Biosynthesis of copper nanoparticles using a bioflocculant from *Alcaligenis faecalis*, characterization and its application

University of Zululand

MSc Dissertation

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Declaration

The research project presented in this dissertation was conducted at the Department of Biochemistry and Microbiology, University of Zululand (UZ) under the supervision of Prof A.K Basson, Prof V.S.R. Rajasekhar Pullabhotla and Prof J.J. Simonis.

I, Nkosinathi Goodman Dlamini declare that this work, aside from the supervisory guidance received, is the product of my own original work and effort.

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Abbreviations

ANOVA	One-way Analysis Of Variance
BOD	Biological Oxygen Demand
BSA	Bovine serum albumin
CVD	Chemical vapour deposition method
COD	Chemical oxygen demand
CuNPs	Copper nanoparticles
Da	Dalton
DOT	Dissolved oxygen tension
DWAF	Department of Water Affairs
DW	Drinking water
EPS	Extracellular polymers
FA	Flocculating activity
FT-IR	Fourier transform infrared spectroscopy
$FeCl_3$	Iron chloride
g	Gram
GW	Ground water
INT	P-iodonitrotetrazodium violet
L	Litre
MBC	minimal bactericidal concentration
mg	milligram
MIC	minimal inhibitory concentration
ml	Millilitre
Mn^{2+}	Manganese(II) ion or Manganous ion
mV	Millivolts
nm	Nanometre
NO^{3-}	Nitrate ion
NWRS	National Water Strategy Resources
OD	Optical density
PVD	Physical vapour deposition method
RE	Removal efficiency
rpm	Revolutions per minute
RSA	Republic of South Africa

SD	Standard deviation
S	Sulphur
SEM	Scanning electron microscope
SPR	Plasmon resonance spectra
SW	Surface water
*T*d	Degradation temperature
TGA	Thermo gravimetric analyser
UN	United Nations
UV	Ultraviolet
V	Volume
W	Weight
WEF	Water Environment Federation
WHO	World Health Organisation
XRD	X-ray Diffractometer

Research output

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Abstract

Nanotechnology offers a great deal of possibility for the efficient removal of pollutants in the area of water purification. Most common method used for nanoparticles formation such as physical (condensation evaporation) and chemical (oxidation and reduction) are very efficient traditional methods. However, both methods have been found to have some shortcomings. One may require a large amount of energy and the other may involve the use of toxic and expensive chemicals which may results in the binding of the nanoparticles on the surface of these chemicals. Naturally occurring secretion secreted by microorganisms (bioflocculants), have attracted considerable interest due to their harmlessness, biodegradability, and negligible secondary pollution. Therefore, in this present study, biosynthesis of copper nanoparticles was successfully achieved by using a bioflocculant from *Alcalegenis feacalis* and copper sulphate salt. Nanoparticles were characterized using a scanning electron microscope equipped with elementary detector (SEM), UV-visible absorption, Thermogravititional analysis (TGA), Fourier Transform Infrared Spectrophotometer (FT-IR) and transmission electron microscopy (TEM). This new synthesis method shows excellent stability and is eco-friendly. Scanning electron microscopy (SEM) images revealed that the huge significant increase of copper compared to the bioflocculant. The synthesized nanoparticles showed a 35.8% copper and 37% oxygen concentration. This was an indication that nanoparticles were successful synthesized. The synthesized copper nanoparticles were shown to have higher flocculating activity and removal efficiency of both BOD and COD when compared with the bioflocculant and commercial flocculant. Moreover, the potential of CuNPs was also observed when compared to the bioflocculant and iron chloride in kaolin clay, mine water, industrial waste water and domestic waste water. The highest flocculating activity was achieved with the lowest concentration of CuNPs (0.002g/L) with 96% and the least activity was 80% at 0.01g/L. The synthesized copper nanoparticles (CuNPs) are cation independent with 96% flocculating activity following 1% behind the most effecting cations (K^+, Mn^{2+} and Li^+) which had 97%. It functioned the best at pH with 7 with 96% FA. It was not as effective at pH 3 as it was least effective with 55% FA. Furthermore, the nanoparticles were found to be thermostable with a flocculating activity was above 90% in a temperature range of 50-100 ℃. A temperature of 100 ℃ was the least effective with 91% flocculating activity. The synthesized copper nanoparticles are also high in removal efficiency of staining dyes, such as safranin (92%), carbol fuchsine (94%), malachite green (97%) and methylene blue (85%). Furthermore, the synthesized CuNPs were shown to possess high removal efficiencies for elements such as sulphur, calcium, aluminium and phosphorus both in mine and waste water. Nutrients such as

nitrate (NO^{3-}) was also removed effectively by the CuNPs. Removal efficiency for both COD and BOD was also high compared to that of $FeCl_3$ and the pure bioflocculant, with a RE of 76% and 80% respectively.

Key words: bioflocculant, biosynthesis, characterization, copper nanoparticles, and removal efficiency

Background of the study

Bioflocculation

A major problem in the world is environmental pollution (Prasertsan *et al*., 2006). The environment, human and aquatic animals are in serious threat due to discharges of wastes from households and industries into the various water bodies without proper treatment (Zaki *et al*., 2014). In various industrial processes flocculants are frequently used for the aggregation of cellular materials and colloidal substances (Salehizadeh *et al.*, 2001).

There are three groups of flocculants: organic synthetic flocculants, inorganic flocculants and naturally occurring flocculants. Polyacrylamide derivatives and polyethylene amine are the examples for organic flocculants. Inorganic flocculants include aluminium sulphate and polyaluminum chloride. Sodium alginate, chitosan and bioflocculants comprised under naturally occurring flocculants (Shojaosadati, 2001; Zhang *et al*., 2002). Polyaluminum salts and polyacrylamide are the most commonly used flocculants among the chemical flocculants in water treatment, industrial downstream processing and drinking water treatment or process (Salehizadeh and Shojaosadati, 2001).

Wu and Ye (2007) described the various features of chemical flocculants high flocculating capability, lower dosage requirements, and their lack of pH effect in waste water treatment, and cost effectiveness. However, even with all these advantages, some of these flocculants for example, acrylamides have been reported to have both neurotoxic and carcinogenic effects on humans, hence their utilization have been reduced (Zheng *et al*., 2008).

Aluminium salts are neurotoxic and known to be one of the causes of Alzheimer's disease (Banks *et al*. 2006). On the other hand ferrite flocculants generally bring out unpleasant metallic taste, odour and decompose easily (Li *et al*., 2008). Due to this inadequacies that limit the application of these chemical flocculants, microorganisms' biopolymers secreted during growth have been considered as possible replacements (Li *et al*., 2008). Bioflocculants are not only safer to the environment and human but they are also easily biodegradable (Deng *et al*., 2003). Bioflocculant flocculating activity is dependent on its characteristics (Gao *et al*., 2009). Composition of bioflocculants are mostly protein, glycoprotein, polysaccharide and nucleic acid (Labille *et al*., 2005). The flocculating activity of some of these bioflocculants have been reported to be higher than that of synthetic chemical flocculants (Kurane *et al*., 1994).

Many bioflocculant producing microorganisms which have been reported in literature are of marine environment origin. For example, *Myxobacterium Nannocystic* sp. NU-2 has been

reported by Zhang *et al.* (2005). Yim *et al.* (2007) also reported p-KG03 bioflocculant produced by a marine dinoflagellate *Gyrodinium impudicum*. In biological systems bridging mechanism is often used to for removal of small suspended solid particles (Zhang *et al.*, 2010). Charge and molecular weight of the biopolymer usually suspended particles, cations and the pH of the waste water usually influence the bridging mechanism (Zhang *et al.*, 2010).

In waste and drinking water treatment, bioflocculants have been widely applied (Deng *et al*., 2003). Bioflocculants can be used in removal of soil solids, inorganic and organic suspended particles (Fujita *et al*., 2000). Zouboulis *et al.* (2004) revealed that the bioflocculant produced by *Rhizomonas* sp. can be used for the removal of humic acids from destabilized landfill leachates. While on another finding by Deng *et al*. (2005) indicates that *Aspergillus parasiticus* bioflocculant could be used to solubilize anionic dyes with high decolourization efficiency in aqueous solution. Wastewater and soy brewery wastewater could be flocculated by a bioflocculant produced from *Serratia fiacaria* (Gong *et al*., 2008). However, the major limitations with the application of bioflocculants are that, large scale production involves high cost and low yields (Kurane *et al*., 1994; Li *et al*., 2009).

Therefore, screening of microorganisms with high flocculating potentials from diverse environments which are capable of utilizing low-cost substrates are crucial factors to be considered in bioflocculation. Furthermore, optimization of fermentation conditions to enhance bioflocculant yields has been a subject of interest for researchers (Wang *et al*., 2007).

Nanoparticles

Nanotechnology is the science that deals with matter at the 1 billionth of meter scale (10^{-9} m = 1 nm), and it also deals with matter manipulation at atomic and molecular scale. The most fundamental component in the fabrication of nanostructures is nanoparticles. Everyday objects described by Newton's law of motions are much bigger than nanoparticles, but it is bigger than an atom or simple molecule that are governed by quantum mechanics (Jinsoo *et al.*, 2005).

The size of nanoparticles range between 1 and 100 nm in general. Physical and chemical properties of metallic nanoparticles are different from their bulk metal counterparts, for instance higher specific surface area, lower melting points, specific optical properties, specific magnetization and mechanical strength. Various industrial applications might find these properties attractive. Preparation of ultrafine particles from ancient times, two approaches have been known (Iwasaki *et al.*, 2004). The first method is top-down, it involves the application of external force to a solid that leads to break-down into smaller pieces. The other method is

bottom-up approach. In this method nanoparticles are produced starting from atoms of liquids or gas based on molecular condensation or atomic transformation (Brinker *et al.,* 1990).

The top-down method can be subdivided into dry and wet grinding. Grain refining method characteristics is that the surface energy increases, which in turn causes the aggregation of particles to increase. The solid substance is grounded as the result of shock in the dry substance or friction. The condensation of small particles takes place simultaneously with pulverization, particles with size 3 µm are difficult to obtain with the grain refining method (Perkampus, 1992). Conversely, a tumbling ball mill, a planter ball mill are used for grinding wet solid substrate. Equated with the dry method, the wet process can be used for the prevention of the condensation of nanoparticles formed (Bernacka-Wojcik *et al.,* 2010).

The bottom-up approach is divided into liquid phase and gaseous phase methods. This involves the use of chemicals, chemical vapour deposition method (CVD), while the physical vapour deposition method (PVD) involves the cooling of evaporated material. The occurrence of organic impurities in nanoparticles is minimized in the gaseous phase methods compare to liquid phase methods. The use of complicated vacuum equipment is necessary which renders this method costly and also the low productivity is a disadvantage. Ultrafine particles of size less than 1 µm can be produced by CVD through chemical reactions occurring in the gaseous phase. The careful control of the reaction can produce nanoparticles with size range between 10 to 100 nm (Gracia *et al.,* 2004).

Heat sources such as laser, plasma process, electrical furnace, or a chemical flame is required to perform the high temperature chemical reaction in the CVD method. The liquid or solid material is evaporated in the PVD method, the resulting vapour is the rapidly cooled, yielding the desired nanoparticles. Arc discharged method can be used to achieve evaporation of materials. Thermal decomposition method has been used in the industries for metal oxide nanoparticles or other nanoparticles production (Sudarshan, 2003).

Chapter 1 Introduction

Surface water, have different constituents which need to be removed in supply water systems. These constituents which needs to me removed can be subdivided into: colloidal solids, settle able suspended solids, and dissolved solids (Kannj and Achi, 2011). Treatment of drinking water mostly consist of: flocculation, coagulation, filtration and disinfection processes. The dosing of a coagulant in water results in the destabilization of negatively charged particles, which results to bigger flocs through a flocculation process which is known as coagulation. Efficient flocculation is described as the gentle water movement which results to gathering of small flocs to form lager masses better suitable for removal by clarification and finally by filtration.

The global growth of waste producing industries is increasingly becoming a problem to the environment and it is a major contributing factor in water pollution (Osibanjio *et al.,* 2011). Synthetic flocculants which seems to be the only option, some of the synthetic flocculants have been found to cause adverse effect to human health and the environment (Zhu *et al.,* 2016).

The aggregation of colloidal and freely suspended particles to form flocs (larger particles) and sedimentation by flocculants is called flocculation (IUPAC, 1997). Flocculants have various biotechnological applications in wastewater, drinking water treatment, the removal of dye solutions, inorganic solid suspensions and industrial downstream processing (Singha, 2012). There are inorganic flocculants, organic flocculants and natural occurring flocculants, such as polyaluminum chloride, and polyacrylamide derivatives respectively (Serdar and Demirsay, 2006). Natural occurring flocculants are usually in a form of extracellular polymers (EPS) of proteins, glycoproteins, lipids, glycolipids, polysaccharides (such as cellulose) and nucleic acids (Cong-Liang *et al.,* 2012).

Bioflocculants have attracted considerable attention with high efficiency, non-toxicity and safety for ecosystems and can replace chemical flocculants (Cong-Liang *et al*., 2012; More *et al*., 2012). Health threats as a result of the use of bioflocculants have not so far been reported.

Nanoparticles are the part of an emerging field of science Nanotechnology, which refers to the synthesis and development of numerous nanomaterials with size ranges from 1-100 nm (Dubchak *et al.,* 2010). Copper nanoparticles (Cu NPs) have attracted a considerable amount of interest to researchers due to the properties they possess such as, low production cost and antibacterial effectiveness. In comparison with precious metals for instance gold, silver or palladium, copper nanoparticles have high surface area to volume ratio, catalytic activity, optical and magnetic properties (Asim *et al.,* 2012). However, their immediate oxidation when

exposed in air becomes the main challenge in their preparation and preservation. Different inert media such as nitrogen, argon have been used by scientist to overcome this problem of oxidation. Reducing agents are also used for protecting or capping copper salt (Feldheim *et al.,* 2002). Reducing and capping agents that are used in the synthesis found to have toxic effects and are very expensive. To overcome the toxicity as a result of reducing agents and to synthesise CuNPs for a green environment, we used bioflocculant in the chemical reduction process.

Bioflocculant work both as reducing and protecting agent, which make the process nontoxic, economical and friendly to the environment. There is an increased demand for several metallic and non-metallic nanoparticles over the past decades. A series of different techniques have been developed to synthesized nanoparticles of different sizes, composition and shapes (Fawcett *et al.,* 2015). These techniques include both physical and chemical methods. The physical method includes laser ablation and high-energy irradiation technique (Shah *et al.,* 2015). The chemical method includes chemical reduction techniques by reducing agents.

The problem with these techniques it is extremely expensive and also involve the use of toxic hazardous chemicals which may pose potential risk to the environment. Studies reveal that there are different factors which affect size, shape and stability of the synthesized nanoparticles. The factors include temperature, reaction time and pH (Shah *et al.,* 2015).

Chapter 2 Literature review

A survey in literature presents a quite enormous and various body of research on nanoparticles synthesis. Most of this literature is ardent to areas of nanoparticles application in medicine, diagnostic tools, and types of method used for nanoparticles synthesis. Major factors affecting the type of nanoparticle produced are also discussed. Furthermore, characterisation and application of nanoparticles in different biotechnological fields are reviewed. Statement, aims and objectives of the study are also listed in this chapter.

2.1 Fresh water and its scarcity

Availability of fresh water in adequate quality and quantity is one of the main challenges that society will face this century (Alcamo *et al.*, 2007). 2.5% of earth's surface water is fresh water and is increasingly threatened by climate change and human (economic) activities (Hoekstra *et al., 2012*). Over the coming decades water shortage will increase ominously and this will result in food security problems, environmental pollution, and decrease in economic development (Hoekstra, 2014)

Water is essential for sustainable development (economic, social and environmental development). Most economic productions are fully dependent on water. Therefore, water usage rate has doubled as the global population increases (Corcoran *et al.*, 2010). Water is a multiple purpose utility which comprise domestic (drinking, cooking and cleaning), industrial (mining, construction industries and manufacturing industries), and agriculture (irrigation) (United Nations Water, 2015). Furthermore, water is essential in ecosystem because it serve as a medium for biochemical reactions.

Improper waste disposal, hydro-power generation, and land drainage are some of the major contributing factors to water scarcity (United Nations Water, 2015). 67% of the global population will live under water stress by the year 2025 (UN Water, 2017) while 20% is assumed to be currently living in water scarce countries. The Republic of South Africa receives mean annual rain fall of 450 mm annual, which is far below the world average of 860 mm (NWRS, 2004) hence we are declared as water scarce country.

Fresh water scarcity

The Figure 2.1 below illustrates geographic map showing global water scarcity. RSA is considered as a country which is approaching physical water scarcity.

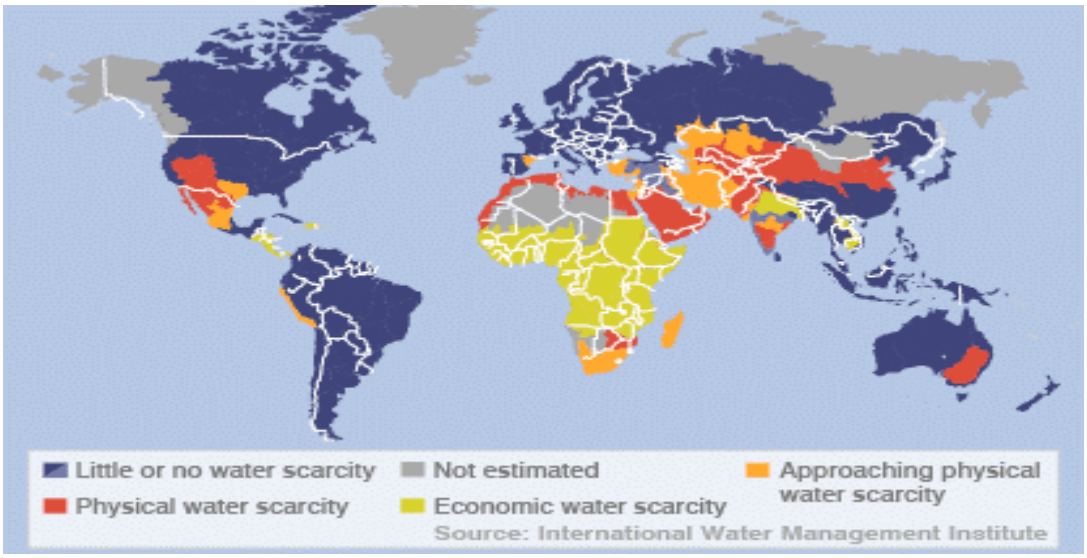

Figure 2.1: Fresh water scarcity (International water institute, 2014).

