INFLUENCE OF CHELATING AGENTS AND NICKEL ON PHENOL UTILIZATION BY *PSEUDOMONAS* SPECIES

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CERTIFICATION

I certify that this work "Influence of chelating agents and nickel on phenol utilization by *Pseudomonas* species" was carried out by Ahumibe, Nkenna Chinonyerem (Reg Number 20094769358) in partial fulfilment of the requirements for the award of the degree of Master of Science (M. Sc) in Environmental Microbiology in the Department of Microbiology of the Federal University of Technology Owerri.

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DEDICATION

This research is dedicated to God Almighty who kept me alive and made this research a success.

Blessed redeemer, I owe my all to You. Everything I am, and all that I have,
I am eternally grateful.

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ABSTRACT

The effects of nickel and four chelating agents, trisodium citrate, ethylenediaminetetraacetic acid, tannic acid and potassium ferricyanide, at different concentrations in nutrient broth and a mineral salts medium supplemented with phenol, on the dehydrogenase activity of a *Pseudomonas* species, as well as the effects of nickel and trisodium citrate on phenol biodegradation were investigated. The results showed that the bacterium was sensitive to nickel with an observed half maximal inhibitory concentration (IC_{50}) of 0.613 mM and 0.0017 mM in nutrient broth and mineral salts medium, respectively. Trisodium citrate and ethylenediaminetetraacetic acid were most efficient in reducing the inhibitory effect of nickel while the effects of tannic acid and potassium ferricyanide were not very pronounced. Nickel inhibited phenol degradation by *Pseudomonas* sp. The addition of trisodium citrate stimulated the growth rate of the bacterium but had no significant effect on phenol degradation rate. The progress of growth was described by the integrated Haldane model with kinetic constants of μ_m 0.0967 h^{-1}, K_s 78.289 mg/l, and K_i 346.687 mg/l at 500 mg/l initial phenol concentration. Maximum specific growth rate (μ_m) decreased in the presence of nickel, and increased with trisodium citrate addition. The results of the study showed that chelating agents could mitigate nickel toxicity. Also, they suggest that trisodium citrate could be used as a possible carbon source in biodegradation studies.

Keywords: Chelating agents, Haldane equation, IC_{50}, Nickel, Phenol, *Pseudomonas* sp.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Soil contamination by metals is quite common, because the soil is both a source of metals and a sink for metal contaminants from various sources. Metals occur naturally in soil in varying concentrations across geographic regions and are also distributed as a result of man's continuous industrial activities and technological development. The total and bioavailable concentration of metals in soils are of great importance with regard to human and microbial toxicology as well as agricultural productivity. The effects of chelating agents, metals and phenols on microorganisms isolated from soils, sediments and aquatic habitats have been published (Campbell *et al*., 2000; Cempel and Nikel, 2006; Cao *et al*., 2007). The total inhibitory effect of a metal depends on both microbiological and environmental factors, however, it is generally accepted that the availability is strongly affected by the metals' speciation (Tandy *et al*., 2004). The speciation of a metal can be described as the distribution of the total metal concentration over all its possible chemical forms. Some peculiar characteristics of metals include their recalcitrance and natural availability at a background level related to weathering of parent rocks, and occurrence as cations which strongly interact with the soil matrix. Metals in soil can thus become mobile as a result of changing environmental conditions (Facchinelli *et al*., 2001). Some metal ions play a number of important roles such as membrane/capsule stabilization and oxidative metabolism in bacteria. They are also essential for maintaining proper health of humans, animals and plants.

The effect of metals on microorganisms has received a great deal of attention in recent years. This is due to the fact that the impact of metals on the microbiological properties of soil depends not only on the degree of contamination, but also on the presence and properties of other types of pollutants and/or organic compounds in the soil environment. Nickel is one of the essential elements found in the earth's crust, occurring at an average concentration of 75 μg/g (Poonkothai and Vijayavathi, 2012). The wide use of nickel in different industries also exposes the environment (soil, air and water) to its uncontrolled emission. Its influence on microbiological properties of the soil is less recognized than that of other metals such as

cadmium or lead. Evidence over the past few years indicates that nickel is a nutritionally essential metal for some plants, bacteria, and invertebrates (Nielson, 1996; Klein and Costa, 2011; López, and Magnitskiy, 2011).

Chemical weathering is attributed to organic chelating agents, *e.g.* peptides and sugars that extract metal ions from minerals and rocks. Most metal complexes in the environment and nature are bound in some form of chelate ring, *e.g.* with a humic acid or protein. Metal chelates are relevant to the mobilization of metals in the soil, as well as their uptake and accumulation into plants and microorganisms. Chelating agents are discharged into the soil from industrial effluents (eg pulp mill effluent) and run-offs from fertilizer applications as metal chelate compounds are common components of fertilizers. However they also occur naturally in soils as plant root exudates or produced by microbes present in the soil matrix (Naidu and Harter, 1998). Others such as tannic acid occur naturally in plants/fruits; citric acid is also found in lime and lemon. Chelation by ethylenediaminetetraacetic acid (EDTA) has been shown to increase cell wall permeability by extracting metals from the cell wall which stabilise lipopolysaccharides, phospholipids and proteins. It binds metal ions through six binding sites, thereby producing very stable metal-chelant complexes. EDTA is released into the environment through wastewaters. Its presence in soils may be due to agrochemical application or to the disposal of products containing EDTA in garbage reservoirs (Oviedo and Rodríguez, 2003).

Apart from metals, there are some compounds that contaminate the environment such as phenolic compounds. They are common constituents of soils contaminated from industrial effluents including oil refineries, as well as pharmaceutical, petroleum, textile and coal refining industries. Phenol and its derivatives are widely used as antimicrobial agents and in many industrial branches such as petrochemical, pulp, paper, tannery and coal refining industries. Thus, it is present in wastewater coming from these industries (Marrot *et al.*, 2006). The toxicity of phenol to the environment and towards humans is well documented and of great concern (Ghadhi and Sangodkar, 1995; Van Schie and Young, 2000). This is because even at low concentrations, phenol has adverse effect on aquatic life, plant and microbial

growth. Due to the widespread distribution of phenol in the environment, some microorganisms have adapted to using the compound as carbon and energy source (Mohite *et al.*, 2010). This utilization/degradation of phenol by microbes especially bacteria has been reported by many researchers (Kanekar *et al.*, 1999; Prieto *et al.*, 2002; Li *et al.*, 2005; Abdullah *et al.*, 2010; Passos *et al.*, 2010).

Soil microbial enzymatic activities are frequently used for determining the influence of various pollutants, including metals, on soil quality (Shen *et al.*, 2005). Enzymatic activities of microorganisms are recognized to be more sensitive bio-indicators of any natural and anthropogenic disturbance than those of plants and animals (Hinojosa *et al.*, 2004). Moreover, soil enzyme assays are considered cheap and easy techniques. The impact of metals on soil enzyme activity depends on the nature and concentration of the metal, and its extent varies from one enzyme to another. At certain concentrations, some metals can stimulate the activity of an enzyme but inhibit it at other concentrations (Nweke and Orji, 2009). Metals affect enzymatic activities through various mechanisms that ultimately influence the microbial community by interacting with the enzyme substrate complexes, denaturing the enzyme protein and active sites (Megharaj *et al.*, 2003). Thus the measurement of microbial enzyme activity has now become an assessment of ecotoxicological impacts of environmental substrates. Dehydrogenase activity is an estimation of the overall microbial activity due to its ubiquity in all microorganisms (Taylor *et al.*, 2002). It is an effective primary test for assessing the potential toxicity of metals to soil microbial activities, planktonic and heterotrophic bacteria from tropical river sediments (Nweke *et al.*, 2006, 2007). The measurement of dehydrogenase activity provides correlative information on the biological activity in an environment. It represents immediate metabolic activities of soil microorganisms at the time of the test.

The progressive accumulation of metals and other pollutants may inhibit the degradation of organic pollutants (such as phenol) or humic substances in the environment (Abou-Shanab *et al.,* 2003). The organic matter content in soil interacts with trace elements present and, in some cases, makes the metal more biologically available. The microbial flora present is usually exposed to these soil constituents (such as chelators, herbicides, metals, pesticides,

phenols) as well as other environmental factors. Despite the numerous reports on the effect of chelating agents and metals on soil microorganisms, effective technological solutions to counter these effects have not been found. This could be due to inadequate quantitative and qualitative definition of the metal contaminants. Metals may be present in a variety of physical and chemical forms, namely, as separate- phase solids, soil-adsorbed species, colloidal solutions, soluble complexed species, or ionic solutes. They can be affected by environmental conditions such as pH, ionic strength of the water phase, soil properties like ion exchange capacity, clay type, and organic matter content. The microbial response varies as a result of differences in biological processes of the microbial consortium present in the soil, available nutrients and concentration of pollutants present, joint action of pollutants and organic compounds present at the time of test.

1.2 Statement of the problem

The presence of heavy metals and phenolic compounds in the environment originates from a range of human activities, which in turn affects microbial, plant and human metabolic activities. This contamination by these compounds is still a world-wide problem that requires effective technological solutions. Hence, this research was carried out to study the efficiency of some chelating agents to bind nickel and evaluate their impact on microbial activity such as phenol degradation.

1.3 Aim and objectives

1.3.1 Aim of the study

This research work aimed at evaluating the influence of nickel and metal chelating agents in biodegradation of phenol by soil *Pseudomonas* species.

1.3.2 Objectives of the study

The objectives of this research work are as follows:

1. To isolate *Pseudomonas* species from soil.

2. To determine the single and mixed effects of different concentrations of nickel and chelating agents on the dehydrogenase activity of the bacterial isolate.
3. To evaluate phenol utilization/degradation by the isolate.
4. To investigate the effects of nickel and a chelating agent(trisodium citrate) on phenol degradation by the bacterial isolate and
5. To model the effects of nickel and chelating agents on the dehydrogenase activity of the isolate.

1.4 Justification of study

Reports of the impact of nickel and phenol on the environment have shown that they have become a threat to public health (Wyszkowska *et al.,* 2005; Wuana and Okieimen, 2011). They are both introduced to the environment mainly by anthropogenic activities such as burning of fossil fuels, composts from different wastes and inorganic fertilizers used on farmlands. Current remediation techniques for metal and phenol polluted environments involves the use of chelating agents and microorganisms capable of degrading phenol. Thus, this study evaluates the capacity of some chelating agents to reduce/increase the toxicity of nickel and its impact on phenol degradation by *Pseudomonas* sp.

1.5 Scope of study

The study investigated the effect of nickel and some chelating agents individually and jointly on the dehydrogenase activity of *Pseudomonas* sp isolated from soil. The ability of the bacterium to degrade phenol in the presence and absence of nickel was evaluated. The dehydrogenase activity at varying concentrations of nickel and chelating agents were evaluated as the absorbance of the formazan extract at 500 nm. The dose response data were fitted into a 3-parameter logistic model. Phenol degradation by the bacterium and its associated microbial growth were represented using the Haldane equation. With the aid of these predictive models, the study determined conditions under which the isolate degraded phenol in the presence of nickel under laboratory conditions. The results added to existing knowledge on phenol degradation in the presence of nickel.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chelating agents and their applications

2.1.1 Nature of chelating agents

Chelation has its origin in the Greek word *chele* meaning claw of a lobster, thus depicting the concept of clinging or holding with a strong grip. Chelating agents are organic or inorganic compounds capable of binding metal ions, to form complex ring-like structures called chelates (Flora and Pachauri, 2010). They are used chemically to remove ions from solutions, medicinally, against microorganisms, as well as in the treatment of metal poisoning and in chemotherapy protocols.Many biological processes rely on chelated metals tightly positioned within the chelated complexes (Bohuslavek *et al.,* 2001). Familiar biological complexes include heme for oxygen transport, cytochromes for electron transport, chlorophylls for photosynthesis and vitamin B12 involved in C-1 metabolism. Some of the chelating agents involved in these complexes are produced by microorganisms (bacteria, algae, fungi) and plants. Synthetic chelating agents are also present in the environment due to their use in household, agricultural products and in many industrial processes (Sillanpaa, 1997).

In order for a compound to be called a true chelating agent, it must have certain chemical characteristics. The compound must consist of at least two sites capable of donating electrons (coordinate covalent bond) to the metal it chelates. It should have high solubility in water, be resistant to biotransformation, posses the ability to reach the sites of metal storage, retain chelating ability at the prevailing pH and the property of forming metal complexes that are less toxic than the free metal ion. For true chelation to occur the donating atom(s) must also be in a position within the chelating molecule such that the formation of a ring with the metal ion can occur. The stability of a chelate is related to the number of atoms in the chelate ring. Monodentate ligands which have one coordinating atom like water (H_2O) or ammonia ($NH_{3)}$ are easily broken apart by other chemical processes, whereas polydentate chelating agents, donating multiple bonds to metal ion, provide more stable complexes. Chlorophyll, the green plant pigment, is a chelate that consists of a central magnesium atom joined with four

complex chelating agents (pyrrole ring). The molecular structure of the chlorophyll is similar to that of the heme bound to proteins to form hemoglobin, except that the latter contains Fe^{2+}ion in the center of the porphyrin. Heme is an iron chelate. Chelation is applied in metal complex chemistry, organic and inorganic chemistry, biochemistry, and environment protection. Chelating agents can form stable water soluble complexes with multivalent metal ions, they prevent undesired interaction by blocking/preventing normal reactivity of metal ions. Chelating agents are often added to various chemical processes to eliminate metal ion contamination in the finished product or to assist in the removal of traces of remaining catalytic metal ions. Some common chelating agents include ethylenediaminetetraacetic acid (EDTA), 2,3-di mercapto propane sulfonic acid (DMPS), thiamine tetrahydro furfuryl disulfide (TTFD), 2,3-dimercaptosuccinic acid (DMSA), nitrilotriacetic acid (NTA), glutamic acid, N,N-diacetic acid (GLDA)and trisodium citrate.Others include humic acids, hydroxyethylenediaminetriacetic acid (HEDTA), diethylenetriaminepentaacetic acid (DTPA), calcium disodium EDTA, disodium dihydrogen EDTA, tetrasodium pyrophosphate, citric acid, monoisopropyl citrate, oxalic acid, phosphoric acid, and monoglyceride phosphate (Sillanpaa, 1997; Kolodynska, 2011).

2.1.2General applications of chelating agents

Chelating agents are used as components or process chemicals in a wide variety of applications (Kołodyńska, 2011). They are applied in the field of agriculture, cosmetics, soap and detergents manufacture, chemical processes (e.g. metal finishing and plating), food and pharmaceutical industries, photography, pulp and paper manufacture, textile dyeing, and in medicine. They have also been implicated in the bioremediation and phytoremediation of contaminated environments (Sun *et al*., 2001; Wuana *et al*., 2010).These applications are further discussed below:

2.1.2.1 Agriculture

Chelating agents are used to prevent the precipitation of important mineral ions (iron, zinc, manganese etc.) essential for maximum crop growth and yields. Otherwise, phosphates, carbonates, silicates and organic materials present in the soil will precipitate and “lock-up”

these trace elements severely limiting their movement to the plant roots and their ultimate uptake and utilization in producing maximum yields.

2.1.2.2. Manufacture of cosmetics

Chelating agents are equally added to cosmetic products. In oil-water emulsions, such as creams and lotions, chelating agents prevent rancid off-odors by tying up traces of catalytic metal ions. Many ingredients are sensitive to trace metal contamination and are protected by the addition of small amounts of chelating agents such as ethylenediaminetetraacetic acid (EDTA).

2.1.2.3 Chemical Processing

Improvement in color often results from the addition of chelating agents to a chemical process. The iron chelates of ethylenediaminetetraacetic acid and hydroxyethylenediaminetriacetic acid(EDTA and HEDTA respectively) are used to effectively and efficiently remove noxious pollutants such as hydrogen sulfide (H_2S), sulfur dioxide (SO_2) and oxides of nitrogen (NO_8, NO_3) from sour natural gas, refinery off-gases and stack gases from coal or oil burning steam-generating plants.

2.1.2.4 Manufacture of detergent compounds

Chelating agents are added to detergents to soften the water by tying up water-hardness ions which tend to impede cleaning and rinsing. EDTA finds particular use in bathtub cleaning products where much of the difficulty lies in removing insoluble calcium soap scum and films. By removing the calcium from the films, the remaining soap is easily rinsed away. Traces of iron are often present in liquid detergents and the addition of HEDTA will prevent the precipitation of unsightly deposits in the package (Oviedo and Rodriguez, 2003).

2.1.2.5 Medicine

EDTA, as an anticoagulant is used for some medical purposes, such as the treatment of hypercalcemia (Kite *et al.*, 2004). It is also used in dentistry to remove inorganic debris of the root canal in preparation for obturation (Walker *et al.*, 2003).

2.1.2.6 Food and Pharmaceutical industries

Discoloration, rancid taste and odor in food products are often caused by the presence of small traces of metal ions such as iron, copper and manganese. The addition of EDTA to products such as mayonnaise, canned and frozen vegetables, shellfish and soft drinks assists in preventing off-taste and color, thereby reducing spoilage losses (Oviedo and Rodriguez, 2003). EDTA also finds use in the pharmaceutical industry as a treatment to remove lead from the body in cases of lead poisoning and in preserving ophthalmic products from certain harmful bacteria.

2.1.2.7 Photography

EDTA-type chelating agents have been used to prevent precipitation of hard water salts in photographic processing solutions and to stabilize developer solutions toward air oxidation. More recently, the iron chelate of EDTA has replaced ferricyanide salts as bleaching agents in the development system for color film (Oviedo and Rodriguez, 2003).

2.1.2.8 Pulp and Paper production

In a like manner to textile bleaching with hydrogen peroxide, chelating agents, primarily diethylenetriaminepentaacetic acid (DTPA) are used in the peroxide bleaching of high-grade pulp. The addition of DTPA increases the brightness levels and reduces brightness reversion upon aging while improving the overall efficiency of the bleaching process. Traces of manganese present in the wood pulp are detrimental to the peroxide system and significantly reduce the brightness of the finished paper. Chelating agents tie up these traces of manganese (Oviedo and Rodriguez, 2003).

2.1.2.9 Textiles

Many dyestuffs used by the textile industry are sensitive to small amounts of water hardness and metal ions. This creates splotchy of mottled colors and the formation of unwanted off-shades. These problems are corrected by the use of chelating agents.
Diethylenetriaminepentaacetic acid (DTPA) is used to stabilize and prevent decomposition of

bleaching baths containing hydrogen peroxide. These result in improved whiteness, fewer pinholes in the fabric and an overall savings in peroxide consumption.

In literature, current remediation technologies have implicated the use of chelating agents for metal-contaminated soils. Chelating agents bind toxic metals from soil solid phases to form strong water-soluble complexes, which can be removed from the soil by plants through enhanced phytoextraction or by using soil washing techniques. They are used for mobilizing metals in enhanced electrokinetic extraction (Kos and Lestan, 2004; Luo et *al.,* 2005).The extent of remediation/extraction using chelating agents depends on factors such as; type of chelating agent used, and concentrations of chelating agents employed. Ethylenediamine tetraacetic acid (EDTA), a strong chelating agent has been studied, and continues to be for soil remediation due to its strong complexes-forming ability. In this study, the chelating agents discussed include; ethylenediaminetetraacetic acid, trisodium citrate, tannic acid and potassium ferricyanide (subsequently presented as EDTA, TSC, TA and $K_3Fe(CN)_6$) respectively. They are further discussed below.

2.1.3 Some examples of chelating agents and their applications

There are many examples of chelating agents which are in use in diverse applications. Some including ethylenediaminetetraacetic acid (EDTA), trisodium citrate (TSC), tannic acid and potassium ferricyanide are here presented.

2.1.3.1Ethylenediaminetetraacetic acid

EDTA, is an amino polycarboxylic acid and a colourless, water-soluble solid. EDTA and its salts are substituted diamines. It can form four or six bonds with metal ions, and is used to dissolve lime scale. It has high affinity for a wide range of cationic metals (Fe, Mn, Cu, Zn, and Mg) and is very effective in the acid-neutral pH range 5 - 7.5 (Kolodynska, 2011). EDTA is produced as several salts, notably disodium EDTA and calcium disodium EDTA. It is produced industrially by the reaction of ethylenediamine with sodium cyanide and formaldehyde in the presence of sodium hydroxide (Kolodynska, 2011). After being bound by EDTA, metal ions remain in solution but exhibit diminished reactivity.

EDTA has been reported as one of the most economical of the commonly used chelating agents, and found to inhibit the growth of some bacterial species, due to its chelating properties (Lanigan and Yamarik, 2002; Chudzick *et al*., 2007). It has been reported to be a potential agent against biofilm formation (staphylococcal biofilm) (Juda *et al*., 2008). It is useful because of its role as a hexadentate ("six-toothed") chelating agent.EDTA has a remarkable ability to form stable, water-soluble complexes with many metal ions, thus it forms complexes with Mn^{2+}, Cu^{2+}, Fe^{3+}, Co^{3+}, Ca^{2+} and Mg^{2+}. The complex forms a larger chelated ion that reduces the ability of the micronutrient to react with the soil, making the micronutrient more available for plant/microbial uptake. The chelating agent keeps the micronutrient in solution even when the soil pH is higher than desired for a specific organism/crop. As an anticoagulant, it is used for some medical purposes, such as the treatment of hypercalcemia (Kite *et al*., 2004). It is also used in dentistry to remove inorganic debris of the root canal in preparation for obturation (Walker *et al*., 2003).

EDTA has been reported as a reusable resource for remediation and recovery of heavy metals from contaminated soils (Luo *et al*., 2005; Yeh and Pan, 2012). EDTA is recalcitrant to biodegradation in soil (Yeh and Wu, 2009). The environmentally measurable concentrations and recalcitrance of EDTA has raised concern about the environmental fate of EDTA. It also has been speculated that EDTA can enhance the toxic effects of heavy metals and radionuclides (Sillanpaa, 1997). These chelating agents are cytotoxic and weakly genotoxic, but not carcinogenic. The chemical structure of EDTA is presented in Figure 2.1.

