ASSESSMENT OF BIOFLOCCULANT PRODUCTION BY ACTINOMYCETES FROM RIVERS AND DAMS OF THE EASTERN FREE STATE PROVINCE OF SOUTH AFRICA AND THEIR POTENTIAL IN WASTEWATER TREATMENT.

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Declaration

I, the undersigned, declared that this doctoral research dissertation submitted to the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State is my original work with exemption to the citations. I vouch that this work has not been submitted at any other University in partial or entirety for the award of any degree. This dissertation does not contain other scientist's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other scientists.

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Summary

Bioflocculation is a process whereby stable aggregates are formed as a result of extracellular polymers produced by certain bacteria and algae. The health related issues associated with the use of chemical synthesized flocculants have necessitated for the use of microbial flocculants which are biodegradable and harmless to the environment in the treatment of water and wastewaters. Actinomycetes are Gram-positive microorganism with high guanine cytosine ratio. These organisms have been implicated in antibiotic production and also has flocculating potential. Their application in the treatment of water and wastewater is yet to be validated. Hence, this study evaluated the isolation of actinomycetes from rivers and dams of the Eastern Cape province of South Africa and validated their potential in wastewater treatment. The optimum medium culture conditions for enhancing bioflocculant production were validated to ascertain optimum flocculating activity. Furthermore, this study confirmed that metal ions supported flocculating activity and showed that the bioflocculants were cation dependent. The selected actinomycetes producing bioflocculant exhibited a flocculating activity exceeding 80% and their identitities were confirmed using 16S rRNA gene sequencing. Basic Local Alignment Search Tool (BLAST) analysis of the nucleotide sequence of the 16S rRNA revealed the bacteria isolated from Sterkfontein dam to have 99% similarity to *Streptomyces platensis* strain HBUM174787; 98% similarity to *Terrabacter* sp. MUSC78T and their sequence were deposited in the Genbank as *Streptomyces platensis* with accession number FJ 486385.1 and *Terrabacter* sp. with accession number KF682157.1 respectively. Similarly, the BLAST analysis of the nucleotide sequence of 16S rRNA confirmed the isolated strain from

Monotsha river to have 99% similarity to *Arthrobacter humicola* strain R1 and the sequence was deposited in the GenBank as *Arthrobacter humicola* with accession number KC816574.1. The partial purified bioflocculants were able to flocculate dairy, meat processing, sewage wastewaters and river water. Interestingly, there was a significant removal of chemical oxygen demand, biological oxygen demand, nitrate, turbidity and suspended solids after treating the river and wastewaters with the partial purified bioflocculants. Fourier analysis suggested the presence of carboxyl and hydroxyl functional groups in the purified bioflocculants which serves as the adsorption positions for suspended particles and has been opined to be the best choice of functional groups for flocculation process. In addition, pyrolysis profile and thermal stability analysis of the bioflocculants suggested that the main backbone is a polysaccharide. Interestingly, the partial purified bioflocculant exhibited better flocculating efficiency when compared with the chemical synthesized flocculants in river and wastewaters treatment. Thus, confirming the bioflocculants as an important tool in biotechnology.

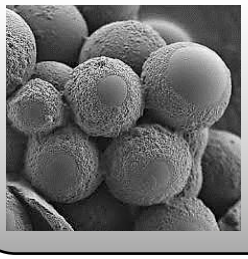
Key words: Flocculating activity, Wastewaters, *Streptomyces platensis*, *Arthrobacter humicola*, *Terrabacter* sp. FTIR, Polysaccharides, Bioflocculation.

Motivation for the study

Globally, the discharge of untreated or poorly treated wastewaters have led to issues related to different diseases and environmental pollution. Wastewater contains high level of suspended solid materials of corn, milk, protein, sugar and products that are made of textile (Kurane & Matsuyama, 1994). The process of recovering such suspended solids does not only reduce the amount of pollutants for discharge. However, it also serves as a source of income for the company as the recovered solids can serve as feed additives for the animals. Flocculants are usually employed in facilitating the effective settling of suspended solids in various types of wastewaters. The use of chemical flocculants including aluminium, sulfate, ferric chloride and polyacrylamide (PAM) have been employed in wastewater treatment prior to their discharge into the environment. Despite their usage in flocculation, there has been a setback which is associated with their toxicity and the cost of purchase of the synthesized flocculants. Consequently, biodegradable microbial flocculants which are innocuous and environmentally friendly have been considered in wastewater treatment because of their safety to humans. However, there is a dearth of information in the scientific literature on the usage of microbial flocculants that are produced by actinobacterial strains in wastewater treatment. Therefore, it is imperative to validate the potential of the actinomycetes strains in wastewater treatment.

Reference

Kurane, R. & Matsuyama, H. (1994). Production of a bioflocculant by mixed culture. Biosci. *Biotechnol Biochem* **58,** 1589‑1594.



CHAPTER ONE

A review on the application of bioflocculants in wastewater treatment

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1.0. Abstract

The rate of increase in industrialization and human day to day activities has resulted in tremendous increase in the discharge of waste and wastewater containing organic and inorganic pollutants into the environment. Coagulation-flocculation technology has been widely employed in water/wastewater treatment as a convenient and reliable technique in removing colloids, particles and cell debris. Organic and inorganic flocculants have also been used in fermentation industries, in waste and water treatment due to its potential to flocculate efficiently at a minute dosage. However, their usage has been restricted as a result of their low efficiency, associated health risks and non-biodegradability. As a result, the health implication of chemical flocculants has necessitated for environmentally friendly biodegradable bioflocculants in wastewater treatment in terms of dye, colour, solids and turbidity removal. Industrial applications of some microorganisms implicated in bioflocculation has been established. However, application of actinomycetes that has been isolated, screened and confirmed for bioflocculation is yet to be validated. Hence, this paper reiterates actinomycetes that has been implicated in flocculation and elaborate on the need for isolation and screening of novel actinomycetes with better removal and cost efficiency in order to

enhance their production in large scale medium and establish their industrial application.

1.2. Introduction

Flocculation is an essential process employed for the removal of suspended solids in domestic and industrial wastewater treatment. Flocculation is accomplished with the help of flocculants, which are either natural or synthetic substances that facilitate the aggregation of particles to form flocs. Flocculants have attracted a wider attention as a means of separation technique in portable, domestic and industrial wastewater treatment plants. Flocculants have been employed in recovery of suspended solutes from solution (Patil *et al.,* 2011). The usage of flocculants in raw water treatment, surface treatment, industry, petroleum refinery effluent and paper industry has been well documented (Shih *et al.,* 2001). Coagulation-flocculation approach is widely used in water and wastewater treatment. Also, flocculants has been known in the removal or separation of colloids and suspended particles of natural organic matter and metal ions. Additional applications of flocculating agents include the elimination of toxic metals, anions, color and odour in wastewater treatment. The usage of chemically synthesized flocculating agents such as aluminum sulfate, polyaluminum chloride, ferric chloride and polymers like polyacrylamide derivatives have been frequently employed in wastewater treatment and fermentation industries as a result of their cost effectiveness and strong flocculating efficiency. However, the application of inorganic and organic flocculating agents has drastically reduced due to the fact that large quantities are required to achieve effective flocculation. The commonly used organic flocculants are polyacrylamide, polyacrylic acid, poly (diallyl dimethyl ammonium chloride) and

polyamine (Singh *et al.,* 2000). Flocculation efficiency is usually measured or quantified based on turbidity removal, chemical oxygen demand and colour removal.

Bioflocculation is the process whereby stable aggregates are formed by extracellular polymers produced by living cells. Recently, the use of microbial flocculants have become a promising substitute for chemical flocculants; because of their safety and biodegradability efficiency which have put them on high demand (Jang *et al.,* 2001). Bioflocculants and metal ions have equally played tremendous role in forming and settling sludge in anaerobic and aerobic treatment systems (Houghton *et al.,* 2001). In addition, they can serve as an alternative to centrifugation and filtration for harvesting microbial cells from broth in food and fermentation industries (Kumani *et al.,* 2011). Since bioflocculants are generally biodegradable in nature, their usage in wastewater treatment, downstream processing and fermentation processes have increased (Salehizadeh & Shojoasadati, 2001). The choice of adopting biotechnological methods for the production of bioflocculant solely depends on the possibility of using different microorganisms to synthesize extracellular substances with different compositions. In recent years, it has been documented that many microorganisms such as fungi, bacteria (including actinomycetes) and algae has the ability of producing environmentally safe extracellular polymers which act as bioflocculants (Salehizadeh & Shojoasadati, 2003). On the contrary, according to literature, researchers have proved the usage of some chemically synthetic flocculants substances to be detrimental to aquatic life and the environment. For instance, acrylamide monomer which is non-degradable has been implicated in causing cancer and Alzheimer's disease in humans

(Zheng *et al*., 2008). Hence, there is a need to substitute or replace the exploration of synthetic flocculants with bioflocculants produced by microorganisms.

Also, an outstanding economic factor is that many developing countries can hardly afford the high costs of purchasing imported chemicals which are used for water and wastewater treatment. This has therefore, necessitated exploration of new microorganisms which could produce flocculants with high flocculating activity and optimizing their fermentation processes in order to improve their productivity. Bioflocculants have been used in treatment of waste and industrial wastewater as both pure and mixed cultures. Microbial flocculants have also been implicated in adsorption of heavy metals (Gong *et al*., 2008). Also, the bioflocculant produced by *Serratia ficaria* was able to remove chemical oxygen demand and turbidity at efficiencies of 64.1-80.7% and 91.8-93.7% respectively in agricultural wastewater (Gomma, 2012). In addition, bioflocculant MMF1 was reported by Zhang *et al.* (2007) in removing COD in indigotin printing and dyeing wastewater. On the other hand, turbidity and COD removal from swine wastewater were achieved at flocculating efficiency of 91% and 42% respectively when treated with bioflocculant (xn11 + xn7) (Zhang *et al.,* 2012a). The bioflocculant produced by the mixed culture of *Halobacillus* sp. and *Oceanobacillus* sp. exhibited a significant efficiency when tested on waste and river water (Cosa & Okoh 2014). Their findings confirmed that the consortium of the mixed bioflocculant eliminates COD and turbidity in brewery wastewater, dairy wastewater and river water at efficiencies greater than 90% when compared with polyacrylamide and aluminum chloride which are used as conventional flocculants (Cosa & Okoh 2014). Based on literature, fungi, bacteria and algae has been greatly implicated in flocculation and in treatment of waste and

industrial water (Zhang *et al.,* 2002; Sheng *et al.,* 2006). However, actinomycetes has also been employed in flocculation, but their production on a large scale medium and application in treatment of waste and industrial wastewater is yet to be established. In this paper, we reviewed actinomycetes that has been implicated in flocculation and elaborate on the need for isolation and screening of novel actinomycetes with increased removal and cost efficiency that will exhibit the potential of treating both waste and industrial wastewater. Some of the reported microorganisms that have been implicated in flocculation and their industrial applications are summarized in Table 1.

Table 1. Applications of some bioflocculants producing microorganisms

Application	Microorganism	Remarks	Reference
Biomass recovery and cell removal	*Paenibacillus polymyxa*	Removed *Scenedesmus* sp.	Patil *et al*., 2011
	Solibacillus silvestris	Removed *Nannochloropsis oceanica*	Wan *et al*., 2013
	Klebsiella pneumoniae	Removed *Acanthamoeba* cysts	Zhao *et al.,* 2013
Water and wastewater treatment	*Oceanobacillus* and *Halobacillus*	Treated brewery, dairy wastewater and river water	Cosa & Okoh 2014
	Azotobacter indicus	Treated dairy, woolen, starch and sugar industry wastewater	Patil *et al.,* 2011
	Cobetia sp. and *Bacillus* sp.	Treated river water, dairy and brewery wastewater	Ugbenyen *et al*., 2014
Decolorization	*Rhodococcus erythropolis*	Remove disperse dye solutions	Peng *et al*., 2014
	Serratia ficaria	Decolourized pulp effluent	Gong *et al*., 2008
	Chryseomonas luteola	Decolourized dye wastewater	Syafalni *et al*., 2012
Mining and other applications	*Rhodopseudomonas sphaeroides*	Flocculated coal slurry	Zhang *et al*., 2012b
	Bacillus subtilis	Synthesis of Ag nanoparticles (60 nm)	Sathiyanrayanan *et al*.,2013
	Halomonas Maura	Synthesis of mauran/chitosan nanopareticles (30-200 nm)	Raveendran *et al*., 2013

Literature has affirmed that actinomycetes have been isolated, screened and implicated in flocculation. However, their industrial applications is yet to be established (Mabinya *et al.,* 2012; Su *et al.,* 2012; Ntsaluba *et al.,* 2013; Nwodo *et al.,* 2013; Nwodo *et al.,* 2012; Shimofuruya, 1996; Mona 2014). The actinomycetes genera that has been implicated in flocculation are listed below: The genus *Arthrobacter* (Mabinya *et al.,* 2013; Su *et al.,* 2012) the genus *Actinobacterium* (sic) (Ntsaluba *et al.,* 2013); the genus *Brachybacterium* (Nwodo *et al.,* 2013); the genus *Streptomyces* (Nwodo *et al.,* 2012; Shimofuruya *et al*., 1996) and the genus *Nocardiopsis* (Mona, 2014). Their culture conditions and flocculating efficiencies are summarized in Table 2.

Table 2. Culture conditions and flocculating efficiencies of actinomycetes spp that has been implicated in flocculation

Isolate	Culture conditions	Component	F/A	References
Arthrobacter sp.	Lactose, urea, glucose, Mg^{2+} & pH 7.0	Glycoprotein	84%	Mabinya *et al.,* 2012 & Su *et al.,* 2012
Actinobacterium sp.	Sodium carbonate, Ammonium sulfate, urea, yeast extract, Ca^{2+} & pH 8.0	Polysaccharide	91%	Ntsaluba *et al.*, 2013
Brachybacterium sp.	Maltose, urea & $MgCl_2$ & pH 7.2	Glycosaminoglycan	91.17%	Nwodo *et al.*, 2013
Streptomyces sp.	Glucose, ammonium sulfate, $MgCl_2$ & pH6.8	Proteoglycan	89%	Nwodo *et al.*, 2012
Nocardiopsis aegyptia	Glucose, peptone, $CaCl_2$ & pH 7.0	Polysaccharide	89%	Simofuruya, 1996
Streptomyces grieseus	Yeast extract & pH 7.0	NA	NA	Mona, 2014

F/A – Flocculating activity
NA- Not applicable

Culture conditions have played an important role in bioflocculant production. Researchers have confirmed that carbon and nitrogen source requirement varies with different isolates (Suh *et al.,* 1998; Salehizadeh & Shojoasadati, 2001). Considering the table above, nitrogen and carbon sources required by each actinomycetes differs from one strain to another and this parameter is one of the determining factors in bioflocculant production. Furthermore, researchers have confirmed that bioflocculant producing bacteria thrives well in a medium that contain organic carbon. For instance,

Mabinya *et al.* (2012) reported that *Arthrobacter* sp. uses lactose, glucose and urea as the best carbon and nitrogen substrates for optimum flocculating efficiency. Based on the table above, each actinobacterium has attained there optimum flocculating efficiency by utilizing different carbon and nitrogen substrates.

Initial pH is also one of the major parameters that influence bioflocculant production and flocculating activity. Salehizadeh and Shojaosadati (2001) has reported that initial pH of medium depicts the electric charge of cells and oxidation reduction capability which may hinder or support the adsorption of nutrients and enzymatic reactions in different organisms. Wang *et al*. (2011) has reported that cations has enhanced or facilitated initial adsorption of flocculants in suspended colloids/particles by a means of reducing or minimizing the negative charge on polymers and colloids.

Cations have aided flocculation in actinobacteria by neutralizing & stabilizing negative charge of some functional groups thereby resulting in bridge formation between colloids (Salehizadeh & Shojoasadati, 2001). Addition of cations to suspension or particles stimulates the rate of floc formation, thereby resulting into sedimentation of particles.

1.3. Isolation of actinomycetes on cultivation media

Actinomycetes have been isolated from both soil sediment and water samples using a serial dilution spread plate technique. These organisms thrive well on mostly selective medium such as starch casein agar, glucose yeast malt extract agar, actinomycetes isolation media, soil extract agar, glycerol-asparagine agar, colloidal chitin, and M3 agar (Si *et al.,* 2004). Antibiotic such as cyclohexamide, nalidixic acid, nystatin, rifampicin

and fluconazole are usually employed to supplement the media in order to inhibit the growth of other bacteria and fungi.

1.3.1. Identification of actinomycetes

The concept of characterizing actinomycetes to genus level has been validated by Lechevalier & Lechevalier, (1980) and Goodfellow, (1989) by using a combination of cultural, morphological and chemical properties. Conversely, characterization of these bacteria to specie level is more cumbersome. The availability of gene sequencing has revolutionized the taxonomy of the aerobic actinomycetes and has become an important yardstick for the identification and characterization of clinical isolates. The 16S rRNA gene sequencing has been employed to identify diversity of actinomycetes, which corroborates the method of chemical and morphological taxonomy.

1.3.2. Actinomycetes

Actinomycetes are gram positive, aerobic and mycelial prokaryotes organism with high guanine cytosine ratio (Garrity *et al.,* 2001). They are unicellular organisms and their means of reproduction is by special spores, fission or conidia. Most are free living, saprophytic bacteria found widely distributed in soil and water (Pandey *et al.,* 2008). They resemble fungi morphologically and bacteria physiologically (Sultan *et al.,* 2002).

They are related to true bacteria in terms of classification, but they are highly considered as higher filamentous bacteria (Siefert & Fox, 1998). Actinomycetes from the genus *Streptomyces* account for two thirds of antibiotic production while the genus *Micromonospora* takes the second place. Vobis (1992) reported that *Micromonospora* produced wide range of broad-spectrum antibiotic substances. The genera

Micromonospora and *Streptomyces* are widely and evenly distributed in aquatic ecosystems and are largely more abundant than other groups of actinomycetes (Jiang & Xu, 1996).

The genera *Actinomadura, Actinoplanes, Actinosynnema, Dactylosporangium, kibdelosporangium, Kitasatosporia, Microbisporia, Micromonospotra, Nocardia, Saccharopolyspora, Streptoalloteichus, Streptosporangium, and Streptoverticillium* have also being implicated in antibiotic production (Zaitlin & Watson, 2006).

