BIOPRODUCTION AND ANTIBACTERIAL PROPERTIES OF SILVER NANOPARTICLES FROM BACILLUS THURINGIENSIS ISOLATED FROM SOIL

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NIGERIA.

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DEPARTMENT OF MICROBIOLOGY
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DECEMBER, 2021

DECLARATION

I declare that the work in this dissertation entitled 'BIOPRODUCTION AND ANTIBACTERIAL PROPERTIES OF SILVER NANOPARTICLES FROM BACILLUS THURINGIENSIS ISOLATED FROM SOIL'has been performed by me at the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

Esther Moradeke AFOLAYAN ______________________ ______________________
P17LSMC8038 Signature Date

CERTIFICATION

This thesis entitled 'BIOPRODUCTION AND ANTIBACTERIAL PROPERTIES OF SILVER NANOPARTICLES FROM BACILLUS THURINGIENSIS ISOLATED FROM SOIL' by Esther Moradeke AFOLAYAN meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University Zaria, and is approved for its' contribution to knowledge and literary presentation.

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ABSTRACT

Multidrug resistant (MDR) organisms have continued to evolve daily. Hence, the urgent need for more effective antimicrobials. The production of metallic nanoparticles, in particular silver nanoparticles (AgNPs) is crucial to the field of nanotechnology and medicine due to their known antimicrobial property. The use of microorganisms to synthesize nanoparticles has continuously been applauded as it proffers easier, cheaper and safer route for production of silver nanoparticles. This study sought to bioproduce AgNPs using *Bacillus thuringiensis* (Bt) isolated from soil and determine their antibacterial activity. Isolation, identification and confirmation of *Bacillus thuringiensis* was carried out by sodium acetate selection, biochemical and molecular methods respectively. Bioproduction of AgNPs was carried out by treating 1mL of *Bacillus thuringiensis* supernatant with 30mL of 1mM silver nitrate at 28^{o}C for 24 hours. Characterization of AgNPs was carried out using UV-Vis Spectroscopy, Fourier Transform Infrared (FTIR) and Scanning Electron Microscopy (SEM). The antimicrobial activities of the synthesized AgNPs were determined by agar well diffusion method against four potentially pathogenic bacteria. Of the 10 presumptive isolates, 6 were confirmed as Bt, the average Bt index was 0.33. The highest Bt index was recorded for samples from Cow Range (0.5) while the lowest (0.1) was from Farmland. The AgNPs have maximum absorbance at 434.5 and 440 nm for AgNPs from isolate CR2^{3} and MRS2^{1} respectively, with FTIR peaks at 3302 - 3379 cm^{-1}, 2137 cm^{-1}and 1643 cm^{-1} indicating that proteins were the capping and stabilization molecules in the synthesis of AgNPs. The particles were irregular/anistropic in shape. Inhibition of *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pnemoniae* was achieved at 25µg/mL with MIC between 25-50 µg/mL and MBC of 75-100 µg/mL. This study confirms that *Bacillus thuringiensis* isolated from soil can mediate synthesis of silver nanoparticles and act as source of antimicrobial agent to MDR bacteria.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The use of silver and silver salts have been from the inception of human civilization but the fabrication of silver nanoparticles (AgNPs) has only recently been recognized. They have been specifically used in agriculture and medicine as antibacterial, antifungal and antioxidants (Siddiqi *et al.*, 2018).

Nanoparticles (NPs) are particles having one or more dimensions in the nanometer range (Thakkar *et al.*, 2010). NPs can be natural (nucleic acids and carbon nanotubes), inorganic (silver, gold, and palladium) and composite (nano-shells, nano-bars, nano-wires, nano-gels and nano-emulsions) in nature (Arshad, 2017). Nanoparticles can be produced by two methods, bottom-up and top-down approach. The former involves the buildup of a material from the bottom: atom by atom, molecule by molecule or cluster by cluster while the latter is the slicing or successive cutting of a bulk material to get nano-sized particle (Husen and Siddiqi, 2014). The physical characteristics of NPs may vary from those of their parent materials (Zargar *et al.*, 2011). NPs have a large surface area to volume ratio unlike their bulk counterpart, therefore having advantageous applications; such as being a potential antimicrobial agent at low concentration and hence have attracted great attention (Kato, 2011; Saxena *et al.*, 2012). There are different nanomaterials having several structures and properties with extensive applications in different aspects of our life such as science, technology, medicine, industry, and environment (Shameli *et al.*, 2012).

Biological synthesis of NPs combines biological principles (i.e. reduction/oxidation) by microbial enzymes with physical and chemical approaches to produce nano-sized particles**.** Biosynthesis can be classified into intracellular and extracellular synthesis based on the site of nanoparticle synthesis (Mann, 2001; Yurtluk *et al*., 2018). For the intracellular method, ions are transported into the microbial cell to form nanoparticles in the presence of enzymes while the extracellular synthesis involve trapping the metal ion on the surface of the microbial cell and reducing ions to form nanoparticles in the presence of an enzyme; nitrate reductase (Zhang *et al*., 2012). Extracellular synthesis is preferred because prior removal of the bacterial cells simplifies recovery of the nanoparticles (Singh *et al*., 2015). In the bacterial synthesis of AgNPs, NADH and NADH–dependent enzymes especially nitrate reductase is induced by $AgNO_3$ ions and reduces the silver ions to form AgNPs (Singh *et al*., 2015). In addition to nitrate reductase, some other peptides/proteins also contribute to the reduction of Ag^+ to AgNPs (Yurtluk *et al*., 2018).

Silver nanoparticles (AgNPs) are currently the most important candidates of choice for solving various medical problems due to their biocompatibility, inertness, oxidation resistance and wide spectrum of antimicrobial activity against diverse range of bacteria and fungi **(**Krishna *et al.*, 2017).

Silver ions and silver nanoparticles (AgNPs) have inhibitory and lethal effects on several bacterial species. The formation of complexes for silver ions is limited and their effect as antimicrobial agent remains only for a short period (Méndez- Vilas, 2011). This has been resolved by the application of the silver nanoparticles which have greater antibacterial properties, promoting the synthesis of reactive oxygen species such as hydrogen peroxide (Mohammed, 2015).

AgNPs have been used in different ways such as in the treatment of burns, dental materials, textile fabrics, water treatment, and antimicrobial paint and as sunscreen lotions (Duran *et al*., 2007; Kumar *et al*., 2008). Oei *et al*. (2012) also reported their uses in biomedicine due to their anti-fungal, anti-inflammatory, and anti-permeability activities. Nanoparticles of silver have been studied as medium for antibiotic delivery and for the synthesis of composites used for disinfecting filters and coating materials (Jain and Pradeep, 2005).

Bacillus thuringiensis of the Division Firmicutes is a ubiquitous Gram-positive, sporulating rod-shaped bacterium. It has been isolated worldwide from a great diversity of ecosystems including soil, water, dead insects, and dusts from silos, diverse conifers, and insectivorous mammals (Fenandez-chapa *et al*., 2019; Malovichko *et al*., 2019).

Silver nanoparticles (AgNPs) synthesized from *Bacillus thuringiensis* is different from other biological approaches. This is because the tolerance capacity of this bacterium is higher than that of certain other bacterial groups like *Bacillus subtilis, Bacillus vulgaris* and *Escherichia coli* (Kumar, 2014). Pourali *et al*. (2016) also reported that after screening several soil samples for microorganisms with the ability to produce AgNPs, *Bacillus thuringiensis* showed the highest potential. Jain *et al.* (2010a) have also reported the swift mediation of AgNPs by *B. thuringiensis* spore crystal mixture.

1.2 Statement of Research Problem

In present times, high-scale production of nanomaterial is an inevitable requirement for our society. In order to meet this demand, many methods of synthesizing AgNPs have been documented such as laser ionizing irradiation, beam electron irradiation, solvothermal and sonochemical methods (Li and Zhang, 2010; Gasaymeh *et al*., 2010; Wani and Ahmad, 2013 and Ahmad *et al.,* 2013). These methods, especially the chemical synthesis of NPs need high pressure, temperature, use of toxic chemicals and are also quite expensive (Ingle *et al*., 2009; Nabikhan *et al*., 2010; Gnanadesigan *et al*., 2012; Ragaa *et al*., 2018). The residual toxic chemicals may accumulate on the NP with potential adverse effects when used for medical purposes. The requirement of high energy is fulfilled by fossil fuels burning; which releases greenhouse gases (GHGs) (Dorcheh and Vahabi, 2016). These have adverse effect on the environment and human health, hence the need for ecofriendly and sustainable methods for the synthesis and assembly of nanoparticles (Kowshik *et al*., 2002).

The increase in the emergence and the spread of antimicrobial resistance is a major global public health threat that is gaining more attention. Developing countries such as Nigeria are facing a huge public health and economic burden from various diseases caused by superbugs. In addition to the search for new drug leads from natural products, studies have demonstrated the potential of nanoparticles in treating infections caused by multidrug resistant pathogens (Pal *et al*., 2007; Awosan *et al*., 2018). The antimicrobial activity of silver nanoparticles against multidrug resistant (MDR) bacteria has also been reported (Zaidi *et al*., 2017; Farouk *et al.*, 2020).

1.3 Justification for the Research

Increase in demand for NPs has necessitated for large-scale production with safer and cheaper alternatives, this has led to the development of economic, non-toxic and eco-friendly methods which reduces energy intake, greenhouse gases and hazardous waste production (Ahmed and Ikram, 2016).

Microorganisms such as bacteria and fungi are mostly preferred for NP biosynthesis due to their fast rate of growth, ease of cultivation and their ability to grow at ambient conditions of temperature, pH and pressure (Fariq *et al*., 2017). The “biogenic” approach is further supported by the fact that the majority of the bacteria inhabit ambient conditions of varying temperature, pH, and pressure (Wei *et al.*, 2012). The NPs generated by biological processes have higher catalytic activity, greater specific surface area, and an improved contact between the enzyme nitrate reductase and metal salt due to the bacterial carrier matrix (Bhattacharya and Mukherjee, 2008).

Many microbes are known to produce nanostructured mineral crystals and metallic nanoparticles with properties similar to chemically synthesized structure while exercising strict control over structure, size and composition of particles (Jain *et al*., 2010). Among the prokaryotes, bacteria have gained high attention for the synthesis of nanoparticles. The use of microorganisms for the bioproduction of nanoparticles also has an advantage of a mass production which is not seasonal unlike what is obtainable from plants. These biosynthesized nanoparticles are also known to be toxic against multidrug resistant organisms (Jain *et al*., 2010).

Silver nanoparticles (AgNPs) have proved to be the most effective of the metallic NPs, with broad spectrum of antimicrobial activity which encourages their use in biomedical applications, water and air purification, food production, cosmetics, clothing and numerous household products (Marambio-Jones and Hoek, 2010).

Pourali *et al*. (2016) analyzed different soil samples for bacterial strains with the highest ability of AgNPs production. Two of their isolates with the highest ability of AgNPs production were identified by PCR to be *B. thuringiensis* and *Enteobacter cloacae.* Several soil microorganisms, such as *Bacillus* spp SBT8, *B. thuringiensis*, *Enterobacter cloacae*, *Pseudomonas* spp, and *Bacillus safensis*, have been isolated for extracellular synthesis of AgNPs (Lateef *et al*., 2014; Pourali *et al*., 2016; Yurtluk, 2018). Therefore, this research focus on the bio-production of AgNPs, using the most efficient AgNPs producing *B. thuringiensis* isolated from soil, characterization of AgNPs and assessing its antimicrobial activity on some selected pathogenic bacteria. The research findings could contribute to the commercial bioproduction of AgNps locally to address the public health problem of antimicrobial resistance locally and globally.

1.4 Aim of the Study

The aim of this study was to bio-produce silver nanoparticles using *Bacillus thuringiensis* isolated from soil and evaluate its antibacterial activity.

1.5 Objectives of the Study

The objectives of this research were to:

1. isolate and characterize *Bacillus thuringiensis* from Soil samples.
2. screen and select the *Bacillus thuringiensis* isolate with the highest potential for silver nanoparticle production.
3. bio-produce silver nanoparticles using the selected *Bacillus thuringiensis* isolates.
4. characterize the silver nanoparticles produced.
5. determine the antibacterial activity of the silver nanoparticles produced on some potentially pathogenic bacteria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Nanotechnology

The word nanotechnology was introduced by Prof. Norio Taniguchi of Tokyo Science University in 1974 while the concept of nanotechnology was first presented by Richard Feynman through his lecture, titled "There's a plenty of room at the bottom" at the American Institute of Technology (Hulkoti and Taranath, 2014). Nanotechnology is a field of science that involves the synthesis, characterization, application of structures, devices, and systems by controlling shape and size at the nanometer scale (Madhuri *et al*., 2012; Edhaya Naveena and Prakash, 2013). Nanotechnology also involves synthesis of nanoparticles of size ranging from 1 to 100 nm (Adlakha-Hutcheon *et al*., 2009). This technology involves a unique combination of scientists from different fields including physicists, chemists, engineers and biologists (Saklani *et al*., 2012).

Nanotechnology, alongside nanostructured materials, play an ever increasing role in science, research and development as well as in every day's life, as more and more products based on nanostructured materials are introduced to the market (Dash, 2013). In the past few years nanotechnology has taken leaps and proven to be of potential advantage to health care. Nanotechnologies also have a huge potential to bring benefits in areas such as drug development, water decontamination, information and communication technologies, and the production of stronger, lighter materials. Human health-care nanotechnology research can definitely result in immense health benefits. The genesis of nanotechnology can be traced to the promise of revolutionary advances across medicine, communications, genomics, and robotics. A complete list of the potential applications of nanotechnology is too vast and diverse to discuss in detail, but without doubt, one of the greatest values of nanotechnology will be in the development of new and effective medical treatments (Merehan, 2013).

2.1.1 Nanotechnology in Nigeria

According to nanotechnology research and innovation forum 2020, Egypt is currently the top nanotechnology research country in Africa, while South Africa is the African country which has filed the most patents and established the most nanotechnology companies and institutions. The South African Nanotechnology Initiative is a national network of academic researchers involved in areas such as nanophase catalysts, nanofiltration, nanowires, nanotubes, and quantum dots. In the guardian news in August 13th, 2020 the minister of Science and Technology in Nigeria Dr. Ogbonnaya Onu has said that Nigeria was set to domesticate nanotechnology to enhance the socioeconomic development in the country. He said this when he received the report of National Steering Committee on Nanotechnology (the committee was inaugurated in 2018) policy at the ministry headquarters in Abuja on 11th August, 2020. He mentioned that Nigeria must fully embrace and domesticate nanotechnology as well as commence further research to enable it to be in leadership role in areas of technology and revamp the health sector. He also encouraged more patents as he said that was one yardstick for assessing progress in scientific research and also that it will encourage more youth to be interested in science. The earliest record of effort made to domesticate the technology was in 2006 when the Nigeria Nanotechnology Initiative (NNI) was spearheaded by the National Agency for Science and Engineering Infrastructure (NASENI) (Adewoye, 2006). Although nanotechnology research is at its infancy in Nigeria, there exist some form of government support, and national funding programs are being developed (Alo, 2017). Very few Nanotechnology studies have been carried out in Nigeria in comparison to other countries such as India, USA, and China. An Annual report by StatNano (2020) ranked Nigeria (529 articles) 48th out of 63 countries based on Number of Indexed Nanotechnology articles while China, USA and India were the top three in the world (78314, 24425 and 17212 articles respectively). Ekekwe (2010) advocated that nanotechnology was advancing with potentials to radically affect key aspects of human existence. He presented that it was an emerging technology that is transforming industries like electronics, materials and medicine. It also has capabilities for low cost, high efficiency and high capacity in tools, industrial processes and products.

Many Scholars expect it to be significant as the steam engine, the transistor, and the internet in terms of societal impacts (Ekekwe, 2010). The significance of nanotechnology has manifested in increase in commercial interest which is growing exponentially across a range of industries (Ekekwe, 2010).

2.2 Nanoparticles

The prefix “nano” in the term nanotechnology is derived from a Greek word nanos, which means “dwarf”. Tolochko (2015) presented that nanoparticles are characterized at least in one of three measurements by nanometer scale concerning both the sample of a material as a whole and its structural elements. One nano meter is extremely small length corresponding to one billionth of 1 meter (m), one millionth of 1 millimeter (mm), or one thousandth of 1 micrometer (μm) (Hosokawa *et al*., 2012). Nanoparticles are interesting nanoscale systems because of the ease with which they can be produced in different shapes (Hulkoti and Taranath, 2014).

Metal particles in the nanometer size range exhibit physical properties that are different from both the ion and the bulk material. This makes them exhibit remarkable properties such as increased catalytic activity due to morphologies with highly active facets (Singh *et al.*, 2008).

Nanoparticles exhibit novel properties which depend on their size and morphology which enable them to interact with plants, animals and microbes (Siddiqi and Husen, 2017) and impart enhancements to engineered materials, including better magnetic properties, improved electrical activity, and increased optical properties (Prabhawathi *et al.*, 2014; Rasulev *et al.*, 2014). Nanoparticles are being currently used as electronics, optoelectronics, in biomedical, environmental, material and energy related areas, as cosmetics, pharmaceuticals, and catalysts (Siddiqi *et al.*, 2018).

2.2.1 Silver Nanoparticles

Silver nanoparticles are one of the promising products in the nanotechnology industry. The development of consistent processes for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. Silver nanoparticles can be synthesized by several physical, chemical and biological methods (Dell'Aglio *et al*., 2015; Siddiqi and Husen 2017).

Over the years, various rapid chemical methods have been replaced by green synthesis to increase quality and reduce toxicity. Silver nanoparticles have unique optical, electrical, and thermal properties and are incorporated into products that range from photovoltaic to biological and chemical sensors, including pastes, conductive inks and fillers which utilize silver nanoparticles for their high electrical conductivity, stabilization and low sintering temperatures. There is also an increase application in the use of silver nanoparticles for antimicrobial coatings, and many textiles, wound dressing, and biomedical devices contain silver nanoparticles that continuously release a low level of silver ions to provide protection against bacteria (Siddiqi and Husen 2017).