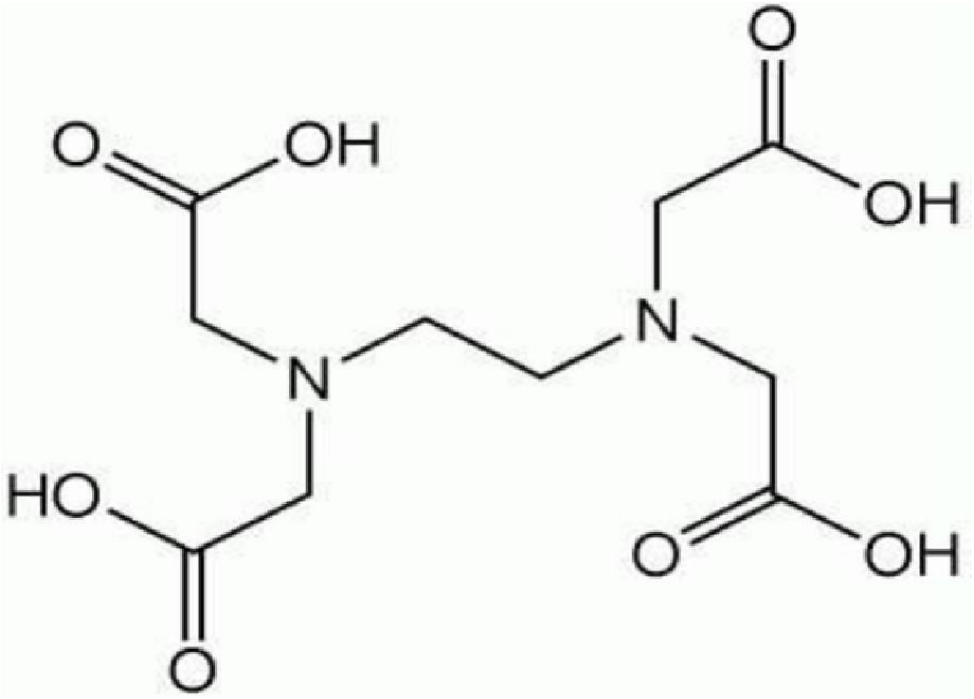

Fig 2.1 Structure of EDTA (source; Kandeel *et al*., 2013)

In the literature, EDTA (Na_2EDTA) is the most frequently cited chelating agent for extracting potentially toxic trace metals from soils, because of its efficiency, availability and relatively low cost. Study by Kos and Lestan (2004) has shown that the addition of 10 mMol/kg of EDTA chelant enhanced plant lead uptake the most.

2.1.3.1.1Applications of ethylenediaminetetraacetic acid

The synthetic aminopolycarboxylic acid, ethylenediaminetetraacetic acid (EDTA), has a variety of industrial applications. It is applied in medicine and laboratory for analytical purposes.

2.1.3.1.1aIndustry

In industry, EDTA is mainly used to sequester metal ions in aqueous solutions. It prevents metal ion impurities from modifying colours of dyed products in the textile industry. In the pulp and paper industry, it inhibits the ability of metal ions, especially Mn^{2+}, from catalyzing

the disproportionation of hydrogen peroxide, which is used in "chlorine-free bleaching." (Jones and Williams, 2002).It is used as a food preservative or stabilizer to prevent catalytic oxidative decoloration by metal ions. In soft drinks containing ascorbic acid and sodium benzoate, it mitigates formation of benzene (a carcinogen). The reduction of water hardness in laundry applications rely on EDTA, thus it is normally used in detergents and soaps because it forms complexes with magnesium ions and calcium. It is used in cosmetic formulations. In shampoos, cleaners and other personal care products its salts are added as chelators to improve their stability in air.

2.1.3.1.1b Medicine

EDTA binds metal ions in the practice of chelation therapy, e.g., for treating mercury and lead poisoning (Lanigan and Yamarik, 2002). It is used in a similar manner to remove excess iron from the body. Dentists and endodontists use EDTA solutions to remove inorganic debris (smear layer) and lubricate the canals in endodontics.

2.1.3.1.1cLaboratory applications

In analytical chemistry, EDTA is used in complexometric titrations and analysis of water hardness or as a masking agent to sequester metal ions that would interfere with the analyses. It can also be used to test for bioavailability of heavy metals in sediments.

2.1.3.2 Trisodium citrate

Trisodium citrate which is sometimes referred to simply as sodium citrate, occurs as white, granular crystals or as white, crystalline powder. It is odourless, possesses a saline, mildly tart flavour and has a cool, salty taste. Trisodium citrate has the chemical formula of $Na_3C_6H_5O_7$, molar mass of 258.06 g/mol, melting point of 300 °C and density of 1.70 g/cm³. It is soluble in water. It occurs in two forms: the crystal form called trisodium citrate dihydrate, and the anhydrous form called trisodium citrate anhydrous. It is slightly deliquescent in moist air, freely soluble in water and practically insoluble in ethanol (96 %).Sodium citrate can refer to any of the three sodium salts of citric acid (Vijaya et al., 2013). Industrial releases of citric acid may occur from the sites of production and through use in industrial processes. Consumers are directly exposed to citric acid or its salts in diluted concentrations in many

applications from soft drinks and processed food to common household cleaners, detergents, washing powders etc.; there are no acceptable daily intake levels. The chemical structure of trisodium citrate is presented in Figure 2.2.

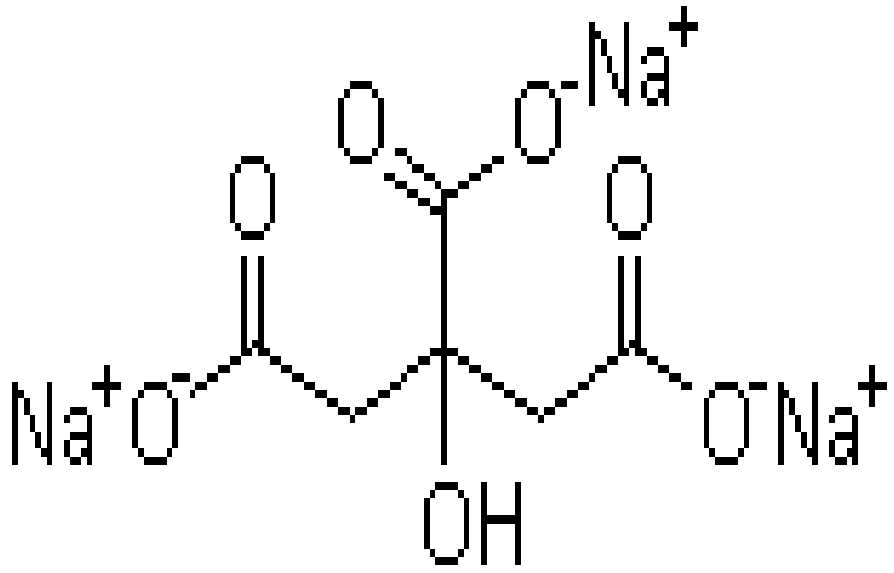

Fig 2.2. Structure of trisodium citrate (source; Vijaya *et al*., 2013)

Trisodium citrate dihydrate is a tribasic salt of citric acid. It is produced by complete neutralisation of citric acid with high purity sodium hydroxide or carbonate and subsequent crystallisation. It is widely used in foods, beverages and various technical applications mainly as a buffering, sequestering or emulsifying agent. Trisodium citrate dihydrate is a non-toxic, neutral salt with low reactivity. Trisodium citrate dihydrate is fully biodegradable and can be disposed of with regular waste or sewage.Trisodium citrate has been reported to remove or reduce metal concentration at different concentrations 200 –1000 mg/L (Azelee *et al*., 2013). The rate of metal removal was affected by length of time of treatment, temperature and pH.

2.1.3.2.1 Health hazards associated with trisodium citrate

2.1.3.2.1a Hypernatremia

As the name implies, sodium citrate contains the mineral sodium in it. Elevated sodium in the blood also known as hypernatremia, is a possible side effect associated with sodium citrate. While those with normal kidneys should be able to tolerate sodium citrate with no change to their normal levels of sodium, some individuals with damage to their kidneys may not tolerate this medication. If the kidneys are not working normally, the sodium may not be excreted; instead, it will build up in the bloodstream and lead to hypernatremia. Symptoms of mild hypernatremia include drowsiness and weakness. As the levels of sodium in the blood increase, symptoms become more severe and may include seizures or even death.

2.1.3.2.1bMuscle Spasms

Tetany is the word used to describe sudden-onset, intensely powerful contractions of muscles. The medical reference up to date lists these muscle spasms as one of the possible side effect of sodium citrate. Although sodium citrate is generally considered a safe medication, especially for patients with healthy, functioning kidneys, the experience of uncontrolled muscle spasms and contractions may be a very painful one.

In human (as well as in animal and plant) physiology, citric acid is a very common intermediate in one of the central biochemical cycles, the Krebs or tricarboxylic acid cycle, which takes place in every cell. It completes the breakdown of pyruvate formed from glucose through glycolysis, thereby liberating carbon dioxide and a further four hydrogen atoms which are picked up by electron transport molecules. Thus, in man approximately 2 kg of citric acid is formed and metabolized daily. This physiological pathway is very well developed and capable of processing very high amounts of citric acid as long as it occurs in low concentrations.

2.1.3.2.2 Applications of trisodium citrate

Many of the following industrial applications utilized trisodium citrate due to its environmentally friendly properties. Citric acid is found throughout nature in many fruits and vegetables and is a natural component in metabolic processes. It is highly biodegradable.

2.1.3.2.2a Fertilizer Micronutrients

Citric chelates of iron, copper, magnesium, manganese and zinc are used to correct soil deficiencies because they are soluble in water at normal soil pH. Metal sulphates are dissolved in water and citric acid followed by neutralization with ammonia. The soluble citrate chelate promotes the transfer of the metal nutrient into the plant's root or leaf system, the biodegradability of citric acid is important for this application.

2.1.3.2.2b Fossil-Fuel Power Plants

Ammoniated citric acid is used to clean metal oxides from the water side of steam boilers with a two-step single-fill operation the resulting surface is clean and passivated. The cleaning solution can easily and safely be disposed of by incineration or liming.

2.1.3.2.2cFood Industry

- Beverage: Citric acid is the acidulant of choice in the beverage industry. It is used extensively in carbonated and non-carbonated beverages as flavouring agents and buffers. The high solubilities of citric acid and citrate salts make them ideal for syrup concentrates. They also increase the effectiveness of anti-microbial preservatives. Modifications such as juice-added beverages, low calorie beverages, and thirst quenchers use citric acid alone and in combinations with citrate salts.
- Canning Industry: Citric acid lowers the pH to reduce heat processing, chelates trace metals to prevent enzymatic oxidation and colour degradation, and also enhances the flavour.
- Confections: Citric acid is utilized in the confection industry. Citrates control sugar inversion, optimize gel-setting characteristics, provides tartness and enhance flavour.

- Coffee Creamers: Sodium citrate is used in dairy and non-dairy creamers to stabilize the casein. This prevents feathering of the creamers when added to hot beverages.
- Dietary Calcium Supplements: Citric acid in the form of calcium citrate is used as a dietary calcium supplement. Studies have shown that calcium citrate is more bio-available than calcium carbonate.

2.1.3.2.2d Detergents

The largest industrial application for citrates is the detergent industry. In liquid detergents, sodium citrate is used as a builder, to increase the effectiveness of the surfactants, due to its high solubility and bio-degradability. Citrate acts as a scale-dissolver and a buffer. In powder detergents, sodium citrate is used as a co-builder and processing aid. It also contributes alkalinity to enhance surfactant performance. The environmentally friendly nature of sodium citrate is a major factor in the use of citrates in the detergent industry.

2.1.3.2.2e Environmental Aspects

Enhanced Oil Recovery: Aluminium citrate is used for *in-situ* gelling of polymers in polymer flooding operations. The citrate controls the rate of availability of the aluminium ion. It has also been reported as a potential metal chelating agent thereby promoting biodegradation of aromatic compounds. Though there are uncertainties as to the optimal choice or concentration for full-scale application (Wuana *et al*., 2010; Qian *et al.,* 2015).

2.1.3.3 Tannic acid

Tannic acid is a plant polyphenol which is found, along with other condensed tannins, in the bark and fruits of many plants (Lopes *et al*., 1999), particularly in bananas, spinach, grapes, pears, berries, nettle and persimmons (Wu *et al*., 2004). It is also found in several beverages such as red wine, beer, coffee, tea(black and green), and many foodstuffs such as sorghum, black-eyed peas, lentils and chocolate (King and Young, 1999). High levels of tannic acid are also found in some plant galls, chinese galls and oak wood. Tannic acids are water soluble phenolic compounds having molecular weights between 500 and 3000, and having the ability to precipitate alkaloids, gelatine and other proteins. It is a polymer of gallic acid molecules and glucose. It will hydrolyze into glucose and gallic or ellagic acid units. It is odourless but

has a very astringent taste. Pure tannic acid is a light yellowish and amorphous powder.It is used as a food additive andits safe dosage ranges from 10 to 400 µg, depending on the type of food to which it is added (Chen and Chung, 2000). It has a molar mass of 1701.19g and the chemical formula $C_{76}H_{52}O_{46}$.

Gallotannin, or common tannic acid, is the best known of the hydrolyzable tannins. It is produced by extraction with water or organic solvents from Turkish or Chinese nutgall. Tara, the pod from *Caesalpinia spinosa,* a plant indigenous to Peru, contains a gallotannin similar to that from galls and has become an important source for refined tannin and gallic acid. The European chestnut tree (principally *Castanea sativa*) and the American chestnut oak (*Quercus prinus*) yield hydrolyzable tannins important in leather manufacture. Tannic acids posses' metal-binding capacities and can form complexes with metals such as iron, copper and zinc. They can bind proteins, scavenge free radicals and inhibit microbial growth, possibly through tannin-polymer complexation, membrane disruption and/or chelation of metal ions. They enter the soil solution in the leachate from leaves, stems and soil organic matter (Kraus *et al*., 2003).

They are among the most abundant organic compounds in plants, but knowledge of their concentrations in soil is vague due to differences in physical, chemical, and biotic properties of the soils and thus is likely to affect the physiology of microorganisms inhabiting the soil. Tannic acid has been shown to possess antioxidant (Wu *et al*., 2004; Andrade *et al*., 2005), antimutagenic (Chen and Chung, 2000) and anticarcinogenic properties (Horikawa *et al*., 1994). The antioxidant mechanism of tannic acid is not yet fully understood. For example, in the presence of copper ions, tannic acid acts either as a prooxidant, promoting DNA damage (Khan *et al*., 2000), or as an antioxidant, suppressing hydroxyl radical formation (Andrade *et al*., 2005).Tannic acid is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues as seen in Figure 2.3 below.

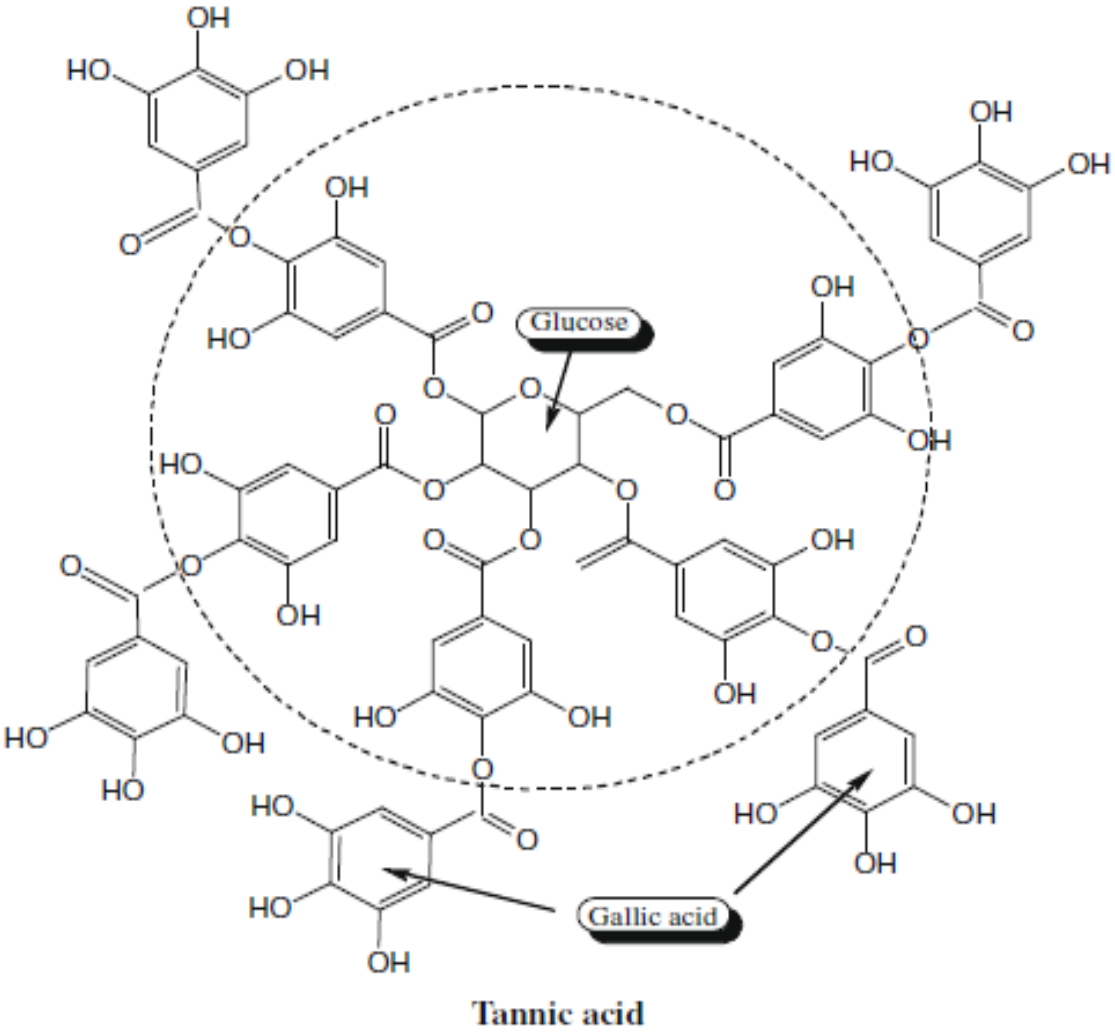

Figure 2.3 Chemical structure of tannic acid (source; Gulcin *et al*., 2010)

2.1.3.3.1 Applications of tannic acid

Tannic acid is used in many industrial applications. The best known is the tanning of leather (as agents for converting animal hides to leather). In addition to its principal applications in leather manufacture and dyeing, they are used in the clarification of wine and beer. It is also used as a constituent to reduce viscosity of drilling mud for oil wells, and in boiler water to prevent scale formation.

2.1.3.3.1a Universal antidote

Historically, Tannic acid was used along with activated charcoal and magnesium oxide in the "universal antidote," formerly used against poisoning. These three ingredients in combination were believed to work better at absorbing poisons than any of the ingredients alone. These days, people apply tannic acid directly to the affected area to treat cold sores and fever blisters, diaper rash, prickly heat, poison ivy, ingrown toenails, sore throat, sore tonsils, spongy or receding gums, and skin rashes, and to stop bleeding. Tannic acid is also taken by mouth and applied directly to bleeding, chronic diarrhea, dysentery, bloody urine, painful joints, persistent coughs, and cancer.

2.1.3.3.1b Foods and beverages

In foods and beverages, tannic acid is used as a flavoring agent and is far more widespread and significant amounts are used as process aids in beer clarification, aroma compound in soft drinks and juices. Equally important are its applications in the wine industry, where it finds use as a natural clarifying agent, color stabilizer and taste enhancer.

2.1.3.3.1c Textile and wood manufacturing

Tannins are a basic ingredient in the chemical staining of wood, and are already present in woods like oak, walnut, and mahogany. Tannic acid can be applied to woods low in tannin so chemical stains that require tannin content will react. The presence of tannins in the bark of redwood (*Sequoia)* is a strong natural defense against wildfire, decomposition and infestation by certain insects such as termites. They are also used for tanning hides and manufacturing ink.

2.1.3.4 Potassium ferricyanide

Potassium ferricyanide is the chemical compound with the formula $K_3Fe(CN)_6$. This bright red salt consists of the coordination compound $(Fe(CN)_6)^{3-}$ ion. It is soluble in water and acid but only slightly soluble in alcohol. Its chemical structure is composed of octahedrally coordinated ferricyanide ions. In solution, it shows some green yellow fluorescence.Potassium ferricyanide appears as bright red, lustrous crystals made by oxidizing potassium ferrocyanide

with chlorine. Sometimes, it appears in the form of small pellets. It is odorless and tasteless. Although this substance is stable, it can decompose when heated and it is sensitive to light. The compound itself has low toxicity but decomposes on strong heating to evolve highly toxic fumes (Mbadcam *et al*., 2010).It is also called red prussiate of potash. Unreacted cyanide salts like potassium ferricyanide are not combustible: however, contact with acids will liberate highly toxic, flammable hydrogen cyanide (HCN) gas.

The iron-cyanide complex ferricyanide, ($(Fe(CN)_6)^{3-}$) from anthropogenic sources can also be found in natural soil environments and water. The potential source of small amounts of this iron cyanide complex in soil and aquifers are industrial wastes from coal gasification, blast furnace sludge from pig iron production, paper de-inking sludge from paper recycling and road salt which contains berlin blue, as anticaking agent. It is potentially hazardous because it is converted to free cyanide ion, CN^- (aq) and hydrogen cyanide, HCN (g, aq.) when transported to surface water and exposed to sunlight. Free cyanides are extremely toxic to higher animals and humans. Cyanide inactivates cytochrome oxidase, an enzyme which is essential for the fixation of oxygen. This inactivation leads to cellular asphyxiation, suspension of all vital functions and subsequent cellular death (Mbadcam *et al*., 2010). The structure of potassium ferricyanide is shown in Figure 2.4.

2.1.3.4.1Applications of potassium ferricyanide

It is used in photography, as an oxidizing agent to remove silver from negatives and positives, a process called dot etching. In colour photograph, potassium ferricyanide is used to reduce the size of colour dots without reducing their number, as a kind of manual colour correction.

It is often used in physiology experiments as a means of increasing a solution's redox potential, as such it can oxidize reduced cytochrome C in intact isolated mitochondria. It is also used in amperometric biosensors as an electron transfer agent replacing an enzyme's natural electron transfer agent such as oxygen as with the enzyme glucose oxidase. It is used as this ingredient in many commercially available blood glucose meters for use by diabetics (Mbadcam *et al*., 2010).

Potassium ferricyanide is used as a mild oxidizing agent in organic synthesis. It is also used in the production of pigments, cyanotyping, laboratory reagents and electroplating.

