POTENTIAL OF *Lysinibacillus fusiformis* 5B AND ITS IMMOBILIZED UREASE FOR DETECTION AND BIOSORPTION OF SELECTED HEAVY METALS

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ABSTRACT

Ureases are a group of enzymes that hydrolyse urea producing carbon dioxide and ammonia. Urease producing bacteria have been hypothesized to have inherent bioremediation abilities. The aim of this research was to detect heavy metals in solution using immobilized urease produced by *Lysinibacillus fusiformis* 5B and determine its potential to biosorp lead (Pb), chromium (Cr), cadmium (Cd) and nickel (Ni). Urease produced by *L. fusiformis* 5B was immobilized on 1cm x 6cm strips using tris-acetate buffer, and phenol red in 10 % glutaraldehyde solution for detection of the heavy metals. *L. fusiformis* 5B was screened for the potential to utilize 5 ppm of each heavy metal using agar dilution method. Broth of *L. fusiformis* 5B was inoculated to 10, 15, 20 and 50 ppm of the heavy metals. The rate of biosorption was determined by atomic absorption spectroscopy (AAS) after 0, 7, 14, 21, 28 and 35 days. The percentage (%) biosorption was determined by Beer Lambert's equation. Detection strips showed varying degrees of magenta compared to standard color chart for the quantification of the heavy metals in solution. *L. fusiformis* 5B was able to tolerate 5 ppm concentration of all the heavy metals. There was an increase in biosorption rate as the time (days) progressed. The highest biosorption for 10, 15, 20 and 50 ppm was chromium with 99.97 %, lead with 99.89 %, chromium with 99.93 % and cadmium with 97.23 %, respectively, after 35 days. There was a significant difference ($P<0.05$) between the biosorption capacity of the isolate as the time (days) progressed. The results of this study showed that immobilized urease produced by *L. fusiformis* 5B was able to detect Pb, Cr, Cd and Ni and can be developed for real-time testing and sensors device to detect these heavy metals in solution. In addition, *L. fusiformis* 5B possessed the capacity to biosorp Pb, Cr, Cd and Ni and can be developed as biosorption agent for these heavy metals.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Metalloids or metals that are five times denser than water or with atomic density greater than 4 g/dm^3 are regarded as heavy metals. Heavy metals are known to be poisonous or toxic even if the concentration is low. Examples of the platinum and heavy metals group elements include: As, Fe, Zn, Ag, Pb, Cd, Ni, and Cr. Due to their presence in trace amount, heavy metals are sometimes called trace elements. Their amount varies from ultratrace (1 μgkg^{-1}) to trace (10 mgkg-10) quantities. Among the heavy metals, Cd, Pb, Hg and Cr(Iv) are ecotoxicologically dangerous. For decades now, heavy metals present in aquatic environment has posed a serious threat to biological systems since they are considered biologically available both in dissolved ionic and organic forms (Petra and Lindholm-lehto, 2019).

Heavy metals have been found to be present in the environment naturally. However, their presence can also be enhanced by human activities and industrialization, which causes serious contaminantion whenever they find their ways to ground or surface water. Industrialization has been the epicenter of environmental contamination by heavy metals. Many sectors including but not limited to Pharmaceutical, marine, aviation, mining, and textile industries contribute immensely to the influx of heavy metals into the environment. However, the main sources/channels by which heavy metals pollution occur is through one of the following: smelting, metal plating, pigment manufacture, tanneries, battery production, petroleum refining, paints production, pesticides application, and mining (Salman *et al*., 2014).

The health of the environment and the biological things in it is seriously threatened by pollution arising from heavy metals. Varying types of heavy metals are utilized in different industries, one way or another these heavy metals are being discharged into the environmental as untreated waste water, which are deleterious to environmental health. Although living things (i.e. animals and humans) requires heavy metals (e.g. zinc, iron and copper) in trace amount/quantity to perform a particular function, they can become toxic whenever they exceed levels at which they are regarded as safe owing to their bioaccumulation characteristics (Mustapha and Halimoon 2015). As a result of this, there is an increase in public awareness on the impacts heavy metals have on the ecosystem and man. As such, industries that serve as a primary source through which heavy metals are released into the environment are becoming more intentional in the treatment of effluent or waste water to reduce their concentration before releasing them into our water bodies. Various conventional waste water treatment techniques have been employed in the past still yet reports of environmental contamination by heavy metal keeps reoccurring (Salman *et al*., 2014).

The presence of heavy metals in humans often come via the consumption of heavy metals polluted food and/or water. Plants also serve as a major link through which heavy metals get into human system since they have the ability to take up these metals from contaminated soil, which gets accumulated in plants. Whenever such plants are consumed the heavy metals present get transferred into the body of humans. It is important to state that excretion rates of heavy metals in humans are very low. Although, humans needs some of these heavy metals to carry out some biological and physiological functions. However, when it is taken and gets accumulated in the human system, it becomes toxic and poses a serious threat such as mental retardation, seizures and encephalopathy among many others (Salman *et al*., 2014).

One of the heavy metals with wide applications is copper. Copper is majorly used by electroplating industries and in electrical appliances. High concentration of copper in the environment is extremely deleterious to life in general. Copper (II) ions are known to cause severe toxicological problems affecting the liver, skin, myocardium, pancreases and the brains of humans. Activities such as fiber production, metallurgical, printed circuits, pipe corrosion, mining and metal plating releases heavy metal into the environment. Industries such as petroleum, paper and pulp, as well as mining industries release large amounts of copper into the environment. Other means by which copper get into the environment is through some human activities such as, use of wood preservatives, fertilizer and pesticides application among many others. Copper have been reported to contaminate food such as nuts, mushrooms, and shell fish. Water, drinks and food packaged in containers made of copper are potential sources of copper poisoning. In some cases, food poisoning by copper has led to enlarged liver, jaundice and are often times linked to increase in the occurrence of cancer of the lung among workers who are constantly exposed to it (Salman *et al*., 2014).

Stable chromium exists in two oxidation states in the environment. They are: Cr (VI) and Cr (III) and their bioavailability, mobility and toxicities vary. Compounds of chromium possessing oxidation state of Cr^{6+} act as power oxidants; they are often used in pyrotechnics, paints, plastics, dyes, inks and as pigments in photography. Cr^{6+} can also be utilized in the manufacture of stainless steel, dyes used in textile industries, in wood preservation, anticorrosion coatings and for tanning of leather. Cr^{6+} is readily mobile and moves across the ecosystem (i.e. from soil to aquatic environment). Cr^{6+} is a strong oxidant, which is readily absorbed into the skin of humans. However, Cr^{3+} is relatively immobile and innocuous, and it is required for normal lipid and carbohydrate metabolism (Salman *et al*., 2014).

Cadmium in high concentration is deleterious to human health, It has been reported to cause "itai-itai" a severe outcome of Cd toxicity characterized by severe pains in the bone. Hypertension, hepatic damage and kidney dysfunction are among the health disorders caused by Cd in humans (Salman *et al*., 2014). Accumulation of heavy metals in vital organs of humans as a result of consuming heavy metal contaminated food has been reported by Abioye *et al*. (2018). These contaminations often arises due to the utilization of tannery effluents in irrigating agricultural fields allowing plants to take up heavy metals present in such effluent. Once these contaminated plants are consumed it becomes a problem to humans.

Heavy metal health implications can cause various degree of illnesses based on chronic or acute exposure. They include cholera, cancer, skin irritation, and kidney dysfunction. As mentioned earlier, tannery effluent poses a serious environment health problem since they contain toxic heavy metals such as Pb, Cu, Cd, Cr and Zn, which are oftentimes absorbed and get accumulated in various parts of plants as free metals. Continuous accumulation of heavy metals by such plants can affect the normal function of plant tissues and cells ultimately affecting their growth and metabolisms. It is important to note that nickel and chromium causes diseases in both humans and cattle.

Biosorption as defined by Petra and Lindholm-lehto (2019) is the ability/potential of any biomass to bind and sequester heavy metals ions from a dilute aqueous solution. The cell wall of such biomass is structured in a way to permit the binding of heavy metals through some binding forces. A typical biosorption model involves a liquid phase holding the dissolved metal ions to be sorbed and a solid sorbent. An efficient up take of metal can vary from minutes to few hours in order for the biomass, which acts as ion exchanger to achieve biosorption. In order to tackle the growing reports of heavy metal contamination of the environment by industrial waste

effluents, various conventional technologies have been exploited. However, they have not been efficient in the removal of toxic metals from effluents. This causes a shift in search of novel technology to help alleviate heavy metal polluted environment towards biosorption on the basis of metal binding capacity already exhibited by numerous biological materials such as microorganisms. Nwidi and Agunwamba (2015) reported yeast, fungi, algae and bacteria to be good metal biosorbents, since they can accumulate heavy metals from industrial effluents through physico-chemical pathways.

The utilization of microorganisms such as bacteria for heavy metal biosorption in aqueous solutions has proven to be cheap with lots of promising potentials. In the past, microorganisms have been reported by Salman *et al*. (2014) to carry out biosorption of radio active compounds and heavy metals through physico-chemical interactions between the cellular compounds of the biomass and the metal ions. As a result, varying species of microorganisms have been exploited over the past two decades for biosorption potentials (Salman *et al*., 2014). With the exception of metal cations that are alkaline in nature such as potassium and sodium ions, all biological materials can be useful one way or another in the sequestration of metals although the process could be significantly passive or active occurring either in living or dead cells of microorganisms. Generally, biological materials are good sorbent materials used in biosorption of metal ions. Aside microorganisms, other cheap sorbent materials exist, which include but not limited to plants, polysaccharide materials, agricultural wastes and industrial wastes (Mustapha and Halimoon, 2015).

The genus *Lysinibacillus* are motile, ubiquitous, rod-shaped Gram positive bacteria, which can exist as aerobes and in the absence of oxygen can assume a facultative mode of aeration. *Lysinibacillus* sp. belongs to the family Bacillaceae where Firmicutes is the Phylum. They are

characterized by the possession of endospores. This genus *Lysinibacillus* has the ability to absorb heavy metals and has been tested for potentials in bioremediation studies. *Lysinibacillus fusiformis* produces biosurfactants, which are characterized on the basis of their emulsifying properties with diesel, engine oil, mobil oil and petrol (Christian *et al*., 2018).

1.2 Statement of the Research Problem

Because of the toxicity and their abilities to bioaccumulate in both plants and animals tissues including man, pollution caused by heavy metals is one of the most important problem, facing the environment today. Heavy metals are difficult to remove since they are biologically and chemically non-degradable. Waste effluents containing heavy metals, which are oftentimes released into water ways cannot undergo biodegradation but can undergo chemical or microbial transformations. The pollution of the environment with heavy metal is an issue of serious concern on human and environmental health. Heavy metal leads to many health issues in human including jaundice, enlarged liver, and increase in lung cancer among exposed workers, seizures and mental retardation. Heavy metals are of great concern since many metal processing industries discharged their waste water into the environment (Salman *et al*., 2014).