2.2.2 Production Approaches of Nanoparticles

Nanotechnology involves the precise manipulation and control of atoms and molecules, to create novel materials with properties controlled at the nanoscale; billionths of a meter. There exist a large number of techniques available to synthesize different types of nanomaterials (Kulkarni, 2015). The intersection of various fields in nanotechnology has culminated in the development of physical, chemical, biological and hybrid techniques to synthesize nanoparticles. Owing to the wide range of applications offered by nanoparticles in different fields of science and technology, different protocols have been designed for their synthesis (Rai *et al.*, 2011). Ramsden (2011) reported that one can use either a top- down or bottom-up approach. The decision on which to adopt depends, of course, on which can deliver the specified properties, and on cost.

2.2.2.1 Top-down approach

The “top-down” approach involves slicing or successive cutting of a bulk material to get nano-sized particle. However, the “top-down” approach generally works with the material in its bulk form, and the size reduction to nanoscale is achieved by specialized controlled physical conditions, for instance thermal decomposition, mechanical grinding, etching, cutting, and sputtering. The main demerit of the top-down approach is the surface structural defects. Such defects have significant impact on the physical features and surface chemistry of metallic nanoparticles (Sidiqqi *et al.,* 2018). Another limitation of the technique is that it produces simpler structures than the bottom-up approach. More importantly, both approaches can work within both biological and non-biological systems, bridging important divides between the biological and non-biological worlds (Horton and Khan, 2006).

Preparation of nanoparticle materials can be carried out by physical methods, such as: CO_2 laser radiation, spray drying, physical vapor condensation, electrical explosion wire, energy ball milling method, microwave pulsed electron evaporation, arc discharge, laser ablation and current magnetron sputtering (Kotov *et al.*, 2004; Okuyama *et al.*, 2006; Abou El-Nour *et al.*,2010; Bagazeev *et al.*, 2010; Wright *et al.*,2011; Ferrer *et al.*, 2012; Sokovnin *et al.*, 2013; Chaitoglou *et al.*, 2014; Dell'Aglio *et al.*, 2015). Physical methods do not require lethal and highly reactive chemicals, generally have a fast processing time and does not produce byproducts as impurities. The physical method is one of the best ways to prepare the pure colloid or nanoparticle. Physical methods have another advantage over chemical methods in that the AgNPs produced have a narrow size distribution, while the main demerits are consumption of high energy and cost (Asanithi *et al.*, 2012; Natsuki *et al.*, 2015). The most prospective physical method for synthesis AgNps is high energy ball milling. The physical method for AgNps synthesis needs physical or mechanical energy. It is commonly used for preparing AgNps powder in the narrow size.

2.3.2.2 Bottom-up approach

The "bottom-up" approach involves the buildup of a material from the bottom: atom by atom, molecule by molecule or cluster by cluster (Husen and Siddiqi, 2014). The "bottom-up" approach is usually a superior choice for the nanoparticles preparation involving a homogeneous system wherein catalysts (for instance, reducing agent and enzymes) synthesize nanostructures that are controlled by the catalyst itself (Siddiqi *et al.*, 2018). This method involves physically manipulating small numbers of the basic building blocks, either individual atoms or more complex molecules, into structures typically using minute probes. This technology is limited to low-volume, high-value applications such as high-performance chip manufacture, but the range of bottom-up techniques and the areas of application are growing rapidly (Horton and Khan, 2006).

2.3.2.2.1 Chemical method

Chemical method of synthesis can be subdivided into chemical reduction, electrochemical, irradiation assisted chemical and pyrolysis methods (Zhang *et al*., 2012). AgNPs synthesis in solution requires metal precursor, reducing agents and stabilizing or capping agent. Commonly used reducing agents are ascorbic acid, alcohol, borohydride, and sodium citrate and hydrazine compounds. Sotiriou and Pratsinis (2010) have shown that the AgNPs supported on nanostructured SiO_2 were obtained by flame aerosol technology, which allows close control of silver content and size. Also, silver/silica nanoparticles with relatively narrow size distribution were obtained by flame spray pyrolysis (Sotiriou *et al*., 2011).

2.3.2.2.2 Biological method

Biological synthesis of AgNPs from herbal extract and/or microorganisms has appeared as an alternative approach with several advantages over the chemical and physical methods of synthesis. It is also a well-established fact that these routes are simple, cost-effective, eco-friendly and easily scaled up for high yields (Husen and Siddiqi, 2014). Biosynthesis of metal and metal oxide nanoparticles using biological agents such as bacteria, fungi, yeast, plant and algal extracts has gained popularity in the area of nanotechnology (Siddiqi and Husen, 2017; Siddiqi *et al*., 2016; Siddiqi and Husen 2017). Extracellular synthesis of AgNPs comprises of the trapping of metal ions on the outer surface of the cells and reducing them in the presence of enzymes or biomolecules, while intracellular synthesis occurs inside the microbial cells. It has been suggested that the extracellular synthesis of nanoparticles is cheap, favors large-scale production and requires simpler downstream processing. Thus, the extracellular method for the synthesis of nanoparticles is preferable (Duran *et al.*, 2007) in comparison to the intracellular method. Ganesh Babu and

Gunasekaran (2009), and Kalimuthu *et al.* (2008) have demonstrated that the intracellular synthesis requires additional steps, for instance ultrasound treatment or reactions with suitable detergents to release the synthesized silver nanoparticles.

2.3.2.2.2.1 Plant synthesized nanoparticles

Plant-mediated biosynthesis of nanoparticles is considered a widely acceptable technology for rapid production of metallic nanoparticles for successfully meeting the excessive need and current market demand as it results in a reduction in the use or generation of hazardous substances to public health. Similar to microbes which have been used as “bio-factory” in the synthesis of metallic nanoparticles, plants are also the natural “chemical factories” which are economical and require minimal maintenance (Nyoman *et al*., 2013). In plants or plant derived materials, a wide range of metabolites with redox potentials are determined, which play a principal role as a reducing agent in the biogenic synthesis of nanoparticles. In comparison to the microbial synthesis of nanoparticles, highly stable nanoparticles are synthesized by plant or plant extracts with higher rate of production. The advantages of plant-mediated preparation of metal nanoparticles lead researchers to search of further exploration of the bio-reduction mechanism of metal ions by plants and the possible mechanism of formation of metal nanoparticle in and by the plants (Ahmad and Sharma, 2012). In recent years, biosynthesis of metal nanoparticles, especially silver and gold nanoparticles, using plant extracts as nano-factories becomes an important subject of researches in the field of bio-nanotechnology (Iravani, 2011). Generally, the bio-reduction mechanism of metal nanoparticle in plants and plant extracts includes three main phases (Makarov *et al*., 2014). The activation phase in which the reduction of metal ions and nucleation of the reduced metal atoms occur. The growth phase, referring to the spontaneous coalescence of the small adjacent nanoparticles into particles of a larger size, accompanied by an increase in the thermodynamic stability of nanoparticles, or a process referred to as Ostwald ripening and the termination phase in which the final shape of the nanoparticles formed.

2.3.2.2.1.2 Algae Synthesized Nanoparticles

Eco-friendly reducing agent, particle-stabilizing capping agent and environmentally acceptable solvent system are the three principal criteria for totally green metallic nanoparticle synthesis (Sau and Murphy, 2004). Algae-mediated biological synthesis of metal nanoparticles is one of them. Algae are eukaryotic aquatic oxygenic photoautotrophs (Castro, 2013). Bioreduction of algae showed large potential in the development of clean green synthesis of different metallic and metal oxide nanoparticles, such as gold, silver, platinum, palladium, copper oxide, zinc oxide, cadmium sulfate, among others (Lengke *et al*., 2006; MubarakAli *et al*., 2012; Jena *et al*., 2013; Azizi *et al*.,2014; Momeni and Nabipour, 2015). Although, quite a number of algae have been found capable of synthesizing different NPs, methodologies for controlling the size and shape of the products, and the identification of the principles’ involved, is still incomplete (Sau and Murphy, 2004).Therefore, many researchers are interested in exploring the biological synthesis of algae mediated nanoparticles.

2.3.2.2.1.3 Fungi synthesized nanoparticles

Nanoparticles can be obtained using fungi compared to bacteria, fungi could be used as a source for the production of large amount of nanoparticles because they can secret larger amounts of proteins which directly translate to higher productivity of nanoparticles (Mohanpuria *et al*., 2008). Yeast, belonging to the class ascomycetes of fungi has shown to have good potential for the synthesis of nanoparticles. The extracellular secretion of the microorganisms offers the advantage of obtaining large quantities in a relatively pure state, free from other cellular proteins associated with the organism with relatively simpler downstream processing. The hypothesis indicated that proteins, polysaccharides and organic acids released by the fungus were able to differentiate different crystal shapes and were able to direct their growth into extended spherical crystals (Balaji *et al*., 2009). Promising synthesis of nanoparticles appears by the use of specific enzymes secreted by fungi. This would lead to the possibility of genetically engineering microorganisms to over express specific reducing molecules and capping agents and thereby control the size and shape of the biogenic nanoparticles (Balaji *et al*., 2009). The mechanism of silver nanoparticle production by fungi is said to follow the following steps: trapping of Ag+ ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system (Mukherjee *et al.*, 2001).The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. Though the exact mechanism involved in silver nanoparticle production by fungi is not fully deciphered, it is believed that the abovementioned phenomenon is responsible for the process.

2.3.2.2.1.4 Actinomycetes synthesized nanoparticles

Actinomycetes are microorganisms that share some characteristics of fungi and prokaryotes such as bacteria. Actinomycetes have been generally used for the synthesis of extracellular enzymes and secondary metabolites (Manimaran and Kannabiran, 2017). They have also been adopted for the biosynthesis of nanoparticles as they have unsurpassed capacity for the production of various bioactive compounds and contain high protein content. Actinomycetes synthesize nanoparticles via both intracellular and extracellular pathway, but extracellular reduction is the most common pathway and has more commercial applications in different fields. Intracellular biomineralization of silver ions was thought to be the result of enzymes present on the cell wall, resulting in production of silver nuclei (Gahlawat and choudhury, 2019). In an effort to elucidate the mechanism or the processes favouring the formation of nanoparticles with desired features, Ahmad *et al*. (2003) studied the formation of monodisperse gold nanoparticles by *Thermomonospora sp.* and concluded that extreme biological conditions such as alkaline and slightly elevated temperature conditions were favorable for the formation of monodisperse particles. Based on this hypothesis, alkali tolerant actinomycete, *Rhodococcus sp,* has been used for the intracellular synthesis of monodisperse gold nanoparticles (Ahmad *et al*. 2003). In this study, it was observed that the concentration of nanoparticles were more on the cytoplasmic membrane. This could have been due to the reduction of metal ions by the enzymes present in the cell wall and on the cytoplasmic membrane.

2.3.2.2.1.5 Virus Synthesizied Nanoparticles

An interesting property of viruses is their thick outer surface coating of capsid proteins which provide a highly suitable platform for interaction with metallic ions (Kobayashi *et al*., 2012). These protein cages can build monodispersed units that are highly robust and mouldable through genetic engineering. Viruses can be modified to serve as templates for material deposition or engineered to create three-dimensional vessels for targeted drugs delivery (Zeng *et al*., 2013).Viruses can be employed for the synthesis of nano-conjugates and nanocomposites with metal nanoparticles which are important bioengineering materials in drug delivery and cancer therapy. The plant viruses are being proved to be safe for nanotechnology applications due to their structural and biochemical stability, ease of cultivation, non-toxicity and non-pathogenicity in animals and humans. In one study, low concentrations of tobacco mosaic virus (TMV) and bovine papilloma virus (BPV) were used as additives along with extracts of various plants i.e *Nicotiana benthamiana, Avena sativa* and *Musa paradisiaca* (Love *et al*., 2017). The TMV and BPV not only helped in the reduction of size, but also significantly enhanced the numbers of the nanoparticles in comparison to the non-virus control. Cao *et al*. (2014) employed red clover necrotic mosaic virus (RCNMV) for the synthesis of nanoparticles for the controlled delivery of doxorubicin drug for chemotherapy. Le *et al*. (2017) investigated the potential of potato virus X nanoparticles for the delivery of doxorubicin drug for cancer treatment. Potato virus X has the capability to synthesize elongated filamentous nanoparticles which exhibit enhanced penetration power in comparison to spherical ones. However, synthesis of nanoparticles by viruses still faces various drawbacks such as involvement of host organism for protein expression, under-developed processes for synthesis and limited research on large scale application.

2.3.2.2.1.6 Bacteria Synthesized Nanoparticles

According to Pantidos and Horsfall (2014), researches have focused on Bacteria as a means to synthesize metal nanoparticles due to their abundance in the environment and their ability to adapt to extreme condition. The synthesis of nanoparticles using bacteria usually involves the intracellular and extracellular synthesis. In the intracellular method, the bacterial cell filtrate is treated with metal salt solution and kept in a shaker in dark or light conditions at ambient temperature and pressure conditions (Rai, *et al,* 2011) while in the extracellular synthesis of nanoparticles using bacteria, the bacterial culture is centrifuged and the supernatant is mixed with metal salt solution (Rai, *et al*., 2011).

The first evidence of silver nanoparticles synthesis using bacteria was that of *Pseudomonas stuzeri A*g 259 which was isolated from a silver mine (Ingale and Chauhari, 2013; Bansal *et al*., 2014;). When *Pseudomonas stuzeri* was added to silver nitrate solution, it produced silver nanoparticles. By taking a cue from this, researchers have since established the viability of using numerous bacteria such as *Escherichia coli, Lactobacillus* Species, *Klebsiella pneumoniae, Streptococcus thermophiles, Bacillus spp SBT8, Bacillus safensis and Staphylococcus aureus* to synthesize nanoparticles (Ojo *et al*., 2016; Sabri *et al*., 2016; Yurtlurk *et al*., 2018). Prakash *et al*. (2010)

reported using *Bacillus megaterium* for the extracellular synthesis of silver, lead and cadmium nanoparticles with sizes in the range of 10-20nm. Another species, *Bacillus laterosporus* was used to synthesize selenium nanoparticles of spherical shape with size ranging from 40-70nm (El-Batel *et al*., 2014).

The most widely accepted mechanism of silver biosynthesis is the presence of the nitrate reductase enzyme. In in-vitro synthesis of silver using bacteria, the presence of alpha-nicotinamide adenine dinucleotide phosphate (NADPH) dependent nitrate reductase would remove the downstream processing step that is required in other cases. During the reduction, nitrate is converted into nitrite and the electron is transferred to the silver ion; hence, the silver ion is reduced to silver (Ag^+ to Ag^0). This has been said to be observed in *Bacillus licheniformis* which is known to secrete NADPH and NADPH-dependent enzymes like nitrate reductase that effectively converts Ag^+ to Ag^0 **(**Vaidyanathan *et al*., 2010).

The mechanism was further confirmed by using purified nitrate reductase from *Fusarium oxysporum* and silver nitrate along with NADPH in a test tube, and the change in the color of the reaction mixture to brown and further analysis confirmed that silver nanoparticles were obtained (Kumar *et al*., 2007). There are also cases which indicate that there are other ways to biosynthesize silver nanoparticles without the presence of enzymes. It was found that dried cells of *Lactobacillus sp.* A09 can reduce silver ions by the interaction of the silver ions with the groups on the microbial cell wall (Fu *et al* ., 2000). The ionized carboxyl group of amino acid residues and the amide of peptide chains were the main groups trapping $(Ag (NH_4)^{2+}$ onto the cell wall and some reducing groups such as aldehyde and ketone were involved in subsequent bio reduction. However, it was found that the reaction progressed slowly and could be accelerated in the presence of OH (Fu *et al*., 2006).

2.3.2.2.1.6.1 Biosynthesis of AgNPs Using *Bacillus* Spp

Quite a number of studies have reported the synthesis of AgNPs by reduction of Ag^+ from aqueous solution of $AgNO_3$ extracellularly or intracellularly using Bacillus *spp* as shown in Table 2.1.

Table 2.1: Reports of several authors on the synthesis of AgNP using *Bacillus* spp

Organism	Location of synthesis	Size of NPs(nm)	Reference
***Bacillus* strain CS 11**	Extracellular	42-92	Das *et al.* (2014)
***Bacillus safensis*, extracellular keratinase**	Extracellular	5-30	Lateef *et al.* (2014)
Bacillus pseudomycoides	Extracellular		Agrawal and kulkarni (2017)
Bacillus cereus	Extracellular	4-5	Ganesh-Babu and Gunasekaran (2009)
Bacillus pumilus	Extracellular	20.12-29.49	Mahmoud *et al*. (2016)
Bacillus methylotrophicus	Extracellular	10-30	Wang *et al.* (2015)
Bacillus licheniformis	Intracellular	50	Kalimuthu *et al*. (2008)
Bacillus thuringiensis	Extracellular	92-142	Bamu *et al*. (2014)
Bacillus thuringiensis	Extracellular	15	Jain *et al.* (2010)
Bacillus thuringiensis	Extracellular	420-1000	Dash (2013)
Bacillus thuringiensis	Intracellular	17-21	Kumar (2014)

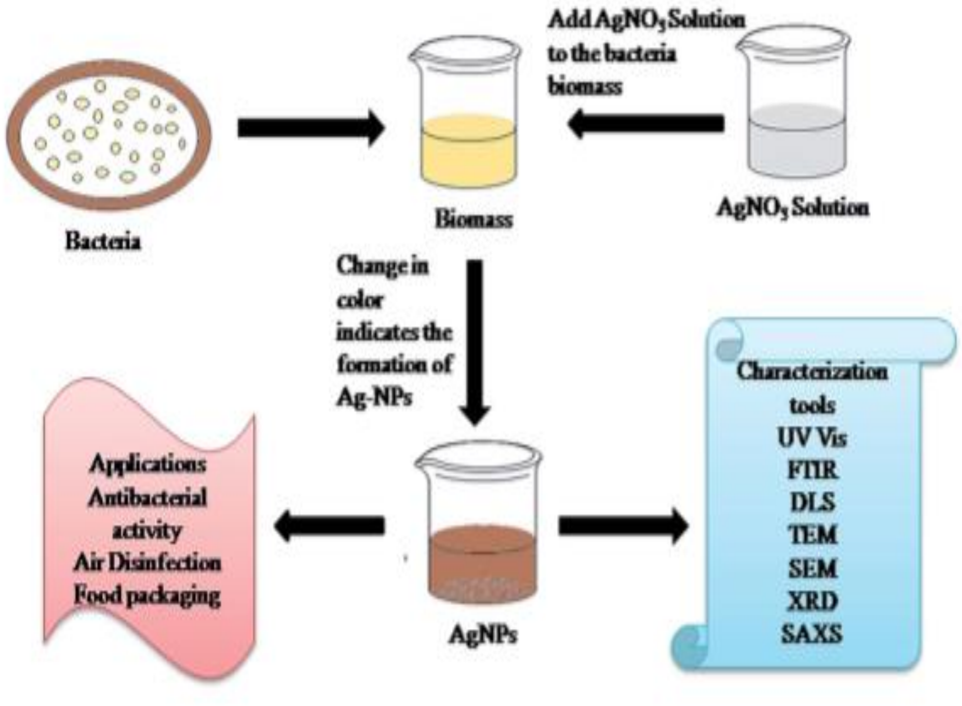

Figure 2.1: Flowchart of biological synthesis of silver nanoparticles from bacteria.
Source: Tarannum *et al*. (2019).