Literature has revealed that ecology of this bacterium is not well documented. Actinomycetes are found in many aquatic environments. They have been isolated from marine and fresh water bodies (Bruns *et al.,* 2003; Terkina *et al.,* 2002). The most common actinomycetes isolated from fresh water environments include: *Actinoplanes, Micromonospora, Rhodococcus, Streptomyces* and *Thermoactinomyces* (Goodfellow and Williams, 1983).

1.3.2.1. The genus *Streptomyces*

This is the largest group of actinomycetes and it belongs to the family Streptomycetaceae (Kämpfer, 2006). Streptomycetaceae is a family of actinomycetes, which constitute the monotypic suborder Streptomycineae. *Streptomyces* are gram positive, aerobic, spore forming microorganism and they are found majorly in soil sediment and marine environment. Goodfellow and Williams (1983) reported that they possess highly branched substrate and aerial mycelium, spores on aerial mycelium and occasionally on substrate medium. Currently, 600 species and 38 subspecies of *Streptomyces* bacteria have been described. The most unique attributes of

Streptomyces is the ability to produce secondary metabolites and bioactive compounds which are important in human, veterinary medicine and agriculture (Keiser *et al.,* 2000). The genus *Streptomyces* has served as basic source of many antibiotics, namely streptomycin and this was the first antibiotic produced against tuberculosis. They have been implicated in production of natural based antibiotics which includes cypemycin, grisemycin, bottromycins and chloramphenicol (Eschbach *et al.,* 2003)

1.3.2.2. The genus *Arthrobacter*

The genus *Arthrobacter* belongs to the family *Micrococcaceae*. Based on quinone system and peptidoglycan structure, the genus *Arthrobacter* can be subdivided into two groups. *Arthrobacter* is a bacteria usually found in soil and some have been recovered from clinical specimens. They are non-sporulating, gram-positive and obligate aerobes that shows respiratory metabolism with the exception of *Arthrobacter globiformis* and *Arthrobacter nicotianae* that are attributed with anaerobic metabolism (Shen *et al.,* 2009). Based on their unique metabolic diversity, *Arthrobacter* species have been explored in industrial applications in the treatment of contaminated wastewater (Carmago *et al.,* 2003). *Arthrobacter nitroguajacolicus* has been reported as a strain with the ability to transform acrylonitrile into acrylic acid (Meyer, 1989). *Arthrobacter crystallopoietes* has been implicated in reduction of hexavalent chromium in contaminated soil, indicating its biotechnological role in bioremediation (Montalvo *et al.,* 2005)

1.3.2.3. The genus *Nocardiopsis*

The genus *Nocardiopsis* are aerobic, spore-forming actinomycetes that are known for the production of branched, vegetative mycelium and aerial hyphae. Species of the genus *Nocardiopsis* may be characterized based on the color of their mature aerial and substrate mycelia, their ability to break down different compounds, and their ability to utilize different carbon sources (Meyer, 1989). The genus *Nocardiopsis* has been currently divided into seven distinct species namely: *Nocardiopsis alborubidus, N. albus, N. antarcticus, N. dassonvillei, N. halophila, N. listen* and *N. lucentensis. Nocardiopsis albus* includes two subspecies, *Nocardiopsis albus* subsp. *albus* and *Nocardiopsis albus* subsp. *prasina. Nocardiopsis* sp. has been reported to be prolific in the production of secondary metabolites (Selvin *et al.,* 2009; Collins *et al.,* 1988) and thought to contribute immensely to the chemical defense mechanisms of their host against predators with biologically active compounds and biofouling (Chou *et al.,* 2007).

1.3.2.4. The genus *Brachybacterium*

The genus *Brachybacterium* is Gram positive, coccoid to ovoid shaped, non- motile bacteria. This genus belongs to the family *Dermabacteraceae* and located in the class actinobacteria (Bolton & Gregory 2007). The genus *Brachybacterium* encompasses ten recognized species namely: *Brachybacterium alimentarium, B. conglomeratum, B. faecium, B. fresconis, B. muris, B. nesterenkovii, B. paraconglomeratum, B. rhamnosum, B. sacelli* and *B. tyrofermentans.* These bacteria contain MK-7 as the major component of menaquinone and the polar lipid profile is composed of diphosphatidylglycerol, phosphatidylglycerol and unidentified phospholipid and glycolipids (Lee *et al.,* 2014). *Brachybacterium* has been proved to be highly effective in removing manganese from solutions, converting it into insoluble manganese oxides.

This bacterium did not only oxidize the manganese, but the resulting oxides themselves also absorbed the metal from the culture solution. Thus, posing *Brachybacterium* sp. a potentially useful strain in bioremediation and cleaning up pollution. Also, this genus has been characterized as a cellulose decomposing microorganism because of their unique attribute in converting large amount of photosynthetically produced cellulosic materials into industrial substrates (Biggs *et al.,* 2000).

1.4. Coagulation-flocculation technology in wastewater treatment

This technology is usually employed in portable water and wastewater treatment to overcome the forces stabilizing the suspended particles, thereby facilitating the collision of particles and formation of floc. Coagulation is a process whereby particle destabilization and charge neutralization occurs as a result of addition of a positively charged ion of metal salt. On the other hand, flocculation refers to collision that occurs as a result of agitation that allows the particles to agglomerate into a larger flocs. Land erosion, dissolution of minerals, decaying of vegetation from domestic and industrial waste discharges have been responsible for suspended materials present in water and wastewater. Such materials may comprise of dissolved organic and inorganic matters and biological organisms such as bacteria, algae or viruses. This materials have to be removed or eliminated as it's responsible for turbidity in water and posing various health risks in human beings. Most of the suspended materials or particles are smaller in size and they also carry negative charges in aqueous medium. As a result, to facilitate the process of settling, the particles have to come together to form a larger flocs. However, this procedure is tedious as a result of electrostatic repulsion forces that hinders the particles from coming together coupled with the negative charge on the material.

Therefore, it requires a longer time for settling and this problem can be solved by destabilizing the particles with the aid of a coagulant. Destabilization can be achieved either with one or combination of two or more of the following mechanism after the addition of a coagulant agent (Duan & Gregory., 2003; Crittenden *et al.,* 2005):

(a) Compression of the electrostatic double layer

(b) Adsorption and charge neutralization

(c) Adsorption and inter particle bridging

(d) Enmeshment in precipitate using excess coagulant dose.

Having destabilized the particles that are present in the wastewater, flocculation then facilitates the aggregation or conglomeration of flocs after the addition of an appropriate flocculating agent. Finally, particles must collide and this can take place under a natural circumstances, (perikinetic floc formation) whereby aggregation is achieved by thermal motion of fluid molecules. It can also be achieved by dissipation of mixing energy (orthokinetic floc formation) (Crittenden *et al.,* 2005).

1.5. Mechanism of flocculation

The concept of flocculation mechanism has been classified into charge neutralization, electrostatic patch and polymer bridging (Blanco *et al.,* 2002). Flocculation mechanism for different types of flocculants and some of the examples of the mechanism has been illustrated in Table 3 and 4. Organic flocculants that have been implicated in different industrial processes includes polyacrylamide (PAA), polyethylene amine and poly diallyl dimethyl ammonium chloride) Singh *et al.,* 2000 & Kang *et al.,* 2007). Acrylamide derivatives are major groups of organic synthetic polymers which are widely used as a

flocculating agents because of their efficiencies and effectiveness (Moussas & Zouboulis 2009). These organic polymers are derived from oil-based or non-renewable raw materials (Suopajarviet *et al.,* 2013). These organic polymers are made up of high molecular weight and numerous charges (polyelectrolytes) in their molecular chain which stimulates their rate of flocculation (Lee *et al.,* 2014). Acrylamide is crystalline in nature and extremely stable in water and many organic solvents (Wong *et al.,* 2006). However, the monomers of polyacrylamide are not biodegradable and have been associated with health related issues. Hence, the use of environmentally microbial flocculants have attracted urgent attention

Table 3. Flocculation mechanism for different types of flocculants.

Flocculants category	Flocculants type	Flocculation mechanism
Chemical coagulants	Inorganic metal salts	Charge neutralization
Chemical flocculants	Polyelectrolytes with low MW & low CD	Charge neutralization
	Poly electrolytes with high MW & low CD	Bridging
	Poly electrolytes with low MW & high CD	Electrostatic patch
	Polyelectrolytes with high MW & high CD	Electrostatic patch + bridging
Bioflocculants	Cationic chitosan	Charge neutralization + bridging
	Anionic cellulose, tannin & sodium alginate	Bridging
	Anionic/neutral plant-based flocculants	Bridging
Grafted flocculants	Amphoteric/cationic/anionic graft copolymers	Charge neutralization + bridging

Duan & Gregory 2003

Table 4. Examples of flocculation mechanism

Flocculant type	Characteristics of flocculant	Flocculation medium	Flocculation mechanism	Reference
Quaternary ammonium based derivative of polyacrylamide (cationic)	High MW (16 X 10^6) High CD (100%)	Colloidal dispersion of anionic polystyrene latex particles	Bridging	Blanco *et al*., 2002
Cationic polyacrylamide (C-PAM) Polyethyleneimine (cationic) polyDADMAC (cationic)	High MW, low CD Low MW, high CD medium,MW, medium CD	Suspension of calcium carbonate	Bridging Electrostatic patch Charge neutralization	Lee *et al*., 2012
Cationic copolymers of acrylamide/diallyldimethyl ammonium chloride	Medium MW (3 x 10^5), low CD (10%) Medium MW (1.2 x 10^5), medium CD (40%) Medium MW (1.2 x 10^5), high CD (100%)	Suspension of silica particles	Bridging Charge neutralization + bridging/ bridging	Zhou & Franks 2006
Cationic polyacrylamide (C-PAM)	High MW, low CD High MW, high CD	Suspension of calcium carbonate	Bridging Electrostatic patch	Rasteiro *et al*., 2007
Cationic polyacrylamide (C-PAM)	High MW (7.2 x 10^6), high CD (80%) High MW (13 x 10^6), medium CD	Suspension of calcium carbonate	Electrostatic patch + bridging bridging	Rasteiro *et al*., 2008

(50%)

1.5.1 Charge neutralization

This refers to the cancellation of negative charge when positively charged colloids or ions come in contact with a negative charge of a particle. Resultantly, electrostatic repulsion between the particles disappears and it enhances the process of coagulation. It also occurs when colloids particles and flocculants are of opposite charge. For instance, in a case where colloidal particles in industrial wastewaters are negatively charged, the use of inorganic flocculants and cationic polyelectrolytes are recommended (Lee *et al.,* 2012).

1.5.2 Electrostatic patch mechanism

This involves charged polymer or colloids binding to a particle of opposite charge. Particles attract with each other through patches of opposite charge causing coagulation of the suspension.

Flocculation can be induced by several methods. Metal such as alum and ferric chloride are commonly used coagulants. They usually dissociate in water and the metal ions can cause flocculation through the process of charge neutralization.

1.5.3 Bridging

This is a process whereby charged colloids come together to the surface of two different particles to form a bridge between the particles. This allows the particle to come together and is responsible for flocculation. High molecular weight polymers with a low charge density adsorbs on a surface in a way that long loops extends to the second surface. This allows polymers that are hanging to interact or attach together, thereby

forming a bridge between the particles or colloids (Mishra & Bajpai, 2005; Mishra & Bajpai, 2006; Zhou & Franks, 2006). Polymer bridging has been reported as the mechanism for flocculation in treatment of textile wastewater with *Plantago psyllium* mucilage and *Tamarindus indica* mucilage (Rasteiro *et al.,* 2007; Rasteiro *et al.,* 2008). The length of polymers is a major determining factor that activates proper bridging.

1.6. Assessment of flocculation efficiency

Addition of flocculants into a cylinder or flask results in the formation of an interface. The upper phase known as the supernatant contains the liquid while the sediments settles below the container. The Jar test and settling test are the parameters used in assessing flocculation efficiency.

1.6.1. Jar test

This technique involves the addition of flocculant to the solution and the dose of flocculant usually varies from 0.025 ppm to 1 ppm. The working principle is achieved by stirring at a uniform speed. First, at a speed of 75 rpm for 2 minutes and then at a slow speed of 25 rpm for 5 minutes. Finally, 10 minute settling time will be allowed before the measurement of turbidity of the supernatant using turbidometer. In a situation where flocs are widely or evenly distributed, it is necessary to explore a higher velocity to suspended solids in order to obtain larger flocs.

1.6.2. Settling test

In the case of a settling test, 100 ml graduated measuring cylinder and a stop watch is usually employed. This involves the addition of suspension sample and the flocculants

in the same cylinder. The cylinder is shaken properly to ensure thorough mixing. Thereafter, the measuring cylinder is positioned uprightly and height of interface between water and settled sediment is measured over time. Jar test has been proved to be more accurate in terms of accuracy than the settling test.

1.7 Physico-chemical parameters in wastewater treatment

1.7.1. Chemical oxygen demand (COD)

This is a parameter whereby chemical oxygen demand measures or determine the required oxygen needed for the chemical oxidation of organic matter with the aid of a strong chemical oxidant. This test is usually employed in determining the organic strength of effluents. The presence of organic materials in wastewater which are very hard in nature or cannot be decompose biologically have necessitated the use of COD which will indicate the amount of oxygen that would be needed after the complete oxidation of all organic materials. The appropriate method is the dichromate method which involves the acidification of the water sample with sulphuric acid and the addition of silver sulfate.

1.7.2. Biological demand (BOD)

This parameter indicate the amount of oxygen needed by bacteria to breakdown organic compounds in wastewater. This test is widely employed to measure the level of contamination of both domestic and industrial wastes with respect to oxygen required when discharged into water body where there is aerobic condition. It’s usually

expressed in milligrams of oxygen consumed per litre of sample during the interval of 5 days of incubation at 20 ^{o}C.

1.7.3. Suspended solids (SS)

These are particles that are left in form of suspension in water as a colloid. It's usually employed in water quality as an indicator and it's abbreviated as SS.

1.7.4. Total suspended solids (TSS)

This parameter plays a vital role in wastewater treatment and their presence in water sample usually results in exhaustion of oxygen level. It measures the content of organic and inorganic materials which may be present in molecular and ionized suspended form. In this case, the solids must be very small in size to be able to survive filtration through the sieve size of 2 micrometer.

1.8. Current challenges and future direction

Environmental pollution has become one of the world major problems. Water pollution caused by industrial pollutants has become public menace, making both private and government sectors interested in mitigating this problem. Coagulation-flocculation technology is an important physicochemical step in the treatment of wastewater. This has been employed to eliminate or reduce suspended colloidal particles accountable for the presence of organic matter and turbidity in wastewater which contributes to biological oxygen demand (BOD) and chemical oxygen demand (COD) content of the water (Sarkar *et al.,* 2006). At present, flocculants are prevalent in the variety of

industrial processes such as wastewater treatment, drinking water purification and downstream process in fermentation process (Shih *et al.,* 2001). Even though some flocculating agents have been established in removing various pollutants from wastewater in a laboratory scale, there is still a necessity to improve their efficiency in the removal of suspended colloids, particles and other forms of organic and inorganic pollutants before the wastewater is discharged into the environment. Though, chemically synthetic flocculants are playing dominant roles in waste and water treatment, yet they are nonbiodegradeable and toxic to the environment. Some of these synthetic flocculating substances poses threat to public health and increase environmental risks. For example, polyacrylamide, one of the most popular flocculants includes acrylamide monomers which are verified as both neurotoxin and strong carcinogens to human beings (He *et al.,* 2010). On the other hand, aluminum salts are by far the most widely used coagulants in water and wastewater treatment. However, several disadvantages of using aluminum salts including Alzheimers disease and similar health related problems associated with residual aluminum in treated waters have been identified (Banks *et al.,* 2006). A significant major global economic factor is that many developing countries can hardly afford the high costs of imported chemicals for water and wastewater treatment. As a result, they depend on inorganic and organic synthetic flocculants which are toxic and non-degradable (Ndabigengesere & Narasiah, 1998). The potential application of bioflocculants in the treatment of different wastewaters, decolorisation of dye wastewaters, cell removal and biomass recovery has been well investigated and established (Kim *et al.,* 2011; Cosa & Okoh, 2014; Ugbenyen *et al.,* 2014). However, in spite of all the bioflocculants recently identified and explored, none

has been practically applied in the industry because of poor productivity and exorbitant cost of production (He *et al.,* 2009). Hence, it is desirable that other cost effective, new biodegradable and environmentally friendly bioflocculant with strong flocculating activity is isolated and screened to supplement, if not replace alum, ferric salts and synthetic polymers. Many of the bioflocculants employed in flocculation has been used as a pure culture. There is an urgent need to explore the interactions between actinomycetes microorganisms in a mixed culture to enhance better flocculating efficiency in the treatment of wastewater.

Till date, application and efficiency of screened actinomycetes in flocculation is yet to be validated especially in large scale medium production and application in wastewater treatment. Thus, for the sake of producing actinomycetes in a large scale medium, coupled with better and higher efficiency in flocculation, the screening of actinomycetes has become a subject of paramount and urgent research in our laboratory.

1.9. Aim and objectives

The overall aim of this study is to assess bioflocculant production of actinomycetes in rivers and dams of the Eastern Free State Province of South Africa and to validate their potential in wastewater treatment.

The specific objectives include:

- To isolate, screen and assess the effect of culture conditions on bioflocculant production in the selected isolates. (Chapter 2).
- To purify and characterize the bioflocculant compound(s) produced (Chapter 3)

- To select the most efficient producers of bioflocculant and validating their potential in the treatment of wastewaters and removal of heavy metals. (Chapter 4)

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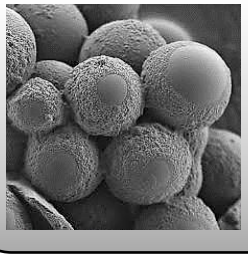


CHAPTER TWO

Isolation, screening and optimization of culture conditions of actinomycetes strains for bioflocculant production.

2.0. Abstract

Total number of 70 actinomycetes strains were isolated from rivers and dams of the Eastern Free State province and were tested against kaolin clay suspension to validate their flocculating efficiencies. Among the strains screened for flocculation, three strains isolated from Monotsha river and Sterkfontein dam exhibited flocculating efficiency above 80%. 16S rRNA confirmed the identities of the positive strains as *Streptomyces platensis*, *Arthrobacter humicola* and *Terrabacter* sp. and their accession numbers have been deposited in the Genbank. The effects of culture conditions which includes initial pH, inoculum size, carbon source, cations and nitrogen source on bioflocculant production were investigated. *Streptomyces platensis* and *Arthrobacter humicola* attained their optimal flocculating efficiencies when glucose and peptone were used as carbon and nitrogen sources at pH 7 and 12 respectively while, *Terrabcter* sp. utilizes galactose and ammonium sulfate as carbon and nitrogen source at pH 8 to attain its optimum flocculating activity. Calcium chloride as a representative of cations enhanced the flocculating activity in the bioflocculant produced by all the positive bioflocculants.