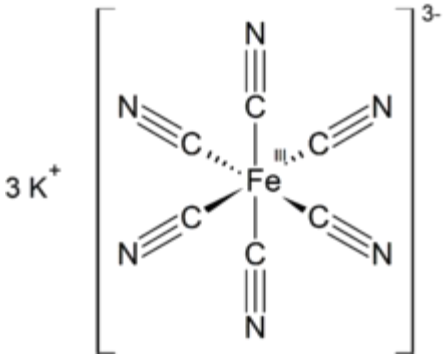

Fig 2.4 Structure of potassium ferricyanide (source; Mbadcam *et al*., 2010)

2.1.3.4.2 Health issues and toxicity

Inhalation of potassium ferricyanide may cause irritation to the respiratory tract with symptoms such as coughing and shortness of breath. Cyanide anions (CN^-) inhibit the body cells use of oxygen by causing metabolic asphyxiation. Prolonged anoxia (reduced level of oxygen in the blood) causes central nervous system (CNS) damage. Early symptoms of exposure to potassium ferricyanide are typical CNS effects like weakness, headache and confusion. Continued exposure causes a weak and irregular heartbeat, unconsciousness, convulsions, coma, and death eventually. As little as a few breaths of HCN vapor may stop respiration and cause collapse. Cyanides are fast acting and highly poisonous by ingestion. Large doses may cause gastrointestinal upset with nausea, vomiting, diarrhea, and possible cramping. Chronic effects include dermatitis and skin ulcers.

2.2. Toxicity of nickel to microorganisms

2.2.1 Chemical nature of nickel

Nickel (Ni) is the 24th most abundant element in the earth crust and has been found in different media in all parts of the biosphere (Cempel and Nikel, 2006; Bhadra *et al*., 2006). It is a metallic element belonging to group VIII b of the Periodic Table. It is hard, silvery-white, malleable, ductile, lustrous and found in nature as a component of silicate, sulphide and arsenide ores. Its atomic number is 28, atomic weight is 58.71, and melting and boiling points of about 1,455°C and 2,732°C respectively. It has high electrical and thermal conductivity and is resistant to corrosion at ambient environmental temperatures between -20°C and +30°C and is therefore often electroplated as a protective coating (Chau and Cordeiro, 1995).It has a specific density of 8.90 g/cm^3.

Although it assumes other valences (-1, 0, +1, +2, +3 and +4), it exists principally in the divalent state (Ni^{2+}) and it is the stable form in the environment. In biological systems, nickel in its ionic state forms stable complexes with various chelators and other organic materials. It dissolves slowly in dilute acids but, like iron, becomes passive when treated with nitric acid. Most nickel compounds are blue or green and finely divided nickel absorbs hydrogen.

2.2.2 Occurrence of nickel in the environment

At level of dynamics in soil, nickel is an abundant metal in the earth crust with about 3% of the composition of the earth. In agricultural soils, typical contents of this element vary from 3 to 1,000 mg/kg, however, the soils derived from basic igneous rocks can contain from 2,000 to 6,000 mg/kg of nickel (Iyaka, 2011; López and Magnitskiy, 2011).Levels in natural waters, range from 2 - 10 µg/l (fresh water) and from 0.2 - 0.7 µg/L (marine). Nickel combines readily with other metals such as iron (Fe), copper (Cu), chromium (Cr) and zinc (Zn) to form alloys.

Massoura *et al*. (2006) observed in a number of weathered European soils that goethite, serpentine and talc were the most frequently found nickel-bearing minerals. Nickel forms divalent salts with virtually every anion and has an extensive aqueous chemistry based on the

green coloured hexahydrate cation ($Ni(H_2O)_6^{2+}$). Nickel is produced from laterite ores by smelting with and without sulphur, or by chemical leaching with either ammonia or sulphuric acid. The metal is mined in Russia, Australia, New Caledonia, Cuba, Canada and South Africa.

2.2.3 Sources of nickel to the environment

Nickel is a naturally occurring element in the earth's crust and is present in igneous rocks (Chauhan *et al*., 2008). It is emitted into the environment from both natural and man-made sources. Natural sources of nickel include dusts from volcanic emissions and the weathering of rocks and soils (Kasprzak *et al*., 2003; Iyaka, 2011). The highest concentrations are found in basic igneous rocks with much lower levels found in sedimentary rocks including shales, clays, limestones and sandstones. Oats, chocolate, soy beans, nuts and other whole grains are also good sources of nickel (Salniko *et al*., 2003).

Although nickel occurs naturally, higher concentrations found in the environment are as a result of anthropogenic input such as depositions from the burning of fossil fuels, energy supplying power stations (coal burning power plants, petroleum combustion, nuclear power stations and high tension lines) (Verkleji, 1993).Others include chemical industries (pigment manufacturing, plating/metal finishing, cement manufacturing) and metallurgical industries (steel manufacturing, ore refining and alloy manufacturing). The lubricants which are antiwear protectants for vehicles emit nickel from inefficient engines during transportation (Sharma and Agarwal, 2005). Atmospheric nickel is considered to exist mainly in the form of aerosols. A part of atmospheric nickel entering into the environment originates from meteoric dusts, smoke particles from forest fires, volcanic ash, windblown soil dusts and aerosols from oceanic dusts. Fertilization of sewage sludge as well as composts from different wastes, and wide utilization of this metal in the aforementioned industries are causes of local contamination of the environment with nickel. Inorganic fertilizers particularly phosphate fertilizers also have variable levels of nickel depending on their resources (Sharma and Agarwal, 2005).Nickel is used as a catalyst in oil refining process, in cryogenic containers, in pollution abatement equipment and also as a component of some plumbing materials. When

pipes and other materials corrode, nickel can be released to drinking water and may cause damage to human health, but the release from this source is small.

2.2.4 Biological importance of nickel

Nickel is considered to be an essential element in animals, microorganisms, plants and a constituent of enzymes and proteins. Nickel is a functional constituent of seven enzymes, which are present in bacteria and animals, while one urease (urea amidohydrolase, EC 3.5.1.5), occurs in plants. Nickel is essential for the active synthesis of urease in plant cells. In several species of higher plants such as jack beans (*Canavalia* sp.), soybeans (*Glycine max*), rice (*Oryza sativa*), and tobacco (*Nicotiana tabacum*), it is required for effective urea metabolism. Nickel deficiency in legumes and other dicots causes a decrease in the activity of enzyme urease, which results to accumulation of toxic levels of urea and is manifested as necrosis at the tip of the leaves (Bai *et al*., 2006).

In soybean, low levels of nickel reduced nodulation (Zobiole *et al*., 2010) and seed yield, a phenomenon that is explained by the involvement of nickel in hydrogenase activity of bacteroids. Application of nickel to crops protects them from certain yield limiting diseases, thus potentially reducing pesticide usage and improving crop yield. It is a key factor affecting the production of secondary plant metabolites and thus influencing plant resistance to disease (Wood and Reilly, 2007). High concentrations of nickel have been reported to inhibit the formation of indole acetic acid (IAA), tryptophan and simultaneously promote the formulation ofphenolic and terpenoid inhibitors.

Some bacteria require nickel as a trace element for enzymes such as urease, CO-dehydrogenase and hydrogenase. In acetogenic bacteria the reduction of carbon monoxide to acetate is dependent on nickel which is needed for activation and synthesis of carbon monoxide dehydrogenase (Drake, 1982). Nickel stimulates the growth of *Acetobacterium woodii* on the medium supplemented with fructose. Nickel is the core metal in the tetrapyrrole ring of methanogenic bacteria which is essential for the growth of these microbes.

Khan and Moheman (2006) reported that nickel interacts with the iron found in haemoglobin, helps in oxygen transport, stimulates the metabolism and is regarded as a key metal in several plant and animal enzyme systems. Nickel is involved in the transmission of genetic code (DNA, RNA) and also present in certain enzyme systems that metabolize sugars. Nickel can substitute for calcium in excitation process and in binding with membrane ligands such as the phosphate groups of phospholipids in the process of nerve transmission, muscle excitation and contraction. Nickel has an essential function in the action or formation of cyclic guanosine monophosphate (cGMP), a signaling agent that regulates various physiological processes such as blood pressure control, sperm physiology, sodium metabolism and cardiovascular health. Nickel is consistently present in RNA and is bound to several biological substances such as proteins (keratin, insulin), amino acids and serum albumins. It also activates enzymes like arginase, trypsin, acetyl coenzyme A, carboxylase and synthetase (Yokoi *et al.,* 2002).

2.2.5 Applications of nickel

- The primary use of nickel is in the production of alloys to make items such as coins, jewellery, valves, heat exchangers, stainless steel, laboratory apparatus, medical instruments, and to cover metal objects. Nickel alloys impart corrosion resistance, heat resistance, hardness and strength. It is also used in nickel plating, manufacture of nickel containing products such as batteries and welding electrodes, and in the production of chemicals containing nickel such as nickel sulphate, nickel chloride, and catalysts (Nickel oxide).
- Nickel compounds are used to colour ceramics, and in the production of magnetic components.
- Nickel alloys and nickel plated items have been used extensively in transport, consumer, industrial and construction sectors including the production of motor vehicles, consumer goods and bathroom fittings, kitchen and tableware, electronics, food processing, textiles, fasteners, wires, and cables.
- Nickel is easy to work and can be drawn into wire. It resists corrosion even at high temperatures and for this reason it is used in gas turbines and rocket engines. Monel is an alloy of nickel and copper (70% nickel, 30% copper with traces of iron, manganese

and silicon), which is not only hard but can resist corrosion by sea water, so that it is ideal for propeller shaft in boats and desalination plants.

- Finely divided nickel is used as a catalyst for the hydrogenation of vegetable oils. Adding nickel to glass gives it a green color.

2.2.6 Toxicity of nickel

Nickel is a potent skin sensitiser and as many as 1 – 4 % of men and 8 – 20% of women in the general population may be nickel-sensitive (Environment Agency, 2009). Ingestion of nickel can cause skin reactions in previously sensitised individuals. The other main concern for oral exposure to nickel is its developmental toxicity potential, which has been observed in experimental animal studies (Environment Agency, 2009). Soluble nickel salts and the mixture of nickel sulphides and oxides present in refinery dust are carcinogenic to the lung and nasal tissues in humans (Environment Agency, 2009). Other toxic effects of nickel observed following inhalation exposure include chronic bronchitis, emphysema, reduced vital capacity and asthma. Both oral and dermal exposure to nickel can cause hypersensitivity reactions of the skin and both exposures should be assessed together. Inhalation exposure may be considered separately for its effects on the respiratory system but it potentially also contributes systemically to skin effects in sensitised individuals (Environment Agency, 2009).

Literature data (Šmejkalová *et al*., 2003, Vasundhara *et al*., 2004) indicate that metals are considered inhibitors of the microbiological and biochemical activity of soil. The impact of heavy metals on microorganisms and on enzymatic activity depends, among others, on soil pH, content of organic and mineral colloids, as well as on the type of heavy metals and their chemical properties has been reported (Kucharski *et al*., 2009). Nickel inhibits enzymatic activity, DNA replication, transcription and translation by binding to proteins and nucleic acids (Salnikow *et al*., 1997). Its influence on microbiological properties of the soil is less recognized than that of other metals. High nickel concentrations on sandy soils can clearly damage plants and high nickel concentrations in surface waters can diminish the growth rates of algae. Microorganisms can also suffer from growth decline due to the presence of nickel, but they usually develop resistance to nickel after a while.

2.2.7 Factors affecting nickel toxicity

Nickel toxicity has been reported to be dependent on pH, and the nature and binding capacity of humic substances present in the system (Sauve *et al*., 2000). The existence of other ions or organic matter which can lead to the formation of metal-complex can reduce nickel toxicity. In surface and sludge amended soils, nickel may be increasingly bound to organic matter, a part of which forms easily soluble chelates. In the presence of fulvic and humic acids, these complexes are much more mobile and may be more important than the hydrated divalent cation in soil solution chemistry. Soil pH is the most important factor controlling nickel solubility, sorption and mobility with the clay, iron manganese mineral, and soil organic matter content being of secondary importance (Ge *et al*., 2000). The solubility and mobility of nickel increases with decreasing pH (Tye *et al*., 2004). Many nickel compounds are soluble at a pH less than 6.5 (IPCS, 1991). In a study of soils from disused railyards, Ge *et al*. (2000) found that above pH 8, nickel carbonate was the major species in soil solution but fulvic acid complexes were significant across the pH range. Compared with other heavy metals such as cadmium and zinc, nickel is rather weakly sorbed to clay and iron minerals, and likely to be more mobile. Nickel adsorption by soils may decrease with complexation, for example in the presence of EDTA and increasing levels of soil organic matter.

2.3 Toxicity of phenols to microorganisms

2.3.1 Nature of phenols

Phenolic compounds make up one of the major families of secondary metabolites in plants, and include a diverse group of compounds. They are required by plants for pigmentation, growth, reproduction, resistance to pathogens and many other functions. They can be divided into non soluble compounds such as condensed tannins, lignins and cell wall bound hydroxycinammic acids, and soluble phenolics such as phenolic acids, phenylpropanoids, flavonoids and quinones. They also have a major contribution to the sensory characteristics of wines, namely colour, mouth feel, bitterness and astringency. Phenols sometimes called phenolics are a class of chemical compounds consisting of a hydroxylgroup (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol also called

carbolic acid (C_6H_5OH). The molecule consists of a phenyl ($-C_6H_5$), bonded to a hydroxyl (-OH) group. Phenol is a colorless-to -white crystalline solid. It contains a six-membered aromatic ring, bonded directly to a hydroxyl group (OH) having chemical formula C_6H_5OH (Prpich and Daugulis, 2005). The chemical structure is presented in Figure 2.5. Phenol is hygroscopic, slightly acidic by nature and has a distinctive odour that is sickeningly sweet and tarry. Its molecular weight is 94.11, density is 1.072 g/cm^{3}and the boiling point is 181.9°C. Its other names are carbolic acid, benzenol, phenylic acid, hydroxybenzene, (Annadurai *et al.*, 2007). They are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators. It is mildly acidic but requires careful handling due to its propensity to cause burns. It evaporates more slowly than water, and a moderate amount can form a solution with water.

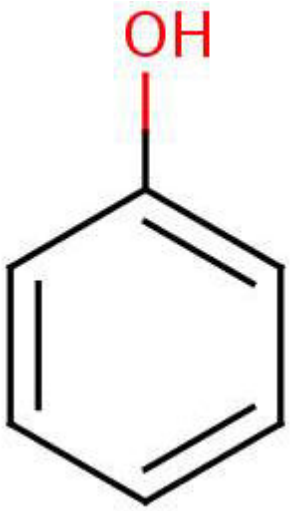

Fig 2.5 Structure of phenol (source; Mahiuddin *et al.,* 2012)

2.3.2 Sources of phenol to the environment

Phenol is both a natural substance and a manufactured chemical. It is produced on a large scale as a precursor to many materials and useful compounds. Natural sources of phenol include forest fire, natural run off from urban area where asphalt is used as the binding

material and natural decay of lignocellulosic material. It is also found in decaying dead organic matters like rotting vegetables and in coal. Phenol is present in the atmosphere as an emission from motor vehicles and as a photooxidation product of benzene. Phenolic compounds are characteristic pollutants in wastewater and effluents from chemicals, pharmaceuticals, textiles, and steel industries. Thus the main sources of phenol to the environment are from wastewaters from coal gasification plants, coke-oven batteries, oil refineries, petrochemical plants and other industries, such as synthetic chemicals, herbicides, pesticides, pulp-and-paper, and photo developing chemicals (Bandyopadhyay *et al.,*1998; Marrot *et al*., 2006).

2.3.3 Occurrence of phenols

Phenol is produced through both natural and anthropogenic processes. It occurs naturally in some foods, human and animal wastes, and in decomposing organic materials (Jame *et al*., 2010). Some phenols may be formed as a result of natural processes like the formation of phenol and p-cresol during decomposition of organic matter or synthesis of chlorinated phenols by fungi and plants (Prpich and Daugulis, 2005).The occurrence of phenols in the environment stems mainly from the production and use of numerous pesticides, in particular phenoxyherbicides like 2.4-dichlorophenoxyacetic acid (2.4-D).

2.3.4 Biological importance of phenol

Phenolic compounds are the most abundant secondary metabolites in plants and are classified into soluble compounds such as phenolic acids, phenylpropanoids quinones, flavonoids and non-soluble compounds such as lignins, condensed tannins, cell wall bound hydroxycinammic acids. These groups of phenols are all involved in many processes in plants and animals. The flavonoids for example play a role in flower and seed pigmentation, plant fertility and reproduction. They have also been shown to regulate polar auxin transport and are thus implicated in plant growth and development (Marrot *et al*., 2006).

2.3.5 Applications of phenol

Phenol has found use in the manufacture of many products such as phenolic resins, nylon, detergents and other synthetic fibers. It is also used in slimicides (chemicals that kill bacteria and fungi in slimes), as a disinfectant and antiseptic. Phenols are key for building polycarbonates, epoxies, and pharmaceuticals.

2.3.5.1 Medicine

Phenol has antiseptic properties and is used for aseptic (germ-free) techniques in surgery and sanitation purposes. It is also used in medicinal preparations such as mouthwash and sore throat lozenges.

2.3.5.2 Industries

It is used in the production of drugs (aspirin), herbicides, and synthetic resins etc. Aqueous phenolic effluents are relatively common industrial wastes, being produced in several industries and operations such as herbicides and synthetic resins industries, petroleum refineries, gas and coke oven industries, phenolic resins, explosive manufacture, plastic and varnish industries, textiles units using organic dyes, smelting and related metallurgical operations, etc (Mahadevaswamy *et al*., 1997; Bandyopadhyay *et al*., 1998; Jayachandran and Kunhi, 2008).

2.3.5.3 Laboratory

Phenol is used along with chloroform (a commonly-used mixture in molecular biology for DNA and RNA purification from proteins) and also used for cell disruption and lysis purposes.

2.3.5.4 Production of cosmetics

Phenol is also used in the preparation of cosmetics including sunscreens, hair dyes, and skin lightening products.

2.3.6 Toxicity and degradation of phenol

Phenols stick to soils and sediments at the bottom of lakes, rivers, or streams and can rapidly enter the body through the skin and gastrointestinal tract. Acute exposure of phenol causes

disorders of the central nervous system, hypothermia, myocardial depression, burning effect on skin, irritation of the eyes, and gastrointestinal disturbance as reported by some researchers (Chakraborty *et al*., 2010). It also causes hepatic damage (Bandyopadhyay *et al*., 1998; Annadurai *et al*., 2007; Arutchelvan *et al*., 2006).Phenol is toxic to bacteria and fungi, and thus used as a slimicide and disinfectant.

Phenol is a membrane-damaging microbiocide that can cause changes in the lipid-to-lipid and lipid-to-protein ratios in the membrane, membrane permeability and activity of membrane-associated proteins. The loss of cytoplasmic membrane integrity leads to disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function and subsequent cell death. Owing to this high toxicity, carcinogenicity and wide distribution in industrial wastes leading to great harm to human beings and microorganisms, they have become of great concern.

A wide variety of microorganisms have been reported as capable of degrading phenol mainly at low concentrations under aerobic and/or anaerobic conditions (Kujawski *et al*., 2004). Biodegradation process offers more opportunities to completely destroy the pollutants if possible or at least transform them to innocuous substances (Vidali, 2001). It is relatively of low cost, no chemicals are employed, and has high public acceptance. Biodegradation of phenol is often inhibited by the toxicity exerted at high concentrations (Oboirien *et al*., 2005; Okpokwasili and Nweke, 2006). This often leads to inhibition of microbial growth. Research on microbial degradation of phenols has intensified in recent years because it is a sustainable way to clean-up contaminated environments (Diaz, 2010). Microbes can adapt rapidly and grow using these compounds as carbon and energy sources, in soils and waste waters. Many microbes belonging to the genus of *Pseudomonas* have been reported as good degraders of phenol. The pure culture of *Pseudomonas* strains are often utilized for metabolic pathway studies evaluating the degradation of many aromatic compounds such as phenol (Dowling and O'Gara, 1994; Timmis *et al*., 1994).

2.3.7 Factors affecting phenol toxicity

Phenol toxicity is affected by many factors such as environmental conditions (pH, and temperature), phenol concentration, presence of other contaminants or compounds in the environment, types of micro organisms present (Jame *et al*., 2010; Abdel-Razek *et al*., 2015). From their studies, phenol degradation was inhibited at low pH (2.5) while at pH range of 3.5-8, phenol degradation was 100% after 72 h. The degradation rate was optimal at pH 7.0, where it reached 80% within 24 h. They also reported that at higher temperatures bacterial activity was inhibited hence inhibiting its biodegradation capabilities. It is believed that sudden exposure to temperatures higher than 35°C may have detrimental effect on the bacterial enzymes. On the other hand, exposure to lower temperatures is expected to slow down the bacterial activity.

The rate of phenol degradation decreased with increase in phenol concentration, where complete inhibition of phenol degradation occurred at 1000 mg/l. There were variations in the biodegradation rate of phenol due to the presence of metallic compounds (cobalt and copper). The degradation was generally delayed with high metal concentrations inhibiting degradation completely (Abdel-Razek *et al*., 2015).

In this study, growth assay and mathematical model were used to investigate the effect of the metal, nickel, on *Pseudomonas* sp. The effects of the chelating agents; trisodium citrate, calcium EDTA (CaEDTA), tannic acid and potassium ferricyanide were determined. In addition, the combined/joint effect of the chelators and nickel was equally assessed in nutrient rich and nutrient deficient media. *Pseudomonas* sp. was chosen due to its widespread behaviour in nature and reported ability to utilize phenol as a carbon source.

CHAPTER THREE

MATERIALS AND METHODS

3.1Sample Collection

Soil sample was collected with an auger at a depth of 30 cm, from a farm location at Ihiagwa, Owerri West Local Government Area of Imo state and transported inside a sterile polyethylene bag within one (1) hour of collection to the laboratory for analyses.

3.2Testchemicals and media used for the bioassay

All reagents and chemicals used in the study were of analytical grade and were used without any further purification. Chemicals (2,3,5-triphenyltetrazolium chloride), triton X, salts of the mineral medium, nutrient broth, nutrient agar, agar No.1,ammonium hydroxide(25%), potassium ferricyanide, 4-aminoantipyrine,phenol and amyl alcohol required for all biochemical assays were obtained from BDH England, Lab M, Fluka, Kemie Lab, Riedel-de haen ag seelze-Hannover, Burgoyne Burbidges & Co or Sigma-Aldrich Co. Ltd., UK.

