleum hydrocarbons may affect soil microbial populations, stimulating the growth of certain microorganisms and exerting toxic effects and inhibiting the growth of others (Rodriguez-Blanco *et al*., 2013).

2.6 Enhancement of Biodegradation

There are two major approaches employed in enhancing the process of biodegradation; they include biostimulation and bioaugmentation

2.6.1 Biostimulation

This process involves the stimulation of native microorganisms to break down the contaminants. The microbial degradation of many pollutants in aquatic and soil environments is hampered by the unavailability of nutrients such as nitrogen, phosphorus and oxygen.

Reports have shown that the addition of nitrogen, phosphorus and oxygen or aeration of the process tends to stimulate the microbial population leading to an increase in the breakdown of pollutants (Smith, 2004; Trindade *et al.*, 2005). Without sufficient supply of oxygen and nutrients, the existing supplies are been depleted by the microbes, thus limiting the degradation level. The addition of fertilizers has also been reported to address the slow nature of biodegradation and the biodegradability of microbes (Margesin and Schinner, 2001; Van Hamme *et al.,* 2003).

2.6.2 Bioaugmentation

Bioaugmentation is the introduction of either indigenous or exogenous microorganisms into a contaminated environment in order to supplement the indigenous microbial population for the purpose of improving biodegradation process (Vidali, 2001; Smith, 2004; Mrozik and

Piotrawska-Seget, 2009). Wang *et al*. (2008) reported that individual isolates possess lower ability than microbial consortia in biodegradation, especially for complex mixtures of compounds such as diesel and crude oil. Some reports claimed that bioaugmentation proved ineffective in stimulating degradation of petroleum hydrocarbon pollutants (Prince, 1997; Swannell *et al*., 1996); a study by Ruberto *et al*., (2003) on the bioremediation of a hydrocarbon polluted Antarctic soil showed a 75 % removal of the hydrocarbon when the contaminated soil was bioaugmented with a psychrotolerant strain (B-2-2) and process improved the bioremediation efficiency. Also, recalcitrant components in crude oil such as PAHs has been broken down and rendered harmless by microbes that have been engineered (Nilanjana, and Preethy, 2011**)**.

2.7 Microbial Degradation of Petroleum Hydrocarbons

Microbial degradation is an environmentally friendly process when compared to other processes such as adsorption, volatilization, photolysis and chemical degradation because, it involves breakdown and complete utilization of hydrocarbons (Haritash and Kaushik, 2009). Microorganisms are numerous and can survive in any form of harsh weather, and they play important roles by contributing to the geological cycle of elements and transformation of natural chemicals in the natural environment (Trindade *et al.,* 2005; Oteyza *et al.,* 2008). Antwi-Akomeah (2011), reported that some microorganisms have unique and naturally occurring microbial catabolic diversity to degrade or transform a large number of compounds, including hydrocarbons, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pharmaceutical active compounds, radionuclide and heavy metals.

Microbial degradation of petroleum hydrocarbon pollutants is the major and the best natural mechanism for its clean up from the environment (Atlas, 1992; Lal and Khanna, 1996). Petroleum can be degraded by several microorganisms which include bacteria, fungi, yeast and microalgae (Bundy *et al.,* 2002), but bacterial are known to play a major role in the degradation of hydrocarbons.

However, microorganisms bring about the biodegradation in order to meet their cell growth and energy needs (Oteyza *et al.,* 2008; Trindade *et al.,* 2005). Bacteria become adapted by acquiring the ability to convert the hydrocarbon in petroleum to the only source of energy and carbon for metabolic activities when such bacteria are exposed to hydrocarbons. The bacteria break down the components of the petroleum and also derive nutrients from it and thus rendering the contaminants innocuous (Ranjan *et al.,* 2014).

The natural population of microorganisms via biodegradation is one of the primary mechanisms through which petroleum hydrocarbon contaminants are removed from the environment (Leahy and Colwell, 1990). Mixed cultures carry out more rapid and extensive biodegradation of petroleum than pure cultures (single species) (Ghazali *et al.,* 2004; Gerdes *et al.,* 2005).

The bacteria and fungi capable of degrading hydrocarbon are mainly responsible for the mineralization of oil pollutants and are diversely distributed in the ecosystems (Leahy and Colwell, 1990). Microbial species when exposed to hydrocarbon get adapted and show selective enrichment, they also bring about genetic changes which results in the high proportion of hydrocarbon utilizing bacteria. This exposure also results in plasmids encoding hydrocarbon catabolic genes (Leahy and Colwell, 1990). Most active agents in

petroleum degradation are the bacterial strains which work as primary oil degraders of spilt oil in the environment, and most of these organisms have genotypic ability to utilize the hydrocarbons as a sole carbon source, but they are grown in an environment contaminated by hydrocarbons (Vamsi, 2012). Adebusoye *et al.,* (2007), reported that bacterial strains such as *Pseudomonas, Fluorescens, P.aeruginosa, Bacillus subtilis, Bacillus sp., Alcaligenes sp., Acinetobacter iwoffi, Flavobacterium sp., Micrococcus roseus* and *Corynebacterium sp.* isolated from a tropical stream contaminated by petroleum hydrocarbons in Lagos, Nigeria was used to clean up the oil contaminants in the same environment.

The major effect of a given density of oil-degrading microorganism to the removal of oil is dependent on their inherent metabolic capability, that is, the heterotrophic potent and its expression as permitted by the environmental conditions (Atlas and Bartha, 1998). Also, Singer and Finnerty (1984) reported that oxidation of the substrate by oxygenases, which require molecular oxygen is the first step in the catabolism of hydrocarbons by bacteria and fungi. This suggested that aerobic conditions are important for microbial oxidation of hydrocarbons through catabolism.

Moreover, some studies have shown that anaerobic degradation of petroleum hydrocarbons by microorganisms occur at negligible rates and as such, its ecological significance is said to be minor (Atlas, 1992; Bailey *et al.,* 1973; Bossert and Bartha, 1984). *Bacillus subtilis* has been reported to have the potential of degrading hydrocarbon owing to its own growth in high concentrations of petrol. This was confirmed by studying the effect of changes in factors such as pH, optical density and CO_2. The result showed an initial high rate of

biodegradation which was attributed to increases in microbial biomass and nutrient availability (Dibble and Bartha, 1991).

Lin *et al.* (2015) investigated the biodegradation of tetradecane using *Acinetobacter venetianus* immobilized on bagasse. The study showed a high rate of degradation was retained by microorganisms strongly immobilized on bagasse and the bagasse was also found to protect the microorganisms from pollution stress. Also, Wilson and Bradley (1996) studied the biodegradation of petrol in an aqueous medium using both free suspension and immobilized *Pseudomonas sp*. The result of the study showed that there is increased contact between microorganisms and hydrocarbons droplets and level of rhamnolipids production was enhanced. Moreover, studies have also shown that the biodegradation rate of crude oil is enhanced by immobilized bacterial cells compared with free-living cells in a different culture medium (Diaz *et al.*, 2002). The immobilization can be done in either batch mode or continuous mode.

The degradation of petroleum hydrocarbons can be initiated by specific enzyme system. Figure 2.2 shows the initial attack on xenobiotics by oxygenases, other mechanism involve attachment of microbial cells to the substrates and production of biosurfactants (heterogeneous group of surface active chemical compounds produced by a large variety of microorganisms). These surfactants enhance solubilisation and removal of contaminants and it also increase the oil surface area thus making the oil available for bacteria to utilize it (Nalanjana and Preethy, 2011). Research has shown that *Pseudomonas* is the most efficient bacteria capable of utilizing hydrocarbons as carbon and energy sources and still producing biosurfactants (Cameotra and Singh, 2008).

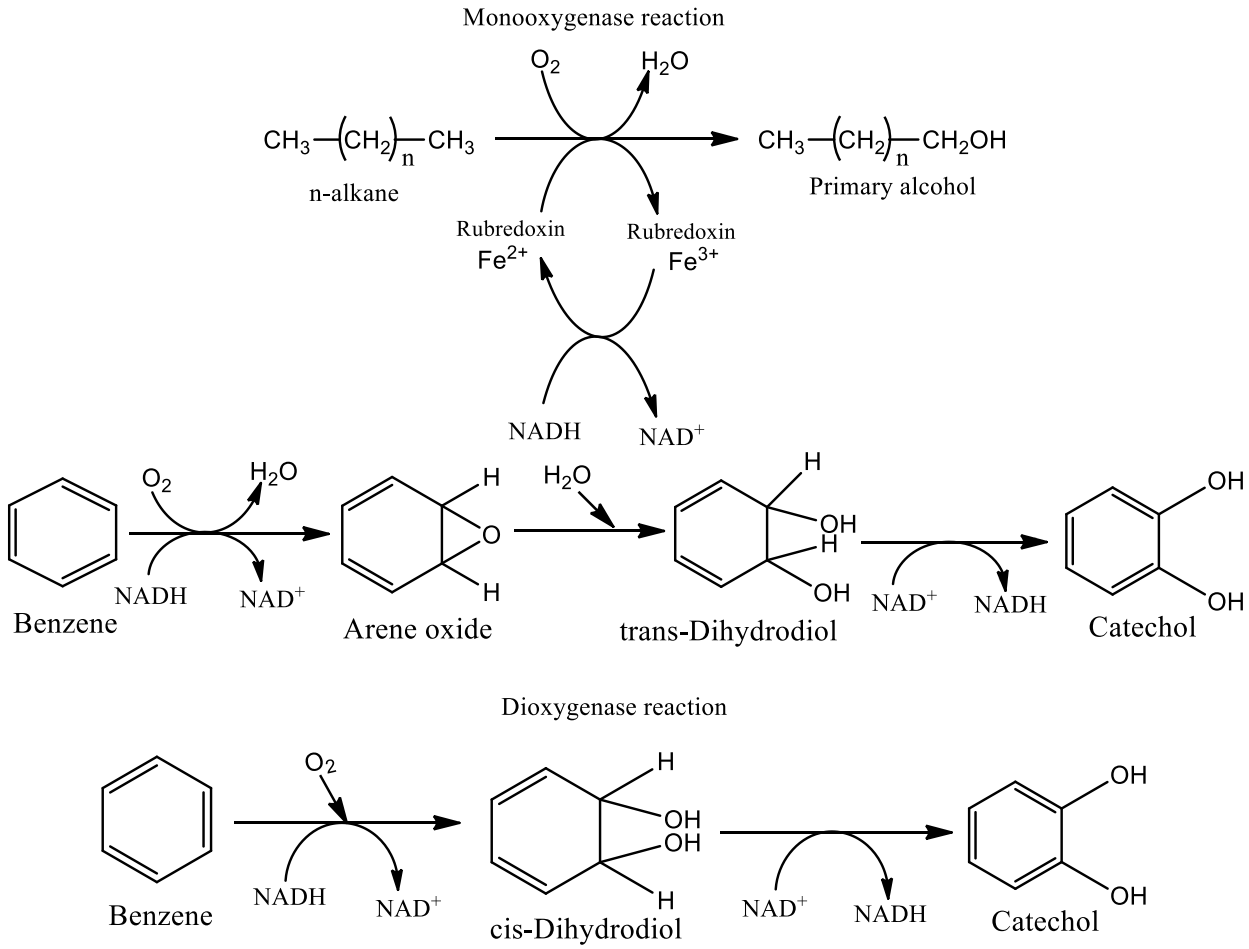


Figure 2.2: Enzymatic reactions involved in the processes of hydrocarbons degradation (Nilanjana and Preethy, 2011).

2.8 Africa Giant Snail (*Achatina achatina*) Shell

Achatina achatina commonly called African giant snail is a species of very large, air-breathing land snail belonging to the phylum Mollusca and class Gastropoda in the family of Achatinadae. This snail is believed to be native to West African countries such as Nigeria, Liberia, Togo, Ivory Coast, Sierra Leone, Benin Republic and Ghana. They are most abundant in the rainy season and very active at night (Egbon *et al.*, 2006). Snail has various applications; the edible part is used as food, while the shell has been used as fillers in paper, paint and ceramic industry owing to its rich calcium content (Sunday and Magu, 2017). Also, the snail shell use in the local manufacturing of jewels, buttons and collections

of arts, and water treatment have been reported (Jatto *et al.,* 2013).Snail shell is composed majorly of calcium carbonate (97.5 %). Other compounds such as calcium phosphate, calcium silicate, magnesium carbonate, magnesium oxide, iron oxide and other organic substances are also present (Srinivaskannan and Abubakar, 2004). Due to the chemical composition of a snail shell, it can be used as a coagulant or as an adsorbent in wastewater treatment (Gumus and Okpeku, 2015).

2.8.1 Solid support

Microorganisms have been incorporated on different solid media for the degradation of organic pollutants. This includes agricultural wastes, diatomaceous earth, activated carbon, polyurethane foam, sand, glass, and chitosan (Agarry *et al.,* 2013; Lin *et al.,* 2015). Solid support known as bio-carriers have been used in several studies which serves as the attachment providing a surface for bacterial growth, aimed at enhancing the potential of removal efficiency of organic pollutants (Agarry *et al.,* 2013). The presence of powdered activated carbon was found to facilitate the biological treatments of toxic organic pollutant (Barragan *et al.*, 2007).

2.9 Equilibrium Studies

2.9.1 Adsorption and adsorption-biodegradation isotherms

This study involves adsorption and simultaneous adsorption-biodegradation which are both isothermal processes. Adsorption isotherms show the distribution of adsorbed molecules (amount of adsorbate) between the liquid and the solid phase when the adsorption process reaches equilibrium.

Tan *et al.,* (2008) reported that to find a suitable model which can be used for design purposes, the analysis of the isotherm data by fitting them to different isotherm models is of

paramount importance. Adsorption models are used to describe the equilibrium established between the adsorbed component on the adsorbent and unadsorbed component in solution represented by adsorption isotherms (Agarry and Aremu, 2012). To investigate an interaction of adsorbate molecules and adsorbent surface, two well-known models; the Freundlich and Langmuir isotherms were selected to explain naphthalene–snail shell interaction in this study.

2.9.1.1 Langmuir isotherm model

The basic assumption of Langmuir is that the sorption of monolayer takes place on the surface of the adsorbent, indicating that only an adsorbate molecule could be adsorbed on one adsorption site and the intermolecular forces decrease with the distance(Meroufel *et al.*, 2013). It is also assumed that the surface of the adsorbent is homogeneous in character and possesses identical and energetically equivalent adsorption sites (Kannan and Meenakshisundaram, 2002; Crini *et al.*, 2007). The Langmuir equation is represented by the following equations:

$$q_e = \frac{q_{max} b C_e}{1 + b C_e} \qquad (2.0)$$

The Langmuir equation is expressed in linear form as below

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \frac{1}{b q_{max} C_e} \qquad (2.1)$$

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \frac{1}{b q_{max}} * \frac{1}{C_e} \qquad (2.2)$$

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 ofq_{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 $\frac{1}{C_e}$ (Gupta and Rastogi, 2007; Sarahi *et al.*, 2010).

The essential features of the Langmuir isotherm may be expressed in terms of equilibrium parameter, R_l, which is a dimensionless constant referred to as separation factor or equilibrium parameter. This is defined in the equation (2.3) below:

$$R_l = \frac{1}{1 + bC_o} \qquad (2.3)$$

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

2.9.1.2 Freundlich isotherm model

Freundlich isotherm model is an empirical relationship which describes the sorption on the heterogeneous surface. That is, adsorption of solutes from a liquid to a solid surface and assumes that different sites with several adsorption energies are involved (Hutson and Yang, 2000). The empirical Freundlich equation based on sorption on the heterogeneous surface can be deduced assuming a logarithmic decrease in the enthalpy of adsorption with

the increase in the fraction of occupied sites (Meroufel *et al.,* 2013). The empirical equation proposed by Freundlich is given by equation (2.4) below:

$$q_e = K_f C_e^{1/n} \qquad (2.4)$$

The equation can be rearranged to obtain a linear form by taking logarithms of equation (2.4) as shown below:

$$\log q_e = \log K_f + \frac{1}{n}\log C_e \qquad (2.5)$$

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) (Martins *et al.,* 2015).

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 $\log C_e$.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Snail shell collection and identification

The snail shell was obtained from a dumpsite within National Research Institute of Chemical Technology (NARICT) junior staff quarters, Basawa road, Zaria.The shells were identified as African Giant snail (*Achatina achatina*), "cabinet 7B" at the Department of Biological Sciences, Ahmadu Bello University, Zaria.