Heavy metal stands out among the most recalcitrant and poisonous substances troubling the environment in this present generation. The scientific community are finding it difficult to evaluate the extent of damage heavy metals caused to biological system. More people become victims of these toxins without knowing it, until it is too late. Presence of heavy metal in the ecosystem has ruined lives of unborn babies and young ones. It is important to detect heavy metals easily before they cause adverse effects. Heavy metals have psychological and physiological effects so they are one of the feared toxins (Bañares *et al*., 2015).

The physical and chemical remediation often leads to generation of toxic products (secondary pollution). Different types of conventional methods and technologies have been developed and are currently in use industrially to reduce the level of heavy metal toxicity in order to minimize its adverse effects on the environment. Utilization of such conventional methods although efficient to some level, but require high operational cost since there must be a continuous inclusion of chemicals making the whole process unsustainable in a long run. Various methods have been designed and installed to tackle the stress caused by heavy metals on the environment; they include chemical and physical methods. In mine sites, precipitation method is commonly used. However, precipitation is not efficient in neutralizing the harmful effects caused by heavy metal since its actions is often impaired by presence of ion or salts of other metals as well as presence of acidic pH. Evaporation often results in the release of wastes and sludges with hazardous potentials (Vershima *et al*., 2015).

Environmental pollution has been a great concern in Nigeria due to their early industrialization stage, poor implementation of regulations and policies to help protect the environment. Many Nigerian vegetables and food crops are contaminated through natural and anthropogenic sources. Plant cells and tissues absorb and concentrate heavy metals on the basis of pH, available moisture content, nutrient, organic matter, and temperature (Nkwunonwo *et al*., 2020).

Humans are often exposed to mercury poisoning whenever they consume fishes whose habitats have been contaminated by the mercury brought about by running water. These contaminants get accumulated in fishes up to high concentrations capable of causing severe health disorders when feed upon by man. As such, it is pertinent to remove and/or recover heavy metals from effluents before discharging them into the water bodies or the environment so as to ensure a safe and clean environment (Vershima *et al*., 2015).

1.3 Justification for the Study

Detection of heavy metals using rapid techniques in water, waste water, liquid food samples and other environmental agents will help to control hazards associated with heavy metal consumption. Current methods used in detection of heavy metals in these elements are expensive, specific, laborious, time consuming, difficult and require expertise. The immobilizing of urease on strip to detect heavy metals, however, will make it easier and faster to check for the presence of heavy metals in the environment. The advantage of the current method is that it is applicable for heavy metal detection, it is not expensive, doesn't require expertise, and is user friendly. The use of microorganism for biosorption is sustainable as they have rapid growth and are ecofriendly. Microorganisms can not only biosorp heavy metals they can also transform it to a less toxic form. The use of microorganisms to biosorp is cheaper than other physicochemical methods (Cao *et al*., 2015). There are several advantages obtained while using biosorption over other conventional methods such as minimization of biological sludge or chemical, it is cheap but highly efficient, does not require additional nutrient, the microbial biomass are easily generated and the recovery of biosorp heavy metal is feasible (Salman *et al*., 2014). It is indicated that urease producing bacteria can be used for biosorption and bioremediation. Immobilizing of urease on paper strip to detect heavy metals will make it easier and faster to check for the presence of heavy metal in the environment (Cao *et al*., 2015).

1.4 Aim and Objectives

The aim of this study was to determine the potential of *Lysinibacillus fusiformis* 5B and its immobilized urease for detection and biosorption of selected heavy metals in the environment

The objectives of this research study were to:

i. Confirm the purity of *Lysinibacillus fusiformis* 5B

ii. Produce urease from *Lysinibacillus fusiformis* 5B

iii. Immobilize urease on strip for detection of Pb, Cd, Cr, and Ni.

iv. Screen *Lysinibacillus fusiformis* for potential to tolerate Pb, Cd, Cr, and Ni.

v. Biosorp Pb, Cd, Cr, and Ni using *Lysinibacillus fusiformis* 5B

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Heavy Metals

There are studies to determine the level of heavy metals; Cadmium, Copper, Chromium, Zinc and Lead in groundwater located in the vicinity of an oil depot in Nigeria and compare with recommended standards. There are loads of heavy metals information which focuses on the exploration areas of oil by researchers in Nigeria and paucity of data or information on the surrounding pollution of especially groundwater around the depot or facilities where the refined oil products are being stored disseminated (Oyeleke and Okparaocha, 2016). In UAE there is long-term consumption of contaminated herbs. As such, it is necessary to install a regular program that monitors and test the quality of both imported and local herbs sold in markets of UAE. Some of these herbs are polluted with heavy metals (Dghaim *et al*., 2015).

The analysis and evaluation of effluents released by industries into the environment especially water system is very critical owing to the roles they play in varying processes. Some of these processes includes: cloud stability, bioaccumulation, surface soil and water loading, increase in water and air-borne diseases as well as atmospheric catalysis. There is a continuous increase in the level of trace metals available in the atmosphere; this is caused by both human and natural events such as storms, evaporation, winds, volcanic eruption, biomass burning, ore smelting, fossil combustion among many others. Trace metals in varying compartment of the environment such as groundwater, soil, air, surface water as well as living organisms differs in concentration and their presence is determined by a number of processes in biogeochemical cycles. Likewise human interaction with the compartments of the environment can also alter the concentration and distribution of heavy metals (Garcia *et al*., 2016).

Various conventional techniques such as physical and chemical methods have been applied in minimizing the adverse effects of heavy metals in the environment. However, their usage comes with lots of constraint including but not limited to high cost, rigorous experimental set-up and incomplete treatment of effluents often result in post treatment effects. Some of the chemical and physical methods in conventional treatment of waste water from industries include sulphide treatment; chrome precipitation, specific coagulation, chemical flocculation, and filtration among many others have been widely employed across industries. In spite of all these available conventional methods of detoxifying heavy metals from waste water, there is still need for complete inactivation or removal of heavy metals from effluents. As such, attention is gradually shifted to biological means as feasible, low cost, environmentally free techniques of alleviating the environment from heavy metals pollution. These biological means include the use of algae, fungi and bacteria (Abioye *et al*., 2018). The use of lower animals such as earthworms has been demonstrated to adsorbed, degrade and move heavy metals such as lead and copper from a particular region, thus, reducing its concentration ultimately reducing the overall toxicity (Zhitong *et al*., 2012).

2.2 Removal of Heavy Metals

Bioremediation is equally a cost effective although time consuming and sometimes being affected by geological and climatic conditions of the contaminated site. Due to a change in the oxidation state of heavy metals; biodegradation can only achieve their transformation from a toxic state to a non-toxic one (Chibuike and Obiora, 2014). The conventional method of removing landfill may create significant risks of contamination during transport, difficulty in finding new sites and expensive. The cap and contain method still requires maintenance and monitoring of removed or isolated soils for a long period of time. If possible, the better approach

is to destroy the pollutants completely or better still the transformation of the toxic substances into innocuous ones (Singh *et al*., 2018).

Atomic absorption spectrometry (AAS) is a method utilized in the measurement of metal concentration by passing it through a specific wavelength of light emitted through a radiation source of a specific element from a cloud of atoms in a given sample. A hollow cathode lamp (HCL) is used in the emission of light that will be absorbed by atoms of the element. In any given sample, the concentration of the element in it is often ascertained by measuring the intensity of light energy reaching the inbuilt detector. AAS is suitable for analysis of various metal (approximately 70) concentrations (Helaluddin *et al*., 2016).

Just like the name, chemical precipitation process involves adding chemical reagents, which is succeeded by separation of solids precipitated from the water. In the precipitation of metals, certain coagulants such as organic polymers, lime, alum and other iron salts are added to the liquid effluents. A membrane process such as electro dialysis (ED) is used to transport ions through semi permeable membrane caused by difference in electric potential. Whereas in ultrafiltration method, waste water effluents are being passed through membrane with pore size ranging from 0.1 - 0.001 micron. Obviously, ultrafiltration will stop substances with high molecular weight such as colloidal materials, inorganic and organic polymeric substances. Other treatment methods such as flocculation and coagulation are important in treatment plants used in the treatment of drinking water.

A cellophane-lime membranes are utilized in reverse osmosis to separate contaminated water from a purified one (Dimple 2014). Other technologies used involves chemical decomposition and incineration at high temperature. They also have several drawbacks such as lack of acceptance by the general public in terms of incineration, which is supposed to destroy the

contaminants end up in releasing it into the atmosphere risking not only the workers but also nearby residents (Singh *et al*., 2018).

Adsorption is a technique used widely for industrial purposes, which could occur in chemical, physical or biological systems. Adsorption involves processes that causes the accumulation of solutes of liquid or gas on an adsorbent (surface of liquid or solid) thereby causing the formation of atomic or molecular film (i.e. adsorbate) (Dimple, 2014). Various technologies have been exploited for remediation of heavy metal from waste water. One of which is bioremediation. Bioremediation simply involves the use of biological systems to remediate polluted environment. One of the technologies utilized in bioremediation is phytoremediation. Phytoremediation involves the use of plants especially those found naturally in wetland ecosystem to sequester metalloids and heavy metals. Phytoremediation and microbial bioremediation of heavy metals have been successfully used in treatment of effluents. Wild and genetic modified plants such as Woody species, herbs, grasses, and forbs are mainly used due to high sensitivity they exhibit against heavy metals. However, in a commercial scale, phytoremediation is limited owing to it slow growth rate, dependent on climatic factor and more importantly time consuming. Heterogeneous Catalysts are cleanups enhanced by catalyst and have been demonstrated in various laboratories in the treatment of waste water (Ravindra *et al*., 2014)

Electrocoagulation employs an electrode that acts as cathode and anode allowing reduction and oxidation reaction. This technique is user friendly, cheap and can be implemented in an industrial scale. However, the high cost of resin makes the whole process costly when applied industrially but it is efficient in treating pollutants to the lowest ppb (Ravindra *et al*., 2014). Bioaccumulative processes are less practically used compared to biosorption, because it does not require addition of nutrients neither does it require maintenance of healthy microbial biomass owing to the high

toxicity of metal among several environmental factors. Different bacterial strains were used for the removal of different metal ions (Mustapha and Halimoon, 2015).SH, OH, R-OH, NH2, COOH, R-O-R, R-S-R and S-OH groups enable microbe-metal interactions. They negatively charge species on the cell wall/membrane of microorganisms (Hansda *et al*., 2015). Yeast, fungi, bacteria and algae have over the past proven to be good biosorbent materials for heavy metal biosorption. Biosorption as defined by Salman *et al.* (2014) is the ability of any biomolecule/biomass to sequester and bind specific disolved molecules of metal ions from an aqueous solution

Biosorption is influenced by two factors, the intrinsic factors which are the structure and composition cell surface, Secondly physicochemical factors in the environment where the cell develops (Aly *et al*., 2018). Biosorption removes residual or minute concentrations of contaminants. The effects of heavy metals are felt even at ppb levels (Lakshmi *et al*., 2018).