2.1. Introduction

The rate at which flocculants are being used in wastewater treatment, food and fermentation industries are increasing on a daily basis due to their efficiency and low cost (He *et al.,* 2009; Zhang *et al.,* 2007). Even though these flocculants have been used extensively, studies have confirmed that some of these inorganic chemicals and polymers are not easily degradable and that they produce carcinogenic substances during the course of degradation (Kwon *et al.,* 1996). For instance, synthetic flocculants including aluminum salts and polyacrylamide derivatives have been reported to be

associated with health problems such as Alzheimers disease (Banks *et al.,* 2006; Lee *et al.,* 2001). Therefore, using these flocculants could aggravate environmental and health concerns (Salehizadeh & Vossoughi 2000; Zheng *et al.,* 2008). Hence, the development of safe and biodegradable flocculants is of necessity. Over the years, some microorganisms including algae, bacteria, actinomyces and fungi have been reported to produce bioflocculants (Sheng *et al.,* 2006; Zhang *et al.,* 1999; Huang *et al.,* 2005). In recent years, several kinds of microorganisms which secreted flocculating biopolymers have been screened and isolated from activated sludge, soil and wastewater (Zhang *et al.,* 2002). For example, the bioflocculant produced by *Bacillus* sp. and *Alcaligenes latus* B-16 were isolated from soil (Yokoi *et al.,* 1996; Suh *et al.,* 1997). While, Saccharomycete STSM1 strain have been isolated from activated sludge (Cheng *et al.,* 2004). A benthic filamentous cyanobacterium known as *Phormidium* J-1 isolated from drainage was efficient in producing a high molecular polymer which can effectively coagulate bentonite particles from suspension (Fattom & Shiloh, 1984). In addition, *Aspergillus parasiticus* could produce a bioflocculant that can flocculate kaolin suspension and water-soluble dyes (Deng *et al.,* 2005). In this study, actinomycetes strains were isolated and screened against kaolin clay suspension to validate their flocculating capability. In addition, the optimal culture medium composition and various factors influencing flocculation efficiency were investigated.

2.2. Materials and methods

2.2.1. Study sites and sample collection

Soil sediment and water sample were collected aseptically from seven different sites (Figure 1). The river sites includes: Wilge, Sekoto, Monotsha and Namahadi while the

dam sites includes: Metsimatso, Fikapatso and Sterkfontein. The samples were collected from the rivers and dam aseptically using sterile containers and air-tight bottles. The sample bottles and containers were kept in a cooler box containing ice pack and were subsequently transported to the laboratory for processing.

2.2.2. Cultivation and isolation of bioflocculant- producing actinomycetes

Exactly 20 g of each sediment sample was air-dried at room temperature for two to three days, crushed, sieved through a 2 cm mesh and kept refrigerated (4 ^{o}C) until use. Approximately 5 g was used from the air dried sample and serial dilutions were performed and cultivation of actinomycetes from the processed soil sediment and water sample were performed as previously described (Jensen *et al.,* 1991) using Yeast Malt Extract agar (YMA) supplemented with 50 mg L^{-1} cyclohexamide and 20 mg L^{-1} nalidixic acid to inhibit the growth of fungi and bacteria respectively at pH 7. The composition of the media includes 0.4% yeast extract, 1% malt extract, 0.4% glucose and 1.6% of bacteriological agar in distilled water. An aliquot (100 µL) of the sample was spread over the cultivation medium and incubated at 28 ^{o}C for one to two weeks. After the incubation period, typical colonies (specific size, shape, aerial mycelium and colour) were purified by streaking on fresh YMA plates and purified colonies were stored on YMA slants in 20% glycerol at -80 ^{o}C in the freezer.

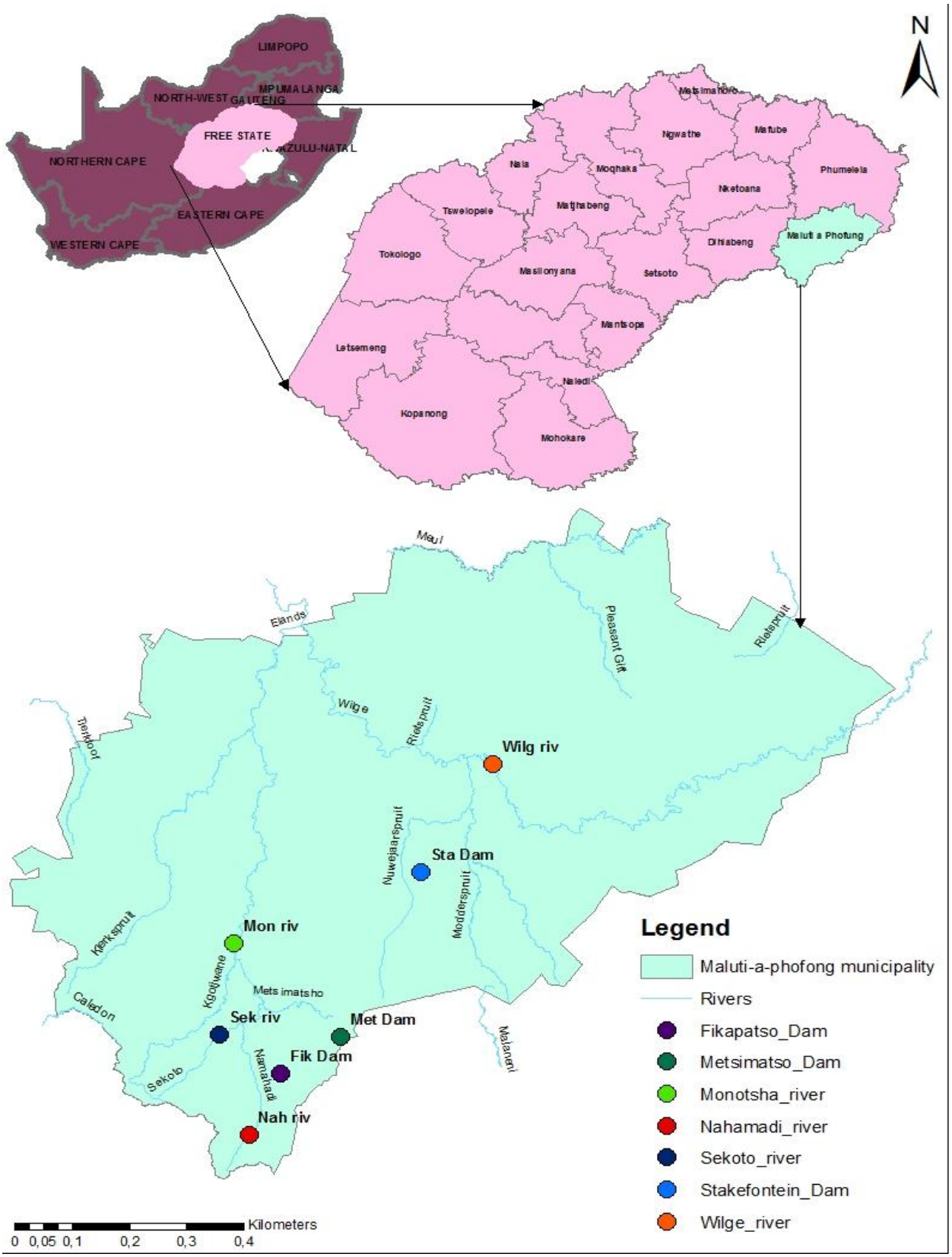


Fig 1: Map showing sampling sites

2.2.3. Screening and culturing of bioflocculant-producing actinomycetes.

Total number of 70 isolated actinomycete strains were screened for bioflocculant production in accordance with the protocol of Xia and co-workers (2008) with adaptation. The isolates were inoculated into McCartney bottles containing 5 mL of screening medium and incubated for 48 h on a rotatory shaker at 30 ^{o}C and 160 rpm at pH 7. The composition of the screening medium was 2% glucose, 0.05% urea, 0.05% yeast extract, 0.02% $(NH_4)_2$ SO_4, 0.01% NaCl, 0.02% $MgSO_4.7H_2O$, 0.5% K_2HPO_4 and 0.2% KH_2PO_4 in distilled water. This pre-culture procedure was then used as the standard inoculum preparation for subsequent experiment. A McCartney bottle containing 5 mL production medium was inoculated with 100 µL pre-culture of the strain and incubated at 25 ^{o}C in a rotatory shaker (Inco shake, Labotec) at 130 rpm for 48 h at pH 7. The production medium composition include 1% glucose, 0.1% peptone, 0.03% $MgSO_4.7H_2O$, 0.5% K_2HPO_4 and 0.2% KH_2PO_4 in distilled water. After incubation, the broth were centrifuged at 3000 rpm for 30 min at 15 ^{o}C and the cell free culture supernatants were collected and used to determine bioflocculant production against kaolin clay suspension. Finally, the strain that displayed highest flocculating efficiency was selected for further investigation.

2.2.4. Determination of flocculating activity

Kaolin clay suspension was used as test material in assessing the efficiency of bioflocculant production. Kaolin suspension was prepared by dissolving 4 g of kaolin clay in 1 L distilled water. A volume of 100 mL of this kaolin suspension was added to 2 mL of cell-free supernatant and 3 mL of calcium chloride (1% w/v) in 250 mL conical

flask. The mixture was thoroughly shaken by hand for 30 s at room temperature, gently poured into 100 mL measuring cylinder and allowed to stand for 5 min. The optical density (OD) of the clarifying solution was measured using a spectrophotometer UV/Visible Biowave II and Biowave II, $^{+}$ England at 550 nm. The control experiment was done in the same way, but cell free supernatant was replaced with 2 mL of production medium. The flocculating activity (FA) was calculated using the equation:

$$FA(\%) = \frac{B - A}{B} \times 100$$

Where: A and B are the respective absorbance of the sample and control experiment at 550 nm.

2.2.5. Identification of organism

The genomic DNA of actinomycetes was extracted using a ZR fungal/bacteria DNA preparation kit (Zymo Research) according to the manufacturer's instructions. The 16S rRNA gene was amplified by using universal primers 27f: (5` - GAGTTTGATCCTGGCTCAG - 3`) 1492r: (5` – GGTTACCTTGTTACGACT - 3`). (Lane, 1991) PCR amplification was carried out in 20 µL reaction volume containing 10 µL of Econo PCR master mix, 1 mM of each primer, 1 µL of template DNA. Sterile 8 µL distilled PCR grade water was added to a final volume of 20 µL. PCR programme used was an initial denaturation (94 ^{o}C for 5 min), 45 cycles of denaturation (94 ^{o}C for 30 s), annealing (55 ^{o}C for 30 s), extension (72 ^{o}C for 1 min, 30 s) and final extension for (72 ^{o}C for 10 min). Thermoscientific thermal cycler PCR machine was used to run the reaction. Afterwards, 5 µl of PCR products and 3.5 µl of GeneRuler DNA ladder was electrophoresed on 1% agarose gel in 1 x Tris Borate EDTA buffer stained with gel red and was visualized under UV transilluminator to confirm that a fragment of the correct

size had been amplified. Automated sequencing of 16S rRNA genes of the organism was done using the sequencer AB 35100 x L genetic analyzer with 24 capillaries. Sequencing reactions was performed according to the manufacturer's protocol using Big Dye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) with 27f and 1492r primers. Sequences was aligned in the Geneank database using the BLASTN program at the National Centre for Biotechnology information (NCBI) and percent homology score was obtained to identify the organism.

2.2.6. Optimization of culture parameters on bioflocculant production

Optimum medium culture conditions for enhancing bioflocculant production and flocculating activity were validated by varying carbon and nitrogen sources, initial pH of the culture medium, effect of inoculum size and cations on bioflocculant production**.** To determine the effect of carbon and nitrogen sources on bioflocculant production, organic and inorganic sources were evaluated. Glucose as an organic carbon source was used to replace xylose, maltose, lactose, sodium acetate, sodium carbonate and phthalate which were representative of organic and inorganic carbon sources respectively. Ammonium chloride as an inorganic nitrogen source was used to replace yeast extract, peptone and casein which serves as organic nitrogen source. The size of inoculant was evaluated on bioflocculant produced by the test organism and was assessed as described by Zhang *et al.* (2007). Flasks containing 50 mL production medium were inoculated separately with 0.5, 1.0, 1.5 and 2.0 ml pre-culture of the test bacteria which was cultivated at 28 ^{o}C at 160 rpm for 72 h. The fermentation broths were centrifuged at 8000 g for 30 min to separate the cells and the resulting supernatants were subsequently assessed for flocculating activity. The effect of initial pH (3-12) of the

production medium on bioflocculant production was assessed at acidic, basic and neural media. Monovalent, divalent and trivalent cations sources (Na^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Fe^{3+} and Ca^{2+}) were also investigated for optimum bioflocculant production.

2.2.7. Statistical analysis

Results were expressed as means ± standard deviation of three replicate determinations and were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range tests to determine significant differences in all the parameters using SPSS 16.0. Values were considered statistically significant at P-values of less than 0.05.

2.3. Results and discussion

A total of 39 and 31 actinobacterial strains isolated from four river sites and three dam sites respectively were screened against kaolin clay suspension for flocculating activity and the results are shown in Table 1.

Table 1: Isolates screened for flocculating activity

Sample site	Screened isolates	Positive isolates
Sekoto river	11	-
Monontsha river	16	1
Wilge river	5	-
Namahadi river	7	-
Total	**39**	**1**
Sterkfontein dam	15	2
Metsimatso dam	6	-

Fikapatso dam	10	-
Total	**31**	**2**

Subsequent to screening of the isolates against kaolin clay suspension, it was confirmed that a strain isolated from Monotsha river exhibited a flocculating efficiency of 83% (Table 2) and the two strains isolated from Sterkfontein dam displayed a flocculating activity exceeding 82% (Table 2). Thus, confirming Sterkfontein dam and Monotsha river as reservoirs for actinomycetes producing bioflocculants. To further confirm the identities of the positive actinomycetes that flocculated kaolin clay suspension, 16S rRNA gene sequencing was used and this yielded a product of expected size (approximately 1.5 kb). Basic Local Alignment Search Tool (BLAST) analysis of the nucleotide sequence of the 16S rRNA revealed the bacteria isolated from Sterkfontein dam to have 99% similarity to *Streptomyces platensis* strain HBUM174787; 98% similarity to *Terrabacter* sp. MUSC78T and their sequence were deposited in the Genbank as *Streptomyces platensis* (SFD 07) with accession number FJ 486385.1 and *Terrabacter sp.* (SFD 11) with accession number KF682157.1 respectively. Similarly, the BLAST analysis of the nucleotide sequence of 16S rRNA confirmed the isolated strain from Monotsha river to have 99% similarity to *Arthrobacter humicola* strain R1 and the sequence was deposited in the Genbank as *Arthrobacter humicola* (MON 05) with accession number KC816574.1.

2.3.1. Full length sequences of the positive bioflocculants

2.3.1.1. Arthrobacter humicola MON 05 Contig (1,387 bp)

CCTCCCACAAGGGGTTAGGCCACCGGCTTCGGGTGTTACCAACTTTCGTGACTTG

ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTG

CGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACT

GAGACCGGCTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCTTTGTACCGG

CCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCGT

CCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGAGTCCCCGCCATTACG

CGCTGGCAACATGGAACGAGGGTTGCGCTCGTKGCGGGACTTAACCCARCATCTC

ACGACACGAGCTGACGACAACCATGCACCACCTGTGAACCGGCCCCAAAGGGGA

ASSMCWGTTTCCAGCCCGGTYCGGTCCMTGTCMAGCCTYGGTAMGRTTCTTCGC

GTTGCWTCGAATKAATCCGCATGCTCCGCCGCTTGTGCGGGCSCCCGTCARKTCC

YYTGAGTTTTMSYYTTGCGKYCGTMCTCSCSRSGCGGGGCMCTTRRTGSGTYASY

TACKGCGYGRRAWACGKSGARKGWMMCSYASAMCTAGYSCCMMACGTWKWSSS

CAWSGWYTACGRCRGKRWCTAMYCSKGTWYSYWCCCCWKGYYKYYSCYCMTSM

KYKYSAKYTWMAGCSYCARKWMMTGCCYASASAMYYGSTKTYSCYMYCGRTRTYY

SYSCTKWTMTCYGCKCMWYYCRSMGYTMCASYMKSMCCTCCAKYCTYCCMTACW

KCMCKCTASTCYSSCMGTACCCRMCKCARAYCCGGAGTTKAGCCCSGGACTTYCA

CGRCASACSCGACAAAYCGYYTACGAGCTCTTTACGCCCAATAATTCSGGAYAACG

CTTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTC

TGCAKKTACCGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGG

CCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATT

CCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC

GGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTRAGCCATTACCTCACC

AACAAGCTGATAGGCCGCGAGTCCATCCAAAACCACAATAAAGCTTTCCACCCCCC

ACCATGCGATGAGGAGTCATATCCGGTATTAGACCCAGTTTCCCAGGCTTATCCCA
GAGTTAAGGGCAGGTTACTCACGTGTTACTCACCCGTTCGCCACTAATCCCCGGT
GCAAGCACCGGATCAT

2.3.1.2. *Terrabacter* sp. SFD 11 Contig (1,385 bp)

GTCGAACGGTGACGATCAAGCTTGCTTGGTCTGATCAGTGGCGAACGGGTGAGTA
ACACGTGAGCAACCTGCCCCAGACTCTGGGATAACCCCGGGAAACCGGAGCTAAT
ACCGGATATGACACTCGCACGCATGTGCTGGGTGTGGAAAGTTTTTCGGTCTGGG
ATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGAT
GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCA
GGGAAGWAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTMGGGTGCGAGCGTTGKCCGGAATTATTGKGCGTAA
AGAGCTKGTAKGMGGWTTGTCKCGTCTGYTGTGAAARTCCGGGGCTCAACCCCG
GRSTTGCASYSGGTACKGGCAGGSKAGASKGTSRKAGGRGAGWSTGGAATTSSW
GRYGKRRCGTYGKARTGCRSAGRTRKMAKGMGSAGMWMYSAKGRSGAACRCMG
RTCKCTRRGSCASKWCTGWSGCTSASWASYGAMRSYRWGGRGMGMAARCAKGG
GKAGMKAMCMGGATWRGMTAYSCYGGWARYSYWKGCCGYWAACKKTGGGCGCT
AKKYGTCGGAYYCCKTSCMCSRGTTMCGKGCYGCAGCTMMCGCMTTRRGCRCCC
CGSCYGGGGRGTWCRRCCKCAAGGSWAAAACTCRRRGGMMTYGMCGGGGGCC
CGCACAAGCGGCGGAGCATGCGGATTAMTWMGATGCAACGCGAAGAWCCTTAYC
AAGGCTTGACATACACCGGARKCASTCAGAGATGGGTGSGTCTTCGGACTGGTGT

ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGKCGTGAGATSTTGGGTTAAGTCCC

GCAACGAGCGCAACCCTCGTTCYATGTTGCCAGCACGTGATGGTGGGGACTCATA

GGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATG

CCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCGA

AACCGTAAGGTGGAGCGAATCCCAAAAAACCGGTCTCAGTTCGGATTGGGGTCTG

CAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCG

GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTA

ACACCCGAAGCCGGTGGCCCAACCCTTG

2.3.1.3. *Streptomyces platensis* SFD 07 Contig (1,371 bp)

TGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCC

TGGAAACGGGGTCTAATACCGGATATGACACACGACCGCATGGTCTGTGTGTGGA

AAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGA

TGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACT

GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA

CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGG

TTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTAYCTGCAGARGAAG

CGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTARGGCGCAAGCGTTGT

CCGSAATTATTGGGCGKRAAGAGCTCGTAGGCGGMTTGTCGCGKCGKAWGTGRA

AGCCCGGGGCTTAACCCCGKGTCTGCWTTMRAYMCGGGCAKGCTMSMGTKCGGT

ASGGGAGATMSGRRYTSSTGGTGTWKCGGTRARRKGSGCAGATATYMSGRGGAW

MRCSGKKRRMKAMGSMGRWWYYCKGRGSCAMWMCKGWCGCKRAGGAGSGAAW

GYGKGSSGAKMSWRMMGGWKWRGAKMSMMWGSKWGGSSASSSMAYARRMKTW

GRKACCYWGGTRKKGGMSRCMKTMMACGTYGGSMRCKMSGYRTSGRMSRCATT

MMAYKYCSYSCSTGGSGCAKMYRRCGSAWTRRGTWMMCCKCMWRGGGAGTRCG

GSSGCAMGGCTMAAACTCMAAGGAATKGRCSKGGGCCCGCACAAGCASCGGARC

AYGTRGCTWRRTTYGACGCAACSCGAARAMCCTTRSCAAGGSKTGMCMYACACC

GGAARCGKCWGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGG

CTGTCGTCMGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC

CTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTG

CCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTAT

GTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGA

GGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA

CCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATAC

GTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGA

AGCCGGTGGCCCAACCCC

Table 2: Flocculating activities of isolates screened from Sterkfontein dam and Monontsha river

Isolate code (MR)	F/A (%)	Isolate code (SD)	F/A (%)
MON 01	32 ± 2.1	SFD 01	12 ± 1.3
MON 02	41 ± 1.4	SFD 02	28 ± 1.9
MON 03	19 ± 1.9	SFD 03	07 ± 0.6
MON 04	51 ± 0.5	SFD 04	35 ± 0.9
MON 05	**83** ± 1.**2**	SFD 05	42 ± 2.6
MON 06	28 ± 0.7	SFD 06	31 ± 1.3
MON 07	61 ± 1.1	**SFD 07**	**85 ± 1.1**
MON 08	13 ± 0.3	SFD 08	59 ± 0.3
MON 09	63 ± 1.6	SFD 09	36 ± 2.5
MON 10	08 ± 2.4	SFD 10	48 ± 1.0
MON 11	16 ± 1.8	**SFD 11**	**82 ± 0.7**
MON 12	54 ± 0.6	SFD 12	18 ± 1.2
MON 13	23 ± 1.0	SFD 13	41 ± 2.9
MON 14	57 ± 1.3	SFD 14	12 ± 1.8
MON 15	55 ± 0.8	SFD 15	36 ± 0.5
MON 16	06 ± 1.2	-	-

MR: Monontsha river, **SFD:** Sterkfontein dam, **F/A:** Flocculating activity

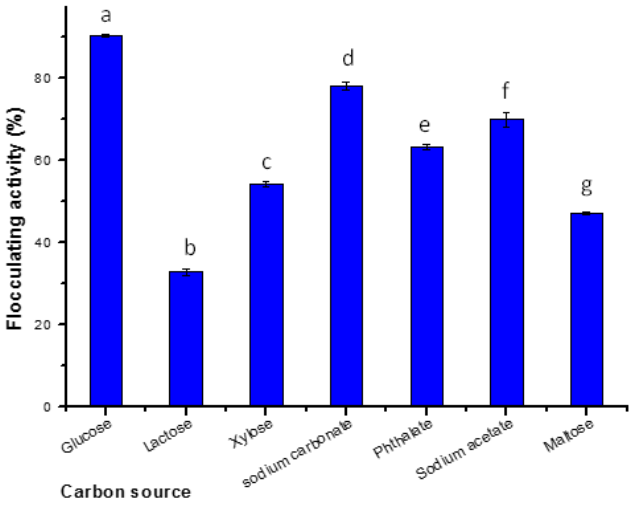
2.4. Factors affecting the bioflocculant activity

2.4.1. Effect of carbon and nitrogen sources

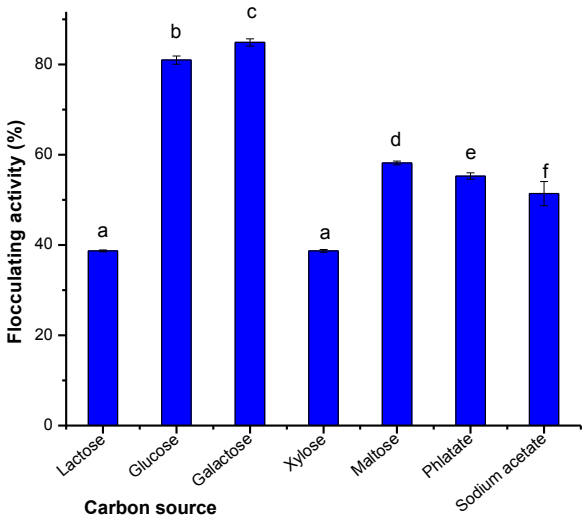
The culture medium composition and conditions have been reported to have impact on bioflocculant production (Xia *et al.,* 2008). Optimization of carbon and nitrogen sources could result in attaining maximum flocculating activity within the shortest time of incubation (Xiong *et al.,* 2010). Fig 2A revealed the bioflocculant production potential of *Streptomyces platensis* utilizing different carbon sources. Glucose, sodium carbonate, phthalate and sodium acetate appeared to support cell growth and bioflocculant production at a flocculating activity of over 60%, with glucose showing the maximum activity (90%). Likewise, the effect of carbon sources in bioflocculant produced by *Arthrobacter humicola* showed that glucose, galactose, sodium acetate and phlatate enhanced flocculating activity above 55%, with galactose resulting in maximum flocculating activity (88%) (Fig 2B). On the other hand, maltose, xylose and lactose resulted in weaker flocculating activity by the bioflocculant produced by *Streptomyces platensis* and *Arthrobacter humicola* as shown in Fig 2A and B. This finding is consistent with the report of (Xia *et al*. (2008) and Cosa *et al*. (2013) where glucose was found favourable for bioflocculant production by *Proteus mirabilis* TJ-1, *Bacillus* sp. and *Virgibacillus* sp. Data obtained with respect to the impact of nitrogen sources on bioflocculant production by *Streptomyces platensis* in this study revealed that the organic (yeast extract and peptone), and the inorganic (urea, ammonium sulfate and ammonium chloride) nitrogen sources supported the growth and production of the bioflocculants with peptone eliciting the most prominent flocculating activity (90%) (Fig 3A). Peptone, yeast extract and urea significantly enhances flocculating activity of *Arthrobacter humicola* with peptone facilitating optimum activity whereas, ammonium

sulfate, ammonium chloride and casein were poorly utilized by the test organism (Fig 3B). Therefore, the highest flocculating activity observed with utilization of organic nitrogen source (peptone) by the test organisms in this study could be attributed to the availability of vitamins and trace elements (Sanjukta *et al.,* 2014). Studies have reported that certain bioflocculant producing strains have effectively utilized peptone or yeast extract as sources of nitrogen, thereby validating peptone as one of the best and cost effective nitrogen sources (Li *et al.,* 2010; Aljuboori *et al.,* 2013). Carbon and nitrogen source requirement differs with different strains. Different result was observed with the effect of carbon and nitrogen sources on bioflocculant produced by *Terrabacter* sp. as shown in Fig 2C & 3C. Glucose, phlatate, sodium acetate and galactose were both utilized by the test organism with glucose stimulating the maximum flocculating activity. Conversely, lactose, xylose and maltose were poorly utilized by the test organism which resulted in weak flocculating activity.

(A)


Flocculating activity (%)
0
20
40
60
80
a
b
c
d
e
f
g
Glucose
Lactose
Xylose
sodium carbonate
Phthalate
Sodium acetate
Maltose
Carbon source

(B)


Flocculating activity (%)
0
20
40
60
80
a
b
c
a
d
e
f
Lactose
Glucose
Galactose
Xylose
Maltose
Phlatate
Sodium acetate
Carbon source

(C)

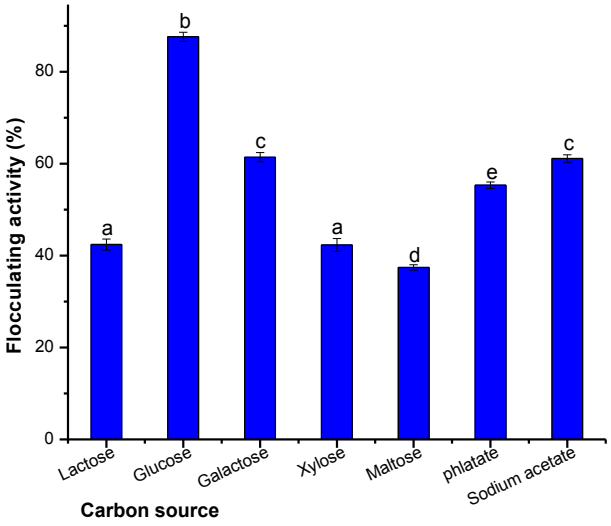
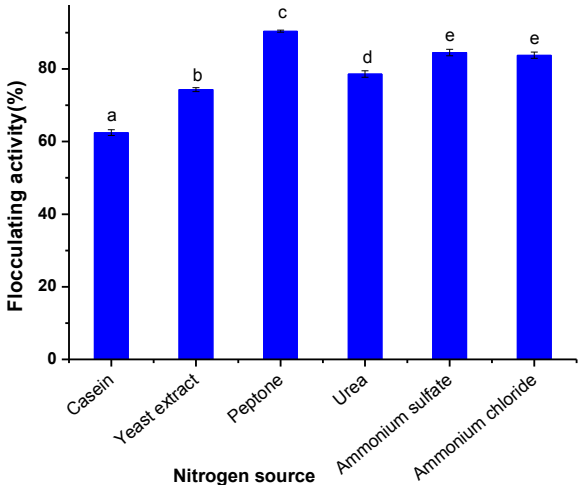


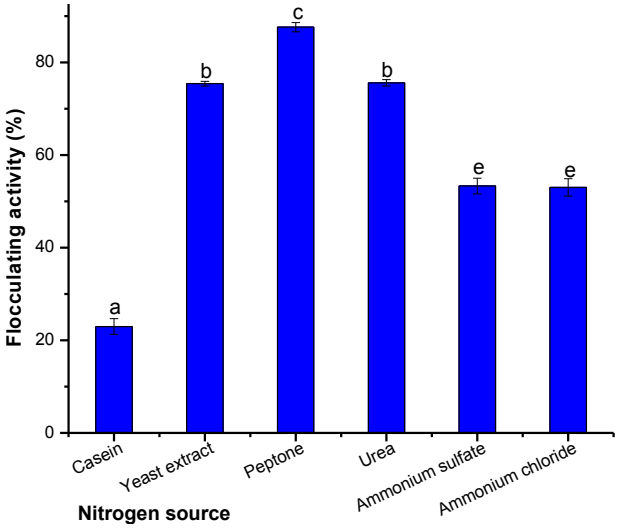
Fig. 2- Effect of various carbon sources on the flocculating activity of (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$.

On the other hand, all nitrogen sources tested with the exception of casein supported flocculating activity with ammonium sulfate resulting in maximum activity (Fig 3C). Furthermore, Li *et al.* (2003) reported that carbon and nitrogen source requirements vary with different organism and this was not only well demonstrated in the present study but also consistent with assertions from previous works. For instance, sucrose, starch and ethanol were suitable for carbon sources that resulted in highest flocculating activity in *Bacillus licheniformis* X14 while ammonium chloride as a nitrogen source was effectively utilized by the organism. The presence of amino, hydroxyl, carboxyl and carbonyl groups in microbial flocculants which are complex polymers, reiterates the

need for the availability of complex carbon and nitrogen sources (Xiong *et al.,* 2010; Salehizadeh & Shojaosadati, 2003).

(A) **(B)**


Flocculating activity(%)
0
20
40
60
80
a
b
c
d
e
e
Casein
Yeast extract
Peptone
Urea
Ammonium sulfate
Ammonium chloride
Nitrogen source


Flocculating activity (%)
0
20
40
60
80
a
b
c
b
e
e
Casein
Yeast extract
Peptone
Urea
Ammonium sulfate
Ammonium chloride
Nitrogen source

(C)

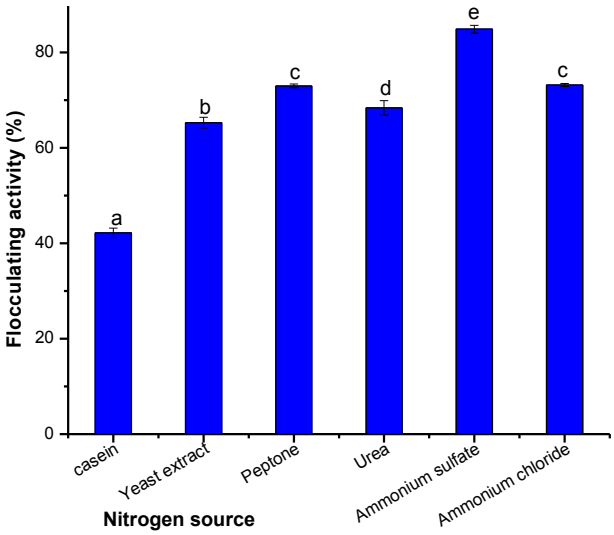

Flocculating activity (%)
0
20
40
60
80
a
b
c
d
e
c
casein
Yeast extract
Peptone
Urea
Ammonium sulfate
Ammonium chloride
Nitrogen source

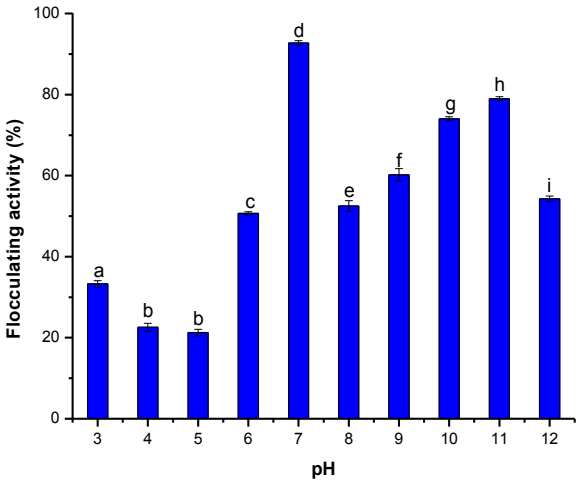
Fig. 3- Effect of various nitrogen sources on the flocculating activity produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$.

2.4.2. Effect of initial pH medium

The initial pH of the medium greatly plays a crucial role in influencing the production and flocculating activity of the bioflocculant. The effects of pH of the medium (at the range of 3-12 scale) on bioflocculant activity are shown in (Fig 4A-4C). The results showed that the optimal bioflocculant activity were observed at pH 7 and pH 8 in bioflocculant produced by *Streptomyces platensis* and *Terrabacter* sp. respectively. It is noteworthy that the basic medium supported the activity at an average flocculating activity of 55% compared to the weakly displayed activity at the acidic medium (Fig 4A & 4B). The poor flocculating activity at the acidic medium could be attributed to excessive concentration of hydrogen ions that alters the electric charge. This finding corroborates the report of Giri *et al.* (2015) and Gong *et al.* (2008) where optimum bioflocculant production by *Bacillus subtilis* F9 and *Serratia ficaria* was attained at neutral pH. As shown in Fig 4B, maximum flocculating efficiency was observed at pH 8. Wan and co-workers (2013) reported that *Solibacillus silvestris* was grown in pH range of 7-9 and optimum flocculating efficiency of micro algal cells was obtained at pH 8. The variations in flocculating activity could be as a result of changes in pH which is liable to affect the change in status of bioflocculant surface characteristics of the suspended particles (Zhang *et al.,* 1999). Electric charge of cells and oxidation-reduction potential which

may likely affects nutrients absorbtion and enzymatic reaction are determined by initial pH (Salehizadeh and Shojoasadati, 2001). This study demonstrates that acidic medium (pH 4 & 6) partially supported flocculating efficiency by the bioflocculant produced by *Arthrobacter humicola*, with the most pronounced activity observed in the basic medium (Fig 4C). This observation corroborates the fact that bioflocculants exhibit varying degree of electrical states at different pH which impact on the flocculating activity of the bioflocculant for kaolin particles (Pan *et al*., 2009). The best and most potent flocculating activity (91%) was observed at the highest pH scale value (pH 12) and this agrees with the report of Li *et al.* (2014) where bioflocculant produced by *Arthrobacter* sp. B4 attained its maximum flocculating activity at pH 12. In another study, the alkaline pH range of 7-12 favoured the bioflocculant produced by *Bacillus megaterium* and maximum yield of bioflocculant was obtained at pH 9, while it was inhibited in an acidic culture medium (Zheng *et al*., 2008).

