BIOSORPTION OF MANGANESE BY *Bacillus subtilis* AND *Pseudomonas aeruginosa* ISOLATED FROM WASTE DUMP SITE

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, NASARAWA STATE UNIVERSITY KEFFI, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE IN ENVIRONMENTAL MICROBIOLOGY

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DECLARATION

I hereby declare that dissertation has been written by me and it is a report of my research work. It has not been presented in any previous application for a degree. All quotations are indicated and sources of information specifically acknowledged by means of references.

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CERTIFICATION

The dissertation title **'Biosorption of Manganese by *Bacillus Subtilis* and *Pseudomonas aeruginosa* isolated from waste dump site'** meets the regulations governing the award of the School of Postgraduate Studies, Nasarawa State University, Keffi, and is approved for its contribution to knowledge.

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DEDICATION

This research work is dedicated to Almighty God for his unending grace and unconditional love upon my life and my family.

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ABSTRACT

In the present study, we isolated and assessed the biosorption of manganese by *Bacillus subtilis* and *Pseudomonas aeruginosa* from waste dump site. The abilities of the organisms to take up manganese were evaluated using atomic absorption spectrophotometer (AAS). The organisms were isolated using standard microbiological culture techniques. The results showed that there was higher sorption rate at temperature 37^{o}C than at 25^{o}C. The biosorption percentage was highest at pH 5 for both *Pseudomonas aeruginosa* and *Bacillus subtilis* with percentage biosorption of 80.4% and 75.2% respectively. The optimum pH for manganese biosorption was found to be 5. The percentage biosorption increases from pH 1to 5 and thereafter decreased from 6 to 9.Percentage biosorption for *Pseudomonas aeruginosa* at 37^{o}C was 89.5% and at 25^{o}C was 69.7%. Also, percentage biosorption for *Bacillus subtilis* at 37^{o}C gave 78.4% while at 25^{o}C, 58.3%was achieved. It was observed that *Pseudomonas aeruginosa* has a higher uptake than *Bacillus subtilis*. One-Way ANOVA test was used in this study as statistical analysis. One-Way ANOVA test for manganese biosorption by *Pseudomonas aeruginosa* in days has the F-value of 0.0430, P-value of 0.0507 and the treatment degree of freedom (df) of 3 while for *Bacillus subtilis* has the F-value of 12.1876, the P-value of 0.0024 and the treatment degree of freedom (df) of 3. The effect of pH on percentage (%) manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* has the P-value of F-value of 2.0558, 0.5005 and the treatment degree of freedom (df) of 1. The effect of temperature on percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* has the F-value of 0.3747, P-value of 1.8743 and the treatment degree (df) of 1. The use of these microorganisms with modifications using biotechnology for biosorption of heavy metals should be encouraged**.**

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Biosorption is a physicochemical process that occurs naturally in certain biomass which allows it to passively concentrate and bind contaminants onto its cellular structure (Ahalya *et al.,* 2003). It is also the ability of microorganisms to accumulate heavy metals from waste through metabolically mediated pathways of uptake. The use of biomass in environmental cleanup has been in practice for a while, scientists and engineers are hoping this phenomenon will provide an economical alternative for removing toxic heavy metals from industrial wastewater and aid in environmental remediation (Ahalya *et al.,* 2003). Heavy metals released into the environment by anthropogenic activities tend to persist indefinitely. They eventually become serious threat to the environment (Vijayaraghavan *et al.,* 2004). Researchers have been looking for cheaper and more effective methods to remediate heavy metal-contaminated waters and reduce the growing public health risk. Biosorption is proven to be quite effective at removing metal ions from contaminated solution in a low-cost and environment-friendly manner (Suh and Kim, 2000). The release of heavy metals into the environment is increasing as a result of high rate of industrial activities and technological development. This poses significant threat to the environment and public health due to their toxicity, accumulation in the food chain and persistence nature. It is therefore important to develop new methods for metal removal, recovery from dilute solutions and the reduction of heavy metal ions to very low concentrations (Gadd *et al.,* 2010). It is often inefficient and very expensive to use conventional technologies, such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery for this purpose. In recent years, the biosorption process has been studied extensively using microbial biomass as biosorbents for heavy metal removal. The use of microbial cells for biosorption of heavy metals has been

recognized as a potential alternative to existing technologies for recovery of heavy metals from industrial waste streams (Ramos *et al*., 2015). Gadd *et al.,* 2010 stated that the use of laboratory-grown microorganism, biomass generated by the pharmacology industries, food processing industries and treatment plants have been involved in most studies of biosorption for metal removal. Increasing industrialization has resulted in an alarming increase in the discharge of heavy metals and other pollutants into the environment. Microbial populations in metal-polluted environments contain microorganisms which have adapted to toxic concentrations of heavy metal and become metal resistant. These microorganisms can be used to remove heavy metals from the environment by various approaches like bioaccumulation and bioabsorption, oxidation and reduction, methylation and demethylation (Gadd *et al.*, 2010).

Ramos *et al*., 2015 reported that certain types of microbial biomass could retain relatively high quantities of metal ions in a process known as biosorption. Metals are directly and / or indirectly involved in all aspects of microbial growth, metabolism and differentiation (Gadd *et al.,* 2010). Elements can interact with microbial cells and be accumulated as a result of physiochemical mechanisms and transport systems. These can be of varying specificity which can be independent or dependent (directly or indirectly) on metabolism (Gadd *et al.,* 2010). Some of these processes are of biotechnological importance. They are relevant to metal removal and recovery from mineral deposits and industrial effluents for industrial use or environmental bioremediation. There is more than one variable affecting the biosorption process, such as temperature, pH, agitation rate and metal concentration. Some of these factors (e.g. pH and metal concentration) have greater influence on metal removal by this process (Yetis *et al.*, 2000). Manganese is a mineral element that is both nutritionally essential and potentially toxic (Ramos *et al*., 2015).

Manganese is a pinkish-gray, chemically active element. It is a hard metal and is very brittle. It is hard to melt, but it's easily oxidized. Manganese is reactive when pure, and as a powder it will burn in oxygen, it reacts with water (it rusts like iron) and dissolves in dilute acids.

Environmental problems associated with heavy metals are very difficult to solve in contrast to organic matters because most of heavy metals have toxic effects on living organisms when exceeding a certain amount of concentration (Brady and Duncan, 2010). The cities of third world countries are growing at very rapid rates compared to those in the developed nations. The increasing growth of cities therefore has implications for municipal waste management among other social services required in the urban communities (Yetis *et al.*, 2000). Data from many of the cities in Africa shows inadequacy in urban social services like shelter, provision of safe drinking water and efficient management of solid wastes. The cities are therefore littered with 'mountains' of rubbish in landfills and open (in most cases illegal) waste dumps which are covered with flies. These also serve as breeding grounds for rodents and mosquitoes which are carriers of diseases (Yetis *et al.*, 2000). The present study is aimed at investigating the biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from waste dump site.

1.2 Statement of the Problem

Environmental problems associated with heavy metals are very difficult to solve in contrast to organic matters because most of heavy metals have toxic effects on living organisms when exceeding a certain amount of concentration. In recent years, heavy metal pollution has become one of the most serious environmental problems. Presence of heavy metals even in traces is toxic and detrimental to both flora and fauna (Brady and Duncan, 2010). With the rapid development of many industries (mining, surface finishing, energy and fuel producing, fertilizer, pesticide, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leather, photography, electric appliance manufacturing, metal surface treating) and aerospace and atomic energy installations, wastes containing metals are directly or indirectly being discharged into the environment causing serious environmental pollution and even threatening human life (Brady and Duncan, 2010) cause Parkinson, lung embolism and

bronchitis. When human beings are exposed to manganese for a longer period of time they may become impotent.

1.3 Research Questions

i. Can the biosorption rate of manganese at different concentrations by the isolates be determined?

ii. Can the effects of pH and temperature on biosorption processes be determined?

1.4 Objectives of the Study

1.4.1 Aim

The aim of this study was to assess biosorption of manganese by Bacteria isolated from Waste Dump site.

1.4.2 Objectives

The specific objectives are:

i. To determine biosorption rate of manganese at different concentrations by isolated microorganisms

ii. To determine the effect of pH and temperature on biosorption processes

1.5 Statements of the Hypotheses

1.5.1 Null Hypotheses

i. The biosorption rate of manganese at different concentrations by the isolates cannot be determined.

ii. The effects of pH and temperature on biosorption processes cannot be determined.

1.5.2 Alternate Hypotheses

i. The biosorption rate of manganese at different concentrations by the isolates can be determined.

ii. The effects of pH and temperature on biosorption processes can be determined.

1.6 Justification of the Study

Manganese is a very common compound that can be found everywhere on earth. Manganese is one out of three toxic essential trace elements, which means that it is not only necessary for humans to survive, but it is also toxic at very high concentrations in the human body (Yetis *et al.*, 2000). Various epidemiological studies of workers exposed to Manganese at average levels below 5mg/m^3 have shown neurobehavioral, reproductive, and respiratory effects, both by objective testing methods and by workers' self-reported symptoms on questionnaires (Yetis *et al.*, 2000).

The uptake of Manganese by humans mainly takes place through food, such as spinach, tea and herbs. After absorption in the human body, manganese will be transported through the blood to the liver, the kidneys, the pancreas and the endocrine glands. Manganese effects occur mainly in the respiratory tract and in the brains. Symptoms of manganese poisoning are hallucinations, forgetfulness and nerve damage. Manganese can also cause Parkinson, lung embolism and bronchitis. When men are exposed to manganese for a longer period of time they may become impotent (Yetis *et al.*, 2000). Biosorption is proven to be quite effective in removing metal ions from contaminated solutions in a low-cost and environment-friendly manner (Sharma *et al*., 2007).

1.7 Scope of the Study

This study is focused on the biosorption of manganese by *Bacillus subtilis* and *Pseudomonas aeruginosa* isolated from waste dump sites.

CHAPTER TWO

LITERATURE REVIEW

2.1.1 Manganese as an essential element

Manganese is an essential metal for the human system and many enzymes are activated by this element. Manganese has a variety of applications in ceramics, dry battery cells, electrical coils and many alloys (Sharma *et al.*, 2007). In addition to the disposal of untreated discharge from the above applications into water bodies, another major source of manganese pollution is the burning of coal and oil (Sharma *et al*., 2007). The intake of higher concentrations of manganese causes manganese psychosis, an irreversible neurological disorder. It is characterised by uncontrollable laughter, sexual excitement and impotence (Sharma *et al*., 2007).