Biosorption of heavy metals are recommended over the conventional methods, this is because the biomaterials used are cheap and renewable; rapid kinetics to treat large volume of water, ability to handle mixed waste and multiple heavy metals, reducing residual metals, and can be used across wide arrays of physicochemical conditions such as pH, temperature and presence of other metal ions. They are also low in terms of operational cost, capital investment and reduces the volume of toxic substances generated at the end (Salman *et al*., 2014).

2.3 *Lysinibacillus fusiformis*

Bacterial cells have been reported in the past to possess inherent ability to survive in an environment polluted by varying contaminants such as petroleum and heavy metals (Alex 2012). Their survival have been attributed to their ability to respond adequately to stress from the environment through production of extracellular substances such as enzymes, fatty acids as well

as polysaccharides making researchers to search for such microorganisms in an environment filled with heavy metal contaminants (Verma, and Kuila, 2019), among which bacteria genera such as *Bacillus*, *Micrococcus*, *Streptomyces*, *Pseudomonas* and *Lysinibacillus* have shown great potentials (Ansari *et al*., 2011).

The enzyme urease is present in varying organism except man. These organisms include plants, invertebrates, fungi and bacteria. They can also be in form of soil enzymes in soil. The activities of urease increases the pH of the surrounding environment via the production of two molecules of ammonia; one acting as a basic molecule while the other as a product (Sujoy and Aparna, 2013). As such, drugs that inhibit the action of urease are essential in treatment and elimination of infectious diseases caused by microorganisms that produces urease. Also, urease inhibition is essential to maintain balance in and ensure plant's efficiency in the uptake of nitrogen from urea (Amtul *et al*., 2002).

Lysinibacillus is a genus that is typically characterized as rod when viewed under the microscope (Light microscope), which contains valid published names of species including *Lysinibacillus massiliensis, L. parviboronicapiens, L. tabacifolii, L. boronitolerans, L. odysseyi, L. xylanilyticus, L. sinduriensis, L. sphaericus, L. chungkukjangi, L. macroides* and *L. fusiformis* (Coorevits *et al*., 2012).

2.4 Urease

Urease which acts as a catalysis in the hydrolysis of urea to yield carbamate and ammonia is a metalloenzyme which depends on nickel. Carbonic acid and other molecule of ammonia are spontaneously hydrolyzed from carbamate (Aygul *et al.,* 2018). Urease was first believed to be exogenous as a metabolite produced by microorganisms in response to their acidic environment. However, some studies have indicated that urease is found in abundance in the cytoplasmic fluid

of yeast and bacterial cell. Initially, it was assumed that pathogenic bacteria such as *Proteus mirabilis, Helicobacter pylori, Campylobacter pyloridis* and *Staphylococcus saprophyticus* uses this enzyme as a potent virulent factor (Mahernia *et al.*, 2015).

2.5 Sources of Heavy Metals

The environment often experience deposition of heavy metals both from man made and natural events. Man-made is the main source of heavy metal pollution. Heavy metals are emitted both in form of compounds (inorganic and organic) and elements. The anthropogenic sources are mining sites, foundries and smelters, combustion by-products and traffics. In mining sites where well are bored, the water often contains concentration of heavy metal above safe levels stipulated by WHO (Duruibe 2007). Heavy metal can be found in contaminated soils through addition of manures, biosolids, pesticides, waste water irrigation, industrial wastes, milling and mining processes (Raymond *et al*., 2011). The natural sources include aerosols particulate, urban runoffs, volcanic erosion, and soil erosion. Heavy metals deteriorate the quality of soil and waters (Ravindra *et al*., 2018). The concentration and amount of heavy metals present in soil is affected by the soil's physicochemical. Environmental monitoring is a priority objective that involves understanding and identifying the origin of these metals, how they interact with the soil and how they get accumulated (Roozbahani *et al*., 2015).

Human activities such as the discharge of industrial waste into water ways are the major source through which heavy metals enter the aquatic environment. Heavy metals from aquatic environment can find their ways into human system through the food chain. Unfortunately, once any aquatic animal (i.e. fish) is consumed by man the already bioacummulated heavy metal in the fish tissues are being transferred into the human system. Once their concentration is beyond the safe level it causes severe health disorders (Mehana *et al*., 2020).

Although plants requires heavy metals for growth and metabolisms. However, these metals can become harmful when it is very much abundant. The ability of plant to acquire essential metals allows them to equally acquire the nonessential metals. Heavy metals affect plants both directly and in directly. Direct effects include damage to cell structures, impairment of cytoplasmic enzymes as well as developing oxidative stress. Indirect effects heavy metals have on plants include replacement of essential nutrients at cation exchange sites of plants for example reduction of beneficial soil microorganisms. These toxic effects lead to a decline in plants growth which sometimes leads to death (Chibuike and Obiora, 2014).

2.6 Methods of Detection of Heavy Metals

There are three main categories used in detection of heavy metals; spectroscopic detection techniques, electrochemical detection techniques and optical detection techniques (Malik *et al*., 2019). Detection of pollutants with spectroscopic and chromatographic techniques requires laborious sample pretreatment and trained personnel. An e-tongue is a multisensory array based on non-specific or low-selective sensing units, taking advantage of their cross-sensitivity to identify a liquid medium, mostly using electro analytical methods. E-tongue is a technology that is developed to address challenges encountered by other technologies in the detection of toxins and heavy metals. Since 2000, the use of membranes of organic origin has been in use in e-tongues, however, not until 2008 before it was fully used in heavy metal detection in seawater (Shimizu *et al*., 2019).

Spectroscopic detection, electrochemical methods of detection and optical methods of detection are methods used in detection of heavy metals. Spectroscopic techniques are widely applicable in detecting and dermination of ion concentration of heavy metals having detection limits that are low. Some disadvantages of spectroscopic detection is that, they are expensive, complex and

requires highly trained personnel to use it effectively (Malik *et al*., 2019) Electrochemical technique however, are cost effective, easy to use, and involves procedures that are simple and reliable to monitor with reduced analytical time. However, sensitivity of electrochemical techniques is quite low, and its detection limit is high compared to optical or spectroscopic techniques (Malik *et al*., 2019).

Optical method of heavy metal detection utilizes conventional methods of luminescence, reflection and absorption spectrometry. In order to apply an appropriate correction factor in optical technique, the pH of the medium needs to be controlled. Since numerous non-selective optical indicators reacts with two or more metal ion likewise metal ion indicators combines with hydrogen molecules. An instrument designed to detect metal ion is known as metal ion detector. This instrument helps in detecting and sometimes quantifies the presence of metal ion in a given environment (Malik *et al*., 2019).

Most analytical conventional methods used in detecting the presence of heavy metal in an environment is versatile, sensitive, precise and have an excellent detection limit. However, these instruments are expensive, involves complex analytical processes, often challenging on pretreatment samples, which are difficult to implement for monitoring purposes. An example is seen with electrochemical analysis, this method accuracy is high likewise its sensitivity; it is simple with wide range of measuring capacity and it is cost effective. However, electrochemical analysis is typically poor in terms of specificity. Piezoelectric biosensors are devices used in detection of heavy metals. In Piezoelectric biosensors, elements used for biorecognition are integrated with Piezoelectric materials, which serves as transducers. There are varying materials from natural and synthetic source that exhibit piezoelectric effects. The commonly used materials in piezoelectric biosensors is quartz crystals, which function based on enzyme

immobilization. Quartz crystals are cost effective, chemically stable, and can withstand high temperature in aqueous medium. However, only a few enzymes are sensitive to heavy metals while using this instrument (Eddaif, *et al*., 2019).

Several other techniques are available to detect as well as estimate the quantity of heavy metal present in a given environment (Morais *et al*., 2012). However, detection technique or device with high sensitivity capable enough to detect traces of metal ions, and are time and cost effective, environmentally friendly, high sensitivity, and user-friendly needs to be developed. Likewise, a device or technique equipt to detect all heavy metal ions needs to be developed (Malik *et al*., 2019).

2.7 Toxic Effects of Heavy Metals

The harmful damage caused by heavy metals to tissues of humans comes as a result of chemical reactions between the metal ions mammalian enzymes, structural proteins and membrane system. Reports of cancer caused by heavy metal have been reported on workers who are constantly exposed to heavy metal poisoning (Obi *et al*., 2017). Lead when absorbed into the human system is mostly transported via the blood cells to the kidneys and liver and subsequently deposited in the bone, teeth and hair as salt of phosphate. Cadmium however, binds to albumin and blood cells and subsequently with metallothionein in liver and kidney tissues. Manganese is most times distributed to lungs by blood cells and get diffused as manganese vapor into central nervous system (CNS) and the lungs. Lipid soluble organic salts of manganese are deposited in the intestine where they undergo fecal elimination. However, water soluble inorganic manganese salts get distributed in plasma and kidney where they undergo renal elimination (Godwill *et al*., 2019). Depending on the type of chemical compound of the heavy metal ion (i.e. volatility,

valency, lipid solubility) and route of exposure; the organs often targeted are those that have the ability to accumulate these metals to the highest concentration possible (Obi *et al*., 2017).

Cell damage and apoptosis are often caused by heavy metal interaction with cells and organelles of humans even at low time of exposure. In humans, the major pathway through which heavy metal get into the system is through dermal contact, inhalation and ingestion. The extent of damage caused by heavy metals on humans depends on the type of metal ion, dose and the duration of exposure. The effects ranges such as cytotoxic, mutagenic, teratogenic, carcinogenic and neurotoxic. Heavy metal poisoning can cause deafness, loss of fertility, blindness, damage of brain cells and ultimately death (Lakshmi *et al*., 2018). In animals, heavy metal poisoning is often witness to cause damage to germ cells of both female and male animals as well as mutations and encourages tumor cells.

Inhalation of heavy metal polluted air; consumption of contaminated drinking water and food crops grown in heavy metal contaminated soils. Cadmium and lead are the most abundant and poisonous heavy metals (Sardar *et al*., 2013). Presence of heavy metals in soils has been demonstrated to cause regression in plant growth. Heavy metals such as Ni, Cd and Pb are of great health importance since they are greatly toxic at low concentration. Some metal ions however, are needed in minute amount for plant biosynthesis, catabolism and anabolisms at great amount (Sardar *et al*., 2013)

Lead (Pb) is one of the most abundant natural substances on earth. In terms of useage it ranks the fifth on the list of metals. It is used in mining, smelting, refining, battery manufacturing, fertilizers, pesticide, part of sewage sludge. All these applications as lead to contamination of ground water resources (Jan *et al*., 2015). Lead can cause neurological, cardiovascular, renal,

gastrointestinal, haematological and reproductive effects on the body due to its systemic toxicity. Blood sampling is used to test lead exposure. Primarily, human get in contact with heavy metal through inhalation of contaminated dust particles, and the ingestion of contaminated water and food stuffs (Obi *et al*., 2017). In pregnant women, high levels of exposure to lead may cause miscarriage. Exposure to high lead levels can severely damage the brain and kidneys and ultimately cause death (Martin and Griswold, 2009).