The metal investigated in this work was obtained as a metallic salt of Fisons laboratory reagents, as nickel sulphate hexahydrate ($NiSO_4.6H_2O$). Chelating agents used were di-sodium ethylenediaminetetraacetic acid(Na_2-EDTA, 99+%, (BDH Ltd, England), potassium ferricyanide ($K_3Fe(CN)_6$), (BDH Ltd, England),tannic acid (Burgoyne burbidges &Co, India), and trisodium citrate dihydrate, ($C_6H_5Na_3O_7.2H_2O$), (BDH Ltd, England).

Mineral salts medium used for the bioassay consisted of the following salts: dipotassium hydrogen orthophosphate (K_2HPO_4) 2.10 g/l; potassium dihydrogen orthophosphate (KH_2PO_4) 0.4 g/l; ammonium nitrate (NH_4NO_3) 0.50g/l; magnesium sulphate ($MgSO_4.7H_2O$) 0.2 g/l; calcium chloride ($CaCl_2.2H_2O$) 0.023g/l; ferric chloride ($FeCl_3.6H_2O$) 002g/l; phenol solution-100mg/l (Alexander and Lustigman, 1966).

3.3Preparation and sterilization of materials

Glass wares used were cleaned by immersing them in detergent solutions for one hour, after which they were washed and rinsed in 25% sodium hypochloride solution, and further rinsed with distilled water twice and then air dried via drainage. It was sterilized by autoclaving at temperature of 121^{o}C and pressure of 15psi for 15min. Sterile plastic disposable tips were used throughout the study.

The various culture media used were prepared by dissolving required amounts of the commercially prepared powdered (hydrated) forms of the media and salts in specified volumes of distilled water and sterilized by autoclaving at temperature of 121^{o}C, and pressure of 15psi for 15 min in well stoppered conical flasks.

3.4 Soil enrichment with phenol

One kilogram (1kg) of the soil sample weighed using Mettler PT320 digital weighing balance was transferred into a 250ml conical flask.Phenol stock solution 10,000 mg/l was prepared by dissolving 10 g of phenol in one litre of water. Then 1ml of the phenol stock solution was transferred into a sterile 100 ml Erlenmeyer flask and made up to 100 ml mark to give a final phenol concentration of 100 mg/l. This was introduced into the 250 ml Erlenmeyer flask containing the soil sample, mixed thoroughly and incubated for a period of three weeks. This was to enable the microorganisms present in the soil sample adapt and synthesize phenol degrading enzymes.

3.5 Isolation of bacteria

One gram (1.0 g) of the enriched soil sample was transferred into a 50 ml Erlenmeyer flask containing 20 ml of sterile water. The soil suspension was stirred and allowed to settle for 30 min after which 10-fold serial dilutions were made up to dilution 10^{-6}. Then 0.1ml aliquots of dilutions10^{-4}-10^{-6} were spread on the mineral salts - phenol agar medium and then incubated at 37^{o}Cfor 72h. The bacterial colonies that developed on the medium were purified by sub culturing further on nutrient agar after which the pure colonies were aseptically transferred into nutrient agar slants and stored at 4^{o}C. This served as the stock culture for bacterial identification and toxicity tests (Media components and preparation are shown in Appendix I).

3.6 Identificationof bacterial isolates

Characterizations were done using standard microbiological methods as described by Cappucino and Sherman (2010); Brown (2007); Cheesbrough (2006); Fawole and Oso, (2004). Colonial properties considered were; colour, elevation, shape, etc. These were followed by biochemical and carbohydrate fermentation tests on bacterial isolates, such as; Gram staining, motility test, catalase, citrate, coagulase, glucose, hydrogen sulphide (H_2S) production/utilization, indole, lactose, mannose, methyl red, nitrate (NO_3) reduction, oxidase, sucrose, Voges Proskauer, and xylose tests.

3.6.1 Citrate Utilization test

The method as described by Cheesbrough (2006) was adopted. This test shows the ability of bacterial species to utilize citrate as a sole source of carbon and ammonia as the only source of nitrogen in which CO_2 is produced as the end product.

Slants of Simmon's citrate agar were prepared in test tubes. With the use of a sterile platinum wire, the slopes of the media were streaked and then the butts were stabbed. The inoculated media were then incubated at 37^{o}C for 48 h. A positive citrate utilization test was given by the development of a deep royal or Prussian blue colour in the slants after 48 h of incubation. A negative citrate utilization test retains the green colour of the medium in the slant.

3.6.2 Indole test

This test distinguishes between bacteria that have the ability to split the amino acid tryptophan into indole and pyruvic acid through the action of tryptophanase, the hydrolytic enzyme and those that do not. The method as described by Cheesbrough (2006) was adopted.

The test was carried out by dispensing 5 ml amount of peptone water into test tubes and sterilized by autoclaving for 15min at 121^{o}C.

The test colonies were inoculated by picking with sterile inoculating needle and stabbing into the broth. Thereafter, the broth was incubated for 48 h at 37^{o}C after which 0.5 ml Kovac's reagent was added to each inoculated tube and the tube shaken gently. The appearance of a red surface layer indicated a positive result while a negative result was shown by no colour formation.

3.7.3. Catalase test

This test is used to differentiate catalase producing bacteria from non-catalase producing bacteria. The enzyme catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. The method as described by Cheesbrough (2006) was adopted in the study.
This test was carried out by picking a little amount of the test organism from the stock agar slant with a sterile wire loop. This was immersed in a 3% aqueous solution of hydrogen peroxide (2-3 ml) contained in a test tube.
A Catalase positive result is evidenced by bubbling occurring immediately in the test tube as oxygen gas is released while a negative result is indicated by no activity in the test tube.

3.7.4. Methyl Red (MR) test

The method as described by Prescott *et al*., (2005) was adopted. This test is based on the ability of some bacteria to ferment glucose with the production of enough acid to lower the pH of the medium. The low pH is detected from the colour change from red to yellow.
Sterile glucose phosphate peptone broth (5 ml) contained in 20 ml test tubes were inoculated with the bacterial isolate. The test tubes were incubated for 48 h at 37^{o}C. Two to three drops (2-3) of methyl red indicator (0.04%) were added into the test tube, mixed and observed for colour change. A bright red colouration indicated a positive reaction while a yellow colouration indicates a negative reaction.

3.7.5 Voges-proskauer test

The method as described by Cheesbrough (2006) was used. This test determines the ability of some organisms to produce acetylmethylcarbinol from organic acids that results from glucose metabolism. The acetylmethylcarbinol is oxidized to diacetyl in the process, and then reacts with a guanine group (α-naphtol) under alkaline conditions to give a pink coloration (Prescott *et al*., 2005).
Five (5) ml of sterile glucose phosphate peptone broth contained in test tubes were inoculated with the bacterial isolates and incubated for 48 h at 37^{o}C. Then, 0.6ml of 5% alcoholic α-naphtol and 0.2ml of 40% potassium hydroxide were added, the mixture was shaken and observed for colour change.

The production of a pink color within 2-5 min shows a positive Voges-Proskauer test reaction while a negative reaction is indicated by no colour change. The composition and preparation of the glucose phosphate peptone broth medium are shown in Appendix I.

3.7.6 Oxidase test

The method as described by Cheesbrough (2006) was adopted. This test assists in the identification of bacterial species, which produce the enzyme cytochrome oxidase (Cheesbrough, 2006).

This test was carried out by soaking a piece of filter paper with 2 or 3 drops of freshly prepared oxidase reagent (Para-amino dimethyl phenylenediamine) in a clean Petri dish. A piece of sterile bent glass rod was used to remove a colony of the test organism and smeared on the filter paper. Where the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour within a few seconds.

3.7.7 Coagulase test

The method as described by Cheesbrough (2006) was adopted. This test is unique to organisms which produce the enzyme coagulase. The enzyme coagulase causes plasma to clot by converting fibrinogen to fibrin. Bound coagulase (clumping factor) which converts fibrinogen to fibrin without requiring a coagulase reacting factor, can be detected by the clumping of bacterial cells in a rapid slide test.

A loopful of the desired colony was placed on 2-3 drops of physiological saline on a clean grease-free glass slide to make a smear of the organism. A few (1-2) drops of human plasma was added to the suspension, stirred for 5 sec and observed for reaction. Clumping within 10 sec indicated a positive coagulase test, while no clumping within 10 sec indicated a negative coagulase test.

3.7.8 Urease test

This test was carried out according to Cheesbrough (2006). The test is important in differentiating enterobacteria from non enterobacteria, and is contingent on the ability of the

bacterium to produce the enzyme urease which breaks down urea to ammonia and carbon dioxide.

The test organism was cultured in a medium which contained urea, with phenol red indicator. When the strain is urease-producing, the enzyme breaks down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in the colour of the indicator to pink-red (Cheesbrough, 2006).

The test organism was heavily inoculated using a sterile wire loop into a bijou bottle containing 3ml sterile Christensen's modified urea broth. (Media components and preparation are shown in Appendix I). This was incubated at 35^{o}C for 24 h in a water bath. A pink colour in the media indicated a positive urease test while no pink colour shows a negative urease test.

3.7.9 Nitrate reduction

Many bacteria have the ability to reduce nitrate to nitrite with the aid of the enzyme nitrate reductase or reduce nitrate to nitrogenous gases. A positive reaction is indicated by the development of a red colouration while no colour change indicates a negative result. This test uses the presence or absence of nitrite (after incubation) as an indicator of nitrate reduction. The results are complicated by the possibility that nitrite might be further reduced to other compounds.

Tubes of nitrate broth were inoculated with the bacterial isolate and incubated for 2-6 days at 35^{o}C, alongside an uninnoculated tube as the control. After 48 h, 1ml each of sulfanilic acid and dimethyl-naphthylamine were added to the test tubes including the control. The development of a red colour indicated the reduction of the nitrate to nitrite. No color change indicates the absence of nitrite. This can happen either because nitrate was not reduced or because nitrate was reduced to nitrite, then nitrite was further reduced to some other molecule. The composition and method of preparation of the media are shown in appendix 1.

3.7.10 Hydrogen sulphide production

This test is used in detecting hydrogen sulfide production by microorganisms based on their ability to reduce sulfur-containing compounds such as amino acids to sulfides. The method

described by Johnson and Case (2007) was adopted. The test can be carried out using lead acetate paper/agar, Kligler iron agar, triple sugar iron agar, or peptone iron agar.

The bacterial isolate was inoculated into a tube of peptone iron agar by the stab method and incubated at 35 ± 2°C for 18-48 h. The tubes were then observed for growth and hydrogen sulphide production. The presence of any blackening of the medium along the line of stab or throughout the butt indicates hydrogen sulphide production.

3.7.11 Gram staining

This is a differential staining technique that distinctly separates bacteria into two major groups (Gram positive and Gram negative) based on their chromogenic responses to the dyes used in the staining procedure. The responses are as a result of the bacterial cell wall's ability to absorb strongly or weakly these dyes due to the composition of the cell wall (Fawole and Oso, 2004).

It was carried out by making a smear of the organism on clean grease free glass slide, air-dried and then heat fixed. This smear was covered with drops of crystal violet for 30 sec, quickly rinsed off with water, washed with Gram's iodine and allowed for 60 sec before rinsing off gently under the tap. The slide was washed with 95% ethanol to decolourize. The smear was then counter stained with Safranin for 30 sec, then rinsed off with water, air-dried and subsequently examined under the microscope using oil immersion objective (×100). Gram-positive bacteria stained purple to violet while Gram-negative stained pink to red.

3.7.12 Spore staining

The method as described by Fawole and Oso (2004) was adopted. Certain bacteria particularly members of the genera *Bacillus* and *Clostridium* produce endospores. The endospores of some bacteria cannot be stained by simple staining and Gram staining because the dyes do not penetrate the wall of the endospores (Cappuccino and Sherman, 2010).

Smears of the test bacteria were prepared on a clean grease-free glass slide and heat fixed properly by passing the slide several times over a Bunsen burner flame. The slides were flooded with Malachite green stain and heated over a beaker of boiling water for 10 min. The slides were then rinsed under running tap water. Safranin stain was flooded over the slides and

allowed for 20 sec. The Safranin stain was also washed off using water after which the slides were examined under the microscope using the oil immersion objective (×100).The positive spore forming bacteria stained green while the vegetative cell bacteria stained pink to red.

3.7.13 Motility test

The method as described by Cappucino and Sherman (2010) was adopted. This technique was used to differentiate between motile and non-motile organisms. The stab culture technique was used in this study in which sterile test tubes containing semi-solid motility medium were inoculated by plunging a sterile straight platinum wire charged with the inocula from the culture isolates vertically into the centre of the medium. Stabbed cultures were examined after 24 h incubation for motility test.

Positive motility test was shown by the occurrence of turbidity in the inoculated tubes which indicated freely motile organism. The appearance of brush-like projections from the stabbed line indicated weakly motile organisms which maintained their growth strictly along the line of stab.

3.7.14 Carbohydrate fermentation test

This test is performed to check the ability of bacteria to ferment sugars. Aliquots of the basal medium (Peptone-sugar water) were dispensed into test-tubes and filled by gently inverting Durham's tubes into the medium. The test cultures (24 h old) were then inoculated into the sterilized medium and incubated for 48 h at 37^0C. A colour change from red to yellow (with phenol red indicator) and air space in the Durham's tubes was considered positive, indicating acid production alone or both acid and gas production respectively (Fawole and Oso, 2004).

A stock solution contained in 500ml Erlenmeyer flask of peptone water and phenol red indicator was properly mixed and dispensed into different test tubes each containing a Durham tube respectively. Into 100ml peptone water, 1g of the test sugar was added (Glucose, Sucrose, Arabinose, Lactose, Mannose and Xylose) and corked properly. Sterilization of the media was by steaming for 30 min and allowed to cool. Using a sterile wire loop,a pure colony of the test organism (24 h old) was inoculated into the sterile medium and incubated at 35^oC for 48 h.

A colour change from red to yellow and air space in the Durham's tubes showed a positive reaction, indicating both acid and gas production respectively. The medium remained red in colour for negative sugar fermentation test.

3.8 Growth inhibition test of *Pseudomonas* species by chelating agents and nickel.

The growth inhibition test of *Pseudomonas* was done using the dehydrogenase assay method as described by Nweke *et al.,*(2007). The dehydrogenase activity (DHA) was determined using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as the artificial electron acceptor, which was reduced to the red colored triphenyl formazan (TPF).

3.8.1 Preparation and standardization of bacterial inocula for the toxicity assays

Microbial cells used for the assay were grown in liquid medium for 24 h in nutrient broth and four days (96 h) in mineral salts medium. A loopful of cells from the stock culture was inoculated into 25 ml sterile nutrient broth and 25 ml sterile mineral salt medium (supplemented with phenol) contained in 100ml Erlenmeyer flasks. The flasks were incubated at 28±2^{o}C with intermittent shaking on a rotary incubator at 150 rpm. Cells were harvested by centrifugation (4000rpm for 10 min). The harvested cells were washed twice in 10ml sterile distilled water in same screw-capped test tubes by centrifugation at 4000rpm for 10min and re-suspended in 10ml sterile distilled water, swirled vigorously to form cell suspension. Three (3) ml from the cell suspension was transferred into a clean glass cuvet to determine cell optical density using a spectrophotometer. The cell suspension was standardized to 0.1 OD and 0.2 OD at 540 nm and used as standard inocula in the dehydrogenase activity assay and phenol degradation assay respectively.

3.8.2. Media

Two types of media were used; nutrient brothand mineral salts medium supplemented with phenol as carbon source. The composition and preparation of the mineral salts medium and nutrient brothused are shown in Appendix I.

3.8.3. Preparation of metal and chelating agents

The chelating agents used in the experiments were prepared by dissolving the required mass of anhydrous metal salt and chelating agents (weighed using the digital weighing balance Mettler PT320) into sterile distilled water contained in 100ml volumetric flasks. Thereafter the solutions were made up to the 100ml mark with distilled water. Ten (10) mM of nickel was prepared by dissolving 0.263g of $NiSO_4.6H_2O$ salt in 10 ml distilled water and made up to 100 ml. Similarly,(8000) mg/l each of EDTA, TSC, $K_3Fe(CN)_6$ and TA were prepared by dissolving 0.8g each of the respective chelating agents in 50 ml of distilled water contained in 100ml volumetric flasks and the volume made up to 100ml. Stock solutions were at concentrations at which no precipitation was observed, and the final chelating agent and metal concentrations used in experiments was obtained by the dilution of the corresponding stock solutions.

3.8.4 Effect of chelating agents on *Pseudomonas* sp

The effect of the individual chelating agents (0 – 5000 mg/l) on the bacterium was tested in two different media, nutrient broth and mineral salts medium supplemented with 100 mg/l phenol. The chelating agents investigated include Ethylenediaminetetraacetic acid, tannic acid, trisodium citrate and potassium ferricyanide (which would be subsequently represented as EDTA, TSC, TA, and $K_3Fe(CN)_6$, respectively). The bacterial inocula and chelating agents used were prepared as described in sections 3.8.1 and 3.8.3 respectively.

The assay was done in 2 ml final volumes consisting of nutrient broth medium and/or mineral salts medium. Individual chelating agents were assayed singly in screw capped test tubes and inoculated with about 0.1 ml of the standardized bacterial suspension. The reactions were done in duplicate culture tubes as shown in Appendix III. The control consisted of the isolates and the media without any chelating agent. The set ups were incubated statically at room temperature (28 ± 2^0C) for 24 h and 96 h for the reaction mixtures containing nutrient broth and msm respectively. Thereafter, 0.1 ml of 0.2% (w/v) INT in deionized distilled water was added to each of the culture tubes and shaken properly. The reaction mixtures were further

incubated statically in the dark at room temperature (28 ± 2ºC) for another 24 h. The cell membrane were solubilized by adding 1% (v/v) Triton X-100 to the culture and allowed to stay for 10 min.

The INT-formazan produced was extracted by adding 4ml of amyl alcohol to each reaction tube and determined spectrophotometrically at 500nm (λmax). Dehydrogenase activity was expressed as absorbance of INTF formed per 0.1ml of suspension of cell in tube per 24 h. The protocols used for the chelating agent assay are attached in Appendix III.

3.8.5 Effect of nickel on *Pseudomonas* sp

The effect of nickel (0.05 - 2 mM) on the bacterium was evaluated in the two medium, nutrient broth and mineral salt medium. The bacterial inocula and chelating agents used were prepared as described in sections 3.8.1 and 3.8.3 respectively.

The assay was also done in 2 ml final volumes consisting of nutrient broth medium and/or mineral salt medium. Nickel at different concentrations was assayed in screw capped test tubes and inoculated with about 0.1ml of the standardized bacterial suspension. The reactions were done in duplicate culture tubes as shown in Appendix III. The control consisted of the isolates and media without nickel. The set ups were incubated statically at room temperature (28 ± 2^0C) for 24 h and 96 h for the reaction mixtures containing nutrient broth and msm respectively. Thereafter, 0.1 ml of 0.2% (w/v) INT in deionized distilled water was added to each of the culture tubes and shaken properly. The reaction mixtures were further incubated statically in the dark at room temperature (28 ± 2ºC) for another 24 h. The cell membrane was solubilized by adding 1% (v/v) Triton X-100 (TX) to the culture and allowing it to stay for 10 min.

The INT-formazan produced was extracted by adding 4ml of amyl alcohol to each reaction tube and determined spectrophotometrically at 500nm (λmax). Dehydrogenase activity was expressed as absorbance of INTF formed per 0.1ml of suspension of cell in tube per 24 h. The protocols used for nickel assay are attached in Appendix III.

3.8.6 Effect of chelating agents and nickel to *Pseudomonas* sp

The joint effect of chelating agents and nickel on the bacterium was equally investigated. Four different concentrations of each chelating agent were selected. They include EDTA,(400, 1000, 2000, and 4000 mg/l), TSC (100, 1000, 2000 and 4000 mg/l), TA (5, 10, 20 and 40 mg/l)($K_3Fe(CN)_6$ 20, 40, 80 and 100 mg/l). Nickel (0.05 - 2 mM) was assayed jointly with the different chelating agent concentrations. The bacterial inocula used were prepared and standardized as previously described in section 3.8.1. Nickel and chelating agent solutions were prepared as previously described in section 3.8.3. The assay was carried out as described in section 3.8.4 with little modification by mixture of individual chelating agent and nickel in each of the different media. The protocols used for nickel and chelating agent assays are shown in Appendix III.

3.9 Evaluation of phenol degradation by the isolate

3.9.1 Construction of calibration curve for phenol determination

Equal volumes of phenol standard solution were transferred into series of 10 ml screw capped tubes and then diluted to volume (4 ml) with distilled water so that the final concentration was in the range of 1-20 mg/ml. Then 0.2 ml of 2N ammonium hydroxide solution, 0.1 ml of 2% 4-aminoantipyrine solution, and 0.1 ml of 8% potassium ferricyanide solutions were added to each tube. The absorbance values at 500 nm were measured against a reagent blank and then plotted against phenol concentration to obtain the calibration curve.

3.9.2 Standardization of innoculum

The microbial cells used for the assay were standardized as described earlier in section 3.8.1.

3.9.3 Media

The media used were as described in Appendix I with some modifications. The mineral salts medium used for this assay was supplemented with 200 mg/l and 500 mg/l phenol.

3.9.4 Assay

The assay was carried out by preparing 100 ml volumes of mineral salt medium supplemented with 200 and 500 mg/l phenol in duplicate 250 ml Erlenmeyer flasks. This was done by measuring out 2 and 5 ml of 10, 000 mg/l phenol stock solutions, which was added to 97 and 94 ml of the mineral salt medium respectively. Thereafter, 1 ml of cell suspension was added. The resultant culture contained phenol concentration of 200 and500 mg/l. The cultures were incubated at room temperature (28 ± 2^{o}C) on a rotary shaker at 120 rpm. Samples were withdrawn periodically for biomass and phenol analysis. The optical densities at (540 nm) of the cultures were determined spectrophotometrically. Residual phenol was determined quantitatively using 4-aminoantipyrene colorimetric method as modified from Folsom *et al.* (1990). The withdrawn culture was centrifuged at 4000 rpm for 15 min to remove the cells. Into 4ml of the sample supernatant (or smaller aliquot of the supernatant diluted to 4 ml) contained in 15-ml screw-capped culture tubes were added 0.2 ml of 2N NH_4OH and 0.1 ml of 2 % 4-aminoantipyrene. The tubes were shaken to mix the contents. Thereafter, 0.1 ml of 8 % $K_3Fe(CN)_6$ was added and the contents were mixed. The absorbance (A_{500}) of the resultant red-coloured solution was determined. Phenol concentrations were calculated by making reference to the standard curve.