2.4 Genus *Bacillus*

According to UK standard for microbiology investigation 2018, Bacillus is a very diverse genus with 268 species and the bacteria belonging to this genus are rod-shaped, often arranged in pair or chains with rounded or square end with a single endospore. Bacillus has been divided into three morphological groups based on spore shape and swelling of the sporangium (Berkeley and Logan 1997; UK standard for microbiology investigation (UKSMI) 2018). Group I is characterized by the presence of ellipsoidal spores that do not swell the mother cell (Priest 1993). This group comprises a large number of species living in soil such as *Bacillus thuringiensis*, *Bacillus sphaericus, Bacillus subtilis, Bacillus anthracis,* and *Bacillus cereus*. Some of these species are very closely related and form different groups within the group I. One of these subgroups includes the *B. cereus* group.

2.4.1 *Bacillus cereus* Group

The *B. cereus* group includes; *B. cereus, Bacillus mycoides, Bacillus thuringiensis, Bacillus anthracis, Bacillus pseudomycoides,* and *B. weihenstephanensis* (Helgason *et al*. 2000; Chen and Tsen, 2002). The first three species are considered as subspecies of *B. cereus* because they are closely related (Leonard *et al*. 1997). The genetic and phenotypic characteristics of *B. thuringiensis* are very similar to *B. cereus* (Priest, 2000).

2.4.1.1 *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a Gram-positive, rod shaped, spore forming saprophytic soil bacterium that was first isolated from diseased larvae of *Bombyx mori* (an economically important insect, called the silkworm) in Japan (Ishiwata, 1901). The author named it Bacillus sotto, which means soft and flaccid, in reference to the appearance of the infected larvae (Fernández-Chapa *et al*., 2019). He noted that young bacterial cultures were not pathogenic to larval insects, in contrast old cultures that were sporulating were highly toxic. However, the first valid description was in 1911, when German scientist; Ernst Berliner, isolated the organism from diseased larvae of the flower moth *Anagasta kuehniella*. He named it *Bacillus thuringiensis*, which derives from Thuringia, the German town where the moths were found (Melo *et al*., 2016). Bt is a ubiquitous bacterium with a large enzyme complement, which allows it to be found in a variety of sites, such as: soil, insects and their habitats, stored products, plants, forest, and aquatic environments. It can remain latent in the environment even in adverse conditions for its development (Azevedo *et al*. 2000; Fiuza, 2001).

Under aerobic conditions, Bt grows in a simple culture medium such as nutrient broth. After nutrients are depleted, it produces a spore along with one or several parasporal crystals. When the spore matures, cells lyse and spores are free and crystals are released into the environment (Asano *et al*. 2003). This bacterium has filamentous appendages (or pili) on the spores (Des Rosier and Lara, 1981; Smirnova *et al*., 1991; Zelansky *et al.*, 1994). Colonies have a dull or frosted glass appearance, with fried egg appearance and often with an undulated margin from which extensive outgrowths do not develop (Sneath 1986; Rampersad and Ammons, 2005).

During sporulation Bt produces one or more proteinaceous parasporal crystals (cry). Globally more than 770 cry genes distributed in about 74 classes have been described. The nomenclature of the cry genes encoding Bt cry proteins was first published by Crickmore *et al*. (1998) and has been constantly updated. According to the recently revised nomenclature, all the cry genes have now been regrouped into 16 classes (Crickmore *et al.*, 2020).

2.5 Isolation of *Bacillus thuringiensis* from Environmental Samples

Screening samples from different environments may be useful to obtain Bt strains with broader host ranges and new toxic properties (Höfte and Whiteley 1989). The abundance of the bacterium depends mainly on the type of environmental sample. Soil has been shown as the main source of Bt novel isolates (De Lucca *et al*., 1981; EL-Kersh *et al*., 2011) as it has been recovered from 70% of soil samples from all over the world (Martin and Travers 1989). There are some suggestions about the high recovery of Bt from soil. First, while collecting sample, the surface should be rejected and the material taken from at least 5 cm under the surface where UV light damage is not possible and temperature is more stable (Trindade *et al*., 1996; Renganathan *et al*., 2011; EL-Kersh *et al*., 2011). Second, the soil can act as a reservoir of spores (Akiba, 1986; Martin and Travers 1989; Meadows 1993; Ohba and Aratake 1994; Lereclus 1996; Raymond *et al*., 2010). The efficiency of isolation also depends on the method used. Enrichment techniques are not useful because it has a lower detection limit which is about 10^3 bacteria per gram of soil. Immunofluorescence-based methods also have a lower detection limit of 10^5 bacteria per gram of soil in spite of their direct enumeration. The most efficient isolation method so far has been the sodium acetate selection, combined with heat treatment. This method eliminates most spore forming and all non-spore forming bacteria. Germination of Bt spores is selectively inhibited by sodium acetate, which allows the germination of other spore-formers. At that time, Bt remains in the quiescent state. Then, heat treatment is applied to kill all undesired bacteria, which have entered the vegetative stage (Martin and Travers 1987; Mukhija and Khanna, 2018).

2.6 Morphological and Phenotypic Characterization of *Bacillus thuringiensis*

Bt has a vegetative cell of 1.0–1.2 μm wide and 3.0–5.0 μm in length, usually motile by means of peritrichous flagella, naturally not numerous. The spore of this bacterium has an ellipsoidal shape but mostly cylindrical and is located in the central or paracentral region when inside the mother cell. The main characteristic that usually distinguishes this species from others of the same genus is the intracellular presence of a protein crystal (Höfte and Whiteley, 1989; Glare and O'Callaghan 2000). However, the protein crystal is plasmid borne and the exchanges of cry genes generate *B. cereus* with a new binding of cry that leads to the similarity of *B. cereus* and *B. thuringiensis* (Fiuza, 2015). Most strains are catalase positive, oxidase negative, casein, gelatin and starch are hydrolyzed, Voges- Proskauer positive and citrate is utilized as sole carbon source. Nitrate is reduced and tyrosine is decomposed. Phenylalanine is not deaminated. Most strains utilize saccharose and other sugars, but *Bacillus thuringiensis* serovars *israelensis* do not ferment this disaccharide (De vos *et al*., 2009). There are wide range of colonial morphology, both within and between species, with medium composition and other incubation conditions having strong influences.

2.7 Genotypic Characterization of *Bacillus thuringiensis*

Identification of microorganisms has moved from phenotypic to genotypic method as they yield more sensitive and accurate results (Narwade *et al*., 2014). Polymerase Chain Reaction (PCR) is a molecular method widely used to characterize the insecticidal bacterium Bt. It provides the determination of the presence of a target gene by the amplification of specific DNA fragments. This method has largely substituted bioassays used in preliminary classification of Bt collections because of its rapidity and reliability (Porcar and Juárez-Pérez, 2002).

Bt produces insecticidal proteins, which are the main type of Crystalline (cry) proteins (Kutasi *et al*., 2016). However, there are over 700 *cry* genes and they possess different sequence homologies. Designing a biomarker for the translated product varies with the different categories of cry protein; hence, the detection will be complex. The 16S ribosomal RNA gene sequences based on universal primers showed high similarity (>99%) index between *B. cereus* and *B. thuringiensis* (Böhm *et al*., 2015), which cannot be classified using genetic and phenotypic assays (Peng *et al*., 2015). Further, there has been a discussion since year 2000 regarding whether the entire B. cereus group should be treated as a complex species of diverse bacteria (Helgason *et al.,* 2000; Bartoszewicz and Marja´nska, 2017). There are also suggestions that phylogeny of these bacteria better fits to their ecological properties (psychrotolerance, virulence) than to taxonomic affiliation (Drewnowska and Swiecicka, 2013; Bartoszewicz and Marja´nska, 2017). To resolve this problem, Wei *et al*. (2019) targeted the transcriptional regulator (*XRE*) gene to detect *B. thuringiensis*, which controls the major type of crystal protein production and it was able to discriminate with high precision *B. cereus* and Bt.

2.8 Characterization of Nanoparticles

Nanoparticles are commonly characterized on the basis of size, shape, disparity and surface area (Many *et al*., 2014). Characterization of nanoparticles is crucial in order to appreciate and control nanoparticles synthesis and applications. Techniques particularly used for characterization include UV-Visible Spectrophotometry, Electron Microscopy (Transmission Electron Microscopy or/and Scanning Electron Microscopy), Fourier Transform Infrared Spectroscopy, Powder X-ray Diffraction, Dynamic Light Scattering amongst several others (Ingale and Chaudhari, 2013).

2.8.1 Detection of colour change by visual inspection

Preliminary confirmation of biosynthesis of silver nanoparticles has reportedly been carried out by visual inspection through detection of colour change typically from colourless to brown (Ojo *et al*., 2016; Yurtluk *et al*., 2018). The silver nanoparticles synthesized exhibit a brown colour in water due to excitation of Surface Plasmon vibration in metal nanoparticles (Matei *et al*., 2015).

2.8.2 UV-visible spectrophotometry

The UV-Visible Spectrophotometry is a generally acceptable technique used for the confirmation of successfully synthesized nanoparticles. It is established that the adsorption of specific metal nanoparticles suspended in solution, peaks at a particular wavelength due to surface plasmon resonance when placed in a UV-Visible spectrophotometer (Rai *et al*., 2011). Majority of studies on biosynthesis of silver nanoparticles adopts the use of UV-Visible spectrophotometry as the first characterization technique after visual detection of colour change (Ojo *et al*., 2016; Lateef *et al*., 2014).

2.8.3 Dynamic light scattering

Oldenburg (2016) presents that further characterization of nanoparticles can be achieved by ascertaining the aggregation state of the particles by techniques such as dynamic light scattering (DLS). The equipment used for DLS is Zetasizer nano which operates using the mechanism of Brownian motion coupled with Stokes-Einstein equation in the determination of the important parameters. Laser diffraction particle size analyzer provides the detail about the particle nature, such as monodispersed, didispersed and polydispersed (Sabri *et al*., 2016).

2.8.4 Electron microscopy

The Electron microscopy is used for morphological characterization of nanoparticles in terms of size and shape. The technique is used for further confirmation of successfully synthesized nanoparticles (Sabri *et al*., 2016). This is agreed by Oldenburg (2016) who reported that the size and shape of metal nanoparticles are typically measured by analytical techniques such as Transmission Electron Microscopy (TEM), scanning electron microscopy (SEM) or atomic force microscopy (AFM). TEM in particular has been used in several studies to ascertain the size and shape of silver nanoparticles (Omidi *et al*., 2014). Plate 2.1 shows scanning electron micrograph of different shaped of silver nanoparticles.

2.8.5 Fourier Transform-Infrared Spectroscopy

The FTIR is an instrumental analysis tool necessary in characterization of silver nanoparticles as it ascertains the nature of the molecules capping/ stabilizing the AgNPs produced. It also reveals the interaction between protein and AgNPs and the functional group of biomolecules responsible for the reduction of silver ions (Krishna *et al*., 2017; Elamawi *et al*., 2018).

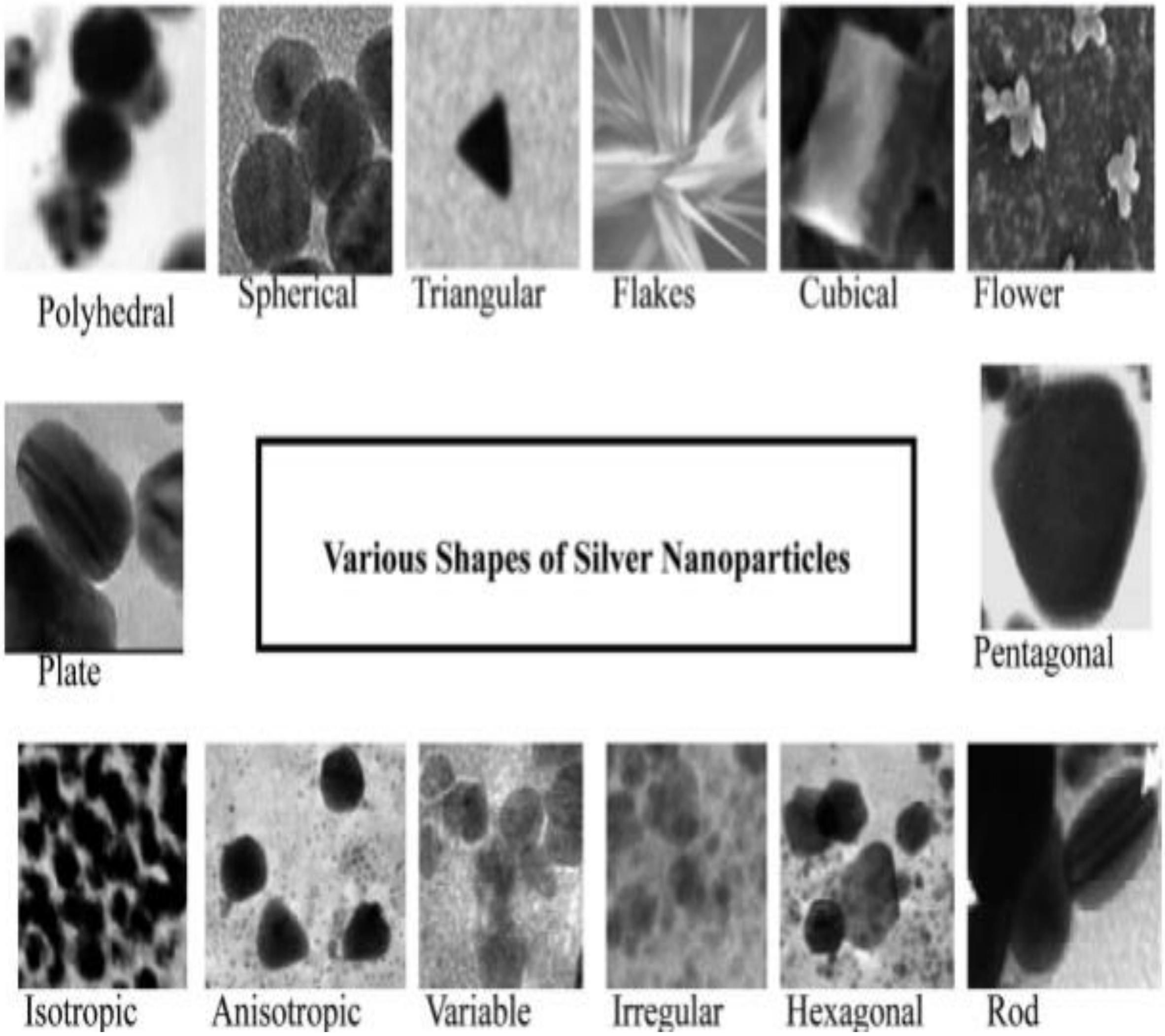

Plate 2.1: Scanning electron micrograph showing shapes of silver nanoparticles

Source: Tarannum *et al*. (2019)

2.9 Applications of Silver Nanoparticles

AgNPs have attracted considerable attention from analysts due to their uncommon attributes. They are used in different fields such as biomedical (fast diagnosis, imaging, tissue regeneration and drug delivery, and development of new medical products), textile industry, food packaging, cosmetic industry, catalysis, sensors, biology, optoelectronics, antimicrobial activities, DNA sequencing, climate change and contamination control, clean water technology, energy generation, and information storage (Tarannum *et al*., 2019). Few of these uses are discussed below.

2.9.1 Nanoparticles as drug carriers

Delivering the drugs precisely and safely to their target sites at the right time to have a controlled release and achieve maximum therapeutic effect is a key issue in the design and development of novel drug delivery systems. Targeted nano-carriers must navigate through blood-tissue barriers to reach target cells. They must enter target cells to contact cytoplasmic targets *via* specific endocytotic and transcytotic transport mechanisms across cellular barriers (Fadeel *et al.,* 2010). Because of their small size, nanoparticle drug carriers can bypass the blood-brain barrier and the tight epithelial junctions of the skin that normally impede delivery of drugs to the desired target site. Secondly, as a result of their high surface area to volume ratio, nano carriers show improved pharmacokinetics and bio-distribution of therapeutic agents and thus minimize toxicity by their preferential accumulation at the target site (Vaidyanathan *et al*., 2009). They improve the solubility of hydrophobic compounds and render them suitable for parenteral administration. Furthermore, they increase the stability of a variety of therapeutic agents like peptides and oligonucleotides (Emerich and Thanos, 2006).