Cadmium is toxic to living things such as humans, plants and many microorganisms. Humans are exposed to cadmium by inhalation via tobacco smoking and occupational exposure (lung damage), ingestion and dietary exposure (kidney and bone damage). Various human activities also releases cadmium into the environment. The main human organ affected by cadmium exposure is the kidney. Cadmium affects the skeletal as a secondary response to kidney damage or direct action on the bone (Obi *et al*., 2017).

Nickel is naturally in food. Vegetables from polluted soils will boost nickel uptake. Humans may be exposed to nickel by breathing air, drinking water, eating food or smoking cigarettes. Smokers have a higher nickel uptake through their lungs. Nickel can be found in detergents. Nickel is needed in human health in trace amount however, when the quantity or concentration is high can cause severe damage to human health. Reports have been made concerning some health damages caused by nickel gas inhalation such as lung embolisms, asthma, respiratory failure, birth defects, heart disorders, chronic bronchitis, pneumonitis, birth defects and allergic skin reactions most times from jewelry (Obi *et al*., 2017).

Chromium (VI) compounds are toxins and known human carcinogens, whereas Chromium (III) is an essential nutrient. Breathing high levels can cause irritation to the lining of the nose. Skin contact can cause skin ulcers. Long term exposure can cause damage to liver, kidney circulatory

and nerve tissues, as well as skin irritation. Even at low concentration cadmium have been demonstrated to be extremely toxic to humans and the ecosystem. Their ability to bioaccumulate in biological system and their half-life reaction in human body ranges from 10 - 33 years.

Long term exposures to Cadmium also induces renal damage. So cadmium is monitored in most countries and international organizations (Mohod and Dhote, 2013). Regulatory safe limits for heavy metals set by World Health Organisation (WHO) for waste water, soil and drinking water is showed in Standard sets aside by different regulatory agencies for heavy metals is shown in Table 2.1.

Table 2.1 Acceptable limits by WHO, SON and FEPA for heavy metals

Heavy metals	Wastewater (ppm)	soil (ppm)	Drinking Water (mg/L)
Cadmium	0.003	0.003	0.003
Chromium	0.05	0.1	0.05
Lead	0.01	0.1	0.01
Nickel	0.02	0.05	0.02

Source: (Geoffrey *et al*., 2020)

Keys: ppm: parts per million; mg: milligram; WHO: World Health Organization; FEPA: Federal Environmental Protection Agency; SON: Standards Organization of Nigeria,

Untreated or inadequately treated heavy metal contamination causes variety of health and environmental issues. Heavy metals greatly reduces the number of living organisms in aquatic ecosystems, The presence of heavy metal pollutants on soil causes great threat to the soil and plants growing on it, with the consumption of such plants by animals and humans leading to

severe detrimental effects (Ravindra *et al*., 2018). Utilization of food crops contaminated with heavy metals is a major food chain route for human exposure (Singh *et al*., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Test Isolate

The microorganism for biosorption and urease production was *Lysinibacillus fusiformis* 5B, which was obtained from Department of Microbiology, Federal University of Technology Minna. The bacterium was isolated from cement samples in Minna, Nigeria by Yakubu (2019) and characterized by Hussaini (2020).

3.2 Preparation of Media

3.2.1 Preparation of nutrient agar medium

Nutrient agar (NA) was prepared in accordance with the manufacturer's instruction. Analytical balance was used to weigh 2.8 g of powdered NA into 250 mL conical. Hundred millimeters (100 mL) of distilled water was used to dissolve the powdered NA which was preheated using a heating mantle before sterilizing in the autoclave at 121 °C for 15 minutes at 15 psi; it was allowed to cool to body temperature before dispensing into petri plates (Cheesbrough, 2006).

3.2.2 Preparation of urea agar base media

Urea Agar Base media was prepared according to manufacturer’s instruction. Analytical balance was used to weigh 2.1 g of the media which was dissolved in 90 mL of distilled water and preheated using the heating mantle before sterilizing using the autoclave at 121 °C for 15 minutes at 15 psi. Urea solution was not added into the urea agar base during sterilization as heat converts urea to ammonia which is toxic to microbial growth. Four grams (4 g) of urea was weighed and dissolved into 10 mL of sterile distilled water. After sterilizing the urea agar base media, it was allowed to cool to 37 °C before adding the dissolved urea solution aseptically using

a sterile filter paper and mixed gently before dispensing into the Petri dishes and was allowed to gel before used (Jeong *et* al., 2017)

3.3 Confirmation of the Test Isolate

3.3.1 Characterization and identification of bacterial Isolate

Gram staining, catalase test, oxidase test, citrate test, hydrogen sulphide production test, starch hydrolysis test, indole test, methyl red test and voges proskauer test were carried out. Characterization of isolates was carried out based on their morphology, cell, motility, catalase, citrate, starch hydrolysis, nitrate reduction, oxidase and gelatin test was performed by standard method. Sugar fermentation test was also performed using standardized method for isolates**.**

3.3.1.1 Gram staining

Using a sterile wire loop, a loop full of water was placed on a clean, grease free slide. The wire loop was flamed using the Bunsen burner before picking the inoculum and smeared on the slide. A thin smear was made in other to enhance viewing under the microscope as thick smear prevents ascertaining if a cultured plate is pure or not. The slides were air dried before heat fixing by passing it 2-3 times over the flame. The slides will be placed over a staining rack and crystal violet (a primary stain) was applied on the slides and allowed to stay for 60 seconds which will then be flooded with running water. Lugol's iodine (a mordant) was applied on the slides and allow to stay for 60 seconds before flooding with water. Alcohol (95 %) was applied on the slides and allowed for 5-8 seconds in other to decolorize the crystal violet-iodine complex formed. Safranin (a secondary stain) was applied and allowed for 60 seconds before flooding with water. The slides were allowed to dry before adding immersion oil and the slides were viewed using the microscope at x100 objective (Cheesbrough, 2006).

3.3.1.2 Catalase test

It was performed by placing 3 drops of 3 % hydrogen peroxide (H_2O_2) on a clean grease free slide. The isolates were smeared on the H_2O_2 using a sterile glass rod and production of bubbles were observed within first 10 seconds. Production of bubbles indicates the microorganism is positive to catalase test (Cheesbrough, 2006).

3.3.1.3 Oxidase test

This was performed by impregnating filter paper with oxidase reagent (tetramesthyl-p-phenylenediamine) after which the test isolate which was 24 hours old will be picked using a sterile glass rod and smeared on the filter paper. Positive to oxidase test indicated by purple/blue coloration within 10-15 seconds (Cheesbrough, 2006).

3.3.1.4 Citrate test

Simmon's citrate agar (SCA) slants was used in carrying out this test. The sterilized media was inoculated with test isolates by stabbing through the slants using a sterile straight wire and incubated in the incubator at 37 °C for 24 hours. The color of the media was observed for color change. Citrate positive indicated by blue coloration and negative retained the initial green color (Cheesbrough, 2006).

3.3.1.5 Hydrogen sulphide (H_2S) production test

Triple sugar iron (TSI) agar was used in performing this test. The test isolates was inoculated into the TSI agar slants by stabbing using a sterile straight wire and incubated at 37 °C for 24 hours. Production of black color indicates production of Hydrogen Sulphide (Cheesbrough, 2006).

3.3.1.6 Starch hydrolysis test

Sterilized nutrient agar containing soluble starch was inoculated with test isolate by aseptically streaking on the gelled surface of the agar medium using a sterile wire loop after which the plates was incubated at 37 °C for 24 hours. Lugol's iodine was used to flood the plates and the excess was poured out of the Petri dish and observed for color formation and zone of clearance within the streaked line. A positive result indicated by zones of clearance within the streaked line (Mondal *et al*., 2008).

3.3.1.7 Indole test

Test isolate was inoculated into sterilized peptone water using a wire loop aseptically. The test tubes was incubated for 48 hours at 37 °C in the incubator. After incubation period, 0.5 mL of Kovac's reagent was added to the cultured broth and allowed to stand for 15 minutes. Formation of indole ring at the top of the broth indicates positive whereas no indole ring formation indicates negative (Mondal *et al*., 2008).

3.3.1.8 Methyl red test

Methyl red -Voges Proskauer (MR-VP) broth was used for MR test. The test isolate was inoculated into a sterilized MR-VP broth aseptically using a sterile wire loop and incubated for 4 days at 37 °C with their caps loosely on. After the incubation period, 0.5 mL of methyl red was added into the culture broth and shaken gently which was then be allowed to stand for 15 minutes. Red coloration indicates positive to methyl red whereas no color change indicates negative to methyl red test (Cheesbrough, 2006).

3.3.1.9 Voges Proskauer test

Similar to Methyl red test, MR-VP broth was used for this test. The sterilized MR-VP broth was inoculated with the test isolates aseptically using a sterile wire loop which was then be incubated

at 37 °C for 4 days after which 0.6 mL of Barit's reagent was added into the cultured broth and allowed to stand for 15 minutes. Production of red coloration after 15 minutes indicates positive to VP test whereas no Colour change indicates negative (Cheesbrough, 2006).

3.4 Production of Urease using the Isolate

3.4.1 Confirmatory test for urease production

Urea agar base media was used to screen for isolate that was able to produce urease to utilize urea. The isolate was aseptically streaked onto urea agar base media and incubated at 37 °C for 18-24 hrs. After the incubation period, the isolate was observed for urease production. A change in the agar medium to pink indicates hydrolysis of urea by urease enzyme.

3.4.2 Large scale preparation of crude urease enzyme

For a large scale production of the crude urease from the bacterial isolate, an inoculum of the isolate was seeded into a fermentation medium to enhance the enzyme production (Agereh *et al.,* 2019).

3.4.3 Inoculum preparation

The bacterial isolate was transferred from a solid agar medium to a broth medium. A nutrient broth medium that served as the basal medium was prepared in a 250 ml conical flask by measuring according to the manufacturer's instruction in a 100 ml distilled water, autoclaved at 121 ^{o}C for 15 minutes and allowed to cool. A sterile wire loop was used to aseptically pick the bacteria isolates from the slants and inoculated into the broth. The conical flask was corked, swirled for proper mixing and incubated for four days in a rotary shaker at 150 rpm at a temperature of 30 ^{o}C and observed for growth.

3.4.4 Fermentation medium

The fermentation medium for urease production was carried out in a chemically defined basal medium composed of the following: glucose 5.0 g/L, disodium hydrogen phosphate Na_2HPO_4 2.0 g/L, magnesium sulphate $MgSO_4 \cdot 7H_2O$ 0.5 g/L, calcium carbonate $CaCO_3$ 0.1 g/L, monopotassium phosphate KH_2PO_4 0.8g/L, Sodium Chloride 2.5 g/L, calcium chloride, cement 5 g/L and 1 L of H_2O. The medium was autoclaved at 121°C for 15 minutes, after which the isolate from already prepared inoculum was inoculated into the medium using 1:20. It was measured in a 1000 ml conical flask for 15 minutes at 121^oC, after which the isolate from already prepared inoculum was added in 1:20. The culture was incubated for five days at 37 °C. After the completion of the incubation period, the final liquid culture will be centrifuged for 10 minutes at 7000 rpm at 4°C in order to remove the bacterial cells. The resulting supernatant was separated and then filtered using a 0.22 μm pore size filter membrane in order to obtain a cell-free supernatant. The cell-free supernatant was taken as the crude urease enzyme produced, and stored in the refrigerator at freezing temperature (Agereh *et al.,* 2019).