3.9.5 Effect of nickel on phenol degradation by the isolate

The effect of nickel (0.05, 0.2, 0.5 and 1 mM) on phenol (500 mg/l) degradation was evaluated in the presence as well as absence of trisodium citrate.

The assay was carried out by preparing 100 ml volumes of mineral salts medium supplemented with 500 mg/l phenol in five 250 ml Erlenmeyer flasks. The four different concentrations of nickel were also accommodated in the different flasks and well labeled. The one without nickel was used as the control.

This was done by measuring out 5 ml of 10, 000 mg/l phenol stock solutions and requisite volumes of nickel solution into a flask containing 50 ml of the mineral salts medium and made up to the 100 ml mark. Thereafter, 1 ml of cell suspension was added. The resultant culture contained phenol concentration of 500 mg/l. The cultures were incubated at room temperature (28 ± 2^{o}C) on a rotary shaker at 120 rpm. Samples were withdrawn periodically

for biomass and phenol analysis as described in section 3.9.4 above. The set up was repeated incorporating 100 mg/l TSC in all the flasks.

3.9.6 Estimation of phenol degradation parameters

The phenol degradation rates (Qs) were calculated as described by Nweke *et al.*(2014) from plots of $S_o - S$ (amount of phenol degraded) versus time of incubation ($t - t_o$). Similarly, the specific growth rates (μ) were taken from $\ln(X/X_o)$ versus $t\text{-}t_0$plots for each initial phenol concentration. In each case, values were taken as the maximum slope in the respective plots. The yield coefficients (Y) were determined from the mass balance by plotting $X\text{-}X_o$ versus $S_o\text{-}S$.

$$Q_s = \frac{S_o - S}{t - t_o}$$

$$\mu = \frac{\ln(X/X_o)}{t - t_0}$$

where Qs = phenol degradation rate, μ = specific growth rate, S_o= initial phenol concentration at time t_o(mg/l), S = phenol concentration at time t (mg/l), X = biomass concentration (A_{500}) at time t; X_o= biomass concentration (A_{500}) at time t_o.

3.10 Data Analysis

3.10.1 Determination of the inhibitory concentrations (IC_{50})

The dehydrogenase activities at varying concentrations of the chelating agents and nickel were recorded as the absorbance of the formazan extract at 500 nm. The dose response data were fitted into 3-parameter logistic model (equation 1).

$$E[Y] = \frac{a}{1 + \left(\frac{x}{x_o}\right)^b} \qquad (1)$$

where E[Y] is the absorbance of formazan extract at 500 nm, x is the concentration of the chemical, a is the maximum response (of untreated control), b is parameter determining the slope, x_o is IC_{50}.

3.10.2 Progress curve analysis of biodegradation and biomass production

The microbial growth can be represented by a Haldane equation:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_i}} \qquad (2)$$

where μ is the specific growth rate (h^{-1}), S is the substrate concentration (mg/l), μ_m is the maximum specific growth rate (h^{-1}), K_s is the half saturation coefficient (mg/l), K_i is the inhibition coefficient (mg/l).

Biomass production is given by the expressions:

$$\frac{dX}{dt} = \mu X \qquad (3)$$

Substituting for μ (in equation 2) into equation 3:

$$\frac{dX}{dt} = \frac{\mu_m S X}{K_s + S + \frac{S^2}{K_i}} \qquad (4)$$

According to mass balance:

$$S = S_o - \frac{(X - X_o)}{Y} \qquad (5)$$

where:

X = biomass concentration,

Y = Yield coefficient (x/s),

S = substrate concentration,

t = time,

X_o= initial biomass concentration,

S_o = Initial substrate concentration.

Substituting for S into equation 4 yields equation 6

$$\frac{dX}{dt} = \frac{\mu_m \left(S_o - \frac{(X - X_o)}{Y} \right) X}{K_s + \left(S_o - \frac{(X - X_o)}{Y} \right) + \frac{\left(S_o - \frac{(X - X_o)}{Y} \right)^2}{K_i}} \quad (6)$$

Integrating equation 6 yields equation 7

$$\mu_m t = \left[\frac{K_s Y}{YS_o + X_o} + 1 + \frac{YS_o + X_o}{K_i Y} \right] \ln \frac{X}{X_o}$$

$$- \frac{K_s Y}{YS_o + X_o} \ln \frac{YS_o + X_o - X}{YS_o} \qquad (7)$$

The rate of biodegradation can be represented as:

$$\frac{dS}{dt} = -\mu \frac{X}{Y} \qquad (8)$$

Substituting for μ (in equation 2) into equation 8:

$$\frac{dS}{dt} = -\frac{\mu_m SX}{\left(K_s + S + \frac{S^2}{K_i}\right)Y} \qquad (9)$$

According to mass balance:

$$X = Y(S_o - S) + X_o \qquad (10)$$

Substituting for X into equation 9 yields equation 11

$$\frac{dS}{dt} = -\frac{\mu_m S\left[Y(S_o - S) + X_o\right]}{\left(K_s + S + \frac{S^2}{K_i}\right)Y} \qquad (11)$$

Integrating equation 11 yields equation 12

$$\mu_m t = \left[\frac{K_s Y}{YS_o + X_o} + 1 + \frac{YS_o + X_o}{K_i Y}\right] \ln \frac{YS_o + X_o - YS}{X_o}$$

$$- \frac{K_s Y}{YS_o + X_o} \ln \frac{S}{S_o} \qquad (12)$$

The biomass production phenol biodegradation data were fitted into integrated Haldane models (equation 7 and equation 12 respectively) and the kinetic parameters were estimated by iterative process implemented in Table Curve 2D v5.01.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Identification of Isolate

The morphological and biochemical characteristics of the isolate used for the study are shown in Table 4.1.The organism was a Gram negative, oxidase positive, motile bacterium capable of fermenting glucose. Based on the characteristics, the most probable identity of the organism was *Pseudomonas* species. It was grown in nutrient broth for 24 h and in a mineral salts medium incorporated with phenol as a sole carbon source, for 96 h, harvested, washed and stored on slants. All subsequent experiments in this study were performed using this organism.

Table 4.1: Morphological and Biochemical Characteristics of the bacterial isolate

TESTS	RESULTS
Cell Morphology and Microscopic Characteristics	
Cell Morphology: Circular, smooth, wet, convex	
Motility	+
Gram Stain	–
Spore	–
Flagellum	+
Capsule	–
Biochemical and Carbohydrate Fermentation Characteristics	
Catalase	+
Oxidase	+
Coagulase	–
Indole Production	–
MethylRed	+
Voges Prauskeur	–
Citrate Utilization	+
Hydrogen sulphide production	–
Nitrate Reduction	+
Urease	+
Triple sugar	Nd
Glucose	+
Sucrose	–
Maltose	–
Lactose	–
Mannose	+
Xylose	+
Probable isolate	*Pseudomonas* sp

(+ = Positive test; - = Negative test; Nd = Not determined)

4.1.2 Effect of chelating agents on the dehydrogenase activity of *Pseudomonas* species

The results of the effect of different concentrations of the chelating agents on dehydrogenase activity of *Pseudomonas* sp. in the two (nutrient broth and mineral salts medium) media used in this study are shown in Figures 4.1 and 4.2 respectively. The figures depict the stimulatory and/or inhibitory effects of the individual chelating agents on the dehydrogenase activity of the organism at various concentrations (0 – 5000 mg/l).

In both media, EDTA and trisodium citrate (TSC) showed stimulatory effects on the dehydrogenase activity of the organism while tannic acid (TA) and potassium ferricyanide ($K_3Fe(CN)^6$) showed inhibitory effects. The stimulatory effect of EDTA and TSC on the dehydrogenase activity of the bacterium increased with increasing concentrations of both chelating agents. At 5000mg/l EDTA and TSC, dehydrogenase activity (measured as absorbance at 500 nm) of 1.17 and 1.02 respectively was still detectable in nutrient broth. A similar pattern was observed in mineral salt medium, dehydrogenase activity of 0.059 and 0.66 were detected at 5000 mg/l EDTA and TSC respectively.

The inhibitory effects of tannic acid and potassium ferricyanide increased with increase in concentration of the chelating agents in both media. In nutrient broth, tannic acid completely inhibited dehydrogenase activity of the bacterium at 5000 mg/l while $K_3Fe(CN)_6$ retained residual activity. However in mineral salts medium, lower concentrations of tannic acid and $K_3Fe(CN)_6$showed stimulatory effects on the dehydrogenase activity of the bacterium. Dehydrogenase activities were highest at 52 mg/l and 28 mg/l of $K_3Fe(CN)_6$ and TA respectively. This assay also served as a range finding experiment for the chelating agents to determine maximum concentrations to be used in the metal- chelator assays.

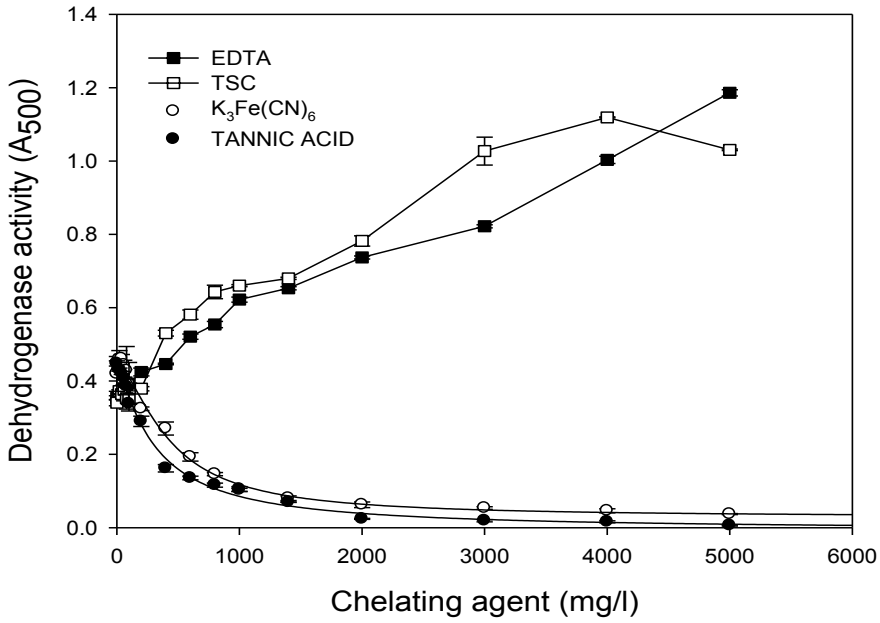

Fig. 4.1: Effects of metal chelators on dehydrogenase activity of *Pseudomonas* species in nutrient broth

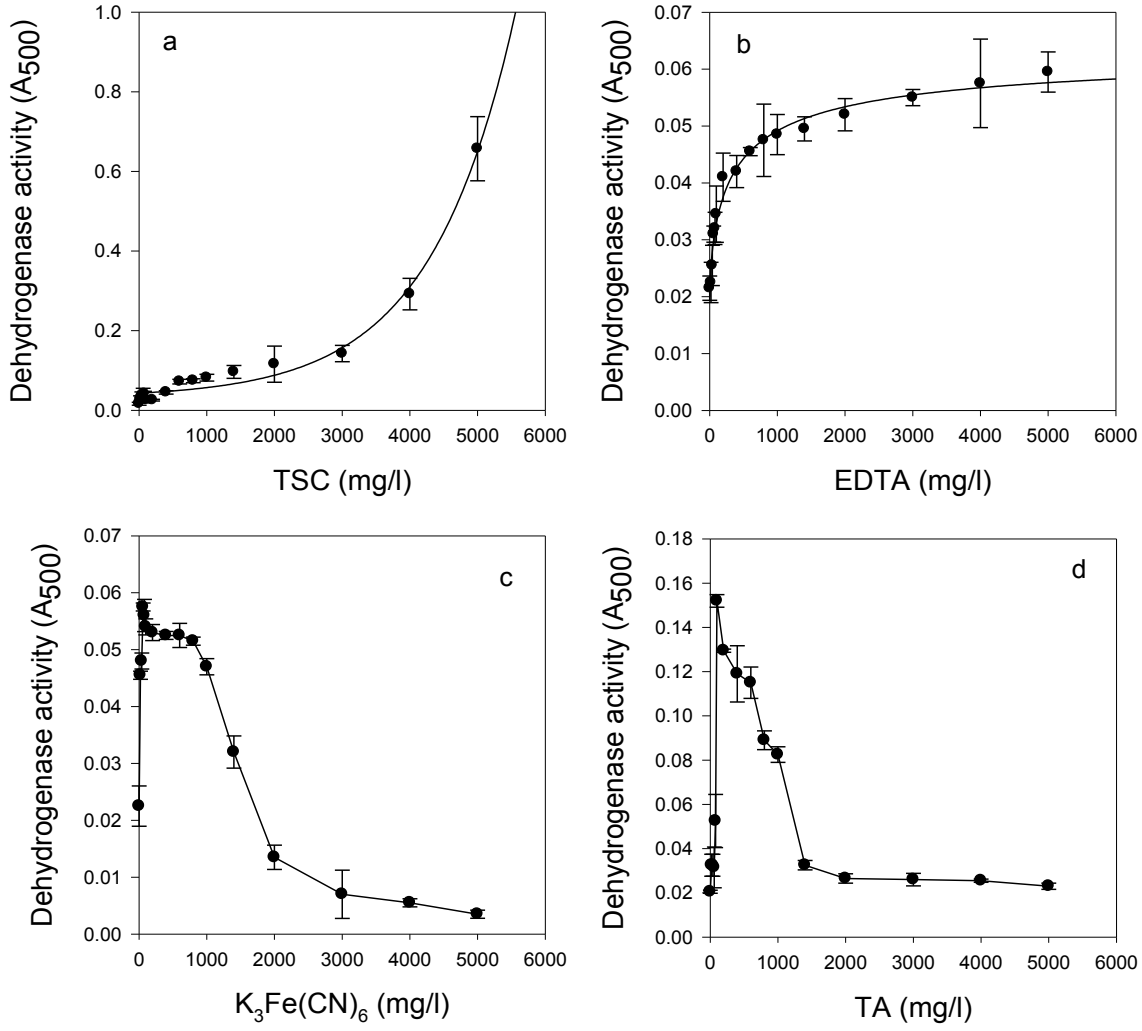

Fig. 4.2 Effects of chelating agents on dehydrogenase activity of *Pseudomonas* sp in mineral salts medium (TSC = Trisodium citrate; EDTA = Ethylenediamine tetraacetic acid; TA = Tannic acid; $K_3Fe(CN)_6$= Potassium ferricyanide)

4.1.3 Effect of nickel on the dehydrogense activity of *Pseudomonas* sp. in nutrient broth and mineral salts medium

The results of the effect of nickel on the dehydrogenase activity of the bacterium are shown in Figure 4.3. In both media, dehydrogenase activity of the organism reduced with increasing concentrations of nickel.

In the mineral salts medium, dehydrogenase activity was completely inhibited at nickel concentrations of 1.2 mM and above. In the nutrient broth, dehydrogenase activity progressively decreased with increase in concentration of nickel. At 2 mM nickel, the dehydrogenase activity as measured by absorbance at 500 nm was 0.06. The toxic effect of nickel was more pronounced in mineral salts medium than in nutrient broth. The determined IC_{50} values of both nickel and chelators in both media are summarized in Table 4.2

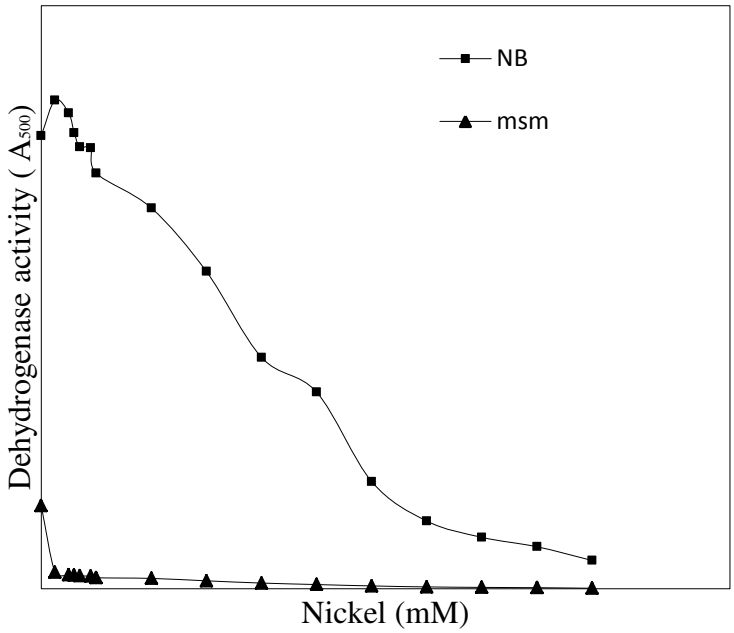

Fig. 4.3 Effect of nickel on the dehydrogenase activity of *Pseudomonas* sp in mineral salts medium (msm) and nutrient broth (NB)

4.1.4 Effects of chelating agents on nickel toxicity to the dehydrogenase activity of *Pseudomonas* species

The results of the effect of chelating agents and nickel on the dehydrogenase activity of the bacterium in nutrient broth and mineral salt medium are shown in Figures 4.4 - 4.11.

i. Effects in mineral salts medium

The results of the effects of trisodium citrate and nickel on the dehydrogenase activity of *Pseudomonas* sp. is depicted in Fig 4.4. Trisodium citrate reduced nickel toxicity to the dehydrogenase activity of the bacterium. It was observed that increase in concentration of TSC, reduced nickel toxicity. At nickel concentration of 1 mM, dehydrogenase activity at TSC concentrations of 100 mg/l, 1000 mg/l, 2000 mg/l and 4000 mg/l were 0.054, 0.124, 0.292 and 0.577 respectively.

Fig 4.5, illustrates the effect of EDTA on nickel toxicity to the dehydrogenase activity of the bacterium. The observed dehydrogenase activities at EDTA concentrations of 400 mg/l, 1000 mg/l, 2000 mg/l and4000 mg/l at 1 mM nickel were 0.009, 0.014, 0.049 and 0.021 respectively.

The effects of potassium ferricyanide and tannic acid are shown in Figs. 4.6 and 4.7 respectively. The dehydrogenase activities at 1 mM nickel with 20 mg/l, 40 mg/l, 80 mg/l and 100 mg/l $K_3Fe(CN)_6$ were 0.002, 0.110, 0.010 and 0.009 respectively. While that of 5 mg/l, 10 mg/l, 20mg/l and 40 mg/l TA were 0.011, 0.006, 0.012 and 0.017 respectively.

ii. Effects in nutrient broth

The results of the effects of the chelating agents on nickel toxicity to the dehydrogenase activity of the bacterium are shown in Figs 4.8-4.11. In Figure 4.8, trisodium citrate showed stimulatory effect on the dehydrogenase activity of *Pseudomonas* sp in the presence of nickel. TSC concentrations of 100 mg/l, 1000 mg/l, 2000 mg/l and 4000 mg/l at 1 mM nickel had dehydrogenase activities of 0.59, 0.718, 1.119 and 1.544 respectively. Dehydrogenase activity increased with increasing concentrations of TSC.

Figure 4.9 illustrates the effect of EDTA and nickel on the dehydrogenase activity of the bacterium. Nickel toxicity on dehydrogenase activity reduced in the presence of EDTA. At EDTA concentration of 2000 mg/l and 4000 mg/l, dehydrogenase activities at 1 mM nickel were 0.630 and 0.680 respectively. While at 2 mM nickel, dehydrogenase activities were 0.1 and 0.06 respectively.

The effects of TA on nickel toxicity to the bacterium are shown in Fig 4.10. Observed dehydrogenase activities at TA concentrations of 5 mg/l, 10 mg/l, 20 mg/l and 40 mg/l were 0.433, 0.322, 0.231 and 0.215 respectively at 1 mM nickel. In Fig 4.11, the effect of $K_3Fe(CN)_6$on nickel toxicity to the dehydrogenase activity of *Pseudomona*s sp. was illustrated. Dehydrogenase activities at 20 mg/l, 40 mg/l, 80 mg/l and 100 mg/l $K_3Fe(CN)_6$were 0.383, 0.465, 0.585 and 0.638 respectively at nickel concentration of 1 mM.

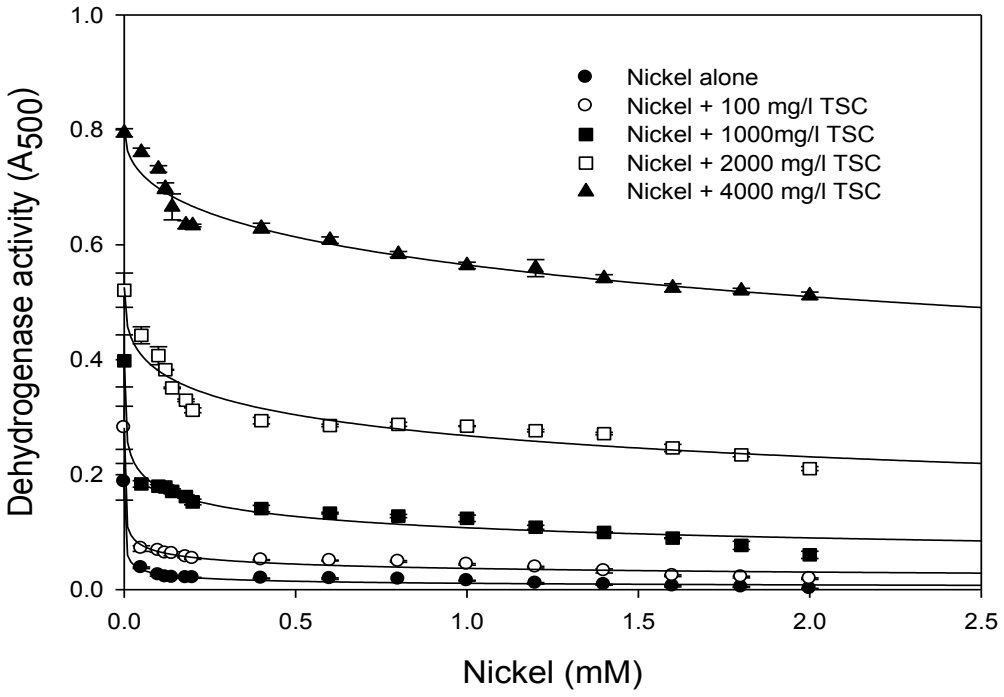

Figure 4.4 Effects of trisodium citrate (TSC) on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp in mineral salts medium.