2.9.2 Improved catalysis with nanoparticles

Magnetic nanoparticles have been used to improve the microbiological reaction rates. Coated microbial cells of *Pseudomonas delafieldii* with magnetic Fe_3O_4 nanoparticles have been used to fulfill desulfurization of dibenzothiophene (Hildebrand *et al*., 2008).The high surface energies of nanoparticles resulted in their strong adsorption on the cells. In fact, magnetic nanoparticles were utilized not only for their catalytic function but also for their good ability to disperse. The application of an external magnetic field ensured that the cells were well diffused in the solution even without mixing and enhanced the possibility to collect cells for reuse (Abu-Dief and Abdel-Fatah, 2017).

Organic pollutant such as 4-Nitrophenol and its derivatives used to manufacture herbicides, insecticides, and synthetic dye have adverse effect on the ecosystem. Due to its toxic and inhibitory nature, 4-nitrophenol is a great environmental concern. Therefore, the reduction of these pollutants is crucial. The 4-nitrophenol reduction product, 4-aminophenol, has been applied in diverse fields as an intermediate for paracetamol, sulfur dyes, rubber anti-oxidants, preparation of black/white film developers, corrosion inhibitors, and precursors in antipyretic and analgesic drugs. The simplest and most effective way to reduce 4-nitrophenol is to introduce $NaBH_4$ as a reductant and a metal catalyst such as Gold nanoparticles, silver nanoparticles, copper oxide nanoparticles and palladium nanoparticles (Sharma *et al*., 2015; Lim *et al*., 2016; Rostami-vartooni, 2016; Gopalakrishina *et al*., 2017). Metal NPs exhibit admirable catalytic potential because of the high rate of surface adsorption ability and high surface area to volume ratio. Nevertheless, the viability of the reaction declines as a consequence of the substantial potential difference between donor ($H_3BO_3/NaBH_4$) and acceptor molecules (nitrophenolate ion), which accounts for the higher activation energy barrier. Metallic NPs can promote the rate of reaction by increasing the adsorption of reactants on their surface, thereby diminishing activation energy barriers (Singh *et al*., 2017).

2.9.3 Improvement in cancer management

Nanotechnology has shown a lot of promise in cancer therapy over the years. Several nanoparticle types are being used today for molecular imaging. Nanoparticles used in cancer management such as semiconductors, quantum dots and iron oxide nanocrystals possess optical, magnetic or structural properties that are less common in other molecules (Popescu *et al*., 2015). Different anti-tumor drugs and biomolecules including peptides, antibodies or other chemicals, can be used with nanoparticles to label highly specific tumors, which are useful for early detection and screening of cancer cells (Singh, 2019). For cancer diagnostics, imaging of tumor tissue with nanoparticles has made it possible to detect cancer in its early stages.

The development of nanotechnology is based on the usage of small molecular structures and particles as tools for delivering drugs. Nano-carriers such as liposomes, micelles, dendritic macromolecules, quantum dots, and carbon nanotubes have been widely used in cancer treatment. Their improved pharmacokinetic and pharmacodynamic properties, have contributed to improved cancer diagnosis and treatment. Nanotechnology allows targeted drug delivery in affected organs with minimal systemic toxicities due to their specificities. However, as with other therapeutic options, nanotechnology is not completely devoid of toxicities and comes with few challenges with its use including systemic and certain organ toxicities, hence, causing setbacks with their clinical applications. Given the limitations with nanotechnology, more advancements must be done to improve drug delivery, maximize their efficacy while keeping the disadvantages to the minimum.

2.9.4 Antimicrobial property

Various studies have been carried out to improve antimicrobial functions because of the growing microbial resistance. According to *in vitro* antimicrobial studies, the metallic nanoparticles effectively obstruct several microbial species (Dizaj *et al*., 2014). The antimicrobial effectiveness of the metallic nanoparticles depends upon two important parameters: (i) material employed for the synthesis of the nanoparticles and (ii) their particle size. Microbial resistance to antimicrobial drugs is a major threat to public health. For instance, antimicrobial drug resistant bacteria contain methicillin-resistant, sulfonamide-resistant, penicillin-resistant, and vancomycin-resistant properties (Fair and Tor, 2014). New antibiotics are needed for combating multidrug-resistant mutants and biofilms. The effectiveness of antibiotics is likely to decrease rapidly because of the drug resistance capabilities of microbes. Hence, even when bacteria are treated with large doses of antibiotics, diseases will persist in living beings. Biofilms developed by microorganisms, contribute to providing multidrug resistance against heavy doses of antibiotics. The most promising approach for abating or avoiding microbial drug resistance is the utilization of nanoparticles (Yeh *et al*., 2020). Due to various mechanisms, metallic nanoparticles can preclude or overwhelm multidrug-resistance and biofilm formation by microbes. Nanoparticles can fight drug resistance because they operate using multiple mechanisms. Therefore, microbes must simultaneously have multiple gene mutations in their cell to overcome the nanoparticle mechanisms. However, microbes are unlikely to have multiple mutated genes, so it is much more difficult to develop resistance to NPs. (Jayaraman, 2009; Wang *et al*., 2020). Silver nanoparticles are the most admired inorganic nanoparticles, and they are utilized as efficient antimicrobial, antifungal, antiviral, and anti-inflammatory agents (Zinjarde, 2012). The antimicrobial potential of silver nanoparticles can be described in the following ways: (1) denaturation of the bacterial outer membrane (Lok *et al*., 2006), (2) generation of gaps in the bacterial cell membrane leading to fragmentation of the cell membrane (Iavicoli *et al.*, 2013; Yun *et al*., 2013), and (3) interactions between AgNPs and disulfide or sulfhydryl groups of enzymes disrupt metabolic processes; this step leads to cell death (Egger *et al*., 2009). The shape of the NPs also contributes to their antimicrobial activity. For example, truncated triangular nanoparticles have highly reactive high-atom-density surfaces which enhanced antimicrobial activity. Tak *et al*. (2015) reported that green synthesized nanoparticles show enhanced antimicrobial activity compared to chemically synthesized nanoparticles. This is because the plants [such as *Ocimum sanctum* (Tulsi) and *Azadirachta indica* (neem)] employed for synthesis of nanoparticles have medicinal properties in themselves (Ramteke *et al*., 2013; Verma and Mehata, 2016).

2.9.5 Removal of pollutant dyes

Organic dyes play a very imperative role due to their gigantic demand in textiles, plastic, leather, food, printing, and pharmaceuticals industries. In textile industries, about 60% of dyes are consumed in the manufacturing process of pigmentation for many fabrics (Carmen and Daniel, 2012). After the fabric process, nearly 15% of dyes are wasted and are discharged into the hydrosphere, and they represent a significant source of pollution due to their recalcitrant nature (Ratna, 2012). The pollutants from these manufacturing units are the most important sources of ecological pollution. They produce undesirable turbidity in the water, which will reduce sunlight penetration, and this leads to the resistance of photochemical synthesis and biological attacks to aquatic and marine life (Dutta *et al.*, 2014). The need for hygienic and safe drinking water is increasing day by day. Considering this fact, the use of metal and metal oxide semiconductor nanomaterials for oxidizing toxic pollutants has become of great interest (Nakkala *et al.,* 2015; Fowsiya *et al*., 2016; Varadavenkatesan *et al*., 2016). Semiconductor nanomaterials have superior photocatalytic activity relative to the bulk materials and have been applied preferentially for the photocatalytic activity of synthetic dyes (Bhuyan *et al*., 2015; Stan *et al*., 2015; Thandapani *et al*., 2017). The merits of these nanophotocatalysts (e.g., ZnO and TiO_2 nanoparticles) are due to their high surface area to mass ratio to enhance the adsorption of organic pollutants. The surface energy of the nanoparticles increases due to the large number of surface reactive sites available on the nanoparticle surfaces. This leads to an increase in rate of contaminant removal at low concentrations. Consequently, a lower quantity of nanocatalyst will be required to treat polluted water relative to the bulk material (Dror *et al*., 2005; Astruc, 2008). Like metal oxide nanoparticles, metal nanoparticles like silver nanoparticles also show enhanced photocatalytic degradation of various pollutant dyes.

2.9.6 Heavy metal ion sensing

Heavy metals (like Ni, Cu, Fe, Cr, Zn, Co, Cd, Pb, Cr, Hg, and Mn) are known for being air, soil, and water pollutants. There are innumerable sources of heavy metal pollution such as mining waste, vehicle emissions, natural gas, paper, plastic, coal, and dye industries (Zhang *et al*., 2012). Some metals (like lead, copper, cadmium, and mercury ions) show enhanced toxicity potential even at trace levels (Que *et al*., 2008). Therefore, the identification of toxic metals in the biological and aquatic environment has become a vital need for effective remedial processes (Nolan and Lippard, 2008; Ray, 2010; Aragay *et al*., 2011). Conventional techniques based on instrumental systems generally offer excellent sensitivity in multi-element analysis. However, experimental set-ups to perform such analysis are highly expensive, time consuming, skill-dependent, and non-portable. Due to the size and distance-dependent optical properties of metallic nanoparticles, they have been preferably employed for the detection of heavy metal ions in polluted water systems (Maiti *et al*., 2016). The advantages of using metal NPs as colorimetric sensors for heavy metal ions in environmental systems/samples include simplicity, cost effectiveness, and high sensitivity at sub ppm levels. Karthiga and Anthony (2013) synthesized AgNPs using various plant extracts and used the synthesized AgNPs as colorimetric sensors for heavy metal ions like cadmium, chromium, mercury, calcium, and zinc (Cd^{2+}, Cr^{3+}, Hg^{2+}, Ca^{2+}, and Zn^{2+}) in water. Their synthesized Ag nanoparticles showed colorimetric sensing of zinc and mercury ions and lead ions (Zn^{2+}, Hg^{2+} and Pb^{2+}).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling Sites and Sample Collection

Soil sampling sites were the *Zango Shanu* farm land, cow ranch and *Yankarfe* metal refuse dump site in Zaria. The surface of the soil was scrapped off and a 10g soil sample was collected from a depth of 5 to 10cm in ten random sampling spots within each site. The ten soil samples from each site were then pooled to obtain representative composite soil samples (Stefani *et al*., 2015). The soil samples collected were placed in a clean polythene bag, transported to Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna State and stored at 4^{o}C until further use.

3.2 Isolation of *Bacillus thuringiensis* (Bt)

The isolation of *Bacillus thuringiensis* from soil was carried out by the sodium acetate selection method as described by Travers *et al.* (1987). From each composite sample, 1g soil was added to 10 ml of sterilized Luria Bertani (LB) broth buffered with 0.25 M sodium acetate (pH 6.8) in 125 ml conical flask (presumably, sodium acetate selectively inhibits the germination of *B. thuringiensis* spores) and was incubated for 4 hours at 30^{o}C with shaking at 250 revolutions per minute (rpm) in a shaker. After incubation, aliquots of 2ml of thoroughly mixed culture broth was transferred to a pre-warmed sterile slant bottles and heated to 80^{o}C for 10 minutes and tenfold of serial dilutions up to10^{-5} was prepared and 1ml of the various dilutions was spread on LB agar and incubated at 28 ± 2^{o} C for 48 hr. After 48 hours of incubation, bacterial colonies on the plates were visually examined. Colonies showing morphological features such as being white, spread out, and have a fried egg appearance characteristics typical for the *Bt* group, were subcultured on T3 agar which partially inhibits the growth of other *Bacillus* species (Travers *et al*., 1987, Rampersad and Ammons, 2005).

3.3 Identification of *Bacillus thuringiensis*

The cultural and biochemical characteristics of the isolates were used for a presumptive characterization of the *Bacillus thuringiensis* (Cowan and Steel, 2003; Bergey, 2004) and then confirmed based on the polymerase chain reaction targeting the transcriptional regulator (XRE) gene and cry2 gene. The details are provided below.

3.3.1 Morphological Characterization of the isolates

The pure isolates were Gram stained and the shape and arrangement of the cells were examined. Also, endospore staining was carried out to determine spore formation by the isolates.

3.3.1.1 Gram Staining

A thin smear of a 24-hour culture was made on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 seconds and rinsed with water. The smear was again flooded with Lugol’s iodine for 30 seconds and rinsed with water; and then decolourized with 70% alcohol for 15 seconds and rinsed with distilled water. The slides were counter-stained using 2 drops of Safranin for 60 seconds and finally rinsed with water, then allowed to air dry. The stained slides were mounted on a microscope and observed under oil immersion objective lens (Fawole and Oso, 2004).

3.3.1.2 Endospore Staining

This test was to detect the presence of bacterial endospores. Heat-fixed smears from 24hour culture of the bacterial isolates were prepared on slides and flooded with 5% Malachite green solution and steamed for a minute. The stain was washed off with water and counter stained with 2 drops of safranin solutions for 20 seconds. The slides were air-dried and examined under oil immersion objective lens. Endospores stain green while vegetative cells will stain pink (Cheesbrough, 2006).

3.3.1.3 Motility

This test was carried out by inoculating the motility medium with colonies from a 24hour culture of the isolates. A stab was made with an inoculating needle to a depth of about one third the total volume of the medium. The culture was then incubated at 37^{o}C for 24 h. Cultures that turned cloudy (turbid) after incubation, meant the organism was motile but cultures that had growth restricted only to the line of inoculation and the rest of the culture remained clear, meant the organism was non-motile (Cowan and Steel, 2003).

3.3.2 Biochemical characterization of Presumptive *Bacillus thuringiensis* Isolates

The following biochemical tests were carried out to characterize presumptive *Bacillus thuringiensis.*

3.3.2.1 Catalase

The isolates were grown on Luria Bertani agar at 37^{o}C overnight. Catalase activity was detected by adding a drop of 3% hydrogen peroxide solution onto an isolated colony in a test tube. Immediate and vigorous bubbling indicated a strong catalase reaction whereas scant or no bubble formation indicated a negative test (Çelenk, 2005).

3.3.2.3 Oxidase

A piece of sterile filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a suspected *B. thuringiensis* colony and smeared on the filter paper. Organisms that were oxidase producing oxidized the phenylenediamine in the reagent to a deep purple colour (Cheesbrough, 2006).

3.3.2.4 Voges-Proskauer test

A loopful of Isolates were grown in 5ml methyl red- Voges-Proskauer medium (MR-VP medium) for 24 h at 37^{o}C. After the incubation, 3 drops of α-naphthol was added and shaken gently, and then 200 ml KOH solution was added and shaken. The tubes were open tubes and slanted to increase contact with air. Development of a cherry red coloration at the surface within 30-45 min was considered as a positive indication for Voges-Proskauer test (Celenk, 2005).

3.3.2.5 Methyl Red Test

Test tubes containing 5ml of MR-VP medium were inoculated with the suspected *B. thuringiensis* colony using wire loop. The tubes were incubated at 37^{o}C for 24 hrs. After incubation, three drops of methyl red indicator was added. Formation of red colour on addition of the indicator signified a positive methyl red test and a yellow colour signified a negative test (Cowan and Steel, 2003).

3.3.2.6 Amylase Activity

A plate of starch-nutrient agar plate was inoculated with the organism. After incubation for 24 h at 37^{o}C, plates were flooded with 5-10 ml of Gram’s iodine solution. Clear area around the growth of the culture indicated the breakdown of starch by the organism due to its production of amylase. Unhydrolyzed starch will form a blue colour with the iodine (Çelenk, 2005).

3.3.2.7 Citrate Test

The test was carried out by inoculating colonies of the isolates over the surface of a slope of Simmons' citrate medium and examined daily for up to 7 days for growth and colour change. Blue colour and streak of growth signified that citrate was utilized while original green colour signified citrate not utilized (Cowan and Steel, 2003).

3.3.3 Molecular Detection of *XRE and CRY2* Using PCR

The pure isolates of presumptive *Bacillus thuringiensis* from the biochemical tests were further confirmed using PCR. The DNA was extracted using Qiagen DNAeasy kit (Jiangsu Mole Bioscience Co., Ltd, China). Table 3.1 shows the primer sets used. The PCR condition for amplification of the XRE gene of *Bacillus thuringiensis* was according to Wei *et al* (2019). PCR reaction was carried out in a volume of 25 µL comprising of 12.5µL of master mix, 0.5µL of each primer (20 pmoL/mL) and 8µL of DNA. The PCR conditions for the amplification of the transcriptional regulator (XRE) were: initial denaturation at 94^{o}C for 3 minutes, followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 49^{o}C for 30 seconds and elongation at 72^{o}C for 30 seconds, and a final elongation at 72^{o}C for 10 minutes. Amplifications conditions for crystal protein (Cry 2) are: initial denaturation at 94^{o}C for 3min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 30 s and elongation at 72^{o}C for 1 min, and a final elongation at 72^{o}C for 10 min. After DNA amplifications the gene banding patterns were visualized through 1.5% agarose gel electrophoresis.

Table 3.1: Primer Sets Used for the PCR of target genes

Target gene	Primer sequence (5'-3')	Product size (bp)	References
Transcriptional regulator (XRE)	AAGATATTGCAAGCGGTAAGAT (Forward)	246	Wei *et al*., (2019)
	GTTTTGTTTCAGCATTCCAGTAA (Reverse)		
Crystal gene (*cry2*)	GTTATTCTTAATGCAGATGAATGGG (Forward)	689	Ben-Dov *et al*., (1997)
	CGGATAAAATAATCTGCGAAATAGT (Reverse)		

3.4 Screening of *Bacillus thuringiensis* Isolates for Bio-production of Silver Nanoparticles

In order to screen and select the most efficient bacterial strain for the bio-production of AgNPs, the method described by Agrawal and Kulkarni (2017) was used. *Bacillus thuringiensis* isolated confirmed by PCR were cultured on LB supplemented with 1mM concentration of $AgNO_3$ at 28^{o}C to screen for the bio-production of AgNPs. The isolates that grew vigorously on the medium were selected and further screened for optimal bio-production of AgNPs by methods described by Kumar *et al*. (2018). Six confirmed *Bacillus thuringiensis* isolates were used for a preliminary assessment of bio-production of silver nanoparticles. The absorbance of the AgNPs produced by each isolate were observed at 420nm in a spectrophotometer (752N model). The isolate which produced AgNPs with the highest absorbance during the growth conditions was considered the most efficient in the bio-production of AgNPs.