(A)


Flocculating activity (%)
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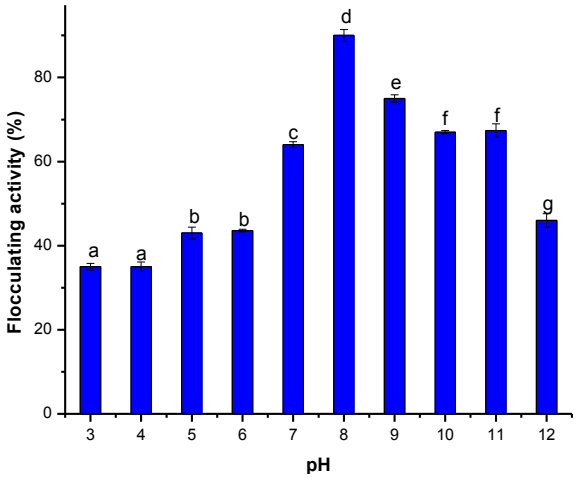
(B)


Flocculating activity (%)
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(C)

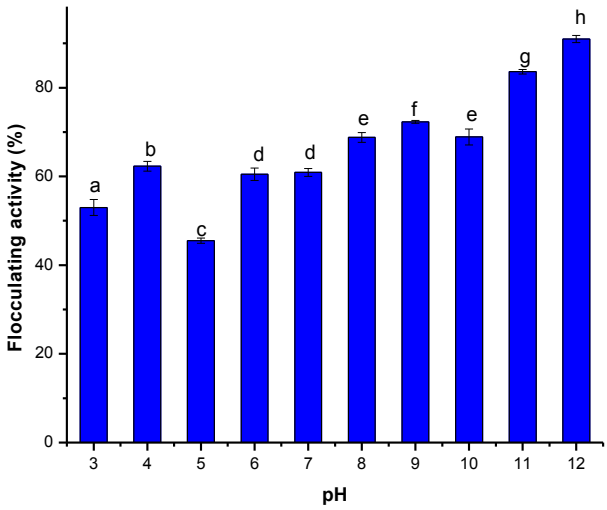
Flocculating activity (%)
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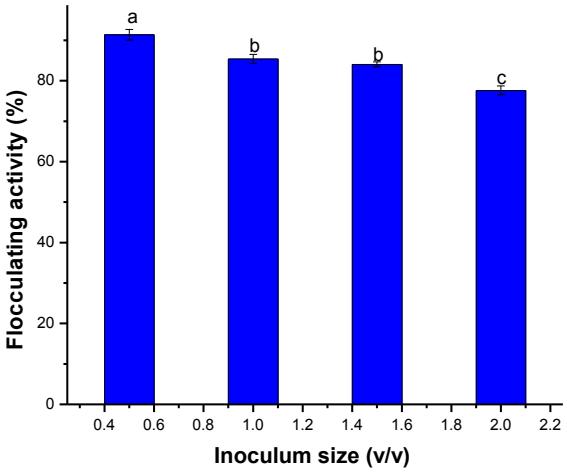
Fig. 4- Effect of pH on the flocculating activity produced by (A) *Streptomyces platensis*, (B) *Terrabacter*, and (C) *Arthrobacter humicola*. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$

2.4.3. Effect of Inoculum size

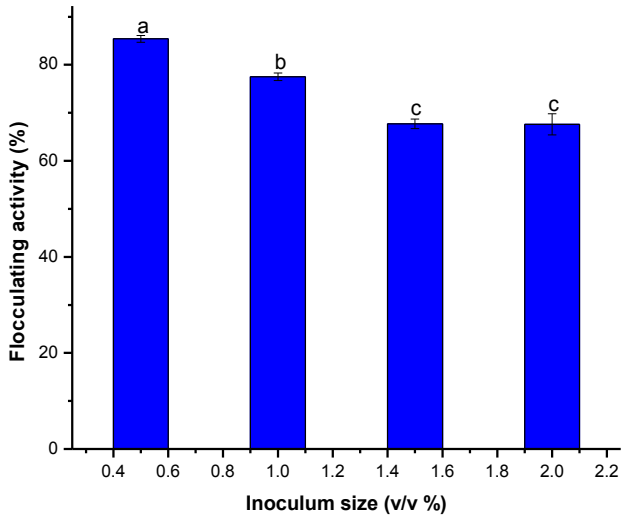
The data on the effect of inoculum size on bioflocculant activity by *Streptomyces platensis* and *Arthrobacter humicola* revealed that the optimum flocculating activity was attained at 1% (v/v) inoculum size. Further increase in the inoculum size led to a steady decrease in the flocculating activity (Fig 5A & 5B). The inoculum size is an important parameter that enhances better flocculating activity. Reports have demonstrated optimum and facilitated flocculating activity of the test organism when inocular size of 1% (v/v) are used and this is in agreement with the result of our finding in this study. Specifically, previous works (Yim *et al.,* 2007; Gong *et al.,* 2008 and Li *et al.,* 2009) have earlier implicated inoculum size of 1% (v/v) in facilitating maximum flocculating activity in bioflocculant produced by *Serratia ficaria*, *Bacillus licheniformis* and for the multi-microorganism consortia containing *Staphylococcus* sp. and *Pseudomonas* sp. On the other hand, highest flocculating activity was recorded when inoculum size of 2% (v/v) was used in the bioflocculant produced by *Terrabacter* sp. (Fig 5C) and this corroborates the report of Aljuboori *et al.* (2013) and Nwodo *et al*. (2013), where bioflocculant produced by *Aspergillus flavus* and *Brachybacterium* sp. attained their maximum flocculating activities at inoculum size of 2% (v/v). It has been established that

small inoculum sizes prolong the lag phase while large inoculum size will make niches of the strain to overlap, thereby hindering bioflocculant production. (Salehizadeh & Yan, 2014). This is well illustrated in the present study where the best and enhanced activity was observed at the lowest investigated inoculum size contrary to the relatively attenuated activity at the higher inoculum sizes. Our findings is contrary to the report of Ugbenyen *et al.* (2014), where inoculum size of 3% (v/v) supported the bioflocculant production of *Bacillus* sp.

(A)



(B)



(C)

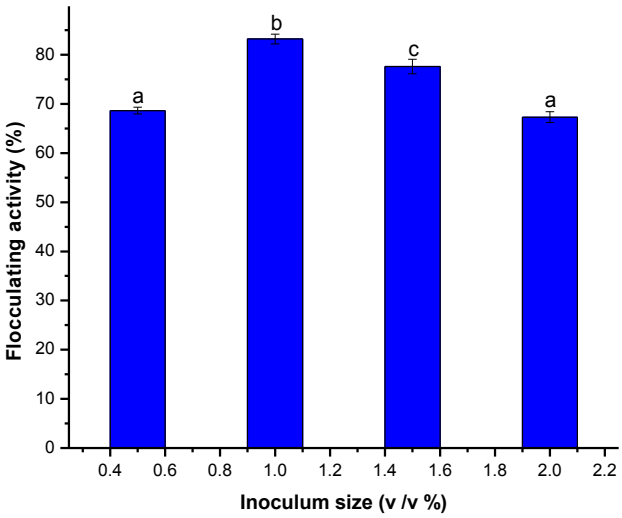


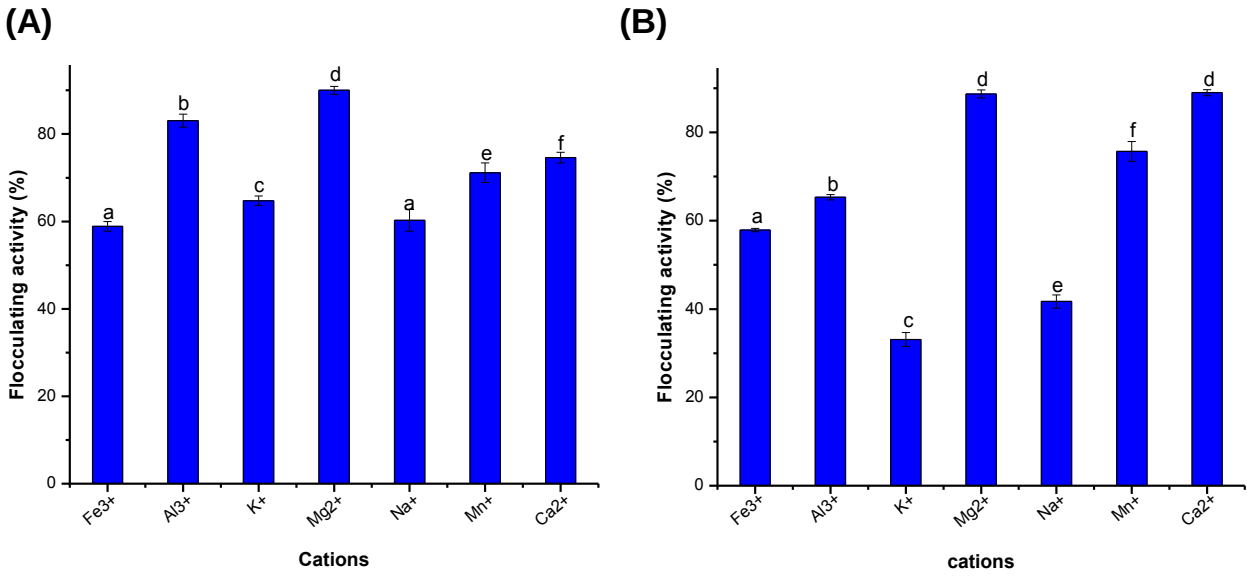
Fig. 5- Effect of inoculum size on the flocculating activity produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$.

2.4.4. Effect of metal ions

The results of the effect of metal ions on the flocculating activity of *Streptomyces platensis* are shown in Fig 6A. Of the cations investigated in this study, the divalent cations appeared to support bioflocculant activity with flocculating activity of over 74% (74.57-91.05%) and optimum activity of 90% observed when $MgCl_2$ was utilized. On the other hand, the monovalent ions partially support flocculating activity exceeding 64% (64.74-71.15%). Also, Al^{3+} as a representative of trivalent cation enhanced the flocculating activity at 83% while, Fe^{3+} resulted in a weak flocculating activity of 58% as shown in Fig 6A. Cations enhance the initial adsorption of bioflocculants on to the surface of the suspended particles, thereby minimizing the negative charge on the polymer and the particles (Zhang *et al.,* 2007). Yim *et al.* (2007) reported that contribution of metal ions to the flocculating activity of a bioflocculant could be due to

neutralizing and stabilizing the residual charge of functional groups and then forming bridges between particles. The weak flocculating activity of Fe^{3+} in the present study is consistent with its inhibitory role against the flocculating activity of the flocculant from the test organism. Our submission is in agreement with the report of Okaiyeto *et al.* (2016) and Ugbenyen *et al.* (2014) where the flocculating activity of bioflocculants produced by two *Bacillus* spp. were both inhibited by Fe^{3+}. Furthermore, the significantly higher value shown by the Mg^{2+} is not only suggestive of its neutralizing and stabilizing effect on the charge of functional groups of the flocculant but also consistent with previous assertion of Nwodo *et al.* (2012) where Mg^{2+} enhanced the flocculating activity of the bioflocculant produced by *Streptomyces* sp. Cations have played important roles in flocculation by neutralizing and stabilizing the negative charges of both functional groups of kaolin clay suspension and bioflocculant (Wu & Ye 2007). The data obtained with respect to the effect of cations on the bioflocculant produced by *Arthrobacter humicola* as shown in Fig 6B demonstrated that all the cations tested stimulated the flocculating activity of the bioflocculant produced against kaolin clay suspension with the exception of monovalent cations. Interestingly, the highest flocculating activity was observed with divalent cations (Ca^{2+} and Mg^{2+}) which agrees with the report of Kumar *et al.* (2004) where flocculating efficiency of bioflocculant produced by a haloalkaliphic *Bacillus* sp. was enhanced in the presence of Ca^{2+}, Cu^{2+} and Zn^{2+} which are representatives of divalent cations. It is noteworthy that the divalent cations appear to enhance the neutralization of negative charges on kaolin clay suspension and the bioflocculant thereby minimizing the gap in between and enhancing the adsorption of bioflocculant to the surface of the kaolin clay which led to agglomeration of flocs

development and better sedimentation of the kaolin clay (Zheng *et al.,* 2008). Although, the addition of cations to suspended particles is known to induce effective flocculating capabilities and their effect may also vary with flocculating activity of each bioflocculant (Wu & Ye 2007). For instance, metal ions have also been reported to inhibit flocculating activity or have no effect at all on the flocculating activity of bioflocculant (Zhang *et al.,* 2007). As shown in Fig 6C, Ca^{2+} resulted in optimum flocculation efficiency of 85% in bioflocculant produced by *Terrabacter* sp. Flocculation efficiency was inhibited below 50% when Na^{+}, Mg^{2+}, Mn^{+} and K^{+} were used. The poor flocculating activity observed when Na^{+} and K^{+} were used might be due to the fact that monovalent cations could be responsible for the formation of bonds that are loose in structure (Patil *et al.,* 2011). This finding is consistent with the report of Zhang *et al.* (2007), where the bioflocculant produced by *Proteus mirabilis* was enhanced with the addition of Ca^{2+} while, KCl resulted in little or no flocculating activity. In addition, bioflocculant produced by *Bacillus subtilis* was stimulated in the presence of Al^{3+} and Fe^{3+} (Wan *et al.,* 2013).



(C)

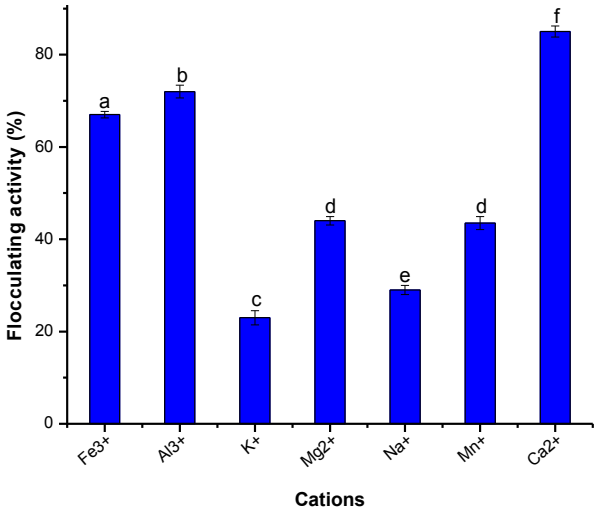


Fig.**6**- Effect of cations on the flocculating activity produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$

2.5. Conclusion

It was confirmed that Monotsha river and Sterkfontein dam are good site for actinomycetes producing bioflocculant. The positive bioflocculants attained optimum flocculating activity at different culture conditions and 16S rRNA confirmed the identities of the choice of the targeted organism. Optimum culture conditions for bioflocculant produced by *Streptomyces platensis* include 1% (v/v) inoculum size, glucose and peptone as carbon and nitrogen sources of choice at neutral pH 7. Furthermore, galactose, peptone, basic pH 12 and 1% (v/v) inoculum size supported the maximum flocculating activity of bioflocculant produced by *Arthrobacter humicola* while, glucose and ammonium sulfate as representatives of carbon and nitrogen sources supported

the bioflocculant produced by *Terrabacter* sp. at pH 8 and 2% (v/v) inoculum size. Having established the flocculating activities of the positive bioflocculants against kaolin clay suspension and the best culture conditions that favoured bioflocculant production, their purification and characterization were evaluated in the next chapter prior to their application in river and wastewaters treatment.

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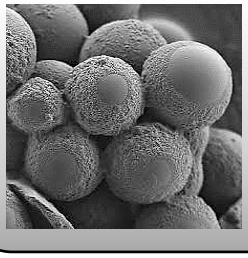


CHAPTER THREE

Characterization of the bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter sp.*

3.0. Abstract

The crude bioflocculants were purified to determine the yield of the bioflocculant. The functional group of the bioflocculants were investigated using Fourier transform infrared spectroscopy. This revealed that the main components of the bioflocculants were carboxyl, amino and hydroxyl group which enhance flocculating activity. Chemical analysis also confirmed the protein and carbohydrate composition of the bioflocculants. Thermal stability and pyrolysis profile of the bioflocculant confirmed the bioflocculant to be heat stable and suggested that the main backbone of the bioflocculant is a polysaccharide. Energy dispersive X-ray analysis detected the presence of C, O, N, S, P, Na and K as the elements that were present in the bioflocculant and scanning electron microscope was done to determine the morphological structure of the bioflocculant and it confirmed that flocculation was formed through bridging.

3.1. Introduction

Microbial flocculants can be defined as biopolymers which enhances particle to particle flocculation through the process of forming bridges which result in the aggregation and settling of suspended materials. The major components of bioflocculants include extracellular polymeric substances such as polysaccharides, proteins, glycoproteins or nucleic acids (Lazarova and Manem, 1995; Gao *et al*., 2006). For instance *Bacillus* sp. I-471 *Vagococcus* sp. W31 and *Halomonas* sp. produce polysaccharide bioflocculants (Mabinya *et al.,* 2011; Kumar *et al.,* 2004; Gao *et al.,* 2006), while *Nocardia amarae* YK-1, *Bacillus licheniformis* and *Rhodococcus erythropolis* produce protein bioflocculants. (Koizumi *et al.,* 1991; Shih *et al.,* 2001; Tadeka & Kurane 1991) and *Arcuadendron* sp. TS-4, *Arathrobacter* sp. as well as *Halobacillus* sp. produce glycoprotein bioflocculants

(Lee *et al.,* 1995; Wang *et al.,* 1995; Cosa *et al.,* 2012). Bioflocculants that possess polysaccharides and proteins in their structure are meant to facilitate cell-cell interactions or adherence of cells to surfaces (Zhang *et al.,* 2002). They are versatile set of materials and may act as new biomaterials that have probable applications in many segments of the economy (Jamil & Ahmed 2008). Availability of hydroxyl and carboxyl groups in the molecules of bioflocculants could stimulate hydrogen bonds formation which might contribute to the stability of bioflocculant. Furthermore, these functional groups and amino groups could act as a binding sites for cations which could facilitate flocculating activity by causing a bridge between the bioflocculant and the suspended kaolin suspension. Thus, they are crucial in the process of flocculation (Wang *et al.,* 2011; Comte *et al.,* 2006). The aim of this chapter is to purify and characterize the purified bioflocculants by investigating their functional groups, pyrolysis profile, elemental and chemical composition.