3.1.2 Collection of refinery effluent samples

Refinery effluent samples were collected from Kaduna Refining and Petrochemical Company (KRPC) by using a clean cup to draw the samples from the effluent ponds into new clean 1 litre plastic containers (Oaikhena *et* al., 2016). The samples were transported at ambient temperature to the Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

3.2 Chemicals and Reagents

All chemicals and reagents used were of analytical grade. Distilled and deionized water were used in this study. The hydrocarbon (Naphthalene, 99% ultra pure chemical grade) a product of Sigma- Aldrich, USA. All chemicals used as the mineral salt medium are of an analytical or biochemical grade, the Microgen® *Bacillus and Enterobacteriaceae* (GNA+GNB) kits were obtained from Microgen Bioproducts, United Kingdom. All glassware was washed with liquid soap, rinsed with water and then soaked in 10 % HNO_3 overnight. The items were then rinsed with ultra-pure water followed by acetone in order to

remove any organic compounds that might adhere to them (Adeniyi and Afolabi; 2002). Afterwards, they were rinsed with distilled water and dried in a Memmert drying oven at 80 ^{o}C for 5 hours (Todorovic *et al.,* 2001).

3.3 Preparation and Pre-treatment of Adsorbent (Snail shell)

The snail shell was washed thoroughly with detergent and rinsed with tap water to remove adhered impurities, rinsed with distilled water and sun-dried. They were then crushed in a miller machine and oven dried at 100 ^{o}C for 12 hours, ground to powder and screened with 0.125 mm particle size. The ground shell was divided into two, labelled as treated (AT) and untreated (AU).

3.3.1 Carbonization process

Hundred grams (100 g) of the snail shell labelled 'AT' was weighed into a crucible and introduced into a Muffle Furnace and was carbonized at a temperature of 600 ^{o}C for 2 hours. This was allowed to cool at room temperature and transferred into a 100 cm^3 beaker half filled with water. The excess water was drained and the sample was air-dried at room temperature for 96 hours. This process was repeated until a substantial amount of carbonized sample was obtained. The resulting sample was stored in sealed plastic containers for activation.

3.3.2 Activation process

Preparation of reagents:

Standard hydrochloric acid (0.1 M): A volume of 8.60 cm^3 of concentrated HCl (sp. Gr. 1.18, 36 %) was pipetted into a 1000 cm^3 volumetric flask containing little amount of water; it was then made up to mark with distilled water.

Standard H_3PO_4 (0.5 M): A volume of 30.50 $cm^3$of phosphoric acid (sp. Gr. 1.88, 85 %) was pipetted into a 1000 cm^3 volumetric flask containing little amount of water; it was then made up to mark with distilled water.

Procedure:

The snail shell powder (50 g) carbonated was leached with 0.1 M HCl (200 cm^3) and washed with distilled water. Activation of the samples was carried out using 0.5 M orthophosphoric acid (H_3PO_4). The sample was placed in a crucible and 0.5 M H_3PO_4 (500cm^3) was added, mixed and heated until a paste was formed. It was then subjected to a temperature of 700 ^oC for 3 hours and then cooled to room temperature. The activated samples were washed with distilled water to remove excess acid and oven-dried at 100 ^oC for 12 hrs. Each sample was then stored in an air-tight container prior biodegradation study (Ambali *et al.*, 2015).

3.4 Determination of Physicochemical Properties of the Snail Shell

3.4.1 pH

Preparation of reagent:

Standard buffer solution: one buffer tablet each of pH 4.0, 7.0 and 9.2 was dissolved in distilled water separately and made up to 100 cm^3.

Procedure:

Ten grams (10 g) of the air-dried and sieved (0.125 mm) snail shell was weighed into a glass container and 10 cm^3 of distilled water was added. The mixture was allowed to stand for 1 hour. The pH meter was turned on and allowed to warm for 15 minutes. The pH meter

was calibrated according to manual's instructions and the pH values to the nearest 0.1 unit were taken (AOAC, 1995).

3.4.2 Bulk density

The bulk density was determined using the method described by Abechi (2011). A 25 cm^3 measuring cylinder was filled to a specific volume with snail shell that had been dried in an oven at 80 °C overnight. The measuring cylinder was tapped for at least 1-2 minutes to compact the carbon and then weighed. The bulk density was calculated using equation 3.1:

$$\text{Bulk Density } (\text{gcm}^{-3}) = \frac{\text{Weight of the dry material}}{\text{volume of packed dry material}} \qquad (3.1)$$

3.4.3 Moisture content

The moisture content was determined by the method of Ilaboya *et al*. (2013). Exactly 1g of the dried snail shell was weighed and placed in washed, dried and weighed crucible. The crucible and its content was placed in an oven and dried at 105 °C to constant weight. The percentage moisture content (MC %) was calculated using equation 3.2:

$$\text{Moisture Content } (\%) = \frac{\text{Loss in Weight on Drying (g)}}{\text{Initial Sample Weight (g)}} \times 100 \qquad (3.2)$$

3.4.4 Ash content

The ash content was determined by the method described by Ilaboya *et al*. (2013). Exactly 1 g of the snail shell was taken, dried in an oven at a temperature of 105°C for 1 hour. The final weight after drying was measured and recorded as (Xg = Oven dry weight). Thereafter the dried snail shell was placed in a cold Muffle furnace and the temperature was allowed to rise until it reaches 500 °C. After 1 hour, it was removed and allowed to cool in a desiccator to room temperature and reweighed again and its weight was again

recorded as (Yg = Ash weight). The percentage ash content was then calculated from equation 3.3:

$$\text{Ash (\%)} = \frac{\text{Ash Weight (g)}}{\text{Oven Dry Wt. (g) of sample}} \times 100 \quad (3.3)$$

3.5 Analysis of Snail Shell

The analysis of the treated and untreated snail shell for major and minor components were performed with an Oxford instrument X-ray Fluorescence spectroscopy (X-Supreme 8000).

3.6 Isolation of *Bacillus subtilis* and *Pseudomonas putida* from the Refinery Effluent.

Preparation of Reagent:

Mineral salt medium: The mineral salt medium (MSM) was prepared with the following composition per 1000 cm^3: KH_2PO_4 (2.0 g), $NaNO_3$ (2.0 g), NaCl (0.8 g), KCl (0.8 g), $Na_2HPO_4.12H_2O$ (2.0 g), $MgSO_4$ (0.2 g), $FeSO_4.7H_2O$ (0.001 g). The salts above were dissolved in 1000 cm^3 of distilled water and sterilized by autoclaving at 121 ^0C for 15 minutes (Eniola *et al.*, 2014; Musa *et al.*, 2015).

3.6.1 Inoculation

This was carried out by following the method reported by Naga *et al*. (2016). The effluent sample was shaken vigorously and 200 cm^3 was dispensed into a beaker and allowed to settle at room temperature on a thoroughly disinfected laboratory workbench for 4 hours to concentrate the sample by sedimentation. The supernatant was discarded leaving the sediment to about 20 cm^3 volume followed by vigorous shaking to re-suspend the sediment. Ten (10) cm^3 of the concentrated effluent sample was dispensed into a 250 cm^3 conical

flask containing 90 cm^3 of mineral salt medium supplemented with 1 % v/v of crude oil. This preparation (culture) was incubated at ambient temperature on a shaker at 150 rpm for 14 days. A portion (50 cm^3) of the culture was dispensed into a sterile 100 cm^3 conical flask and heat shocked (heated) in a water bath at 80 ^oC for 1 hour to eliminate all non-spore formers. Both the heat shocked and non-heat shocked culture were serially diluted to a factor of five (five-fold dilution). Then 0.1 cm^3 from the diluents of the heat shocked culture was inoculated on the surface of centrimide agar, while the non-heat shocked sample was inoculated on the surface of nutrient agar using spread plate method. The resulting bacterial colonies were examined for size, shape, margin consistency and pigmentation. Distinctive colonies were sub-cultured unto nutrient agar plates by streaking for purification. The resulting pure colonies were sub-cultured onto a nutrient agar slant medium for further studies and preserved at 4-5 ^oC (Ekhaise and Nkwelle, 2011).

3.6.2 Identification of the isolates

The isolates were identified with a combination of microscopic and biochemical techniques. Isolates presumed to be *Bacillus subtilis* and *Pseudomonas putida* were confirmed using Microgen® *Bacillus and Enterobacteriaceae* (GNA+GNB) kits according to the manual's instruction. For the microscopic identification, the isolates were gram stained and examined microscopically to determine their gram reaction and cellular morphology. All the gram-negative rods and gram-positive rods were subjected to the Microgen kit test for identifying the bacteria.

3.6.3 Screening the capacity of *Bacillus subtilis* and *Pseudomonas putida* to degrade hydrocarbons.

A volume of 1000 cm^3 mineral salt agar medium was prepared each in different conical flasks and enriched separately with 0.5 %, 1 %, 1.5 % and 2 % crude oil and sterilized by autoclaving. The medium was dispensed into Petri dishes and allowed to solidify at room temperature, after which they were inoculated with 18 hours culture of the *Bacillus subtilis* and *Pseudomonas putida* isolates separately by streaking. The preparation was incubated at ambient temperature for 48 hours. The capacity of the isolates to degrade hydrocarbons were determined by the ability of the organisms to grow on the medium which was categorized as luxuriant growth, medium growth, scanty growth and no growth (Ekhaise and Nkwelle, 2011). Isolates that grew well on the various concentration of the crude oil media was used for the study.

3.7 Preparation of Inoculum for Biodegradation

Preparation of reagent:

Naphthalene stock and standard solutions: A 100 mg of Naphthalene was dissolved in 300 cm^3 acetone and was made up to 1000 cm^3 mark in a standard volumetric flask with distilled water (100 mg/dm^3). Standard solutions of various concentrations of 10 mg/dm^3, 20 mg/dm^3, 30 mg/dm^3, 40 mg/dm^3 and 50 mg/dm^3 were prepared by serial dilutions of 10 cm^3, 20 cm^3, 30 cm^3, 40 cm^3 and 50 cm^3 of stock solution respectively in a 1 dm^3 volumetric flask, and they were all made up to mark with distilled water (Crisafully *et al.*, 2008).

Procedure:

The method as used by Agarry and Aremu (2012) was adopted for this study. A primary culture was prepared by transferring 2 loops full of microorganisms (bacteria) from an agar slant culture into 100 cm^3 of feed medium (containing 20 cm^3 Mineral Salt Medium and 80 cm^3 of 60 mg/dm^3 of Naphthalene solution in 250 cm^3 Erlenmeyer flask). The flask was then cultivated in a gyratory shaker incubator programmed at 120 rpm and 30 ^oC for 48 hours. Thereafter, a secondary culture was prepared by transferring 10 cm^3 of the primary culture into another 100 cm^3 feed medium in a 250 cm^3 Erlenmeyer conical flask and the incubation process was repeated. The secondary culture was 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.

3.8 Batch Adsorption and Biodegradation Studies

Preparation of reagent:

Synthetic wastewater: The synthetic water was prepared by dissolving 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. The solution was divided into three and the pH was adjusted to 5, 7 and 9 respectively using 1 M NaOH and 0.5 M H_2SO_4. The wastewater was then stored in a clean bottle for the adsorption and biodegradation study.

Procedure:

The biodegradation was carried out by adding a known volume (100 cm^3) of the prepared inoculums of the bacteria into two glass bottles containing a known amount of treated and untreated snail shell respectively. Exactly 150 cm^3 of synthetic wastewater with known

amount of naphthalene concentration was added to the respective flasks, the water was maintained at a known pH of 5, 7 and 9 respectively. The flask was 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. The *Pseudomonas putida* immobilized on untreated snail shell (AU) and treated snail (AT) were labelled PPU and PPT respectively, while the *Bacillus subtilis* immobilized on both untreated and treated snail shell were labelled BSU and BST respectively. A similar process was followed for the control; adsorption without microbes incorporated on the surface of the snail shell, both the untreated snail shell (AU) and treated snail shell (AT). The naphthalene concentrations were determined by UV-spectrophotometer (Agarry and Aremu, 2012).

The amount of the naphthalene adsorbed, q_e, was calculated from equation 3.4;

$$q_e = \frac{V(C_o - C_e)}{m} \qquad (3.4)$$

Where, qe is the amount of adsorbate (naphthalene) adsorbed in milligram per gram of the adsorbent, Co 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.mis the mass in gram of the adsorbent, V is the volume of the solution in dm^3. The percentage naphthalene removal was calculated using the equation 3.5:

$$\% \text{ Removal Efficiency} = \frac{C_o - C_e}{C_o} \times 100 \qquad (3.5)$$

3.8.1 Effect of initial naphthalene concentration

The effect of initial naphthalene concentration was also studied by varying the initial concentration of the naphthalene used for the adsorption/biodegradation. The concentrations of interest were 10, 20, 30, 40 and 50 mg/dm^3. The experiment was carried out also as above but at constant pH of 7 and adsorbent dose of 2.0 g. The concentration of the residual naphthalene remaining in the solution was determined using Ultraviolet/Visible Spectrophotometer. Thus, the optimum concentration of the naphthalene was determined.

3.8.2 Effect of adsorbent dose

The effect of adsorbent dose was studied by varying the adsorbent dose used for adsorption. The dosages of interest in grams were 2, 3 and 5. The experiment was carried out as above, but at a constant concentration of 50 mg/dm^{3}of naphthalene and pH of 7, while the adsorbent dose was varied. The concentration of the residual naphthalene remaining in the solution was determined using Ultraviolet/Visible Spectrophotometer. Thus, the optimum adsorbent dose was determined.

3.8.3 Effect of pH

Preparation of reagents

Standard H_2SO_4 (0.5 M): A volume of 18.40 cm^3 of conc. H_2SO_4 (sp. Gr. 1.84, assay 98 %) was carefully transferred into 1000 cm^{3}volumetric flask and diluted to 1000 cm^3 with distilled water. It was thereafter standardized against a primary standard, Na_2CO_3.

Standard Na_2CO_3 (0.01 M): 5.3 g of A.R. grade Na_2CO_3 was dissolved in 1000 cm^3 volumetric flask with distilled water, which gives a concentration of 0.1 M Na_2CO_3. 100 cm^3 of this solution was diluted with distilled water to get 0.01 M, which was used for the standardization of 0.5 M H_2SO_4.

Standard sodium hydroxide (1 M): A 40 g of NaOH pellets were dissolved in a small quantity of distilled water in a 1000 cm^3 volumetric flask, the flask was made up to mark with distilled water.

Procedure:

The effect of pH was studied by varying the pH used for both adsorption and biodegradation at the optimized concentration. The pH of interest was 5, 7 and 9. The experiment was carried out as stated above at constant adsorbent dosage of 2 g and optimum naphthalene concentration of 50 mg/dm^3. The pH of the synthetic water was adjusted with 0.1 M H_2SO_4 or 0.1 M NaOH. The concentration of the residual naphthalene remaining in the solution was determined using Ultraviolet/Visible Spectrophotometer. Thus, the optimum pH of the adsorbate was determined.

3.9 Sample Analysis

The naphthalene concentrations, elemental analysis and FTIR analysis of its metabolites were determined using Agilent Cary Series UV-VIS spectrophotometer (CARY 300 UV-VIS, USA), X-ray Fluorescence spectroscopy (X-Supreme 8000, UK) and FTIR spectroscopy (CARY 630, USA) respectively.

3.10 Statistical Treatment of Data

The data obtained were subjected to Graph pad prism (version 5). Then, Microsoft Excel computer software package (Microsoft corp., 2013 version) was used for plotting the charts. Analysis of variance was performed using a One-way ANOVA to test if significance difference existed between mean concentrations of treated and untreated snail shell at 95 % confidence level ($P<0.05$), Tukey's multiple comparison test and student t-test

was also used to test for significant difference between different groups of treatment. All the analyses were conducted in triplicates and expressed as mean data ±SD (standard deviation) (Duncan, 1955).

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Properties of the Snail (*Achatina achatina*) Shell

Table 4.1 shows the results of the analyses of the physicochemical parameters of the snail shell. The pH obtained were 6.94 and 7.88 for treated and untreated snail shells respectively. Also, the bulk density values obtained for both the untreated and treated snail shells were 0.72 g/cm^3 and 0.55 g/cm^3 respectively. Similarly, the moisture content obtained were 0.23 % and 0.15 % respectively for untreated and treated snail shell. The values of the ash content for the untreated and the treated snail were 11.65 % and 7.94 % respectively.

4.2 Composition of the Snail (*Achatina achatina*) Shell

The snail shell was characterised using X-ray Fluorescence (XRF). The X-ray fluorescence (XRF) analysis of the snail shell for the major and minor components are given in Table 4.2. About eleven elements with their oxides were identified. The snail shell was substantial with CaO; this showed calcium as the major element making up to 96.13 % and 96.71 % respectively for untreated and treated snail shell.