The figure below shows water crisis global due to water pollution, drought, and high consumption

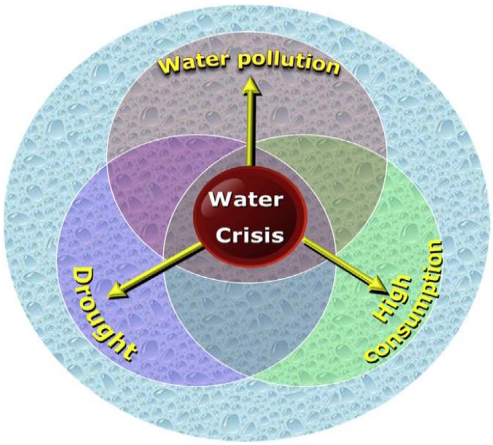

Figure 2.2: Global water crisis sources.

Scarcity of water is a key global problem, and a perfect relationship between drinking water (DW) and the health of the public. Manufacturing activities, swift urbanization and human deeds are the main contributing factors for life-threatening toxic contaminants discharge into several water resources (Zhang *et al.*, 2011). Incidents of water pollution are reported all over the world due to release of toxic pollutants into DW suppliers, this is due to fast growth of urbanization (Zhang *et al.*, 2011a; Hanna-Attisha *et al.*, 2016).

Various organic, inorganic, and biological contaminants are major contributing factors to ground water (GW), surface water (SW), and drinking water (DW) contamination. Arsenic (As) is most the notorious heavy metal among the inorganic compounds with an estimation of 140 million people exposed to it globally (Ravenscroft *et al.*, 2009). Most people who are affected are people living in developing countries due to the absence of water quality supplies, public awareness, and regulatory surveillance (Chopra and Ruhi, 2016).

A number of new synthetic chemical compounds and materials are produced yearly due to the advancement of science; most of these chemical compounds/materials are released into water during their application or dumping process. Chemical or biological transformation occurs to the released substances which in turn results in more toxic by-products that are detrimental to both human health and animals (Zhu *et al.*, 2016).

2.2. Coagulation-flocculation process

The aggregation of colloidal and freely suspended particles to form flocs (larger particles) and sedimentation by flocculants is called flocculation (IUPAC, 1997). Due to its efficiency and simple operation, coagulation-flocculation is widely used for waste water treatment (Zhu *et al.*, 2004). Moreover, the process also has various biotechnological applications in drinking water treatment, food processing, dye solutions, inorganic solid suspensions and industrial downstream processing (Singha, 2012).

2.3. Factors affecting flocculation process

Flocculation can be affected by numerous factors viz. water pH, flocculant concentration, ionic strength, molecular weight, shear, polymer type, types of solids (slurry), and process conditions. These factors have a great influence on flocculation, individually or collectively.

Change in concentration at which the flocculant is added to a slurry can significantly improve the flocculation process (Connelly *et al.*, 1990). At low concentration, the flocculant distribution in the slurry improves, thus improving performance. Nonetheless, dilution system determines the degree in which the flocculant should be diluted. Higher flocculant concentration may results to “shear”, which is the mechanism in which the flocculants protects or covers suspended solids, thereby preventing the flocculation process. Distribution of the flocculant is very essential, as well as overmixing can cause damage and cause floc shear. Hence, a balance between floc shear and flocculant distribution must be found (Connelly *et al.*, 1990).

The pH of the water to be treated plays a significant role in the flocculation process. The choice of the flocculant to be used is highly dependent on pH. In acidic conditions non-ionic polymers show an increase in flocculating activity (Richardson *et al.,* 1984). While anionic polymers are coiled up just like non-ionic polymers but they have very low flocculating activity. With anionic flocculants, the functional group (amide) are replaced by carboxylate group which is more inert, thus the number of hydrogen bonging sites are reduced. Hence, at lower pH non-ionic flocculants perform better (Richardson *et al.,* 1984).

2.4. Types of flocculants

There are inorganic, organic and natural occurring flocculants (Serdar *et al*., 2006). Natural occurring flocculants are usually in a form of extracellular polymers (EPS) of proteins, glycoproteins, lipids, glycolipids, polysaccharides (such as cellulose) and nucleic acids (Cong-Liang *et al.,* 2012). Depending on the charge of the flocculant they can either be anionic, cationic, and neutral.

2.4.1. Inorganic flocculants

Alum, calcium hydroxide, aluminium chlorohydrate, aluminium sulphate, Iron (II) sulphate (ferrous sulphate), Iron (III) chloride (ferric chloride), calcium oxide, sodium aluminate, and sodium silicate are examples of inorganic flocculants.

2.4.2. Organic flocculants

They can flocculate well at wide range of pH, have high molecular weight but generally have low charge density (Lachhwani, 2005). There are two main groups of these flocculants namely:

- Synthetic organic flocculants: these include numerous monomers such as acrylic acid, styrene sulphonic acid and diallyldimethyl ammonium chloride.
- Natural organic flocculants: these comprises of natural polymer such as cellulose, starch, natural gums and mucilage and their derivatives.

2.4.3. Naturally occurring flocculants

Natural occurring flocculants are less toxic, cheap but have low flocculating efficiency when matched with organic and inorganic flocculants. Examples of naturally occurring flocculants include; *Moringa oleifera* seeds (horseradish tree), gelatine, chitosan, isinglass, guar gum and aliginates (Ahmad *et al*., 2015).

2.5. Bioflocculation process

The natural process in which bioflocculants are used to flocculate, settle and remove particles, suspended solids and to remove colour in waste water is called bioflocculation (Tsao *et al.*, 2006). Macromolecules secreted by microorganisms in their environment as a result of substrate metabolism, microbial growth, and degradation of microorganisms are potential bioflocculants (Carlos *et al*., 2011). Prokaryotic as well as eukaryotic microorganisms can secrete microflocculants (Wingender *et al*., 1999).

Microorganisms, especially bacteria have shorter generation times, are versatile and mostly produce bioflocculants (Adewale *et al.,* 2012). Microbial flocculants serves a basic functions in bacteria, such as aggregations of bacterial cells, adherence to surfaces, formation of flocs and biofilms, and for water retention to reduce dehydration of the cell. Bioflocculants are absorbing organic, and inorganic compounds as well (Wingender *et al.,* 1999; More *et al*., 2014).

Bioflocculants are defined as capsular, slimy, loosely bound or tightly bound according to the nature of their association with the cells or the method used to extract and separate them from the cells (Hendricks, 2006). Microbial strains from algae, actinomyces, bacteria, and fungi have been proven to produce bioflocculant (He *et al*., 2012). Over a 100 species of bioflocculant producing microorganisms have been reported and their bioflocculant nature or activity characterized (Ahmad *et al*., 2015). The bioflocculants have higher flocculating efficiencies even at low concentrations (Lin and Harichund, 2011). This is due to the special microbial flocculant components, microbial flocculant matrix that show adsorption abilities, hydrophobicity, hydrophilicity and biodegradability.

It is much safer to use bioflocculants as compared to chemically synthesised flocculants. Some microorganisms are beneficial in different biotechnological applications. However, some may be opportunistic and pathogenic through their secondary metabolites (Spellman and Gordon, 2014). Toxic compounds can cause damage to immune cell functioning, cell lysis and even cell-death. This can directly or indirectly cause more occurrences of human infections and diseases (Mims *et al.,* 2004). Thus, for biosafety reasons, only bioflocculants from bacterial strains can be used for food, feed production or wastewater treatment. Bioflocculant from *Alcaligenis faecalis* will be used in the current study.

There is a need to test these bioflocculants for their toxicity before they can be used (Zhonga *et al*., 2014). In this study, the cytotoxicity and potential genotoxicity of the harvested, purified

bioflocculant will be determined by diphenyl-tetra-zolium bromide (MTT) assay and Ames test, respectively (Zhonga *et al*., 2014).

2.6. Factors affecting bioflocculant production

2.6.1. Inoculum size

Inoculum size is an important parameter in the production of a bioflocculant. (Mabinya *et al*., 2011). An inoculum size prolongs the stagnant phase of bacteria growth and a large inoculum size forms a niche and inhibits bioflocculant production (Zhang *et al.,* 2002). Insufficient inoculum size prolongs the lag phase of microbial growth there by delaying bioflocculant production. Bioflocculants are produced during the late phase of exponential growth. However, large inoculum sizes deplete nutrients faster due to competition amongst microorganisms (Yokoi *et al.,* 1997).

2.6.2. Carbon sources

The organic carbon sources include glucose, sucrose, fructose, maltose, starch, lactose, xylose and molasses. All microorganisms require nutrients as their energy source for growth (Liu *et al.,* 2009). Different carbon sources vary in the extent to which they affect bioflocculant production by different microorganisms (Deng *et al*., 2005). The bioflocculant produced by a consortia of *Provivencia rettger*i and *Bacillus megaterium*, prefer glucose, xylose, maltose, starch, lactose, fructose and sucrose as their carbon sources.

2.6.3. Nitrogen sources

Zaki *et al.* (2014) documented that nitrogen sources are important nutrient factors that enhance bioflocculant production whereby microorganism utilise either organic or inorganic nitrogen sources, or both, to a produce bioflocculant. Examples of organic nitrogen sources were listed as peptone, urea, yeast extract and casein. While the inorganic nitrogen sources incluedes ammonium chloride, ammonium sulphate and sodium nitrate. Liu *et al.* (2009) reported that *Chryseobacterium daeguense* W6 produces bioflocculant by using organic nitrogen sources for which tryptone was the most preferred, resulting in flocculating activity of more than 90%, while all inorganic nitrogen sources resulted in poor flocculating activity. In another study, Piyo *et al*. (2009) observed that *Bacillus* sp. Gilbert utilised an inorganic nitrogen source such as ammonium chloride effectively, to produce bioflocculant with a flocculating activity of 91%, while organic nitrogen sources like urea and peptone were poorly utilised.

2.6.4. Effect of temperature on bioflocculant production

Temperature plays an important role in the production of a bioflocculant as different microorganisms prefers different growth temperatures (Gao *et al.,* 2009). Flocculation rate has been reported to increase with an increase in growth temperature (Yang, *et al.,* 2007). The metabolism of microorganisms has a direct relationship with the cultivating temperature (Kurane and Nakata, 1991). Literature reports that most of the bioflocculant-producing microorganisms prefer a temperature range between 25-35 ℃ for bioflocculant production. Enzymatic reaction are directly affected by optimal temperature used which in turn influence the production of a bioflocculant (Kurane and Nakata, 1991). In another study it was reported that production of bioflocculant by multiple microorganism consortia was optimal at 30 ℃ (Zhang *et al*., 2007). The consortia of *Aspergillus parasiticus*, *Arthrobacter* sp. Raats, *Cobetia* sp., produced their bioflocculants at 28 ℃ in a study reported by Deng *et al*. (2005) and Ugbenyen *et al*. (2012).

2.6.5. Effect of initial pH on bioflocculant production

The initial pH for bioflocculant production varies with different microorganisms (Salehizadeh and Shojaosadati, 2001). According to He *et al.* (2010), the flocculating activity of a bioflocculant produced by *Halomonas* sp. V3a was above 80% in a pH range of 3 to 11 and the highest flocculating activity of 97% was achieved at pH 7. Contrary to the above observations, *Anabaera* sp. produced a bioflocculant in a medium of pH of 2 (Choi *et al.*, 1998). It was stated in literature that at a low pH both the bioflocculant and the kaolin particles are likely to absorb the hydrogen ions (H^+), consequently weakening the bioflocculant-kaolin complex mediated by Ca^{2+}. A similar effect was also observed at high pH values due to OH^- ions (He *et al.*, 2008).

2.6.6. Effect of shaking speed on bioflocculant production

Shaking speed enhance the flocculating activity by increasing the aeration process where nutrients are evenly distributed in the growth media (Maliehe *et al.,* 2015). Bioflocculant production is directly proportional and dependent on the dissolved oxygen tension (DOT) of the culture medium. The increase or decrease of respiration of microorganisms is highly dependent on the increase or decrease in DOT of the culture broth, the nutrient absorption and enzymatic reaction depends on DOT (Salehizadeh and Shojaosadati, 2001). Choi *et al.* (1998) reported that the bioflocculant production of different microorganisms require different speed. Bioflocculant production improved by *Paecilomyces sp.* at 200 revolutions per minute (rpm). In another study Kurane and Nokata (1991) observed that the bioflocculant by *Alcaligenes*

latus was produced at 300 rpm. Contrary to these, bioflocculant TJ1 was produced at 130 rpm by *Proteus mirabilis* (Zhang *et al.*, 2010).

2.7. Bioflocculation process and the contributing factors in flocculating efficiency

2.7.1. Effect of bioflocculant dosage on flocculation process

To achieve maximum flocculating activity of the bioflocculant, dosage size is one of contributing factors which needs to be taken into consideration (Zufarzaana *et al.,* 2012). Researchers are interested in finding a cost effective bioflocculant that must be effective at low dosage. Different dosage sizes of different microorganisms have been documented in the past decades. Takeda *et al.* (1991) reported on a bioflocculant which required 20 mg/l dosage to achieve the highest flocculating activity, produced by *Rhodoccus erythropolis* and *Enterobacter sp.* Lee *et al.* (2010) suggest that a bioflocculant produced by *Arcualendron* sp. require 2 mg/l to reach the highest flocculating activity.

A consortium bioflocculant produced by *Pestalotiopsis* sp., *Bacillus* sp and *Gyrodinium impudicum* KG03 was effective at a low dosage of 1 mg/l for maximum flocculating activity (Lu *et al*., 2005). In another study Zhang *et al.* (2002) reported that the optimum dosage which was required to attain maximim flocculating activity was at 30 mg/l from *Sarongium cellusum*. Contrary to this findings, 0.1 mg/l bioflocculant was sufficient to achieve highest flocculating activity when a bioflocculant from *Bacillus mucilaginosus* was used (Deng *et al.,* 2003). Lu *et al.* (2005) documented the highest dosage of 90 mg/l when a bioflocculant by *Enterobacter aerogenes* was observed with kaolin clay.

2.7.2. Cations effect on bioflocculation

Cations stimulate the flocculating activity of microorganisms. (Okaiyeto *et al.,* 2014). Microorganisms utilize different cations, some prefer trivalent, divalent and other monovalent. Cations play a vital role in the flocculation processes by neutralizing and stabilising the residual negative charge of both functional groups and the surface charge of the suspended particles. This weaken electrostatic repulsion between particles, and enhancing flocculation activity (Yang *et al*., 2007).

2.7.3. Thermal stability of the bioflocculant

In a study conducted by Kurane *et al.* (1994) and Salehizadeh *et al.* (2001), *Rhodococcus erythropolis* and *Bacillus firmus* produced bioflocculant, after it was subjected to boiling water for 15 min. the flocculating activity decrease to 50%. He *et al*. (2008) stated that the bioflocculant REA-11 did not have any significant decrease in flocculating activity after it was

heated at 80 ℃ for 1 h but further increase in temperature up to 100 ℃ drastically reduced the flocculating activity.

Gong *et al.* (2008) documented that the bioflocculant produced by *Aeromonas sp.* decreased by only 9.2% after it was heated at 100 ℃ for 1 h. A bioflocculant produced from *Serratia ficaria* decreased flocculating activity by 15% after it was heated at 100 ℃ for 15 min and 20% reduction in flocculating activity after it was subjected to 50 ℃ for 30 min (Gong *et al*., 2008). A bioflocculant mainly polysaccharide produced from a consortium of *Rhozobium radiobacter* and *Bacillus sphaeicus* retained 90% flocculating activity after heated at 100 ℃ for 30 min (Wang, 2011). Thermostable bioflocculant was reported to have maintain 78% flocculating activity after heated for 25 min. at 100 ℃.

2.7.4. Molecular weight effect on flocculating activity of the bioflocculant

Bioflocculants with different molecular weights have been documented in literature (Li *et al.*, 2009). Molecular weight of the bioflocculant contributes to its flocculating efficiency (Shojaosadati, 2001). Purified bioflocculant produced from *B. subtilis* was 1.5 x 10^5 Da in the study documented by Yokoi *et al.* (1997). A bioflocculant TKF04 with a molecular weight of 3.2 x 10^5 Da was produced from *Citrobacter* sp. (Fujita *et al*., 2000). A thermal and alkaline stable biopolymer with a molecular weight of 8.1× 10^4 Da was produced from *Agrobacterium* sp. M-503. Molecular weight is a significant factor in supporting the bridging mechanism in flocculation of kaolin suspension (Zhang *et al*., 2010 and Li *et al*., 2008).

2.8. Nanoparticles

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 1-100 nm (Langer, 2005). Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Biodegradable polymeric nanoparticles have been made in recent years, and used for drug delivery devices, particularly those coated with hydrophilic polymer such as poly ethylene glycol (PEG). Furthermore, their ability to circulate for a prolonged time and target particular organs, carry DNA in gene therapy, and having the ability to deliver proteins, has great benefit (Lee *et al*., 2010).

Nanoparticles can be classified into three categories, namely: One dimensional nanoparticles 1D Nano Structure Materials (NSMs) which are thin films (sizes 1–100 nm) or monolayer is now common in the field of solar photovoltaic cells offering, different technological applications. Two dimensional nanoparticles (2D NSMs) are particles which have two dimensions outside of the nanometric size range. 2D NSMs with certain geometries exhibit

unique shape-dependent characteristics and subsequent utilization as building blocks for the key components of nanodevices (Jun *et al.,* 2005; Kim *et al.*, 2009; Bae *et al.,* 2010).

Three dimensional nanoparticles (3D NSMs) have a large specific surface area and other superior properties over their counterparts arising from quantum size effect, a system which allows a particles time evolution (Ren *et al.*, 2000; Mastragostino *et al.,* 2007). Moreover, the 3D NSMs have recently attracted research interests because these nanostructures have higher surface area and supply enough absorption sites for all involved molecules in a small space (Shen, 2005).