Heavy metal ion biosorption by biological materials such as bacteria and fungi presents few problems when operated in a continuous mode; however, solid/liquid separation is a major constraint in such methods. Even though immobilisation may solve this problem, chemical costs and mechanical strength should be taken into consideration. For these reasons, recent research has focused on the use of low-cost waste materials as adsorbents (Vijayaraghavan *et al*., 2005). The adsorption capacity of several low-cost adsorbents has been investigated, mainly using biopolymers which are obtained from renewable sources and adsorb metallic ions selectively (Sharma *et al*., 2007). Of such biopolymers, chitosan is a linear polysaccharide based on a glucosamine unit. It is obtained from the de-acetylation of chitin, which is the major component of crustacean shells. As a consequence, it is one of the most available biopolymers in Nature. The potential use of chitosan as an adsorbent has been demonstrated, particularly for the removal of transition and post-transition elements from

aqueous waste (Zamin *et al*., 2004), with the use of chitosan as a chelating ion-exchanger for the removal of metal ions being well established in the literature. Although, its amino and hydroxyl groups can act as chelating sites (Qi and Xu, 2004; Dambres *et al*., 2000), these active binding sites are not readily available for sorption when it exists as a gel or in its natural form.

Several investigators have attempted to modify chitosan to facilitate mass transfer and to expose the active binding sites in order to enhance its adsorption capacity (Hassan *et al.*, 2006; Boddu *et al.*, 2003). Hassan *et al*., 2006 and Ramos *et al*., 2015noted that the maximum uptake of chitosan flakes towards molybdate ions was approximately one-half that obtained with chitosan beads.

Some researchers have recognised that this biosorbent requires further modification and development before it can be used commercially. Thus, Boddu *et al.*, 2008 evaluated the sorption of As (III) and As (V) ions by a chitosan-coated biosorbent and an iron–chitosan composite, respectively. Similarly, Tao *et al*., (2009) studied the removal of Pb (II) ions by a chitosan/TiO2 composite. However, for such materials, it is also necessary to provide a physical support and to increase the accessibility of the metal ion-binding sites for process applications (Vijayaraghavan *et al*., 2005).

2.1.2 Environmental effect of manganese

Manganese compounds exist naturally in the environment as solids in the soil and small particles in the water. Manganese particles in the air are present in dust particles. These usually settle back to earth within a few days.

Humans increase Manganese concentrations in the air by industrial activities and through burning fossil fuels. Manganese that comes from human sources can also enter surface water, groundwater and sewage water. Through the application of manganese pesticides, manganese will enter soil.

For animals, manganese is an essential component of over 36 enzymes that are used for their carbohydrate, protein and fat metabolism. When animals eat too little manganese, their growth, bone formation and reproduction will be affected. For some animals the lethal dose is very low, thus they have little chance of surviving when their uptake of manganese exceeds the essential dose (Vijayaraghavan *et al*., 2005). Manganese substances can cause lung, liver and vascular disturbances, reduced blood pressure, development failure in animal foetuses and brain damage. Laboratory tests on animals have shown that severe manganese poisoning can also cause tumour development within animals.

In plants, manganese ions are transported to the leaves after uptake from the soil. Manganese can cause both toxicity and deficiency symptoms in plants. When the pH of the soil is low, manganese deficiencies usually occur. Plants' mechanisms will be disturbed when they can't absorb sufficient manganese from the soil. Highly toxic concentrations of manganese in soils can also do harm to plants, causing swelling of cell walls and withering and brown spots on leaves. Deficiencies can also cause these effects (Vijayaraghavan *et al*., 2005).

2.1.3 Health effects of Manganese

Manganese is a very common compound that can be found everywhere on earth. Manganese is one out of three toxic essential trace elements, which means that it is not only necessary for humans to survive, but it is also toxic when too high concentrations are present in the human body (Vijayaraghavan *et al*., 2005). When people do not live up to the recommended daily allowances, it impairs their health while high intake is also injurious to health. Various epidemiological studies of workers exposed to manganese at average levels below 5mg/m3 have shown neurobehavioral, reproductive, and respiratory effects, both by objective testing methods and by workers' self-reported symptoms on questionnaires (Vijayaraghavan *et al*., 2005).

The uptake of manganese by humans mainly takes place through food, such as spinach, tea

and herbs. After absorption in the human body manganese will be transported through the blood to the liver, the kidneys, the pancreas and the endocrine glands. Manganese effects occur mainly in the respiratory tract and in the brains. Symptoms of manganese poisoning are hallucinations, forgetfulness and nerve damage. Manganese can also cause Parkinson, lung embolism and bronchitis. When men are exposed to manganese for a longer period of time they may become impotent. A syndrome that is caused by manganese has symptoms such as schizophrenia, dullness, weak muscles, headaches and insomnia(Davidson,2015).

Because manganese is an essential element for human health shortages of manganese can also cause health effects: fatness, glucose intolerance, blood clotting, skin problems, lowered cholesterol levels, skeleton disorders, birth defects, changes of hair color, Neurological symptoms (Vijayaraghavan *et al*., 2005). Chronic manganese poisoning may result from prolonged inhalation of dust and fume. The central nervous system is the chief site of damage from the disease, which may result in permanent disability. Symptoms include languor, sleepiness, weakness, emotional disturbances, spastic gait, recurring leg cramps, and paralysis. A high incidence of pneumonia and other upper respiratory infections has been found in workers exposed to dust or fume of Manganese compounds. Manganese compounds are experimental equivocal tumorigenic agents (Eriksson *et al.*, 2007).

Studies of the neuropathological bases for manganism have pointed to the involvement of the corpus striatum and the extra pyramidal motor system (Eriksson *et al.,* 2007). Neuropathological lesions have generally been associated with the basal ganglia, with neuronal degeneration in the putamen and globuspallidus (Eriksson *et al.,* 2007). Brain imaging studies have also recently begun to provide additional insight into the neuropathology of manganese toxicity (Eriksson *et al.,* 2007).

2.2.1 Toxicity

Manganese toxicity may result in multiple neurologic problems and is a well-recognized health hazard for people who inhale manganese dust, such as welders and smelters (Eriksson *et al.,* 2007). Unlike ingested manganese, inhaled manganese is transported directly to the brain before it can be metabolized in the liver (Eriksson *et al.,* 2007). The symptoms of manganese toxicity generally appear slowly over a period of months to years, in its worst form, manganese toxicity can result in permanent neurological disorder similar to those of Parkinson's disease, including tremors, difficulty walking, and facial muscle spasms. This syndrome often called manganism, is sometimes preceded by psychiatric symptoms, such as irritability, aggressiveness, and even hallucination (Eriksson *et al.,* 2007). Additionally, environmental or occupational inhalation of manganese can cause inflammatory response in lungs (Han *et al.,* 2009). Clinical symptoms of effects to the lung include cough, acute bronchitis, and decreased lung function (Frisbie *et al.,* 2012).

2.2.2 Ingested manganese

Limited evidence suggests that high manganese intakes from drinking water may be associated with neurological symptoms similar to those of Parkinson's disease. Severe neurological symptoms were reported in 25 patients who drank water contaminated with manganese, and probably other contaminants, from dry cell batteries for two to three months (Frisbie *et al.,* 2012). Water manganese level were found to be 14mg/liter almost two months after symptoms began and may have already be declining. A study of older adults in Greece found high level of neurological symptoms in those exposed to water manganese level of 1.8-2.3mg/liter (Frisbie *et al.,* 2012). More recent study has shown that children exposed to high level of manganese through drinking water experience cognitive and behavioural deficits (Frisbie *et al.,* 2012).

2.2.3 Intravenous manganese

Manganese neurotoxicity has been observed in individuals receiving total parenteral nutrition, both as a result of excessive manganese in the solution and as an incidental contaminant (Frisbie *et al.,* 2012). Neonates are especially vulnerable to manganese-related neurotoxicity (Frisbie *et al.,* 2012). Infants receiving manganese-containing TPN can be exposed to manganese concentrations about 100-fold higher than breast-fed infants (Aschner and Ashner, 2005).

2.2.4 Adequate intake

No recommended dietary allowances (RDA) for manganese have been established. When there are no RDAs for a nutrient, the Adequate Intake (AI) is used as a guide (Frisbie *et al.,* 2012). The AI is the estimated amount of the nutrient that is used by a group of healthy people and assumed to be adequate. The daily Adequate Intake (AI) levels for manganese are: infants birth to 6 months, 3 mcg; 7 to 12 months, 600 mcg; children 1 to 3 years, 1.2 mg; 4 to 8 years 1.5 mg; boys 9 to 13 years, 1.9 mg; boys 14 to 18 years, 2.2 mg; girls 9 to 18 years, 1.6 mg; men age 19 and older, 2.3 mg; women 19 and older, 1.8 mg; pregnant women age 14 to 50, 2 mg; breastfeeding women, 2.6 mg (WHO, 2011; Frisbie *et al.,* 2012)..

2.2.5 Sources of manganese

Manganese (Mn) is an element widely distributed in the earth's crust. It is considered to be the twelfth most abundant element and the fifth most abundant metal. Manganese does not occur naturally in a pure state; oxides, carbonates and silicates are the most important manganese-containing minerals. The most common manganese mineral is pyrolusite (MnO2), usually mined in sedimentary deposits by open-cast techniques (WHO, 2011). Manganese occurs in most iron ores. Its content in coal ranges from 6 μg/g to 100 μg/g; it is also present in crude oil, but at substantially lower concentrations Frisbie *et al*., (2012).

Manganese is mainly used in metallurgical processes, as a deoxidizing and desulfurizing

additive and as an alloying constituent. It is also used in the production of dry-cell batteries, in chemical manufacturing, in the manufacture of glass, in the leather and textile industries, and as a fertilizer. Organic carbonyl compounds of manganese are used as fuel-oil additives, smoke inhibitors and anti-knock additives in petrol (Frisbie *et al.*, 2012).

Crustal manganese enters the atmosphere by a number of natural and anthropogenic processes, which include the suspension of road dusts by vehicles and wind erosion and the suspension of soils, particularly in agricultural, construction and quarrying activities. Manganese can be distributed widely. The most common forms of manganese compounds in coarse particles of crustal origin are oxides or hydroxides of oxidation state +2, +3 or +4, and manganese carbonate (Frisbie *et al.*, 2012).