4.3 Identification of *Bacillus subtilis* and *Pseudomonas putida* Isolates based on Microgen®*Bacillus and Enterobacteriaceae* (GNA+GNB) Kits

Table 4.3 shows the result of the bacteria isolated. The probability of the respective organism as revealed by the microgen® kit confirmed that the organisms are *Pseudomonas putida* and *Bacillus subtilis*. The isolate's number, probability and the identity of the bacteria were all listed in Table 4.3.

Table 4.1: Physicochemical Properties of the *Achatina achatina*

Parameters	Untreated Snail Shell	Treated (Activated) Snail Shell
Colour	Milky white	Ash
pH	7.88 ± 0.12	6.94 ± 0.11
Moisture content (%)	0.23 ± 0.02	0.15 ± 0.02
Ash content (%)	11.65 ± 0.16	7.94 ± 0.11
Bulk density (g/cm^3)	0.72 ± 0.03	0.55 ± 0.02

Table 4.2 Composition of *Achatina achatina*

Component	Weight Composition (%)	
	Untreated snail shell	Treated snail shell
MgO	0.05	0.07
Al_2O_3	0.65	0.74
SiO_2	1.97	1.83
P_2O_5	0.15	0.14
SO_3	0.18	0.19
Cl	0.07	0.06
K_2O	0.14	0.11
CaO	96.13	96.21
TiO_2	0.01	0.01
Fe_2O_3	0. 10	0.08
SrO	0.56	0.54

Table 4.3: Identification of *Bacillus subtilis* and *Pseudomonas putida* Isolates based on Microgen kit

Isolate's Code	Prophile Number	Probability (%)	Identity
TE1	73710427	99.68	*B. subtilis*
TE2	74370622	95.75	*B. subtilis*
TE3	74370622	95.75	*B. subtilis*
REC1	640522001	90.50	*P. putida*
REC2	640722001	90.50	*P. putida*
REC3	640722001	90.50	*P. putida*

4.4 Crude Oil Utilization by the Isolates of *Bacillus subtilis* and *Pseudomonas putida*

The result obtained on the ability of wild strains of *Bacillus subtilis* and *Pseudomonas putida* isolates to grow on mineral medium containing varying concentrations of crude oil as the sole carbon source was presented in Table 4.4. *Bacillus subtilis* isolate TE1 and *Pseudomonas putida* REC2 showed luxuriant growth in Mineral medium containing 0.5 %, 1 % and 1.5 % concentrations of crude oil but medium growth on that containing 2 % crude oil. All the isolates of *Bacillus subtilis* and *Pseudomonas putida* showed luxuriant growth on medium containing 0.5 % crude oil except isolate REC1 which had medium growth on 0.5 % crude oil medium. Also, the isolates TE2, TE3, REC1 and REC3 showed medium growth on 1 % crude oil and scanty growth on medium containing 1.5 % crude oil respectively. Also, isolate REC1 showed no evidence of growth on the mineral medium containing 2 % crude oil while all the other isolates of both *Bacillus subtilis* and *Pseudomonas putida* showed scanty growth.

4.5 Adsorption and Biodegradation of Naphthalene

4.5.1 Effect of initial naphthalene concentration

Figure 4.1 shows the effect of concentration on the adsorption and biodegradation of naphthalene. This was investigated over a concentration of 10 – 50 mg/dm^3 at constant pH of 7 and adsorbent dosage of 2 g. It was observed that as the initial naphthalene concentration increases, there was a corresponding increase in the percentage removal of the naphthalene for both the adsorption and the biodegradation process. The naphthalene removal was predominant with the *Pseudomonas putida* and *Bacillus subtilis* immobilized on both treated (activated) and untreated snail shell.

Table 4.4: Growth of *Bacillus subtilis* and *Pseudomonas putida* Isolates on Mineral Medium Containing Crude Oil

Isolate's code	organism	Crude Oil Concentration (%)			
		0.5	1.0	1.5	2.0
TE1	*B. subtilis*	+++	+++	++	++
TE2	*B. subtilis*	+++	++	+	+
TE3	*B. subtilis*	+++	++	+	+
REC1	*P. putida*	++	++	+	-
REC2	*P. putida*	+++	+++	++	+
REC3	*P. putida*	+++	++	+	+

KEY: + = Scanty growth, ++ = Medium growth, +++ = Luxuriant growth, - = No growth

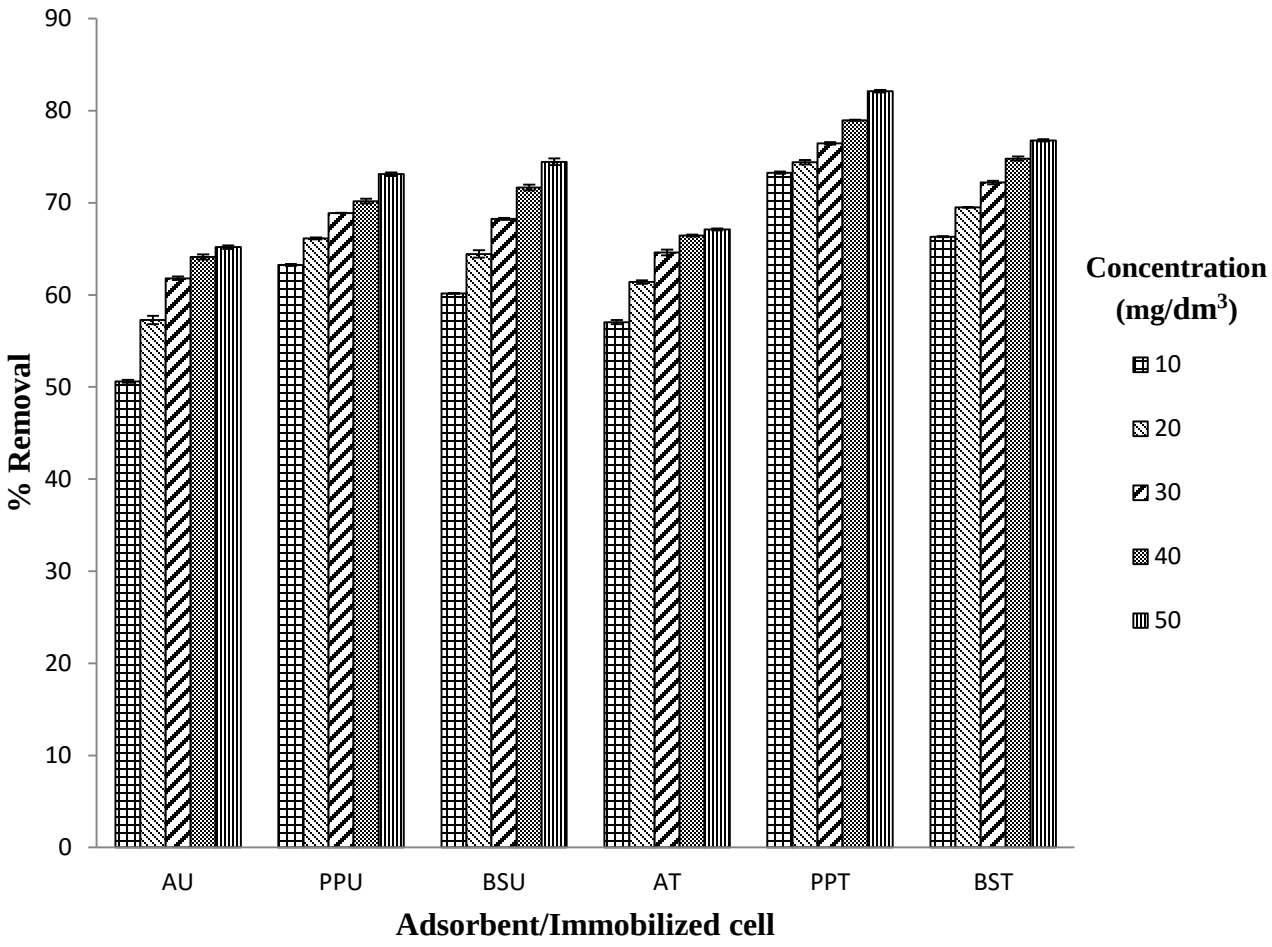


Figure 4.1: Effect of Initial Concentration on the Adsorption/SAB of Naphthalene

Key:

AU = untreated snail shell

AT = treated snail shell

PPU = *Pseudomonas putida* incorporated on untreated snail shell

PPT = *Pseudomonas putida* incorporated on treated snail shell

BSU = *Bacillus subtilis* incorporated on untreated snail shell

BST = *Bacillus subtilis* incorporated on treated snail shell

4.5.2 Effect of adsorbent dosage

Figure 4.2 shows the percentage of naphthalene adsorbed and biodegraded at varying adsorbent doses set at 2, 3 and 5 g respectively at fixed naphthalene concentration (50 mg/dm^3) and pH of 7. It was observed that there was a gradual decrease in the amount of naphthalene adsorbed and biodegraded per unit mass of the adsorbent dose increases from 2 to 5 g.

4.5.3 Effect of pH

Figure 4.3 is an illustration of the effect of pH on the adsorption and biodegradation of naphthalene at constant naphthalene concentration of 50 mg/dm^3 and optimum dosage value of 2 g. It shows a contrasting trend on the adsorption and biodegradation of naphthalene. The effect of pH on the adsorption of naphthalene using both treated (activated) and untreated snail shell shows a corresponding progressive increase in the percent removal of naphthalene as the pH was increased from 5 to 9, with the optimum percent removal of 65.32 % and 67.51 % occurring at pH 9 respectively for both the treated (activated) and untreated snail shell. It was evident as shown in figure 4.3, that increasing the pH of the synthetic wastewater from 5 to 7, there was an increase in the percent removal of naphthalene. But, the percent removal began to decrease as the pH moved from pH 7 to pH 9.

4.6 FTIR Analysis of Naphthalene and its Metabolites

Figure 4.4 shows the FTIR of naphthalene before degradation with major peaks showing aromatic C-H bond at 3047 cm^{-1}, aromatic ring at 1581 cm^{-1} and C=C bond at 1381 cm^{-1}. Figure 4.5 also shows the peaks obtained from the FTIR of the naphthalene metabolites

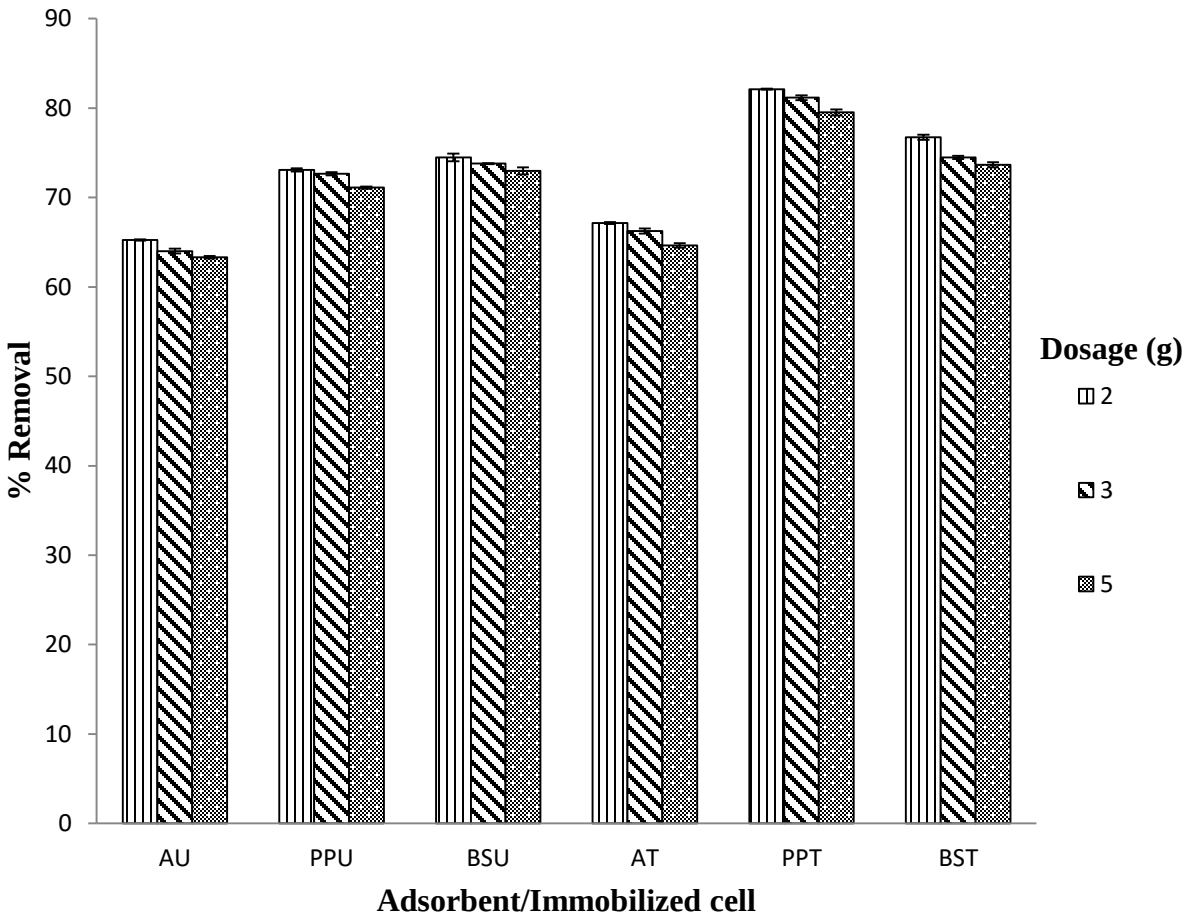


Figure 4.2: Effect of Adsorbent Dosage on the Adsorption/SAB of Naphthalene

Key:

AU = untreated snail shell

AT = treated snail shell

PPU = *Pseudomonas putida* incorporated on untreated snail shell

PPT = *Pseudomonas putida* incorporated on treated snail shell

BSU = *Bacillus subtilis* incorporated on untreated snail shell

BST = *Bacillus subtilis* incorporated on treated snail shell

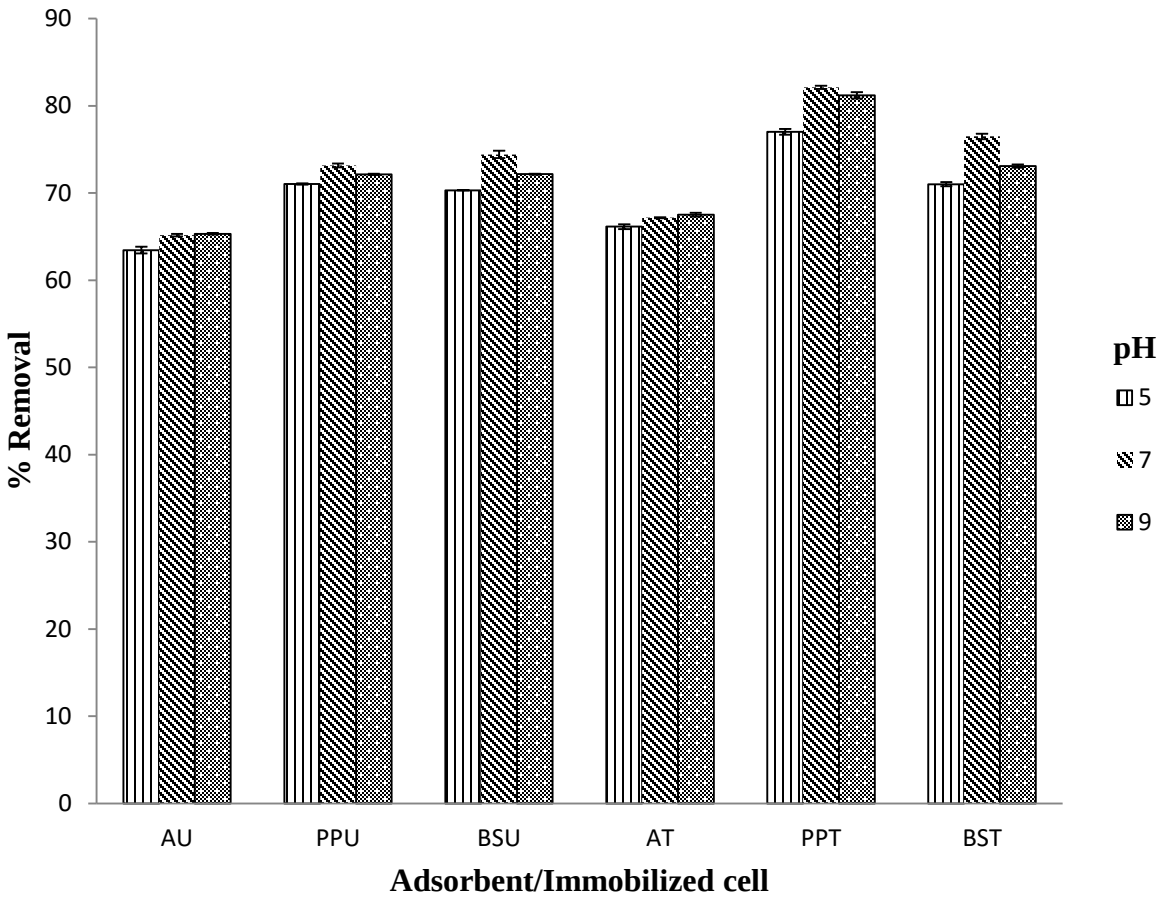


Figure 4.3: Effect of pH on the Adsorption/SAB of Naphthalene

Key:

AU = untreated snail shell

AT = treated snail shell

PPU = *Pseudomonas putida* incorporated on untreated snail shell

PPT = *Pseudomonas putida* incorporated on treated snail shell

BSU = *Bacillus subtilis* incorporated on untreated snail shell

BST = *Bacillus subtilis* incorporated on treated snail shell.