3.5 Immobilized Urease *Lysinibacillus fusiformis* 5B for Heavy Metals (Pb) Detection

3.5.1 Determination of urease activity

Ten milliliter (10ml) of fresh 1% urea salt solution was prepared and dispensed into each of the two clean conical flasks. Two milliliters (2 ml) of urease solution was added and mixed thoroughly and Incubated at 50 ^oC for half an hour. Three (3) drops of phenolphthalein was added to give a faint pink colour indicating that the urease is breaking down the urea into NH_3. to the solution was added 15 ml of neutralized formaldehyde solution. This liberates H+ from NH_4^+, with the formation of hexamethylene tetrates:- (a colourless solution). The solution was titrated against 0.1N Sodium Hydroxide to a pink end point (titre value) (Cao *et al*., 2015).

The urease activity was calculated using the endpoint value of the titration as :

$$\text{urease activity} = \frac{1.4 \times \text{titre value}/}{1800 \text{ (seconds)} \times 10 \text{ mL}} \quad \text{Equation (i)}$$

3.5.2 Immobilization of urease on paper strip for heavy metal identification

Paper strips were cut into 1cm x 6cm strips and prewashed with tris-acetate buffer and (0.1M pH 6.5) and dried. The strips were dipped in a mixture of equal volume (10 ml each) of urease solution and a solution of 0.6mg/ml phenol red prepared in 10 % glutaraldehyde solution. After agitation for 5 minutes, the strips (all yellow in colour) were removed from the enzyme - phenol red mixture and dried under vacuum. The test strips were dipped in a solution of Pb (to inhibit or deactivate the urease) and dried with the other strip as control. The negative and positive control were not dipped in the Pb solution. Both test and positive control strips were dipped in 1 % urea salt solution (Arvind, 2019).

3.5.3 Determination of urease inhibition by Pb using varying degree of urease activity

A 10 mL of freshly prepared 1 % urea salt solution was measured into each of five clean conical flasks. Two millilitres (2 ml) of urease solution was added and mixed thoroughly and 2 mL of varying Pb concentration was added. This was incubated at 50 ^{o}C for half an hour. Three (3) drops of phenolphthalein was added to give a faint pink color. The urease breaks down urea to release varying concentration of NH_3 –which is alkaline, the addition of phenolphthalein translates to varying degree of pink which is informed by the level of inhibition by the varying Pb concentration present in the solution. Fifteen millilitre (15 ml) of neutralized formaldehyde solution was the added. This liberates H^+ from NH_4^+, with the formation of hexamethylene tetramine:- (a colourless solution). The solution was titrated against 0.1N Sodium Hydroxide to a pink end point (titre) (Cao *et al*., 2015).

The urease inhibition by Pb was calculated using the endpoint value of the titration as :

$$\text{Urease inhibition} = \frac{1.4 \times \text{titre value}}{1800 \text{ (seconds)} \times 10\text{ml}} \quad \text{Equation (ii)}$$

3.6 Biosorption of the Heavy Metal Using *Lysinibacillus fusiformis*

3.6.1 Preparation of heavy metal solutions (lead, chromium, cadmium and nickel)

In order to get the stock solution of lead, 0.0157 g of lead acetate was dissolved in 100 mL of distilled water. To prepare the stock solution of cadmium, 0.018 g of cadmium sulphate was dissolved in 100 mL of distilled water (Abioye *et al*., 2018). The stock solution of nickel sulphate was prepared by dissolving 0.026g in 100ml of distilled water. The prepared stock for the metal solution (lead, chromium, cadmium and nickel) was agitated for 15 minutes and then allowed to stand for a period of 24 hours in order to obtain a complete dissolution of salt (Abioye *et al*., 2017).

3.6.2 Screening of *Lysinibacillus fusiformis* for the potential to utilise heavy metals

A concentration of 5 ppm of the heavy metal (lead, chromium, nickel and cadmium) was prepared using agar dilution method utilizing nutrient agar. Development of bacterial colonies signifies that the isolates can withstand the heavy metals whereas absence of visible colonies indicates the inability of test isolates to withstand heavy metal (Abioye *et al*., 2018).

3.6.3 Biosorption of heavy metals (lead, Chromium, Cadmium and Nickel)

The heavy metal nutrient broth culture medium was prepared in different concentrations (10, 15, 20 and 50 ppm) using the prepared stock solutions. The culture broth containing the varying concentration of heavy metals was then sterilized at 121 °C for 15 minutes, after which the culture broth was allowed to cool before inoculating 5 mL of 24 h old culture, where cells of *L. fusiformis* 5B have attained 1.5×10^6 cfu/mL with the exception of the blank, which was used as

control. The heavy metal culture broths were incubated aerobically in an incubator with shaker at 37°C for 35 days (Abioye *et al*., 2018).

3.6.4 Wet digestion for the determination of Total Cd, Pb, Ni and Cr using atomic absorption spectroscopy

A quantity (0.5 grams) of the sample was weighed into a 100 mL volumetric flask, 30 mL of wet digestion acid (650 mL of nitric acid in 1 L beaker, 80 mL of perchloric acid and 20 mL of sulfuric acid) were measured, added and stirred to mix. The sample was placed on a fume cupboard and digested until the sample reduced to 20 mL. The heating was continued until white fumes of nitric acid disappeared and sample reduced to 10 mL. The sample was transferred quantitatively to a 50 mL volumetric flask and made to mark with dH_2O. It was then shaken vigorously and filtered through a Whatman 0.45 µm filter paper. One milliliter (1 mL) of the clear digest was pipetted into another 50 mL volumetric flask and made to mark with dH_2O. Samples were read using Atomic Absorption Spectrophotometer (AAS) (AA WIN 500 PG) at 7 days interval starting with zero reading (day 1) using wavelengths 359.4 nm, 326.1 nm, 283.3 nm and 231.1 nm for chromium, cadmium, lead and nickel, respectively. The percentage of biosorption was determined by measuring the amount of heavy metal removed from the medium through estimation of the residual metal concentration using AAS. Beer Lambert's law (Equation 1) was used to achieve the percentage biosorption (Furr and Bretherick, 2006).

% Biosorption = [(Initial metal concentration – final metal concentration) / Initial metal concentration] x 100 Equation (iii)

3.6 Data analysis

Statistical package for Social Science (SPSS 24) utilizing one-way analysis of variance (ANOVA) was used to analyze the data generated from this study in order to determine the

significant difference ($P<0.05$) between the biosorption capacity of the isolate as the time (days) progressed.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Identity of *Lysinibacillus fusiformis*

Morphological and biochemical characteristics of the isolate which were used to confirm *L. fusiformis* are presented in Table 4.1.

4.1.2 Urease inhibition by Pb, Cd, Cr and Ni using varying degree of urease activity

The different concentration of heavy metals (Pb, Cr, Cd, Ni) solution (100 ppm, 200 ppm, 400 ppm, 600 ppm) gave different shades of magenta and serve as a standard colour chart (Figure 4.1 – Figure 4.4). The inhibition of urease is inversely proportional to the amount of NH_3 released from the breakdown of urea. Urease activity was inversely proportional to the concentration of the heavy metal. The test strips showed magenta/pink colour lesser shade than the positive control, depending on the concentration of the lead solution. The test strips are used to check the standard color chart to confirm the concentration of the lead, cadmium, chromium and nickel present.

Table 4.1 Identity of *Lysinibacillus fusiformis*

Test	*L. fusiformis*
Morphology	
Size	Small
Margin	Irregular
Texture	Rough
Elevation	Flat
Colour	Whitish
Shape	Bacilli
Gram's reaction	+
Urease	+
Citrate	-
H_2S	-
Motility	+
Starch utilization	-
Methyl red	+
Voges-Proskauer	-
Spore	+

Keys;+Positive, -Negative

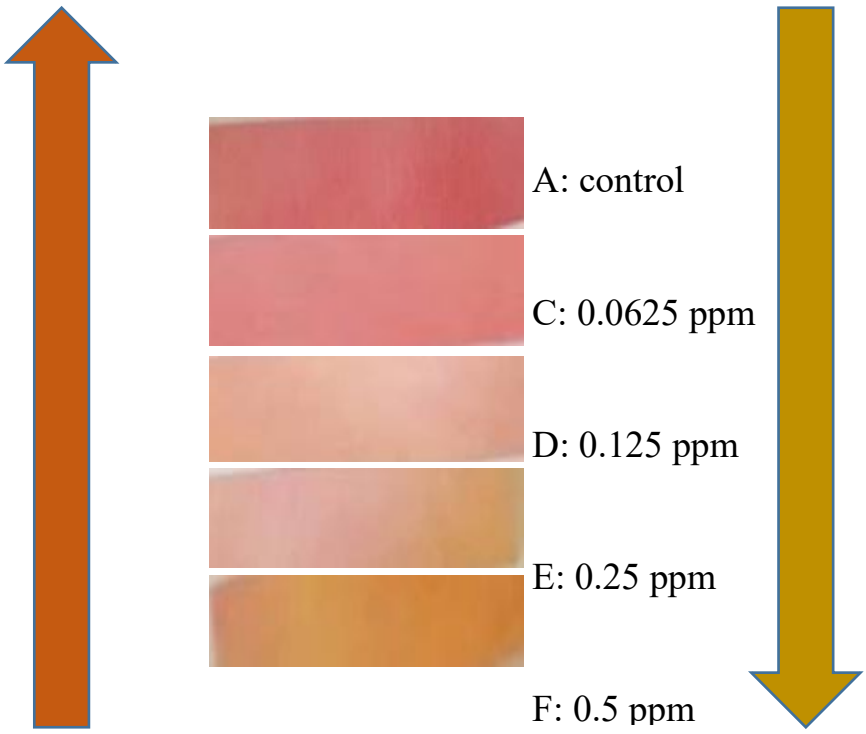

Figure 4.1: Standard colour chart of immobilized strip for Lead (Pb)