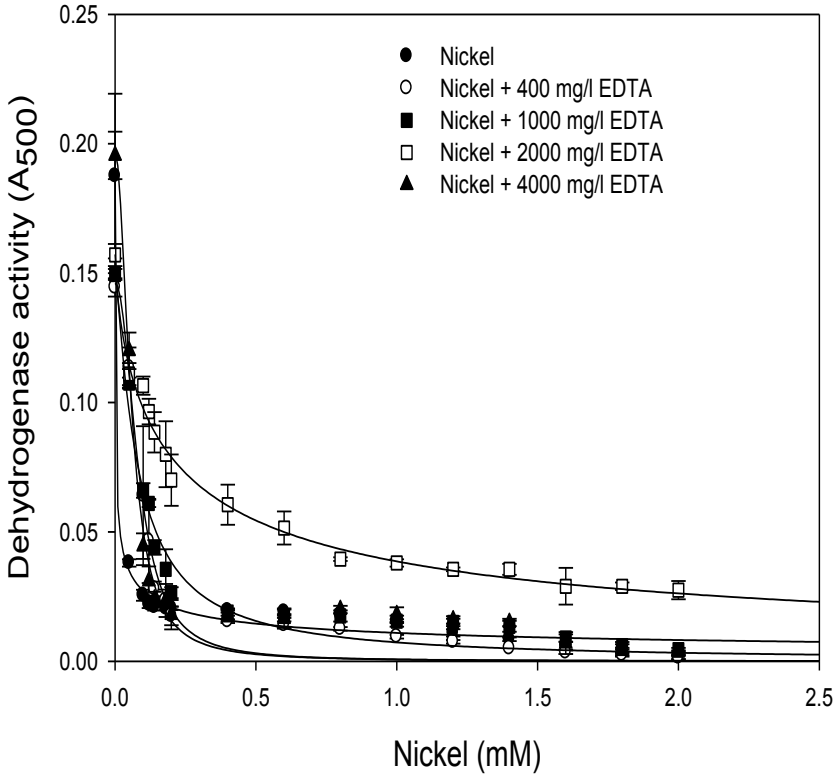

Figure 4.5 Effects of ethylenediaminetetraacetic acid (EDTA) on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp in mineral salts medium

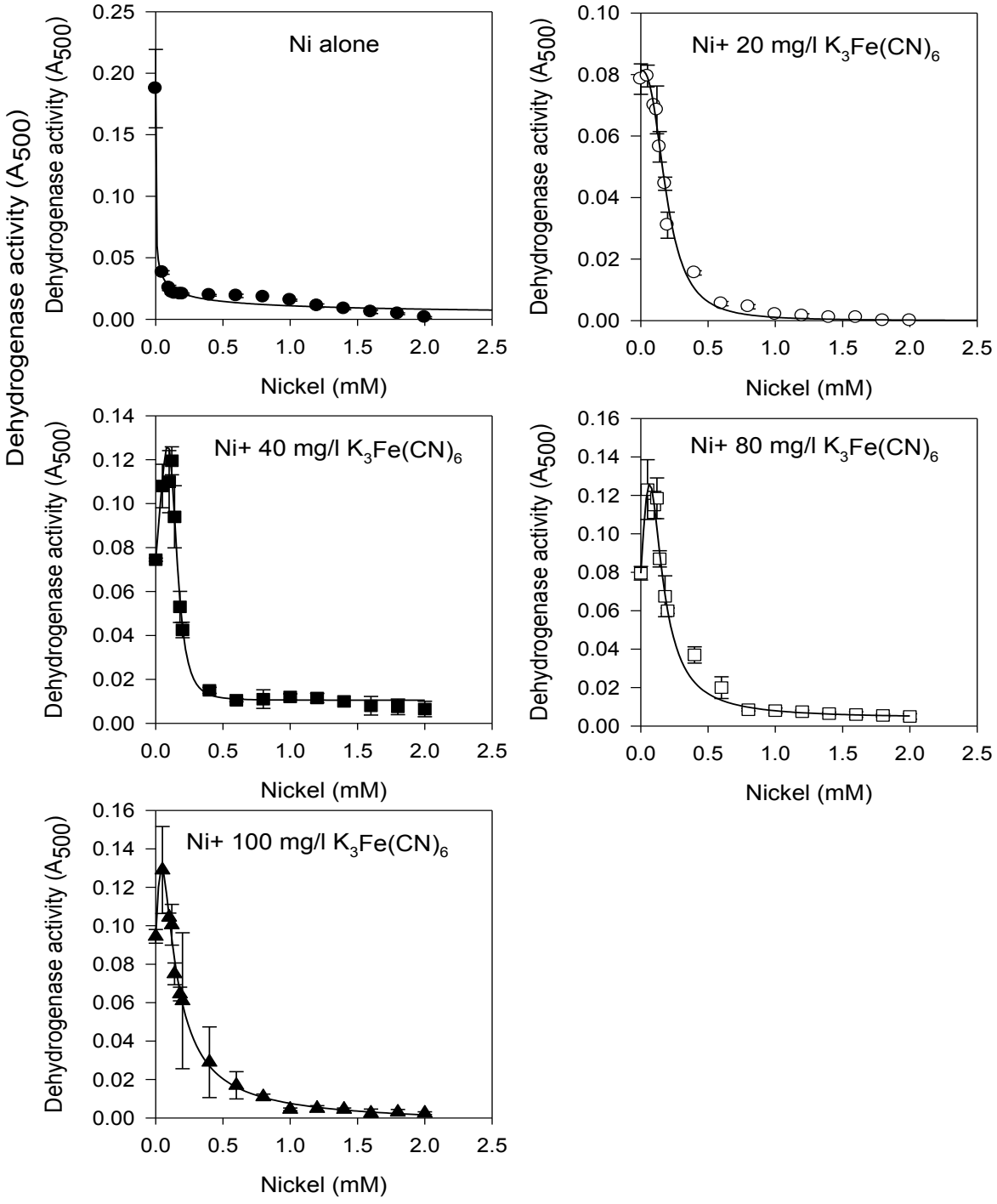

Figure 4.6 Effects of potassium ferricyanide ($K_3Fe(CN)_6$)on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp in mineral salts medium

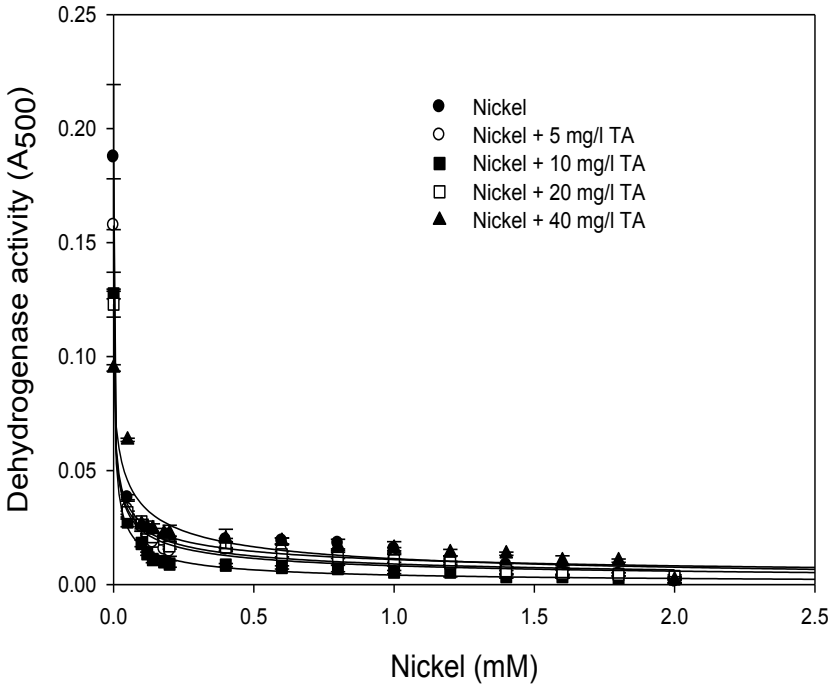

Figure 4.7 Effects of tannic acid (TA)on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp in mineral salts medium

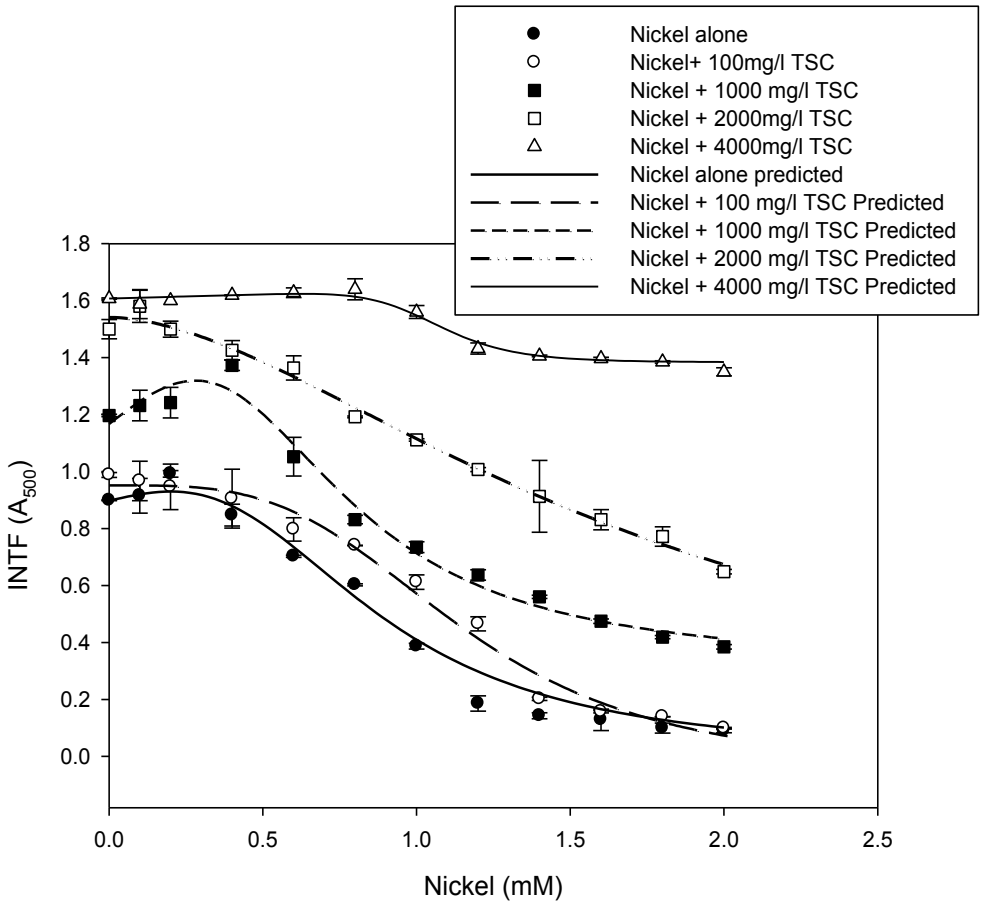

Figure 4.8 Effect of trisodium citrate (TSC) on nickel toxicity to dehydrogenase activity of *Pseudomonas* spin nutrient broth

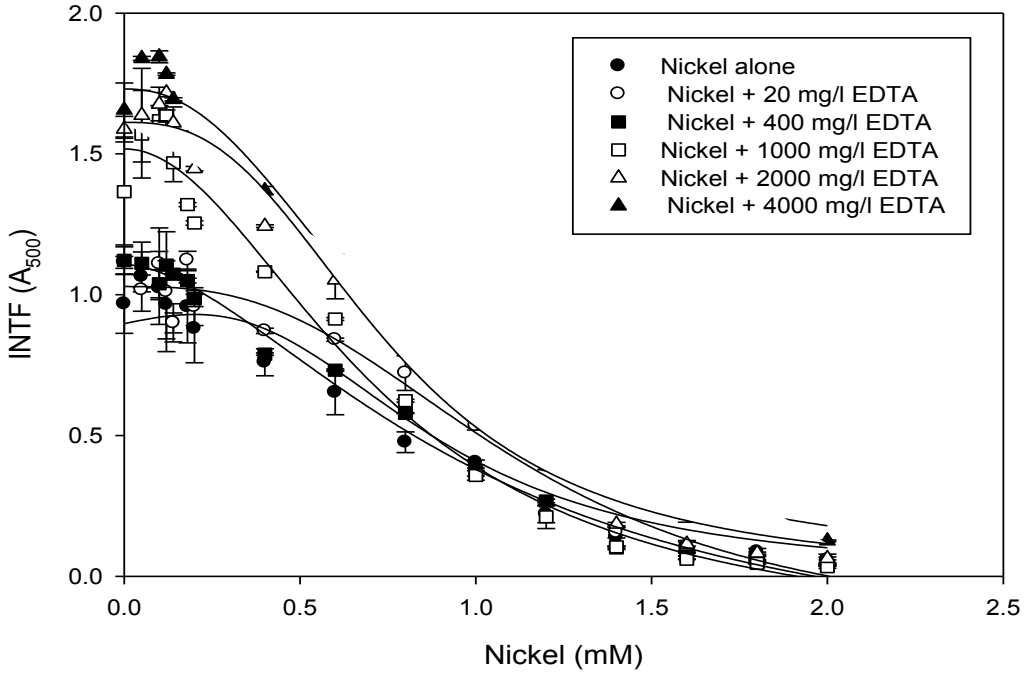

Figure 4.9 Effects of ethylenediaminetetraacetic acid on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp. in nutrient broth

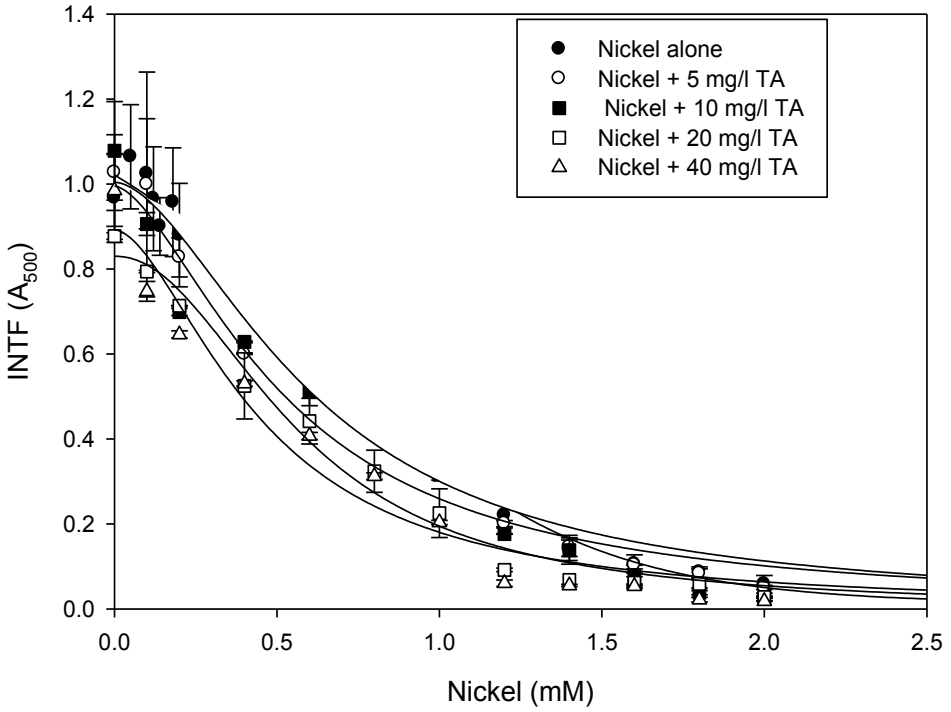

Figure 4.10 Effects of tannic acid on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp. in nutrient broth

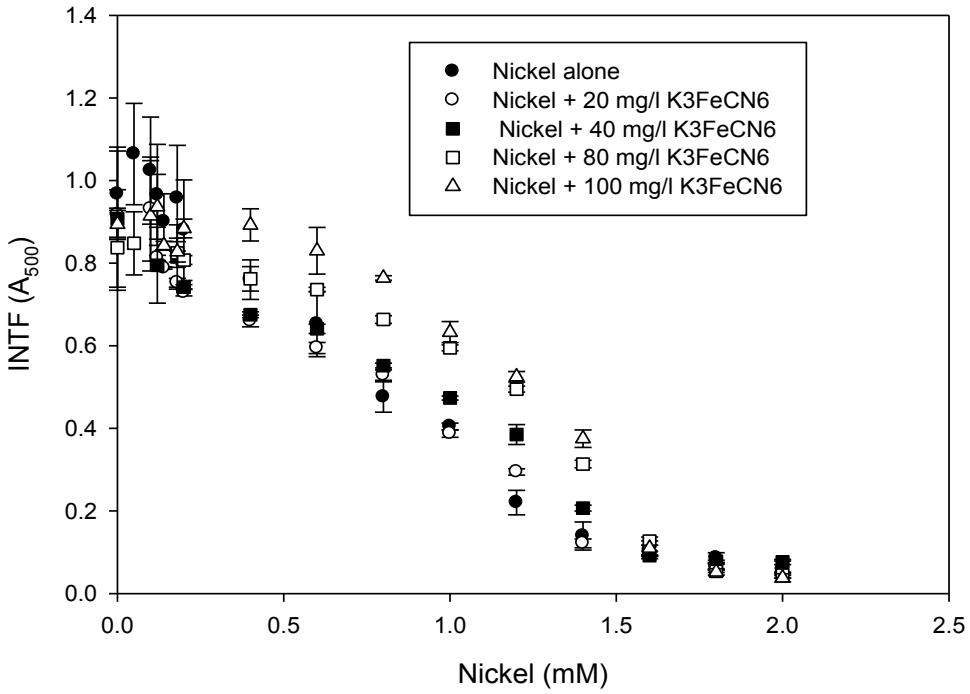

Figure 4.11 Effects of potassium ferricyanide on nickel toxicity to dehydrogenase activity of *Pseudomonas* sp. in nutrient broth.

4.1.5 Determination of median inhibitory concentration (IC_{50}) of nickel in combination with chelating agents

The result of the IC_{50} of the single and joint effects of nickel and the chelating agents are shown in Table 4.2. Nickel had inhibitory effects on the dehydrogenase activity of the organism with observed IC_{50} of 0.613 mM and 0.0017 mM in nutrient broth and msm respectively. The highest toxic effect was nickel in mineral salt medium.

The chelating agents exhibited various effects on the dehydrogenase activity of the *Pseudomonas* sp. In both media, TSC and EDTA did not show any inhibitory effect up to the maximum concentration administered. The IC_{50} of nickel in the presence of tannic acid and potassium ferricyanide were 308.50 ± 27.39 mg/l and 427.10 ± 35.58 mg/l in nutrient broth respectively. Trisodium citrate progressively increased the IC_{50} with increasing concentrations. The same phenomenon was observed with potassium ferricyanide and EDTA in both media. However IC_{50} at 2000 mg/l EDTA was higher than at 4000 mg/l.

Tannic acid increased the IC_{50} with increasing concentrations in mineral salt medium while in nutrient broth it decreased with increasing concentrations.

Table 4.2Toxicity threshold of Nickel and the chelating agents

TREATMENT	IC50 in NB	IC50 in MSM
Nickel Alone	0.613± 0.106 (mM)	0.0017±0.0011 (mM)
TSC alone	5000 mg/l	> 5000 mg/l
EDTA alone	5000 mg/l	> 5000 mg/l
TA alone	308.50± 27.39mg/l	> 5000 mg/l
$K_3Fe(CN)_6$ alone	427.10±35.58mg/l	2604.17 ±0.20 mg/l
Nickel + TSC 100 mg/l	1.154±0.102	0.0022± 0.0013
Nickel + TSC 1000 mg/l	1.176±0.518	0.0562 ±0.0157
Nickel + TSC 2000 mg/l	1.730±0.041	1.0910 ±0.274
Nickel + TSC 4000 mg/l	> 2.0	6.8180 ±1.396
Nickel + EDTA 400 mg/l	0.649±0.082	0.0848±0.0068
Nickel + EDTA 1000 mg/l	0.713±0.017	0.0795±0.0098
Nickel + EDTA 2000 mg/l	0.750±0.044	0.2019±0.0192
Nickel + EDTA 4000 mg/l	0.739±0.019	0.0583±0.0068
Nickel + TA 5mg/l	0.615±0.077	0.0034±0.0016
Nickel + TA 10 mg/l	0.528±0.080	0.0059±0.0017
Nickel + TA 20 mg/l	0.572±0.043	0.0071±0.0017
Nickel + TA 40 mg/l	0.450±0.070	0.0427±0.0167
Nickel + $K_3Fe(CN)_6$20 mg/l	0.754±0.122	0.1921±0.0084
Nickel + $K_3Fe(CN)_6$40 mg/l	0.978±0.076	0.2020±0.0110
Nickel + $K_3Fe(CN)_6$80 mg/l	1.247±0.025	0.2270±0.0293
Nickel + $K_3Fe(CN)_6$ 100 mg/l	1.260±0.027	0.2560±0.042

EDTA=Ethylenediamine tetraacetic acid; TSC = Trisodium citrate; TA = Tannic acid;$K_3Fe(CN)_6$= Potassium ferricyanide; NB= Nutrient broth; MSM =Mineral salt medum; IC_{50}=50% inhibition concentration; mg/l = milligram per litre; mM = millimolar

4.1.6 Biodegradation of phenol and biomass production

4.1.6.1 Effects of trisodium citrate on phenol degradation

The bacterium utilized phenol as sole carbon source at initial concentrations of 200 mg/l and 500 mg/l phenol. The resident time varied according to initial level of phenol. Figure 4.12 illustrates the time course of *Pseudomonas* sp. growth and phenol depletion at 200 mg/l and 500 mg/l phenol in the presence of 50 mg/l and 100 mg/l trisodium citrate. The bacterium spent 75 hand 120 h to completely degrade 200 mg/l and 500 mg/l phenol respectively. The time spent to completely degrade 200 mg/l and 500 mg/l phenol in the presence of 50 mg/l trisodium citrate were 72 h and 120 h, while at 100 mg/l trisodium citrate, the bacterium completely degraded phenol in 96 h and 123 h respectively. The maximum optical density attained was 0.562 at 500 mg/l phenol alone. Figure 4.12 also depicts the typical cell growth curve. The cell growth curve has typical exponential and stationary phases with increased lag phase at 500 mg/l phenol. However trisodium citrate addition was observed to shorten the lag phase of the cell growth.

Table 4.3 depicts the estimated specific growth (μ) and degradation (Q_s) rate of the bacterium during phenol degradation in the presence of trisodium citrate. The highest specific growth rates attained were 0.062 h^{-1} at 200 mg/l phenol and 0.060 h^{-1}at 500 mg/l phenol with 100 mg/l trisodium citrate.