3.5 Bio-production of Silver Nanoparticles Using Selected Bt

The bio-production of AgNPs was carried out as described by Ojo *et al.* (2016) with slight modification. One millilitre of standardized inoculum of *Bacillus thuringiensis* prepared according to McFarland's scale 0.5 (1.5×10^8 cells/ml) was inoculated in 30ml LB broth and incubated for 24 hours at 37°C. Then, the culture was centrifuged at 4000 rpm for 15 min and the cell-free supernatant was used for the bio-production of AgNPs**.** Thirty milliliters of aqueous silver nitrate solution (1 mM) was mixed carefully with 1ml of cell-free supernatant in 250 ml conical flask for the reduction of silver nitrate at 28^{o}C and static conditions for 24hours. The control without the $AgNO_3$ (cell-free supernatant) was held at the same conditions, change in colour was observed visually (Lateef *et al*., 2014).

3.6 Characterization of silver nanoparticles

3.6.1 UV-Vis Spectra Analysis

About 1 ml of the sample suspension was collected followed by dilution of the sample with 2 ml of deionized water and subsequent scan in UV-visible (Vis) spectra, between wave lengths of 250nm to 800 nm in a spectrophotometer (Lateef *et al*., 2014).

3.6.2 Fourier Transform Infrared Spectroscopy

The FTIR spectroscopy is very important for characterizing the interaction between proteins and AgNPs. It can also quantify secondary structure in metal nanoparticle–protein interaction (Elamawi *et al*., 2018). The AgNPs solution was centrifuged at 4000 rpm for 20 min. The residue obtained was used for FTIR analysis (Lateef *et al*., 2014). Fourier Transform Infrared (FTIR) spectroscopy analysis was carried out using Shimadzu FTIR-8400S Fourier transform infrared spectrophotometer scanning 700 to 4000 cm^{-1}.

3.6.3 Scanning Electron Microscopy Analysis

AgNPs solution was centrifuged at 4,000 rpm for 15 min, and the supernatant was discarded while the pellets were used for the SEM analysis. The pellet was mixed properly and carefully placed on a stud. The analysis was done using Phenom ProX SEM (Phenom World Eindhoven, Netherlands). The image of AgNPs was obtained using a desktop computer connected to the scanning electron microscope.

3.7 Antibacterial Effect of Silver Nanoparticles on Selected Pathogenic Bacteria

Agar well diffusion was used for testing the effect of silver nanoparticles synthesized by *Bt* isolated from soil on four selected pathogenic bacteria namely; *Escherichia coli* (test pathogen 1), *Escherichia coli* (test pathogen 2), *Staphylococcus aureus* and *Klebsiella pneumonia* with varying antibiotic resistance patterns as shown in Appendix V. Standardized suspension of each selected test organism (0.5 x 10^6 CFU/ml) was swabbed uniformly onto sterile Muller-Hinton Agar (MHA) plates using sterile cotton swabs. Wells of 9 mm diameter were bored into the agar medium using gel puncture. An aliquot of 100 µl containing silver nanoparticles at different concentration (100µg/ml, 75µg/ml, 50µg/ml and 25µg/ml) was added into each well. After incubation at 37˚C for 24hr, zones of inhibition were measured. Supernatant of *Bt* culture and ciprofloxacin (antibiotics) were used as controls for antimicrobial activity.

The Minimum Inhibitory Concentration was determined in Mueller Hinton broth using serial two-fold dilutions of AgNPs at concentrations ranging from 25 to 100µg/ml with normalized bacterial concentrations (1 × 10^8 CFU/mL, 0.5 McFarland's standard). The positive control contained Mueller Hinton broth with tested bacteria, and the negative control contained only broth. The samples were incubated for 24 h at 37^{o}C. The MIC is the minimum concentration of AgNPs that visually inhibits 99% of bacterial growth. The MBC was determined by inoculating samples that did not show any growth of pathogenic bacteria on Mueller Hinton agar and observing growth after incubation at 37^{o}C for 24h.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and identification of *Bacillus thuringiensis*

4.1.1 Isolation of *B. thuringiensis* isolates

Thirty (30) bacterial strains were isolated and sub-cultured on fresh T3 agar medium. Only twenty-five (25) isolates grew on T3 agar medium with a characteristic creamy white colour with elevation being either raised or flat. Of the 25 isolates, ten (10) were irregular in shape with undulating margin, while the remaining fifteen (15) were circular in shape with entire margin. The results are shown in Table 4.1.

4.1.2 Identification of *B. thuringiensis*

4.1.2.1 Morphological Identification of the Isolates

Twenty-one isolates were Gram positive, rod shaped, being scattered/clustered as shown in Table 4.2. Endospore test revealed that only fifteen isolates of the twenty-one Gram positive isolates were endospore positive. Table 4.3 shows the variation in the position of the oval shaped spores present in the isolates being either subterminal or central. The isolates were identified based on Bergey's manual of Bacteriology.

Table 4.1: Colonial Characteristics of bacterial isolates on T3 Medium

Isolate code	**Colour**	**Shape**	**Margin**	**Elevation**	**Inference**
F3^{2}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
F1^{1}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
CR1^{3}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
CR2^{2}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
CR3^{2}	Creamy white	Circular	Entire	Raised	*Bacillus cereus* group
MRS2^{3}	Creamy white	Irregular	Undulated	Flat	*Bacillus cereus* group
MRS2^{2}	Creamy white	Irregular	Undulated	Flat	*Bacillus cereus* group
F1^{2}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
MRS3^{4}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
CR2^{1}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
MRS3^{2}	Creamy white	Irregular	Undulated	Flat	*Bacillus cereus* group
MRS2^{3}	Creamy white	Circular	Entire	Raised	*Bacillus cereus* group
CR2^{2}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
MRS2^{1}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
F2^{1}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
F1^{3}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
CR3^{1}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
CR2^{3}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
F2^{4}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
F2^{2}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
CR2^{4}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
F3^{3}	Creamy white	Irregular	Undulated	Flat	*Bacillus cereus* group
MRS1^{3}	Creamy white	Circular	Entire	Raised	*Bacillus cereus* group
MRS2^{3}	Creamy white	Circular	Entire	Flat	*Bacillus cereus* group
CR2^{1}	Creamy white	Irregular	Undulated	Raised	*Bacillus cereus* group
CR2^{2}	-	-	-	-	-
F2^{3}	-	-	-	-	-
MRS3^{3}	-	-	-	-	-
F3^{1}	-	-	-	-	-
MRS1^{2}	-	-	-	-	-

Key: CR- Cow range; F- farmland; MRS- Metal recycling site; - means No growth

Table 4.2: Gram's reaction of presumptive *Bacillus cereus* group isolated from soil

Isolate code	Gram's Reaction	Morphology	Arrangement of cells
F3^2	+	Rods	Scattered
CR1^3	+	Rods	Scattered
CR2^2	+	Rods	Scattered
CR3^2	+	Rods	Scattered
MRS2^3	+	Rods	Scattered
MRS2^2	+	Rods	Scattered
F2^2	+	Rods	Scattered
MRS3^4	+	Rods	Scattered
CR2^1	+	Rods	Clustered
MRS3^2	+	Rods	Scattered
MRS2^3	+	Rods	Clustered
MRS2^1	+	Rods	Clustered
F2^1	+	Rods	Scattered
F1^3	+	Rods	Scattered
CR2^3	+	Rods	Scattered
F2^4	+	Rods	Scattered
F2^2	+	Rods	Scattered
CR2^4	+	Rods	Scattered
F3^3	+	Rods	Scattered
MRS1^3	+	Rods	Clustered
MRS2^3	+	Rods	Clustered
CR3^1	_	Rods	Clustered
F1^2	_	Rods	Clustered
F1^1	_	Rods	Clustered
CR2^1	_	Rods	Clustered

Key: -; Gram negative +; Gram positive

Table 4.3: Endospore characteristics of *Bacillus cereus* group isolated from soil

Isolate code	Position of spore	Shape of spore
F3^{2}	Central	Oval
CR1^{3}	Central	Oval
CR2^{2}	Subterminal	Oval
MRS2^{3}	Central	Oval
MRS2^{2}	Central	Oval
F2^{2}	Subterminal	Oval
CR2^{1}	Subterminal	Oval
MRS3^{2}	Central	Oval
MRS2^{1}	Central	Oval
CR2^{3}	Central	Oval
F2^{4}	Central	Oval
F2^{2}	Subterminal	Oval
CR2^{4}	Central	Oval
MRS1^{3}	Central	Oval
MRS2^{3}	Central	Oval

4.1.2.2 Biochemical Identification of *Bacillus cereus* group Isolates

The distinguishing features of the isolates based on several biochemical tests is shown in Table 4.4. Of the fifteen (15) isolates, only ten (10) were observed to be motile and positive to catalase, starch hydrolysis, citrate utilization, methyl red and Voges-Proskaeur tests, while they showed negative reactions to oxidase and indole tests. Table 4.5 shows the distribution of *Bacillus thuringiensis* isolates in various soil samples after microscopic and biochemical characterization. Cow rangeland had the highest distribution of 50%, followed by metal recycling site (40%) and lastly, agricultural farmland had the lowest (10%).

4.1.2.3 Molecular detection Using *XRE* and *Cry2* Genes

Molecular characterization (PCR) of *XRE* gene using PCR confirmed that the some of the *Bacillus* isolated from several soil samples were *B. thuringiensis*. Plate I shows the result of the polymerase chain reaction using specific primer for *XRE* gene with an amplicon size of 246 bp. Six of the ten isolates show bands corresponding to 246 bp. None of the isolates shows bands corresponding to *cry2* gene with an amplicon size of 689 bp.

Table 4.4: Biochemical characteristics of putative *Bacillus thuringiensis* isolates from selected soil samples

Isolate code	Oxidase	Catalase	Starch hydrolysis	Citrate	MR	VP	Motility	Indole	Presumptive identity
F3^{2}	_	+	+	+	+	+	+	_	Bt
CR1^{3}	_	+	+	+	+	+	+	_	Bt
CR2^{2}	_	+	+	+	+	+	+	_	Bt
MRS2^{3}	_	+	+	_	_	+	+	_	NBt
MRS2^{2}	_	+	+	+	+	+	+	_	Bt
F2^{2}	_	+	_	+	+	_	+	_	NBt
CR2^{1}	_	+	+	+	+	+	+	_	Bt
MRS3^{2}	_	+	+	_	+	+	+	_	NBt
MRS2^{1}	_	+	+	+	+	+	+	_	Bt
CR2^{3}	_	+	+	+	+	+	+	_	Bt
F2^{4}	+	_	_	_	_	_	+	_	NBt
F2^{2}	_	+	_	+	_	_	+	_	NBt
CR2^{4}	_	+	+	+	+	+	+	_	Bt
MRS1^{3}	_	+	+	+	+	+	+	_	Bt
MRS2^{3}	_	+	+	+	+	+	+	_	Bt

Key: -; negative +; positive, MR: Methyl Red test, VP: Voges proskauer, Bt: *Bacillus thuringiensis* NBt: Not *Bacillus thuringiensis*

Table 4.5: Distribution of Presumptive *Bacillus thuringiensis* isolates in Various Soil samples after Morphological and Biochemical Characterization

Sources of soil samples	***Number of *Bacillus*-like isolates**	**Number of Bt-like isolates**	**[a]Bt index**	**Frequency of distribution of Bt (%)**
Farmland	10	1	0.10	10
Cow range	10	5	0.50	50
Metal recycling site	10	4	0.40	40
Total	30	10	0.33[b]	100

***** Creamy white, circular, dry, flat elevation and with wavy margin

[a] Bt Index: *Bacillus thuringiensis* isolation index was calculated by dividing the number of Bt-like isolates by the total number of Bacillus-like colonies obtained.
[b] average Bt index

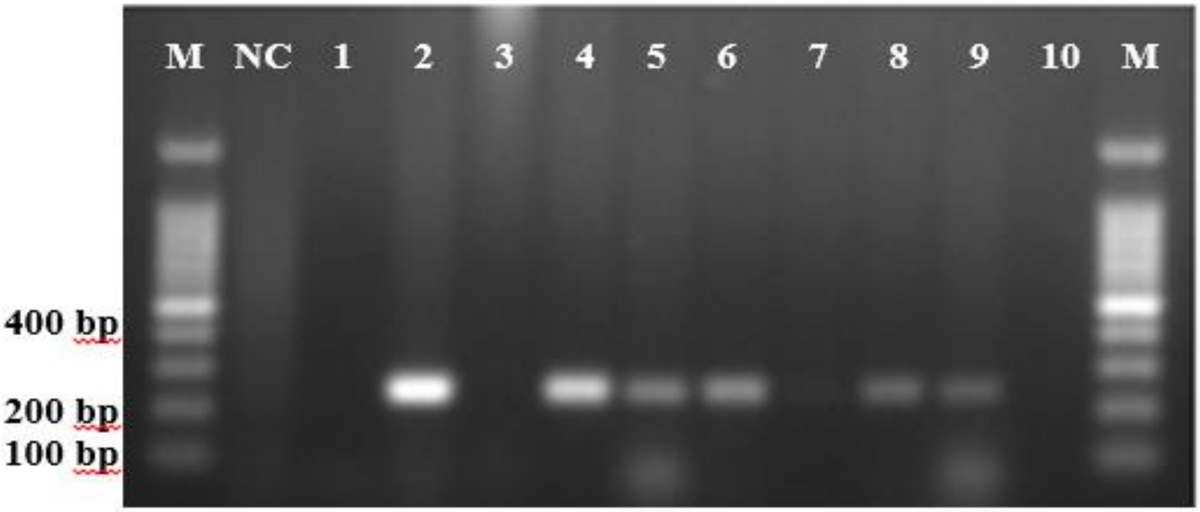

Plate II: Amplicons of *XRE* genes of *Bacillus thuringiensis* isolates from selected soil samples. Lane M: 100 bp DNA ladder marker (Biolabs), Lane NC: Negative control, Lane 1: cow range at Zango CR1[3], Lane 2: Metal recycling site at Yankarfe, MRS2[2], Lane 3: Lambo pepper Farm, Zango F3[2], Lane 4: Metal recycling site at Yankarfe, MRS1[3], Lane 5: Metal recycling site at Yankarfe, MRS2[3] Lane 6: Metal recycling site at Yankarfe, MRS2[1,] Lane 7: cow range at Zango CR2[1] Lane 8: cow range at Zango CR2[2], Lane 9: cow range at Zango CR2[3], Lane 10: cow range at Zango CR2[4], Lane NC: Negative control. The expected molecular sizes of *XRE* gene was at 246bp.

4.2 *Bacillus thuringiensis* Isolates AgNPs production potential

Five of the six isolates showed brown/dark colouration, while the remaining one showed a light ash colouration (Appendix III), all indicating the ability to produce silver nanoparticles. The absorbance readings at 420 nm of the produced AgNPs were as shown in Table 4.6. Isolates MRS2^1 and CR2^3 with the highest AgNPs producing potentials were further used for bioproduction and characterization of the AgNPs.

4.3 Bioproduction of Silver Nanoparticles from *Bacillus thuringiensis*

AgNPs production was characterised with colour formation from the reduction of silver ion by the enzyme; nitrate reductase. The intensity of colour increased as the bio-reduction of silver ion progresses and becomes stable when the reaction was completed. Appendix IV shows the brown solution formed from the reaction of culture supernatant and $AgNO_3$ solution after 24 h of the reaction, while the $AgNO_3$ solution and culture supernatant in the control experiment remained unchanged. The dark brown solution formed was an indication of the synthesis of AgNPs.

Table 4.6: Silver Nanoparticles Production potential by the *Bacillus thuringiensis* Isolates measured by absorbance at 420nm

Isolate code	Absorbance
CR2^3	0.811
MRS1^3	0.303
CR2^2	0.173
MRS2^1	0.879
MRS2^2	0.481
MRS2^3	0.200

4.4 Characterization of Silver Nanoparticles Synthesized by *Bacillus thuringiensis*

4.4.1 UV-vis spectrophotometry

As shown in Figures 4.1 and 4.2, the broad peaks portrayed by AgNPs from each of the isolates of *B. thuringiensis* were within the range of 434-440 nm as shown by the UV- visible absorbance spectra which further gave credence to the formation of silver nanoparticles.

4.4.2 Fourier Transform Infrared (FTIR) Analysis

Figures 4.3 and 4.4 shows the FTIR spectra analysis of the AgNPs produced by isolates $MRS2^1$ and $CR2^3$. The FTIR absorption spectra showed distinct strong peaks at 3379, and 1643cm^{-1} for $CR2^3$, while 3302, and 1643 cm^{-1} were obtained for $MRS2^1$. Other minor peaks at 3942, 3865, 3796, 2407, 2137, 1265 and 1087 cm^{-1} for $CR2^3$, and 3942, 3873, 3788, 2692, 2399, 2137, 1257 and 109 cm^{-1} for $MRS2^1$ were obtained.

4.4.3 Scanning Electron Microscopy (SEM)

The micrograph obtained as shown in Figure 4.5 for AgNPs produced by *B. thuringiensis* isolate $MRS2^1$ showed that the particles were majorly anisotropic in shape and partly irregular with size of 748 nm. The micrograph obtained for AgNPs produced by *B. thuringiensis* isolate $CR2^3$ as shown in Figure 4.6 showed that the particles were majorly irregular in shape with size of 748 nm.