2.9. Synthesis of nanoparticles

2.9.1. Physical synthesis of nanoparticles

Two of the more significant physical approaches for nanoparticles synthesis are laser ablation and evaporation-condensation (Kruis *et al.,* 2000). The advantages of physical synthesis methods are the absence of solvent contamination in the prepared thin films and the uniformity of nanoparticles (NPs) distribution in contrast to chemical synthesis. However, physical synthesis of NPs requires the usage of high amount of energy, and requires time to achieve thermal stability (Jung *et al.,* 2006). Moreover, devices such as a tube furnace consume energy and preheating time is necessary before it reaches stable operating temperature (Magnusson *et al.,* 1999). A demonstration revealed that silver NPs could be synthesized via a small ceramic heater with a localised heating area (Jung *et al.,* 2006).

2.9.2. Chemical synthesis of nanoparticles

Most common approach for nanoparticles synthesis is reduction by organic and inorganic reducing agents (Wiley *et al.*, 2005). Examples of reducing agents which are used in metal ions reduction are as follows: elemental hydrogen, ascorbate, sodium borohydride ($NaBH_4$), sodium citrate, polyol process, Tollens reagent, N, N-dimethylformamide (DMF), and poly (ethylene glycol)-block. Reduction of metal ions such as silver (Ag^+) in aqueous or non-aqueous solutions, results into to a formation of metallic silver (Ag^0), which is followed by cluster formation. Moreover, the formed clusters eventually result into metallic colloidal silver particles formation (Evanoff and Chamanov, 2004).

It is imperative to use protective agents during nanoparticle preparation to stabilize and protect the NPs that can be absorbed on or bind onto nanoparticle surface (Oliveira *et al.,* 2005). The presence of surfactants comprising functionalities such as: amines, acids, and alcohols for interactions with particle surfaces can stabilize particle growth, and protect particles from sedimentation, agglomeration, or losing their surface properties (Evanoff Chamanov, 2004)

2.9.2.1. Chemical reduction

Chemical reduction by reducing and oxidising agents is the most common way for synthesizing nanoparticles. Examples of reducing agents which are used for nanoparticles synthesis include sodium citrate, sodium borohydride ($NaBH_4$), ascorbate and elemental hydrogen. Reducing and oxidizing agents are added to stabilize the nanoparticles, the use of these agents during nanoparticles synthesis is very imperative as they also prevent the occurring of agglomeration (Merga *et al.,* 2007). Moreover, surfactants which comprises of functionalities such as amines, acids, alcohol and thiols, protect the nanoparticles from losing their surface properties.

Compounds that are polymeric, example: poly (vinylpyrrolidone), poly (vinyl alcohol), and poly (methacrylic acid) are reported to be effective protective agents in stabilization of NPs. Synthesis of some metallic NPs can be prepared at room temperature, by mixing metal ion solution with reduced polyoxometalates which aids as a stabilizing and reducing agents (Wiley *et al.,* 2005).

2.9.2.2. Micro emulsion techniques

The technique is based on two-phase aqueous system, which involves the initial spatial separation of reactants (reducing agents and metal precursor) into two immiscible phase. Formed metal clusters at the interface are stabilized, as the results of their surface being coated with stabilizer molecules occurring in the non-polar aqueous medium, and transferred to the organic medium by the inter-phase transporter (Evanoff *et al.,* 2004). The use of highly toxic organic solvents is one of the major shortcomings of this technique. Therefor huge amount of organic solvents and surfactant must be separated and removed from the final product.

2.9.2.3. UV-initiated photo reduction

UV-initiated photoreduction is a simple and effective method, which has been documented for producing NPs with the aid of citrate, poly acrylic acid, collagen and polyvinylpyrrolidone. For example, in a study conducted by Huang and Yang, (2007) NPs were produced via photoreduction of silver nitrate in the presence of citrate which served as stabilizing agent for prevention of NPs aggregation.

The properties of synthesized NPs were studied as a function of UV irradiation time. Larger NPs were attained when irradiated under UV for 3 h. Disintegrated NPs to smaller sizes resulted when further irradiation occurred (Merga *et al.,* 2007). Poly (vinylalcohol) has been used to synthesize NPs at room temperature by UV irradiation photoreduction. Both the concentration

of poly (vinylalcohol) and silver nitrate engage in recreation major role in the growth of the dendrites and nanorods (Evanoff *et al* 2004)

2.9.2.4. Electrochemical synthetic method

Synthesis of NPs using electrochemical synthetic method make it possible to control particle size by adjusting electrolysis parameters and by changing the composition of electrolytic solution homogeneity of the synthesized NPs can be achieved. Electrochemical reduction resulted in a polyphenylpyrrol coated nanospheroids with size range between (3-20 nm) (Johans *et al.,* 2002). Nanospheroids (1-18 nm) were obtained in another study by electrochemical reduction inside or outside zeolite crystals according to silver exchange degree of compact zeolite film modified electrodes (Zhang *et al.,* 2002). Spherical NPs with size range (10-20 nm) with narrow size distributions were appropriately prepared in aqueous solution by an electrochemical method (Ma *et al.,* 2004). Poly N-vinylpyrrolidone helped to prevent agglomeration as it aid as a stabilizer.

2.9.2.5. Irradiation methods

Synthesis of NPs can be achieved through the use of variety of irradiation methods. A well-defined shape and size distribution NPs were synthesized using laser irradiation of an aqueous solution (Abid *et al.,* 2002). Moreover, NPs were synthesized using laser in a photo-sensitization synthetic method. At short irradiation times, 20 nm NPs were produced while 5 nm NPs were produced when irradiation time was increased.

2.9.2.6. Microwave-assisted synthesis

Microwave initiated method is promising and is a better option for synthesizing nanoparticles than for synthesis of silver than a conventional oil bath in terms of consistently yielding, narrower size distributions, nanostructures with smaller sizes and a higher degree of crystallization (Nadagouda *et al.,* 2011). Microwave heating has reduced energy consumption in a sense that it has shorter reaction times, and better product yields which prevents formation of agglomeration of the formed particles (Nadagouda *et al.,* 2011). Furthermore, microwave assisted synthesis reduce waste of chemicals and reaction time in several organic syntheses and chemical transformations (Polshettiwar *et al.,* 2009).

Literature reports that NPs could be synthesized by microwave-assisted synthesis method with the aid of carboxymethyl cellulose sodium as reducing and stabilizing agent. The size of synthesized NPs depends on sodium carboxymethyl cellulose and metal salt concentration. Formed NPs were stable and uniform, and were found to be stable without any visible change

for 2 months at room temperature (Chen *et al.*, 2008). In another study NPs with size range 12 nm were synthesized using starch as a template and reducing agents.

2.9.3. Biological synthesis of nanoparticles

Bacteria, actinomycetes, fungi and algae are the organisms which can be used in biosynthesis of nanoparticles. Mann (2001) described biological synthesis of nanoparticles by microorganisms as an environmentally friendly technology. For the synthesis of metallic nanoparticles such as silver, gold, platinum, zirconium, palladium, iron, cadmium and metal oxides such as titanium oxide and zinc oxide both prokaryotes and eukaryotes are used (Hulakoti *et al.,* 2014). Depending on the location of nanoparticles during synthesis, nanoparticles may be synthesized either intracellular or extracellular surfaces (Hulakoti *et al.,* 2014).

The method of synthesising nanoparticles intercellular by fungi involves the use of enzymes and the transportation of ions into microbial cells to form nanoparticles. Fungi are mostly known for producing nanoparticles extracellularly, this is due to their massive secretory components, which aid in the nanoparticles reduction and capping (Sakthivel *et al.,* 2010). The size of nanoparticles formed inside the organisms are smaller compared to those formed extracellularly. Limitation to the size of the nanoparticle formed within the organism is probably due to particle nucleating inside the organism (Narayanan *et al.,* 2010). Synthesising nanoparticles extracellularly by fungi has more applications as compared with intracellular synthesis as it is without unnecessary connection of cellular components from the cell.

2.9.3.1. Biological synthesis of metallic nanoparticles using bacteria

Frequent exposure of bacteria to different and sometimes harsh environmental conditions in nature requires them to have a survival mechanism. Endurance in these extreme conditions is dependent on their ability to resist environmental stresses (Dhillon *et al*., 2012). To withstand multiplicity of stresses such as high toxicity in the environment as a results of high metallic ions concentrations, natural defence mechanisms exist in bacteria to support overcome these toxic conditions.

Mechanisms such as changes in metal concentration via redox reactions help microorganisms deal with environmental conditions biologically. Moreover, systems such as efflux, intracellular accumulation, and precipitation of metals and extracellular formation of complexes assist microorganisms to survive in high metal concentrated environment (Dhillon *et al*., 2012).

Bacterial species which are mainly used for metallic nanoparticles synthesis comprises of *A. feacalis, Lactobacillus spp., Actinobacter spp*., *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Corynebacterium spp*., and *Pseudomonas spp*. (Iravani, 2014; Sunkar *et al*., 2012; Tollamadugu *et al*., 2011). The biological reduction of Ag^{+} ions to Ag nanoparticles involves an electron transfer process (Ahmed *et al.,* 2003).

Au ions (Au^{+} and Au^{3+}) were reduced to Au nanoparticles by *Pseudomonas aeruginosa* in a study conducted by (Husseiny *et al*., 2007). However, non-involvement of biological enzymes has also been indicated. For example, in a study by Liu *et al.* (2005) Ag nanoparticles were produced from dried cells of *Bacillus megaterium.* (Sneha *et al.,* 2005). They were also able to prove that non-enzymatic reactions did not occur when *Corynebacterium sp* was used in Au particles formation.

Various factors such as appropriate pH, some organic functional groups presence at the cell wall, and temperature are believed to induce nanoparticle reduction (Lin *et al.,* 2001). Fu *et al.* (2000), reported that *Lactobacillus sp*. A09 and *Bacillus megaterium* D01 dried biomass revealed that Ag ions reduction to produce nanoparticle can be as a results of the interaction of functional groups found in cell wall of the bacteria.

Physical parameters such as temperature and pH changes can also significantly influence size, shape, and composition of a nanoparticle (Hulkoti *et al.,* 2013). Therefore, optimization of synthesis parameters during nanoparticle formation is important to improve overall properties of the formed particle. Size and morphology of nanoparticles were shown to be influenced by both pH and metallic salt concentration. At a pH of 6, spherical Au nanoparticles with size range between 10-20 nm were produced at low concentration of $AuCl_4$, when *Rhodopseudomonas capsulata* was used (He *et al.,* 2008).

When the salt concentration was increased above pH 6 Ag nanowires resulted (He *et al.,* 2008). Change in pH resulted in similar results when the pH was changed to 4, both spheres and triangular nanometre scale plates were produced by altering the pH in the solution He *et al.,* 2008). The studies indicate that medium's pH should be controlled to get nanoparticles with desired morphology.

2.9.3.2. The use of plants to synthesize metallic nanoparticles biologically

The properties possessed by plants such as the ability to biologically reduce and hyper-accumulate metallic ions, are environmentally-friendly methods for synthesizing metallic nanoparticles and for application as detoxificant (Kale *et al.,* 2013; Khan *et al.,* 2013).

Bioactive molecules such as alkaloids, phenolic acids, polyphenols, proteins, sugars, and terpenoids contained in plant extracts play a significant role in the reduction of metallic ions and stabilization (Castro *et al* 2011; Marshal *et al.,* 2007). Dissimilarity in composition and interaction between these active biomolecules in plants with aqueous metal ions is believed to result in a range of nanoparticles size and shape.

Importantly, nanoparticles synthesis from reduction of metal salts by plants is a simple process which can be done at room temperature. Metal salt solution is mixed with the plant extract, the reduction of salts start immediately and the formation of nanoparticles is indicated by colour change in the reaction mixture, metal ions are converted from mono or divalent oxidation states to zero-valent states during the initial activation period and is followed by nucleation of the reduced metal atoms (Malik *et al.,* 2014).

The growth period follows immediately, in which small particles amalgamate to form larger nanoparticles that are more thermodynamically stable. Progress in growth results in nanoparticle aggregation to form different morphologies such as triangles, hexagons, pentagons, rods, and wires (Akhtar *et al.,* 2013). Most energetically favourable and stable morphology is determined by the ability of the plant extracts to stabilize the nanoparticle in the final stage of synthesis. Factors such as metal salt concentration, reaction time, pH of the reaction solution, and reaction temperature significantly influence the quality, size, and morphology of the synthesised nanoparticles (Mittal *et al.,* 2013).

2.10. Different factors which affect nanoparticles synthesis

A number of controlling factors such as pH, reactant concentrations, reaction time, and temperature influence biological synthesis of metallic nanoparticles.

2.10.1. Effect of pH on nanoparticle formation

The reaction-medium-pH value has a major role during nanoparticles formation (Gardea-Torresdey *et al.,* 1999). Varying the pH of the reaction medium has resulted in different size and shape of nanoparticles synthesised in studies conducted recently. Lower pH (acidic) values have resulted to a formation of larger nanoparticles compare to high pH (basic) values (Sathishkumar *et al.,* 2010). For example, when *Avena sativa* (Oats) biomass was used at pH 2, rod-shaped Au nanoparticles were synthesized with larger size (25 to 85 nm). Smaller particles with size 5 to 20 nm were observed at pH 3 and 4 (Armendariz *et al.,* 2004). This suggests that between pH 3 and 4 there were more functional groups contained within the

extract accessible for Au nanoparticle formation. At pH 2 particle aggregation resulted, due to less functional groups present in the plant extract.

In a similar study, *Cinnamon zeylanicum* bark extract resulted in an increased number of nanoparticles with an increase in bark extract concentrations. The shape of nanoparticles became spherical at pH 5 and above (Armendariz *et al.,* 2004). During the synthesis of palladium (Pd) nanoparticles from *Cinnamon zeylanicum* bark extract the particle size did not increase with an increase in pH. Below pH 5 the particle size range was between 15 to 20 nm and at higher pH particles range between 20 to 25 nm (Sathishkumar *et al*. 2009).

2.10.2. The effect of reactant concentration on nanoparticles formation

Biomolecules concentration found in plant extract can influence metallic nanoparticle formation. A study where sun-dried *Cinnamomum camphora* (camphor) leaf extract was used revealed that the amount of *Cinnamomum camphora* extract varies the shape of the Au and Ag nanoparticles synthesized in the reaction medium (Huang *et al.,* 2007). For instance, when chloroauric acid was used as a precursor and subjected to cumulative extract concentrations, the shape of the resulted nanoparticle changed from spherical to tetrahedral pyramid-shaped. Similarly, the change in amount of *Aloe vera* leaf extract in the reaction medium comprising of chloroaurate ions the spherical shaped nanoparticles of gold changed from triangular plates (Chandran *et al.,* 2002). The presence of carbonyl compounds in the extract assist in shaping particle growth. The size of the particle was modulated between 50 and 350 nm by the extract concentration. Likewise, variation of *Plectranthus amboinicus* leaf extract concentration in the medium produced different shapes of Ag nanoparticles (Narayanan *et al.,* 2010).

2.10.3. The effect of reaction time on nanoparticles formation

Reaction time is an important factor in the synthesis of spherical Ag nanoparticles (Ahmad *et al.,* 2012). An extract from *Ananas comosus* (Pineapple) gave a quick colour change was observed within 2 min. (Ahmad *et al.,* 2012). Nanoparticles were observed within 2 min. in the reaction medium after a quick reduction of aqueous $AgNO_3$. After 5 minutes of reaction time there was only a slight change in colour observed. Spherical nanoparticles of around 12 nm size were formed.

Dwivedi and Gopal (2007), produced Ag and Au nanoparticles were produced using *Chenopodium album* leaf extract. The synthesized nanoparticle appeared within 15 minutes and continued to form up to 2 h. After a period of 2 h, very few nanoparticles were produced (Prathna *et al.,* 2011). Prathna *et al.* (2011) revealed that an increase in reaction time resulted

in an increase in nanoparticle size in the combination of *Azadirachta indica* leaf extract and $AgNO_3$. The particles size range from 10 to 35 nm with the variation in time between 30 min. and 4 h (Prathna *et al*., 2011).

2.10.4. The effect of reaction temperature on nanoparticles formation

Reaction temperature is generally known as a key factor in any synthesis. Temperature has been found as an important factor in determination of size, shape, and yield of nanoparticles formed by plant extracts (Sathishkumar *et al*., 2010). For example, Ag nanoparticles with an average size of around 35 nm were produced when *Citrus sinensis* (sweet orange) was used at 25 ℃. However, increase in reaction temperature resulted in the decrease in particle size of the nanoparticles to 10 nm at 60 ℃ (Kaviya *et al*., 2011). Likewise, when *Diospyros kaki* (persimmon) leaf extract was used in a study conducted by Song *et al*. (2005) Ag nanoparticles with high stability were produced over 25 to 95 ℃ reaction temperature range.

In a study where *Avena sativa* (oats) biomass was used the thermal variation in the reaction conditions brought about changes in the size and shape of the synthesised Au nanoparticles (Armendariz *et al.,* 2004). According to Gericke (2006), a high rate of formation of Au nanoparticles is promoted by higher temperatures. Spherical Au nanoparticles formation resulted from lower temperatures while formation of plate-like Au nanoparticles resulted from higher temperatures (Gericke, 2006). The rate of reaction and particle formation seems to increase at high or temperature reaction, however, the average particle size decreases and the number of particles increase during the reaction. This all depends on the nucleation process (Armendariz *et al.,* 2004).

2.11. Possible application of nanoparticles

2.11.1. Medicine

Ultrasonography uses sound waves to create an image for many different purposes. These sound waves are transmitted through the body and bounce off of tissue and return to a receiver. The receiver measures the time it takes for the sound wave to reflect and return to the place of origin, which is perceived as a distance and is converted into an electrical signal, which is then converted into an image by the computer. This type of medical imaging is used in many branches of medicine spanning from obstetrics to oncology. Unfortunately, with ultrasonography minor details could be missed because the image may not be of the best quality. Nanoparticles have been found to help increase the contrast of the image produced by the ultrasonography particularly when imaging tumours.

The particles used are called perfluorocarbon emulsion nanoparticles (PFC) and are about 250 nm in diameter. The size of these particles is very important for a detailed image. According to Liu *et al.* (2006), the smaller particles, particularly ones with surface alteration, have an extended half-life in the circulation and increase the number of passes through tumour vasculature. This increase the image of the tumour. Liu *et al.* (2006) were able to test these hypotheses by performing experiments in which they injected intravenously the nanoparticles in a suspension of saline water into mice. By performing these experiments on a living animal model, their results suggest it could have an application in humans. They were able to conclude from these experiments that the brightness, also called the mean grey scale level, increased with concentration and particle size (Liu *et al*., 2006). The increase in image quality from ultrasonography, due to nanoparticles, can help in many branches of medicine because ultrasonography can be considered more economical than other non-invasive imaging technologies and can assist with diagnosis.