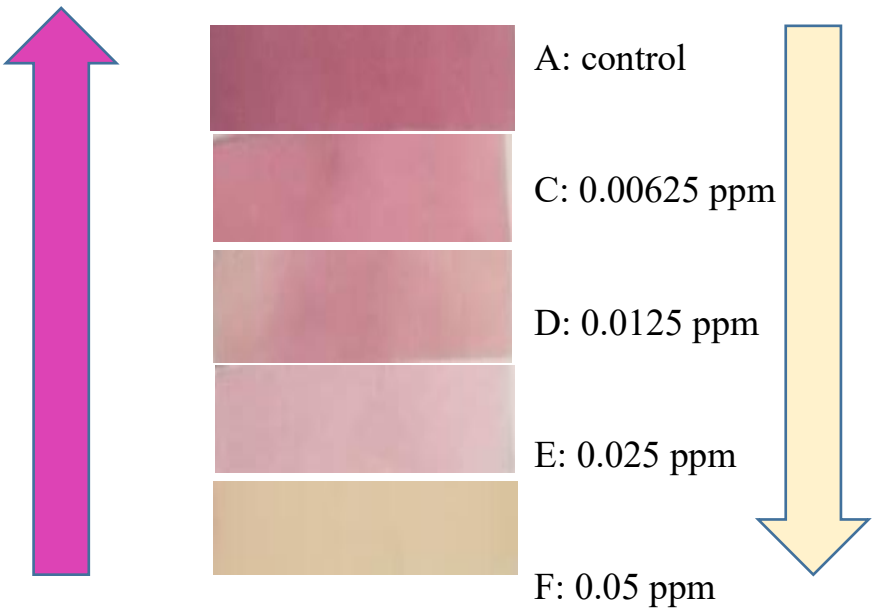

Figure 4.2: Standard colour chart of immobilized strip for Chromium (Cr)

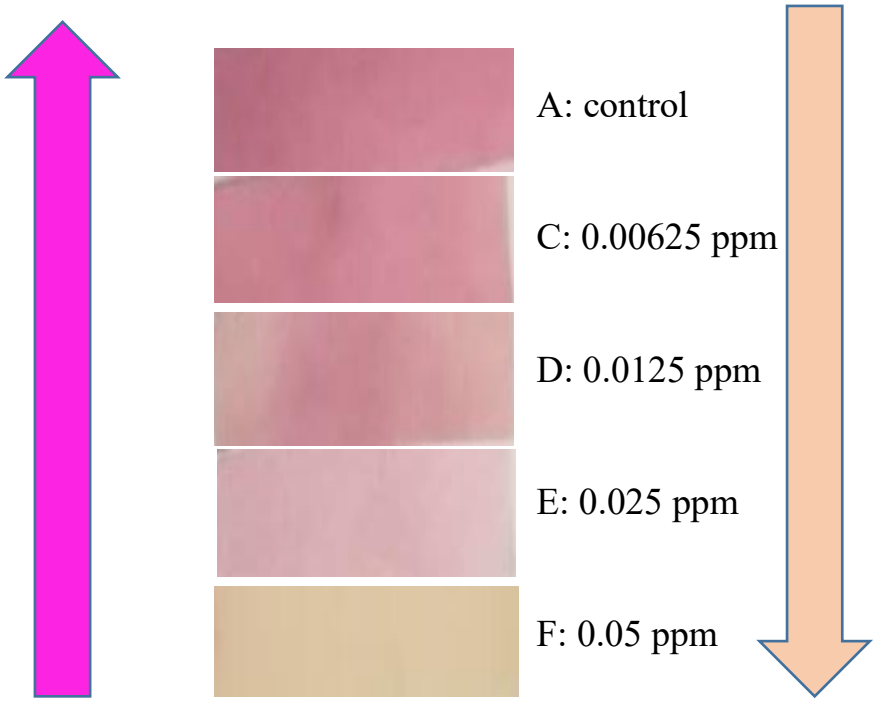

Figure 4.3: Standard colour chart of immobilized strip for Cadmium (Cd)

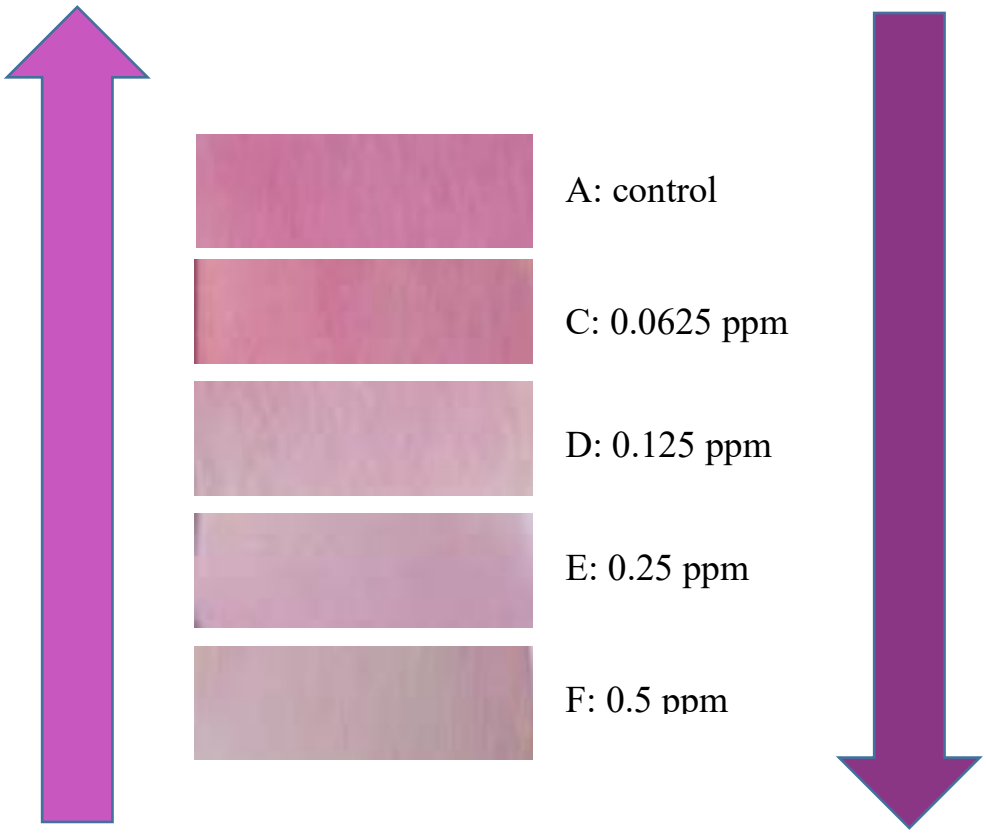

Figure 4.4: Standard colour chart of immobilized strip for Nickel (Ni)

Different concentrations of lead, chromium, cadmium and nickel was used to determine the urease activity. The inhibition of urease is inversely proportional to the amount of NH_3 released from the breakdown of urea. The urease activity (mg/ml/sec) corresponding to the degree of inhibition due to presence of heavy metals is shown in Table 4.2.

Table 4.2. The urease activity (mg/ml/sec) corresponding to the degree of inhibition due to presence of heavy metals

Concentration (ppm)	**Urease Activities (mg/ml/sec)**			
	Pb	**Cd**	**Cr**	**Ni**
Control	$1.92x10^{-3}$	$1.92x10^{-3}$	$1.92x10^{-3}$	$1.92x10^{-3}$
0.0625	$0.85x10^{-3}$	$1.61x10^{-3}$	$1.48x10^{-3}$	$1.21x10^{-3}$
0.125	$1.29x10^{-3}$	$1.31x10^{-3}$	$1.20x10^{-3}$	$1.04x10^{-3}$
0.25	$1.06x10^{-3}$	$0.96x10^{-3}$	$1.11x10^{-3}$	$0.82x10^{-3}$
0.50	$0.81x10^{-3}$	$0.41x10^{-3}$	$0.62x10^{-3}$	$0.58x10^{-3}$

4.1.2 Identification of heavy metal on paper strip

4.1.3 Screening of *Lysinibacillus fusiformis* for the potential to utilize heavy metals

Plate 1 showed that *Lysinibacillus fusiformis* was capable of tolerating lead, chromium, cadmium and nickel. The Plates inoculated with *Lysinibacillus fusiformis* showed growth at 5 ppm.

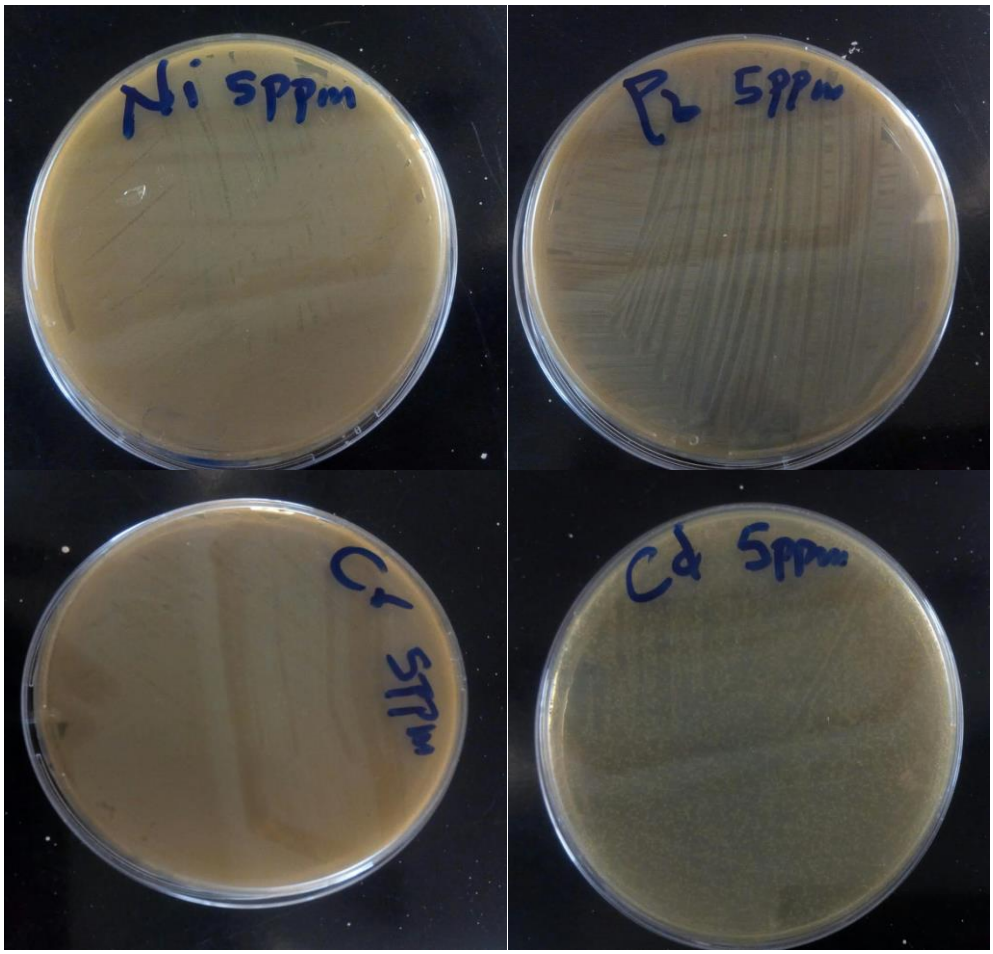

Plate I. Viable growth of *Lysinibacillus fusiformis* on media containing heavy metals

4.1.4 Biosorption of the heavy metals using the *Lysinibacillus fusiformis*

The biosorption of lead by *Lysinibacillus fusiformis* at different concentration and at different intervals is presented in Table 4.3. At 10 ppm lead concentration, the highest biosorption rate was observed on 35th day (99.96 %) and the lowest was on the 7th day (58.05 %). At 15 ppm, the highest biosorption rate was 35th day (89 %) and the lowest was observed on the 7th day (52.37 %). At 20 ppm, the highest biosorption rate was observed on the 35th day (99.24 %) and the lowest was observed on the 7th day with 70.34 %. At 50 ppm lead concentration, the highest biosorption rate was observed on 35th day (86.61 %) and the lowest was observed on the 7th day

with 40.06 %. This result showed that *Lysinibacillus fusiformis* is capable of biosorping lead at different concentration. The result also showed that there was a significant difference ($P<0.05$) between the biosorption capacity of the isolate as the days progress.