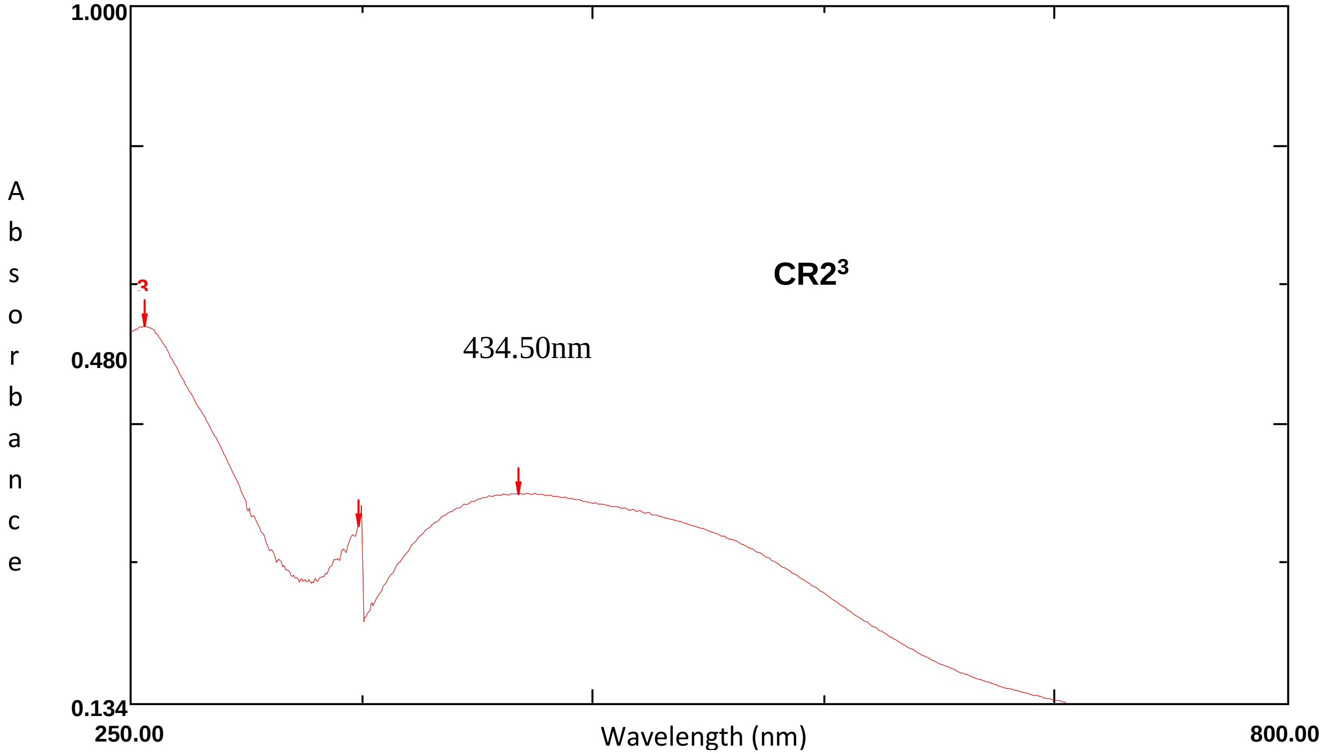

Fig. 4.1: UV absorption spectrum showing characteristic peak at 434.5nm for silver nanoparticles produced by *B. thuringiensis* isolate CR2[3]

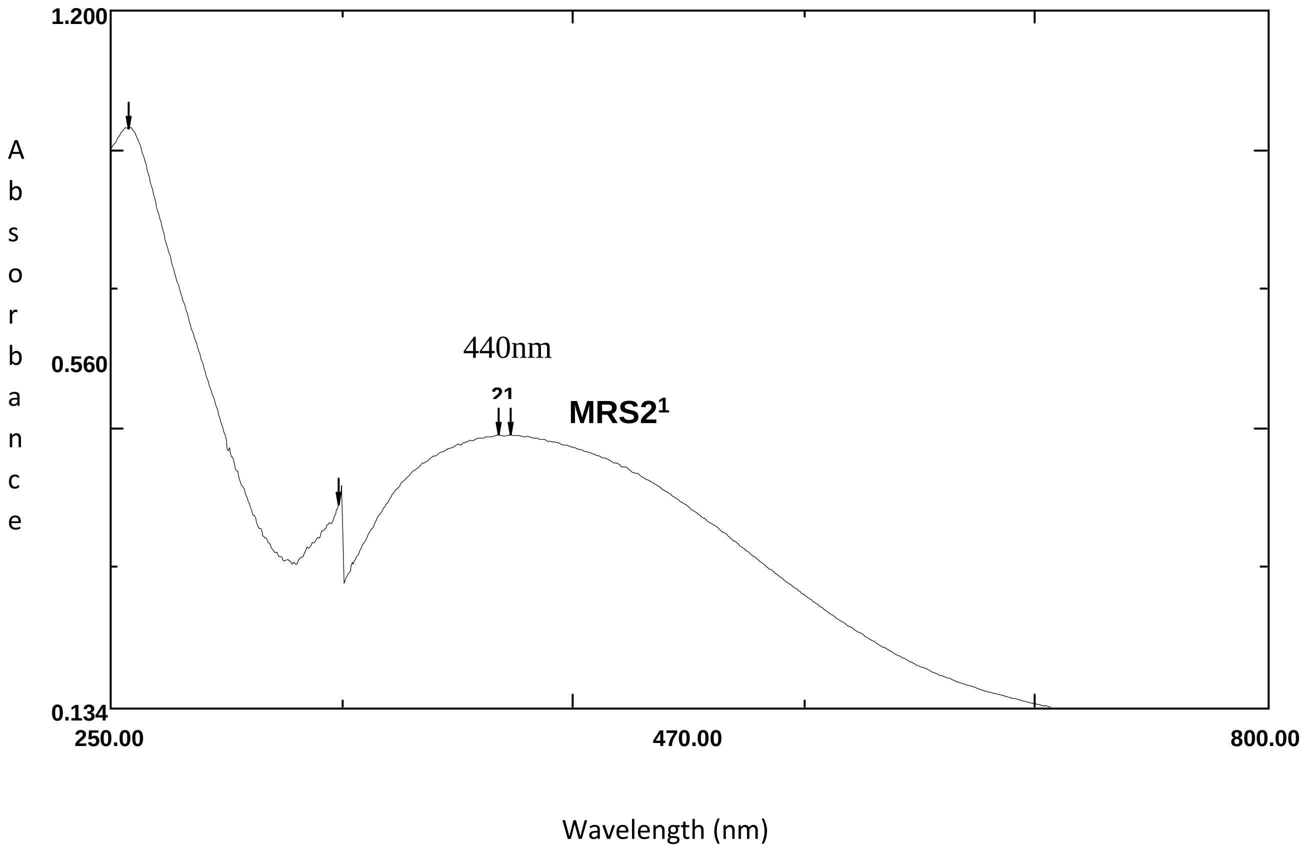

Fig 4.2: UV absorption spectrum showing characteristic peak at 440nm for silver nanoparticles produced by *B. thuringiensis* isolate MRS2^1

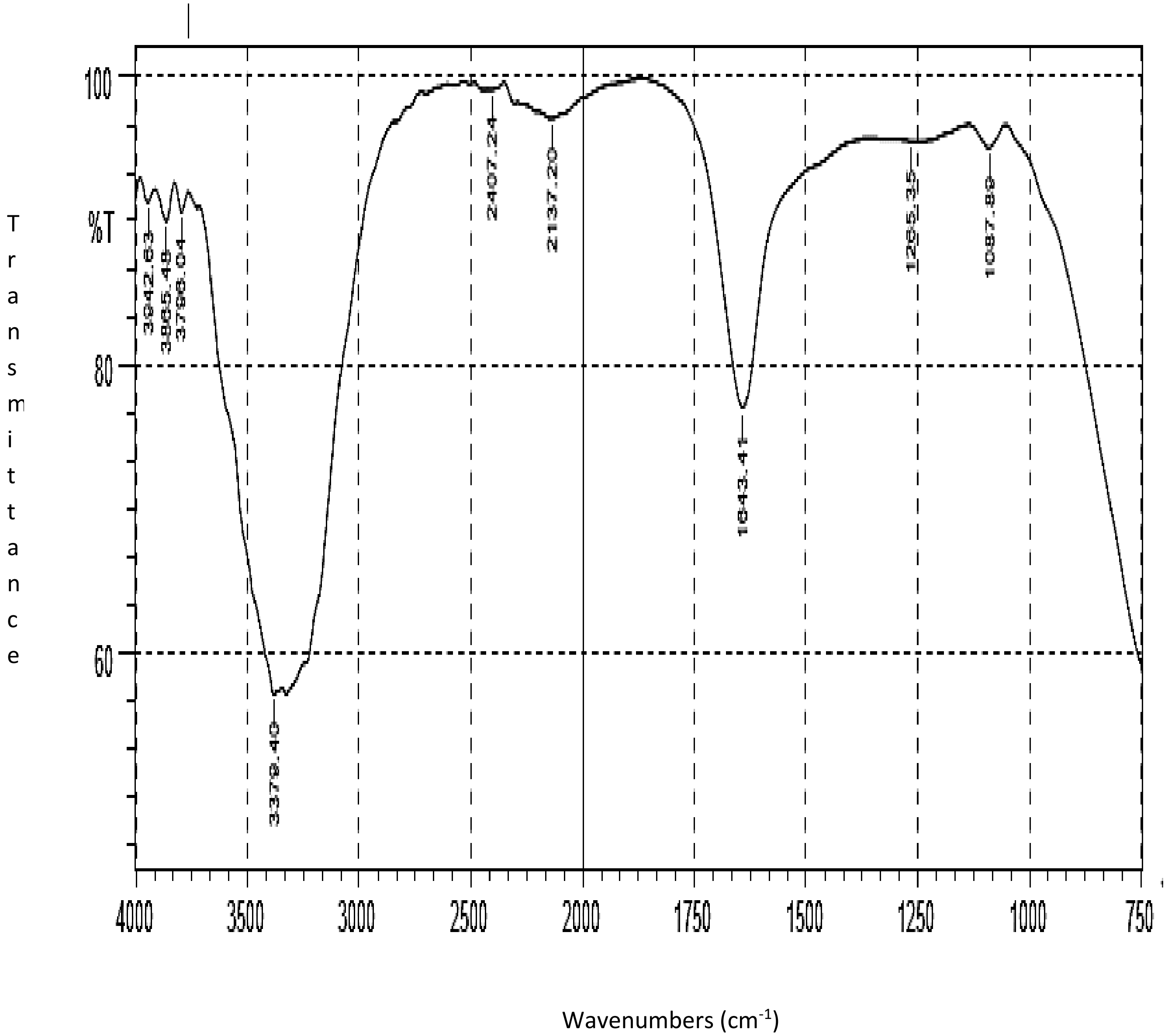

Fig. 4.3: FTIR spectrum of silver nanoparticles produced by *B. thuringiensis* isolate CR2[3]

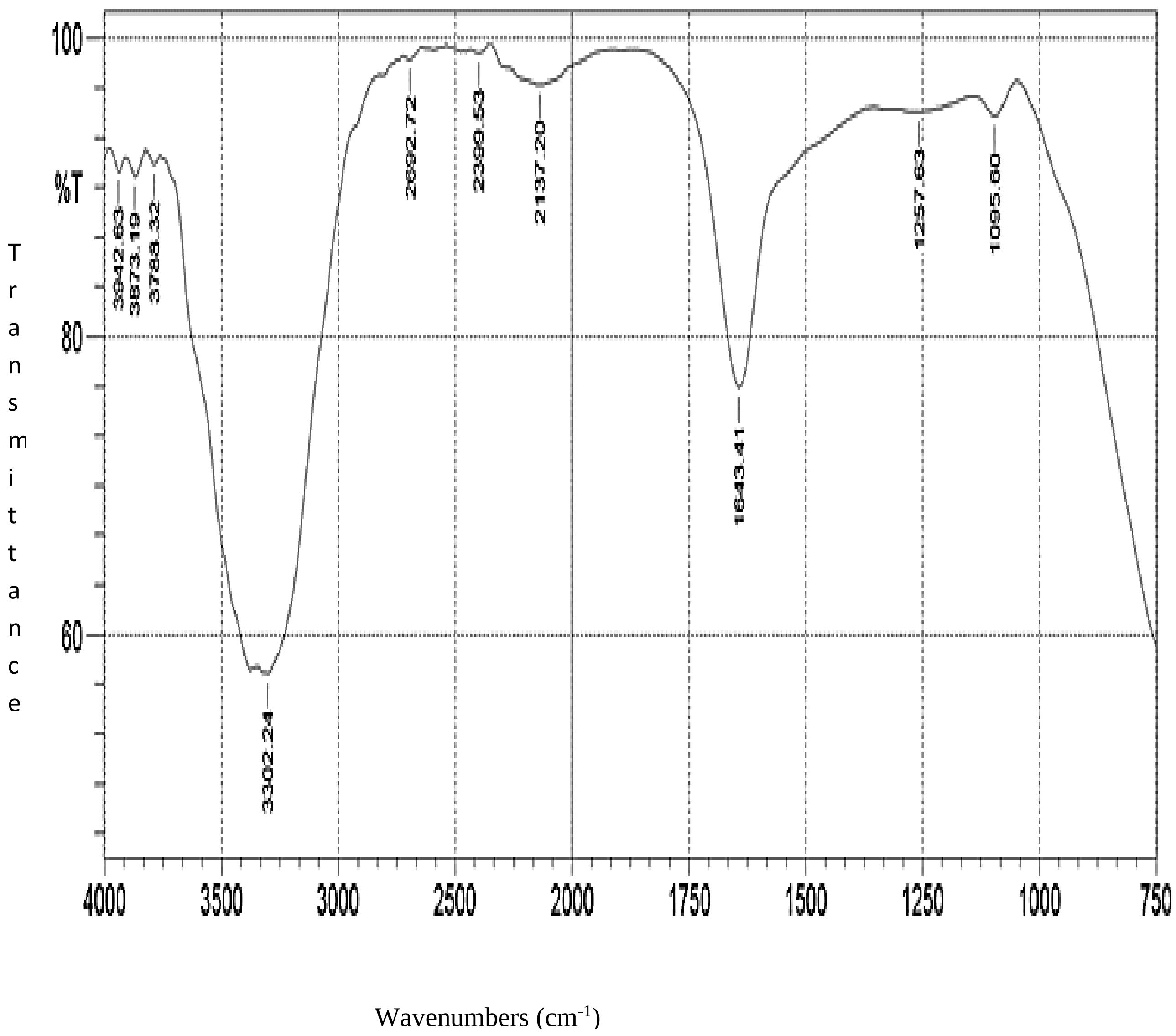

Fig. 4.4: FTIR spectrum for silver nanoparticles produced by *B. thuringiensis* isolate MRS2[1]

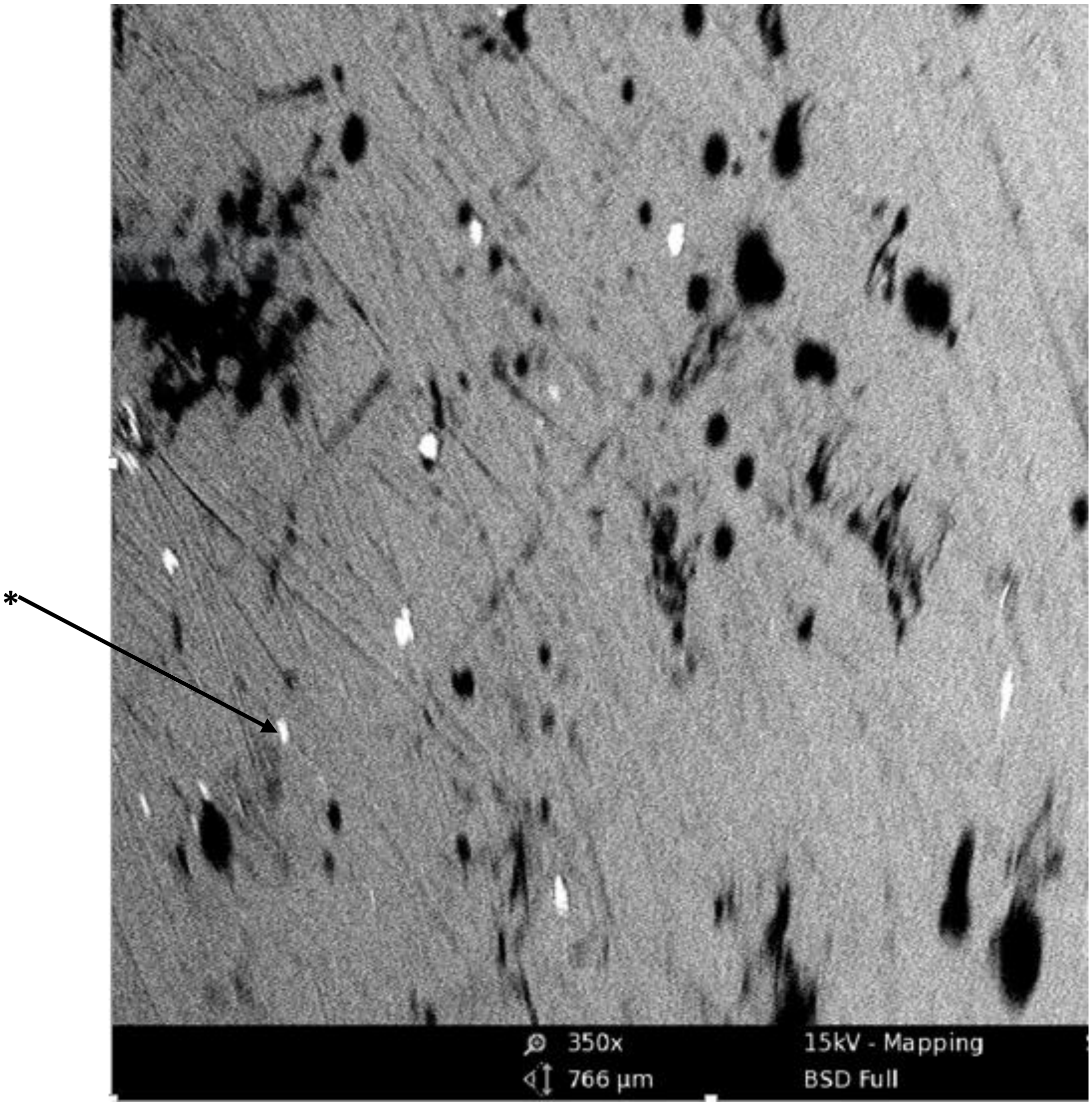

Fig. 4.5: SEM image of AgNPs produced by *B. thuringiensis* isolate MRS2[1]