2.11.2. Nanoparticles in waste water treatment

Most countries today are facing drinking water problems, more especially developing countries. These challenges faced by the world are as the results of depleting fresh water supply due to the following factors: (a) lengthy droughts, (b) growth of the population, (c) more stringent health based regulations and (d) demands from competing range of users (Laboratories, 2003; World Health Organization, 1996; US Environmental Protection Agency, 1996)).

Clean water is crucial to human health. Bacterial contaminated drinking water contributes 80% of infectious disease in countries such as India. The World Health Organization (1996) recommended that there should be 0 fecal and total coliform counts contained in 100 ml of any water intended for drinking. Immediate investigative action should be taken when either of these groups of bacteria is encountered in a sample. The last step in water treatment is the removal or inactivation of pathogenic microorganisms (US Environmental Protection Agency, 1999).

US Environmental Protection Agency (2012), states that defence against possible chemical and biological terrorist acts of water treatment systems is also becoming a serious concern in water resources planning. Chemical and physical disinfection agents such as chlorine and its derivatives, ultraviolet light (Droste, 1997), are techniques which are used in water treatment.

Moreover, the use of halogens such as chlorine (Cl) and bromine (Br) are widely applied as antibacterial agents.

Direct usage of halogens as bactericides in pure form causes problems as they are highly toxic and have high vapour pressure. NH_4^+ is the most common cation affecting human and animal health found in water. Removal of ammonia in drinking water is very important as it prevents oxygen depletion, algae bloom and it is extremely toxic to most fish species (Jung *et al.*, 2006). For this reason it is believed that the application of bioflocculants in water treatment can resolve these adverse effects.

2.12. Rationale of the study

Synthesis of nanoparticles for the treatment of water pollution and antibiotics resistant microorganisms requires methods that are essentially eco-efficient with margin of safety to human and animal health. Different chemicals have been applied to synthesize nanoparticles. Although some of these methods are effective they often resulted in environmental hazards and health threats. The damaging effect of water pollution as well as the physico-chemical treatments used, do not only impose bad impact to the environment and humans but also to the economic viability of any country (Evanoff *et al.,* 2008).

Therefore to ensure the safety of the ecosystem and humans, synthesis of nanoparticles, need novel, effective use of other materials with different or same mechanism of action. Biological methods such as biosynthesis of nanoparticles using a bioflocculant could be a promising approach. Although these biological systems are effective, poor understanding of their mechanism, low efficiency or activity and production costs are inhibitors to their utilization.

This study is proposed to find a low cost method for nanoparticles synthesis. The study will furthermore seek to find the mechanism of action and safety of the bioflocculant produced using a copper nanoparticle. Copper possess an antimicrobial effect, there for this unique property makes it more useful to human health as it can be used to prevent pathogenic microorganisms.

2.13. Problem statement

The most effective and common methods for nanoparticles synthesis are physical and chemical. Both of these methods are known to be efficient and reliable, but they both have some shortcomings. It requires a lot of energy consumption as it uses instruments such as laser ablation while the other may require the use of expensive reducing and oxidising agents to stabilize the nanoparticles. Some chemicals are also carcinogenic and not biodegradable.

The World Health Organization (WHO) recently released a statement stating that there is a scarcity of new antibiotics which can be used to combat the problem of remerging pathogenic microorganisms which are resistant to all commercially available antibiotics (WHO Drug Information, 2011). These microorganisms are known as carbapenemase-producing bacteria, and these pose a serious threat to our water quality. The increase in these emerging pathogenic microorganisms could be due to the misuse of antibiotics.

Several chemical methods have been implemented to reduce the re-emergence of antibiotic resistant microorganisms. However, the problem seems to be unceasing. Some of the chemical treatments used have also shown environmental un-friendliness. The biological synthesis of nanoparticles for the treatments of antibiotic resistant microorganism has the potential as an alternative to this crisis. However, very little about this biological synthesis is understood. Thus, the study is intended to find a solution to the challenges of water quality and improved human health.

2.14. Aim

The aim of the study was to biosynthesize copper nanoparticles using a bioflocculant, characterise and evaluate its application in water treatment

2.15. Objectives

1. Extract, purify and characterize the bioflocculant from *Alcaligenes faecalis*
2. Biologically synthesize copper nanoparticles using purified bioflocculant and characterize biosynthesized nanoparticles
3. Test the flocculating activity of biosynthesized copper nanoparticles using kaolin clay
4. Test for antimicrobial effect of biosynthesized copper nanoparticles
5. Apply biosynthesized copper nanoparticles in waste water treatment and dye removal

Chapter 3 Material and methods

3.1. Source of the bacteria and production medium

The bioflocculant was extracted from *Alcalegenis faecalis* which was previously isolated from the marine environment and was identified as *Alcalegenis faecalis* through Inqaba Biotec, South Africa. The bioflocculant production medium was prepared in accordance to a description by Zhang *et al.* (2007) and it was composed of glucose (20 g), $MgSO_4 \cdot 7H_2O$, (0.2 g), $(NH_4)_2SO_4$ (0.2 g), K_2HPO_4 (5 g), urea (0.5 g), yeast extract (0.5 g) and KH_2PO_4 (2 g) in a litre of filtered seawater at pH 8 and sterilized by autoclaving at 121 ℃ for 15 min. After autoclave the medium was then allowed to cool and was inoculated with fresh culture which was resuscitated the previous day. The broth was then incubated in a shaking incubator for 72 h at 30 ℃ at a speed of 165 rpm.

3.2. Extraction and purification of the bioflocculant

Extraction and purification of the bioflocculant was done following a description by Chang *et al* (1998) where the culture broth was taken out after 72 h of growth and centrifuged at 4,000 rpm at 4 ℃ for 30 min. This was done in order to remove cells and insoluble substances. The supernatant was then transferred into a clean container and the cells were discarded. 1 litre of distilled water was then added into the supernatant and was centrifuged again at 4,000 rpm at 4 ℃ for 15 min.

Two volumes of ethanol were added to the supernatant, agitated and the solution was stored at 4 ℃ for 12 h. After 12 h the precipitate was vacuum-dried and 100 ml of distilled water was added. A mixture of chloroform and *n*-butyl (5:2 v/v*)* was also added. After stirring, the mixture was left to stand for 12 h at room temperature. The supernatant was then vacuum-dried in order to obtain a purified bioflocculant.

3.3. Determination of flocculating activities

4 g of kaolin clay was dissolved into 1 litre of distilled water, after which 100 ml of the solution was then transferred to a 200 ml conical flask and 2 ml of 0.002 g/l of dissolved nanoparticles were added into a flask containing 100 ml kaolin solution and 3 ml of 1 g/l $CaCl_2$ were added and the mixture was shaken at 165 rpm using shaker for 1 min. The mixture was transferred to graduate measuring cylinder and it was allowed to stand for 5 min. The supernatant was then taken for analysis using UV-visible spectrophotometer at 550 nm for optical density. The formula used for the determination of the flocculating activity was as follows:

$$\text{Flocculating Activity (\%)} = [(A - B/A)] \times 100$$

Where A is the optical density of control at 550 nm and B is the optical density of a sample at 550 nm.

3.4. Characterization of the bioflocculant

SEM was used for better image at 1000 keV i.e. wavelengths of resolution. Morphology and elementary analysis of the bioflocculant was determined using scanning electron microscope equipped with elementary detector (SEM-Sipma-VP-03-67). BRUKER D8 Advance X-Ray Diffractometer operated at 40 kV, 40 mA, with graphite monochromatized CuKα1 radiation of wavelength λ=1.5406 Å. The phase composition was investigated and the crystallinity of the bioflocculant. XRD was recorded in the 2θ range from 20° to 80° at scanning steps of 0.03°.

A Perkin-Elmer spectrophotometer was used to investigate UV-vis spectrum of the bioflocculant. 0.1 ml of the sample was taken and diluted with 2 ml of deionized water, as a function of time of reaction the investigation was conducted in the wavelength region 300 to 700 nm operated at a resolution of 1 nm.

Fourier Transform-Infrared (FTIR) spectroscopy was used to identify and confirm the functional groups present in the bioflocculant (Perkin Elmer System 2000, Cambridge, England).

3.5. Thermo-gravimetric analysis (TGA)

The purified bioflocculant degradation was verified using thermo-gravimetric instrument in accordance to Cosa *et al.,* (2013). High temperatures with range 22 to 900 ℃ was used to heat the bioflocculant, at a constant rate of ramping, 10 ℃ min^{-1} and under constant flow of nitrogen gas.

3.6. Biosynthesis of copper nanoparticles

The synthesis of copper nanoparticles was achieved using a method described by SaharZaki *et al.* (2014) with some minor modifications, 0.5 g of purified bioflocculant was added into a 200 ml solution of 3 mM $CuSO_4$. The mixture was agitated until a homogenous solution was achieved. Foil was then used to cover the mouth of the conical flask containing the solution, prevent the interference of foreign material. The solution was left unstirred at room temperature for 24 h. The bioflocculant solution was used as a control for this experiment and synthesis of nanoparticles was confirmed through physical observation and characterization, after 24 h. A blue precipitate was observed and the synthesized nanoparticles were collected using centrifuge at 4 ℃, 4000 rpm for 30 min. and vacuum-dried overnight and sample stored for characterization.

3.7. Synthesised copper nanoparticles characterization

SEM was used for better image at 1000 keV i.e. wavelengths of resolution. Morphology and elementary analysis of the synthesized nanoparticles was determined using scanning electron microscope equipped with elementary detector (SEM-Sipma-VP-03-67). BRUKER D8 Advance X-Ray Diffractometer operated at 40 kV, 40 mA, with copper monochromatized CuKα1 radiation of wavelength λ=1.5406 Å was used to investigate the phase composition and crystallinity of the bioflocculant. XRD was recorded in the 2θ range from 20° to 80° at scanning steps of 0.03°.

Perkin-Elmer spectrophotometer was used to investigate UV-vis spectrum of the synthesized copper nanoparticles. 0.1 ml of the sample was taken and diluted with 2 ml of deionized water. As a function of time of reaction the investigation was conducted in the wavelength region 300 to 700 nm operated at a resolution of 1 nm. Fourier Transform-Infrared (FTIR) spectroscopy was used to identify and confirm the functional groups present in the bioflocculant (Tensor 27, Bruker FT-IR spectrophotometer).

TEM images for the copper nanoparticles were obtained using a JEOL 1010 transmission electron microscope. The specimens were prepared by using a micropipette to place a diluted drop of suspension in toluene on a copper grid (150 mesh). The samples were allowed to dry completely at room temperature. Samples were viewed at 100 kV as the voltage accelerates. The images were captured digitally using a Megaview III camera, stored and measured using Soft Imaging System iTEM software.

The as-synthesized bioflocculant passivated copper nanoparticles decomposition was studied using a thermo-gravimetric instrument in accordance to Cosa *et al.,* (2013). High temperatures with range 22 to 900 ℃ was used to heat the bioflocculant passivated copper nanoparticles, at a constant rate of 10 °C min^{-1} under constant flow of nitrogen gas.

3.8. Application of synthesized copper nanoparticles on waste water treatment

Samples of water from different industries were examined in accordance to a method described by Okaiyeto *et al.* (2014). One sample was collected from a domestic water treatment plant (Vulindlela water treatment plant). The next sample was collected from Tendele coal mine. The last sample was collected from local industries Empangeni. To test flocculating activity of copper nanoparticles against different waste water sample the following procedure was followed. 100 ml of waste water was poured in a flask and 3 ml of 1 % $CaCl_2$ solution was added into the flask containing the waste water samples after which 2 ml of copper

nanoparticles were added into a flask. Mixture was then shaken vigorously for 1 min. using shaking incubator after which the mixture was transferred into graduated 100 ml measuring cylinder. It was allowed to stand for 5 min. before the upper phase was taken for analysis using UV-visible spectrophotometer at 550 nm optical density. The flocculating activity and removal efficiency of dissolved heavy metals of the synthesized nanoparticles was compared to that of bioflocculant and chemical flocculent (iron chloride). All data was collected in triplicates and the results were recorded using graph dap prism (2016) student eddition.

The biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen (N), phosphorus (P), sulphur (S), aluminium (Al) and calcium (Ca) in wastewater were measured with spectro-quant (Pharo 300, Merck KGaA, Germany), before and after application of the copper nanoparticles. The removal efficiency (RE) of the pollutants was calculated by the following equation:

$RE\ (\%) = [C - C/C_0] \times 100$

Where: C_0 is the initial value and C is the final value after the flocculation treatment of the sample.

3.9. Application synthesized copper nanoparticle on dye removal

Decolourization experiments was performed in accordance to description by (Gong *et al.,* 2008). Staining dyes, carbol fuchsine, safranine, malachite green and methylene blue were studied. The dye solution with 5 g/l concentration was prepared. 100 ml of the dye solution was transferred into 250 ml conical flasks after which 2 ml of 0.002 g/ml of copper nanoparticles was added. The mixture was vigorously shaken for 1 min. at 165 rpm and transferred to a graduated measuring cylinder. The mixture was allowed to stand for 10 min. and the upper phase was taken for analysis using UV-visible spectrophotometer at 550 nm. The residual concentration of the dye in the samples was then calculated, and the decolourization efficiency was calculated based on the initial dye and final dye concentrations after treatment (Gong *et al.,* 2008).

$RE\ (\%) = [C - C/C_0] \times 100$

Where: C_0 is the initial value and C is the final value after the treatment of the sample.

3.10. Antimicrobial activity of synthesized nanoparticles

Test bacteria of choice were first resuscitated by inoculation into the sterile nutrient broth and incubated at 37 ℃ overnight. After which, 1 ml from each culture was inoculated into separate test tubes containing 9 ml of sterile nutrient, the test tubes were labelled with *E. coli, B. pumilus, Acinetobacter and K. pneumoniae*. The culture was then incubated overnight at 37 ℃. The absorbance of each organism was then determine at 600 nm using a UV-visible spectrophotometer to ascertain the turbidity of each organism. The turbidity of all the organisms was then adjusted using fresh sterile nutrient broth to attain an absorbance between 0.1-0.5 which is within McFarland accepted standard.

3.10.1. Minimum inhibitory concentration (MIC)

A method described by Eloff (1998), was adapted. The minimal inhibitory concentration (MIC) of the synthesized copper nanoparticles was determined. MIC is described as the lowest concentration of the CuNPs required to inhibit microorganisms. Quantitative determination of the synthesized nanoparticles was achieved through the use of 96-well plates. All the wells of 96-well plates were inoculated with 50 µl of sterile nutrient broth. O.2 g of CuNPs was dissolve into 2 ml of distilled water. The solution of CuNPs (50 µl) was the poured into the first row of 96-well containing nutrient broth and mixed. A 3-fold dilution was then performed whereby (50 µl) from row A was taken to row B of the 96 micro-well plates and it was mixed again and another (50 µl) was taken from row B to other subsequent rows until all the well had the CuNPs in different concentrations. The (50 µl) in the last column was discarded so that the total volumes of all the 96-wells is (50 µl). Selected bacteria strains were then added (50 µl) into corresponding wells. The antibiotic Ciprofloxacin (40 %) was used as a positive control control, while distilled water was used as a negative control.

The plates were then incubated at 37 ℃ overnight. *P-iodonitrotetrazodium* violet (INT) solution was used as an indicator after incubation period. 40 µl 0f 0.2 mg/ml INT solution was added to each well and further incubated at 37 ℃ for 30 min. the presence of a reddish colour indicated the reduction of INT to formazan by metabolic active microorganism. The absence of reddish colour (clear) was an indication of inactivity of microorganisms since INT was not broken down to form formazan. All the tests were conducted in triplicates and mean values were taken.

3.10.2. Minimum bactericidal concentration (MBC)

MBC was determined through using the agar dilution method. A loop full of culture of each strain from the well which indicated no colour change was streak on a Muller Hilton nutrient agar. The plates were incubated at 37 ℃ for 12 h. The lowest concentration of CuNPs that exhibited the complete killing of the test organisms were considered as the MBC (Qalleh *et al*., 2012). All data were collected in triplicates with mean and standard deviation values determined where differences were considered significant at 0.05 at confidence level ($p>0.05$) by the use of graph pad prism version 6.

Chapter 4 Results and Discussion

4.1. Elementary analysis of bioflocculant and copper nanoparticles

These results from the SEM-EDX, show the different elements present in the purified bioflocculant in Fig. 4.1. The bioflocculant is composed of elements such as: O, C, P, Ca, Cl, Na, K, Mg, and S. The element with the highest Wt% was oxygen with 29.0Wt% while sulphur was the least with just 0.6 Wt%.

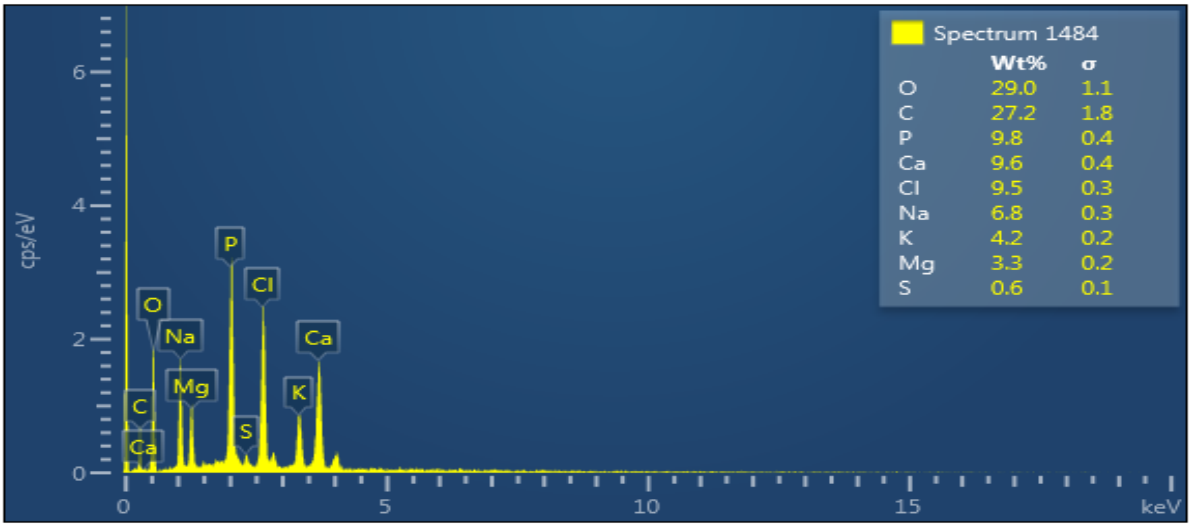

Figure 4.1: Different elements present in the bioflocculant.