Table 4.3 Biosorption percentage of lead by *Lysinibacillus fusiformis*

	Lead concentration (ppm)			
Time (Days)	**10**	**15**	**20**	**50**
7	58.01±0.01[d]	52.36±0.36[d]	70.44±0.44[e]	40.06±0.06[e]
14	76.54±0.54[c]	69.87±0.87[c]	74.44±0.44[d]	50.11±0.11[d]
21	97.40±0.40[b]	94.68±0.68[b]	78.11±0.11[c]	57.29±0.29[c]
28	99.80±0.80[a]	99.41±0.41[a]	88.14±0.14[b]	70.14±1.14[b]
35	99.96±0.96[a]	99.89±0.89[a]	99.24±0.24[a]	86.61±0.61[a]

Values are $\bar{x}$±SEM of duplicate values. $\bar{x}$ with dissimilar letter(s)s are not significantly different from each other according to Duncan Multiple Range Test (DMRT).

ppm; parts per million

The biosorption of chromium by *Lysinibacillus fusiformis* at different concentrations for 35 days is presented in Table 4.4. Table 4.4 shows results obtained from the biosorption of chromium by *Lysinibacillus fusiformis* at different concentrations and at different intervals. At 10 ppm chromium concentration, the highest biosorption rate was observed on 35th day with 99.97 % and the lowest was on the 7th day with 67.33 %. At 15 ppm the highest biosorption ate was 35th day with 99.86 % and the lowest was observed on the 7th day with 64.58 %. At 20ppm the highest biosorption rate was observed on the 35th day with 99.93 % and the lowest was observed on the 7th day with 75.23 %. At 50 ppm chromium concentration, the highest biosorption rate was observed on 35th day with 91.26 % and the lowest was observed on the 7th day with 55.69 %.

This result showed that *Lysinibacillus fusiformis* is capable of absorpting chromium at different concentration.

Table 4.4. Biosorption percentage of chromium by *Lysinibacillus fusiformis*

	Chromium concentration (ppm)			
Time (Days)	**10**	**15**	**20**	**50**
7	67.33±0.33[c]	64.58±0.58[d]	75.23±0.23[c]	55.69±0.69[e]
14	95.95±0.95[b]	83.33±0.33[c]	89.01±0.01[b]	66.62±0.62[d]
21	99.87±0.87[a]	97.29±0.29[b]	98.96±0.96[a]	70.47±0.47[c]
28	99.92±0.92[a]	99.34±0.34[a]	99.86±0.86[a]	86.72±0.72[b]
35	99.97±0.97[a]	99.86±0.86[a]	99.93±0.93[a]	91.26±0.26[a]

Values are $\bar{x}$±SEM of duplicate values. $\bar{x}$ with dissimilar letter(s) are not significantly different from each other according to Duncan Multiple Range Test (DMRT).

ppm; parts per million

The biosorption of nickel by *Lysinibacillus fusiformis* at different concentration and at different intervals is shown in Table 4.5. Table 4.5 shows results obtained from the biosorption of nickel by *Lysinibacillus fusiformis* at different concentrations and at different intervals. At 10 ppm nickel concentration, the highest biosorption rate was observed on 35th day with 98.13 % and the lowest was on the 7th day with 37.91 %. At 15 ppm, the highest biosorption ate was 35th day with 99.33 % and the lowest was observed on the 7th day with 20.99 %. At 20 ppm the highest biosorption rate was observed on the 35th day with 91.70 % and the lowest was observed on the 7th day with 46.81 %. At 50 ppm nickel concentration, the highest biosorption rate was observed on 35th day with 84.24 % and the lowest was observed on the 7th day with 24.60 %. This result shows that *Lysinibacillus fusiformis* is capable of absorpting nickel at different concentration.

The result also showed that there was a significant difference ($P<0.05$) between 7th day and 35th day.

Table 4.5. Biosorption percentage of nickel by *Lysinibacillus fusiformis*

	Nickel concentration (ppm)			
Time (Days)	**10**	**15**	**20**	**50**
7	37.91 ± 0.91^{d}	20.99 ± 0.99^{d}	46.81 ± 0.81^{d}	24.6 ± 0.60^{e}
14	66.27 ± 0.27^{c}	54.72 ± 0.72^{c}	64.14 ± 0.14^{c}	38.4 ± 0.40^{d}
21	$90.47\pm\pm0.27^{b}$	78.27 ± 0.27^{b}	82.49 ± 0.49^{b}	44.9 ± 0.90^{c}
28	92.89 ± 0.96^{b}	96.96 ± 0.96^{a}	80.49 ± 0.49^{b}	64.9 ± 0.90^{b}
35	98.13 ± 0.33^{a}	99.33 ± 0.33^{a}	91.7 ± 0.070^{a}	84.24 ± 0.22^{a}

Values are $\bar{x}\pm$SEM of duplicate values. $\bar{x}$ with dissimilar letter(s) are not significantly different from each other according to Duncan Multiple Range Test (DMRT).

ppm; parts per million

The biosorption of cadmium by *Lysinibacillus fusiformis* at different concentrations for 35 days is shown in Table 4.6 and at different intervals. At 10 ppm lead concentration, the highest biosorption rate was observed on 35th day with 99.94 % and the lowest was on the 7th day with 44.31 %. At 15 ppm, the highest biosorption ate was 35th day with 98.79 % and the lowest was observed on the 7th day with 53.59 %. At 20 ppm the highest biosorption rate was observed on the 35th day with 99.77 % and the lowest was observed on the 7th day with 54.43 %. At 50 ppm cadmium concentration, the highest biosorption rate was observed on 35th day with 97.23 % and the lowest was observed on the 7th day with 60.03 %. This result showed that *Lysinibacillus fusiformis* was able to biosorp cadmium at different concentration considered.

Table 4.6. Biosorption percentage of cadmium by *Lysinibacillus fusiformis*

	Cadmium concentration (pm)			
Time (Days)	**10**	**15**	**20**	**50**
7	44.31±0.31^{c}	53.59±0.59^{e}	54.43±0.43^{e}	60.03±0.03^{e}
14	83.54±0.54^{b}	79.73±0.73^{d}	68.49±0.43^{d}	63.56±0.56^{d}
21	99.53±0.53^{a}	86.94±0.94^{c}	76.29±0.29^{c}	65.29±0.29^{c}
28	99.74±0,74^{a}	94.42±0.42^{b}	91.89±0.89^{b}	82.16±0.16^{b}
35	99.94±0.94^{a}	98.79±0.79^{a}	99.77±0.77^{a}	97.23±0.23^{a}

Values are x̄±SEM of duplicate values. x̄ with dissimilar letter(s) are not significantly different from each other according to Duncan Multiple Range Test (DMRT).

ppm; parts per million

4.2 Discussion

In this study, heavy metal tolerance was exhibited by *Lysinibacillus fusiformis* 5B against 5 ppm concentration of tested heavy metal salts. This was ascertained by the presence of abundant growth on the surfaces of cultured nutrient agar. This is however possible, owing to the components of the cell wall of *Lysinibacillus* species, which contains thick peptidoglycan, teichuronic and teichoic acid bonded by Asp-Lys (Jacob *et al*., 2018). *Lysinibacillus* species also have a mechanism that helps them actively pump out toxic substances from their cells in what is known as efflux pumps. Extracellular and intracellular sequestration of metal ions as well as reduction in membrane permeability are also strategies used by Gram positive bacteria to resist entry of toxic metal substances into their cells (Saurabh *et al*., 2012; Kranthi *et al*., 2018). He *et al*. (2011) reported *Lysinibacillus fusiformis* ZC1 to be highly resistant to chromium. *Lysinibacillus fusiformis* ZC1 showed highest resistance reported so far for chromium as it recorded minimum inhibitory concentration of 60 mM. Likewise studies by Mathivanan *et al*. (2003), which reported high heavy metal tolerance of *Lysinibacillus fusiformis* KMNTT-10 to lead (II) up to a concentration of 500 ppm. There have been many ways of detecting heavy metals in water bodies and in the soil. Immobilization of urease on strip is a positive way of detecting heavy metals in water bodies. It is faster, less costly, and less time consuming. Tests strips also indicate level of concentrations of heavy metal (lead, cadmium, chromium and nickel) that is present (Cao *et al*., 2015).

The use of cellulose acetate paper for the strip has more advantage. The immobilized enzyme would not be washed away when dipping into the reagents used. It helps to give accurate results when it comes to the concentrating using the standard color charts (Cao *et al*., 2015). Biosorption of heavy metal carried out by *Lysinibacillus fusiformis* 5B in this study was observed across all

concentrations (i.e. 10, 15, 20 and 50 ppm). After 7 days of incubation, the result obtained showed high rate (> 40%) of biosorption of heavy metals (Cd, Cr and Pb) across the concentration considered with the exception of nickel (Ni), which showed as low as 20.99 % (15 ppm) and the highest at day 7 being 46.81 % (20 ppm). This could be as a result of varying degree of toxicity of different heavy metal. In the biosorption of heavy metals by bacteria cells, the amount of time in which the bacterial cells are in contact with the heavy metal play a key role in biosorption. This was observed in this study, as the longer time the cells of *Lysinibacillus fusiformis* 5B were in contact with the heavy metal solution, the more the cells adsorb the heavy metal onto their cells. *Lysinibacillus fusiformis* 5B recorded low biosorption of Ni after the seventh day and a high rate of biosorption (>50 %) across all concentration (10, 15 and 20 ppm) with the exception of 50 ppm. This showed that nickel may be more toxic to *Lysinibacillus fusiformis* 5B or the affinity of the functional groups present on the cell wall of *Lysinibacillus fusiformis* 5B was less compared to other heavy metals (Ansari *et al*., 2011). A high biosorption rate (> 55 %) of chromium was recorded at day 7. This showed that the functional groups present on cell surface of *Lysinibacillus fusiformis* 5B have high affinity for ions of chromium present in the solution, which is in line with the observation made by He *et al*. (2011) using *Lysinibacillus fusiformis* ZC1. It is important to note that biosorption of heavy metal reduces with increase in the concentration of the heavy metals (Cr, Ni and Pb). This is evident in this study, as at day 21, a high rate of biosorption (>90 %) was recorded across all the heavy metals at concentration of 10 ppm.