2.2.6 Routes of exposure

2.2.6.1 Air

The degree of respiratory uptake of manganese by inhalation depends primarily on particle size, with fine particles being small enough to reach the alveoli and be absorbed into the bloodstream. Coarse particles tend to be removed from the respiratory tract by mucociliary action that results in their relatively rapid movement to the nasopharynx and ingestion. The water solubility of a manganese compound appears to affect the time course of respiratory tract absorption, but not necessarily the amount ultimately absorbed. One study found no difference between the absorption of 1-μm particles of $MnCl_2$ and Mn_2O_3 in healthy adults (Frisbie *et al.*, 2012). Another study by Frisbie *et al.*, (2012) found that, following intratracheal instillation of $MnCl_2$ and Mn_3O_4 in rats, the soluble chloride cleared four times faster than the insoluble oxide from the respiratory tract; despite this initial difference, however, after 2 weeks the amounts of labelled manganese in the respiratory tract were similar for the two compounds. Extra-thoracic deposition is another possible route of exposure. Some studies have indicated that neurotoxic metals such as Aluminium and

cadmium can be directly transported to the brain olfactory bulbs via nasal olfactory pathways (Frisbie *et al.,* 2012).

2.2.6.2 Drinking-water

Concentrations of manganese in fresh water may vary from less than one to several thousand micrograms per litre, although drinking water generally contains less than 100μg/litre. In 100 of the largest cities in the United States, 97% of the surveyed public water supplies contained concentrations below100μg/litre (Frisbie *et al.,* 2012)

2.2.6.3 Food

Food generally constitutes a major source of manganese intake for humans, but concentrations in foodstuffs vary markedly. The highest concentrations are found in certain foods of plant origin, especially wheat and rice, with concentrations between 10 mg/kg and 100 mg/kg. Polished rice and wheat flour contain less manganese, because most of it is in the bran. High concentrations of manganese have been found in tea leaves, eggs, milk, fruits and meat generally contain less than 1 mg manganese per kg of food (Frisbie *et al.,* 2012). In a study performed in Canada, it was estimated that, of people's total manganese intake via food, 54% came from cereals and 14% from potatoes while meat, fish and poultry provided only 2% of manganese intake (WHO, 2011). However, manganese concentrations may differ for the same items in different countries and areas. Studies indicate that dietary manganese intakes range from 1–2 mg/day in bland hospital diets to around 18 mg/day for diets consisting predominantly of vegetables, nuts and seeds (WHO, 2011). Diets high in nuts and whole grains tend to be high in manganese, whereas highly processed foods tend to be low. In one study, the daily intake for children aged 3–5 years averaged 1.4 mg/day, and 2.18 mg/day for children aged 9–13 years (Frisbie *et al.,* 2012). The daily intake of manganese by bottle-fed and breastfed infants are very low because of the low concentrations of manganese in both breast-milk and cow's milk (Frisbie *et al.,* 2012). Although infant formula

concentrations may be 3–100 times more than those of breast-milk (WHO, 2011).

2.2.7 Relative significance of different routes of exposure

In terms of environmental sources and pathways of exposure, dietary intake of manganese generally dominates other routes of manganese exposure. Assuming an air concentration of 50ng/m3 and 40% absorption, daily manganese intake by inhalation would be in the order of 400ng/day (based on breathing 20 m^3air per day). Water and food manganese concentrations may vary widely, and the percentage absorption may also vary considerably depending on several factors, which includes: age (Frisbie *et al.,* 2012) iron status (Frisbie *et al.,* 2012), other nutrients in the diet, individual differences (Frisbie *et al.,* 2012) and the form of manganese (Frisbie *et al.,* 2012). Typically, about 3–8% of an ingested dose is absorbed, but this figure might be greater in young children. In general, manganese intakes are 0.1–24 μg/day for water and 2–8 mg/day for food.

2.2.8 Factors Affecting Biosorption

The major factors that affect the biosorption processes are initial metal ion concentration, temperature and pH. WHO, 2011 reported that temperature does not influence the biosorption processes in the range of 20°-35°C. However, pH seems to be the most important parameter in the biosorption processes. It affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of the metallic ions (Khalid *et al.*, 2011).

2.2.9 Microbial Biosorbents

2.2.9.1 Bacteria as Biosorbent

Numerous studies have identified a number of potential bacteria species capable of accumulating metals. *Bacillus sp* has been identified as having a high potential of metal sequestration and has been used in commercial biosorbent preparation (Sulaiman, 2015).

Rabbani *et al.*, 2012 studied the application of bacteria (*Pseudomonas sp. Zoogloearamiga* and *Streptomyces sp.)* for the recovery of heavy metals from aqueous environment. The optimum pH values for Chromium (Cr), Pb (Lead) and Cu (copper) biosorption were found to be 2.0, 4.5, and 3.5 respectively. The biosorption properties of bacterial biomass and the effect of environmental factors (pH, metal concentration, contact time etc) on Cr, Cd and Cu biosorption were explored by Sulaiman, 2015. They reported that a species of gram negative bacterium, *Pontoea* TEM 18, isolated from waste water of a petrochemical along with other microorganism exhibit greatest Cu tolerance. The cell walls of gram negative bacteria are somewhat thinner than the gram positive ones; also they are not heavily cross-linked. They have an outer layer of lipopolysacharide (Lps), phospholipids and proteins. Sulaiman, 2015 compared Cd^{2+} biosorption capacities of gram positive and gram negative bacteria. Glycoprotein present in the outer side of gram positive bacteria cell walls were suggested to have more potential binding site for Cd^{2+} than the phospholipids and the Lps and hence are responsible for the observed difference in capacity . Rabbani *et al.*, 2012 reported biosorption of Cr (III) by 17 bacteria strain isolated from Ramsar warm spring, Iran. A new strain of gram positive coccobacilli bacteria (NRC-BT-2) was found to be highly capable for the biosorption of Cr (III). In the batch experiment with various initial concentrations of Cr ions to obtain the sorption capacity and isotherms, the biosorption of Cr (III) or Cr (VI) onto the cell surface of *Pseudomonas aeruginosa* was investigated (Sadeshi and Roux, 2012).

2.2.9.2Cell Wall Characteristics

The primary components of the Gram-positive cell wall are peptidoglycan, teichoic acid, and teichuronic acid (Khalid *et al.*, 2011; Sadeshi and Roux, 2012; Sulaiman, 2015). All three constituents contain functional groups that, when deprotonated, can effectively bind metal cations. Peptidoglycan contains carboxyl, hydroxyl, and amine functional groups, teichoic acid includes phosphoryl groups, and teichuronic acid is similar to teichoic acid, but contains

carboxyl functional groups rather than the phosphoryl groups of teichoic acid. Gram-negative cell walls include a lesser amount of peptidoglycan than Gram-positive cells and have a complex outer membrane, but they do not include teichoic and teichuronic acid constituents (Sadeshi and Roux, 2012). The outer membrane of Gram-negative bacteria contains phospholipids, lipoproteins, lipopolysaccharides, and various proteins. The phospholipids have phosphoryl groups in the same local coordination environment as the phosphoryl groups in teichoic acid (Sulaiman, 2015).

The peptidoglycan structure consists of two sugars, N-acetylglucosamine and N-acetylmuramic acid (NAG, NAM), with a side peptide chain attached to the NAM. The peptide chain includes four amino acid groups with the *D*-glutamic acid and the *meso*diaminopimelic acid (DAP) containing the two carboxyl groups of interest for metal cation adsorption. Peptidoglycan constitutes up to 50 % of the cell wall by weight (Sadeshi and Roux, 2012; Rabbani *et al.,* 2012). Teichoic acids comprise the other major portion of the Gram-positive cell wall. Teichoic acid is linked covalently to the peptidoglycan sugars by a linkage unit containing two sugars and a phosphoryl group. The phosphoryl group in the linkage unit may also be active in adsorption of cations (Sadeshi and Roux, 2012).

The cell walls of Gram-positive bacteria can exhibit a negative charge due to the deprotonation of the carboxyl, phosphoryl, and hydroxyl functional groups (Rabbani *et al.*, 2012). At low pH, the functional groups located on the cell wall are mostly protonated, and, therefore, little to no metal adsorption occurs. As pH increases, the surface functional groups deprotonate successively, resulting in the overall negative charge on the cell wall and an increasing number of sites available for metal adsorption (Sulaiman, 2015).

2.2.9.3 Fungi (Mold) as Biosorbent

Biomaterials like fungi have been proved efficient and economical for the removal of toxic

metal from dilute aqueous solution by biosorption because fungi biomass offer the advantage of having a high percentage of cell wall material, which show excellent metal binding property (Rabbani *et al.,* 2012). Larger quantity of fungi biomass is available from the antibiotic and food industries. Ultimately the biosorption result not only in metal removal but also provide an eco-friendly environment (Rabbani *et al.,* 2012).

Heavy metal uptake by *Aureobasidium pullanans* and *Cladosporium resinae* was studied. There was a distinct two phase uptake of heavy metals, the first consisting of metabolism independent cell wall uptake followed by an energy dependent cellular uptake (Frils and Myers, 2011). The latter was affected by temperature, absence of energy source and metabolic inhibitors. Removal of Cd by nine species of fungi had been investigated by Huang *et al.,* 2008 in both batch culture and continuous reactor. Biomass grown in laboratory was harvested and stored in freeze dry conditions. The uptake was found to be controlled by adsorption and not by surface precipitation. Both fresh and freeze dried biomass also took up heavy metals such as Cu, Pb, Zn and Co (Gadd *et al.,* 2010).