These results from the SEM-EDX, illustrates the elements which were found to be present in the elementary analysis of copper nanoparticles. Copper was the second highest present element, signifying binding of copper onto the bioflocculant see (Fig. 4.2).

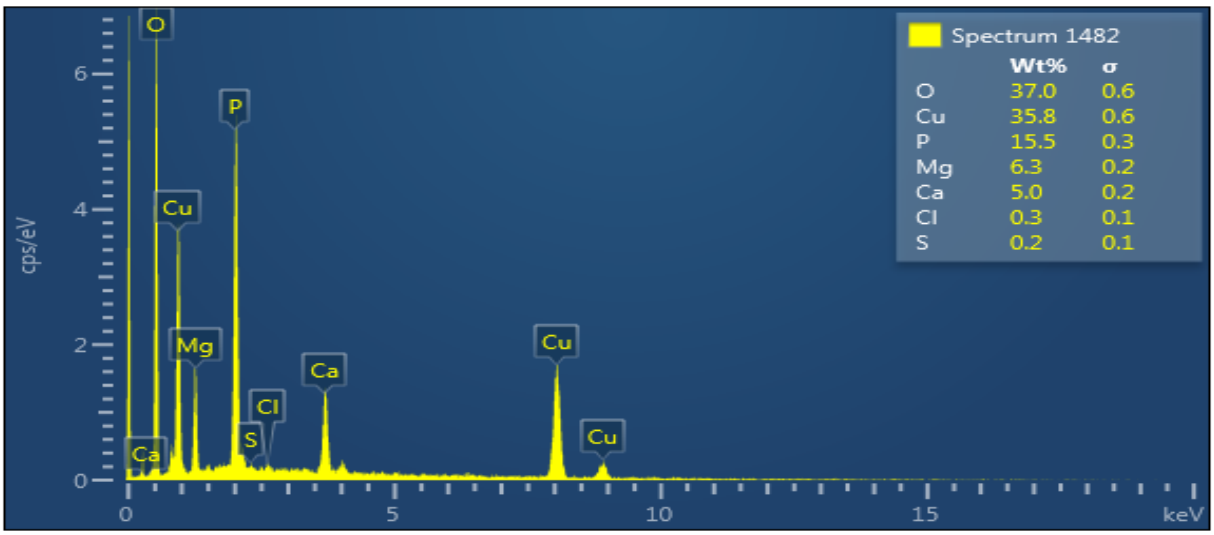

Figure 4.2: Different elements present in the copper nanoparticles.

Elements such as O, C, P, Ca, Cl, Na, K, Mg, and S were found to be present in the purified bioflocculant at the concentration of weight by percentage (Wt %). Oxygen was the highest with 29% while sulphur was the least with just 0.6. The presence of the elements in bioflocculant play the important role in the structure and flocculating activity (Cosa et *al.*, 2013). Furthermore, the presence of various elements bring about flexibility and stability to the bioflocculant and CuNPs. The presence of elements such as oxygen, carbon suggest that the bioflocculant is predominately carbohydrates in nature, also the absence of nitrogen confirms that it cannot be a glycoprotein (Devi *et al.*, 2015). In a study by Okaiyeto *et al.,* (2014), similar results were obtained in a bioflocculant MBF-UFH where elements such as: C, O, Na, Mg, P, S, Cl, K and Ca were found.

In the synthesized copper nanoparticles the elements present were: O, Cu, P, Mg, Ca, Cl, and S. these findings reveal that oxygen with 37 Wt. % followed by copper with 35.5 Wt. % which is an indication that the synthesis of copper nanoparticles was a success. It can be therefore deduced that the presence of copper in the highest percentage is as the results of copper binding onto the surface of the bioflocculant. The results of the bioflocculant alone did not have any copper present Figure 4.1 and 4.2 respectively.

4.2. Surface morphology of both bioflocculant and synthesized copper nanoparticles

Figures 4.3 (A and B) illustrates the surface morphology of both the bioflocculant and nanoparticles. These analysis was done using the SEM. The morphological surface of the bioflocculant is similar to the synthesized nanoparticles but a bigger particle size.

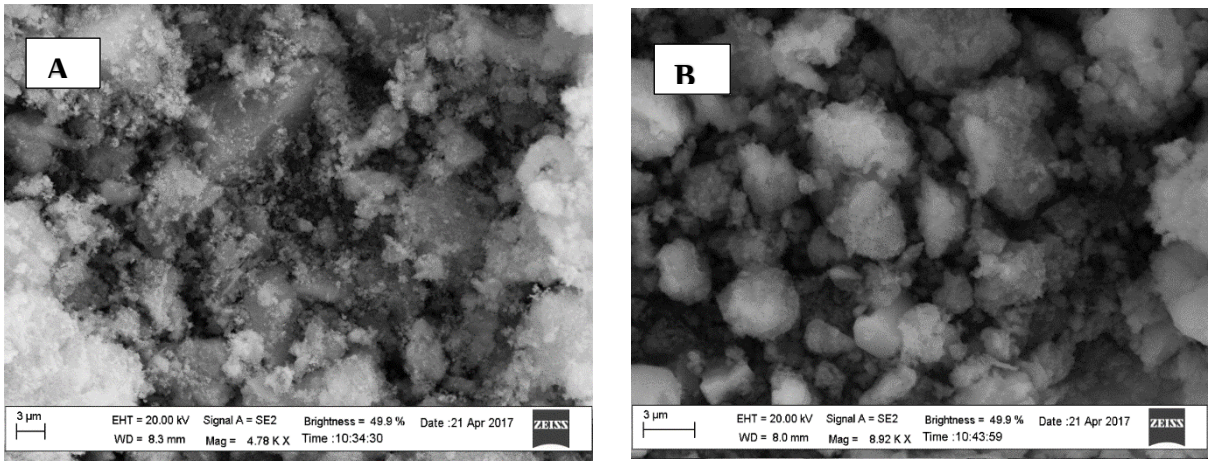

Figure 4.3: SEM image of synthesised copper nanoparticles (A) and bioflocculant (B)

With respect to SEM analysis, Fig. 4.3 A and B shows the scanning electron micrograph of the copper nanoparticles and the purified bioflocculant. The morphology of the synthesized copper nanoparticles and bioflocculant shown is amorphous in structure. The surface morphology of the synthesized copper nanoparticles and the purified bioflocculant were observed using scanning electron microscope as shown in Fig. 4.3. The surface morphology of the flocculants play significant role during the formation of flocs (Zhang *et al*., 2007). Poor or effective formation of flocs may be accounted for by the structure of the flocculant. The structures of bioflocculants reported in literature include amorphous, porous or crystal-like (Cosa *et al.*, 2013). The synthesized copper nanoparticles are agglomerated which could be as the results of electric static force between particles.

4.3. Functional groups found in both the bioflocculant and copper nanoparticles

Figure 4.4 shows the band at 3256 cm^{-1} (bioflocculant) and 3282 cm^{-1} (copper nanoparticles) showed the presence of hydroxyl (OH) group and amine (NH_2) group in the samples. The weak band at 2144 cm^{-1} in both the samples can be designated to the presence of aliphatic bonds. The peak located at 1646 cm^{-1} indicates the presence of an amide group. The vibrational peaks at 1034 cm^{-1} (bioflocculant) and 997 cm^{-1} (copper nanoparticles) are analogous to the C-O stretching in alcohols, which confirms the OH group presence. The vibrational bands observed at 571 cm^{-1} is typical of Cu-O bonds in the as-synthesised copper nanoparticles. The peaks between 1,000–1,100 cm^{-1} suggests the presence of saccharide derivatives.

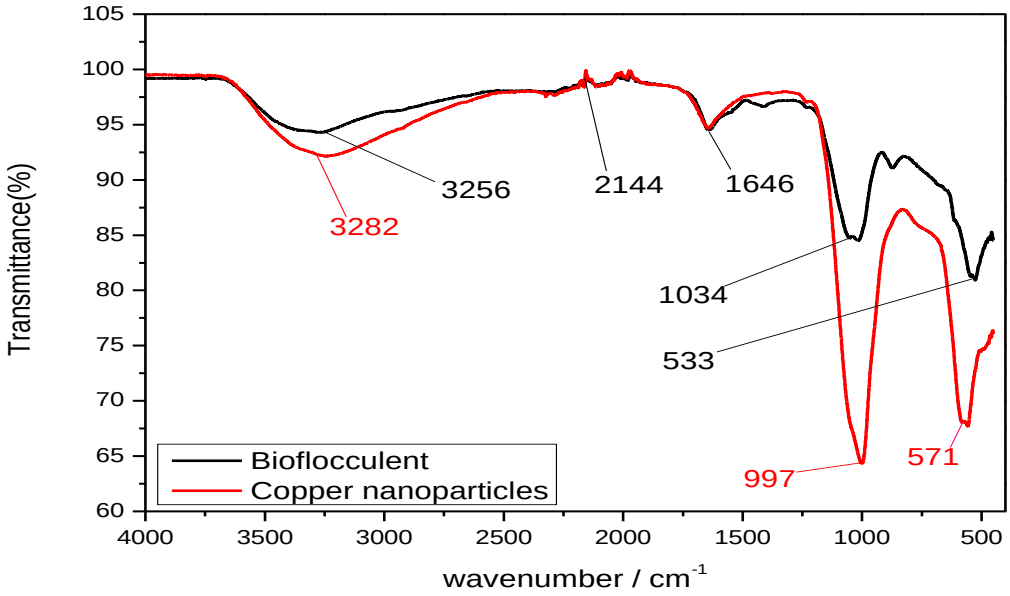

Figure 4.4: FT-IR spectra of bioflocculant and copper nanoparticles showing various functional groups.

Shojaodasati (2001) states that microorganism produce bioflocculant with different composition chemical composition. Purified bioflocculant's flocculating activity solely depends on the chemical structure which is correlated to the functional groups present in the molecule. Xiong *et al.,* (2010) documented that the functional groups present in the molecule also serve as a binding site for different colloids in suspension. Different functional groups present in the molecule were revealed by the Fourier-transform infrared (FTIR) spectroscope analysis. Figure 4.4 shows the band at 3256 cm^{-1} (bioflocculant) and 3282 cm^{-1} (copper nanoparticles) showed the presence of hydroxyl (OH) group and amine (NH_2) group in the samples The weak band at 2144 cm^{-1} in both the samples can be designated to the presence of

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4.4. Thermogravimetric analysis of both the bioflocculant and copper nanoparticles

Thermogravimetric analysis was performed on the bioflocculant and the copper nanoparticles to reveal the behaviour under the heat. This analysis gives information of pyrolytic property of the material when exposed to high temperatures. Figure 4.5 indicates the weight loss of about 20% at about 150 ℃ and about 36% weight loss at 700 ℃. The first weight loss for the bioflocculant could be attributed to the moisture content loss. Similar results were obtained in the case of bioflocculant p-KG03 produced by a marine dinoflagellate *Gyrodinium impudicum* KG03. Where the initial weight loss appeared between 40–230 ℃ and remaining weight loss was noticed at around 310 ℃. The complete decomposition of the bioflocculant is observed at temperatures above 800 ℃.

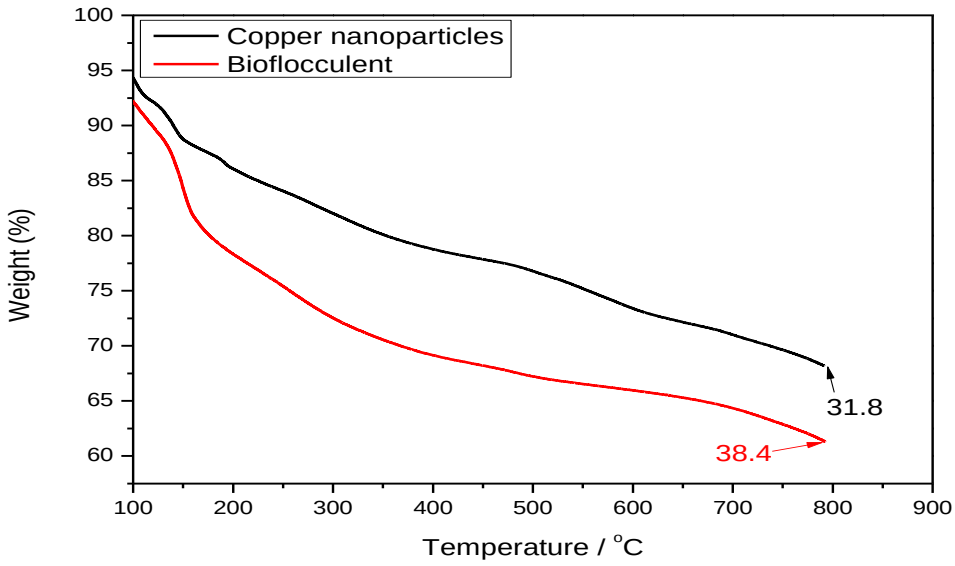

Figure 4.5: Thermogravimetric analysis for the bioflocculant and copper nanoparticles.

Bioflocculant thermal degradation generally occurs in two phases (Kumar *et al.*, 2014). Phase one involves the loss of moisture which is due to increase in temperature up to about 150 which in turn results to weight loss. The second phase is brought about depolymerisation of the structure of the bioflocculant at temperatures above 400 ℃. The behaviour of properties of the

purified bioflocculant were studied using thermogravimetric analysis. This technique enables us to comprehend the pyrolysis of the bioflocculant when exposed to very high temperature.

From Figure 4.5 about 20 % weight was lost at between 100 and 200 ℃ about 29 % loss at 500 ℃. The further increase in temperature resulted in more weight loss. The loss in moisture could be the result of the first weight loss (Kumar, 1998). This findings are similar to the one documented in the study by Okaiyeto (2014). However, the findings are contrary to those documented in another study conducted by Yim *et al.*, (2007), where bioflocculant p-KG03 produced by marine dinoflagellate *Gyrodinium impudicum* KG0, weight loss was initially observed at 40-230 ℃.

The observation seen in Figure 4.5 elicit three phases in a TGA of cooper nanoparticles. The first phase is seen approximately from temperature 40 to 120 ℃, which could be the result of loss in moisture content or drying of residual solvent which was used during purification. The second phase is observed at temperatures around 150 ℃ and below 200 ℃, this could be related to decomposition of polymer which in turn resulted in weight loss. Moreover, further increase in temperature resulted in more weight loss in the synthesized nanoparticles.

This findings suggest that synthesized nanoparticles are thermostable as they are able to maintain weight above 60% even at temperatures above 500 ℃. Contrary to the findings in a study conducted by Lee *et al.* (2008) where weight gain of about 20% was observed at 180 ℃ and stopped at 600 ℃. This was related to thermal oxidation of copper nanoparticles to form copper oxide.

4.5. TEM images of copper nanoparticles

Below are the Figures 4.6 and 4.7 of copper nanoparticles which shows the approximation of size range of nanoparticles. The synthesized copper nanoparticles seems to be spherical in shape and they are agglomerated.

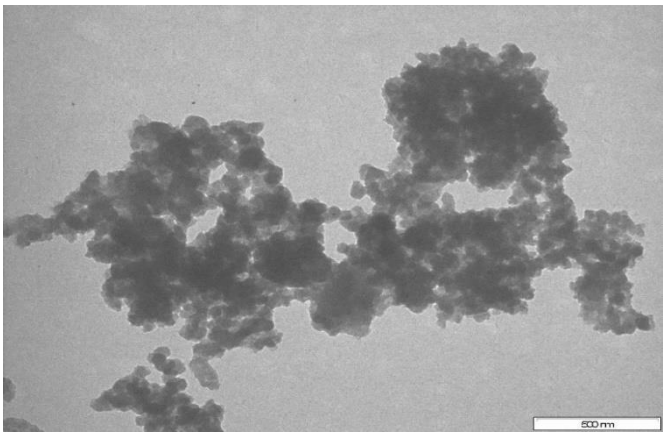

Figure 4.6: TEM image of copper nanoparticles at 500nm scale.

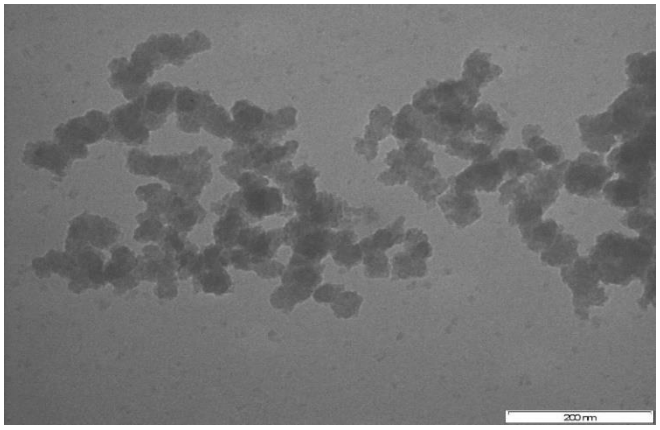

Figure 4.7: TEM image of copper nanoparticles at 200nm scale.

The morphology of the synthesized CuNPs was characterized using TEM. Sample preparation for TEM was achieved by keeping a drop of colloidal solution on a copper grid. Sample was dried at room temperature before placing it in the specimen holder. A thin sample was irradiated with a sharp high-energy electron beam focused by magnetic lenses. The electron intensity distribution of the beam after interaction with the sample was imaged onto a fluorescent screen by the objective lens and the post objective lens system. Images were recorded by a digital CCD camera reproduced or displayed on a computer monitor. The results show that the average particle size was found to be 100 nm, and seems spherical in morphology as shown in Fig 4.6 and 4.7 above

4.6. X-ray diffraction pattern of the bioflocculant and the copper nanoparticles

Figure 4.8 below is an illustration of X-ray pattern of the bioflocculant at angle (2θ) and deep peaks were seen between 20 and 30 angle.