However, biosorption of cadmium in this study disagrees with the general notion that the higher the heavy metal concentration the lower the biosorption as the result obtained for cadmium at day 7 of incubation of *Lysinibacillus fusiformis* 5B showed lowest biosorption (44.31 %) at 10

ppm whereas the highest biosorption (60.03 %) was recorded at 50 ppm. This could be related to the affinity the functional groups present on the surface of *Lysinibacillus fusiformis* 5B have on the metal ions since they all have binding sites, which could either be inhibited or enhanced at varying concentration of the heavy metal. This study observed little percent increase in the biosorption of metals by *Lysinibacillus fusiformis* 5B towards the latter stages of incubation. This could be accounted for as a result of aging in bacterial cells typical of a batch culture having no renewal of nutrients or bacterial cells (Oyewole *et al*., 2019). The results also indicated that *Lysinibacillus fusiformis* has a limit for absorbing these heavy metals, the cell wall as a tolerant range for different heavy metals.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Urease produced by *L. fusiformis* 5B was inhibited by heavy metals and the colour was proportionate to the concentration of heavy metals. Immobilized urease produced by *L. fusiformis* 5B was able to detect Pb, Cr, Cd and Ni in solution. *L. fusiformis* had the ability to tolerate heavy metals (lead, cadmium, chromium and nickel). *L. fusiformis* 5B biosorped Pb, Cr, Cd and Ni with increasing capacity as the time (days) of incubation progressed. Ninety nine percent (99 %) of the heavy metals were biosorped by the test isolate for 10 ppm, 15 ppm and 20 ppm except for 50 ppm with 84 % biosorption.

5.2 Recommendations

It is recommended that;

i. *Lysinibacillus fusiformis* 5B can be explored to biosorp environments contaminated with these heavy metals and thereby help to reclaim these environments of heavy metals toxicity.
ii. The application of urease impregnated strip for heavy detection in water, wastewater, produced water and other elements.
iii. Genetic profiling and modification of *Lysinibacillus fusiformis* 5B for increased biosorption potential.
iv. Development of real time sensor device for heavy metals detection.

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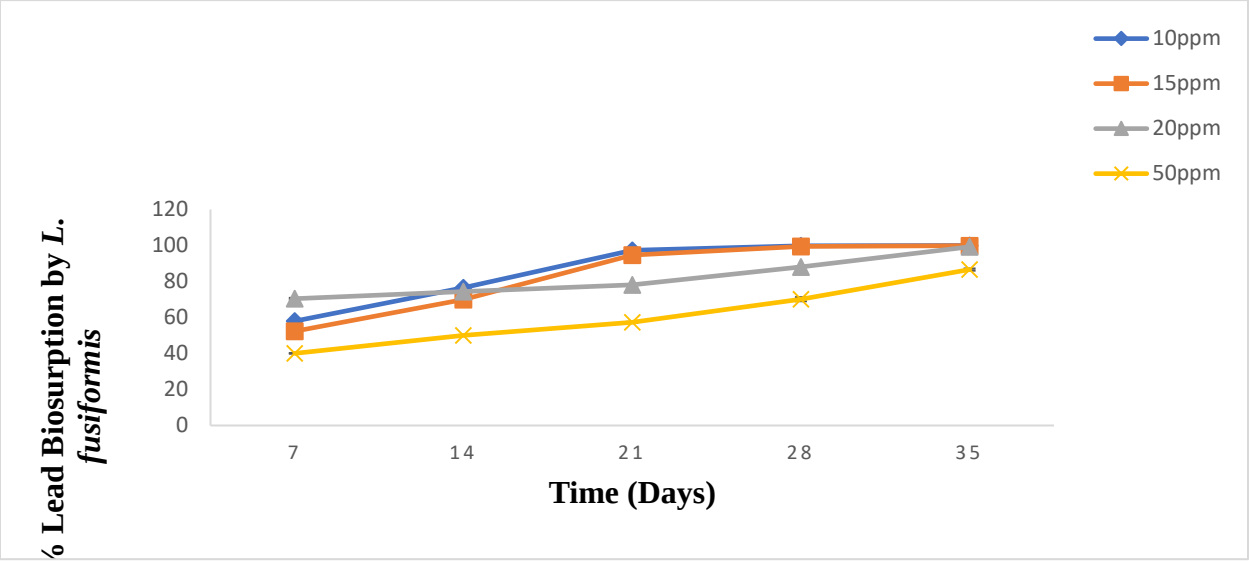
APPENDIX

APPENDIX A: Analysis parameter for biosorption of heavy metals

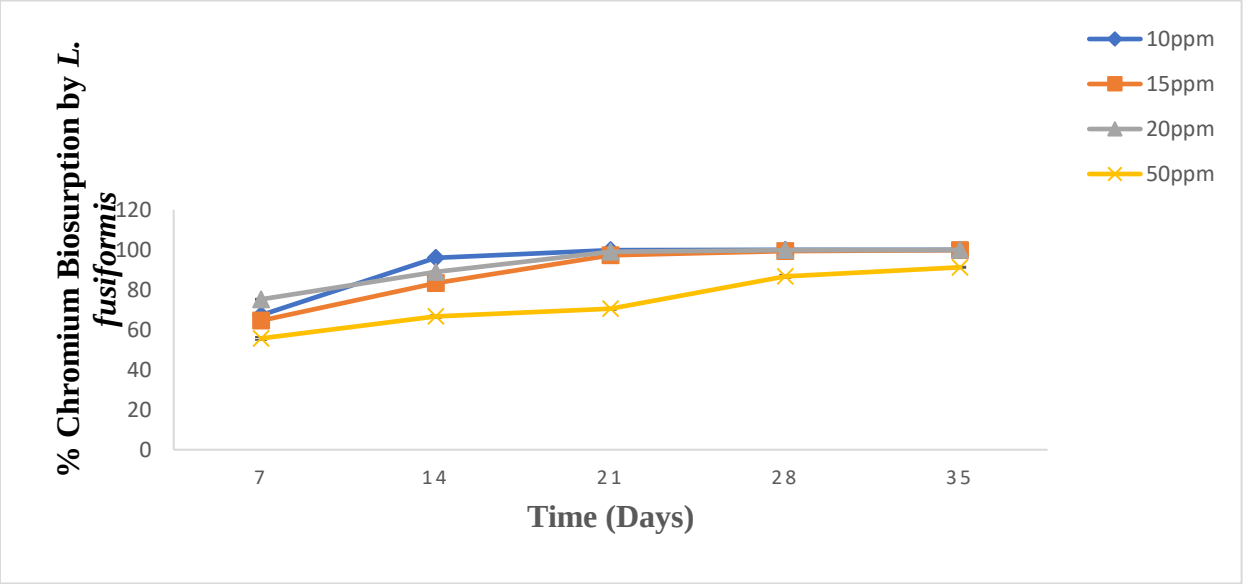
Chromium analysis parameter: are analytical line 357.9nm, bandwidth 0.4nm, filter factor 1.0, lamp current 3.0ma, integration time 3.0sec, background none, flame type air/natural gas,flame setting 400ml/min, senstivity 1.00mg/l, detection limit 0.60mg/l, working range 2,50-150.0mg/l and wavelength 359.4nm. Cadmium analysis parameter are analytical line 228.8nm, bandwidth 0.4 nm, filter factor 1.0, lamp current 2.0ma, integration time 3.0sec, background D2/SR, flame type air/natural gas, flame setting 400ml/min, senstivity 0.020 mg/L, detection limit 0.008 mg/L, working range 0.06-3.20 mg/L and wavelength 326.1nm. Lead analysis parameter are analytical line 217.0 nm, bandwidth 0.4 nm, filter factor 1.0, lamp current 2.0ma, integration time 3.0sec, background D2/SR, flame type air/natural gas,flame setting 400ml/min, senstivity 0.15mg/l, detection limit 0.02mg/l, working range 0.10-14.0mg/l and wavelength 283.3nm.Nickel analysis parameter are analytical line 232.1nm, bandwidth 0.2nm, filter factor 1.0, lamp current 5.0ma, integration time 3.0sec, background D2/SR, flame type air/natural gas, flame setting oxidizing blue, senstivity 0.05mg/l, detection limit 0.008g/l, working range 0.04-8.0mg/l and wavelength 231.1nm (Furr and Bretherick 2006).

Appendix B. Test strips or detection of heavy metals

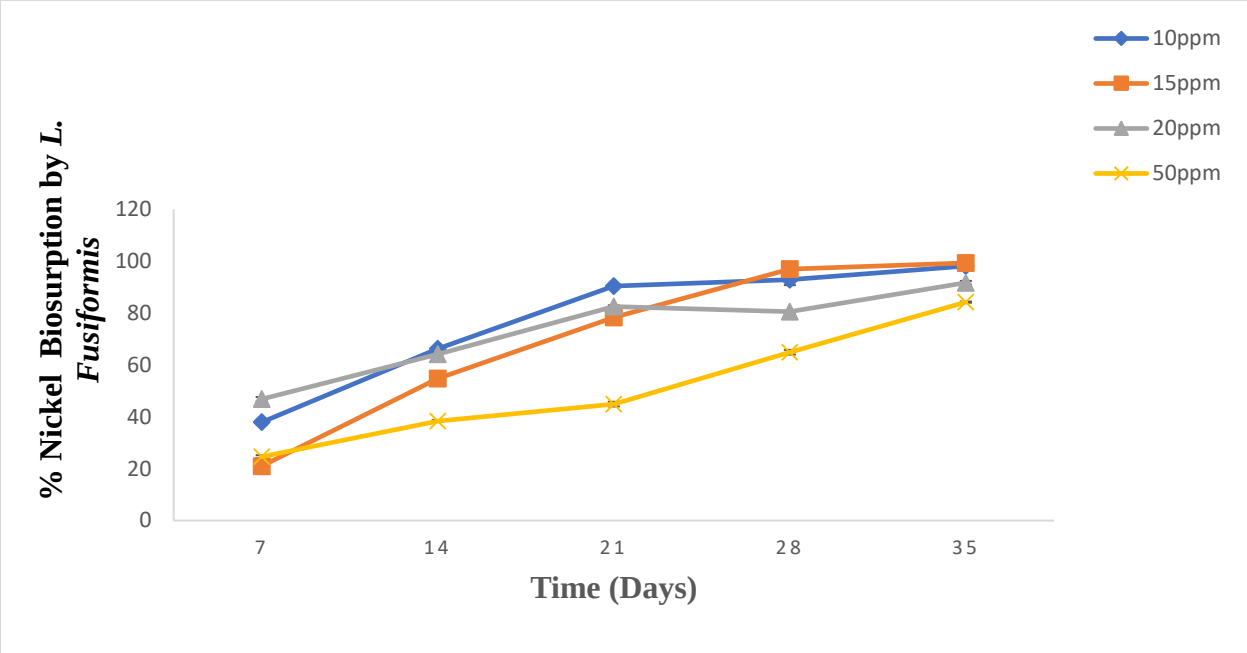
Appendix C: Percentage of lead biosorption by *Lysinibacillus fusiformis*



Appendix D: Percentage of chromium biosorption by *Lysinibacillus fusiformis*



Appendix E: Percentage of nickel biosorption by *Lysinibacillus fusiformis*



Appendix F: Percentage of cadmium biosorption by *Lysinibacillus fusiformis*