Aspergillus niger has been found capable of removing heavy metals, Pb, Cd and Cu. The role played by various functional groups in the cell wall of *Aspergillus niger* was investigated (Gadd *et al.,* 2010). The Biomass was subjected to chemical treatments to modify the functional groups, carboxyl, amino and phosphate to study their role in biosorption of heavy metals. The effect of pre-treatment on Pb biosorption capacity of fungi biomasses such as *Aspergillus versicolor* and *penicillium verrrucosum* was investigated by Cabuk *et al,* (2005). Fungi biomasses were subjected to physical treatments like heat and autoclaving and chemical treatment with Sodium hydroxide (NaOH), formaldehyde, gluteraldehyde, acetic acid, Hydrogen peroxide (H_2O_2), commercial laundry detergent, orthophosphoric acid and dimethyl sulphide. Biosorption of Pb was increased when Biomass of *Aspergillus versicolor*

was pre-treated with dimethyl sulfoxide, H_2O_2 and gluter aldehyde. Rabbani *et al.,* 2012 investigated the absorption of Zn (II) ions by *Rhizopusarrhizus*, a filamentous fungus in a batch reactor. Five morphologically different fungi were isolated from leather tanning effluent in which species of *Aspergillus* and *Hirsutella* had higher potential to remove Chromium (Cr). The potential of *Aspergillus spp* for the removal of Cr was evaluated in shake flask culture at different pH, temperature, inoculums size, and Carbon (C) and Nitrogen (N) source. The maximum Cr was removed at pH 6.0 and 30^0c in the presence of sodium acetate (0.2%) and yeast extract (0.1%) (Grisaro *et al.*, 2011).

Biosorption of heavy metals by micro fungi or mushroom is a known phenomenon nowadays but not much work has been done, since mushroom grow under natural habitat, heavy metal pollutants present in the soil or in the natural substrates are taken up by the fruiting bodies of mush room resulting in accumulation of metals in the mycelia or sporocarp (Khoo and Ting, 2009). Size, texture and other physical characteristics are conducive for development into biosorbent without the need of immobilization or development of sophisticated reactor configuration as needed in the case of microorganism (Khoo and Ting, 2009).

Uptake of Cadmium (Cd), Pb, Cobalt (Co) and Cu by mycelia and sporocarps of an edible mushroom *Volvariella volvacea*, was reported by Purkayasthanitra *et al*., 2009. Metal (CuII) uptake potential mechanism and application of mushroom *Garnoder malucidium*, as a biosorbents has been reported by Khoo and Ting, 2009. Uptake of heavy metal pollutants by edible mushrooms and its effect on their growth, productivity and mammalian system was also studied. Garcial *et al*, 2009 reported the essential use of *Agaricus macrospores* for bioextraction of heavy metals from contaminated waste. The data indicated that *Agaricus macrospores* effectively extracted Cd, Hg and Cu from contaminated waste.

2.2.9.4 Yeast as Biosorbent

Among the promising biosorbent for heavy metal removal, which have been researched

during the past decades, yeast (*Saccharomyces cerevisiae)* has received increasing attention due to its unique nature in spite of the mediocre capacity for metal uptake (Edwards and Ho, 2012). *Saccharomyces cerevisiae* in different forms has been studied in biosorption research. For example, living cell/dead cell, immobilized cell/free cell, wild type/mutant type, flocculent/non-flocculent cell, engineered and non-engineered cell, laboratory culture/waste cells from different industries (Edwards and Ho, 2012).

They compared the removal efficiency for Zn, Cu, and Ni ions at the stage of biosorption, sedimentation and desorption. The results showed that *Saccharomyces cerevisiae* has a mediocre efficiency for or multi metal biosorption systems by comparing the index q_{max} of Langmiur equation with seven types of waste biomass for the removal of Pb ion. Chojnaka, 2007 indicated that Pb uptake capacity by *Saccharomyces cerevisae* is in the middle, in the middle, in comparison to six biomaterials used. Vianna and Yun, 2008 studied the biosorption capacity for Cu, Cd, and Zn, using three kinds of waste biomass from fermentation industries which are *Bacillus lentus, Aspergillus oryzae* and *Saccharomyces cerevisiae.* The results showed that protonated *Bacillus lentus* had the highest sorption capacity for Cu and Cd, followed by protonated biomass of *Aspergillus oryzae* and *Saccharomyces cerevisiae. Saccharomyces cerevisiae* is a unique biomaterial in biosorption research (Edwards and Ho, 2012). In *Saccharomyces cerevisiae,* free cells appear unsuitable in practical application, largely due to solid/liquid separation problem. However, Veglio *et al.,* 2011 pointed out that investigation on the performance of free cells for metal uptake can provide fundamental information on the equilibrium of the biosorption process, which is useful for practical application. Meanwhile, flocculating cell has been suggested for biosorption, attempting to overcome the separation problem of free cells. Brady and Duncan, 2010 proved that the cells of *Saccharomyces cerevisiae* treated with hot alkali were capable of accumulating a wide range of heavy metal cations (Fe^{3+},Cu^{2+}, Cr^{3+}, Hg^{2+}, Pb^{2+},$Cd^{2+,}$ Co^{2+}, Ag^{+}, and Fe^{2+}). Some

toxic metal ion studied in *Saccharomyces cerevisiae* biosorption are Pb, Cu, Zn and Cd, Hg, Co, Ni and Cr. Biosorption of Cr (VI) and As (V) onto methylated yeast biomass has been studied by Sekei and Myers, 2011. *Saccharomyces cerevisiae* can distinguish different metal species based on their toxicity such as Se (IV) and Se (VI), Sb (III) and Hg (II). This kind of property makes *Saccharomyces cerevisiae* useful not only for the bioremediation, removal and recovery of metal ions, but also for their analytical measurement. Mapolelo *et al*., 2004 proved that biosorption of Cd (II), Cr (III), Cr (VI), Cu (II), Pb (II) and (Zn (II) by *Saccharomyces cerevisiae* is dependent on optimum pH values above 5.

2.2.9.5 Algae as Biosorbent

Use of algal biomass as a biosorbent is emerging as an alternative, economical and effective proposition because of certain added advantage of algae over others (Crist *et al.*, 2011). Algae have low nutrient requirement, being autotrophic they produce a large biomass and unlike fungi they generally do not produce toxic substances, binding of metal ion on alga surface depends on different conditions like ionic charge of metal ion, alga species and chemical composition of metal ion solution (Crist *et al.*, 2011).

The uptake of Pb by dried biomass of green algae *Chlorella vulgaris* was investigated in a single-stage batch reactor in the concentration range of 25-200mg/l (Aksu and Kutsal, 2010). Sorption phenomenon at different pH values and temperatures was expressed by the Freundlich adsorption isotherm. Increased Pb uptake values at higher ph values and temperature were observed. Holan and Volesky, 2010) reported the biosorption of Pb and the Ni by biomass of marine algae. The multi-metal sorption system was investigated by Decarvalho *et al.*, 2005 with brown marine algae, *Ascophyllum nodosum.* Using two metal system comprising either (Cu+Zn) (Cu+Cd) or (Zn+Cd), they found that each of the metals inhibited the sorption of others. Biosorption of Cr (VI) from aqueous solution by green algae spirogyra had also been reported. The brown seaweed, Sargassum sp. (Chromophyta) was

used as a biosorbent for Copper ions (Crist *et al.*, 2011). The influence of different experimental parameters such as initial pH, shaking rate, sorption time, temperature, equilibrium condition and concentration of Cu ions on Cu uptake was evaluated (Crist *et al.*, 2011).

Biosorption of Cr (III) by *Sargassum* spp. was studied by Cossich *et al.*, 2004. The result shows that pH has an important effect on Cr biosorption capacity. The biosorbent size did not affect the Cr biosorption rate and capacity. The removal of Cr (VI) by Eclonia biomass, the brown sea weed was examined in a binary aqueous containing Ni. The removal rate was unaffected by the presence of Ni (II). Kiran and Viraraghavan (2011) reported biosorption of Cr (VI) by native isolate of an unexplored algal strain.

2.2.9.7 Conventional methods of metal ion removal and disadvantages

Many procedures have been applied in order to remove heavy-metals from aqueous streams. Among the most commonly used techniques are chemical precipitation, chemical oxidation and reduction, ion-exchange, filtration, electrochemical treatment, reverse osmosis (membrane technologies), evaporative recovery and solvent extraction (Xia and Liyuan, 2002). These classical or conventional techniques give rise to several problems such as unpredictable metal ions removal and generation of toxic sludge which are often difficult to dewater and require extreme caution in their disposal (Xia and Liyuan, 2002). Besides that, most of these methods also present some limitations whereby they are only economically viable at high or moderate concentrations of metals but not at low concentrations (Rabbani *et al.*, 2012), meaning diluted solutions containing from 1 to 100 mg/L of dissolved metal(s) (Cossich *et al.*, 2002). Heavy metal removal by classical techniques involves expensive methodologies.

2.2.9.7.1 Reverse Osmosis

It is a process in which heavy metals are separated by a semi-permeable membrane at a

pressure greater than osmotic pressure caused by the dissolved solids in wastewater. The disadvantage of this method is that it is expensive (Rabbani *et al.,* 2012).

`2.2.9.7.2 Electro dialysis

In this process, Tarangini, 2009 discovered that the ionic components (heavy metals) are separated through the use of semi-permeable ion selective membranes. Application of an electrical potential between the two electrodes causes a migration of cations and anions towards respective electrodes. Because of the alternate spacing of cation and anion permeable membranes, cells of concentrated and dilute salts are formed. The disadvantage is the formation of metal hydroxides, which clog the membrane. The disadvantage is the formation of metal hydroxides, which clog the membrane (Tarangini, 2009).

2.2.9.7.3 Ultra filtration

They are pressure driven membrane operations that use porous membranes for the removal of heavy metals. The main disadvantage of this process is the generation of sludge (Tao *et al*., 2009).

2.2.9.7.4 Ion-exchange

In this process, metal ions from dilute solutions are exchanged with ions held by electrostatic forces on the exchange resin. The disadvantages include high cost and partial removal of certain ions (Ramos *et al*., 2015).

2.2.9.7.5 Chemical Precipitation

Precipitation of metals is achieved by the addition of coagulants such as alum, lime, iron salts and other organic polymers. The large amount of sludge containing toxic compounds produced during the process is the main disadvantage (Ahalya *et al.,* 2003). The above techniques can be summarized as expensive, not environment friendly and usually dependent on the concentration of the waste. Therefore, the search for efficient, eco-friendly and cost

effective remedies for wastewater treatment has been initiated. In recent years, research attention has been focused on biological methods for the treatment of effluents, some of which are in the process of commercialization (Prasad and Freitas, 2003). There are three principle advantages of biological technologies for the removal of pollutants; first, biological processes can be carried out in situ at the contaminated site; Second, bioprocess technologies are usually environmentally benign (no secondary pollution) and third, they are cost effective. Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals (Ramos *et al*., 2015, 1993; Malik, 2004).