* Arrows showing silver nanoparticles synthesized

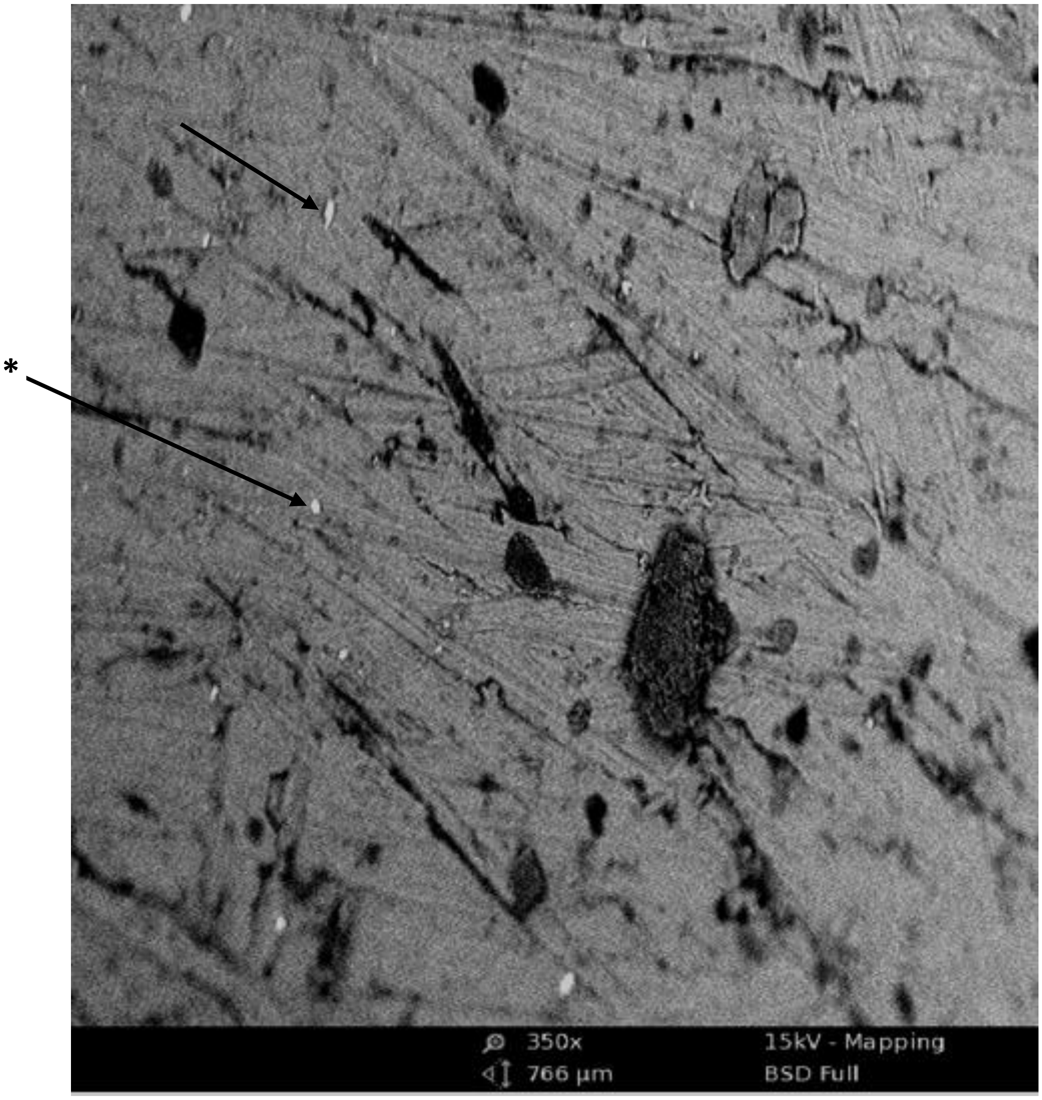

Fig. 4.6: SEM image of AgNPs produced by *B. thuringiensis* isolate CR2[3]

* Arrows showing silver nanoparticles synthesized

4.5 Antimicrobial Activity of the synthesized Silver Nanoparticles

Table 4.7 shows the antimicrobial resistant pattern of the test organisms while Tables 4.8 and 4.9 show the result of the antibacterial activity of the synthesized AgNPs. The synthesized silver nanoparticles exhibited antibacterial activity against all the tested bacteria; *Klebsiella pneumoniae*, *E.coli* (strain 2) and *Staphylococcus aureus* except *E.coli* (strain 1) which was resistant at all AgNPs concentrations tested. The zone of inhibition of 100µg/ml AgNPs produced by Bt isolate MRS2^1 (22mm) against Klebsiella *pneumoniae* was greater than that of ciprofloxacin (10 µg) 20mm.

The MIC were found to be at 50 mg/mL for all tested organisms, except *Klebsiella pneumoniae* that was at 25µg/ml for AgNPs produced by isolate MRS2^1. MBC were recorded at 100 µg/mL for all tested organisms, except *E.coli* (strain 2) that was at 75µg/ml for AgNPs produced by MRS2^1. The Minimum inhibitory and minimum bactericidal concentrations are as presented in Tables 4.9– 4.12.

Table 4.7**: The antibiotics resistant pattern of the test organism**

Test organism	**Resistance pattern**	**MAR index**
***E.coli* (strain 1)**	AMC, TE, CPX,AMP and CFT	0.5
***E.coli* (strain 2)**	AMC, TE and AMP	0.3
Klebsiella pneumonia	CPX, SP, AMP and AU	0.4
Staphylococcus aureus	APX	0.1

AMC- Amoxicillin-clavulanic acid, TE- Tetracycline, AMP- Ampicillin, CPX- Ciprofloxacin, CFT- Ceftriaxone, SP- Sparfloxacin, AU- Augumetin and APX- Ampiclox

Table 4.8: Antibacterial activity of silver nanoparticles synthesized (CR2^3) against some pathogenic bacteria

Test organism	Diameter of inhibition zone produced by silver nanoparticles(nm) (mean±SD)				Diameter of inhibition zone produced by controls(nm) (mean±SD)	
	100 µg/ml	**75 µg/ml**	**50 µg/ml**	**25 µg/ml**	**Ciprofloxacin (10µg/ml)**	**Bt supernatant without AgNO$_3$**
E.coli (strain 1)	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
E.coli (strain 2)	19.00±1.6	13.00±0.4	13.00±1.6	13.00±1.5	30.00±0.8	0.00±0.0
Klebsiella pneumoniae	15.00±0.4	14.00±0.8	14.00±0.8	13.00±0.4	20.00±0.4	0.00±0.0
Staphylococcus aureus	17.00±0.8	15.50±0.4	14.00±0.4	13.50±0.4	40.00±0.8	0.00±0.0

Table 4.9: Antibacterial activity of synthesized silver nanoparticles by *B.thuringiensis* isolate (MRS2^1) against some potentially pathogenic bacteria

Test organism	Diameter of inhibition zone produced by silver nanoparticles(nm) (mean±SD)				Diameter of inhibition zone produced by controls(nm) (mean±SD)	
	100 µg/ml	75 µg/ml	50 µg/ml	25 µg/ml	Ciprofloxacin (10µg/ml)	Bt supernatant without $AgNO_3$
E.coli (strain 1)	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
E.coli (strain 2)	19.00±0.8	15.00±0.7	13.00±1.5	12.50±0.8	30.00±0.4	0.00±0.0
Klebsiella pneumoniae	22.00±0.2	20.00±0.2	19.00±0.8	19.00±0.4	20.00±0.4	0.00±0.0
Staphylococcus aureus	20.00±0.3	16.00±0.3	15.00±0.0	11.00±0.2	40.00±0.0	0.00±0.0

Table 4.10: Minimum inhibitory concentration of synthesized silver nanoparticles by *B. thuringiensis* isolate (MRS2[1]) against some pathogenic bacteria

Test organism	25µg/mL	50µg/mL	75µg/mL	100µg/mL	*B.thuringiensis* culture	Silver nanoparticles
E. coli (Strain 2)	+	**-***	**-**	**-**	+	**-**
Staphylococcus aureus	+	**-***	**-**	**-**	+	**-**
Klebsiella pneumoniae	**-***	-	-	-	+	-

Key: + means **growth; -: no growth, *=MIC**

Table 4.11: Minimum inhibitory concentration of synthesized silver nanoparticles by *B. thuringiensis* isolate (CR2^3) against some pathogenic bacteria

Test organism	25µg/ml	50 µg/mL	75 µg/mL	100 µg/mL	*B.thuringiensis* culture	Silver nanoparticles
E. coli (Strain 2)	+	-*	-	-	+	-
Staphylococcus aureus	+	-*	-	-	+	-
Klebsiella pneumoniae	+	-*	-	-	+	-

Key: + means **growth; -: no growth, *=MIC**

Table 4.12: Minimum bactericidal concentration of synthesized silver nanoparticles by *B. thuringiensis* isolate (MRS2^1) against some pathogenic bacteria

Test organism	25µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
E.coli (Strain 2)	+	+	**-***	**-**
Staphylococcus aureus	+	+	+	**-***
Klebsiella pneumoniae	+	+	+	**-***

Key: + means **growth; -: no growth, *=MBC**

Table 4.13: Minimum bactericidal concentration of synthesized silver nanoparticles by *B. thuringiensis* isolate ($CR2^3$) against some pathogenic bacteria

Test organism	25μg/mL	50 μg/Ml	75 μg/mL	100 μg/mL
E.coli (Strain 2)	+	+	+	**-***
Staphylococcus aureus	+	+	+	**-***
Klebsiella pneumoniae	+	+	+	**-***

Key: +: growth; -: no growth, *=MBC

CHAPTER FIVE

5.0 DISCUSSION

Ten (10) of the isolates were motile, catalase, citrate, MR, VP and starch hydrolysis positive and indole negative. All 10 isolates were assumed to be *Bt* and this result coincides with that of Eswarapriya *et al*. (2010) and Bergey (2004) that reported the strains of *Bacillus thuringiensis* to be positive for catalase production, citrate utilization, starch and casein hydrolysis.

The average Bt index was 0.33 with the highest being for samples from Cow Range (0.5) while the lowest was from Farmland (0.1), Compared to other sampling siteS the high occurrence in cow range site could be attributed to its high organic content (cow dung) in the soil which favors proliferation of Bt (Mukhija and Khanna, 2018). On the other hand, the low Bt index recorded for samples in Farmland could be due to overuse and misuse of pesticide and/or other agricultural practices such as tillage. According to the report of other authors, the average Bt index varies between soil samples across the world (El-Kersh *et al*., 2011; Renganathan *et al*., 2011) and this may be due to variance in nutrient availability, environmental condition, isolation source or geography.

The amplification of *XRE* gene in this study revealed that six of the ten isolates were positive. Wei *et al*. (2019) have reported 97.3% accuracy when using *XRE* gene to differentiate between *B.cereus* and *B. thuringiensis*. However, the *cry2* gene was not amplified in any of the isolates. It has been reported that the *B. thuringiensis* strain may synthesize one or more crystal proteins since several crystal toxin genes have been found for *B. thuringiensis*. The main reason for the diversity of toxin genes is the transfer of plasmids in *B. thuringiensis,* some *cry* genes have been reported in *B.cereus* too (Adang *et al*., 2014). Wei *et al*. (2019) also reported isolates of *B. thuringiensis* harbouring no *cry2* genes. The likely reason for the efficiency of *XRE* gene over *cry2* gene in detecting *B.*

thuringiensis in this study could be due to the fact that *XRE* is a transcriptional regulator that regulates the major type of crystal protein production and entire gene activity.

All isolates grew vigorously on media supplemented with 1mM $AgNO_3$ indicating their ability to synthesize AgNPs. All isolates changed the color of their solution which usually is the indication for the formation of AgNPs. One of the isolates did not show the known characteristic brown colour but a light ash colour, which could be an indication that the isolate did not produce silver nanoparticles or did so only weakly, as suggested by its low absorbance (0.173). Isolates $CR2^3$ and $MRS2^1$ with the highest absorbance (0.811 and 0.879 respectively) were further selected for production and characterization of AgNPs since absorbance is proportional to concentration, the higher the concentration of the AgNPs formed, the higher the absorbance. Also, authors such as Lateef *et al*. (2014) have confirmed that there can be other colors apart from the characteristic known brown color which could be attributed to the variations in the composition of biomolecules found in the media used for culturing the organisms and/or the organisms that mediated the AgNPs synthesis (Lateef *et al*., 2014).

Preliminary confirmation of the synthesis of silver nanoparticles was ascertained by visual color change. The mixture of cell culture supernatant and 1mM $AgNO_3$ changed from cloudy white to brown while the controls (1mM $AgNO_3$ and cell culture supernatant) remained unchanged. Several studies have also reported the appearance of brown colour as preliminary sign that AgNPs have been formed which supports the findings of this study. Mahmoud *et al*. (2016) reported AgNPs of brown colour synthesized from culture supernatant of *Bacillus pumilus*, while Lateef *et al*. (2014) reported dark brown-coloured AgNPs synthesised from keratinase of *Bacillus safensis*. Similar observation was reported for the supernatant of *Bacillus megaterium*, where a pale yellow to brown colour was formed due to the reduction of aqueous silver ions to silver nanoparticles (Saravanan *et al*., 2010) and Elamawi *et al*. (2018) also reported brown colouration from pale yellow when AgNPs synthesis was mediated by *Trichoderma longibrachiatum*. The development of colour as a function of formation of nanoparticles is due to the excitation of surface plasmon resonance of the reduced metal particles, which was facilitated by the bioreductant molecules in the cell-free extract (Ojo *et al*., 2016).

Furthermore, broad peaks at wavelength of 434nm for isolate CR2^3 and 440nm for isolate MRS2^1 following UV visible spectrometry confirmed the production of AgNPs. This agrees with other studies that reported peaks of absorption spectra of AgNPs in the range 391–450 nm which is the characteristic peak for AgNPs. The broad peaks seen in this study are due to presence of polydisperse particles (Jain *et al*. 2010). Jain *et al*. (2010) reported an absorption peak for *Bacillus thuringiensis* spore crystal silver nanoparticle at 450 nm. Biologically synthesized silver nanoparticles using other organism such as *Bacillus licheniformis*, *Aspergillus flavus*, *Bacillus subtilis*, and *Aeromonas sp*, had absorbance peaks in the range of 400 to 440nm (Dhoondia and Chakraborty 2012). Kumar *et al*. (2018) also reported a peak at 436nm from production of AgNPs by *Brevibacterium invocatus* while Prakash *et al*. (2011) reported silver plasmon peak for *Bacillus cereus* at 435 nm. The absorption spectra of silver nanoparticles exhibited an intense absorption peak range due to its surface plasmon excitation, which describes the collective excitation of conduction electron in metal (Thomas *et al*., 2014). The peak at 292 nm in this study could be attributed to the presence of proteins due to the tyrosine and tryptophan residues present in the protein moiety of the media used (Ganesh Babu and Gunasekaran 2009; Lateef *et al.*, 2015). Schmid (2001), also stated that the side chains of aromatic amino acids like tyrosine, tryptophan and phenylalanine absorb in the near region of 240-300nm.

Furthermore, FTIR measurements identified the possible interactions between silver and bioactive molecules, which may be responsible for synthesis and stabilization (capping) of silver nanoparticles. The broad peaks at 3302–3379 cm^{-1} and 1643 cm^{-1} correspond to the existence of amine and amide I group respectively indicating that proteins present in the cell free extract of *B.thuringiensis* culture supernatant were the capping and stabilization biomolecules in the synthesis of AgNPs (Shankar *et al*. 2014). This supports the presence of proteins in the synthesized AgNPs as observed in UV-Vis spectra analysis at 292nm. The broadness of the peak is due to the overlap of both O-H and N-H bond stretching of primary and secondary amines (Ojo *et al*., 2016). The peaks 1257–1265, 2137, and 2399–2407 cm^{-1} are assigned to the O-H vibration of alcohols, $C \equiv C$ stretch of alkynes, and C-N of nitrogen compounds respectively. These indicate that biomolecules also rich in amine (N-H) and hydroxyl (O-H) groups were responsible for the reduction of the metal ions (Ag^+), as well as capping of AgNPs to prevent their agglomeration (Kumari *et al*., 2015; Elamawi *et al*., 2018). FTIR results also revealed that secondary structure of proteins was not affected as a consequence of reaction with silver ions or binding with AgNPs (Jain *et al*., 2010). It is well known that nanoparticle–protein interactions can occur either *via* free amino groups or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzyme proteins (Gole *et al*. 2001; Mandal *et al*. 2005). The carbonyl groups of amino acid residues and peptides had strong ability to bind to silver (Balaji *et al*., 2009). It is important to understand though, that it is not just the size and shape of proteins, but the conformation of protein *molecules* that plays an important role in stabilization of the AgNPs produced (Jain *et al*., 2010a).