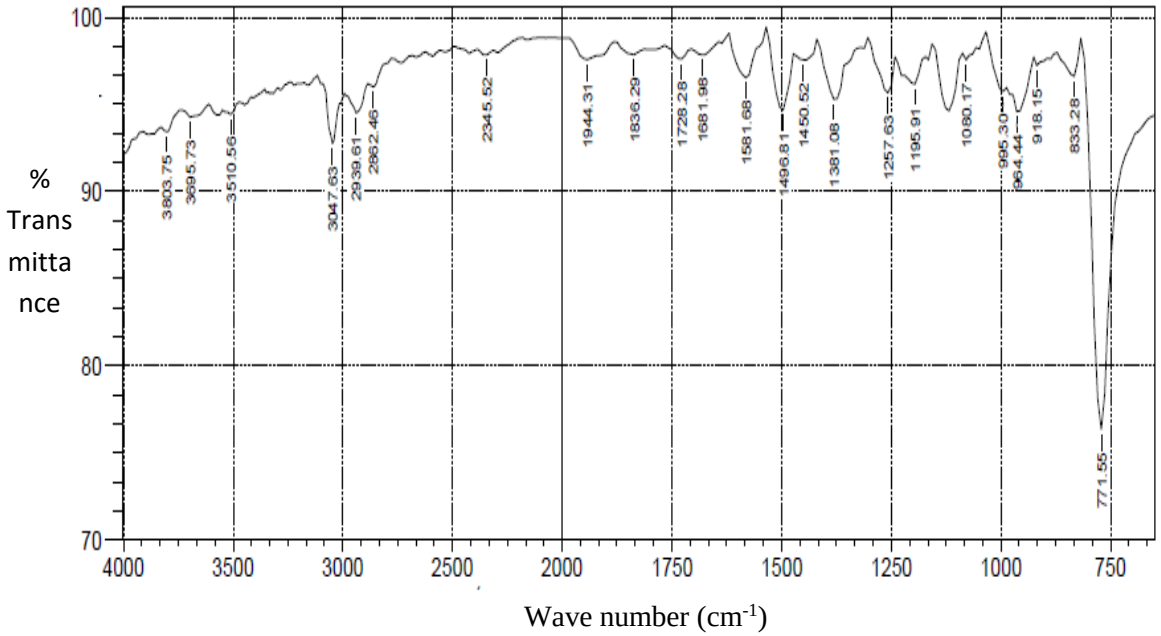


Figure 4.4: FTIR spectrum of naphthalene

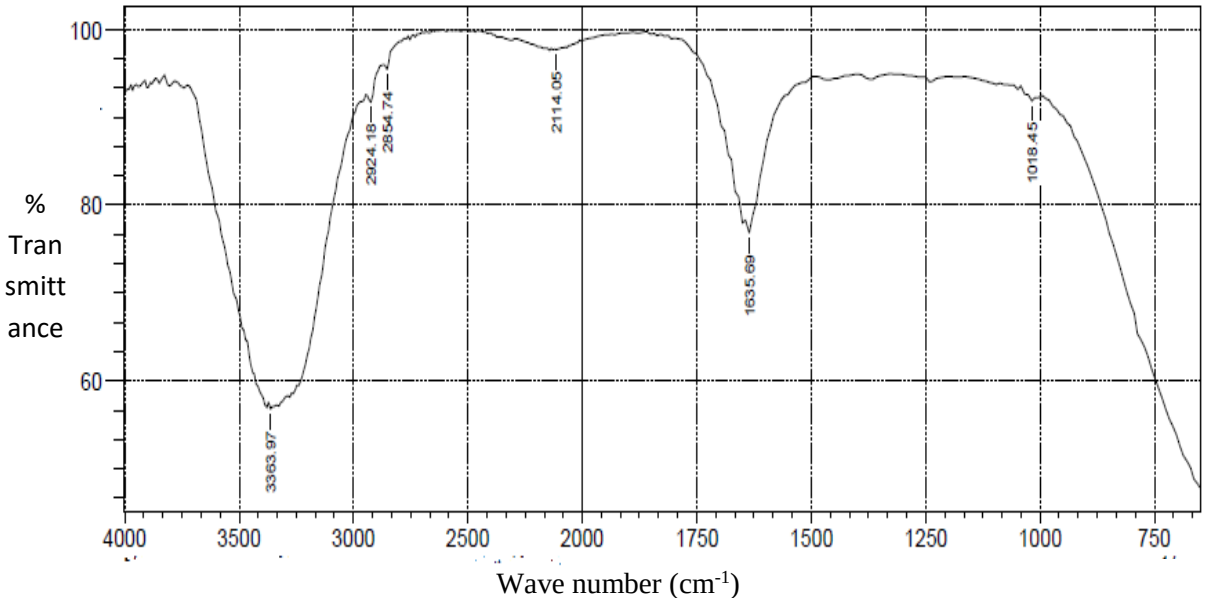


Figure 4.5: FTIR spectrum of naphthalene metabolites

after the biodegradation process. A broad peak of hydroxyl group was obtained at 3363 cm^{-1}, C=O bond 1635 cm^{-1} and C-H bond stretch at 2924 cm^{-1}respectively.

4.7 Comparison of Adsorption of Naphthalene and its Biodegradation

Table 4.5 shows the analysis of variance (ANOVA- one way) for the percentage removal was applied to check for significance between the adsorption and adsorption-biodegradation of naphthalene using both treated and untreated snail shell respectively. For untreated snail shell, there was significant difference ($P<0.05$) between AU, PPU and BSU. The Tukey's multiple comparison test showed that PPU is significantly higher ($P<0.05$) than BSU, and BSU is significantly higher ($P<0.05$) than AU. Also, the treated snail showed significant difference ($P<0.05$) between AT, PPT and BST, and the Tukey's multiple comparison test showed that PPT is significantly higher ($P<0.05$) than BST, and BST is significantly higher ($P<0.05$) than AT.

Moreover, the student t-test for comparing the treated and untreated snail shell showed that AT is significantly higher ($P<0.05$) than AU, PPT was significantly higher ($P<0.05$) than PPU and BST is also significantly higher (0.05) than BSU. The various test of significance indicated the effectiveness of adsorption-biodegradation over adsorption. However, the bacteria immobilized on treated snail showed greater efficiency.

4.8 Adsorption and Biodegradation Isotherms

The Langmuir isotherm constants and coefficient of determination, R^2,were obtained from Figure 4.6 – 4.11, while the calculated R_L values for the optimum initial concentration (50 mg/L) for both the adsorption and biodegradation were all listed in the Table 4.6. The experimental data obtained for adsorption and biodegradation showed correlation

Table 4.5: Comparison of Adsorption of Naphthalene and its Biodegradation

Ads/I.C	Naphthalene concentration				
	10 mg/dm^3	20 mg/dm^3	30 mg/dm^3	40 mg/dm^3	50 mg/dm^3
AU	50.62 ± 0.18^{a}	57.28 ± 0.55^{a}	61.81 ± 0.24^{a}	64.12 ± 0.36^{a}	65.20 ± 0.23^{a}
AT	57.05 ± 0.27^{b}	61.40 ± 0.22^{b}	64.62 ± 0.38^{b}	66.46 ± 0.12^{b}	67.10 ± 0.13^{b}
BSU	$60.17 \pm 0.05^{b,c}$	$64.45 \pm 0.49^{b,c}$	$68.26 \pm 0.15^{b,c}$	$71.65 \pm 0.38^{b,c}$	$74.46 \pm 0.46^{b,c}$
PPU	$63.26 \pm 0.13^{b,c,d}$	$66.13 \pm 0.12^{b,c,d}$	$68.90 \pm 0.02^{b,c,d}$	$70.18 \pm 0.32^{b,c,d}$	$73.11 \pm 0.23^{b,c,d}$
BST	$66.33 \pm 0.07^{b,c,d,e}$	$69.51 \pm 0.02^{b,c,d,e}$	$72.21 \pm 0.25^{b,c,d,e}$	$74.80 \pm 0.28^{b,c,d,e}$	$76.79 \pm 0.14^{b,c,d,e}$
PPT	$73.28 \pm 0.14^{b,c,d,e,f}$	$74.41 \pm 0.30^{b,c,d,e,f}$	$76.46 \pm 0.16^{b,c,d,e,f}$	$78.96 \pm 0.08^{b,c,d,e,f}$	$82.12 \pm 0.16^{b,c,d,e,f}$

Key:

Letters (a, b, c, d, f) showed there is significant difference ($P<0.05$) within columns

Ads = Adsorbent

I.C = Immobilized cells

AU = untreated snail shell

AT = treated snail shell

PPU = *Pseudomonas putida* incorporated on untreated snail shell

PPT = *Pseudomonas putida* incorporated on treated snail shell

BSU = *Bacillus subtilis* incorporated on untreated snail shell

BST = *Bacillus subtilis* incorporated on treated snail shell

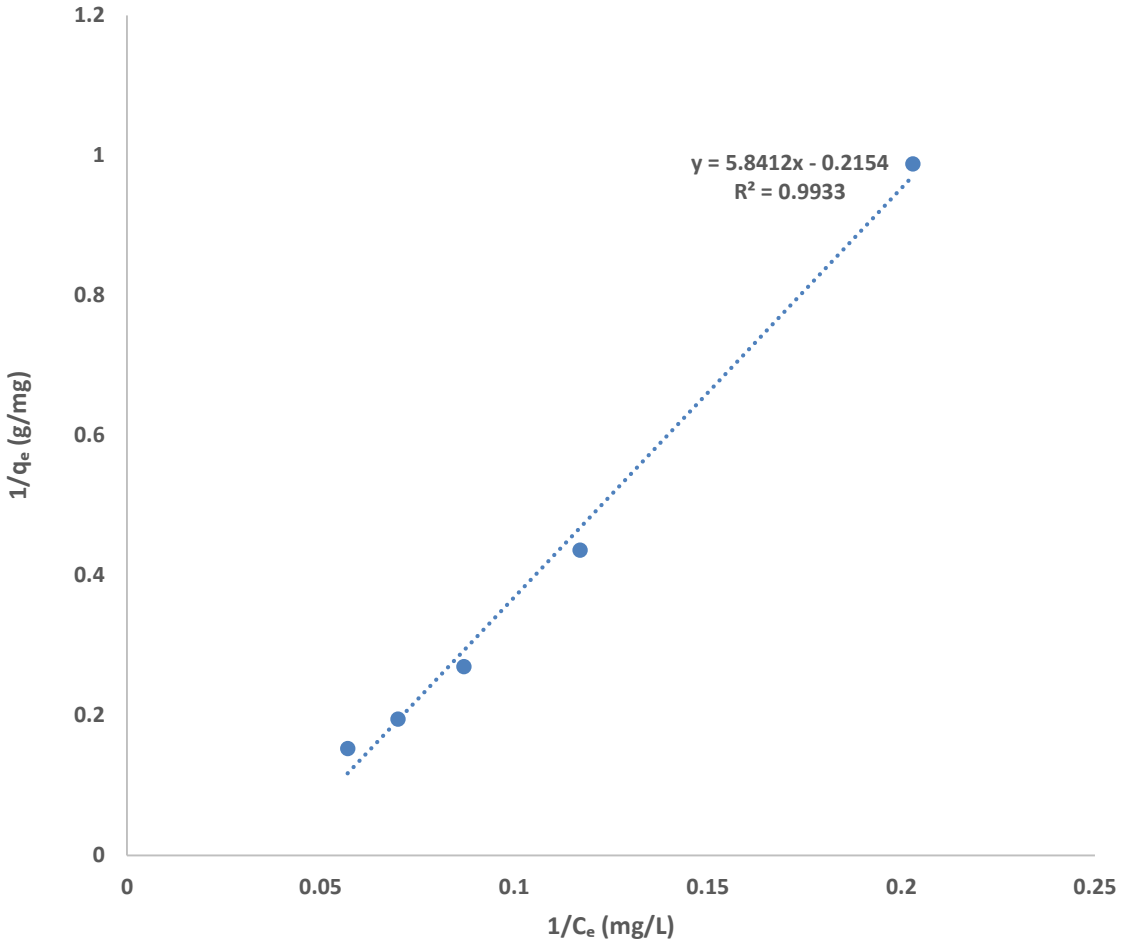


Figure 4.6: Langmuir Isotherm for the Adsorption of Naphthalene with untreated Snail shell (AU)

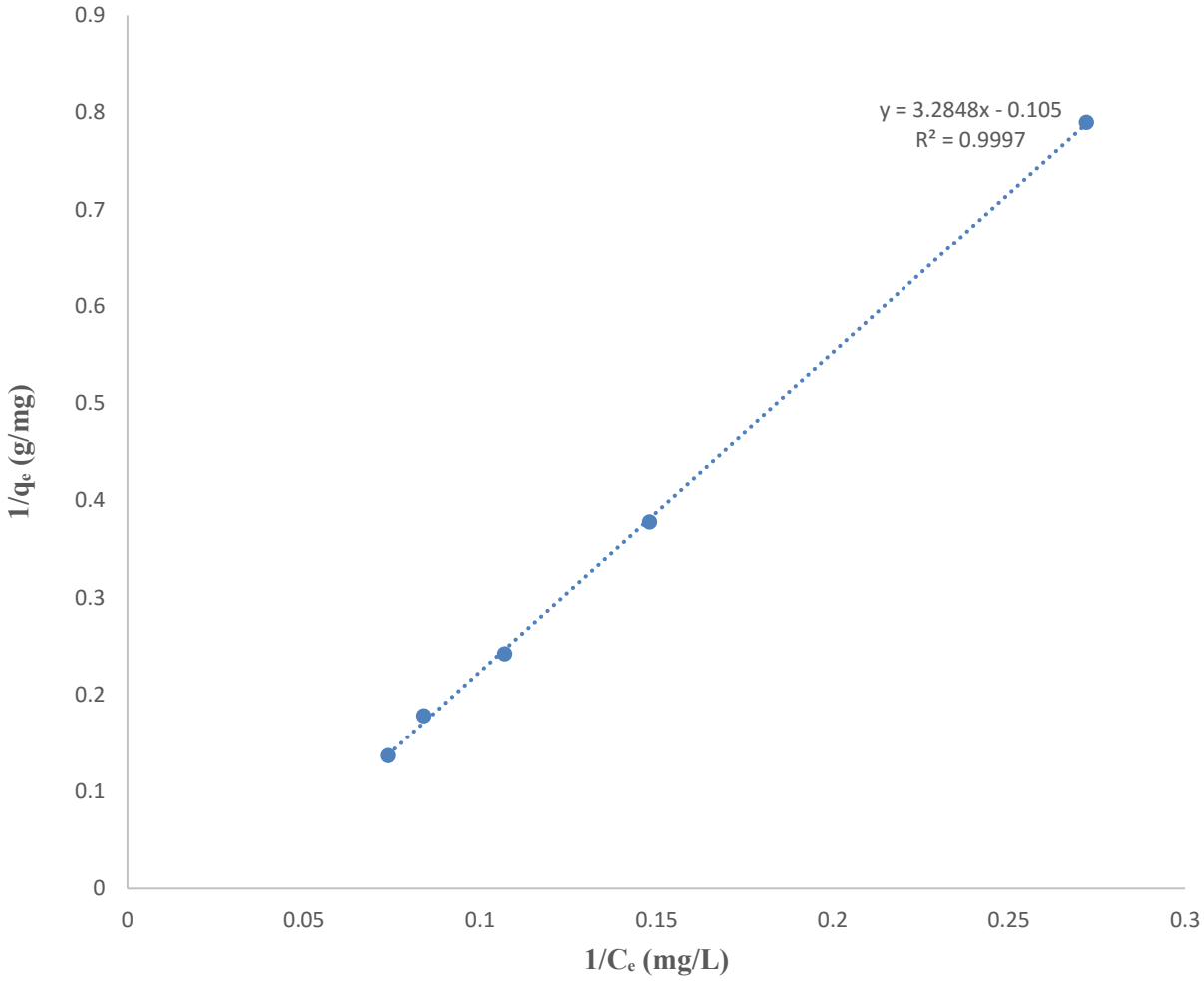


Figure 4.7: Langmuir Isotherm for the SAB of Naphthalene with *Pseudomonas putida* immobilized on untreated Snail shell (PPU)