2.3.1 Biosorption Equilibrium Models

Preliminary testing of solid-liquid absorption system is based on two types of investigations: a) equilibrium batch sorption tests and (b) dynamic continuous flow sorption studies. The equilibrium of the biosorption process is often described by fitting the experimental points with models usually used for representation of isotherm adsorption equilibrium (Gadd *et al.*, 2010).

Recently, some biosorbents have emerged as an eco-friendly, effective and low cost material option (Hoell, 2001). These biosorbents include some agricultural wastes, fungi, algae, bacteria and yeast (Horsfall *et al.*, 2009). Studies using biosorbents have shown that both living and dead microbial cells are able to uptake metal ions and offer potential inexpensive alternative to conventional absorbents (Khoo and Ting, 2009). However, living cells are subject to toxic effect of heavy metals, resulting in cell death. Moreover, living cells often require the addition of nutrient and hence increase the BOD and COD in the effluent. For these reason, the use of non-living biomaterials or dead cells as metal binding compounds has been gaining advantage because toxic ions do not affect them. In addition, dead cells require less care and maintenance, and are cheaper. Furthermore, dead biomass could be easily

regenerated and reused (Mofa, 2011). Ramos *et al*., 2015 reported the biosorption kinetics and modelling. In addition to the biosorption isotherm analysis, biosorption kinetics was carried out by observing the variation of the metal uptake as function of time under different experimental conditions (Plaza *et al*., 2013). The experimental kinetic data were modelled by the pseudo-first and pseudo-second order kinetics. Results of the kinetic analysis indicate that the data fitted well the pseudo-second order rate equation. The pseudo-second order modelling showed that the metal biosorption is predominantly a physiochemical between the biomass and metal ions.

In addition, according to Vijayaraghavan *et al*., 2011, the constants of the pseudo-second order model would be based on the concentrations of the ions in the solution, the pH and the temperature of the solution. Vijayaraghavan *et al.,* 2011 further confirmed that the equilibrium biosorption data which showed that neodymium biosorption was strongly influenced by the pH and temperature. The researchers stated that chemical reactions have the main role in the rate-controlling step and the best correlation coefficient values were obtained by the pseudo-second order chemical reaction kinetics.

Kucuker *et al*., 2017 suggested that neodymium biosorption kinetics on *C. vulgaris* for the maximum uptake were at 21, 35 and 50˚C. The uptake generally consisted of two stages: a fast uptake rate in the first 15 min, where more than 90% of neodymium biosorption occurred, followed by a slower uptake rate as equilibrium approaches. Kucuker *et al*., (2017) agreed that equilibrium was reached within 30 minutes for all tested temperatures. The first phase was attributed to surface adsorption, mainly based on anion exchange with the participation of the carboxyl groups on the cell wall of biomass. A pH of 5 was found as an optimum pH for biosorption of neodymium, because the pKa of the carboxylic acid functional group was 4.8. A number of researchers reported that the carboxylic groups play a

main role for metal uptake from aqueous solution (Ramos *et al*., 2015; Plaza *et al*., 2013; Kucuker *et al*., 2017; Vijayaraghavan *et al*., 2011). Researchers have been looking for cheaper and more effective methods to remediate heavy metal-contaminated waters and reduce the growing public health risk. Biosorption is proven to be quite effective at removing metal ions from contaminated solution in a low-cost and environment-friendly manner.

The release of Heavy metals into the environment are increasing continuously as a result of industrial activities and technological development, thus posing a significant threat to the environment and public health due to their toxicity, accumulation in the food chain and persistence in nature. It is therefore important to develop new methods for metal removal and recovery from dilute solutions and for the reduction of heavy metal ions to very low concentrations. It is often inefficient and/or very expensive to use conventional technologies, such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery (Rabbani *et al.,* 2012). In recent years, the biosorption process has been studied extensively using microbial biomass as biosorbents for heavy metal removal. The use of microbial cells for Biosorption of heavy metals has been recognized as a potential alternative to existing technologies for recovery of heavy metals from industrial waste streams. The use of either laboratory-grown microorganism or biomass generated by the pharmacology and food processing industries or wastewater treatment units have been involved in Most studies of biosorption for metal removal (Rabbani *et al.,* 2012).

Increasing industrialization has resulted in an alarming increase in the discharge of heavy metals and other pollutants into the environment including water resources. Microbial populations in metal polluted environments contain microorganisms which have adapted to toxic concentrations of heavy metal and become metal resistant. These microorganisms can be used to remove heavy metals from the environment by various approaches like

bioaccumulation and bioadsorbtion, oxidation and reduction, methylation and demethylation (Rabbani *et al.,* 2012).

The microbe based approach for removal and recovery of toxic metals from industrial effluents can be economical and more efficient in comparison to physicochemical methods for heavy metal removal.

CHAPTER THREE

MATERIALS AND METHODS

3.1.1 Equipments

The equipments used in this study are: the incubator (HME Global, Model No. DNP-9022A, England), a hot air oven (Townson/Mercer Ltd. Croydon, England), a refrigerator/freezer (Model PRN 1313 HCA, BEKO, Germany), an autoclave (Yamato, USA), microscopes (Wild Herbrug M11, Switzerland and Bright field microscope), a laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station Labcaire product 220/240v), a centrifuge (Eppendorf 5804, Germany), an electronic weighing balance (Model QT 600), a pH meters (Orion Research, Model SA520,USA and Cyber scan 510, Signapore) and an atomic adsorption spectrophotometer (AAnalysat 800, USA)

3.1.2 Media

Bacteriological media used were Nutrient broth, Nutrient Agar, Centrimide Agar, Methyl Red Voges Proskauer Medium, Simmon's Citrate Agar, Phenol red broth, molten starch agar, Urea broth, MR-VP medium, nitrate broth, tryptone broth culture and Peptone Water. The media are products of Sigma Chemicals Ltd. Mueller-Hinton Agar (MHA) were produced by Hi Media, Vadhani, India.

3.1.3 Reagents

Chemical reagents such as Gram's iodine was purchased from Sigma Chemical Ltd., Crystal violet, Kovac's reagent, 40%KOH, Zinc powder, acetone alcohol and safranin were purchased from BDH Chemicals Ltd. Manganese for the experiment was purchased as Manganese (II) sulphate tetrahydrate ($MnSO_4.4H_2O$) from Zels Scientific Store, Keffi.

3.2 Methods

3.2.1 Study Area

This study was carried out in Keffi, a fast growing cosmopolitan town geographically located on longitude $7^{0}50^{0}$ E and latitude $8^{0}3$N, northwest of Lafia (the capital of Nasarawa state, Nigeria) and is situated on an altitude of 850m above sea level. Keffi, though in Nasarawa state, is about 68km from Abuja, the Federal capital of Nigeria (Makut *et al.,* 2014)

3.2.2 Sample collection

Surface Soil (0-20cm deep) was collected from the major dumpsite in Agwan lambu area of the Keffi metropolis, Nasarawa State using sterile polythene bag (Fawzy *et al*., 2017). The soil was sampled (Fawzy *et al*., 2017). The samples were transported in polythene bags in ice pack to Innovative Biotechnology Laboratory, Keffi and stored at 4^{o}C for analysis (Ogunmwonyi *et al*., 2008).

3.2.3 Sterilization technique

The polythene bags were cold sterilized in uv-radiation box for 12hours while glasswares were sterilized in the hot air oven at 160^{o}C for $1^{1/2}$ hours. Growth media and diluents (distilled water) were autoclaved at 121^{o}C for15 minutes at 15 P.S.I. as described by Gowsalya *et al*., (2014) and Ogunmwonyi *et al*., (2008).

3.2.4 Serial dilution

1g of soil sample was measured out using an electronic weighing balance (modelQT600) and placed in test tube 250ml Erlenmeyer flask (previously sterilized) containing 9ml of sterile water (Fawzy *et al*., 2017). The tube flask was tightly capped and shaken thoroughly to ensure adequate washing of the soil. This suspension was the first dilution (10^{-1}). 9 ml of sterile water was measured using sterile disposable pipette and put into different sterile test

tubes to carry out a 10 fold serial dilution. 1ml of the washed soil (aliquot) was transferred to a sterile test tube containing 9ml of sterile water using a fresh sterile disposable pipette to give 10^{-2}. The test tube was shaken to ensure that the solution was thoroughly mixed. This process was repeated for all the test tubes to achieve 10^{-5} (Fawzy *et al*., 2017).

3.2.5 Isolation of *Pseudomonas aeruginosa*

Nutrient agar was prepared by adding 28 g of nutrient broth powder to 1000ml of distilled water which was mixed properly and sterilized by autoclaving (121^{o}C for 15 minutes) as described by Gowsalya *et al*., (2014). For the isolation of *Pseudomonas aeruginosa*, about 20ml of nutrient agar having a temperature of about 45^{0}C was dispensed into Petri dishes and allowed to solidify. The dilutions used were 10^{-2} to 10^{-5}. For each dilution, three plates were inoculated by streaking on the solidified agar. 0.2ml of the aliquot was used. The plates were removed at the end of the incubation period. Pure cultures were obtained by sub culturing on centrimide agar. A smear of the culture was streaked on centrimide agar plates and incubated at 37^{0}C for 24 hours. The presence of greenish to bluish growth on the plates were marked as suspected *Pseudomonas aeruginosa* and further subjected to biochemical tests to ascertain the organism.

3.2.6 Isolation of Bacillus species

For the isolation of *Bacillus subtilis*, the initial dumpsite soil sample was suspended in normal saline solution (Alo *et al*., 2013). Fivefold serial dilutions of the sample were prepared as previously described (Fawzy et al., 2017). Isolation of *Bacillus subtilis* from serial dilution 10^{-2} to 10^{-5} was done using the method of Colinon *et al*., (2013). After preparation and solidification of the agar, 0.2ml of the aliquot was introduced to the media. The Petri dishes were incubated at 37^{o}C for 24 hours. Colonies were selected from the agar plates after 24 hours and streaked on freshly prepared nutrient agar to purify the culture. The plates were further incubated at 37^{o}C for 24 hours to get a pure culture (Colinon *et al*., 2013).

3.2.7 Identification of *Pseudomonas aeruginosa* and *Bacillus subtilis*

3.2.7.1 Cultural and Morphological Identification of *Pseudomonas aeruginosa* and *Bacillus subtilis*

The cultural and morphological identifications of *Pseudomonas aeruginosa* and *Bacillus subtilis* were carried out as described by Tankeshwar, (2018). All tests were carried out using aseptic techniques.