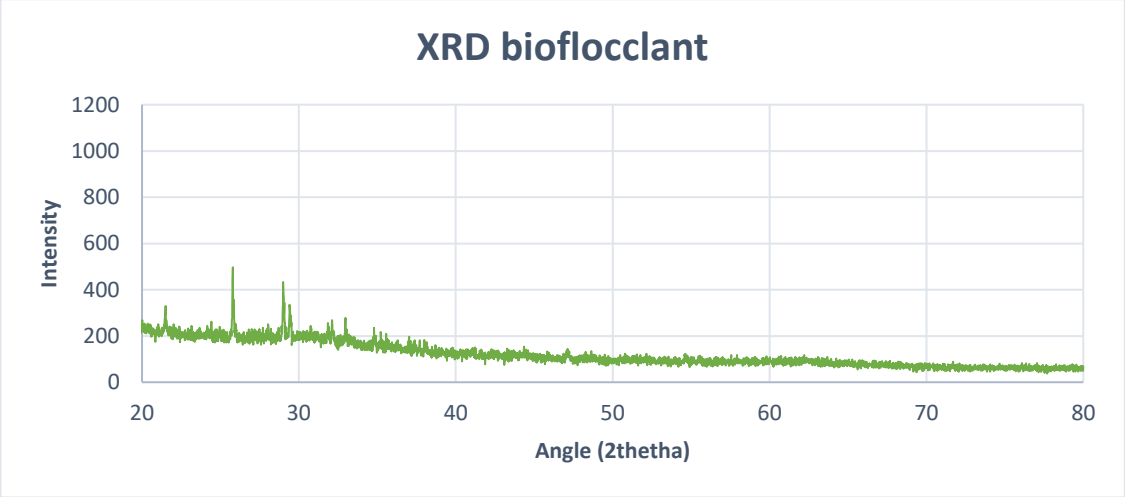

Figure 4.8: X-ray diffraction pattern of the bioflocculant

Figure 4.9 below is an illustration of X-ray pattern of the CuNPs at angle (2thetha) and deep pics were seen between 30 and 50 angle. The pattern looks similar to that of standard copper nanoparticles.

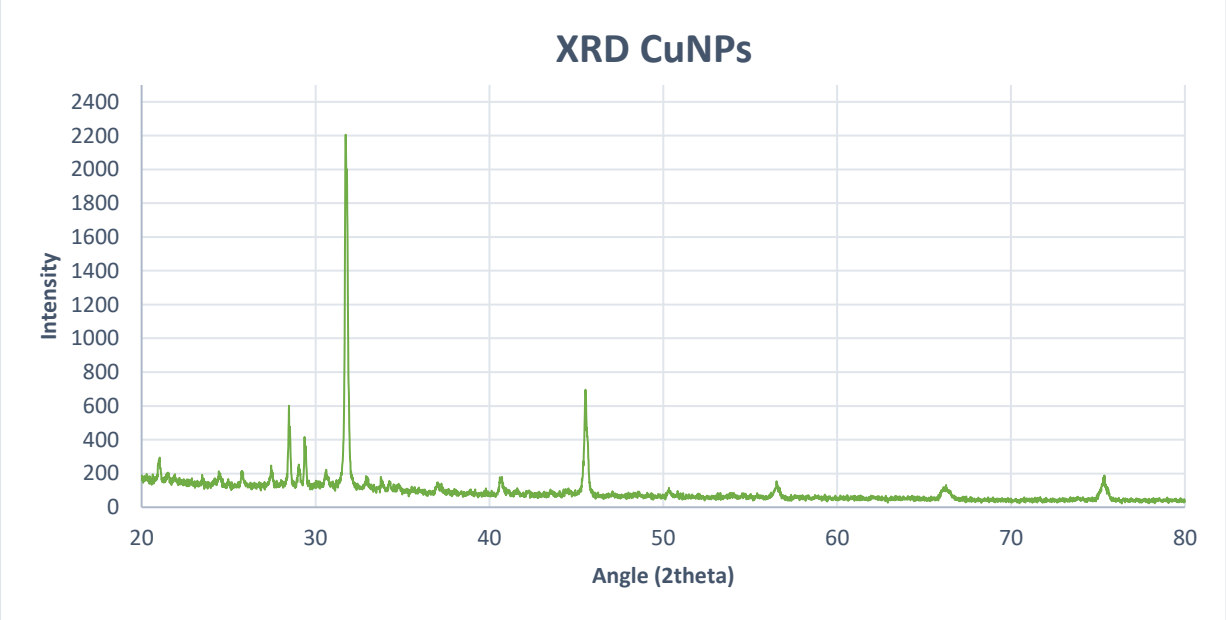

Figure 4.9: X-ray diffraction pattern of copper nanoparticles.

The XRD patterns of the bioflocculant as shown in the Figure 4.8 above, the intense peaks were observed between the 20˚ and 30˚ 2θ angle. In comparison to the CuNPs the bioflocculant shown to have some peaks which may be due to impurities found in the compound. The peaks broadening in the XRD patterns of the solids is generally attributed to particle size effects.

Smaller particle size are signified by broader peaks. Therefore, it can be deduced that the bioflocculant possess much bigger particles compare to the CuNPs as there were no broader peaks in the bioflocculant.

Figure 4.9 shows XRD patterns of copper nanoparticles, synthesized from a bioflocculant. In comparison to a copper standard (JCPDS 04-0836), the characteristic diffraction peaks of copper were observed at around 33˚ and 47˚. They correspond to the (111) and (220) planes of the fcc structure. No other impurity peaks was detected in the sample. Based on the findings it can be therefor deduced that it is possible to produced pure nanoparticles using this method. Sharpening of the diffraction peaks results from moderate temperature. This is an indication of growth of CuNPs and their improved quality. The synthesized copper nanoparticles were revealed to be crystalline in nature.

Intense Bragg reflections suggested that strong X-ray scattering centres in the crystalline phase could be due to the bioflocculant from which the nanoparticles were synthesized. Therefore, XRD result suggests that the crystallization of the bio-organic phase occurred on the surface of the copper nanoparticles or *vice versa*. The peaks broadening in the XRD patterns of the solids is generally attributed to particle size effects. Smaller particle size are signified by broader peaks. Moreover, broader peaks may also signify the effects due to experimental conditions on the nucleation and growth of the crystal nuclei. By using Debye-Scherrer's equation the particles size was calculated. The size of crystallite was in the range of 6-10 nm, which indicating a high surface area and surface area to volume ratio of the nanoparticles.

4.7. UV-visible spectra for both the bioflocculant and copper nanoparticles

Figure 4.10 below represents UV-visible spectra of the bioflocculant and copper nanoparticles at the wave length of 350 nm. The findings reveal no straight pics which could be due to some impurities on the samples.

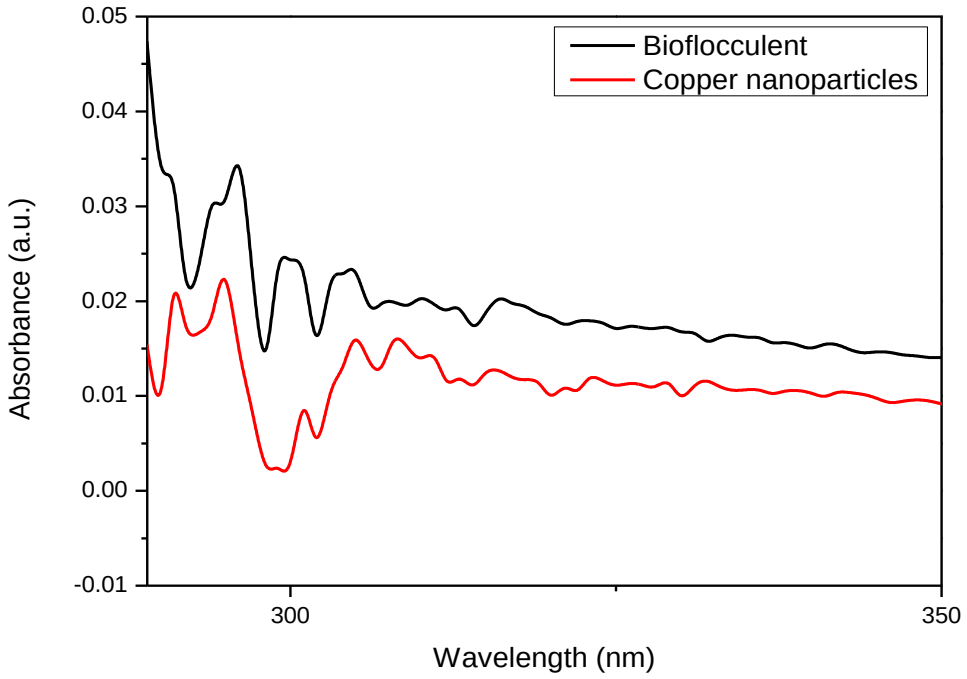

Figure 4.10: UV-visible spectra of the bioflocculant and synthesized nanoparticle.

Figure 4.10 The UV-Vis spectroscopy recorded from both the bioflocculant and copper nanoparticles solution showed the plasmon resonance (SPR) spectra with absorbance 200-700 nm and the pic maxima for both the bioflocculant and the synthesized particles was observed at around 280 nm, which could be ascribed to the formation of Cu nanoparticles. The change in colour of the bioflocculant from white to blue indicated the formation of copper nanoparticles. CuNPs exhibited green colour in a solution due to surface plasmon vibration in CuNPs. Shift in exact position of the SPR band depend on the properties of the individual particle, these properties include shape, size and capping agents.

4.8. The effect of copper nanoparticles and bioflocculant dosage on flocculating activity

Figure 4.11 below represents the results obtained during the determination of the effect the copper nanoparticles concentration on flocculating activity. The flocculating activity of copper nanoparticles decreased proportionally with the increase in dosage concentration.

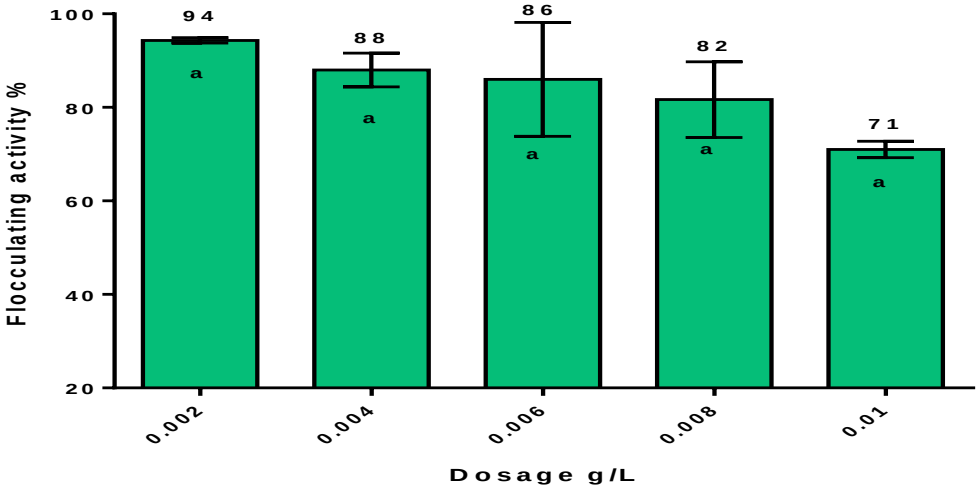

Figure 4.11: Effect of copper nanoparticles dosage on flocculating activity. Values sharing the same letter (a) have no statistical significance. P<0.05.

The amount of nanoparticles powder required for optimal flocculation is called dosage. The different concentration of CuNPs solution were prepared and its flocculating activity was evaluated. Various concentrations were prepared by dissolving different amounts of CuNPs powder in a range of 0.002, 0.004, 0.006, 0.008 and 0.01 g/l. Each of the dosage size were dissolved in 50 ml distilled water. After which one litre of kaolin clay solution was prepared where by 4 g of kaolin clay powder was dissolved in a litre of distilled water.

Three millilitres of 1 % (w/v) $CaCl_2$ and 2 ml of CuNPs solution was added into 500 ml conical flask containing 100 ml of kaolin clay and it was then agitated for one minute and then transferred to graduated 100 ml measuring cylinder and it was left to stand for 5 min. supernatant was taken for analysis at 500 nm. As depicted in the Figure 4.11 the nanoparticles flocculates best at low dosage as the highest flocculating activity was achieved at 0.002 g/l with flocculating activity of 96%. The increase in dosage concentration resulted in decrease in flocculating activity.

The synthesized nanoparticles work better than the bioflocculant from which it was synthesized as the highest flocculating activity was achieved at lowest dosage 0.002 g/l while 0.008 g/l in the bioflocculant was the highest. According to Wang *et al.* (2011), excessive addition of the flocculants results in destabilized kaolin particles suspension which in turn results into

repulsion of negatively charged kaolin particles. Most literature reports on the low dosage within the concentration of 10-50 mg/l as the most effective in terms of flocculating activity (Gao *et al.*, 2006 and Zhang *et al.*, 2002). The increase in dosage concentration results into decrease in flocculating activity, this could be due to blocking of binding site of kaolin particles by excess flocculating agent. Contrary to this findings the bridging phenomena could not be effectively formed when the dosage of the bioflocculant was too low (Gong *et al*., 2008).

Figure 4.12. Illustrates effect of bioflocculant concentration on flocculating activity. The flocculating activity of bioflocculant increased proportionally with the increase in dosage concentration. After 0.008 g/l there was decrease in flocculating activity of the bioflocculant.

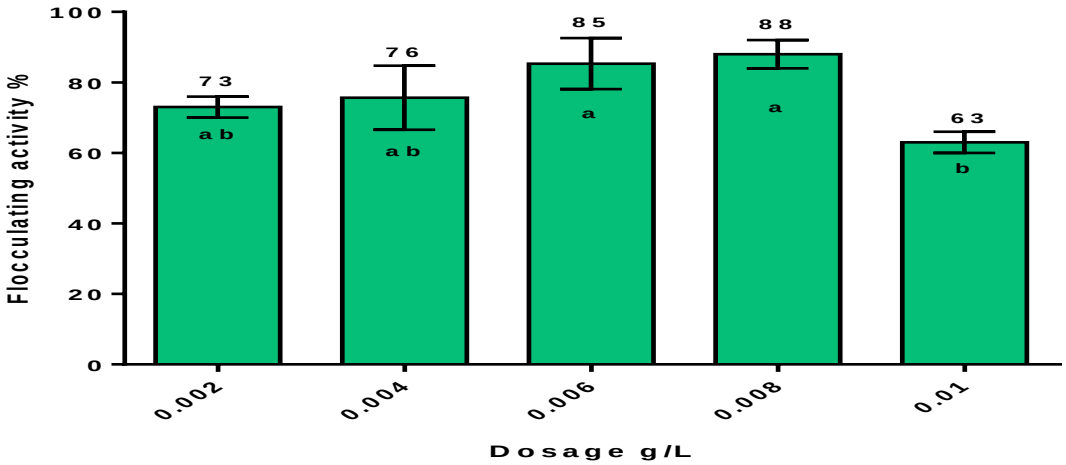

Figure 4.12: Effect of bioflocculant dosage on flocculating activity. Values sharing the same letter (a-b) have no statistical significance. P<0.05.

The amount of purified bioflocculant powder required for optimal flocculation is bioflocculant dosage. Bioflocculant dosage for optimum activity was determined using the method of Wang *et al*. (2010). The effect of bioflocculant dosage on flocculating efficiency was verified in the dosage range of 0.002-0.01 g/l as depicted in Figure 4.12. It was noted that an increase in dosage above 0.008 g/l resulted in a decrease in flocculating activity. The maximum flocculating activity (88%) was reached at the dosage of 0.008 g/l. However, there was no statistical significance between 0.002 and 0.008 g/l hence the lowest concentration was selected for all experiments which followed.

A similar observation was observed by Okaiyeto *et al.* (2014) on the bioflocculant produced by a consortium of *Halomonas sp.* According to the study done by Zulkeflee *et al*. (2012), bridging flocculation mechanism of the bioflocculant will not be affected at low dosage while at high dosage it will result in inhibition of the restabilization of Kaolin particles. Findings

documented by Wang *et al*. (2011) showed that the bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus* F6 showed optimal flocculating activity being attained only at a maximum dosage of 0.012 mg/ml. Therefore, in the current study the bioflocculant worked well at dosage a 0.002g/l, which is in agreement with most studies.

4.9. The effect of copper nanoparticles on staining dye removal

Figure 4.13 illustrates effect of copper nanoparticles on staining dye removal. The synthesized nanoparticles have a high affinity for all examined dyes with removal efficiency above 85%. This copper nanoparticles are effective to remove dyes in waste water from different industries like the clothing industries.

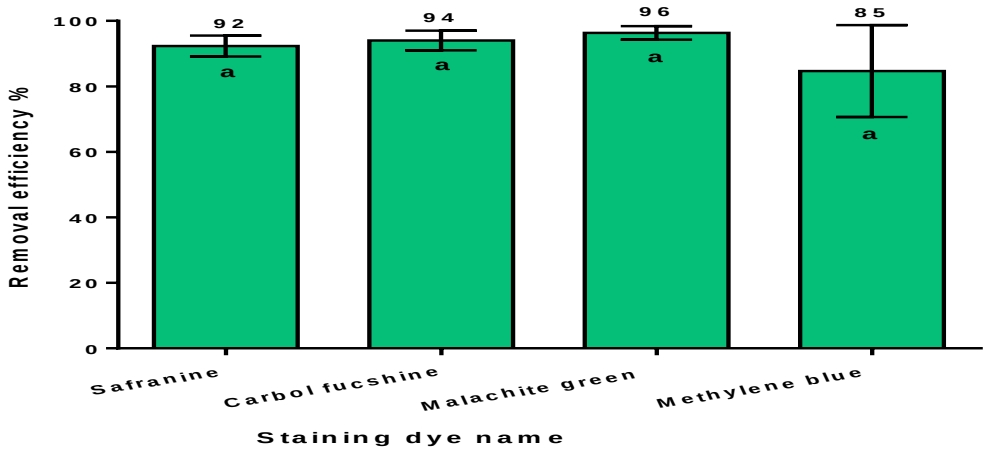

Figure 4.13: Effect of copper nanoparticles on staining dye removal. Values sharing the same letter (a) have no statistical significance. P<0.05.

Figure 4.13 in this study, the dye removal potential of copper nanoparticles synthesized from a bioflocculant was investigated. Synthesized nanoparticles were able to remove all different dyes. This could be by causing aggregation of particles due to bridging and charge neutralization as reported previously (Salehizadeh and Shojaosadati 2001). When the particles extend from the surface into a solution for a distance greater than the distance over which the antiparticle repulsion acts bridging occurs. This results into a biopolymer adsorb into other particles resulting into flocs formation (Hantula *et al.*, 1991 and Levy *et al.*, 1992).