3.2. Materials and methods

3.2.1. Extraction and purification of bioflocculant

The purification was done according to the modified methods of Chen *et al.* (2002) and Piyo *et al.* (2011). After 72 h of fermentation (1 L of flask) using optimum conditions established in Chapter 2, the culture broth was centrifuged at 8000 g for 30 min at room temperature to remove bacteria cells. One volume of sterile distilled water was added to the supernatant phase and centrifuged at 8000 g for 15 min to remove insoluble substances. Thereafter, two volumes of ethanol was later added to the supernatant, stirred and left to stand for 12 h at 4 ^{o}C. The precipitate was vacuum-dried to obtain crude bioflocculant. The crude product obtained was dissolved in water to yield a

solution, to which one volume of a mixed solution of chloroform and n-butyl alcohol (5:2 v/v) was added. Subsequently, the mixture was stirred, poured into separating funnel and allowed to stand for 12 h at room temperature. Finally, the supernatant was discarded and two volumes of ethanol was added to recover the precipitate and then lyophilized to obtain a partially purified bioflocculant.

3.2.2. Characterization of purified bioflocculants

3.2.2.1. Chemical composition analyses

The total protein content of the partially purified bioflocculants were determined by Lowry's method using bovine serum albumin (BSA) as a standard. (Lowry *et al.,* 1951). Lowry solution, which is composed of A [2.85 g NaOH and 14.3 g Na_2CO_3], B [1.42 g $CuSO_4.5H_2O$] and C [2.85 g $C_4H_8Na_2O_8$] were prepared separately in distilled water and the solutions were mixed together in a ratio of 100:1:1 (v/v). BSA standard was prepared by weighing 0.05 g of BSA in 500 mL distilled water, stirred and final concentration of the stock was adjusted to 100 mg BSA $L.^{-1}$ The diluted solutions from the stock of BSA solution were used to prepare the standard curve. The samples were prepared in triplicates the same way the standard was done, thoroughly mixed with a vortex for 5 minutes and 0.5 mL was transferred into a glass tube. Thereafter, 0.7 mL of the Lowry solution was added, vortex and incubated for 20 min at room temperature in the dark. A volume of 0.1 mL of diluted Folin reagent was then added to each tube, vortex and incubated for 30 min at room temperature in the dark. The absorbance of the resulting mixture in each case for the sample and standard was measured at 750 nm.

The calibration curve from the absorbance reading of the standard was prepared and the protein content of the sample in mg BSA L^{-1} was calculated from the curve.

For the total sugar content of the purified bioflocculant, the phenol sulfuric method using glucose as a standard solution was adopted. (Dubois *et al.,* 1956). The working standard solution was prepared by dissolving 100 mg of glucose in 100 mL of distilled water and 10 mL was taken from the stock and further diluted to 100 mL. The standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL of the working standard into a series of test tubes and 0 serves as the blank. The volumes were made up to 1 mL in all tubes including the sample tubes by adding distilled water. A volume of 1 mL of phenol solution was added to each tube and 5 mL of 96% sulphuric acid was then added and shaken thoroughly. The contents in the tubes were later shaken after 10 min and placed in water bath at 25-30 ^{o}C for 20 min. Subsequently, the absorbance was spectrophotometrically measured at 490 nm and the amount of total carbohydrate present in the sample solution was calculated from the standard calibration curve.

3.2.2.2. Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of the purified bioflocculants were analyzed using Fourier Transform Infrared (FTIR) Spectrophotometer (Perkin Elmer System 2000, England). The bioflocculant was pulverized with potassium bromide salt at 25 ^{o}C and pressed into a pellet for FTIR spectroscopy at the frequency range of 4000-650 cm^{-1}

3.2.2.3. Thermo gravimetric analysis (TGA) and thermal stability

Thermo-gravimetric analyses of the purified bioflocculants were done using a thermo gravimetric analyzer (STA 449/C Jupiter, Netzsch, Germany Perkin Elmer TGA7, USA)

over a temperature range of 20 to 600 ^{o}C at a heating rate of 10 ^{o}C /min under a constant flow of nitrogen gas. Heat stability was evaluated by incubating the bioflocculant solutions in water bath at a temperature range of 50, 60, 70, 80, 90 and 100 ^{o}C for 25 min and the residual flocculating activity was determined using the protocol of Gong *et al*. (2008).

3.2.2.4. Scanning electron microscopy (SEM) and energy dispersive X-ray analysis

The purified bioflocculant samples were placed on a carbon coated stub and gold coated using Eiko IB3 ION coater. Afterwards, the scanning electron micrograph of the bioflocculant was obtained using a JEOL JSM-7800F FE-SEM with an Oxford SDD X-Max EDS System which was used to analyze the elemental composition of the purified bioflocculants.

3.3. Statistical analysis

Results were expressed as means ± standard deviation of three replicate determinations and were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range tests to determine significant differences in all the parameters using SPSS 16.0. Values were considered statistically significant at P-values of less than 0.05.

3.4. Results and discussion

3.4.1. Bioflocculant yield and chemical analysis

After 72 hr of fermentation under optimal culture conditions, 4.61 g of purified bioflocculant produced by *Streptomyces platensis* was obtained from 1 L of culture broth. The bioflocculant secreted by *Streptomyces platensis* contained polysaccharide (83%) and (4.6%) protein as its major constituents. With respect to bioflocculant yield of *Terrabcter* sp under optimal conditions, 2.1 g of pure purified bioflocculant was recovered from 1 litre of culture broth while 3.6 g of pure purified bioflocculant was recovered from the bioflocculant produced by *Arthrobacter humicola*. Chemical analysis of the bioflocculant confirmed the total sugar content of the bioflocculant produced by *Terrabacter* sp. and *Arthrobacter humicola* to be 71.6% and 82% respectively, suggesting that the main component of the biofloccualant was polysaccharide. On the other hand, there was no presence of protein in the bioflocculant structure of both tested organism. The higher yield of purified bioflocculants in this study suggested a higher productivity in bioflocculant produced by the test organism within a short period of time as compared with other strains that has been reported in literature. For instance, a bioflocculant yield of 1.33 g L^{-1} was reported after 3 days of cultivation for *Proteus mirabilis* (Xia *et al.,* 2008) while, 1.47 g L^{-1} was recovered from the bioflocculant produced by *Bacillus* sp. strain F19 (Zheng *et al.,* 2008).

3.4.2. Fourier Transform Infrared analyses

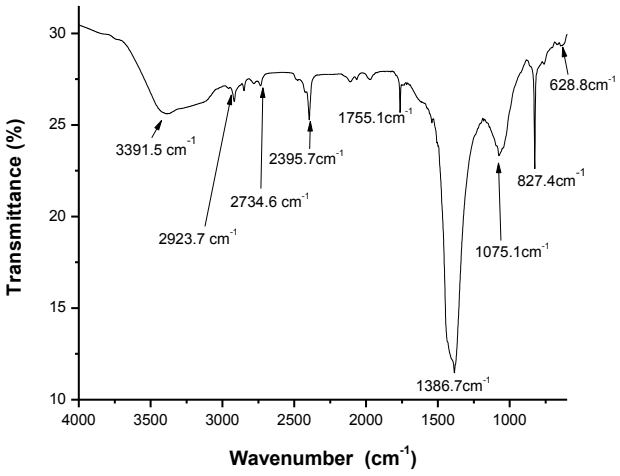
Peaks corresponding to different functional groups at stretching vibration range of 628.8 - 3417.4 cm^{-1} were evident from the FTIR spectrum of bioflocculant produced by *Streptomyces platensis* (Fig. 1A). The FTIR spectrum of the purified bioflocculant analyzed revealed the presence of different functional groups. While the band with the peak at 3417.4 cm^{-1} can be assigned to the stretching vibration of hydroxyl and amino

groups (Fujita *et al.* 2000). The vibration at 2923.7 cm^{-1}, 2734.6 cm^{-1} and 2395.7 cm^{-1} indicated aliphatic C-H bands. The peak at 1755.1 cm^{-1} is characteristics of C=O stretching vibration in carboxylic group and the intense peak at 1386.7 cm^{-1} is a representative of CN stretching group. The peaks at 827.4 cm^{-1} and 628.8 cm^{-1} are known to be characteristics of sugar derivatives and β- glycodisic bonds between sugar monomers (Xiong *et al.,* 2010). The presence of hydroxyl, amino and carboxyl group present in the IR spectrum of *Streptomyces platensis* might be responsible for the significant and excellently elicited flocculating activity in this study. Presented in Figure 1B is the result of the FTIR analysis of the bioflocculant produced by *Arthrobacter humicola*. The spectrum of the purified bioflocculant exhibited a band at 3365 cm^{-1} which is descriptive of a hydroxyl group. The band at 1420 cm^{-1} could be assigned to the symmetrical stretching in the carboxylate, indicating the presence of uronate in the purified bioflocculant (Okaiyeto *et al.,* 2014). The peak at 1073 cm^{-1} is characteristic of C-O groups suggesting the presence of carboxyl group in the purified bioflocculant (Ahmad *et al.,* 2013). Furthermore, the absorption bands at 954 and 816 cm^{-1} respectively are suggestive of sugar derivatives. Xiong *et al.* (2010) reported that the small absorption peak is associated with B-glycosidic linkages and sugar monomers. The occurrence of carboxyl and hydroxyl functional groups suggests adsorption positions for suspended particles and has been opined to be the best choice of functional groups for flocculation process (Daolun & Shinhong, 2008). Interestingly, the results of the biochemical analyses of the purified bioflocculants for carbohydrate and proteins revealed that it contained 82% polysaccharide with no detection of protein in the structure. This observation is not only remarkable but also confirms that the main

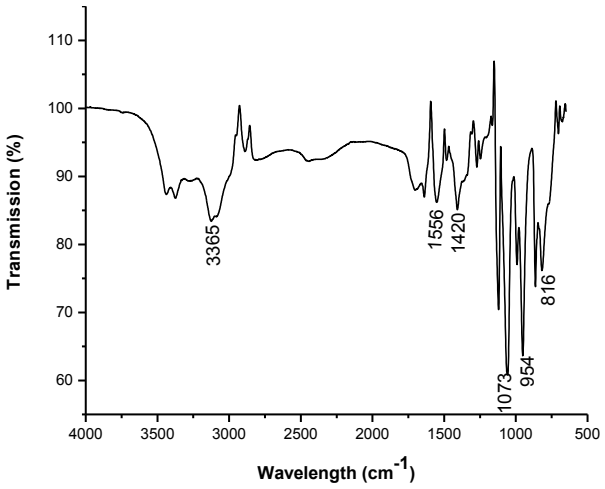
backbone of the purified bioflocculant structure is a polysaccharide, which is supportive of its excellently elicited flocculating activities in this study.

The Fourier Transformer Infrared Spectrogram of the purified bioflocculant produced by *Terrabacter* sp. exhibited many peaks from 4000 to 650 cm^{-1} (Fig. 1C). The broad intense peak at 3362 cm^{-1} suggest the presence of hydroxyl groups in the purified bioflocculant and the small band at 2454 cm^{-1} could be attributed to C-H stretching. The peak at 1646 cm^{-1} and 1552 cm^{-1} is characteristics of carbonyl group stretching in an amide group (Shriner *et al.,* 1998; Wang *et al*., 2011). The peak at 1058 cm-1 which corresponds to C-O stretching vibration in alcohols suggested the presence of hydroxyl group in the bioflocculant (Deng *et al.,* 2005). The peak at 833 cm^{-1} is characteristics of sugar derivatives. The FTIR analysis demonstrated that the presence of carboxyl, hydroxyl and the sugar derivative confirmed that the main component of the bioflocculant is a polysaccharide (Gong *et al*., 2008; Li *et al*., 2009).

(A)


Transmittance (%)
Wavenumber (cm-1)
3391.5 cm-1
2923.7 cm-1
2734.6 cm-1
2395.7cm-1
1755.1cm-1
1386.7cm-1
1075.1cm-1
827.4cm-1
628.8cm-1

(B)


Transmission (%)
Wavelength (cm-1)
3365
1556
1420
1073
954
816

(C)

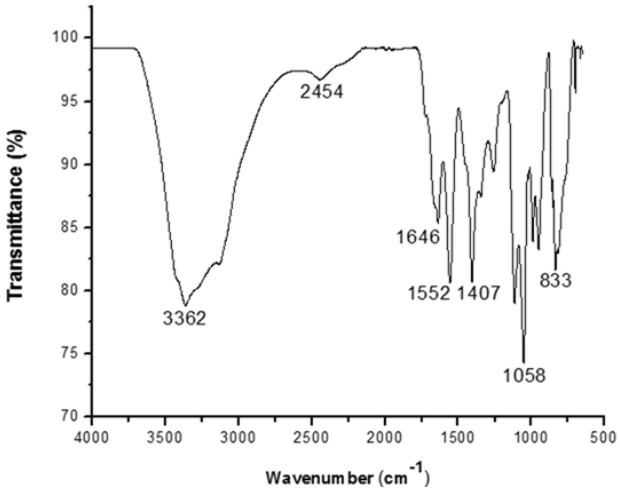

Transmittance (%)
Wavenumber (cm-1)
3362
2454
1646
1552
1407
1058
833

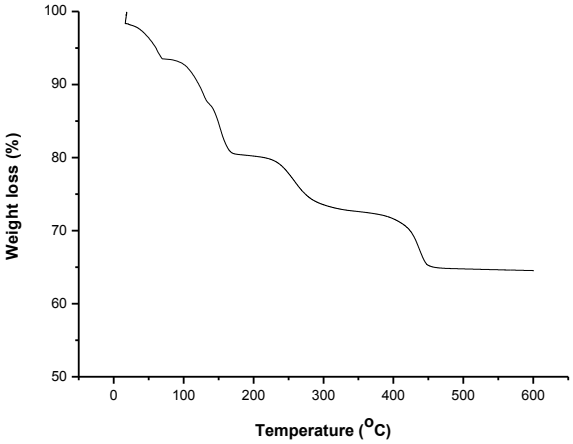
Fig. **1**- Fourier-transform infrared (FTIR) spectrogram of purified bioflocculant produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp.

3.4.3. Thermogravimetric analyses and thermal stability

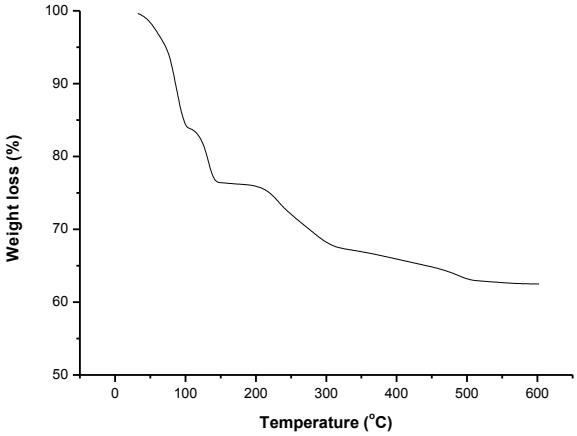
This analysis was performed to monitor the pyrolysis profile of the test bioflocculants (weight loss versus temperature). Fig 2A revealed that over 60% weight was retained following heat treatment on the purified bioflocculant produced by *Streptomyces platensis* at the highest treatment temperature (600 ^{o}C). This is a probable affirmation of the thermally stable nature of the bioflocculants under investigation. From the TGA analysis, while about 20% loss in weight in the temperature range of 20 to 150 ^{o}C was observed in the purified bioflocculant, there was a gradual decomposition of the material at temperature range of 150 ^{o}C to 450 ^{o}C. Over 60% weight was retained after heating the material at 600 ^{o}C (Fig. 2A). As shown in Fig 2B with respect to *Arthrobacter humicola*, there was a decomposition at 50 and 140 ^{o}C and about 77% weight was retained. The initial loss in weight could be attributed to moisture content of the bioflocculant. Furthermore, when the temperature was increased from 150 to 500 ^{o}C, there was a decrease of about 27% mass fraction which could occur as a release of volatile hydrocarbons from the heat decomposition of the polysaccharides in the purified bioflocculant (Yim *et al.,* 2007). However, further increase from 500 to 600 ^{o}C demonstrated that the purified bioflocculant weight was retained. Thus, affirming the main component of the material to be polysaccharide. This behavior is also observed in degradation that occurred when bioflocculant produced by *Terrabacter* sp. was subjected to heat as shown in Fig 2C. There was about 18% loss in weight when the temperature was increased from 50 ^{o}C to 120 ^{o}C. The loss in weight could be attributed

to the moisture content in the purified bioflocculant (Ugbenyen & Okoh, 2014). The moisture content was a result of the availability of carbonyl and hydroxyl group in the molecular structure of the purified bioflocculant (Kumar *et al.,* 2004). Moreover, there was a gradual decomposition of the material at 120 ^{o}C to 500 ^{o}C and about 35% loss was observed. When the temperature was increased from 500 ^{o}C to 600 ^{o}C, the bioflocculant was stable and about 65% weight was retained.

(A)


Weight loss (%)
100
90
80
70
60
50
0
100
200
300
400
500
600
Temperature (°C)

(B


Weight loss (%)
100
90
80
70
60
50
0
100
200
300
400
500
600
Temperature (°C)

(C)

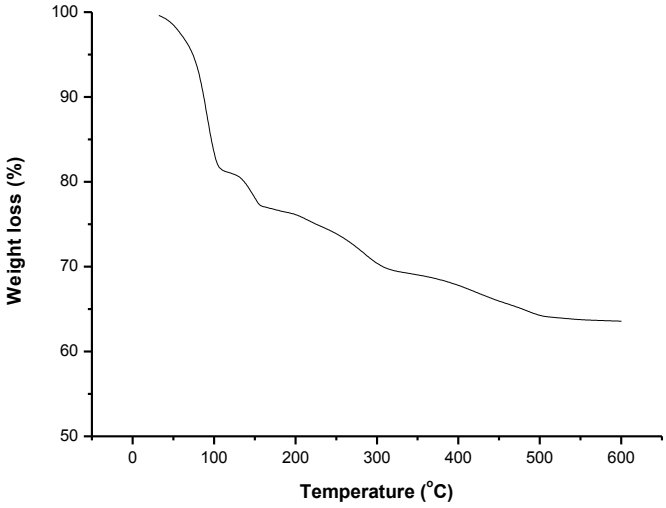
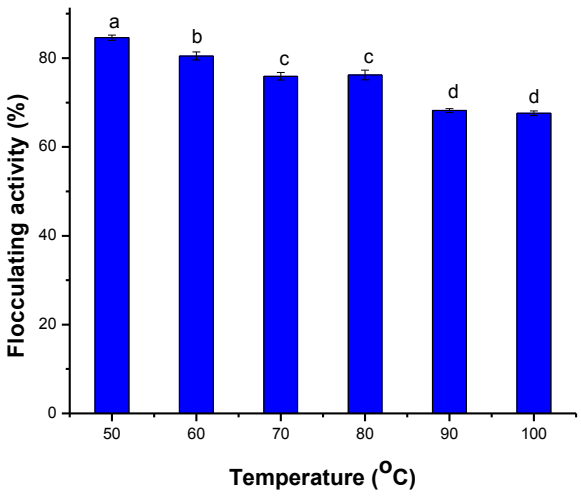

Weight loss (%)
100
90
80
70
60
50
0
100
200
300
400
500
600
Temperature (°C)

Fig. 2- Thermogravimetric analysis of purified bioflocculant produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp.