3.2.8 Gram Staining Examination

A drop of sterile water was placed on a clean grease-free glass slide. An inoculation loop was sterilized in a Bunsen burner flame and allowed to cool. It was then used to transfer light smear of the 24hour old culture on the slide and it was gently stirred into the sterile water until a thin homogeneous film was obtained. The inoculation loop was re-sterilized. The thin homogeneous film was allowed to air dry and heat-fixed by passing through Bunsen burner flame (blue cone flame). The slide was allowed to cool to the touch and flooded with crystal violet for 1 minute. The smear was rinsed off gently with sterile water for 20 seconds. The slide was flooded with the mordant (Gram's iodine) for 30 seconds and carefully rinsed with sterile water for 30 seconds. It was then decolourized rapidly with acetone alcohol (decolourizing agent) for 10-15 seconds until no more violet colour was visible in the draining run-off. The smear was counter stained with safranin for 30 seconds and rinsed with sterile water until no colour appeared in the effluent. The slide was allowed to air-dry. The slide was placed under bright field microscope using the magnification 100x oil immersion for greater clarity. A drop of immersion oil was placed on the slide, avoiding motion during application to prevent bubbles. Gram positive isolates retained the blue/purple colour inferred by the crystal violet trapped within their cell walls, while gram negative isolates appeared red/

pinkish colour since the violet washed through the cell wall maintaining the colour of the secondary dye (Tankeshwar, 2018).

3.2.9 Biochemical tests

3.2.9.1 Indole test

The Indole Test for the suspected organism was carried out as described by Tankeshwar, 2018. A colony of the suspected organism from culture plate was inoculated into a previously sterilized test tube containing 5ml of tryptone broth and incubated at 37°C for 24 hours in ambient air. 0.5ml of Kovac's reagent was added to the broth culture, and shaken. A positive reaction was indicated by the appearance of pink coloured ring in the reagent layer above the broth.

3.2.9.2 Voges-Proskauer test

A test tube containing methyl red-vogues proskauer (MR-VP) broth was inoculated with a pure culture of test organism (24 hours old) and incubated for 24 hours at 35^{o}C. 1ml of the broth was transferred into a sterile test tube. 0.6ml of 5% alpha-naphthol and 0.2ml of 40% KOH (Potassium hydroxide) was added. The tube was shaken gently to expose the medium to atmospheric oxygen and left undisturbed for 10 to 15 minutes Formation of pink/red colour indicated a positive reaction for Voges-Proskauer test while the formation of a yellow colour indicated a negative reaction for Voges-Proskauer test (Tankeshwar, 2018).

3.2.9.3 Methyl red

Two test tubes containing MR-VP broth was inoculated with a pure culture of test organism and incubated for 4 days at 35^{o}C. 5 drops of the methyl red indicator solution was added to the first test tube (for voges-proskauer test) while Barrit's reagent was added to the other tube. Formation of a red colour indicated positive reaction for methyl red (Tankeshwar, 2018).

3.2.9.4 Citrate utilisation test

The citrate utilization test for the suspected organism was carried out as described by Tankeshwar, (2018). A test tube containing Simmons citrate agar (slant surfaced) was inoculated (using inoculating loop) lightly as a single streak with a pure culture of the test organism (24 hours old). It was incubated at 37°C for 24 hours. Blue colour on the medium denotes alkalinisation and meant a positive result while green colour was negative.

3.2.9.5 Catalase test

A small amount of the pure colony of the test organism incubated at 37°C for 24hours was transferred aseptically to the surface of a clean, dry glass slide using an inoculating loop. A drop of 3% Hydrogen peroxide (H_2O_2) was placed on the slide and mixed gently. Rapid evolution of oxygen (bubbling gas) within 5-10 seconds shows positive result while absence of bubbles or only few scattered bubbles shows negative result. Catalase test was performed as described by Tankeshwar, (2018).

3.2.9.6 Nitrate reduction test

Nitrate reduction test was carried out as described by Tankeshwar, 2018. A heavy growth of pure colony of the isolate was inoculated aseptically into test tube containing nitrate broth and incubated at 37°C for 48 hours. One dropper full of Sulfanilic acid and one dropper full of alpha naphthylamine were added to the medium. Red colour indicates positive nitrate reduction test, otherwise, it is negative. A confirmatory test for nitrate reduction was performed by adding Zinc powder to the negative tubes. If there is a colour change, then the result is confirmed negative because it means that the nitrate had been present and was reduced to nitrite. If there is no colour change, it signifies a positive result.

3.2.9.7 Urease test

Urease test was carried out as described by Tankeshwar, (2018). This was done by inoculating a test tube (placed in a slant position) containing the urea broth medium with a loopful of a

pure culture of the test organism (24 hours old). The surface of the agar slant was streaked with the test organism. The cap of the test tube was left loosely and incubated at 35°C for 24 hours. Intense pink/ magenta colour indicated a positive test; otherwise, it maintained it retained its original colour (light orange) meaning the test was negative.

3.2.9.8 Sugar fermentation test

This uses phenol red broth to test for the fermentation of different sugars. Phenol red broth is a general purpose fermentation media that includes the pH indicator phenol red. Seven Test tubes (100 mm) were filled with 5 ml of phenol red carbohydrate broth (each test tube containing one of these sugars: mannose, maltose, galactose, glucose, mannitol, sucrose, lactose and duely labeled). A Durham tube was inserted into each of the test tubes to detect gas production. The media was autoclaved at 121°C for 15minutes for sterilization. The sterilization process also drove the broth into the inverted Durham tube. Inoculating loop was aseptically used to inoculate the test organisms (24hours old) into the broth containing the test sugars and inverted Durham tubes. This was incubated at 37°C for 18 hours.

Acid production result: After incubation, positive result is indicated when the liquid in the tube turns yellow (caused by the change in the color of the phenol red indicator). It indicates that there is a drop in the pH because of the production of the acid during the course of fermentation of the carbohydrate (sugar) present in the media.

Negative result is indicated by no change in colour (the broth maintains its red colour), indicating the bacteria cannot ferment that particular carbohydrate source present in the media.

Gas production result: Positive result is indicated by bubbles (small or big depending up the amount of gas produced) was seen in the inverted Durham tube.

Negative result is indicated when there is no bubble in the inverted Durham tube i.e. test organisms did not produce gas from the fermentation of the carbohydrate present in the media (Tankeshwar, 2018). This test was carried out for both *Pseudomonas aeruginosa* and *Bacillus*

subtilis.

3.2.9.9 Starch hydrolysis Test

Molten starch agar was poured into sterile Petri dish and allowed to solidify. After solidification, the starch agar plate was divided into three equal sectors using a marking pen to mark the bottom of the plate. The plate was labelled with the organisms' names and the date. Two sectors were inoculated separately (using sterilized inoculating loop) at a spot with the test organisms (24 hours old). The plate was inverted and incubated aerobically at 35^{o}C for 48 hours. The plate was removed from the incubator taking note of the location and appearance of the growth before adding the iodine. The growth and surrounding areas were covered with a dropper full of Gram's iodine. The areas surrounding the growth were examined for clearing. The clear zone that appeared indicated a positive result while a blue coloured zone indicated a negative result. This test was carried out for both *Pseudomonas aeruginosa* and *Bacillus subtilis* (Tankeshwar, 2018).

3.3 Choice of metal for biosorption studies

Manganese is mainly used in metallurgical processes, as a deoxidizing and desulfurizing additive and as an alloying constituent. It is also used in the production of dry-cell batteries, in chemical manufacturing, in the manufacture of glass, in the leather and textile industries, and as a fertilizer. The manganese used for this research was gotten in the form of Manganese (II) sulphate tetrahydrate ($MnSO_4.4H_2O$) and the initial concentrations used were 5ppm, 10ppm and 15ppm (Abioye *et al.,* 2015).

3.4 Metal Solution Preparation

Stock solution was prepared by dissolving 0.8111g of Manganese (II) sulphate tetrahydrate ($MnSO_4.4H_2O$) in de-ionised H_2O using 1000ml volumetric flask which gives 100ppm. It was shaken thoroughly for 15 minutes and allowed to stand for 24 hours to obtain complete

dissolution (Abioye *et al*., 2015). The stock solution was adjusted to pH7 using sodium hydroxide (NaOH) (Abioye *et al.,* 2015).

3.5 Determination of Metal Concentration

Determination of manganese concentration was done by using the atomic absorption spectrophotometer. The concentration of manganese used in this research (5pm, 10ppm and 15ppm) were achieved by further diluting the stock solution. All metal concentrations were determined with the use of the atomic absorption spectrophotometer. The processes are detailed below (Abioye *et al.,* 2015).

3.6 Biosorption experiments

These experiments were carried out as described by Hasan *et al*., 2010. Nutrient broth was prepared (as described above). 50ml of nutrient broth was measured and dispensed in different Erlenmeyer flasks containing metal stock solution of different concentrations (5ppm, 10ppm and 15ppm). Two set of each metal stock solution concentration was prepared (one set for *Pseudomonas aeruginosa* and the other set for *Bacillus subtilis*). 2ml of fresh inoculums (24 hours old culture of both *Pseudomonas aeruginosa* and *Bacillus subtilis*) was inoculated separately in each flask using aseptic techniques. These experimental flasks with cultures were incubated at different temperatures of 25^0C, 37^0C and 45^0C for 12days. 2ml of the samples were drawn on 3, 6, 9, and 12 days to determine the final concentration of manganese. The supernatant were separated by centrifugation at 4000 rpm for 25 minutes with a centrifuging machine (Eppendorf 5804, Germany) (Hasan *et al,* 2010). The supernatant was digested with nitric acid (HNO_3) so as to make it clear for analysis (Abioye *et al.,* 2015) using Adsorption Atomic Spectrometer (Analysat 800, USA). This was put in the cuvette of the hollow cathode of the atomic absorption spectrophotometer and set at a wavelength of 279.8nm at 0.2nm band pass with a working range of 0.1-5g/ml for direct reading. All experiments were carried out in triplicates and the results were recorded. The % biosorption

of manganese by *Bacillus subtilis* and *Pseudomonas aeruginosa* was calculated using the following equation (Abioye *et al*., 2015 and Vieira and Volesky, 2000):

$$\% \text{ Removal of Mg ion (\%R)} = \frac{C_1 - C_2}{C_1} \times 100$$

C_1 = The initial concentration of manganese in the solution

C_2 = The final concentration of manganese in the solution

3.6.1 Determination of the Effect of Metal Concentration

The initial metal concentration of 5ppm, 10ppm and 15ppm was observed to have profound effect on the rate at which the organisms used up manganese. Manganese removal by *Bacillus subtilis* and *Pseudomonas aeruginosa* was studied for 12 days with final concentration checked at day 3, day 6, day 9 and day 12 (Fawzy *et al.*, 2017).