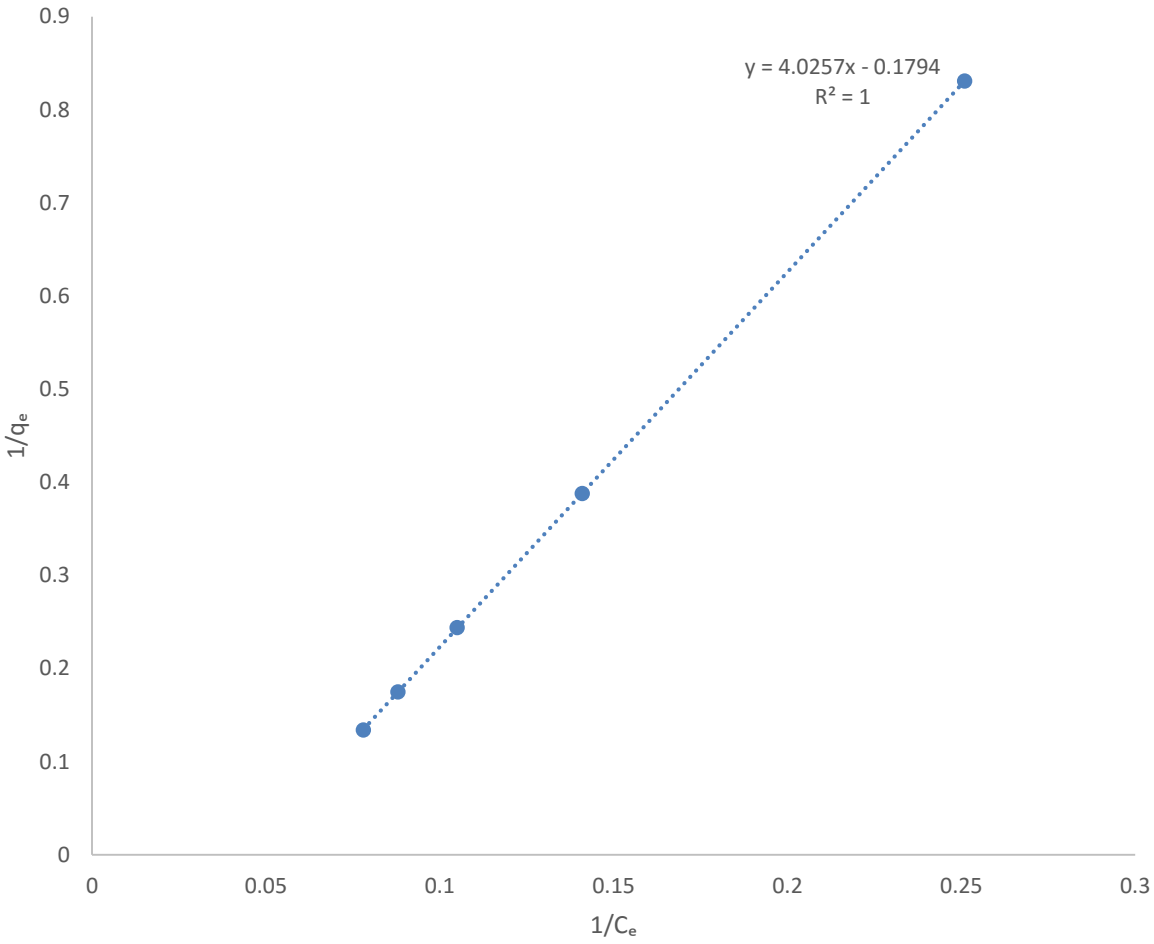


Figure 4.8: Langmuir Isotherm for the SAB of Naphthalene with *Bacillus subtilis* immobilized on untreated snail shell (BSU)

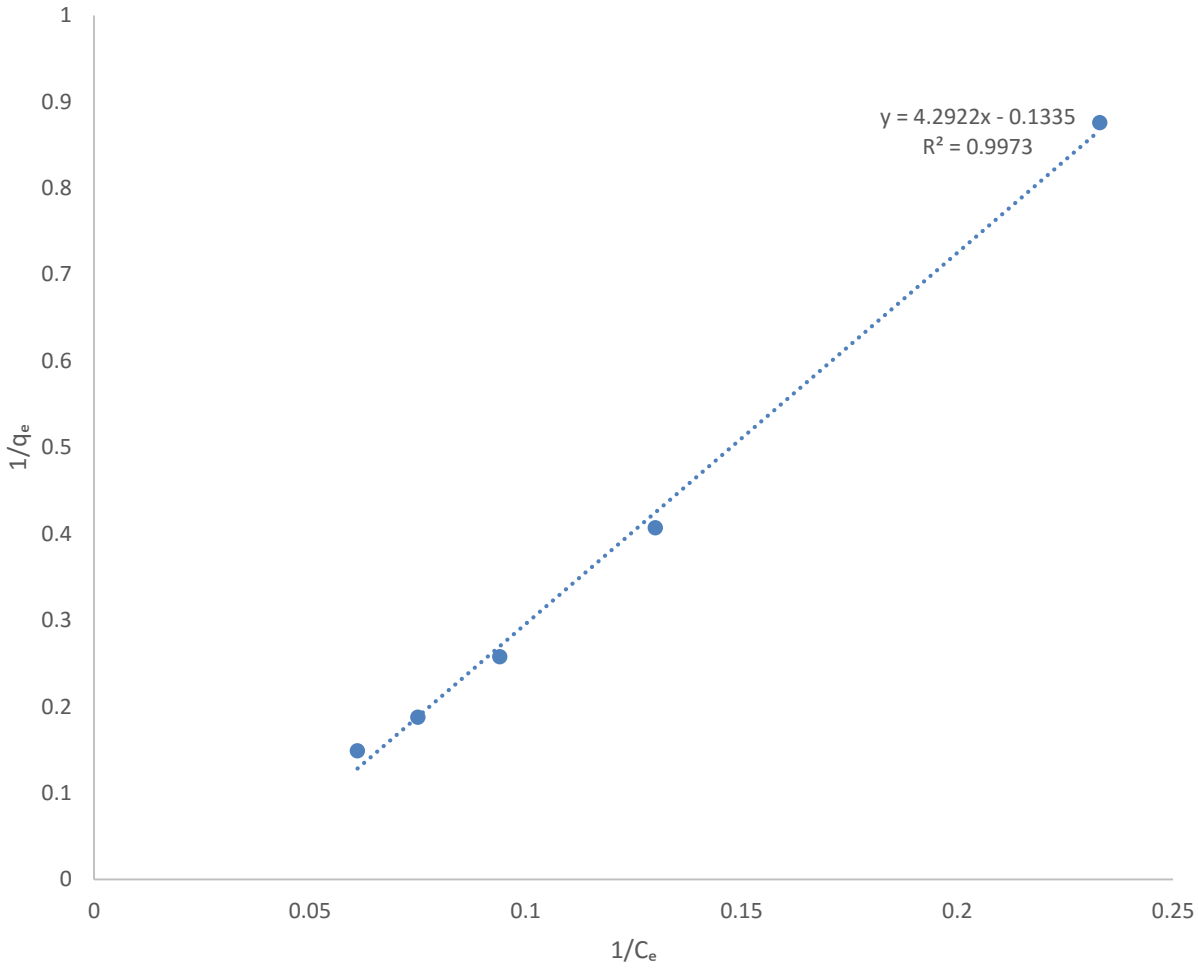


Figure 4.9: Langmuir Isotherm for the Adsorption of Naphthalene with Treated Snail shell (AT)

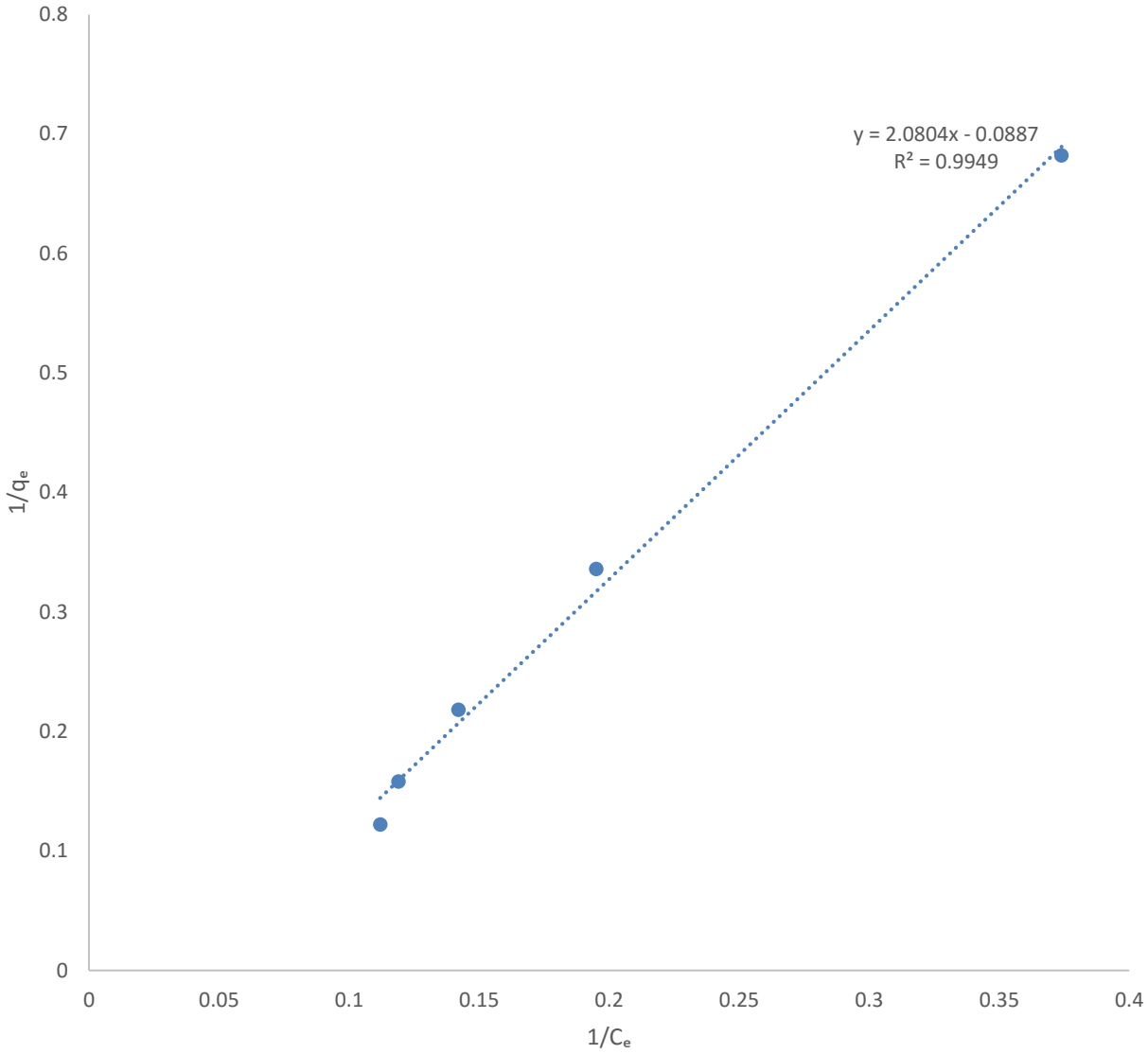


Figure 4.10: Langmuir Isotherm for the SAB of Naphthalene with *Pseudomonas putida* immobilized on Treated Snail shell (PPT)

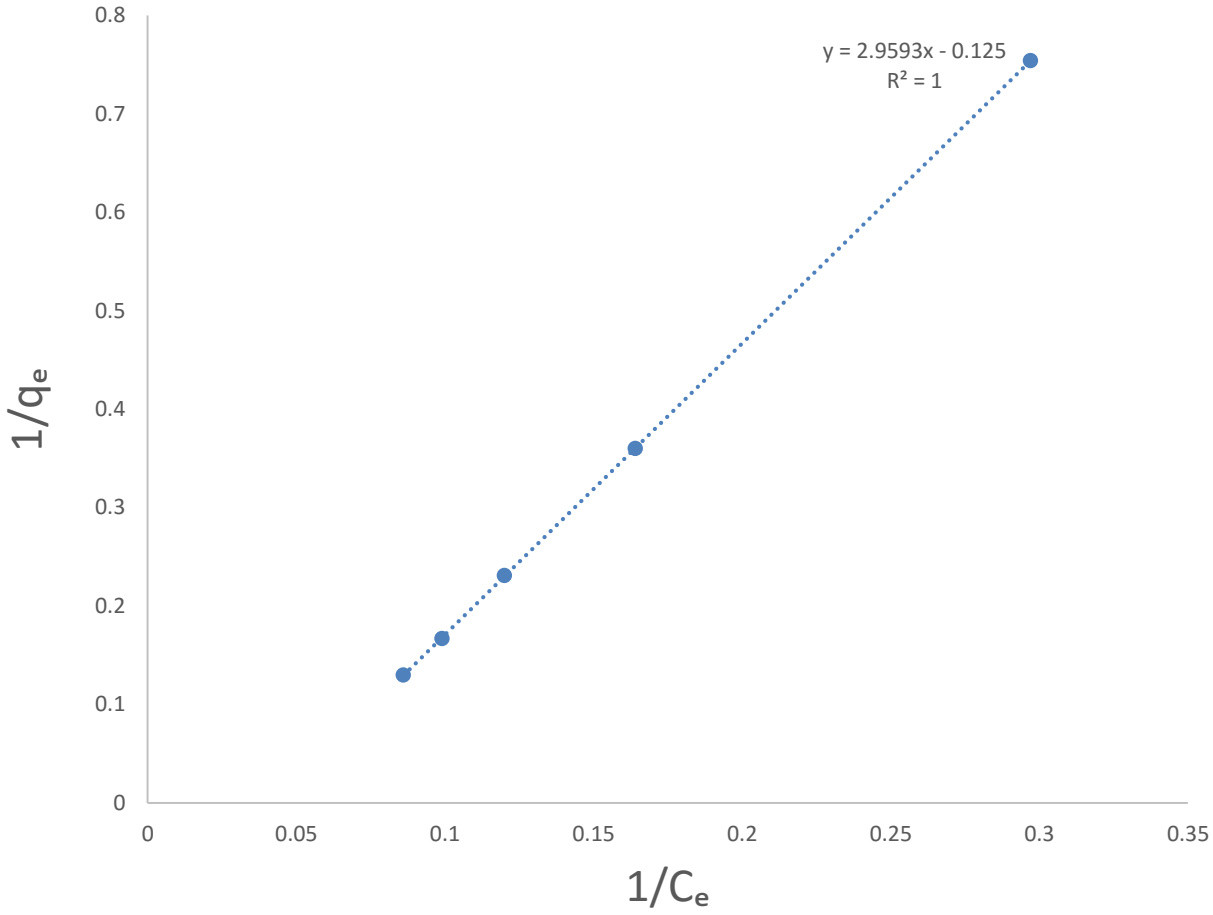


Figure 4.11: Langmuir Isotherm for the SAB of Naphthalene with *Bacillus subtilis* immobilized on Treated Snail shell (BST)

Table 4.6: Correlation Parameters of Langmuir and Freundlich Isotherms

	Langmuir				Freundlich		
Adsorbent/ Immobilized cells	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
AT	-7.49	-0.0311	-1.8018	0.9973	0.75	0.16	0.9994
PPT	-11.27	-0.0426	-0.8850	0.9947	0.74	0.36	0.9721
BST	-8.00	-0.0422	-0.9009	1.0000	0.71	0.23	0.9947

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.

coefficient, R^2, to be 0.9933 (AU), 0.9997 (PPU), 1.0000 (BSU), 0.9973 (AT), 0.9949 (PPT) and 1.0000 (BST) respectively, indicating a good fit to the Langmuir isotherm.