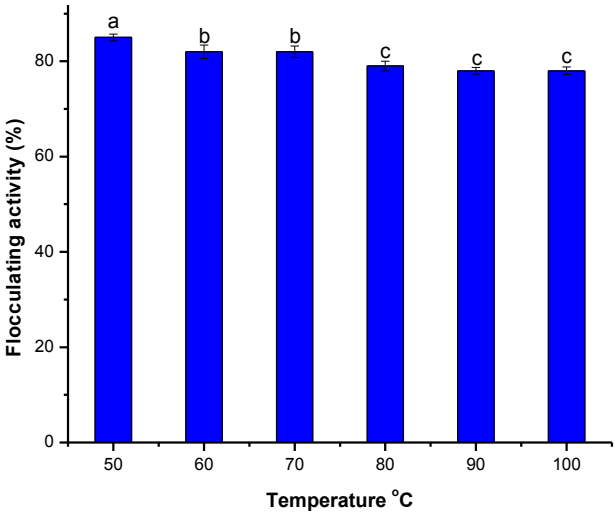
Thermal stability of the flocculating activity was determined to validate the stability profile of the bioflocculants. Although, a temperature-dependent decrease was observed following the heat treatment, the flocculating activity of the bioflocculant culture broth was maintained at around 70% over the entire treatment conditions (50^{o} – 100 ^{o}C) for 25 min (Fig 3A). The heat stability and high flocculating activity (70%) by the bioflocculant from *Streptomyces platensis* over the different treatment conditions could be attributed to the fact that the main backbone is a polysaccharide. Similar behaviour was observed in the case of *Arthrobacter humicola* where fluctuation in the flocculating activity over a temperature range of 50-100 ^{o}C was observed (Fig 3B). It is noteworthy that based on the structure of the bioflocculant, more than 78% flocculating activity was retained after heating the purified bioflocculant for 25 min at the highest temperature (100 ^{o}C). This finding corroborates the report of Giri *et al.* (2015) where the bioflocculant produced by *Bacillus subtilis* is composed mainly of polysaccharides. Also, consistent with our report, the consortium of *Cobetia* sp. and *Bacillus* sp. retained its residual flocculating activity of 87% after heating at 100 ^{o}C for 25 min (Ugbenyen & Okoh, 2014). Likewise, bioflocculant produced by *Aspergillus flavus* was thermostable, retaining 90% flocculating activity within the temperature range of 10-100 ^{o}C. Several studies have been reported that substantiate the thermal stability of different microbial flocculants (Gao *et al.,* 2009 ; Wang *et al.,* 2013). After heating the cultured bioflocculant produced by *Terrabacter* sp. at 50 ^{o}C to 70 ^{o}C for 25 minute as shown in Fig 3C, the bioflocculant

maintained its flocculating activity at over 80%. However, flocculating activity slightly decreased below 60% after heating the bioflocculant at 80 ^{o}C to 100 ^{o}C (Fig 3C). The slight decrease in flocculating activity at higher temperature exhibited by all the positive bioflocculants could be due to the breaking down of the polysaccharide chain which could result into slow formation of bridges with the kaolin particles. The heat stability displayed by the bioflocculant produced by *Terrabacter* sp. is consistent with the reported findings that flocculants rich in polysaccharide are more heat stable than those composed of mainly protein and nucleic acids (Gao *et al.,* 2009; Wang *et al.,* 2010).

(A)


Flocculating activity (%)
0
20
40
60
80
a
b
c
c
d
d
50
60
70
80
90
100
Temperature (°C)

(B)


Flocculating activity (%)
0
20
40
60
80
a
b
b
c
c
c
50
60
70
80
90
100
Temperature °C

(C)

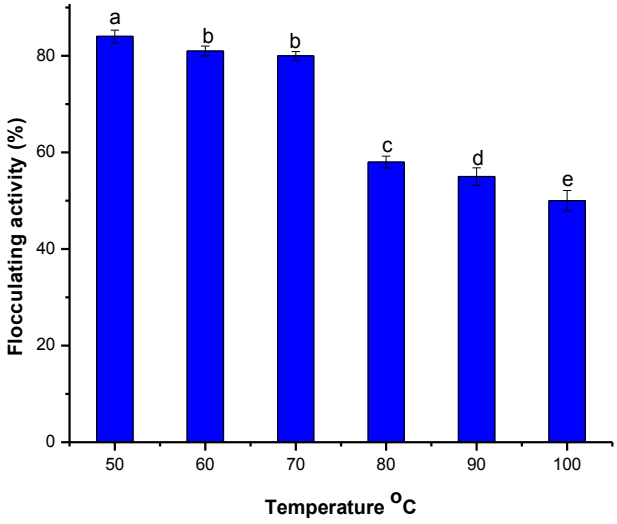

Flocculating activity (%)
0
20
40
60
80
a
b
b
c
d
e
50
60
70
80
90
100
Temperature °C

Fig. 3- Effect of temperature on the flocculating activity produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. Percentage flocculating activities with different alphabetic letters are significantly different $\mathbf{p} < 0.05$.

3.4.4. Scanning electron microscopy and energy dispersive X-ray analysis (EDX)

The surface morphology of the flocculated kaolin clay, partial purified bioflocculant and kaolin clay suspension were observed under SEM (Fig 4, 5, 6). The partial purified bioflocculant of *Streptomyces platensis* appeared as an agglomerate of oval shapes filled with scattered dot (Fig. 4A). Further elemental analysis by EDX indicated C – 21.41; O – 35.59; N- 0.62; S – 26.16, and P – 7.48% w/w as the major constituents of the purified bioflocculant. The elemental constituents revealed by the EDX analysis are further attestation of the confirmed chemical constituents of the purified bioflocculant to be rich in polysaccharides and proteins. Furthermore, the purified bioflocculant of *Arthrobacter humicola* appeared as whitish flakes (Fig. 5A) while *Terrabacter* sp. appeared as a rod like clumpy structure (Fig 6A). The elemental composition of bioflocculant produced by *Arthrobacter humicola* and *Terrabacter* sp. revealed they contain C- 13.9; O- 41.96; Na- 26.79; P-16.61, K-0.74 and C-15.7; K-1.2; O-51.2; S-0.1 and P-1.4 weight percentage respectively. In addition, the EDX analysis revealed that the bioflocculant produced by *Arthrobacter humicola* and *Terrabacter* sp. are rich in polysaccharides with no detection of protein in their structure. During the process of flocculation, kaolin clay adsorbed on to the binding sites of bioflocculant which resulted

into larger flocs formation and sedimentation of the kaolin particles as shown in Fig 4B, 5B, 6B (Wu & Ye 2007). Thus, suggesting that flocculation is achieved through bridging.

A **B** **C**

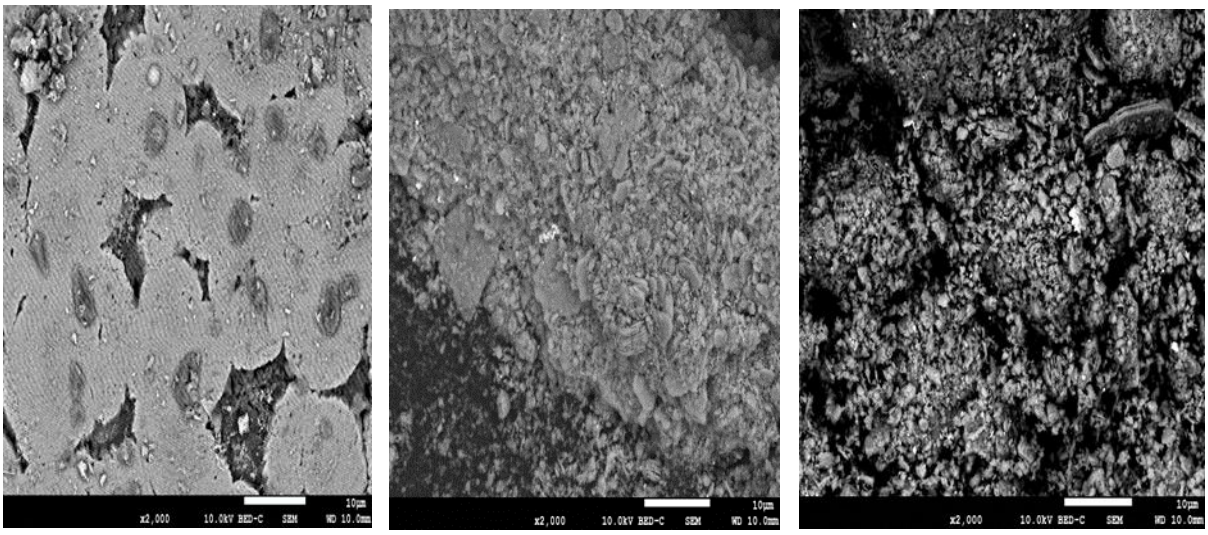


Fig. 4- Scanning electron micrographs of *Streptomyces platensis.* (A) Purified bioflocculant (B) Flocculated and (C) Kaolin clay suspension

A **B** **C**

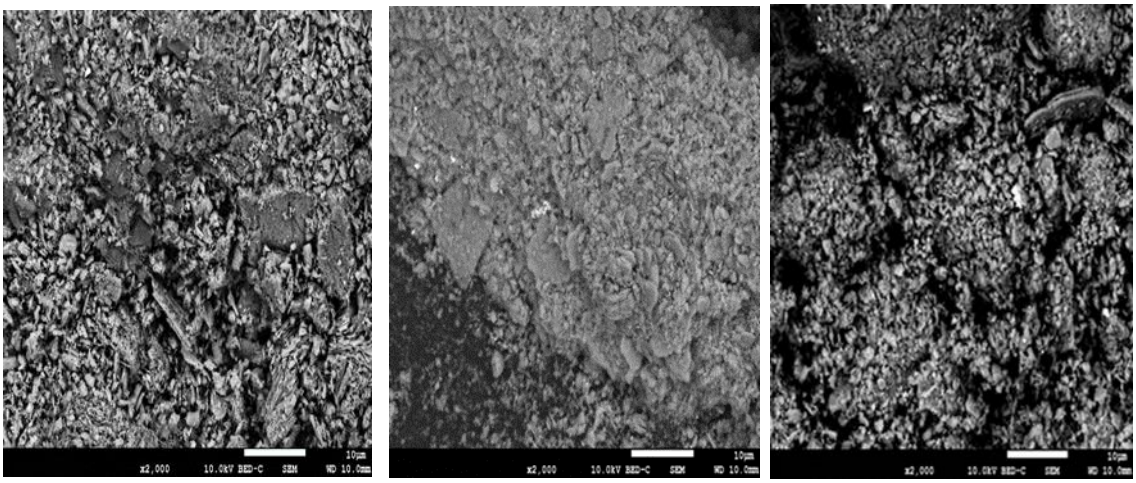


Fig. 5- Scanning electron micrographs of *Arthrobacter humicola.* (A) Purified bioflocculant (B) Flocculated and (C) Kaolin clay suspension

A **B** **C**

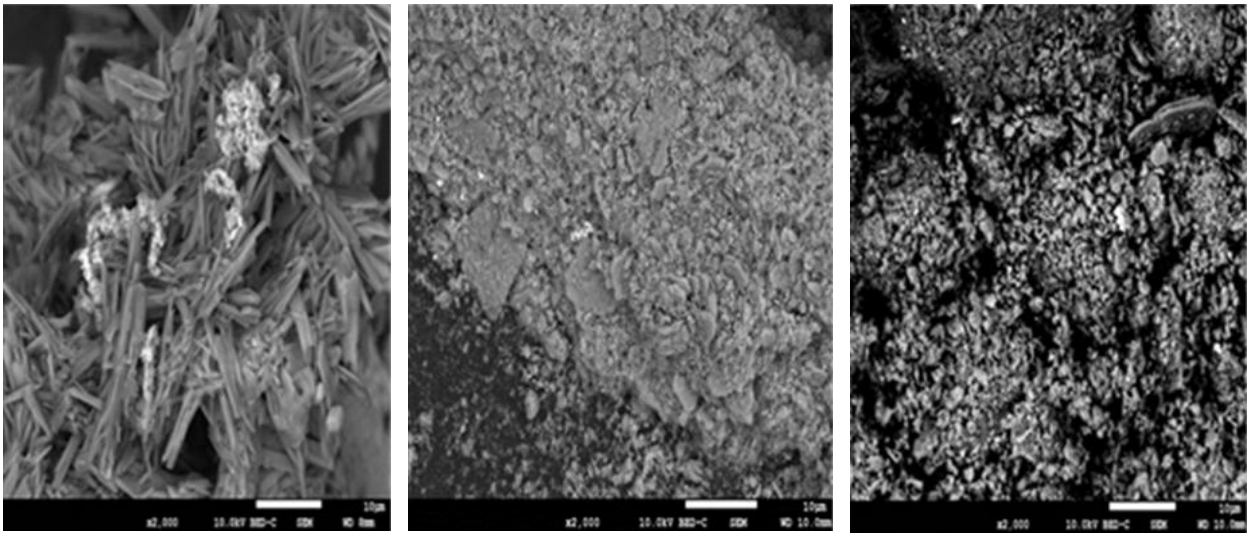


Fig. 6- Scanning electron micrographs of *Terrabacter sp.* (A) Purified bioflocculant (B) Flocculated and (C) Kaolin clay suspension

3.5. Conclusions

In conclusion, it was evident that the main backbone of bioflocculant produced by the test organisms were polysaccharide and it also confirm that flocculation was achieved through bridging. The characterization investigated on the purified bioflocculant produced by *Streptomyces platensis* revealed the stability of the bioflocculants and confirm the presence of carboxyl, hydroxyl and amino groups as the major functional groups which were responsible for facilitating the process of flocculation. In addition, EDX analysis confirmed the presence of sulphur and nitrogen in the bioflocculant produced and affirms the presence of amino group in the bioflocculant produced by *Streptomyces platensis*. On the other hand, EDX and chemical analysis revealed the absence of amino group in the bioflocculant produced by *Arthrobacter humicola* and *Terrabacter* sp. thus, confirming their main backbone as a polysaccharide.

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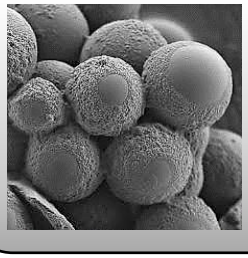


CHAPTER FOUR

Flocculating performance of the bioflocculant produced by *Arthrobacter humicola, Streptomyces platensis and Terrabacter sp.* in river and wastewater treatment

Part of this chapter has been published in *Brazilian journal of Microbiology and BMC Biotechnology*

4.0. Abstract

Microbial flocculants aids the aggregation of suspended particles and solutes in solutions, thus becoming an alternative approach to chemical flocculants which are associated with different health related problems in water and wastewater treatment. Spectrophotometer and BOD meter were used to measure the level of COD, BOD and nitrate removal in the river and wastewater samples. The potential of the bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in river and wastewaters treatment were evaluated. The result revealed that 0.2 mg/ml of the

purified bioflocculant produced by *Streptomyces platensis* was used to achieve maximum flocculating activities with respect to COD and turbidity removal in river and meat processing wastewater. Maximum flocculating activities were achieved by *Arthrobacter humicola* and *Terrabacter* sp at 0.8 mg/ml and 0.5 mg/ml in the treatment of the wastewaters used in this study. The purified bioflocculant were able to remove COD, BOD, nitrate, suspended solids and turbidity in sewage and dairy wastewater respectively. These purified bioflocculants exhibited better flocculating efficiencies when compared with the chemically synthesized and natural flocculants in removing turbidity, COD, BOD, nitrate and suspended solids from the wastewaters. The flocculating abilities exhibited by the bioflocculants in river and wastewater suggested there potentials as a replacement for chemical flocculants.

4.1. Introducion

The rate of increase in industrialization and human day to day activities has resulted in tremendous increase in the discharge of waste and wastewater containing organic and inorganic pollutants into the environment. Wastewaters containing heavy metals are directly or indirectly discharged into the environment and they have posed a serious threat to public health. Cadmium, lead and mercury are the most dangerous metals and they are known as toxic- trio metals. Murthy and co-workers (2013) reported that toxic

metals can adversely affect physiological function by blocking the active sites of enzymes, displacing the major metal ions and altering the conformation of proteins. Coagulation-flocculation technology is widely used in the treatment of wastewaters, especially in the removal of suspended solids, particles and debris (Ghosh *et al.,* 2009; Zhang *et al.,* 2012b). Although, chemical flocculants including polyaluminium chloride, polyethyleneime, aluminium sulfate and ferric chloride have been used in water treatment, they are however not degradable and therefore not environmentally friendly. In addition, aluminium salts and polyacrylamide derivatives have been reported to be associated with health problems such as Alzheimers disease (Arezoo, 2002; Salehizadeh & Shojoasadati, 2002). Therefore, using the synthetic organic flocculants could aggravate environmental and health concerns. Hence, the development and use of safe, biodegradable flocculants have become a necessity. Interestingly, literature has revealed algae, fungi, bacteria and actinomycetes as microbial flocculants that have been used as substitutes for chemical flocculants in wastewater treatment (Cosa & Okoh, 2014). Such bioflocculants have attracted wide attention due to their biodegradability and safety in wastewater treatment. They have been used in the treatment of dye solutions, inorganic solid suspensions, downstream processing, meat processing wastewater and removal of heavy metals (Deng *et al.,* 2005; Gong *et al.,* 2008; Salehizadeh & Shojoasadati, 2003). Although certain strains of actinomycetes have been implicated in flocculation, their industrial application is yet to be explored. As a result, there is the need to explore more actinomycetes strains with enhanced flocculation efficiency to serve as a replacement or substitute to chemical synthesized flocculants and establish its toxicological effect before application. There is dearth of

information in literature on the application of *Streptomyces platensis*, *Arthrobacter humicola* and *Terrabacter* sp. in the treatment of meat processing wastewater, dairy wastewater, brewery wastewater, sewage wastewater and river water respectively.

In this chapter, we reported on the biotechnological importance of the bioflocculant produced by the positive actinomycetes in wastewater treatment and heavy metal removal.