3.6.2 Determination of the Effect of pH

The metal sorption was monitored for pH range 1 to 9. 2ml of inoculums (*Bacillus subtilis* and *Pseudomonas aeruginosa*) was dispersed in 50ml each of the solution containing 5ppm of Manganese metal concentration. All flasks were maintained at different pH values ranging from 1 to 9 for 12 days in an incubator at temperature of 37^0C (Abioye *et al.*, 2015). The pH of the solution was controlled using pH meter (CyberScan 510, Singapore).

3.6.3 Determination of the effect of temperature

Experiments were carried out at room temperature of 25^0C, incubation temperature of 37^0C and at a higher temperature of 45^0C for each culture (*Bacillus subtilis* and *Pseudomonas aeruginosa*). The samples were collected at intervals as above and analyzed for metal concentration using the atomic adsorption spectrophotometer (Abioye *et al.*, 2015).

CHAPTER 4

DATA PRESENTATION AND RESULTS

4.1 Data Presentation

Morphological and Biochemical Characterization of *Pseudomonas aeruginosa* and *Bacillus subtilis* are presented in Table 4.1.1. Table 4.1.2 illustrates the effect of metal concentration on percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* in days. On the 3rd day, the sorption percentage at 5ppm was 50.0% for *Pseudomonas aeruginosa* and 47.3% for *Bacillus subtilis*. This increased rapidly up to 76.3% for *Pseudomonas aeruginosa* and 60.0% for *Bacillus subtilis* by the 6th day. The sorption rate became gradual after 9 days with percentage sorption of 78.9% and 79.9 % for *Pseudomonas aeruginosa* on days 9 and 12 respectively. *Bacillus subtilis* had percentage sorption of 68.8% and 69.2% on days 9 and 12 respectively. These biosorption rates are similar to those reported by Fadel *et al.*, (2017). Initial metal concentration of 5ppm (parts per million) has the highest sorption rate of 79.9% and 69.2% for *Pseudomonas aeruginosa* and *Bacillus subtilis* on day 12 respectively. *Pseudomonas aeruginosa* was effective in manganese removal up to 79.9%, 65.4% and 62.0% for initial metal concentrations of 5ppm, 10ppm, and 15ppm respectively by the last day (day 12). *Bacillus subtilis* was also effective in manganese removal up to 69.2%, 60.2% and 59.3% for initial metal concentrations of 5ppm, 10ppm, and 15ppm respectively by the 12th day. Initial metal concentration of 5ppm (parts per million) has the highest sorption rate of 79.9% for *Pseudomonas aeruginosa* and 69.2% for *Bacillus subtilis* respectively. Figure 4.1.3 depicts the effects of pH on percentage (%) manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis*. This was observed over a range of pH 1 to 9. The biosorption percentage was highest at pH 5 for both *Pseudomonas aeruginosa* and *Bacillus subtilis* with percentage biosorption of 80.4% and 75.2%

respectively. The optimum pH for manganese biosorption was found to be 5. The percentage biosorption increases from pH 1 to 5 and starts to decrease from pH 6 to 9. Figure 4.1.4 shows the effects of temperature on percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis*. The percentage biosorption was 69.7% and 58.3% at temperature of 25^{o}C for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. Percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* was highest at temperature 37^{o}C with percentage biosorption of 89.5% and 78.4% for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. At a temperature of 45^{o}C, percentage biosorption for *Pseudomonas aeruginosa* was 50.0% while *Bacillus subtilis* had a percentage biosorption of 45.0%. The bioload gradually decreases with increase in metal concentration while it remained static with increase pH until pH of 6 was reached. Change in temperatures caused fluctuations in their growth. The metal ions did not affect the viability of the organisms used in this study.

One-Way ANOVA test for manganese biosorption by *Pseudomonas aeruginosa* in days has the F-value of 0.0430, P-value of 0.0507 and the treatment degree of freedom (df) of 3 while the one-way ANOVA test for *Bacillus subtilis* has the F-value of 12.1876, the P-value of 0.0024 and the treatment degree of freedom (df) of 3. The effect of pH on percentage (%) manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* has the P-value of F-value of 2.0558, 0.5005 and the treatment degree of freedom (df) of 1. The effect of temperature on percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* has the F-value of 0.3747, P-value of 1.8743 and the treatment degree (df) of 1.

Table 4.1.1: Morphological and Biochemical Characterization of *Pseudomonas aeruginosa* and *Bacillus subtilis*

S/N	Gram's Reaction	IND	MR	VP	CIT	STH	CAT	NIT	OXI	GLU	URE	Inference
1	Negative rod	-	-	+	+	+	+	NA	-	+	+	*Pseudomonas aeruginosa*
2	Positive rod	-	-	+	+	+	+	+	-	+	+	*Bacillus subtilis*

KEY: IND = Indole; MR = Methyl Red; VP = Voges Proskauer; CIT = Citrate Utilisation; STH = Starch Hydrolysis; CAT = Catalase; NIT = Nitrate Reduction; OXI = Oxidase; GLU = Glucose Utilisation; URE = Urease Production; – = Negative, + = Positive

Table 4.1.2 Percentage Biosorption of Manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* in different concentrations

Initial Concentration of Manganese (ppm)	Percentage (%) Biosorption by *Pseudomonas aeruginosa*				Percentage (%) Biosorption by *Bacillus subtilis*			
	Day 3	Day 6	Day 9	Day 12	Day 3	Day 6	Day 9	Day 12
5	±50.0	±76.3	±78.9	±79.9	±47.3	±60.0	±68.8	±69.2
10	±45.9	±57.5	±64.6	±65.4	±40.4	±54.3	±66.6	±60.2
15	±44.8	±55.1	±61.3	±62.0	±37.9	±50.3	±58.6	±59.3

KEY: ppm = parts per million, % = percentage

Figure 4.1.3
Graphical illustration of Percentage Manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* at different pH

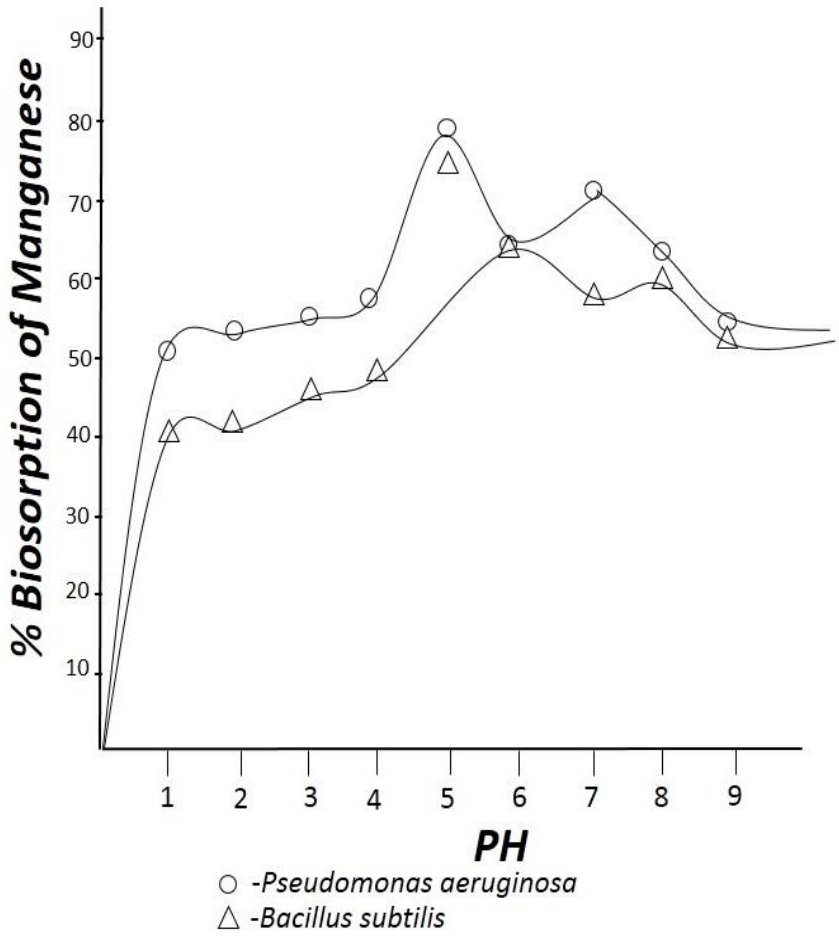
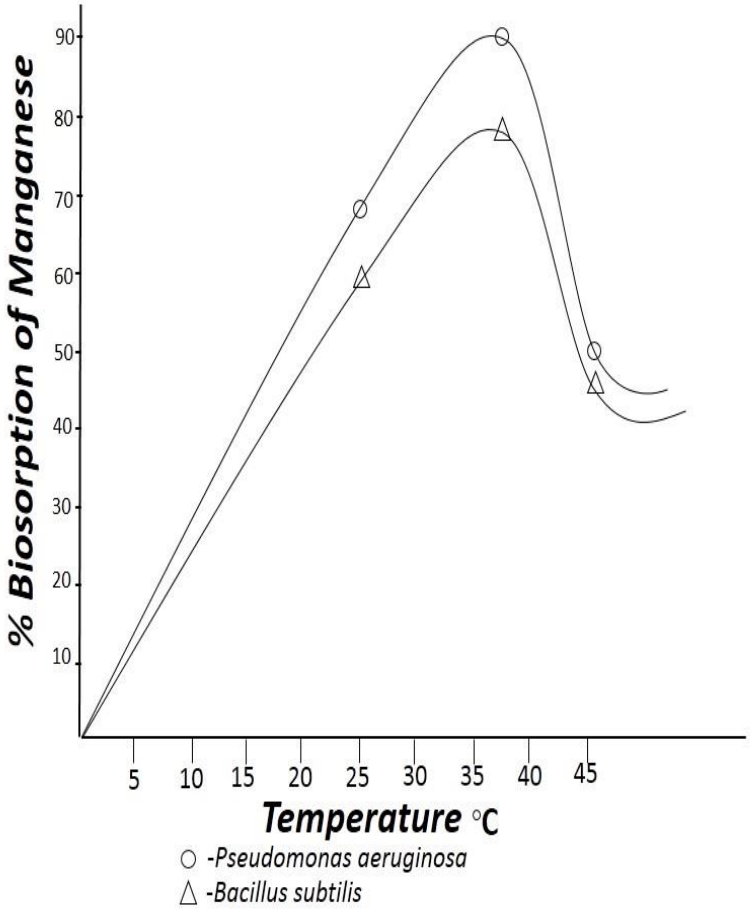


Figure 4.1.4

Graphical illustration of Percentage Manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* at different temperatures



4.2 Discussion of Findings

During the last two decades, extensive attention has been paid on management of environmental pollution caused by hazardous materials such as heavy metals.