The obtained results Figure 4.13 show that the synthesized nanoparticles possess huge potential for removing stain in all dyes which were tested. Concentration of nanoparticles remained constant (0.002 g/l), this demonstrate that the nanoparticles are effective as the removal efficiency was above 80% in all dyes without the addition of cations. Contrary to the findings reported by Shubo *et al.* (2005) where the removal efficiency directly depended on the high

concentration of the bioflocculant. The functional groups present in the polymer must be able to interact with sites on the surface of the colloidal particle in order to be effective.

4.10. Thermostability of the copper nanoparticles

Figure 4.14 show the effect of temperature on flocculating activity of copper nanoparticles. CuNPs were found to be heat stable with a flocculating activity above 90%.

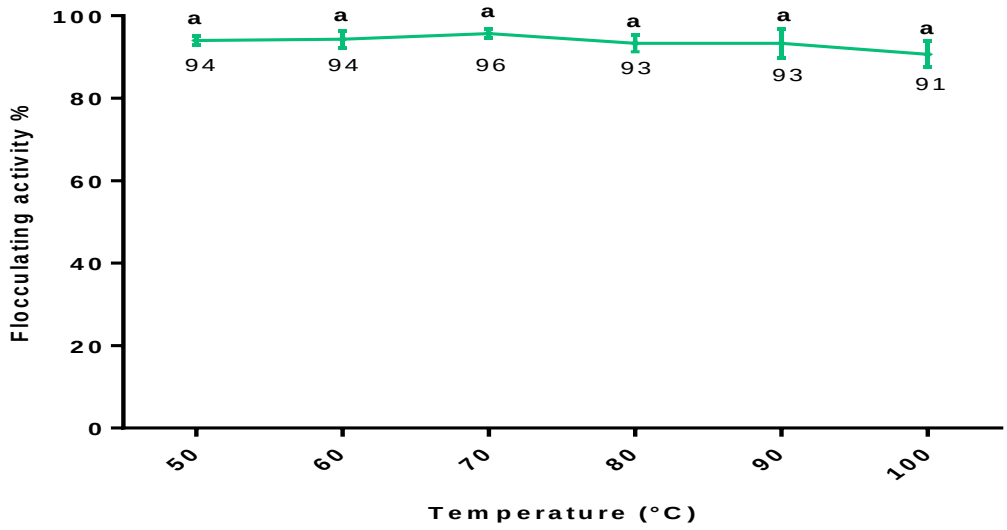

Figure 4.14: Effect of temperature on flocculating activity of CuNPs. Values sharing the same letter (a) have no statistical significance. $p<0.05$

Figure 4.14 the relationship between temperature and flocculating efficiency of the synthesized nanoparticles was examined at a temperature range from 50-100 ℃ (Sun *et al*., 2012). The synthesized nanoparticles were subjected to different temperatures using a water bath for 30 min, this was to ascertain its stability when subjected to higher temperatures. The synthesized nanoparticles retained over 90% flocculating activity suggesting that the nanoparticles were very stable. The highest flocculating activity was achieved below 70 ℃, increase in temperature resulted in slightly decrease in flocculating efficiency, but the difference was not significance in terms of statistical analysis. Therefore, it was deduced that the nanoparticles were thermo-stable and its flocculating activity was not affected when the temperature was elevated. Salehizadeh (2001) reported that the presence of protein or peptide in the structure of a bioflocculant was generally linked to its sensitivity to heat, while sugar containing bioflocculant were more heat-resistant, hence it can be concluded that the bioflocculant from which the nanoparticles were synthesized contains more sugar than protein. The thermal stability of the synthesized nanoparticles may be due to the hydroxyl group found in the

bioflocculant that is responsible in the formation of hydrogen bonds in its structure (Ugbenyen *et al.*, 2014).

Bioflocculants with high carbohydrates are thermal stable compare to those with high protein content (Walker and Wilson, 2005. The was a sharp significant drop observed when the temperature was increase, the bioflocculant flocculating activity was 92 % at 50 ℃ and dropped to 70% when the temperature was 100 ℃ (Maliehe, 2016). The difference of 22% drop in flocculating activity denotes that synthesize copper nanoparticles are more heat stable as the flocculating activity was above 90% at 100 ℃.

4.11. The effect of cations on flocculating activity of copper nanoparticles

Figure 4.15 represents the results obtained during the determination of the effect of cations on flocculating activity of CuNPs. Synthesized CuNPs are cations independent as the flocculating activity was above 95% without addition of cations.

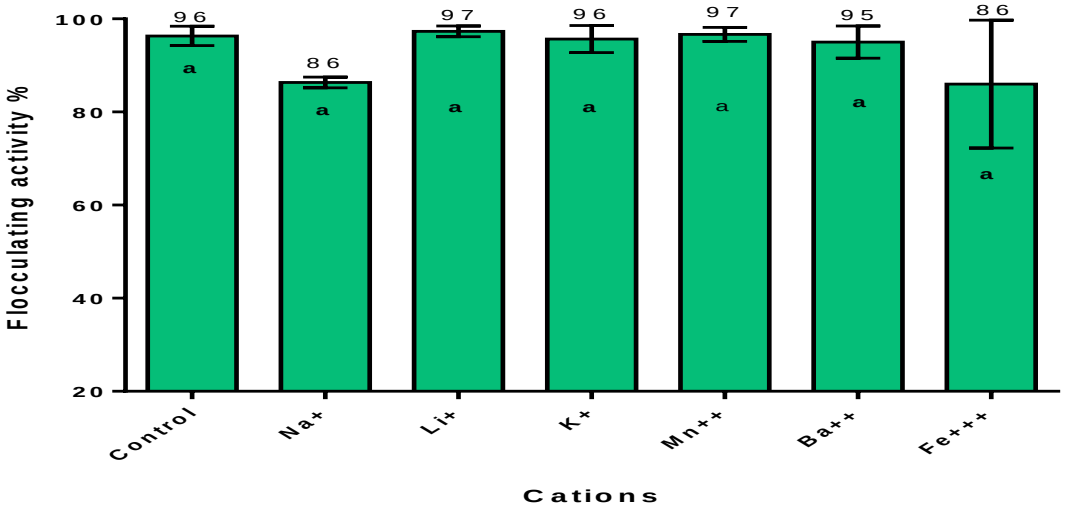

Figure 4.15: Effect of cations on flocculating activity of CuNPs. Values sharing the same letter (a) have no statistical significance. $p<0.05$

The effect of cations on flocculating activity of CuNPs was investigated as shown in Figure 4.15 all verified cations enhanced the flocculating activity above 86%. The synthesized copper nanoparticles can also work best without the addition of cations as the control was the second highest with the flocculating activity of 96%. According to a study conducted by Levy *et al*, (1992) trivalent, divalent and monovalent cations are said to have the effect of stimulating the adsorption of bioflocculant on the suspended kaolin particles by decreasing the negative charge of both the polymer and particles. This stimulation process of flocculating activity was

observed in a bioflocculant produced by *Bacillus licheniforms* and *Bacillus circulans* when Al^{3+} and Ca^{2+} were used (Li *et al.*, 2009).

Cations neutralise and stabilise the negative charge of the functional groups of colloidal kaolin particles in solution and the bioflocculant (He *et al.*, 2010. The bioflocculant is cation dependent with 90% flocculating activity when Ba^{2+} was used. Contrary to this the synthesized copper nanoparticles work effectively without the addition of cation with 96% flocculating activity, this makes the synthesized nanoparticles commercial valuable.

4.12. The effect of pH on flocculating activity on copper nanoparticles

Figure 4.16 show the effect of pH on flocculating activity of copper nanoparticles. CuNPs work best in neutral and alkaline pH.

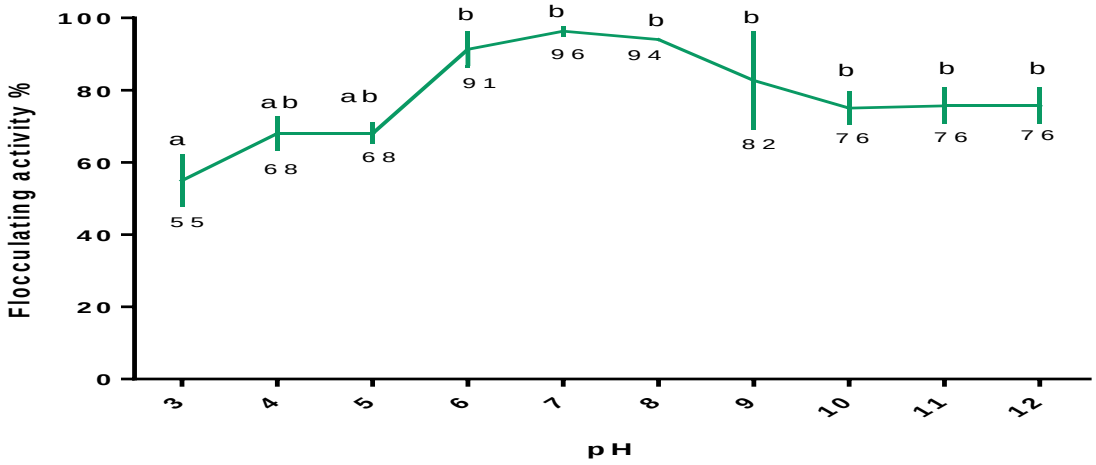

Figure 4.16: Effect of pH on the flocculating activity of synthesized copper nanoparticles. pH values sharing the same letter (a-b) are not significantly different from each other ($p<0.05$).

Key factors which influence the flocculation process include pH of the reaction mixture (Zaki *et al.*, 2013). The flocculant charge status and surface characteristics of the colloidal particles may be altered by pH which in turn may affect flocculating efficiency (Zhang and Lin, 1999). Different flocculants have been reported to produce flocculating efficiency with optimal activity at varying pH values (Wang *et al.*, 2011). NaOH and HCl were used to adjust kaolin solution's pH whenever it was necessary.

Figure 4.16 a strong flocculating activity was observed over a wide range of pH 3-12. Maximum flocculating activity of 96% was achieved at neutral pH 7 implying that the adjustment of pH would not be necessary for achieving high flocculation with this

nanoparticles. However at acidic pH there was low flocculating activity as it was just 55 % at pH 3. This may be due to the bioflocculant which shows different electric states at different pH hence affecting the bridging efficiency of the bioflocculant for clay powder (Yong *et al*., 2009).

At alkaline pH the flocculating activity remained constant from pH 10-12 with flocculating activity above 75 %. Therefore, it can be deduced that spatial charge arrangements for flocculation were not encompassing at pH 3. Similar results were observed for a bioflocculant produced by *Bacillus velezenis* 404 was stable at pH range of 3-9 and reached its maximum stability at pH 7 (Yong *et al.,* 2009). Like many other flocculants reported in literature, the synthesized copper nanoparticles flocculate in quite range of pH suggesting that it is a stable flocculant and its optimal flocculating pH was 7.

Both the bioflocculant and the synthesized copper nanoparticles had the flocculating activity at acid pH (3-5) with the flocculating activity below 80%. However, at neutral pH synthesized copper nanoparticles had the highest flocculating activity of 96%. Moreover, the synthesized copper nanoparticles work best at alkaline pH with 76% flocculating activity compared to bioflocculant which had flocculating activity below 70%.

4.12. The effect of agitation on flocculating activity of synthesized copper nanoparticles

Figure 4.17 shows the effect of shaking speed on flocculating activity of copper nanoparticles. Speed is one of the parameters which influences flocculating activity. Synthesized CuNPs work well in all ranges of speed evaluated.

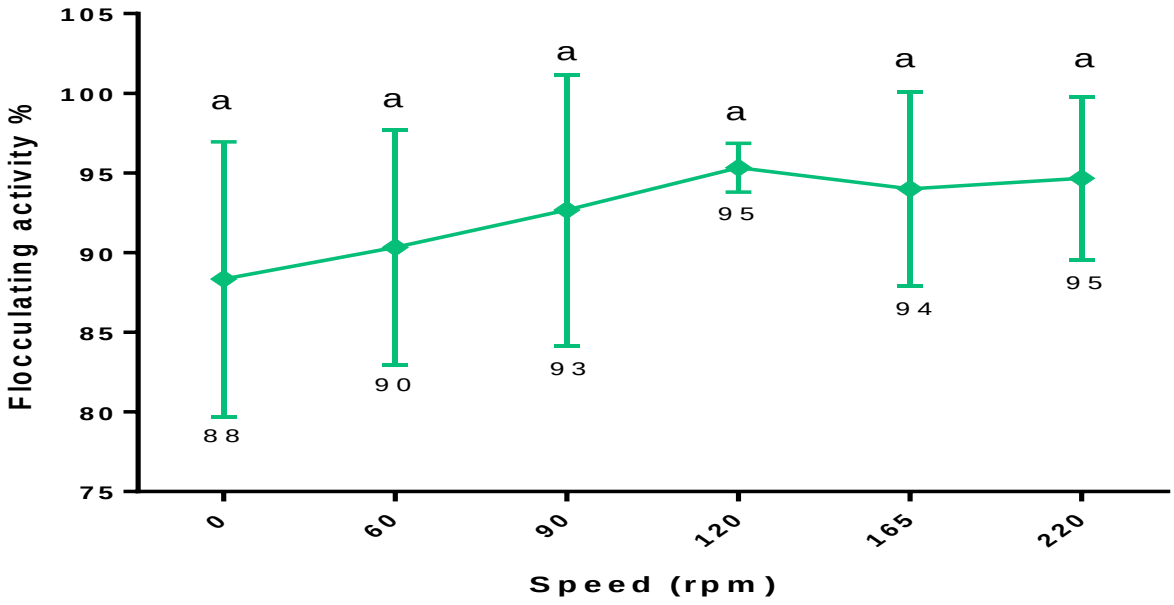

Figure 4.17: Effect of agitation speed on flocculating activity of CuNPs. Values sharing the same letter (a) have no statistical significance. $p<0.05$.

The effect of agitation on the flocculating activity of the synthesized copper nanoparticles is shown in Fig. 4.17 above. The agitation effect on flocculating activity was assed using shaking incubator IncoShake. The solution of 100 ml kaolin clay was mixed with 2 ml copper nanoparticles, the mixture was placed inside the shaking incubator for 1 min. at different speeds and flocculating activity was measured. The highest flocculating activity was observed at 220 rpm. However, the statically difference was not significant in all the speed which indicates that the synthesised nanoparticles can work well without agitation. This results contradict with those of Zu *et la.* (2013) where they found that the agitation and flocculation had the direct proportional relationship.

4.14. Solubility assay of copper nanoparticles

Below are the results of solubility (Table 4.1) assay of the synthesized nanoparticles, water was the only solvent in which the nanoparticles dissolved completely.

Table 4.1: Solubility assay of copper nanoparticles.

Solvents	**Solubility**
Water	+
Ethanol	-
Butanol	-
Chloroform	-

Key: + Soluble and - insoluble

Table 4.1 bioflocculants differ in charge they possess on surface, polar and hydrophobic components they possess which in turn affects the solubility of the synthesized nanoparticles. The synthesized copper nanoparticles were found to be insoluble to all organic solvents tested. Bioflocculants which are hydrophobic mainly consist of protein while hydrophilic bioflocculant consist mainly of carbohydrates (Jordan *et al.*, 1998). The copper nanoparticles were synthesized from a bioflocculant which consist of carbohydrates mostly. The solubility of the CuNPs in water can be attributed to the presence of the hydroxyl group which might have created hydrogen bonds with water molecule (Okaiyeto *et al.*, 2015).

4.15. The effect of sodium ions concentration on flocculating activity

Table 4.2 below represents the results obtained during the determination of the effect on Na^+ concentration on the flocculating activity of CuNPs. The flocculating activity of CuNPs was not affected by the concentration of Na^+. The synthesized nanoparticles did maintain high flocculating activity above (89%) even at high salinity (35 g/l).

Table 4.2: The effect of sodium ions concentration on flocculating activity.

NaCl (g/l)	FA (%) ± SD	
5	95	0.148
10	96	0.331
15	91	0.329
20	95	0.329
25	89	0.156
30	98	0.261
35	97	0.262

High salt concentration denatures flocculants, thus affecting flocculation (Atlas and Bartha, 1987). Table 4.2 higher concentration of sodium ions Na^+ promote loss of functional structures and interfere with charges of bioflocculant. The synthesized copper nanoparticles exhibited salinity stability as they did maintain flocculating activity above 90% even at the highest concentration of NaCl 35 g/l. Sea water is characterised by high salt concentration 33-37% (Abraham and Marteel-Parrish, 2014). The stability of the CuNPs in high salt concentration could be attributed to the bioflocculant from which it was synthesized as its microorganism used was isolated from marine environment.

4.16. The removal efficiency of pollutants in domestic waste water

Table 4.3 below show removal of heavy metals by different flocculants. All the flocculants show to have affinity for N but CuNPs were most effective compare to both iron chloride and bioflocculant.

Table 4.3: The removal efficiency of pollutants in domestic waste water by CuNPs.

Type of flocculants	**Water quality before treatment (control)**			**Water quality after treatment (flocculants)**			**Removal efficiency (%)**		
	P (mg/l)	**S (mg/l)**	**N (mg/l)**	**P (mg/l)**	**S (mg/l)**	**N (mg/l)**	**P**	**S**	**N**
CuNPs	7.6±0.0	1.7±0.1	155±0.0	1.5±0	0.61±0.1	17±0.0	79	64	89
Bioflocculant	7.6±0.0	1.38±0.1	138±0.0	4.1±0	0.96±0	17±0.0	46	42	87
$FeCl_3$	7.6±0.0	0.57±0.1	155±0.0	5.3±0	0.34±0.0	18±0.0	30	40	88

Enrichment of water with N, P and S are the most primary factors to induce water eutrophication (Fang *et al.,* 2004). The chemical formula of alga is C_{106} H_{263} O_{110} N_{16} P. The least portion of chemical formula for algae consist of N and P elements. P is considered as a limiting factor in the growth of algae in water. Mainstone and Parr. (2002) reported that 80% of reservoir and lake eutrophication is limited by phosphorus, while about 10% of lake and reservoir eutrophication is limited by nitrogen. Sulphur can react with hydrogen and form H_2S which has a very unpleasant small and is not good for aquatic life. The synthesized copper nanoparticles showed the huge potential to remove this elements from waste water, this renders the synthesized copper nanoparticles as an efficient alternative to chemical flocculants Table 4.3 above.