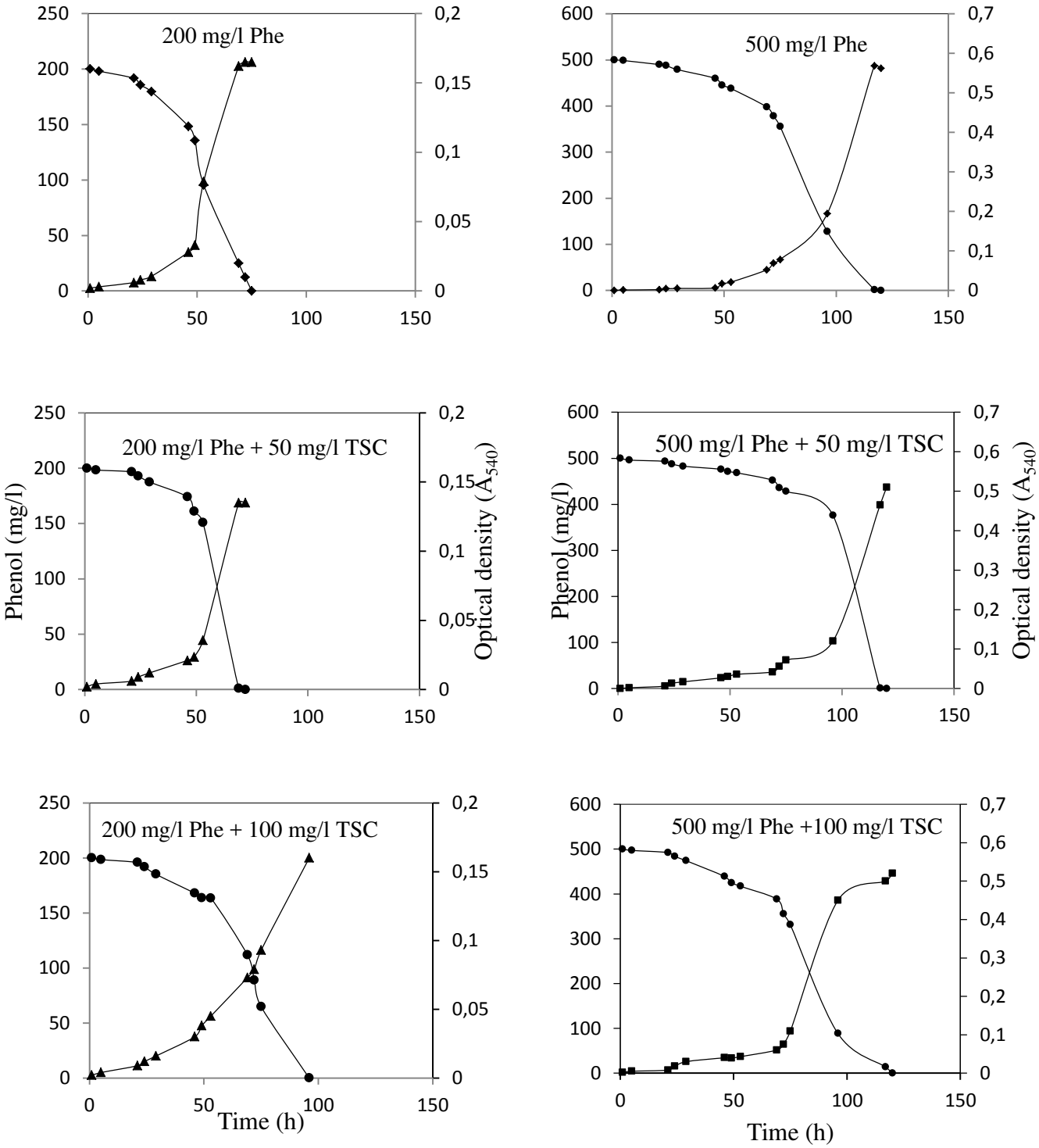

Figure 4.12 Effects of trisodium citrate (TSC) on phenol degradation

Table 4.3 Effect of trisodium citrate on the degradation and specific growth rate of *Pseudomonas* sp. during phenol degradation

Treatment		μ (h^{-1})	Q_s (mg/l/h)
Phenol (mg/l)	Trisodium citrate (mg/l)		
200	0	0.062	1.434
200	50	0.056	0.919
200	100	0.056	0.706
500	0	0.053	2.672
500	50	0.054	0.715
500	100	0.060	1.503

4.1.6.2 Effects of nickel and trisodium citrate on phenol degradation

The inhibitory effects of nickel on the ability of the bacterial isolate to grow and degrade phenol in the absence or presence of trisodium citrate are shown in Figures 4.13 and 4.14. In these Figures, phenol degradation was inhibited by nickel even at low concentration. Optical densities attained at all nickel concentrations were not up to 0.05. The organism did not achieve a complete growth cycle in the presence of nickel. The lag phase took longer time than in phenol alone and in the presence of trisodium citrate. Precipitate and slime formation were observed in the flasks. There was no complete mineralization of phenol in the presence of nickel.

Specific growth and phenol degradation rates of the bacterium in the presence of nickel alone and nickel with trisodium citrate are shown in Tables 4.4 respectively. The determined specific growth and degradation rates reduced generally with nickel addition in both experiments.

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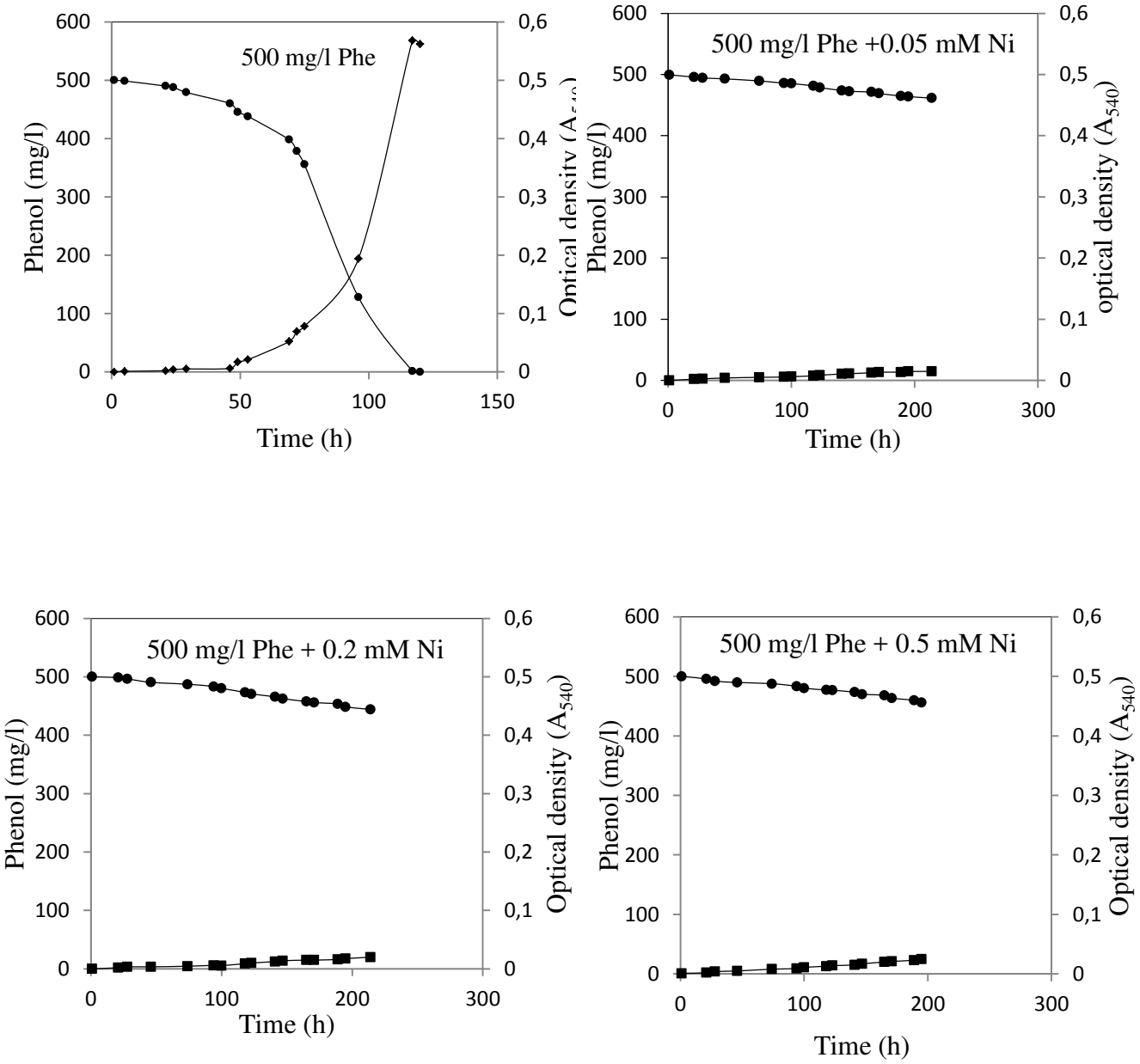

Figure 4.13 Effect of nickel on phenol degradation in the absence of trisodium citrate

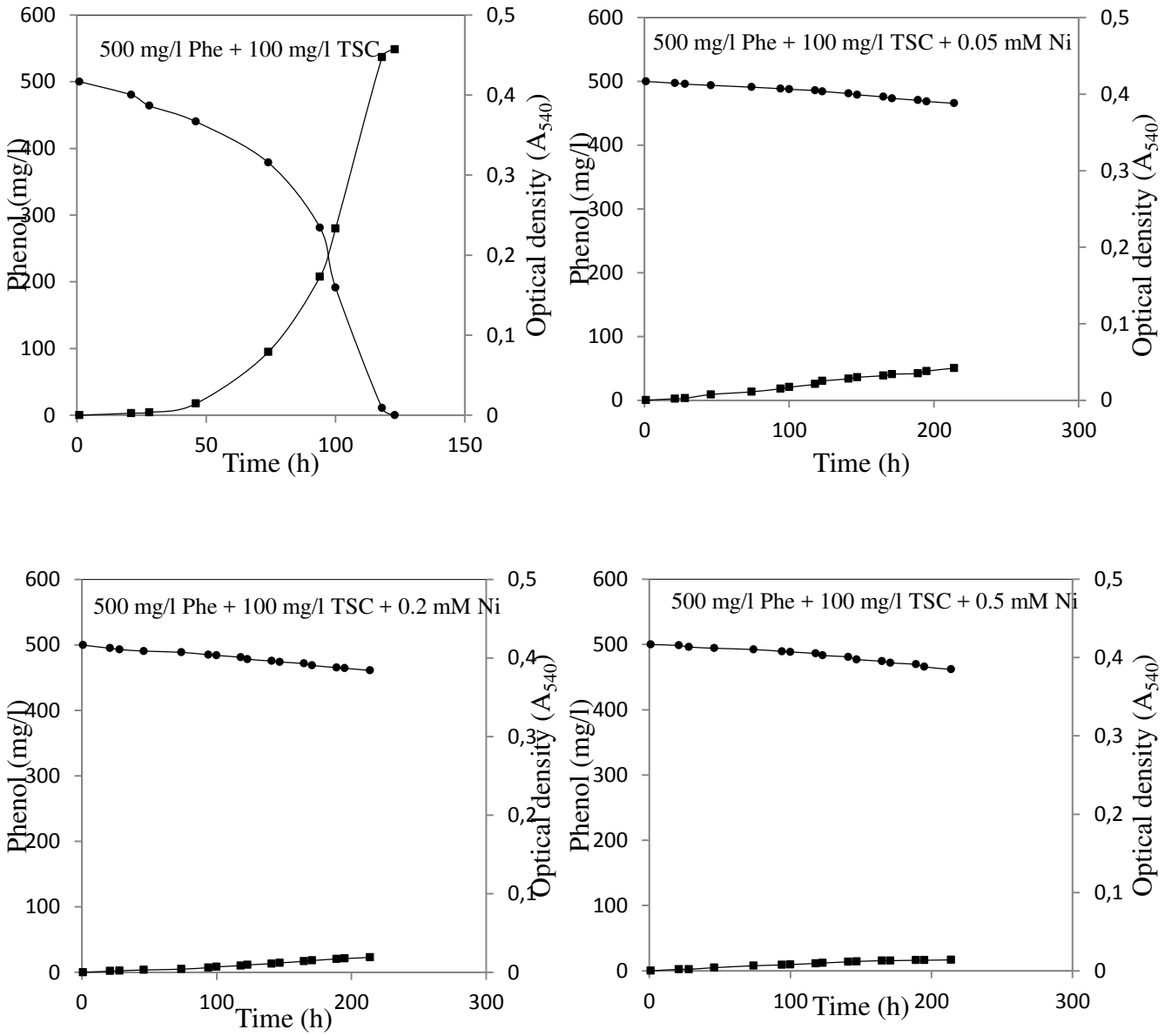

Figure 4.14 Effect of nickel on phenol degradation in the presence of trisodium citrate (TSC)

Table 4.4 Estimated specific growth and phenol degradation rates of *Pseudomonas* species in the presence of nickel and trisodium citrate

Treatment			μ (h^{-1})	Q_s (mg/l/hr)
Phenol (mg/l)	Nickel (mM)	Trisodium citrate (mg/l)		
500	0	0	0.053	2. 672
500	0.05	0	0.022	0.169
500	0.2	0	0.018	0.234
500	0.5	0	0.025	0.277
500	1.0	0	0.025	0.308
500	0	100	0.059	1.258
500	0.05	100	0.029	0.125
500	0.2	100	0.019	0.174
500	0.5	100	0.023	0.122
500	1.0	100	0.022	0.098

(μ = Specific growth rates of organism (h^{-1}); Q_s = Phenol degradation rates (mg/l/hr)

4.1.6.3. Effect of nickel on trisodium citrate utilization by *Pseudomonas* sp

The effect of nickel on trisodium citrate utilization is shown in Fig 4. 14. Optical densities attained with trisodium citrate and nickel varied. Maximum optical densities of 0.017 and 0.042 attained were recorded at 50 mg/l and 100 mg/l trisodium citrate respectively. Nickel inhibited the ability of the bacterium to utilize trisodium citrate. The optical density at 540 nm decreased with nickel addition. The specific growth rates (μ) at 50 mg/l and 100 mg/l trisodium citrate are shown in Table 4.5. The growth rate decreased with addition of nickel.

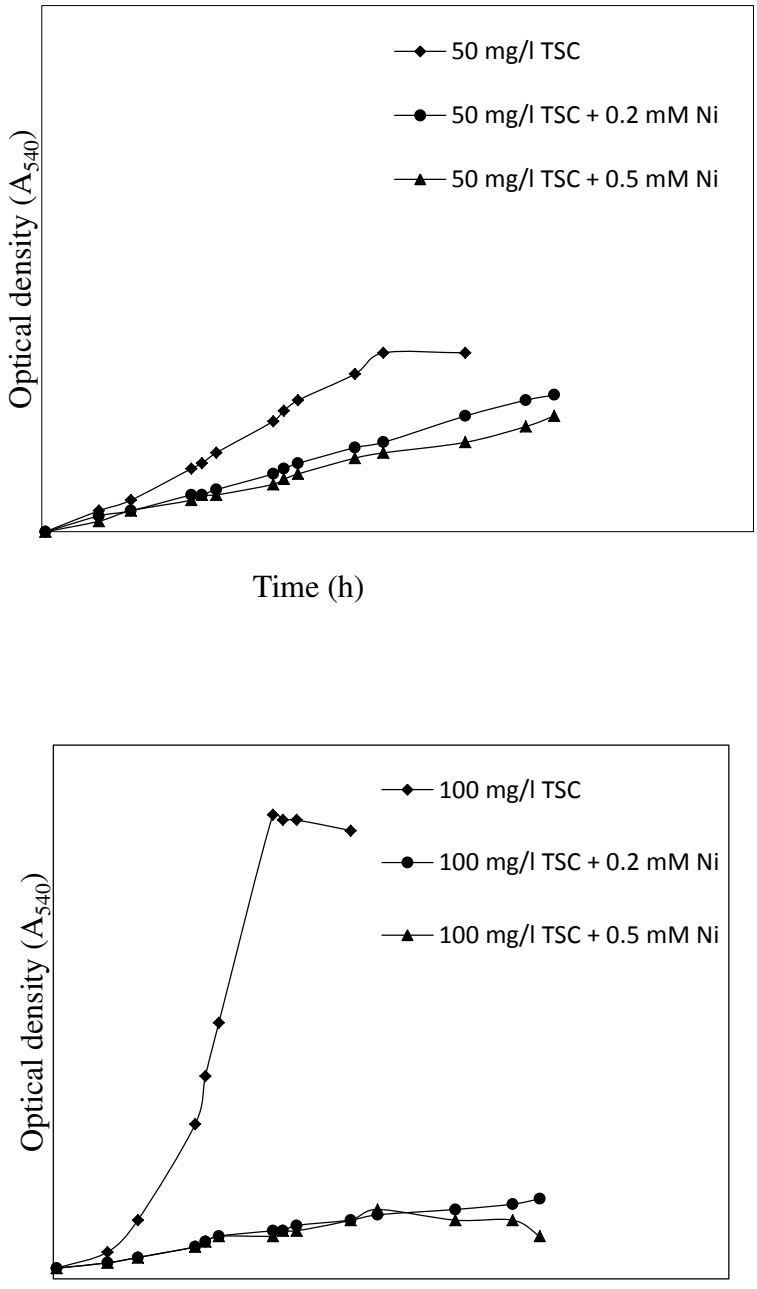

Figure 4.15 Effect of nickel on trisodium citrate utilization by *Pseudomonas* species

Table 4.5 Effect of nickel and trisodium citrate on the specific growth rate of *Pseudomonas* sp

Treatment		μ (h^{-1})
Trisodium citrate (mg/l)	Nickel (mM)	
50	0	0.043
50	0.2	0.028
50	0.5	0.028
100	0	0.066
100	0.2	0.027
100	0.5	0.028

4.1.6.4. Kinetics of phenol degradation and biomass production in the presence of nickel and trisodium citrate

The kinetic parameters from the biomass production data are shown in Table 4.8. From the results, the maximum specific growth rate at 500 mg/l phenol was 0.0967 h^{-1}. This increased with the addition of 100 mg/l trisodium citrate to 0.1736 h^{-1}but decreased with the addition of 0.5 mM and 1.0 mM nickel to 0.0489 h^{-1} and 0.0497 h^{-1} respectively. The progressive curve analysis showed that the growth and degradation data could be described by Haldane's substrate inhibition model with R^2 values greater than 0.9. The half saturation constant (k_s) and yield coefficient (Y) increased with addition of 100 mg/l trisodium citrate.

Table 4.6 Effects of trisodium citrate and nickel on growth kinetics parameter values for phenol degradation

Treatment	S_o (mg/l)	Y (A_{500} units.l/mg)	K_s (mg/l)	K_i (mg/l)	$\mu_m(h^{-1})$	R^2
Phe alone	500	0.00025	78.289	346.687	0.0967	0.9750
Phe +TSC	500+100	0.00090	145.577	236.750	0.1736	0.9854
Phe+Ni	500+0.5mm	0.00094	11. 102	370.325	0.0489	0.9430
Phe + Ni	500+1.0mm	0. 00100	13.395	381.980	0.0497	0.9483

S_0 =substrate concentration (mg/l); Y= yield coefficient ((A_{500}) (mg/l))(mg/biomass or mg/substrate)

K_s = half saturation constant; K_i = inhibition coefficient (mg/l); μ_m = maximum specific growth rate (h^{-1})

R^2= coefficient of determination, phe=phenol; ni= nickel; tsc=trisodium citrate

4.2 Discussion

The Gram - negative bacterium isolated from a farmland in Ihiagwa, Owerri West L.G.A., Nigeria was identified as a species of *Pseudomonas,* through colonial, morphological, microscopic and biochemical characterizations according to Cheesbrough (2006) and Bergey's Manual of Systematic Bacteriology, (2005). It is most probably *Pseudomonas aeruginosa* but for the purpose of this work it would be referred to as *Pseudomonas* sp until further genetic characterization is made outside this work. *Pseudomonas* sp are widely distributed in the soil and possess an innate ability to utilize organic compounds (Nair *et al.*, 2008; Bajaj *et al*., 2009).

Metals affect the growth characteristics of microorganisms, thereby affecting their metabolic activities in the environment. In the present study, nickel was investigated. The effect of different concentrations of nickel on the dehydrogenase activity of *Pseudomonas* species in nutrient broth and mineral salt medium showed that nickel was toxic to the organism, and nickel toxicity to *Pseudomonas* species (rate and extent of attenuation of dehydrogenase activity) increased with increasing concentration. This finding is in line with previous reports of researchers (Wyszkowska *et al.,* 2005; Kucharski *et al.,* 2009). They demonstrated the inhibitory effect of nickel on soil microbial dehydrogenase activity.

The inhibitory effect of nickel occurred at low concentrations and was more pronounced in mineral salts medium than in nutrient broth. The IC_{50} of nickel on *Pseudomonas* sp. in NB (0.613 mM) was 360 times higher than in minimal medium (0.0017 mM). This difference suggests the presence of other substances in the nutrient broth that influenced the availability of nickel. This will mimic the situation in the soil, which is an even more complex environment. Metal contamination is one of the priority environmental problems on earth due to its significant threat to public health.The presence of nickel in the soil therefore will affect the population and activity of *Pseudomonas* sp in the soil, thereby antagonizing its ability to remove phenol from polluted soil.

The availability of nickel and other polluting heavy metals in the soil is affected by the presence of chelating agents which bind metals. In the present study, four chelating agents EDTA, TSC, TA, $K_3Fe(CN)_6$ were assessed on their ability to reduce nickel toxicity. Prior to this, the effect of these four chelating agents were assessed on the dehydrogenase activity of *Pseudomonas* sp which determined whether or not they were toxic to *Pseudomonas* sp. EDTA and TSC alone showed no toxic effects to *Pseudomonas* species either in minimal or in complex medium while the effect of TA and $K_3Fe(CN)_6$were not significant, both considerably higher in msm than in NB. The chelating agents exerted varied effects on the IC_{50} of nickel against the bacterium. They all increased the IC_{50} of nickel except for tannic acid which reduced it in nutrient broth. This shows that the chelating agents were capable of reducing nickel toxicity to the dehydrogenase activity of the organism. The slight stimulatory response may have been due to a surface permeability effect, or metabolism of the chelating agents by the organism (Campbell *et al.,* 2000).The chelating ability of the chelating agents decreased in the order TSC > EDTA > $K_3Fe(CN)_6$> TA.