Scanning Electron Microscopy (SEM) proffered vital data about the size and shape of silver nanoparticles synthesized by Bt isolates. The particles were predominantly irregular or partly anisotropic in shape. These shapes conform to the types of nanoparticles shapes reported by Tarannum *et al*. (2019) and it also supports the result of the UV-visible scan analysis that showed a broad peak indicating the presence of more than one shape. The particles size was 748nm for the isolates clearly indicating the formation of small particles. Several authors have reported various sizes and their findings support this study. Dash (2013) and Murthy *et al.* (2014) reported sizes of silver nanoparticles mediated by *Bacillus thuringiensis* ranging from 450 to 1000nm and 32-1106 nm respectively. However, some studies have reported even smaller sizes of AgNPs such as 5-15 nm (Kumar *et al*., 2018) and 15-25 nm (Krishna *et al*., 2017). The small size of silver nanoparticles facilitates their access through the cell membrane of pathogens hence their medical and pharmacologic applications (Omidi *et al*., 2014).

Finally, this study sought to test for the antibacterial properties of silver nanoparticles synthesized by *Bt* isolated from soil on some potentially pathogenic isolates. The clear zone of inhibition by silver nanoparticles against the isolates; *E. coli* (strain 2), *Klebsiella pneumoniae*, and *Staphylococcus aureus* were observed except for *E.coli* (strain 1). It could be that higher concentrations of AgNPs are needed to inhibit the growth of *E.coli* (strain 1). The antimicrobial activity of the AgNPs synthesized from both isolates were similar, and this could be attributed to the fact that their sizes and shapes were similar. The MIC of AgNPs produced by isolate $MRS2^1$ against the selected isolates was 50µg/ml except *Klebsiella pneumoniae* which was 25µg/ml, while the MBC for all the tested pathogens was 100µg/ml except for *E.coli* (strain 2) which was at 75µg/ml of AgNPs produced by isolate $MRS2^1$. Alsamhary (2020) reported MIC at higher concentration of 300 µg /mL for *Klebsiella pneumoniae* as against MIC of 50 µg /mL recorded in this study.

The antibacterial activity of AgNPs did not differ based on whether the test organisms were Gram negative or positive, this supports the report of Ravishankar and Jamuna (2011).

Silver nanoparticles (100 µg /ml) synthesized by *Bacillus cereus* produced a zone of inhibition of 10mm against *E.coli* (Prakash *et al.*, 2011). Lateef *et al.* (2014) reported AgNPs (150 µg /ml) from *Bacillus safensis* LAU 13 produced zone of inhibition of 8.6 to 12.5 mm against some clinical *E. coli* strains. In another study, the inhibitory effect of AgNPs were tested on *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus*, and 60-100 µg/ml concentrations inhibited these bacteria (Lateef *et al* 2015). Ojemaye *et al*. (2020) reported lower zones of inhibition for *E.coli and Staphylococcus aureus* at 12.40 mm and 7.5 mm respectively at concentration of 60 mg/mL with average AgNPs size of 32nm. The zones of inhibitions in this study were achieved at a lower concentration and this contradicts Elbeshehy *et al*. (2015) who stated that smaller sized NPs are better antimicrobial agent as they can easily penetrate the cell wall considering the fact that the AgNPs produced by the cited authors were smaller in size. However, Yurtluk *et al*. (2018) tested the antibacterial potential of the silver nanoparticles synthesized by *Bacillus sp SBT8* on *S.aureus*, and *E.coli* 0157: H7 and observed zones of inhibition of 11 mm and 8 mm respectively at a lower concentration of 10 µg /mL of synthesised AgNPs than that of this study.

Aside size, the shapes of NPs also accounts for antimicrobial activity. NPs interacting with periplasmic enzymes cause varying gradations of bacterial cell damage with respect to the shape of NPs (Cha *et al*. 2015; Varier *et al*., 2019). Although there are currently no reports on the antibacterial activity of irregular/anisotropic shaped NPs seen in this study, there are reports and activities of other shapes. Actis *et al*. (2015) reported that cube-shaped AgNPs exhibit stronger antibacterial activity than sphere-shaped and wire-shaped AgNPs with similar diameters, due to the specific surface area and facet reactivity. However, Yao *et al*. (2013) compared the antibacterial activity of polymer nano-objects with sheet-like, cylindrical, and spherical shapes and found no significant difference in antibacterial performance across the series.

The possible mechanisms of AgNPs toxicity to bacteria is that AgNPs adhere to the bacteria surface and affect the membrane function by perforating the cell wall and disrupting the integrity of the membrane, which leads to cell death (Liu *et al*. 2010; Bakirde *et al*., 2015). The silver atom is inert and stable, but it becomes reactive when it assumes an oxidation state of +1. The silver ions (Ag^+) bind to proteins in the plasma and nuclear membrane, forming a complex that leads to structural changes in the membrane (Reidy *et al*., 2013; Le Ouay and Stellacci, 2015). Ag^+ are also released *via* the dissolution of AgNPs. The Ag^+ ions react non-selectively with electron-donating groups such as thiols, hydroxyls, imidazoles, and phosphates (Franci *et al*., 2015). AgNPs are also believed to exhibit antibacterial activity by triggering the formation of reactive oxygen species. These species then interact with the glycoproteins on the cell wall before being transferred into the cytoplasm, where they show major antibacterial activity (Pal *et al*., 2007). Sharma *et al*. (2009) suggests that AgNPs may interfere with the permeabilility process and respiratory function of the cell by disturbing the components of the microbial electron transport system.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, six *B. thuringiensis* isolates were identified in this study, of which, 4 (67%) were isolated from metal recycling sites and the other 2 (33%) from cow rangeland. Of the six, isolates MRS2^1 and CR2^3 showed the highest silver nanoparticles (AgNPs) producing potentials after screening. These isolates MRS2^1 and CR2^3 were observed to produce silver nanoparticles (AgNPs) within 24 h. UV- vis spectra, FTIR and SEM analysis used for characterization confirmed that AgNPs were formed with protein acting as capping agent and revealed that the average particle size was 748nm. The AgNPs produced were proven to have inhibitory effect on some potentially pathogenic bacteria including *E.coli, S.aureus* and *K.pneumoniae,* thus implying possible applications in the development of coating agents, medical devices and instruments, wound healing bandages and antibacterial agents/devices.

6.2 Recommendations

1. There is need for further studies to determine conditions that allow for high concentrations of AgNPs using microorganisms to enable mass production.

2. The synergistic effect of silver nanoparticles and antibiotics should also be evaluated as this could greatly improve the efficiency of antibiotics currently in use and reduce drug resistance in microorganisms. In vitro trials should be done to confirm this.
3. There is need to compare the efficacy of the irregular/ anistropic shape of nano particles (NPs) as antimicrobials against other shapes of NPs.

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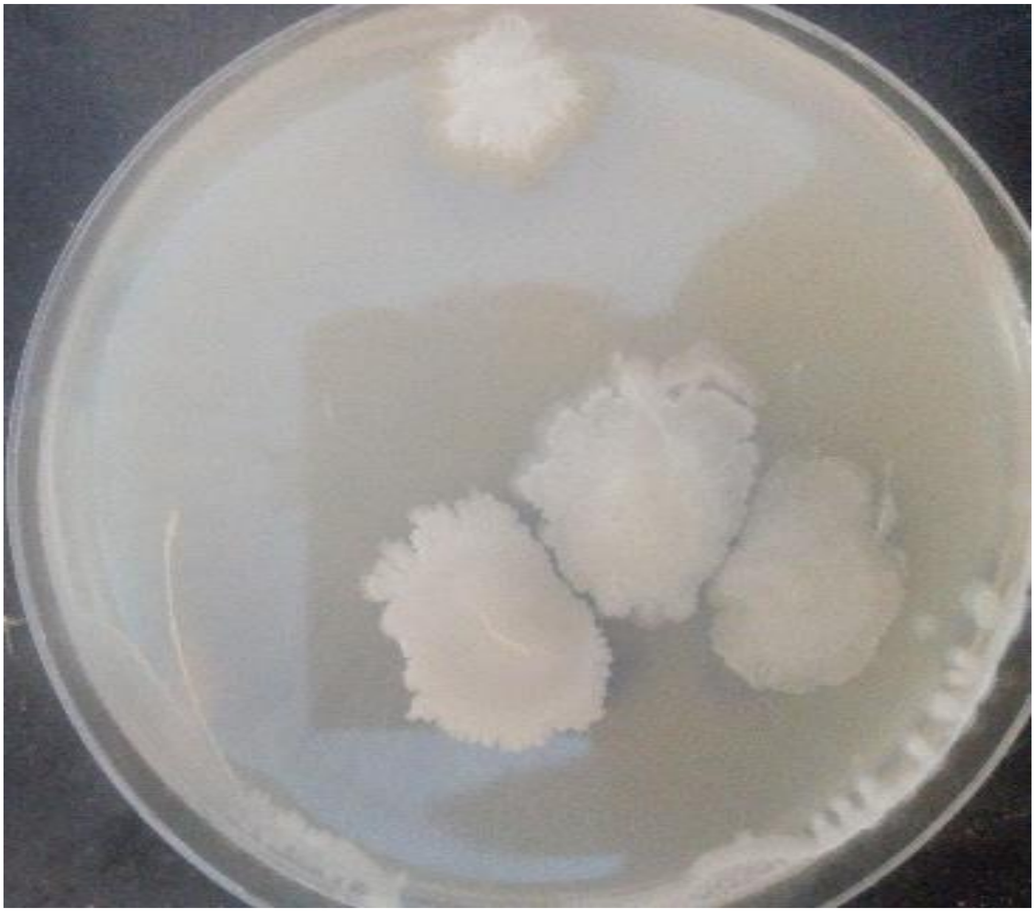
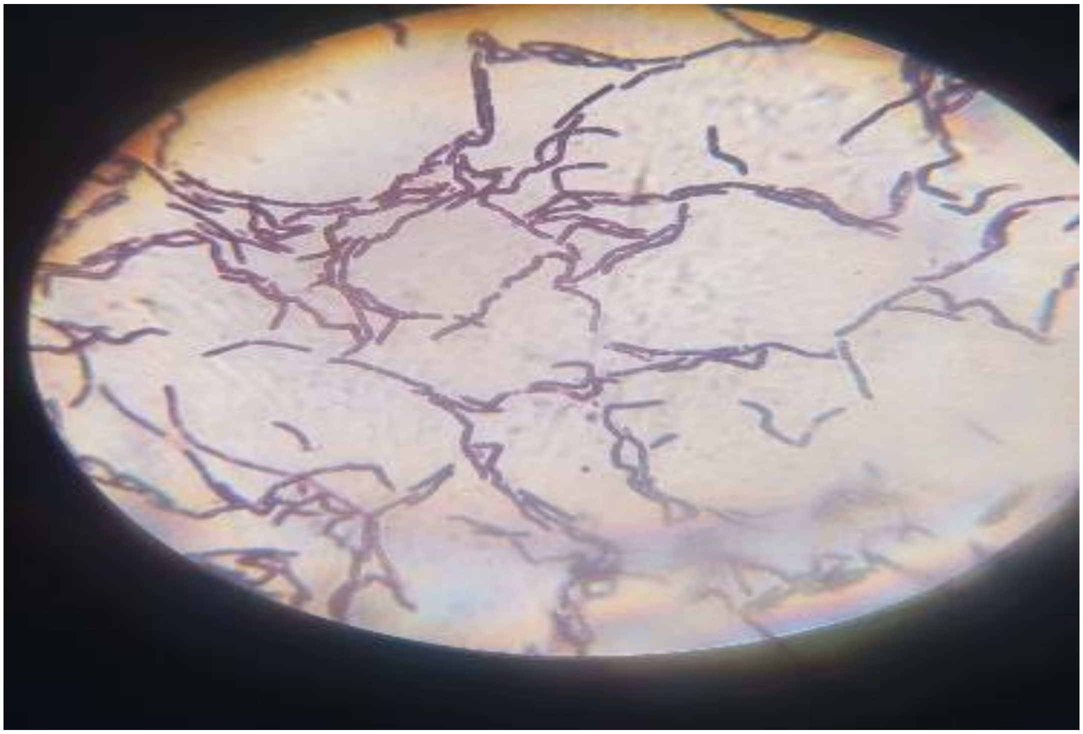
APPENDICES

Appendix I: Pure culture of *B. thuringiensis* on Luria Bertani agar

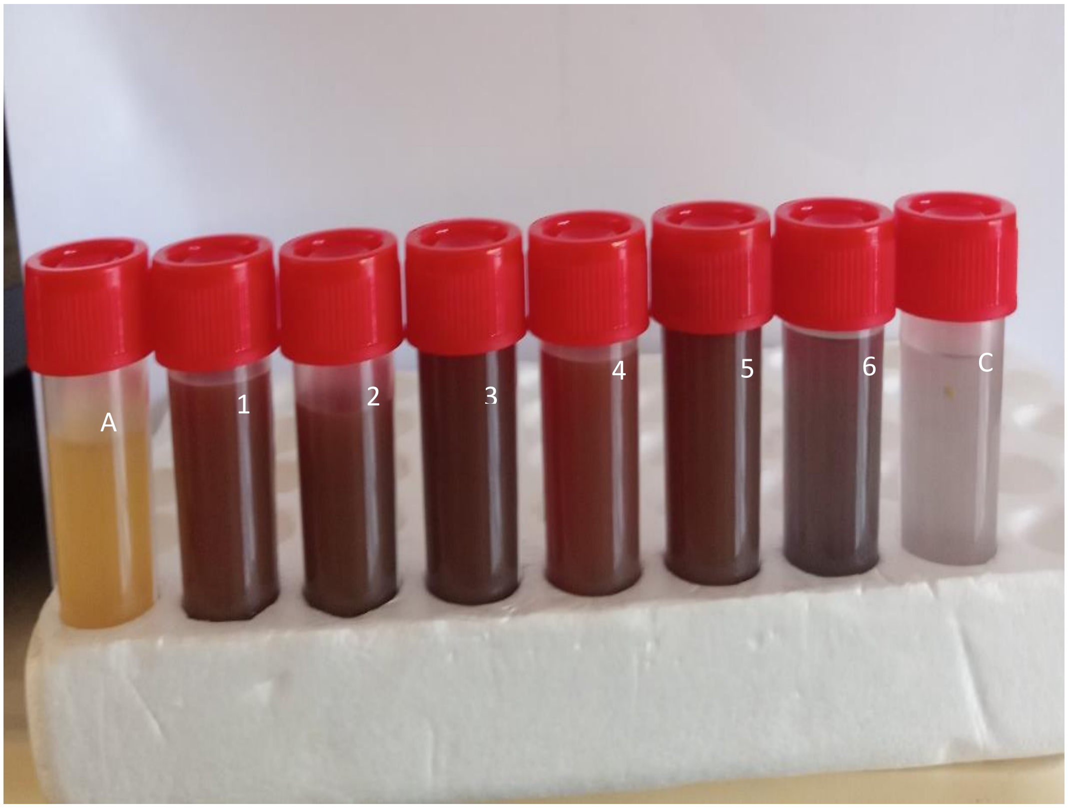


Appendix II: Microscopic characteristics of *B. thuringiensis* after Gram's staining (X100)



Appendix III: **Screening for Silver Nanoparticles production by the six *B. thuringiensis* Isolates.**

A: *Bacillus thuringiensis* supernatant, 1-6: Reaction mixture of *Bacillus thuringiensis* isolates + $AgNO_3$, C: $AgNO_3$ (Control).



Appendix IV: Production of silver nanoparticles by selected *B.thuringiensis* isolates

Key: A: *Bacillus thuringiensis* supernatant (Control); 1-2: Reaction mixture of *Bacillus thuringiensis* isolates + $AgNO_3$; C: $AgNO_3$ (Control).

Appendix V: **Raw Data for the Antibacterial activity of silver nanoparticles synthesized ($CR2^3$) against some pathogenic bacteria**

Test organism	**Diameter of inhibition zone produced by silver nanoparticles(nm) (mean±SD)**				**Diameter of inhibition zone produced by controls(nm) (mean±SD)**	
	100 µg/ml	**75 µg/ml**	**50 µg/ml**	**25 µg/ml**	**Ciprofloxacin (10µg/ml)**	**Bt culture without AgNO3**
	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00
E.coli (strain 1)	0.00	0.00	0.00	0.00	0.00	0.00
	21.00	13.00	13.00	15.00	30.00	0.00
	17.00	12.50	15.00	11.50	29.00	0.00
E.coli (strain 2)	19.00	13.50	11.00	12.50	31.00	0.00
	15.00	14.00	14.00	13.00	20.00	0.00
Klebsiella	15.50	13.00	15.00	12.50	20.50	0.00
pneumoniae	14.50	15.00	13.00	13.5	19.50	0.00
	17.00	15.50	14.00	13.50	40.00	0.00
Staphylococcus	18.00	16.00	14.50	13.00	39.00	0.00
aureus	16.00	15.00	13.50	14.00	41.00	0.00

Appendix VI: **Raw Data for the Antibacterial activity of silver nanoparticles synthesized (MRS2^{1}) against some pathogenic bacteria**

Test organism	**Diameter of inhibition zone produced by silver nanoparticles(nm) (mean±SD)**				**Diameter of inhibition zone produced by controls(nm) (mean±SD)**	
	100 µg/ml	**75 µg/ml**	**50 µg/ml**	**25 µg/ml**	**Ciprofloxacin (10µg/ml)**	**Bt culture without AgNO3**
E.coli (strain 1)	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00
E.coli (strain 2)	20.00	15.50	11.50	13.50	30.00	0.00
	19.00	15.50	15.00	11.50	29.50	0.00
	18.00	14.00	12.50	12.50	30.50	0.00
Klebsiella pneumoniae	22.00	19.70	19.00	19.50	20.00	0.00
	22.30	20.00	21.00	19.00	20.50	0.00
	21.70	20.30	20.00	18.50	19.50	0.00
Staphylococcus aureus	20.30	15.60	15.00	11.00	40.00	0.00
	20.10	16.30	15.00	10.70	40.00	0.00
	19.60	16.10	15.00	11.30	40.00	0.00