4.17. The removal efficiency of metals and nutrients in domestic waste water

Table 4.4 below shows the removal efficiency of dissolved metals and nutrients in domestic water by copper nanoparticles. Copper nanoparticles showed the highest removal efficiency.

Table 4.4: The removal efficiency of metals and nutrients in domestic waste water.

Type of flocculants	**Water quality before treatment (control)**			**Water quality after treatment (flocculants)**			**Removal efficiency (%)**		
	Al (mg/l)	**No_3^- (mg/l)**	**Total nitrogen (mg/l)**	**Al (mg/l)**	**No_3^- (mg/l)**	**Total nitrogen (mg/l)**	**Al**	**No_3^-**	**Total nitrogen**
CuNPs	0.86±0.0	20.6±0.1	12.9±0.0	0.33±0	7.7±0.1	9.6±0.0	62	63	68
Bioflocculant	0.86±0.0	17.7±0.1	12.9±0.0	0.29±0	11.1±0	11.3±0.0	57	37	56
$FeCl_3$	0.86±0.0	17.7±0.1	12.9±0.0	0.54±0	15.4±0.0	10.8±0.0	37	61	61

Over the years there has been the significant increase in nutrients in lakes in response to increase discharge of domestic waste water and pollution from agricultural practices and urban development (Mainstone and Parr, 2002). Nutrients enrichment, especial nitrate and nitrogen has been considered as a main threat to the marine water. This may results to eutrophication which in turn affects the economy development of the country. The synthesized copper nanoparticles had effectively removed aluminium, nitrate and total nitrogen better than both the bioflocculant and iron chloride.

4.18. The removal efficiency of BOD and COD in coal mine waste water by CuNPs

Table 4.5 below shows the removal efficiency for BOD and COD by copper nanoparticles in coal mine waste water. The synthesized nanoparticles had the highest removal efficiency for both BOD and COD compare to bioflocculant and $FeCl_3$.

Table 4.5: The removal efficiency of BOD and COD in coal mine waste water by CuNPs.

Type of flocculants	Water quality before treatment (control)		Water quality after treatment (flocculants)			Removal efficiency (%)	
	BOD (mg/l)	COD (mg/l)	BOD (mg/l)	COD (mg/l)		BOD	COD
CuNPs	123.2±0.0	154±0.0	0±0.0	0±0.0	0.07±0.0	96	89
Bioflocculant		154±0.0	0±0.0	0±0.0	0.09±0.0	75	78
$FeCl_3$	123.2±0.0	154±0.0	0±0.0	0±0.0	0.32±0.0	72	81

High levels of COD and BOD in water do not support aquatic life (Zimmerman, 2010). The presence of N, P and S in higher concentration in water prompt eutrophication (Sigee, 2005). The application of bioflocculant and copper nanoparticles for removal of these pollutants from domestic waste water, coal mine water and industrial waste water, were determined in comparison with iron chloride. The synthesized nanoparticles had the best removal efficiency compared to bioflocculant and iron chloride for COD, BOD Table 4.5.

The removal efficiency of the flocculants could be attributed to the surface structure, chemical components and to the functional groups. The results are contrary to other findings reported in literature (Zhang *et al.*, 2007; Okoh and Ugbenyen, 2014), whereby the purified bioflocculant had the highest removal efficiencies of pollutants in waste water. The findings suggest that the synthesized copper nanoparticles possess high potential for industrial application. Furthermore, the effectiveness of CuNPs recommend that they also have potential to reduce adverse effects of chemical flocculants being used. The ability for CuNPs to reduce the tested water quality parameters signifies their multi functionality.

4.19. Heavy metal removal in coal mine waste water

Table 4.6 below shows the removal efficiency of heavy metals in the water by copper nanoparticles. CuNPs has the highest removal efficiency for phosphorus but trail behind bioflocculant in sulphur and calcium removal.

Table 4.6: Removal of heavy metals in coal mine water.

Type of flocculants	**Water quality before treatment (control)**			**Water quality after treatment (flocculants)**			**Removal efficiency (%)**		
	P (mg/l)	**S (mg/l)**	**Ca (mg/l)**	**P (mg/l)**	**S (mg/l)**	**Ca (mg/l)**	**P**	**S**	**Ca**
CuNPs	2.0±0.0	0.55±0.1	56±0.0	0.3±0	0.13±0.1	17±0.0	85	76	71
Bioflocculant	2.0±0.0	0.55±0.1	56±0.0	1.3±0	0.10±0	15±0.0	35	72	73
$FeCl_3$	2.0±0.0	0.55±0.1	56±0.0	0.5±0	0.24±0.0	18±0.0	75	56	68

Industrialization is good for the economic growth of any country. However, industrial growth in many instance produce a huge amount of waste which end up reaching our water bodies if untreated. Water sample from a local coal mine was use to ascertain the removal efficiency of synthesized copper nanoparticles. Elements such as P, S, and Ca were tested Table 4.6 above. The synthesized copper nanoparticles showed some remarkable ability to remove this elements better than the bioflocculant and synthetic flocculant. The results suggest that the copper nanoparticle cab be a suitable alternative to replace chemical flocculants. The nanoparticles provide properties such as degradability and friendliness to the environment which the chemical flocculants lack.

4.20. Flocculating efficiency of CuNPs, iron chloride and bioflocculant

Below is the Figure 4.18 showing the flocculating efficiency of CuNPs in comparison to that of $FeCl_3$ and bioflocculant. Different waste water samples were examined including kaolin, coal mine waste water, industrial waste water and domestic waste water.

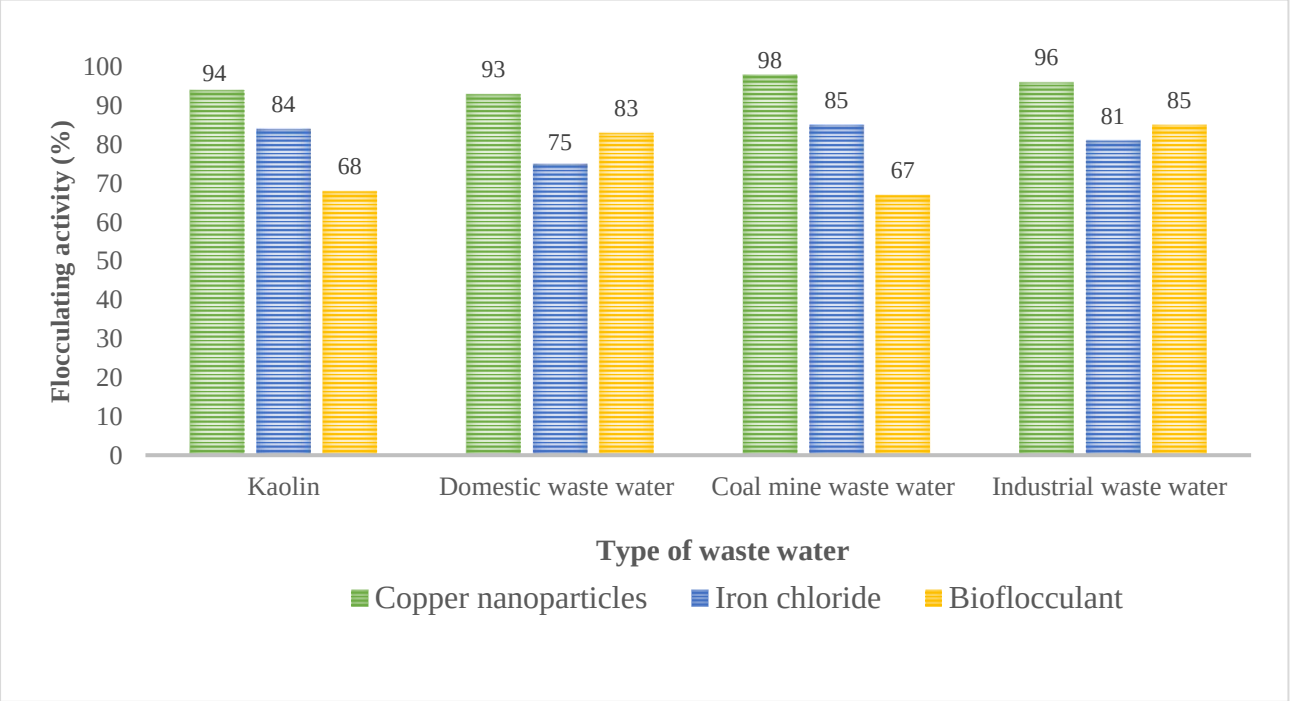

Figure 4.18: Flocculating efficiency of CuNPs compare to Iron chloride and bioflocculant using kaolin clay, coal mine water, domestic waste water and industrial waste water.

Chemical flocculants are considered cost effective due to their high flocculating efficiency, however their secondary pollutants are bad for the environment. Contrary bioflocculants are said to be environmental friendly but they are not as effective compare to chemical flocculants. In this particular study the aim was to compare the effectiveness of CuNPs which were synthesized from bioflocculant against the commercial flocculant (iron chloride) and pure bioflocculant. In comparison to other flocculants ($FeCl_3$ and bioflocculant), the synthesized CuNPs showed a huge potential. The results were outstanding in all the tested waste water as the flocculation activity was above 80 % as shown in Figure 4.18 above. These findings suggested the multifunctionality of the synthesized nanoparticles. However, to obtain maximum efficiency more optimization needs to be done on the synthesized CuNPs.

4.21. Minimal inhibitory concentration, minimal bactericidal concentration in mg/ml for CuNPs

Table 4.7 below shows the MIC and MBC of the synthesized copper nanoparticles compare to ciprofloxacin. The synthesized nanoparticles showed some remarkable properties against both Gram positive and Gram negative organisms, the least MIC and MBC was observed against K. *pneumoniae* and *Acinetobacter.*

Table 4.7: Minimal inhibitory concentration, minimal bactericidal concentration in mg/ml for CuNPs

	CuNPs		Ciprofloxacin	
Bacterial strains	MIC	MBC	MIC	MBC
E. coli	6.25	12.5	3.125	6.25
B. pumilus	3.13	6.25		
A. freundii	12.5	12.5	1.56	3.13
K. pneumoniae	12.5	25.0	1.56	3.13

Ciprofloxacin is an antibiotic used to treat a number of bacterial infections both gram positive and gram negative. Ciprofloxacin (20 µl) was used as a positive control in this study. The inhibitory effect of ciprofloxacin was observed in all the Gram-negative tested microorganisms. However, the Gram-positive *(B. pumilus*) could still grow in the presence of both high and low concentration of ciprofloxacin. Contrary, the synthesized nanoparticles had remarkable effect in both gram positive and gram negative organisms. The antimicrobial activity of CuNPs was determined in accordance to a description by Maliehe *et al.* (2015). 96-well microplates was employed, where four different strains of both Gram-positive and Gram-negative pathogenic organisms were used.

Table 4.7 test organism included *E.coli, B. pumilus, Klebsiella pneumoniae, and Acinetobacter*. Even though the antimicrobial activity of the synthesized CuNPs was more prominent on Gram-negative organisms it also showed some remarkable abilities against Gram-positive *B. pimillus* with the low MIC of 3.13 mg/ml concentration. Gram-positive bacteria lack the outer membrane, the constituents of the CuNPs are directly in contact with the phospholipid bilayer of the cell (Maliehe *et al.*, 2015).

Gram-negative microorganisms have outer membrane which is approximately 7 to 8 nm in addition to a thin peptidoglycan layer. The presence of this layer in Gram-negative bacterial provides protection against antimicrobial and chemotherapy, this is due to the protective

liposaccharides layer that exhibit antigenicity and toxicity against antimicrobial agents (Martinto and Madigan, 2006). The ability of the CuNPs to inhibit the growth of all the Gram-negative bacteria suggest that the synthesized CuNPs may have the potential to overcome resistance of Gram-negative bacteria.

Chapter 5 Conclusion

Copper nanoparticles were successful synthesized using a purified bioflocculant. Synthesized nanoparticles were characterized and applied in waste water treatment, stain removal and its antimicrobial effect was evaluated. The removal efficiencies were determined using different water samples, one from coal mine water the other from domestic and industrial waste water respectively.

The maximum flocculating activity of CuNPs was achieved at the low dosage of 0.002 g/l compare to that of bioflocculant which was achieved at dosage concentration of 0.006 g/l. The synthesized nanoparticles work best in quite a wide range of pH, but neutral and alkaline pH had the best flocculating activities. Moreover, the synthesized nanoparticles revealed to be thermostable as they maintained flocculating activity above 85 % even when exposed to 100 °C temperatures.

In the treatment of waste water, the synthesized nanoparticles worked better compared to both the iron chloride and bioflocculant. The CuNPs possess some good properties in terms of staining dye affinity as it had above 80 % removal efficiency in the staining dye tests. The elemental composition of CuNPs compare to that bioflocculant revealed that, there was huge significant increase in copper with 37.8 Wt. % while there was no copper present in the bioflocculant. Thermogravimetric analysis of both the bioflocculant and CuNPs showed that the synthesized nanoparticles are more thermally stable compared to bioflocculant.

The synthesized particles possessed some antimicrobial properties, to all selected test strain positive results were obtained. The bioflocculant alone did not give any positive results, suggesting that the antimicrobial property was due to copper presence. Furthermore, the nanoparticles were found to be saline stable as they maintained higher flocculating activity of above 80 % even at highest salt concentration NaCl (35 g/l).

Recommendations

For further studies, parameters such as pH, temperature, concentration of the metal salt should be optimized to ensure the efficiency of the synthesized particles. Also more optimization should be conducted on the synthesized CuNPs in order to obtain maximum efficiency of the synthesized CuNPs. The results should also be compared to more than one chemical flocculants to ensure its efficiency.

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Appendix

Raw Data

Table 5.1: Copper nanoparticles inoculum size, Optical density OD: 550 nm

Inoculum size (g)	1st reading	2nd reading	3rd reading	Average
0.002	0.172	0.139	0.152	**0.154**
0.004	0.794	0.123	0.031	**0.316**
0.006	0.171	0.230	0.760	**0.387**
0.008	0.311	0.472	0.748	**0.510**
0.01	0.821	0.819	0.819	**0.819**

Table 5.2: Bioflocculant Inoculum size, OD: 550 nm

Inoculum size (g)	1st reading	2nd reading	3rd reading	Average
0.002	0.666	0.820	0.752	**0.746**
0.004	0.376	0.776	0.841	**0.664**
0.006	0.468	0.210	0.265	**0.314**
0.008	0.539	0.986	0.806	**0.777**
0.01	0.795	0.089	0.090	**0.325**

Table 5.3: Thermostability of CuNPs, OD: 550 nm

Temperature ℃	1st reading	2nd reading	3rd reading	Average
50	0.194	0.137	0.168	**0.166**
60	0.143	0.211	0.094	**0.149**
70	0.131	0.072	0.143	**0.115**
80	0.208	0.108	0.146	**0.154**
90	0.226	0.066	0.156	**0.149**
100	0.300	0.164	0.200	**0.221**

Table 5.4: pH stability of CuNPs, OD: 550 nm

pH	1st reading	2nd reading	3rd reading	Average
3	0.116	0.877	0.891	**0.628**
4	0.727	0.905	0.915	**0.849**
5	0.826	0.914	0.783	**0.841**
6	0.162	0.364	0.170	**0.232**
7	0.070	0.131	0.074	**0.092**
8	0.159	0.155	0.158	**0.157**
9	0.043	0.628	0.652	**0.441**
10	0.509	0.709	0.682	**0.633**
11	0.476	0.657	0.702	**0.611**
12	0.699	0.693	0.492	**0.628**

Effect of cations on flocculating activity of CuNPs, OD: 550 nm

Cations	1st reading	2nd reading	3rd reading	Average
Na^{+}	0.330	0.301	0.355	**0.328**
Li^{+}	0.059	0.052	0.084	**0.065**
K^{+}	0.031	0.077	0.134	**0.080**
Ca^{2+}	0.172	0.139	0.152	**0.154**
Mn^{2+}	0.065	0.042	0.121	**0.076**
Ba^{2+}	0.156	0.033	0.114	**0.067**
Fe^{3+}	0.223	0.457	0.393	**0.357**
Control	0.073	0.135	0.050	**0.086**

Effect of speed on flocculating activity of CuNPs, OD: 550 nm

Speed (rpm)	1st reading	2nd reading	3rd reading	Average
0	0.240	0.100	0.491	**0.277**
60	0.085	0.442	0.165	**0.231**
90	0.098	0.027	0.410	**0.178**
120	0.128	0.154	0.061	**0.114**
165	0.042	0.320	0.073	**0.145**
220	0.092	0.261	0.019	**0.124**

Stain removal, OD: 550 nm

Name of staining dye	1st reading	2nd reading	3rd reading	Average
Safranin	0.089	0.256	0.230	**0.192**
Carbol Fuchsine	0.268	0.077	0.184	**0.176**
Malachite green	0.072	0.048	0.161	**0.094**
Methylene blue	0.370	0.148	0.934	**0.484**

Salinity assay of CuNPs, OD: 550 nm

NaCl g/l	1st reading	2nd reading	3rd reading	Average
5	0.206	0.093	0.097	**0.132**
10	0.228	0.018	0.083	**0.109**
15	0.250	0.015	0.372	**0.212**
20	0.297	0.062	0.039	**0.133**
25	0.255	0.213	0.310	**0.259**
30	0.082	0.052	0.012	**0.049**
35	0.083	0.092	0.060	**0.078**

Flocculating efficiency of CuNPs compare to Iron chloride and bioflocculant, OD: 550nm

Type of suspension		**1st reading**	**2nd reading**	**3rd reading**	**Average**
Kaolin clay	CuNPs	0.172	0.139	0.152	**0.154**
	$FeCl_3$	1.247	0.223	2.132	**1.201**
	Bioflocculant	0.468	0.210	0.265	**0.314**
Industrial	CuNPs	0.175	0.129	0.069	**0.124**
	$FeCl_3$	0.337	0.050	0.865	**0.417**
	Bioflocculant	0.191	0.796	0.733	**0.573**
Domestic	CuNPs	0.254	0.100	0.218	**0.190**
	$FeCl_3$	0.321	0.495	0.790	**0.535**
	Bioflocculant	1.761	0.156	0.967	**0.961**
Mine waste	CuNPs	0.112	0.047	0.031	**0.063**
	$FeCl_3$	0.195	2.598	0.645	**1.146**
	Bioflocculant	0.284	0.044	0.034	**0.121**