This study explored the manganese sorption potentials of B*acillus subtilis* and *Pseudomonas aeruginosa* isolated from soil of waste dump sites. There was significant difference in the percentage of manganese biosorption between *Pseudomonas aeruginosa* and *Bacillus subtilis***.** The initial metal concentration was observed to have a profound effect on the biosorption rate. Metal removal by bacteria was rapid at the initial time of the biosorption with a sharp increase between 3 and 6 days. However Between 9 and 12 days the sorption rate was only marginal which could be due to saturation of binding site. Biosorption kinetics with an initial rapid metal uptake followed by slow uptake was observed, and this kinetic model has been accepted for various biosorbents such as bacteria and fungi (yeast) under similar operation conditions (Goyal *et al*., 2003). These biosorption rates reported in this research are similar to those reported by Fadel *et al*., (2017). The optimum pH for manganese biosorption was found to be 5. The percentage biosorption increases from pH 1 to 5 and starts to decrease from pH 6 to 9. The fluctuation beyond pH 5 could be due to decrease of low availability of surface for biosorption at low pH (Vijayaraghavan and Yun, 2008). It was observed that biosorption by both *Pseudomonas aeruginosa* and *Bacillus subtilis* is optimum at pH 5. This report corresponds with what was reported by Dhankhar *et al*., 2011 for absorption of Cu^{+2}, Zn^{+2} and Fe^{+2} by *Bacillus subtilis.* Hasan *et al*., 2010 also reported that the favourable pH for metals biosorption by bacteria has been found in a range of 3-6 (Vijayaraghavan and Yun, 2008). Hydrogen ion potential (pH) is a significant factor for maintaining the surface characteristics of the biosorbent, sustaining the chemical properties of biosorbate and a vital

controlling aspect in the biosorption process (Debarshi *et al*., 2013). The pH of the solution must be appropriate for interaction. Generally, it was observed that an increase of pH caused protonation of metal ions binding sites exposed by cellular surface. However, a decrease of pH causes competition between protons and positively charged metal ions. This observation is in correspondence with Fadel *et al*., (2017). Metal removal by the organisms was rapid with a sharp increase between 3 and 6 days. However Between 9 and 12 days the sorption rate was only marginal which could be due to saturation of binding site.

Percentage biosorption of manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* was highest at temperature 37^0C with percentage biosorption of 89.5% and 78.4% for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively while the percentage biosorption was 69.7% and 58.3% at temperature of 25^0C for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. This result further confirms the research of Abioye *et al*., (2015).

Higher sorption rate at temperature 37^oC than at 25^oC may be due to shrinkage of cells at lower temperatures which reduce the surface area of contact (Vijayaraghavan and Yun, 2008). In general, *Pseudomonas aeruginosa* has a higher uptake of manganese than *Bacillus subtilis*. This may be due to Cell walls of gram negative bacteria being somewhat thinner than the gram positive ones and are also not heavily cross-linked. Fadel *et al*., 2017 explained the higher tolerance ability of the gram negative bacteria to heavy metals than the gram positive species.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

This study was explored to examine manganese sorption potentials of *Bacillus subtilis* and *Pseudomonas aeruginosa* isolated from soil of waste dump sites. In general, *Pseudomonas aeruginosa* has a higher uptake of manganese than *Bacillus subtilis*. This may be due to Cell walls of gram negative bacteria being somewhat thinner than the gram positive ones and are also not heavily cross-linked (Mohanty *et al.,* 2004). The higher tolerance ability of the gram negative bacteria to heavy metals than the gram positive species was explained by Eagon, 2004 and Hasan *et al*., 2010. The maximum metal tolerance by the gram negative bacterial forms might be due to their abundant sedentary organism and also due to the metal precipitation in their peptidoglycan layers (Parameswari *et al.,* 2009). The lipopolysaccharides nature of the outer membrane of gram negative organisms is also responsible for efficient metal binding capacity (Mohanty *et al.,* 2004).

5.2. Conclusion

From this research, both *pseudomonas aeruginosa* and *Bacillus subtilis* were effective biosorbents in Manganese removal at varying conditions of pH, Temperature, different metal concentrations and time (days) which are the major factors that affect biosorption processes. The pH of 5, temperature of 37^{o}C, 12 days contact time and 5ppm initial metal concentration were the optimum conditions found suitable for the biosorption of manganese by *pseudomonas aeruginosa* and *Bacillus subtilis*. Although, both organisms were effective in Manganese biosorption, *Pseudomonas aeruginosa* was found to be more effective in Manganese biosorption hence a better alternative over *Bacillus subtilis.*

5.3 Recommendation

The following recommendations have been made:

i. Improvement of sorption capabilities of *Pseudomonas aeruginosa* and *Bacillus subtilis* through genetic engineering to enhance higher uptake of manganese as well as other heavy metals at varying conditions of pH, Temperature, Metal concentration, and Biomass concentrations.

ii. Use of microorganisms such as *Pseudomonas aeruginosa* and *Bacillus subtilis* for biosorption of heavy metals should be encouraged**.**

5.4 Limitations of the study

The limitation encountered in this study is the inability to access enough funds to further research into *Bacillus subtilis* and *Pseudomonas aeruginosa*.

5.5 Suggestions for the further study

Genetically modified microorganisms such as *Pseudomonas aeruginosa* and *Bacillus subtilis* should be looked into to be able to adequately biosorp manganese which will be of great advantage in the cleanup process of polluted areas and leaching-prone environments.

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APPENDICES

APPENDIX 1

Media Used

- Nutrient Agar (NA)

  1.0g lab-lemco powder

  2.0g yeast extract

  5.0g peptone

  5.0g sodium chloride

  15.0g Agar No.3 (oxoid)

- Nutrient Broth (NB)

  1.0g lab-lemco powder

  2.0g yeast extract

  5.0g peptone

  5.0g sodium chloride

APPENDIX 2

Reagents Used

- **Safranin**

  1.0g Safranin

  100ml of Distilled Water

- **Crystal Violet Stain**

  0.5g Crystal Violet

  100ml of Distilled Water

APPENDIX 3

Sample Collection Site

Waste Dumpsite in Agwanlambu Area of Keffi, Nasarawa state.

APPENDIX 4

Table of data generated for final concentration of Manganese biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* before conversion to percentage value

Initial Concentration (C1) of Manganese (ppm)	Final concentration (C2) of Manganese biosorption by *Pseudomonas aeruginosa*				Final concentration (C2) of Manganese biosorption by *Bacillus subtilis*			
	Day 3	Day 6	Day 9	Day 12	Day 3	Day 6	Day 9	Day 12
C1=5	±2.5	±1.18	±1.06	±1.1	±2.64	±2.00	±1.56	±1.54
C1=10	±5.41	±4.25	±3.54	±3.46	±5.97	±4.57	±3.34	±3.98
C1=15	±8.28	±6.74	±5.81	±5.70	±9.32	±7.46	±6.21	±6.11

Formula for conversion to percentage is $\frac{C_1-C_2}{C_1} X100$

APPENDIX 5

Table of data generated for Manganese biosorption at various pH by *Pseudomonas aeruginosa* and *Bacillus subtilis* before conversion to percentage values

Ph	Biosorption by *Pseudomonas aeruginosa*	Biosorption by *Bacillus Subtilis*
1	±2.44	±3.00
2	±2.34	±2.88
3	±2.23	±2.75
4	±2.08	±2.63
5	±0.98	±1.24
6	±1.78	±1.83
7	±1.38	±2.08
8	±1.93	±1.99
9	±2.34	±2.34

Formula for conversion to percentage is $\frac{C_1-C_2}{C_1} X100$

APPENDIX 6

Table of data generated for percentage Manganese biosorption at various Biomass concentration by *Pseudomonas aeruginosa* and *Bacillus subtilis*

pH	Biosorption by *Pseudomonas aeruginosa*	Biosorption by *Bacillus Subtilis*
0.5	±54.3	±35.4
1.0	±57.8	±42.1
1.5	±62.3	±55.2
2.0	±65.3	±72.4

APPENDIX 7

Table of data generated for Manganese biosorption at various temperature by *Pseudomonas aeruginosa* and *Bacillus subtilis* before conversion to percentage

Temperature (^{o}C)	Biosorption by *Pseudomonas aeruginosa*	Biosorption by *Bacillus subtilis* (%)
Room temp 25	±1.52	±2.09
Optimum 37	±0.53	±1.08
High temp 45	±2.5	±2.75

Formula for conversion to percentage is $\frac{C_1-C_2}{C_1} X100$

APPENDIX 8

Table of data generated for Percentage (%) Manganese Biosorption by *Pseudomonas aeruginosa* and *Bacillus subtilis* at different pH

pH	Biosorption by *Pseudomonas aeruginosa* (%)	Biosorption by *Bacillus subtilis* (%)
1	±51.2	±40.1
2	±53.3	±42.5
3	±55.5	±45.0
4	±58.5	±47.5
5	±80.4	±75.2
6	±64.5	±63.5
7	±72.5	±58.5
8	±61.5	±60.2
9	±53.2	±53.2

KEY: pH = Hydrogen Ion Potential, % = percentage

APPENDIX 9

Table of data generated for Percentage Biosorption of Manganese by *Pseudomonas aeruginosa* and *Bacillus subtilis* at different temperatures

Temperature (^{o}C)	Biosorption by *Pseudomonas aeruginosa* (%)	Biosorption by *Bacillus subtilis* (%)
25	±69.7	±58.3
37	±89.5	±78.4
45	±50.0	±45.0

KEY: ^{o}C = degree celsius; % = Percentage