Table 4.6 also shows the values of the Freundlich Constants n and K_f, for the adsorption and biodegradation study which were obtained from Figure 4.12 – 4.17. For the adsorption of naphthalene with treated (activated) and untreated snail shell, the n values were 0.7484 and 0.6672 respectively, while their respective $K_f$values are 0.0929 and 0.1624. Also, the n and $K_f$values for the biodegradation were as follows; with *Pseudomonas putida* immobilized on utreated snail (n = 0.7561,K_f = 0.2194) and treated snail shell (n = 0.7397, K_f = 0.3625).For *Bacillus subtilis* immobilized on untreated snail shell n = 0.6495, K_f= 0.1357 and treated snail shell n = 0.7127, K_f = 0.2323.

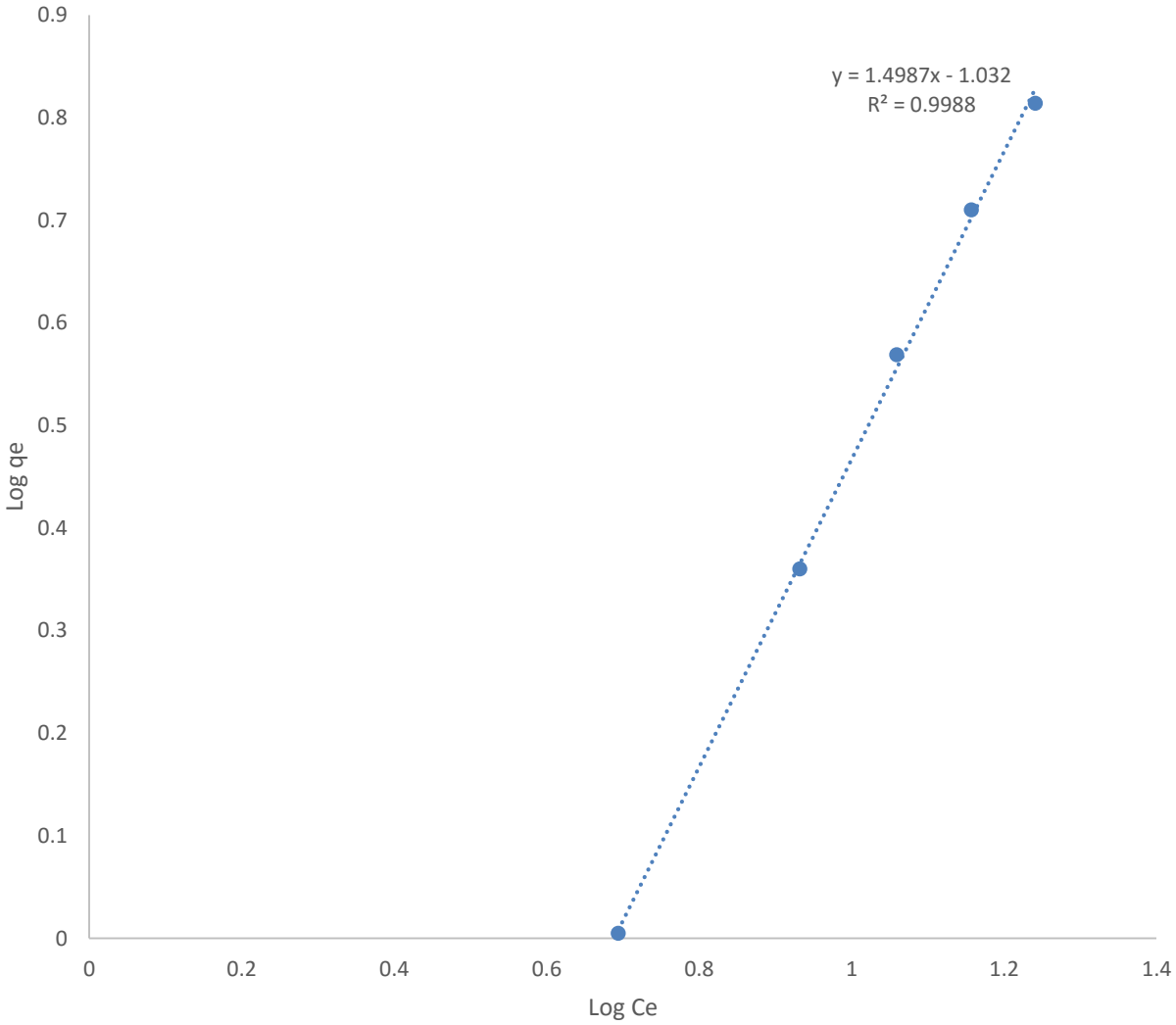


Figure 4.12: Freundlich Isotherm for the Adsorption of Naphthalene with Untreated Snail shell (AU)

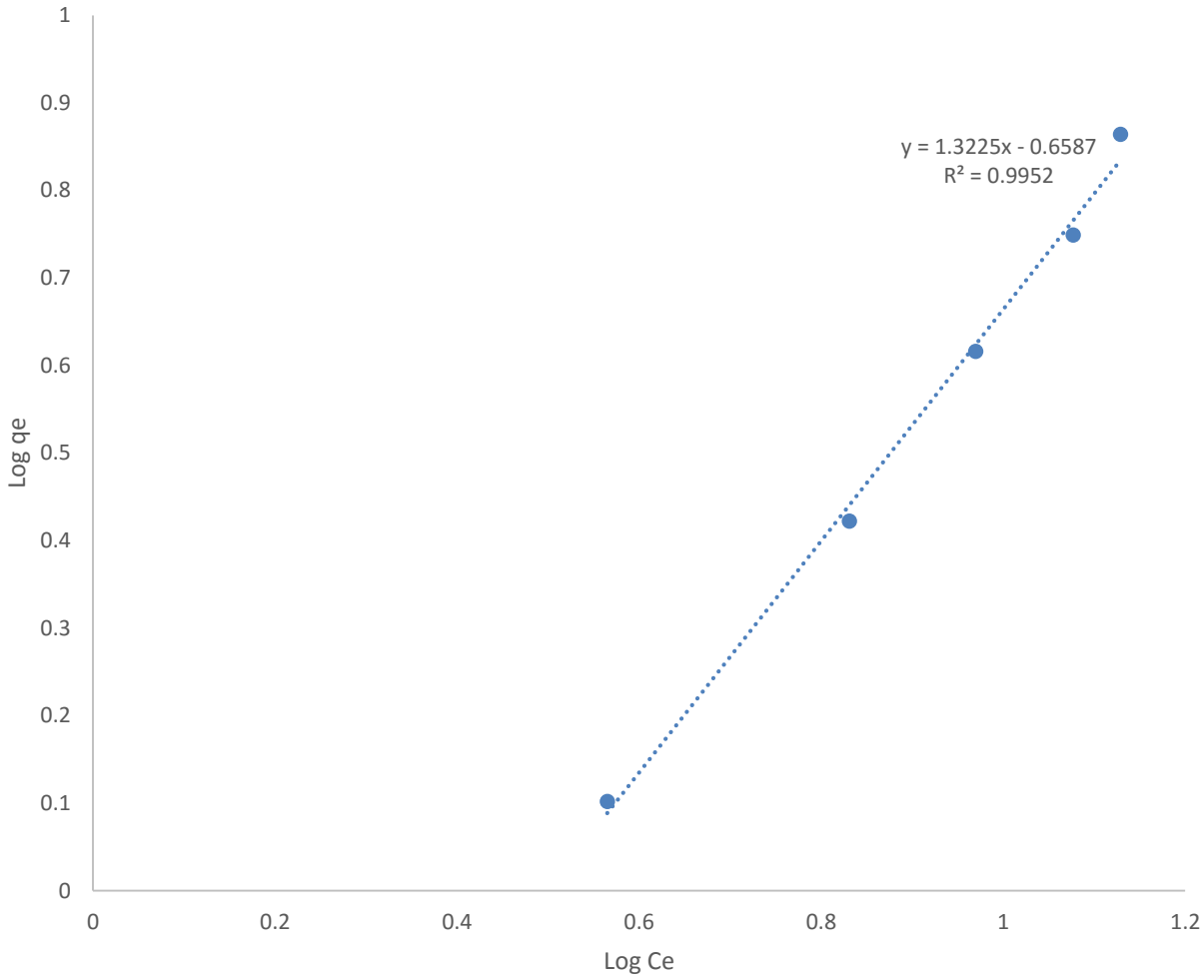


Figure 4.13: Freundlich Isotherm for the SAB of Naphthalene with *Pseudomonas putida* immobilized on untreated snail shell (PPU)

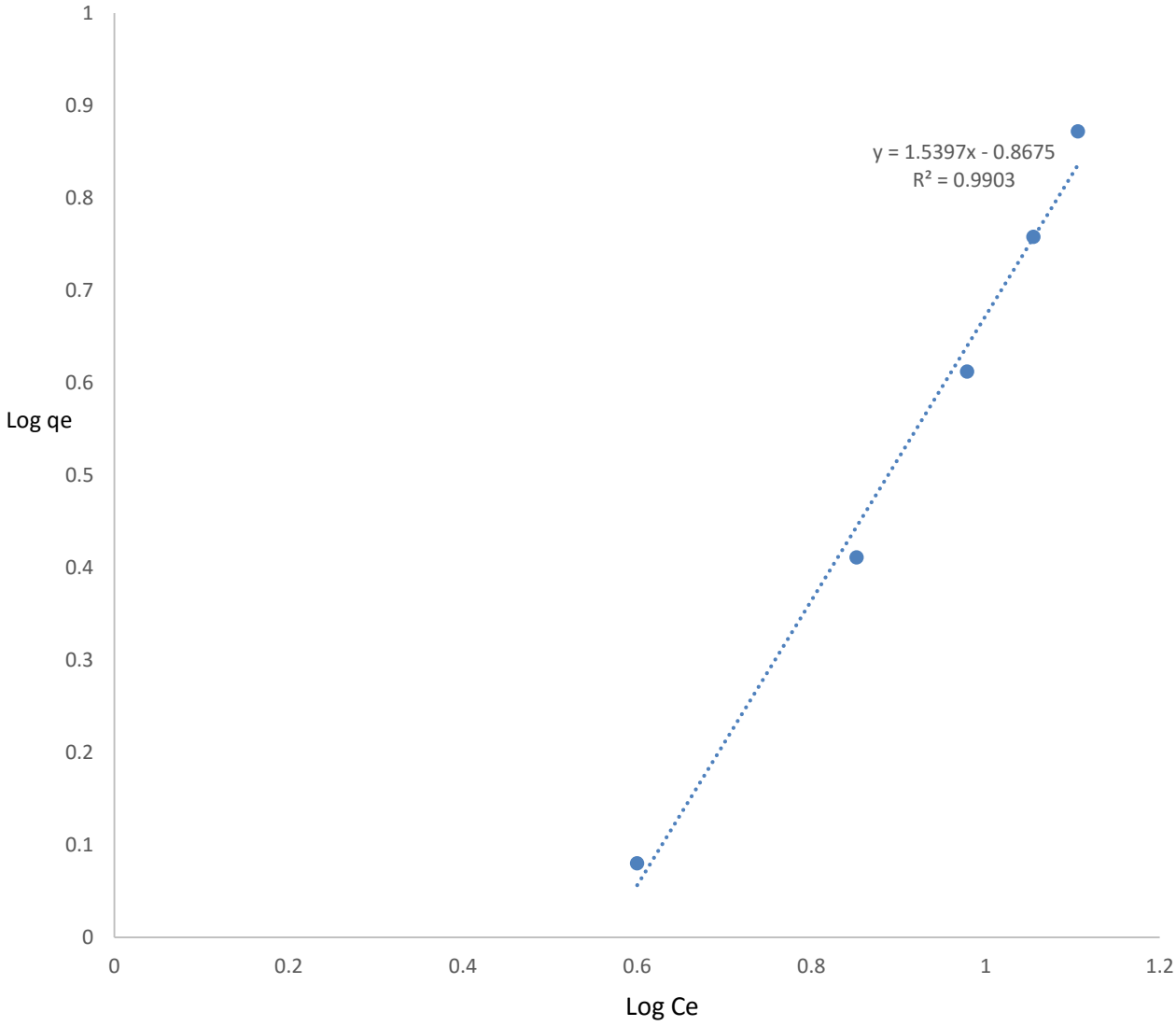


Figure 4.14: Freundlich Isotherm for the SAB of Naphthalene with *Bacillus subtilis* immobilized on Untreated Snail shell (BSU)

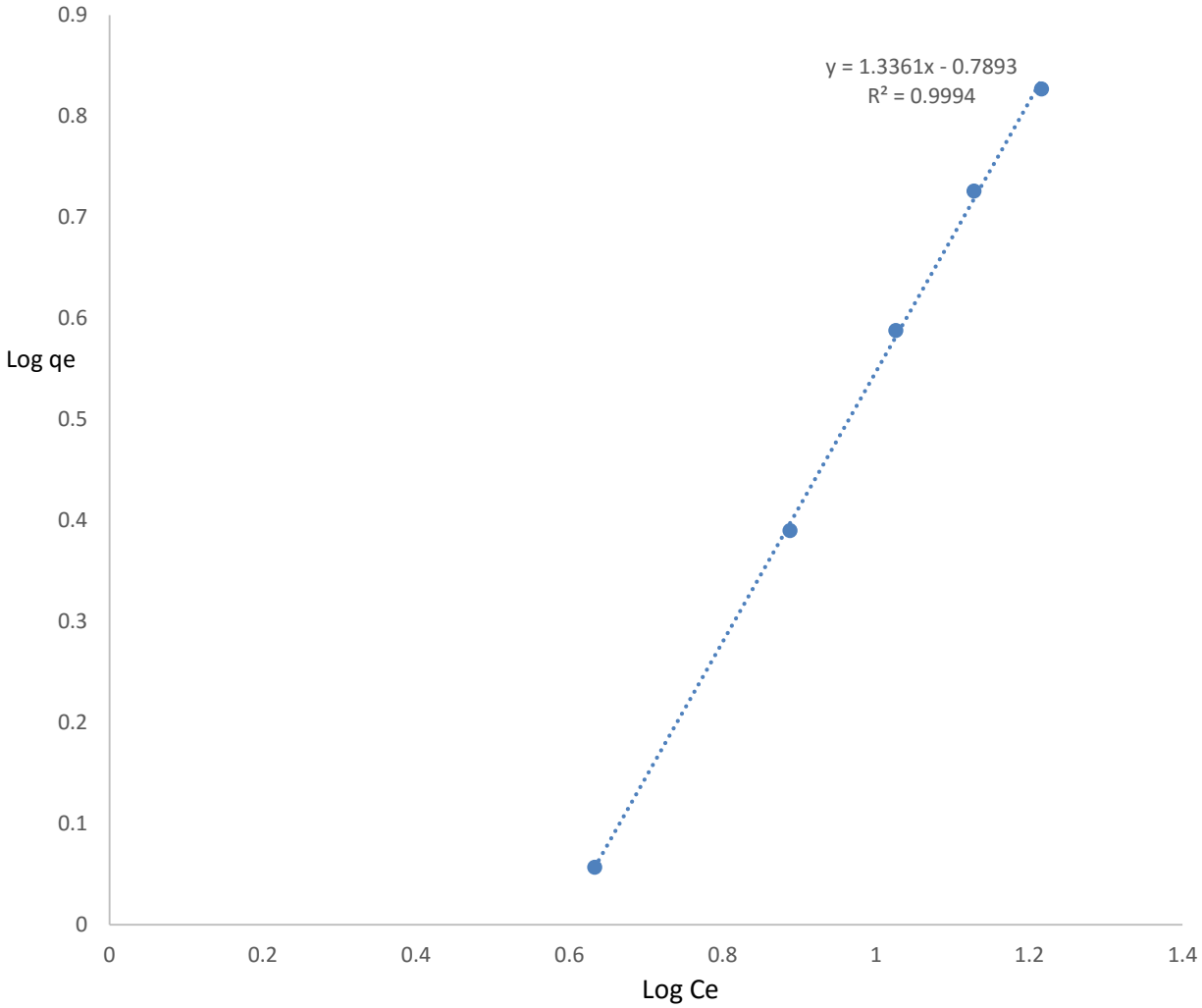


Figure 4.15: Freundlich Isotherm for the Adsorption of Naphthalene with treated Snail shell (AT)