Chelating agents have been reported to bind metal ions thus making them unavailable to both plants and microbes (Kucharski *et al*., 2009; Harrington *et al*., 2012) which in turn inhibits/reduces the toxic effects exerted by the metals. The results from the present study especially in the case of TSC and EDTA support these reports. Wuana *et al*. (2010) in their study reported that EDTA was more efficient in removing heavy metals from soil than citric acid. The results from the present study differ in this regard, but provide dose–response relations, which are regularly used in toxicology, as a quantitative analysis to show the criteria of 50% effective concentration (IC_{50}).

Biodegradation of phenol by *Pseudomonas* species and biomass production

In the present investigation, it was observed that the bacterium utilized phenol as a source of carbon and energy at initial concentrations of 200 mg/l and 500 mg/l, thus possessing an innate ability to degrade phenol. The higher phenol concentration of 500 mg/l exerted some toxic effect on the growth of the organism by inducing a longer lag phase and time course than was observed at 200 mg/l phenol. This supports reports of previous researchers who

found that phenol had inhibitory effect at high concentrations (Kumar *et al*., 2005; Dey and Mukherjee, 2010; Firozjaee *et al*., 2011; Nweke and Okpokwasili, 2014). Inhibitory effect of metals on biodegradation of phenol has been reported (Shweta and Dhandayuthapani, 2013,). Nickel in the present study showed strong inhibitory effect on the growth of the bacterium studied, as well as on phenol degradation even at the lowest concentration administered (0.05 mM). The bacterium found it very difficult to grow in the presence of nickel as it could not attain an optical density of 0.1, thereby preventing complete mineralization of phenol.

The estimated specific growth and degradation rate reduced with nickel addition. This provides evidence that nickel inhibited the growth of the bacterium and its ability to degrade phenol. The addition of trisodium citrate in the presence of nickel did not mitigate nickel toxicity. This may probably be as a result of the trisodium citrate concentration used. Nickel prevented the organism from being able to access phenol and trisodium citrate as carbon sources for its growth. This is in line with the views of Sandrin and Maier, (2003) that the presence of metals inhibits a broad range of microbial processes. These results show that the presence of metals is a huge threat to microbial degradation of phenol. It also shows that the trisodium citrate concentration used had no pronounced effect on nickel inhibition of phenol degradation by *Pseudomonas* sp.

The presence of trisodium citrate in the culture medium reduced the lag phase of the growth cycle of the organism atphenol concentrations tested. This suggests that trisodium citrate increased the tolerance of the organism to high phenol concentration by providing a readily metabolisable carbon source to support cell growth. However, the time interval to completely deplete phenol did not improve with trisodium citrate addition. The bacterium depleted phenol alone faster than phenol with trisodium citrate; thus, suggesting that the stimulatory effect of trisodium citrate was more pronounced on the growth characteristics of the bacterium than on the degradation rate of phenol. Reports on the effect of trisodium citrate on phenol degradation are scarce but the use of other substrates such as glucose, yeast extract and peptone as carbon sources has been reported (Sridevi and Lakshmi, 2009; Basha *et al.,* 2010;

Chakraborty *et al*., 2010). Their results showed that these non-toxic compounds act as growth substrates in the presence of high concentrations of phenol by stimulating the viability of cells and enhancing biodegradation. Chakraborty *et al* (2010) in their study reported that glucose addition up to a specific low concentration could improve the degradation rate, but impeded the degradation process at higher concentrations. The present study depicted an increased maximum specific growth of *Pseudomonas* sp during phenol degradation in the presence of trisodium citrate. This indicates that trisodium citrate could be used as a co-substrate for enhanced degradation of phenol by *Pseudomonas* species. It is worthwhile to explore the limits of trisodium citrate application for enhanced phenol degradation.

Generally the Haldane model described the growth of *Pseudomonas* sp. in this study, both in the presence of nickel and trisodium citrate. The maximum specific growth rate of the organism obtained in this study differs from those reported in available literature. This variation may be due to different factors/physiological parameters that influence the biodegradation activity of microorganisms such as incubation temperature, pH of the medium, carbon or energy source, toxicity of co contaminant present, concentration of micro- and macro nutrient, source of microorganism, method of evaluation and media constituents.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The results obtained in this study provide evidence that *Pseudomonas* sp is susceptible to nickel and is capable of degrading phenol. The study showed that some chelating agents (trisodium citrate and ethylenediaminetetraacetic acid) mitigated nickel toxicity to *Pseudomonas* sp, while others at high concentrations were toxic to *Pseudomonas* sp which in turn increased the inhibitory effect of nickel. The results provide information on the IC_{50} of nickel to *Pseudomonas* sp both in the presence and absence of the evaluated chelating agents; thus demonstrating the appropriate chelating agent for protecting the bacterium against the toxic effects associated with exposure to nickel.

The study showed that nickel inhibits phenol degradation, and suggests the use of trisodium citrate as an additional carbon source to support biodegradation of phenol. However further studies should be carried out to identify the optimum concentration of trisodium citrate that can be employed to reduce the effects of nickel on biodegradation of phenol. Therefore, in the complex soil environment, *Pseudomonas* species could be used to remediate the effect of phenol pollution. The efficiency though will depend on the balance of the components of the environment. The presence of EDTA/TSC in nickel polluted soil would reduce the availability of nickel, allowing proliferation of *Pseudomonas*species and thus improving phenol degradation/utilization.

5.2 Recommendations

The presence of chelating agents in a nickel polluted environment will enable the growth of *Pseudomonas* species thereby promoting their metabolic activities. TSC and EDTA appeared to offer greater potential than TA and $K_3Fe(CN)_6$as chelating agents to use in remediating metal polluted soil. Tannic acid and potassium ferricyanide are, however, recommended in events of moderate contamination. It is recommended therefore that trisodium citrate and EDTA be used as appropriate chelators to mitigate the toxicity of nickel to *Pseudomonas*

species so as to enhance the remedial capacity of *Pseudomonas* species. Further studies should be carried out to ascertain the effect of higher concentrations of trisodium citrate on phenol degradation. This will provide more cost effective applications of bacterial strains for phenol degradation at large scale in industries, where it poses problem due to its detrimental health effects on different organisms and human beings.

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APPENDIX I

Media Preparation

1. Solid Media

a) Nutrient Agar

Formula and preparation

Yeast extract	-	2.0 g
Peptone	-	5.0 g
Agar No.1	-	15.0 g
Sodium chloride	-	5.0 g
Meat extract	-	1.0 g
Distilled water	-	1000 cm^3
pH	-	7.4 ± 0.2

Dissolve 28 g. in 1000.0 cm^3 of distilled water. Boil gently to dissolve the medium completely. Sterilize for 15 minutes at 121^0C. Allow to cool to 40-45^{o}C before dispensing into bottles and plates.

b) Simmons's citrate agar

Formulation and preparation

Magnesium sulphate	-	0.20 g
Ammonium dihydrogen phosphate	-	1.00 g
Dipotassium phosphate	-	1.00 g
Sodium citrate	-	2.00 g
Sodium chloride	-	5.0 g
Bromothymol blue	-	0.08 g
Agar	-	15.0 g
Distilled water	-	1000 cm^3
pH	-	6.8 ± 0.2

Dissolve 24.28 g in 1000 ml distilled water. Boil gently to dissolve the medium completely. Dispense 4.0 - 5.0 ml into tubes. Sterilize at 121^{o}C for 15 minutes. Cool in slanted position (long slant, shallow butt).

c) Peptone Iron Agar

Formula and preparation

Peptone - 15.0 g

Proteose Peptone - 5.0 g

Ferric Ammonium Citrate. - 0.5 g

Sodium Glycerophosphate - 1.0 g

Sodium Thiosulfate - 0.08 g

Agar - 15.0 g

Distilled water - 1000.0 cm^3

1. Dissolve 36 g of the powder in 1000.0 cm^{3}of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Sterilize at 121°C for 15 minutes.

2. Liquid Media

a) Nutrient Broth

Formulation and Preparation;

Beef extract - 1.5 g

Yeast extract - 1.5 g

Peptone - 5.0 g

Sodium chloride - 5.0 g

Distilled water - 1000.0 cm^3

pH - 7.4 ± 0.2

Dissolve 13 g in 1000.0 cm^3 distilled water. Dispense into bottles, sterilize for 15 minutes at temperature of 121^{0}C and pressure of 15 psi.

b) Mineral salt medium

Formulation and preparation;

Dipotassium hydrogen orthophosphate	-	2.10 g
Potassium dihydrogen orthophosphate	-	0.4 g
Ammonium nitrate	-	0.5 g
Magnesium sulphate pentahydrate	-	0.2 g
Calcium chloride	-	0.023 g
Ferric chloride	-	0.002
Phenol	-	0.2 mM
Agar	-	15g
Water	-	1000 cm^3
pH	-	6.8± 0.2

Dissolve salts in distilled water. Sterilize for 15 minutes at 121^0C. To prevent precipitation of salts, they were sterilized separately, allowed to cool to 40-45^{o}C before mixing together and dispensing into bottles and flasks.

c) Koser's citrate broth

Formula and preparation (koser's (1923))

Sodium – ammonium Hydrogen Phosphate	-	1.5 g
Di-potassium Hydrogen phosphate	-	1.0 g
MgSO4	-	0.1 g
Sodium Citrate	-	3.0 g
Distilled water	-	1000.0 cm^3

Adjust pH to 6.8 dispense in 10ml portion in test tubes. Sterilize for 15 minutes at 121^0C

d) MR-VR broth, 3% salt

Formula and preparation

Peptone	-	7.0 g

Glucose - 5.0 g

Nacl - 30.0 g

Di-potassium Hydrogen Phosphate - 5.0g

Distilled water - 1000.0 cm^3

Adjust pH to 6.9. Dispense in 10ml portions in test tubes. Sterilize for 15 min at 121^0C.

e) Peptone water broth

Formula and preparation

Peptone - 10.0 g

Nacl - 5.0 g

Distilled water - 1000.cm^3

Adjust pH to 7.0 – 7.4. Dispense into bottles and sterilize for 15 minutes at 121^0C.

f) Peptone-sugar water

Formula and preparation

Peptone - 10.0 g

Nacl - 5.0 g

Phenol red - 30.0 ml

Sugars - 1g/100 ml

Distilled water - 1000.0 cm^3

1. Dissolve the peptone – salt in the distilled water.

Adjust pH to 7.2 – 7.3. Add phenol red.

2. Dispense into 100ml portion into conical flask containing 1g of the sugar desired. Dispense into test tubes and sterilize by steaming at 110^0C for 30 minutes for 3 days (tyndalization).

g) Nitrate Broth

Peptone - 5 g/l

Meat extract - 3 g/l

Potassium nitrate	-	1 g/l
pH	-	7.0 ± 0.2

Sulfanilic acid (Reagent A)	-	100 g
N,N-Dimethyl-1-naphthylamine (Reagent B)	-	10 ml
Zinc	-	1 g

Preparation of Reagents:

Sulfanilic acid solution (Reagent A): Eight grams of sulfanilic acid was dissolved in 1 litre 5N acetic acid. Reagent A was then stored at room temperature for 3 months in the dark. Reagents may be stored in dark brown glass containers; bottles may be wrapped in aluminum foil to ensure darkness.

α-Naphthylamine solution (Reagent B): Dissolve 6 g of N,N-Dimethyl-1-naphthylamine in 1 litre 5N acetic acid. Store Reagent B at 2 to 8°C for up to 3 months, in dark. Reagents may be stored in dark brown glass containers; bottles may be wrapped in aluminum foil to ensure darkness.

Directions:

Dissolve 9 g of Nitrate Broth in 1 litre distilled water. Dispense 10 ml aliquots of the broth into tubes. Sterilize by autoclaving at 121°C for 15 minutes.

APPENDIX II

Preparation of Reagents

a) Methyl-red indicator

Formula and preparation

Methyl-red	-	0.10g
Ethanol (95%)	-	30.0ml

Dissolve methyl-red in 95% ethanol. Add 200mls distilled water to make up for 500 ml.

b) Oxidase reagent

Formula and preparation

Para-amino dimethylphenylenediamine

Monohydrochloride	-	0.01g
Distilled water	-	100.0ml

Reagents should be prepared and use fresh. Store in dark prior to use.

c) Kovac's (indole) reagent

Formula and preparation

P- Dimethylaminobenzaldehyde	-	5.0g
Concentrated Hydrochloric acid	-	25.0g
Amyl-alcohol	-	75.0ml

Dissolve (i) in (ii) and (iii) and mix thoroughly by shaking.

d) Voges-Proskauer (V-P) reagent

Formula and preparation

Solution A: Alpha Naphthol	-	5.0g
Absolute ethanol	-	100.0ml

Solution B: KOH - 40.0g

Distilled water - 100.0ml

Mix A and B and shake thoroughly to homogenize.

e) Gram's staining reagents

Crystal violet

Solution A: Crstal violet - 20.0g

Ammonium Oxalate - 9.0g

Absolute ethanol - 95 ml

Distilled water - 100ml

Solution B: Safranin (counter stain)

Safranin (Saturated alcohol solution) - 10ml

Distilled water-90ml

Solution C: Lugol's lodine solution

Potassium iodide - 10ml

Iodine - 10g

Distilled water - 1000cm^3

Solution D: Acetone-alcohol decolourizer

Acetone - 500ml

Absolute Ethanol - 475ml

Distilled water - 25ml

f) Bromothymol blue indicator

Formula and preparation

Bromothymol blue	-	0.1g
(N/10) NaCL, 0.1mu/1	-	2.5g
Distilled water	-	45.5ml

Dissolve bromothymol blue in distilled water, mix well and store in the dark.

g) Phenol red indicator

Formula and preparation

Phenol red crystals	-	2.0g
Distilled water	-	30ml

Weigh 2g of phenol red into a beaker containing 30ml of distilled water, stir to dissolve chemical. Transfer to a clean bottle, and mix well.

h) Hydrogen peroxide reagents

Formula and preparation

H_2O_2, 3% aqueous solution

Protect from light and store in a cool place. Keep in bottle closed with a glass stopper.

i) Lactophenol cotton blue

Formula and preparation

Phenol	-	10.0 g
Cotton Blue (aniline blue)	-	0.04 g
Glycerol	-	20.0 g
Lactic Acid	-	10.0 ml

Distilled water - 10 ml

1) Weigh the cotton blue and dissolve in the water. Warming the water will help the stain to dissolve more quickly.
2) Weigh the Phenol in beaker and add the solution. Stir to dissolve the phenol. Transfer to a clean brown bottle.
3) Add the lactic acid and glycerol, and mix well.

J) 2N Ammonium hydroxide solution

Preparation

Measure out 150.2 ml of the 25 % solution and dilute to 1 litre.

This is a 2N solution

k) 8% Potassium ferricyanide solution

Potassium ferricyanide	8 g
Distilled water	100 ml

Dissolve 8 g of potassium ferricyanide in distilled water and volume madeup to 100 ml.

4 Amino antipyrine solution

l) 2% 4 Aminoantipyrine solution

4-aminoantipyrine	2 g
Distilled water	100 ml

2 g of 4-aminoantipyrine was dissolved in distilled water and volume was made up to 100 ml.

APPENDIX III

Protocol for chelating agents and nickel used in the assay

3a: Protocol for studying the effects of trisodium citrate on phenol degradation

Flask number	1	2	3	4	5	6
Phenol (ml)	2	2	2	5	5	5
TSC (ml)	0	12.5	25	0	12.5	25
Media (ml)	60	60	60	60	60	60
Distilled water (ml)	37	24.5	12	34	21.5	9
Culture (0.2 OD) (ml)	1	1	1	1	1	1
Reaction mixture Final volume	100	100	100	100	100	100
Phenol final conc (mg/ml)	200	200	200	500	500	500
TSC final conc (mg/ml)	0	50	100	0	50	100

3b: Protocol for studying the effects of nickel on trisodium citrate on utilization

Flask number	1	2	3	4	5	6	7
Nickel (ml)	0	0	0	5	12.5	5	12.5
TSC (ml)	0	12.5	25	12.5	12.5	25	25
Media (ml)	50	50	50	50	50	50	50
Distilled water (ml)	49	36.5	24	31.5	26	19	11.5
Culture (0.2 OD) (ml)	1	1	1	1	1	1	1
Reaction mixture Final volume	100	100	100	100	100	100	100
TSC conc (mg/ml)	0	50	100	50	50	100	100
Nickel conc (mM)	0	0	0	0.2	0.5	0.2	0.5

3c: Protocol for studying the effects of nickel and 100 mg/l trisodium citrate on 500 mg/l phenol degradation

Flask number	1	2	3	4	5	6	7	8	9	10
Phenol (ml)	5	5	5	5	5	5	5	5	5	5
TSC (ml)	0	0	0	0	0	25	25	25	25	25
Media (ml)	40	40	40	40	40	40	40	40	40	40
Distilled water (ml)	54	52.75	49	41.5	29	29	27.75	24	16.5	4
Nickel	0	1.25	5	12.5	25	0	1.25	5	12.5	25
Culture (0.2 OD) (ml)	1	1	1	1	1	1	1	1	1	1
Reaction mixture Final volume	100	100	100	100	100	100	100	100	100	100
Nickel final conc (mM)	0	0.05	0.2	0.5	1.0	0	0.05	0.2	0.5	1.0

Protocol for effect of chelating agents on *Pseudomonas* sp. in nutrient broth and mineral salt medium

Tube number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Water (ml)	1.4	1.3	1.2	1.1	1	0.9	0.4	1.3	1.25	1.2	1.15	1.05	0.9	0.65	0.4	0.15
C A (ml)	0	0.1	0.2	0.3	0.4	0.5	1	0.1	0.15	0.2	0.25	0.35	0.5	0.75	1	1.25
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Conc (mg/l)	0	20	40	60	80	100	200	400	600	800	1000	1400	2000	3000	4000	5000

Protocol for effect of chelating agents and nickel on *Pseudomonas* sp. after 24 in nutrient broth
Trisodium citrate 100 mg/l and nickel 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	0.9	0.89	0.88	0.876	0.872	0.864	0.86	0.82	0.78	0.74	0.7	0.66	0.62	0.58	0.54	0.5
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
TSC (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final volume (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Trisodium citrate 1000 mg/l and nickel 10 mM

Tube number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Water (ml)	1.15	1.14	1.13	1.126	1.122	1.114	1.11	1.07	1.03	0.99	0.95	0.91	0.87	0.83	0.79	0.75
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
TSC (ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final volume (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Trisodium citrate 2000 mg/l and nickel 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	0.9	0.89	0.88	0.876	0.872	0.864	0.86	0.82	0.78	0.74	0.7	0.66	0.62	0.58	0.54	0.5
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
TSC (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final volume (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Trisodium citrate 4000 mg/l and nickel 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	0.4	0.39	0.38	0.376	0.372	0.364	0.36	0.32	0.28	0.24	0.2	0.16	0.12	0.08	0.04	0
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
TSC (ml)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final volume (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Tannic acid 5 mg/l and nickel 4mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.375	1.35	1.325	1.315	1.305	1.285	1.275	1.175	1.075	0.975	0.875	0.775	0.675	0.575	0.475	0.375
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
TA (ml)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Tannic acid 10 mg/l and nickel 4 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.35	1.325	1.3	1.29	1.28	1.26	1.25	1.15	1.05	0.95	0.85	0.75	0.65	0.55	0.45	0.35
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
TA (ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Tannic acid 20 mg/l and nickel 4 Mm

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.3	1.275	1.25	1.24	1.23	1.21	1.2	1.1	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
TA (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Tannic acid 40 mg/l and nickel 4 mm

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.3	1.29	1.28	1.276	1.272	1.264	1.26	1.22	1.18	1.14	1.1	1.06	1.02	0.98	0.94	0.9
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
TSC (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Edta and nickel

EDTA 400 mg/l AND NICKEL 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.2	1.175	1.15	1.14	1.13	1.11	1.1	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
TA (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol(ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

EDTA 1000 mg/l and nickel 4 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.15	1.125	1.1	1.09	1.08	1.06	1.05	0.95	0.85	0.75	0.65	0.55	0.45	0.35	0.25	0.15
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
EDTA (ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

EDTA 2000 mg/l and nickel 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	0.9	0.89	0.88	0.876	0.872	0.864	0.86	0.82	0.78	0.74	0.7	0.66	0.62	0.58	0.54	0.5
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
EDTA (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

EDTA 4000 mg/l and nickel 10 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	0.4	0.39	0.38	0.376	0.372	0.364	0.36	0.32	0.28	0.24	0.2	0.16	0.12	0.08	0.04	0
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
EDTA (ml)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Potassium ferricyanide and nickel

Potassium ferricyanide 20 mg/l and nickel 4 mM

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1.3	1.275	1.25	1.24	1.23	1.21	1.2	1.1	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3
Metal(ml)	0	0.025	0.05	0.06	0.07	0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
$K_3Fe(CN)_6$(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Potassium ferricyanide 80 mg/l and nickel 10 mm

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water (ml)	1	0.99	0.98	0.976	0.972	0.964	0.96	0.92	0.88	0.84	0.8	0.76	0.72	0.68	0.64	0.6
Metal(ml)	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
$K_3Fe(CN)_6$	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Medium (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Culture (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final vol (ml)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Potassium ferricyanide 100 mg/l and nickel 10mm

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
water	0.9	0.89	0.88	0.876	0.872	0.864	0.86	0.82	0.78	0.74	0.7	0.66	0.62	0.58	0.54	0.5
metal	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
K_3FeCN6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
medium	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
culture	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
final vol	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Nickel 10mm

tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
water	1.4	1.39	1.38	1.376	1.372	1.364	1.36	1.32	1.28	1.24	1.2	1.16	1.12	1.08	1.04	1.0
metal	0	0.01	0.02	0.024	0.028	0.036	0.04	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4
medium	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
culture	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
final vol	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Key; CA = Chelating Agent, TSC = Trisodium citrate, EDTA = Ethylenediaminetetraacetic acid, $K_3Fe(CN)_6$ = Potassium ferricyanide, TA = tannic acid,Conc= Concentration (mg/L)

NB; To accommodate the concentrations chosen for the analysis, two stock chelating agent solutions were prepared, 400mg/l and 8000mg/l stock. Thus concentrations of 0-200mg/l were taken care of using the 400mg/l stock while 400-5000mg/l were taken care of using 8000mg/l stock.