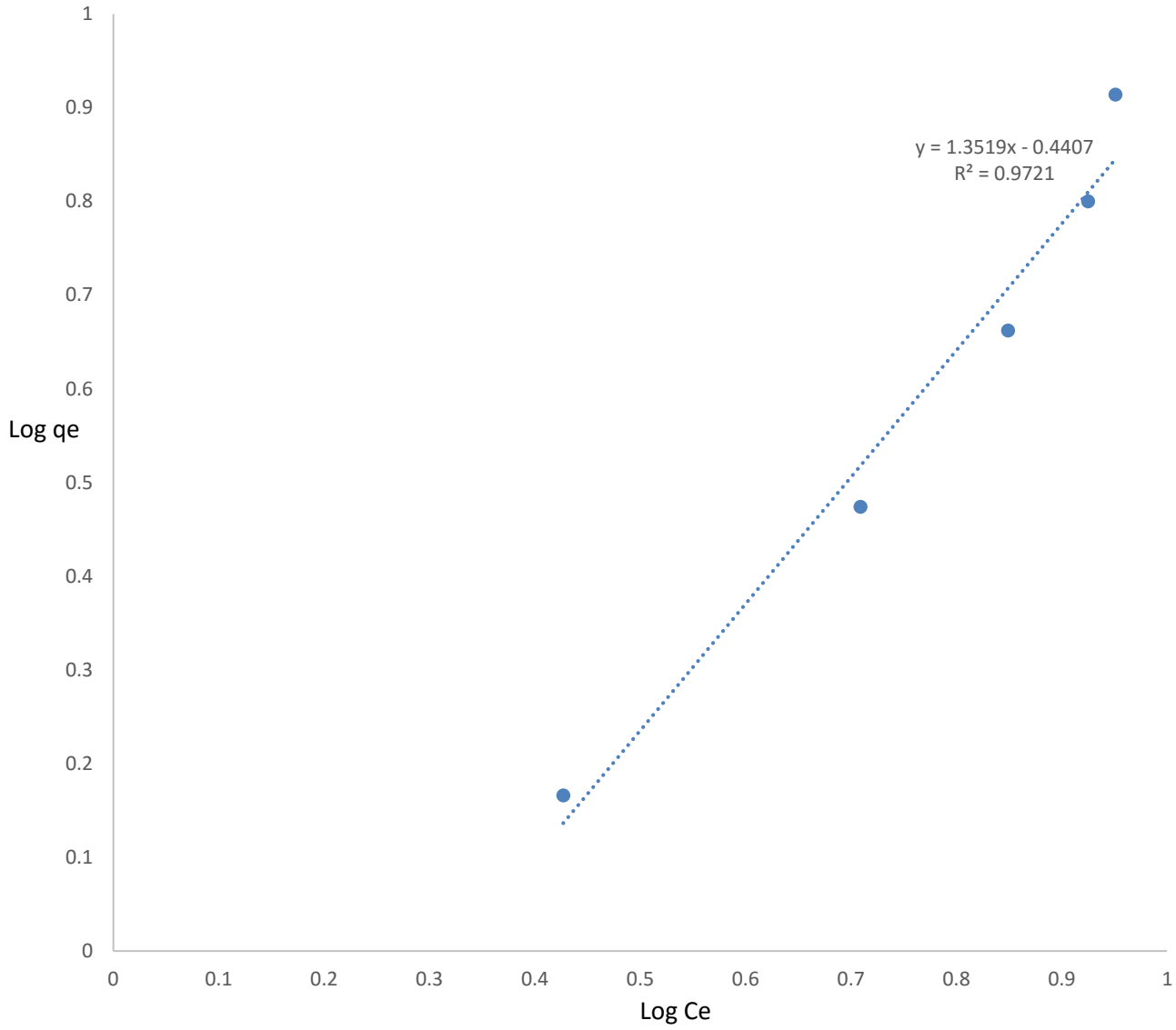


Figure 4.16: Freundlich Isotherm for the SAB of Naphthalene with *Pseudomonas putida* immobilized on Treated snail shell (PPT)

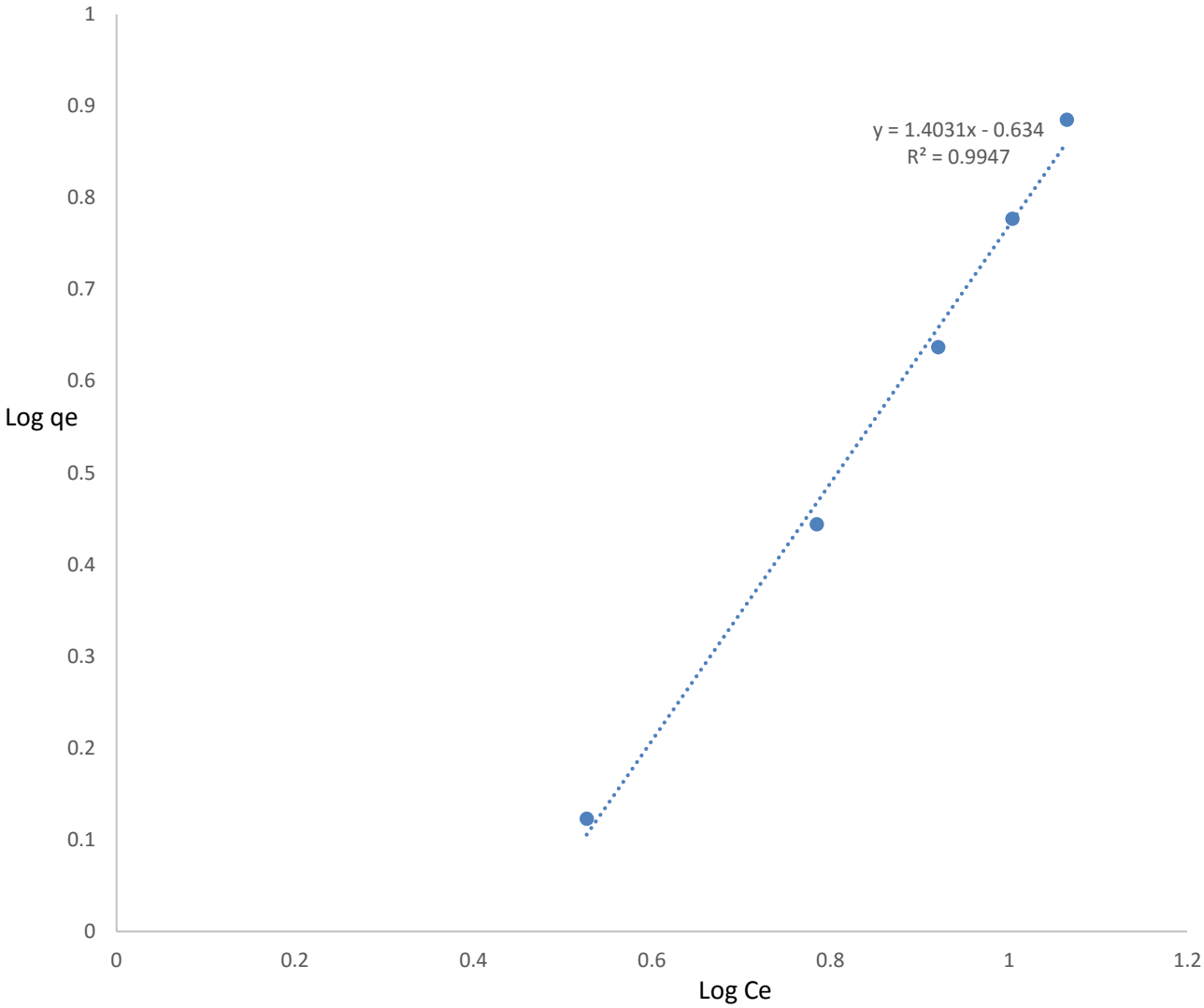


Figure 4.17: Freundlich Isotherm for the SAB of Naphthalene with *Bacillus subtilis* immobilized on Treated Snail shell (BST)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Physicochemical Properties of *Achatina achatina*

5.1.1 pH

The low pH of the treated snail shell (6.94) can be attributed to its treatment with acid and further washing during the preparation process. However, the alkaline pH of the untreated snail shell (7.88) may be due to the presence of inorganic matter content such as calcium carbonate, and also the protein content present in it. This is similar to existing literature where the pH of *Achatina achatina* was reported to be between 2.0 -7.5 (Jatto *et al*., 2010; Udeozor and Evbuomwan, 2014). However, the various pH obtained showed that they are close to neutrality, thus ensuring a good medium for the bacteria to thrive.

5.1.2 Moisture content

The low moisture content values obtained for both treated and untreated snail shell make them better adsorbent for this study. Although the treated snail was more suitable as it had lower moisture content, this corroborates with report that the rate of adsorption of contaminant increases when adsorbents with low moisture contents are employed (Rengaraj *et al.*, 2002).

5.1.3 Ash content

The ash content of the treated (activated) snail shell is relatively lower compared to that of the untreated snail shell. The values of the respective ash content obtained showed that the snail shell had good mechanical strength to be used as medium for microbial cells during biodegradation. The snail shell used for this study had low inorganic constituents as shown

from the values obtained. This agrees with the existing literature that lower ash content in activated carbon increases the carbon yield. (Adib *et al.,* 2016).

5.1.4 Bulk density

The values for bulk density of the untreated and treated snail shell were 0.72 and 0.55 g/cm^3 respectively. These values are similar to those reported by Ambali *et al.,* (2015) for snail shell (*Achatina achatina*). The bulk densities obtained in this study revealed that the snail shell could be of great potential for wastewater treatment as the values are higher than 0.25 g/cm^3, the minimum required for commercial adsorbents as reported by Denver (1991).

5.2 Composition of the Snail Shell

The data obtained indicated variation in the elemental concentrations in both samples, with calcium as the substantial major element making up to 96.21 % and 96.13 % respectively for the treated and untreated snail shell. The major elements present in shell aided microorganisms to produce enzymes needed for the breaking down of contaminants, this agrees with the existing literature that excessive nutrient concentrations are capable of improving microbial growth, thus increasing biodegradation rate (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.

5.3 Utilization of Crude Oil as Source of Carbon by the Wild Strains of *Bacillus subtilis* and *Pseudomonas putida*

The difference in the ability of the various strains of *Bacillus subtilis* and *Pseudomonas putida* to grow on mineral medium containing varying concentrations of crude oil may be attributed to better autochthonous adaptation with corresponding better developed enzyme system for

hydrocarbon metabolism of certain strains than others and the presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species. This is similar to reports by Majid *et al*., 2008 and Onuoha *et al*., 2011.

5.4 Adsorption and Adsorption-Biodegradation (SAB) of the Naphthalene

5.4.1 Effect of initial naphthalene concentration

The results obtained in both the adsorption and simultaneous adsorption-biodegradation (SAB) 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, the percent removal was predominant with the bacteria incorporated on treated and untreated snail shell. Similar observations have been reported by Lin *et al.,* (2015), on the biodegradation of tetradecane using *Acinetobacter venetianus* on bagasse. As the concentration increased, the percent removal was higher in the biodegradation process across all the concentration range than the adsorption. This shows that biodegradation predominated after adsorption had reached equilibrium, thus showing the effectiveness of biodegradation on the removal of naphthalene from wastewater as observed by Agarry and Aremu (2012). Also, the advantage exhibited by biodegradation over adsorption of naphthalene showed that the mechanism works partly due to the fact that naphthalene can be adsorbed onto the snail shell and further degraded by the two bacteria incorporated on the snail shell respectively. More so, the success could also depend on the various major elements present in the snail shell which serves as source of nutrients and as medium that aid the various bacteria. Furthermore, the optimum removal rate for the adsorption study was 67.10 % as obtained with treated snail shell compared to untreated snail shell with 65.20 %. Also, for the simultaneous adsorption-biodegradation; *Pseudomonas*

putida and *Bacillus subtilis* immobilized on treated (activated) snail shell, both have the optimum removal rate of 82.12 % and 76.79 % respectively compared to *Pseudomonas putida* (73.11 %) and *Bacillus subtilis* (74.46 %) immobilized on the untreated snail shell. This is an indication that carbonization and activation of adsorbents improved their adsorptive capacities hence, leading to higher rate of contaminants removal as observed by Owabor and Iyasele, (2006); Owabor and Ogunsakin (2006). This could also be linked to availability of active sites on the snail due to the effect of its treatment by the orthophosphoric acid.

5.4.2 Effect of adsorbent dosage

The percentage removal of naphthalene both for the adsorption and adsorption-biodegradation were found to decrease with increasing quantity of adsorbent from 2 g to 5 g. It is assumed that adsorption capacity increases with increased adsorbent dosage, since the number of particles increased and, more surface would be available for adsorbate binding as reported by Oladunni *et al.,* (2012). However, a further increase in the adsorbent dosage beyond 2 g resulted in a decrease in capacity. This capacity reduction could be due to the overlap of adsorption sites, as a result of overcrowding of adsorbent particles beyond the optimal dose. Also, a high substrate content may inhibit the activity of microorganisms, as excess nutrient aid microbial growth but does not necessarily improve the rate of biodegradation as observed by (Chaineau *et al.,* 2005; Lin *et al.*,2015). Thus, the results show that the adsorbent dose of 2 g has the highest removal efficiency for both the adsorption and simultaneous adsorption-biodegradation study.

5.4.3 Effect of pH

A contrasting trend was observed on the effect of pH for both adsorption and simultaneous adsorption-biodegradation of naphthalene. The optimum removal rate occurred at pH 9 for the

adsorption of naphthalene using both treated and untreated snail shell. It was clear that the adsorption of naphthalene varies with pH, that is, as the pH increased from 5 – 9, there was a corresponding progressive increase in the percentage removal. However, it was a contrasting observation for the simultaneous adsorption-biodegradation of naphthalene with *Pseudomonas putida* and *Bacillus subtilis* incorporated individually on both treated (activated) and untreated snail shell with the optimum achieved at pH 7. This shows that the bacteria are more active at a neutral pH. This observation is similar to the reports that microorganisms achieve better efficiency at pH 6 – 8, with the optimum obtainable at pH 7 (Mentzer and Ebere, 1996; Agarry and Aremu, 2012).

At a low pH (acidic pH), the external surface charges on the adsorbent are positive, thus causing lower adsorption and adsorption-biodegradation of naphthalene at pH 5.Moreover, when the pH was increased from pH 5 - 9, the adsorbent surface becomes more negatively charged, therefore, the maximum adsorption of naphthalene with positive charged was attained at pH 9, while the maximum adsorption-biodegradation capacity occurred at pH 7. This observation agrees with existing reports on other adsorbent systems (Annadurai *et al.*, 2000; Annadurai *et al.*, 2007; Agarry and Aremu, 2012; Darsa *et al.*, 2014).

5.5 FTIR Spectra of Naphthalene and its Metabolites

The sharp peaks observed at 3047 cm^{-1} and 1381 cm^{-1} were indication of the aromatic carbon-hydrogen single bond (Ar C-H) and carbon-carbon double bond (C=C) stretching in the aromatic compound respectively. However, these aromatic rings and bonds were broken down and replaced by new bonds. The broad peak at 3363 cm^{-1} and the peak at 1635 cm^{-1} can be attributed to the oxygen-hydrogen single bond (OH- stretch) and the vibration of the carbon-oxygen double bond (C=O stretch) in the carboxylic acid respectively. Also, the peak at 2924

cm^{-1} was due to the carbon-hydrogen single bond stretch (C-H) within the carboxylic chain. This showed the step by step intermediates conversion of hydrocarbon.

5.6 Comparison of Adsorption of Naphthalene and its Biodegradation

The percent removal of naphthalene significantly increased from 10 – 50 mg/L both for the adsorption and adsorption-biodegradation, but the adsorption-biodegradation were the most significant ($P<0.05$). The higher rate of adsorption-biodegradation can be attributed to the synergistic effects of both the bacteria and snail shells, this was in agreement with the existing literature (Agarry and Aremu, 2012; Lin *et al.,* 2015), and it also agrees with the bio-filtration mechanism where formation of bio-layer is followed by degradation of the compounds thus achieving a higher efficiency (Chaudhury *et al.,* 2003). However, the removal percent with *Pseudomonas putida* and *Bacillus subtilis* immobilized respectively on treated snail shell were significantly higher than *Pseudomonas putida* and *Bacillus subtilis* immobilized on untreated snail shell. This was an indication that adsorptive capacities of adsorbent is improved by its activation by suitable activating agent (Owabor and Ogunsakin, 2006; Owabor and Iyasele, 2006). The removal efficiencies followed the order: PPT > BST > PPU > BSU > AT > AU.

5.7 Equilibrium Studies

5.7.1 Langmuir isotherm model

It was observed that the linearized form of the Langmuir isotherm was linear over the concentration range studied and the result fitted with the model with respect to R^2 values obtained. The high degree of correlation for the linearized Langmuir relationship observed suggests monolayer adsorption on specific sites or single surface reaction. This is was a similar report to Oladunni *et al.,* (2012). The values obtained for the Langmuir isotherm constants were indicative of the surface binding energy and monolayer coverage as stated by

Bhavna *et al*., (2011) and Kiurski *et al*., (2011). This suggests that some heterogeneity in the surface or pores of the treated (activated) and untreated snail shell used for the naphthalene adsorption and biodegradation played a major role.

5.7.2 Freundlich isotherm model

As evidenced by the respective high correlation coefficient(R^2) values obtained from the adsorption and biodegradation study, it showed that the isotherms fitted well with the Freundlich model. The obtained n values were all below one, which is an indication of a cooperative adsorption in sites with different binding energies and indicative of favourable adsorption process. This corroborate with the findings of Salman and Al-Saad, 2012; Abechi *et al*. (2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 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. It was observed that the adsorption and adsorption-biodegradation of naphthalene increased with an increase in initial naphthalene concentration. The optimum adsorbent dosage was found to be 2 g for both processes, while a contrasting result was obtained for their respective pH optimum. The highest percent removal occurred at a basic pH of 9 for the adsorption, while the optimum pH where the highest percent removal took place is at pH 7 for adsorption-biodegradation. The *Pseudomonas putida* and *Bacillus subtilis* immobilized on the respective snail shell showed remarkable removal efficiency on naphthalene compared to the adsorption of naphthalene. The higher efficiency of the immobilized bacteria may be attributed to the synergistic effect of combining simultaneously adsorption and biodegradation. The efficiencies of the snail shell significantly followed the order: PPT > BST > PPU > BSU > AT > AU. Hence, simultaneous adsorption-biodegradation process as observed from this study, offers better results than adsorption.

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 but more appropriate with Freundlich isotherm models. Thus,

Pseudomonas putida and *Bacillus subtilis* immobilized on snail shell is a promising alternative to physicochemical methods of remediating polluted sites.

6.2 Recommendations

Based on the outcome of the study, the following areas are recommended for further studies:

i. The reuse of immobilized snail shell should be studied.
ii. The effect of ratio of bacteria to snail shell should be investigated.
iii. Investigation on the mechanical stability of the immobilized bacteria on the snail shell should be carried out.
iv. Due to cost implications and time involved in one factor at a time, design of experiment (DOE) should be used for further study.

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APPENDICES

Appendix I: Experimental Data for Effect of concentration on the Adsorption of Naphthalene with untreated snail shell (AU)

C_o(mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	4.94 ± 0.02	50.60	50.45	50.81	50.62 ± 0.15
20	8.54 ± 0.09	57.27	56.74	57.83	57.28± 0.45
30	11.46 ± 0.06	61.87	62.01	61.54	61.81 ± 0.20
40	14.35 ± 0.12	64.53	63.86	63.98	64.12 ± 0.29
50	17.40 ± 0.10	64.95	65.25	65.41	65.20 ± 0.19

Appendix II: Experimental Data for Effect of concentration on the SAB of Naphthalene with *Pseudomonas putida* immobilized on untreated snail shell (PPU)

C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	3.67 ± 0.01	63.37	63.12	63.30	63.26 ± 0.11
20	6.77 ± 0.20	66.24	66.00	66.15	66.13 ± 0.10
30	9.33 ± 0.01	68.92	68.90	68.88	68.90 ± 0.02
40	11.93 ± 0.10	70.52	69.89	70.13	70.18 ± 0.26
50	13.45 ± 0.09	73.37	72.95	73.00	73.11 ± 0.19

Appendix III: Experimental Data for Effect of concentration on the SAB of Naphthalene with *Bacillus subtilis* immobilized on untreated snail shell (BSU)

C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	3.98 ± 0.00	60.15	60.23	60.13	60.17 ± 0.04
20	7.11 ± 0.08	63.92	64.57	64.87	64.45 ± 0.40
30	9.52 ± 0.04	68.31	68.09	68.37	68.26 ± 0.12
40	11.34 ± 0.13	71.54	71.33	72.07	71.65 ± 0.31
50	12.77 ± 0.19	74.55	73.97	74.87	74.46 ± 0.37

Appendix IV: Experimental Data for Effect of concentration on the Adsorption of Naphthalene with treated snail shell (AT)

C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	4.30 ± 0.02	56.78	57.06	57.31	57.05 ± 0.22
20	7.72 ± 0.04	61.43	61.61	61.17	61.40 ± 0.18
30	10.62 ± 0.09	64.57	64.26	65.02	64.62 ± 0.31
40	13.42 ± 0.04	66.34	66.46	66.58	66.46 ± 0.10
50	16.45 ± 0.05	67.22	67.12	66.97	67.10 ± 0.10

Appendix V: Experimental Data for Effect of concentration on the SAB of Naphthalene with *Pseudomonas putida* immobilized on treated snail shell (PPT)

C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	2.67 ± 0.01	73.34	73.12	73.37	73.28 ± 0.11
20	5.12 ± 0.05	74.45	74.68	74.09	74.41 ± 0.24
30	7.06 ± 0.04	76.46	76.30	76.61	76.46 ± 0.13
40	8.42 ± 0.03	78.99	78.87	79.01	78.96 ± 0.06
50	8.94 ± 0.06	82.15	82.26	81.95	82.12 ± 0.13

Appendix VI: Experimental Data for Effect of concentration on the SAB of Naphthalene with *Bacillus subtilis* immobilized on treated snail shell (BST)

C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
10	3.37 ± 0.01	66.41	66.27	66.30	66.33 ± 0.06
20	6.10 ± 0.00	69.50	69.53	69.51	69.51 ± 0.01
30	8.34 ± 0.06	71.92	72.37	72.33	72.21 ± 0.20
40	10.08 ± 0.09	74.74	75.10	74.55	74.80 ± 0.23
50	11.61 ± 0.06	76.84	76.90	76.63	76.79 ± 0.12

Appendix VII: Experimental Data for Effect of pH on the Adsorption of Naphthalene with untreated snail shell (AU)

pH	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	50	18.27 ± 0.20	63.76	63.72	62.89	63.46 ± 0.40
7	50	17.41 ± 0.07	65.27	64.97	65.28	65.17 ± 0.14
9	50	17.34 ± 0.05	65.39	65.37	65.19	65.32 ± 0.09

Appendix VIII: Experimental Data for Effect of pH on the SAB of Naphthalene with *Pseudomonas putida* immobilized on untreated snail shell (PPU)

pH	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	14.49 ± 0.04	71.11	70.92	71.05	71.03 ± 0.08
7	13.41 ± 0.11	73.40	73.26	72.88	73.18 ± 0.22
9	13.93 ± 0.04	72.25	72.10	72.06	72.14 ± 0.08

Appendix IX: Experimental Data for Effect of pH on the SAB of Naphthalene with *Bacillus subtilis* immobilized on untreated snail shell (BSU)

pH	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	50	14.85 ± 0.01	70.30	70.29	70.34	70.31 ± 0.02
7	50	12.79 ± 0.21	74.69	74.76	73.83	74.43 ± 0.42
9	50	13.91 ± 0.02	72.19	72.22	72.11	72.17 ± 0.05

Appendix X: Experimental Data for Effect of pH on the Adsorption of Naphthalene with treated snail shell (AT)

pH	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	50	16.92 ± 0.14	66.32	65.76	66.38	66.15 ± 0.28
7	50	16.41 ± 0.03	67.19	67.10	67.25	67.18 ± 0.06
9	50	16.25 ± 0.11	67.42	67.81	67.30	67.51 ± 0.22

Appendix XI: Experimental Data for Effect of pH on the SAB of Naphthalene with *Pseudomonas putida* immobilized on treated snail shell (PPT)

pH	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	50	11.49 ± 0.17	77.49	76.85	76.71	77.02 ± 0.34
7	50	8.95 ± 0.09	81.87	82.14	82.32	82.11 ± 0.18
9	50	9.40 ± 0.19	81.63	80.72	81.24	81.20 ± 0.37

Appendix XII: Experimental Data for Effect of pH on the SAB of Naphthalene with *Bacillus subtilis* immobilized on treated snail shell (BST)

pH	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
5	50	14.50 ± 0.12	71.14	71.21	70.67	71.01 ± 0.24
7	50	11.75 ± 0.15	76.08	76.59	76.81	76.49 ± 0.31
9	50	13.45 ± 0.10	73.29	72.84	73.17	73.10 ± 0.19

Appendix XIII: Experimental Data for Effect of Dosage on the Adsorption of Naphthalene with untreated snail shell (AU)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	17.37 ± 0.03	65.28	65.17	65.33	65.26 ± 0.07
3	50	18.00 ± 0.13	64.25	63.65	64.13	64.01 ± 0.26
5	50	18.34 ± 0.07	63.47	63.12	63.35	63.31 ± 0.15

Appendix XIV: Experimental Data for Effect of Dosage on the SAB of Naphthalene with *Pseudomonas putida* immobilized on untreated snail shell (PPU)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	13.46 ± 0.09	73.26	73.15	72.82	73.08 ± 0.18
3	50	13.68 ± 0.08	72.84	72.67	72.43	72.65 ± 0.17
5	50	14.45 ± 0.07	71.22	71.18	70.92	71.11 ± 0.13

Appendix XV: Experimental Data for Effect of Dosage on the SAB of Naphthalene with *Bacillus subtilis* immobilized on untreated snail shell (BSU)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	12.76 ± 0.21	74.25	75.09	74.13	74.49 ± 0.43
3	50	13.11 ± 0.03	73.84	73.81	73.72	73.79 ± 0.05
5	50	13.51 ± 0.19	72.45	73.19	73.30	72.98 ± 0.38

Appendix XVI: Experimental Data for Effect of Dosage on the Adsorption of Naphthalene with untreated snail shell (AT)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	16.42 ± 0.06	67.19	67.27	67.00	67.15 ± 0.11
3	50	16.88 ± 0.14	66.37	65.87	66.51	66.25 ± 0.27
5	50	17.68 ± 0.12	64.94	64.62	64.35	64.64 ± 0.24

Appendix XVII: Experimental Data for Effect of Dosage on the SAB of Naphthalene with *Pseudomonas putida* immobilized on treated snail shell (PPT)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	8.94 ± 0.03	82.17	82.13	82.04	82.11 ± 0.05
3	50	9.42 ± 0.14	80.79	81.29	81.43	81.17 ± 0.27
5	50	10.24 ± 0.18	79.12	79.45	79.97	79.51 ± 0.35

Appendix XVIII: Experimental Data for Effect of Dosage on the SAB of Naphthalene with *Bacillus subtilis* immobilized on treated snail shell (BST)

Dosage	C_o (mg/dm^3)	C_e (mg/dm^3)	% Removal (i)	% Removal (ii)	% Removal (iii)	% Removal
2	50	11.62 ± 0.15	76.40	77.11	76.75	76.75 ± 0.29
3	50	12.75 ± 0.09	74.25	74.57	74.66	74.49 ± 0.18
5	50	13.17 ± 0.14	73.79	73.92	73.28	73.66 ± 0.28

Appendix XIX: Experimental Data for Langmuir Isotherm Model

	q_e	C_e	$1/q_e$	$1/C_e$
AU	1.012	4.938	0.988	0.203
	2.291	8.544	0.436	0.117
	3.708	11.458	0.270	0.087
	5.130	14.351	0.195	0.070
	6.520	17.398	0.153	0.057
PPU	1.265	3.674	0.790	0.272
	2.645	6.774	0.378	0.148
	4.134	9.330	0.242	0.107
	5.614	11.928	0.178	0.084
	7.311	13.447	0.137	0.074
BSU	1.203	3.983	0.831	0.251
	2.578	7.109	0.388	0.141
	4.095	9.523	0.244	0.105
	5.732	11.341	0.175	0.088
	7.446	12.768	0.134	0.078
AT	1.141	4.295	0.876	0.233
	2.456	7.719	0.407	0.130
	3.877	10.615	0.258	0.094
	5.317	13.416	0.188	0.075
	6.710	16.448	0.149	0.061
PPT	1.466	2.672	0.682	0.374
	2.976	5.119	0.336	0.195
	4.587	7.063	0.218	0.142
	6.317	8.417	0.158	0.119
	8.212	8.940	0.122	0.112
BBT	1.327	3.367	0.754	0.297
	2.781	6.097	0.360	0.164
	4.332	8.338	0.231	0.120
	5.984	10.081	0.167	0.099
	7.679	11.605	0.130	0.086

Appendix XX: Experimental Data for Freundlich Isotherm Model

	q_e	C_e	Log q_e	Log C_e
AU	1.012	4.938	0.005	0.694
	2.291	8.544	0.360	0.932
	3.708	11.458	0.569	1.059
	5.130	14.351	0.710	1.157
	6.520	17.398	0.814	1.241
PPU	1.265	3.674	0.102	0.565
	2.645	6.774	0.422	0.831
	4.134	9.330	0.616	0.970
	5.614	11.928	0.749	1.077
	7.311	13.447	0.864	1.129
BSU	1.203	3.983	0.080	0.600
	2.578	7.109	0.411	0.852
	4.095	9.523	0.612	0.979
	5.732	11.341	0.758	1.055
	7.446	12.768	0.872	1.106
AT	1.141	4.295	0.057	0.633
	2.456	7.719	0.390	0.888
	3.877	10.615	0.588	1.026
	5.317	13.416	0.726	1.128
	6.710	16.448	0.827	1.216
PPT	1.466	2.672	0.166	0.427
	2.976	5.119	0.474	0.709
	4.587	7.063	0.662	0.849
	6.317	8.417	0.800	0.925
	8.212	8.940	0.914	0.951
BBT	1.327	3.367	0.123	0.527
	2.781	6.097	0.444	0.785
	4.332	8.338	0.637	0.921
	5.984	10.081	0.777	1.004
	7.679	11.605	0.885	1.065

Appendix XXI: Analysis of Variance for Untreated Snail shell

Table Analyzed	AU:PPU:BSU				
One-way analysis of variance					
P value	< 0.05				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	713.8				
R squared	0.9958				
ANOVA Table	SS	df	MS		
Treatment (between columns)	150.1	2	75.03		
Residual (within columns)	0.6306	6	0.1051		
Total	150.7	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
AU (%) vs PPU (%)	-7.903	42.22	Yes	***	-8.715 to -7.091
AU (%) vs BSU (%)	-9.260	49.47	Yes	***	-10.07 to -8.448
PPU (%) vs BSU (%)	-1.357	7.248	Yes	**	-2.169 to -0.5445

Appendix XXII: Analysis of Variance for Treated Snail shell

Table Analyzed	AT:PPT:BST				
One-way analysis of variance					
P value	< 0.05				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	8603				
R squared	0.9997				
ANOVA Table	SS	df	MS		
Treatment (between columns)	347.7	2	173.9		
Residual (within columns)	0.1213	6	0.02021		
Total	347.9	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
AT (%) vs PPT (%)	-15.02	183.0	Yes	***	-15.37 to -14.66
AT (%) vs BST (%)	-9.687	118.0	Yes	***	-10.04 to -9.331
PPT (%) vs BST (%)	5.330	64.94	Yes	***	4.974 to 5.686

Appendix XXIII: Student t-test for AU vs AT

Table Analyzed	Data 17
Column A	AU (%)
Vs	vs
Column B	AT (%)
Unpaired t test	
P value	0.05
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=12.41 df=4
How big is the difference?	
Mean ± SEM of column A	65.20 ± 0.1348 N=3
Mean ± SEM of column B	67.10 ± 0.07265 N=3
Difference between means	-1.900 ± 0.1532
95% confidence interval	-2.325 to -1.475
R squared	0.9747
F test to compare variances	
F,DFn, Dfd	3.444, 2, 2
P value	0.4500
P value summary	Ns
Are variances significantly different?	No

Appendix XXIV: Student t-test for PPU vs PPT

Table Analyzed	Data 18
Column A	PPU (%)
Vs	Vs
Column B	PPT (%)
Unpaired t test	
P value	< 0.05
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=56.14 df=4
How big is the difference?	
Mean ± SEM of column A	73.11 ± 0.1325 N=3
Mean ± SEM of column B	82.12 ± 0.09074 N=3
Difference between means	-9.013 ± 0.1606
95% confidence interval	-9.459 to -8.568
R squared	0.9987
F test to compare variances	
F,DFn, Dfd	2.131, 2, 2
P value	0.6388
P value summary	Ns
Are variances significantly different?	No

Appendix XXV: Student t-test for BSU vs BST

Table Analyzed	Data 19
Column A	BSU (%)
Vs	Vs
Column B	BST (%)
Unpaired t test	
P value	0.05
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.435 df=4
How big is the difference?	
Mean ± SEM of column A	74.46 ± 0.2634 N=3
Mean ± SEM of column B	76.79 ± 0.08185 N=3
Difference between means	-2.327 ± 0.2758
95% confidence interval	-3.092 to -1.561
R squared	0.9468
F test to compare variances	
F,DFn, Dfd	10.35, 2, 2
P value	0.1761
P value summary	Ns
Are variances significantly different?	No