4.2. Materials and methods

4.2.1. Source of river and wastewaters tested with positive bioflocculants

The wastewater investigated in this study includes: dairy, brewery, sewage, meat processing wastewaters and river water. Dairy and sewage wastewaters were collected aseptically from dairy factory, Harritsmith and wastewater treatment plant, Puthaditjabha in Eastern Free State Province of South Africa respectively. In addition, meat processing wastewater was collected from an Industrial area situated on the Gauteng East Rand and the river water was collected from Wilge River in the Eastern Free State of South Africa. Each of the bioflocculant was selected under optimum conditions to treat all the wastewater used in this study.

4.2.2. Jar test determination of bioflocculant dosage.

The optimum dose of the purified bioflocculants (obtained as described in Chapter 2) needed for clarification of kaolin clay suspension and wastewater were determined using the Jar test experiment following a standard protocol. Different concentrations of the powdered purified bioflocculant ranging from 0.1 to 1.0 mg/mL were prepared and

their flocculating activities were measured. The standard procedure includes rapid mixing at 160 rpm for 2 min, followed by gradual flocculation period of 40 rpm for 2 min and sedimentation for 5 min. After sedimentation, 2 mL was gently withdrawn from the upper clarifying phase in order to measure the flocculating activity (Lee *et al.,* 2001; Wang *et al.,* 2010).

4.2.3. Comparism of traditional flocculants and the purified bioflocculant

The flocculating efficiencies of polyacrylamide, polyethylenime, alum, ferric chloride and the test bioflocculant were investigated according to the protocol of Ugbenyen & Okoh, (2014). In order to compare the coagulation performance of synthesized flocculants and the purified bioflocculant, each flocculant was prepared at different concentrations with the addition of cations that stimulates flocculation processes and tested against kaolin suspension and wastewater using the jar test. The control experiment was prepared in the same way but the flocculants (chemically synthesized or bioflocculant) was replaced with distilled water. The residual flocculating activity were measured as earlier discussed in section 2.2.4.

4.2.4. Flocculation of meat processing wastewater and river water by bioflocculant produced by *Streptomyces platensis*

The meat processing wastewater was collected from a holding tank at Industrial area situated on the Gauteng East Rand and the river water was collected from Wilge River in the Eastern Free State of South Africa. The meat processing wastewater and river water were collected aseptically using sterile 1 L glass bottle, labelled correctly and transported into the laboratory for processing using cooler box containing ice packs.

Samples were processed immediately on arrival in the laboratory. Optimum dose of 0.2 mg/mL of the purified bioflocculant and 3 mL of 1% $MgCl_2$ were added into 100 mL of river water and meat processing wastewater. The mixture was agitated at 160 rpm for 2 min using Jar test at room temperature and the speed was later reduced to 40 rpm for 2 min to facilitate floc formation. Afterwards, it was allowed to settle for 5 min and the supernatant was gently taken for analysis. The residual COD and turbidity for each sample were determined according to the method of Gong and co-workers (2008). To assay for chemical oxygen demand (COD), 2 mL of raw and treated samples were added into COD vials (150 mgL^{-1}). For the blank, 2 mL of sterile distilled water was added into the COD vial. The caps were tightly closed, rinsed with water and finally wipe with a clean paper towel. The DRB 200 digester was pre heated to 150 ^{o}C before inserting the prepared COD vials in the digester. The vials were gently inverted to mix, inserted into the digester and heated for 2 h. The COD vials were removed after digesting and allowed to cool down at room temperature. After cooling, the samples were measured using a spectrophotometer (DR 3800) at the wavelength of 620 nm. The turbidity of the river water and meat processing wastewater were measured using turbidimeter (HACH, USA). The removal efficiency was calculated as follows:

$$RE = \left[\frac{A_o - A}{A_o}\right] \times 100$$

Where A_0 is the initial value and A is the value after the flocculation treatment.

4.2.5. Flocculation of sewage and dairy wastewater by bioflocculants produced by *Arthrobacter humicola* and *Terrabacter* sp.

The sewage wastewater collected from the wastewater treatment plant, Phuthaditjhaba, Eastern Free State Province, South Africa, was used to validate the flocculating efficiency of *Arthrobacter humicola*. Different physiological parameters like pH, suspended solids, biological oxygen demand (BOD), turbidity, chemical oxygen demand (COD) and nitrate of the water sample were measured before and after treatment. Optimum dose of 0.8 mg/mL of the purified bioflocculant and 3 mL of 1% $CaCl_2$ were added into 100 mL of sewage wastewater. Also, dairy wastewater was collected from dairy factory at Harrismith, Eastern Free State Province of South Africa. A dose of 0.5 mg/ml of purified bioflocculant produced by *Terrabacter* sp. and 3 ml of 1% $CaCl_2$ was added to 100 ml of the dairy wastewater. A six padded stirrer was used to mix the suspension at 160 rpm for 2 min, and them at 40 rpm for 2 min. The treated samples were left to settle for 5 min and the supernatant was used for further physiological analyses. For the sake of comparison, chemically synthesized flocculants (polyethylenimine and polyacrylamide) and naturally occurring flocculant (alum) were also tested. This procedure was conducted in triplicates. The turbidity, suspended solids, COD and nitrate were measured using spectrophotometer DR 3800 and turbidimiter (HACH, USA). To assay for suspended solids, the contents were blended at 200 rpm for 1 min followed by blending at 60 rpm for another 5 min. (Patil *et al.,* 2011). The removal efficiencies were thereafter calculated as:

$$RE = \left[\frac{A_o - A}{A_o}\right] \times 100$$

Where Ao and A are the initial and final values obtained before and after treatment respectively.

To assay for the BOD, 25 mL of raw and 50 mL of treated wastewater samples were added into BOD bottle and the bottles were filled up with BOD buffer (2.25% $MgSO_4.7H_2O$, 2.75% $CaCl_2$, 0.025% $FeCl_3.6H_2O$ and phosphate buffer solution). The BOD buffer was used as the initial working solution. The bottles were incubated at 20 ^oC for 5 days. The initial and final dissolved oxygen (DO) were measured after 15 min and 5 days respectively using a HI5421 BOD Meter (Hanna, USA). The BOD and the percentage BOD removal efficiency were subsequently estimated using the equations:

$$BOD = \frac{D_1 - D_2}{P}$$

D_1 = DO in diluted specimen after preparation

D_2 = DO after 5 days

P = decimal fraction of specimen used

$$RE(\%) = \frac{B_1 - B_2}{B_1} \times 100$$

B_1 = Untreated sample

B_2 = Treated sample

4.2.6. Assay for removal of heavy metals by *Terrabacter* sp.

One hundred ml of dairy wastewater was treated with a bioflocculant dosage of 0.5 mg/ml and 3 ml of 1% $CaCl_2$. The treated and untreated samples were analyzed at The Institute for Ground Water Studies, University of the Free State, South Africa, using Inductible Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Prodigy-7

Teledyne Leeman, USA) to validate the efficiency of the bioflocculant in removing suspected metals present in the samples

4.3. Statistical analysis

Results were expressed as means ± standard deviation of three replicate determinations and were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range tests to determine significant differences in all the parameters using SPSS 16.0. Values were considered statistically significant at P-values of less than 0.05.

4.4. Results and discussion

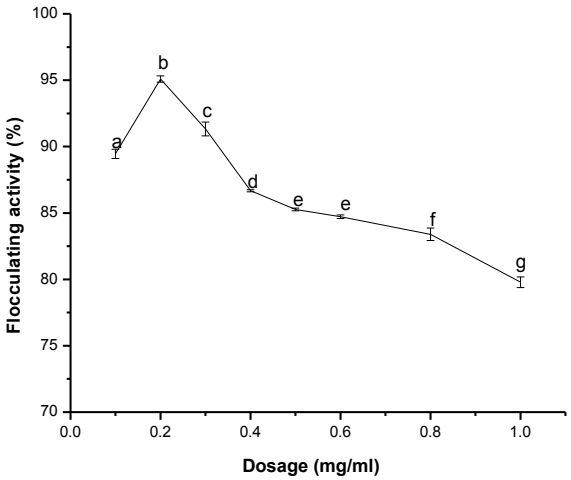
4.4.1. Effect of bioflocculant dosage produced by *Streptomyces platensis, Arthrobacter humicola and Terrabacter* sp. on kaolin clay suspension.

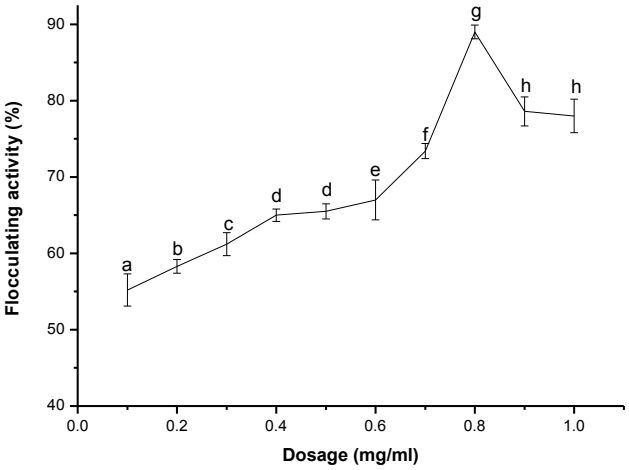
Optimum flocculating activity of 94.88% was recorded when 0.2 mg/mL of bioflocculant dosage produced by *Streptomyces platensis* was used to flocculate kaolin clay as shown in Fig 1A. In contrast, further increase in dosing with the bioflocculant from 0.3 mg/mL to 1.0 mg/mL resulted in decreased removal of turbidity and flocculating activity. Hence, the choice of concentration used in flocculating both the river and meat processing waste water for subsequent experiment. Optimization of coagulant dosage in the treatment of water is important in order to achieve maximum turbidity removal and flocculating efficiency at a cheaper cost. In this study, the enhanced flocculating activity at 0.2 mg/mL bioflocculant dosage suggests that the initial dose of 0.1 mg/mL used may be considered insufficient and ultimately caused incomplete and inefficient coagulation. Although, this report is contrary to the findings of Cosa *et al.* (2013) and Wang *et al.*

(2011) where optimum dosage employed to achieve maximum flocculation was 0.1 mg/mL and 12 mg/mL respectively. The data obtained with respect to the flocculating activity of the purified bioflocculant produced by *Arthrobacter humicola* is shown in Fig 1B. The flocculating efficiency was a bit weaker at lower concentrations (0.1-0.7 mg/mL). It is however noteworthy that the maximum flocculating efficiency (89%) was observed at 0.8 mg/mL dose and this was choose as the best (optimal) concentration for the successive assays. Further increase in bioflocculant dosage resulted in a decline in flocculating activity. Insufficient dosage of bioflocculant will hinder or affect bridging mechanism formation of flocs and over dosage will result into high viscosity formation which will inhibit sedimentation of suspended particles by restabilising the kaolin particles. Hence, establishing the optimum bioflocculant dosage is an important parameter in flocculation. Our assertion in this study on the effect of dosage on flocculation is not only significant but also agrees with the report of Ugbenyen and Okoh (2014), where the bioflocculant produced by the consortium of *Cobetia* spp and *Bacillus* sp attained its optimum flocculating activity of 90% at concentration dosage of 0.8 mg/mL. In contrast to the present study, the bioflocculant produced by *C. daeguense* was more than 90% in the range of 0.3-8.2 mg/L and the maximum value achieved 96.9% at bioflocculant dosage of 1.2 mg/L at optimal pH 5.6 and temperature of 15 ^{o}C (Liu & Chen, 2010). Furthermore, it was shown in Fig 1C that bioflocculant concentration of 0.1 – 0.4 mg/ml resulted in flocculating activity of over 75% in the bioflocculant produced by *Terrabacter* sp. As the dosage was increased to 0.5 mg/ml, optimum flocculating activity of over 85% was observed. Further increase in the bioflocculant concentration resulted in a decrease in flocculating activity, probably due

to obstruction of the site of adsorption which minimises flocculation and formation of flocs (Zulkeflee *et al.,* 2012; Gong *et al.,* 2008; Liu & Chen, 2010). This variation in dosage could be as a result of charge reversal and destabilization of colloidal particles (Patel & Vashi, 2013). Overdosing or insufficient dose would lead to poor performance of coagulant during the process of flocculation. The use of correct dosage in flocculation is an important parameter in determining the optimum conditions for performance of flocculants in coagulation-flocculation technology. Hence, validating and establishing the optimum dose needed at a particular time in treatment of potable water and wastewater in order to reduce cost of production, minimize the risk of overdosage in human beings and minimize formation of sludge in water treatment is imperative (Patil *et al.,* 2011).

(A) **(B)**


Flocculating activity (%)
Dosage (mg/ml)
a
b
c
d
e
e
f
g


Flocculating activity (%)
Dosage (mg/ml)
a
b
c
d
d
e
f
g
h
h

(C)

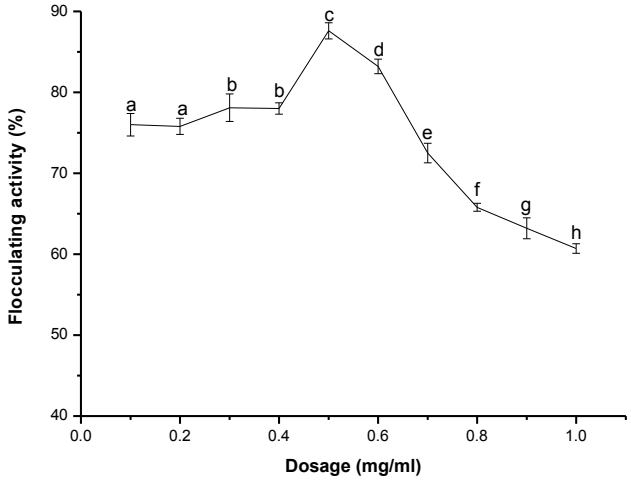

Flocculating activity (%)
Dosage (mg/ml)
a
a
b
b
c
d
e
f
g
h

Fig 1. Effect of bioflocculant dosage produced by (A) *Streptomyces platensis*, (B) *Arthrobacter humicola*, and (C) *Terrabacter* sp. on kaolin clay suspension. Percentage flocculating activities with different alphabetic letters are significantly different ($p<0.05$).

4.4.2. Choice of selected positive bioflocculants against each waste water treatment

As shown in Table 1-5, Result showed that each organism appeared to be waste water specific. In other words, each positive bioflocculants has affinity for different waste water treatment and the bioflocculant that exhibited the highest flocculating activity was choosen. For instance, *Streptomyces platensis* was effective against meat processing waste water and river water with highest flocculating activities of 82 and 91.4% respectively (Table 2 & 4). Likewise, *Arthrobacter humicola* could flocculate sewage wastewater at flocculating efficiency of 89.4% compare to other selected bioflocculant (Table 3) while, *Terrabacter* sp. could flocculate dairy waste water and remove some heavy metals from wastewater (Table 1). However, none of the positive bioflocculants could flocculate and remove COD, BOD, turbidity, nitrate and suspended solids in brewery waste water (Table 5).

Table 1: Screening of bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in dairy wastewater treatment

	(%) flocculating activity		
Bioflocculant dosage (mg/ml)	***Streptomyces platensis***	***Arthrobacter humicola***	***Terrabacter* sp**
0.1	33.4 ± 0.9	-	61.0 ± 2.3
0.2	38.0 ± 1.2	-	61.7 ± 0.9
0.3	37.8 ± 0.4	-	63.0 ± 0.7
0.4	32.5 ± 2.7	41.0 ± 2.8	69.8 ± 0.2
0.5	21.3 ± 1.8	43.8 ± 1.9	**85.1 ± 0.1**
0.6	15.8 ± 1.4	44.8 ± 0.7	79.4 ± 0.1
0.7	15.1 ± 0.1	49.2 ± 1.4	74.0 ± 0.9
0.8	-	36.5 ± 0.8	73.5 ± 1.6
0.9	-	35.7 ± 1.1	71.7 ± 0.7
1.0	-	35.1 ± 0.6	70.8 ± 1.9

(-) denotes no flocculating activity

Table 2: Screening of bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in meat processing wastewater treatment

	(%) flocculating activity		
Bioflocculant dosage (mg/ml)	***Streptomyces platensis***	***Arthrobacter humicola***	***Terrabacter* sp**
0.1	79.0 ± 0.5	-	-
0.2	**82.0 ± 0.1**	-	-
0.3	80.8 ± 0.1	-	-
0.4	77.1 ± 0.9	-	-
0.5	77.6 ± 1.6	-	-
0.6	70.1 ± 2.3	-	-
0.7	68.5 ± 1.3	-	-
0.8	67.1 ± 2.9	-	-
0.9	58.3 ± 0.2	-	-
1.0	58.1 ± 0.8	-	-

(-) denotes no flocculating activity

Table 3: Screening of bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in sewage wastewater treatment

	(%) flocculating activity		
Bioflocculant dosage (mg/ml)	***Streptomyces platensis***	***Arthrobacter humicola***	***Terrabacter* sp**
0.1	18.7 ± 1.5	71.6 ± 2.1	-
0.2	18.5 ± 1.1	72.1 ± 1.3	-
0.3	18.6 ± 0.9	72.0 ± 0.5	-
0.4	22.1 ± 0.2	77.8 ± 0.3	-
0.5	22.4 ± 1.8	77.9 ± 0.8	-
0.6	23.6 ± 0.6	83.2 ± 1.2	-
0.7	25.0 ± 1.4	88.7 ± 0.9	-
0.8	24.9 ± 2.4	**89.4 ± 1.3**	-
0.9	26.1 ± 0.6	88.3 ± 2.6	-
1.0	26.6 ± 1.2	86.5 ± 1.9	-

(-) denotes no flocculating activity

Table 4: Screening of bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in river water treatment

	(%) flocculating activity		
Bioflocculant dosage (mg/ml)	***Streptomyces platensis***	***Arthrobacter humicola***	***Terrabacter* sp**
0.1	89.9 ± 0.2	58.3 ± 1.0	18.1 ± 1.5
0.2	**91.4 ± 1.3**	59.7 ± 0.6	22.6 ± 1.9
0.3	90.9 ± 0.1	61.3 ± 1.3	22.8 ± 0.2
0.4	84.2 ± 1.8	60.5 ± 2.1	24.2 ± 2.9
0.5	83.7 ± 2.1	61.9 ± 1.0	17.5 ± 0.3
0.6	82.1 ± 1.9	66.2 ± 0.8	17.1 ± 1.6
0.7	82.0 ± 1.1	48.7 ± 2.3	15.9 ± 0.8
0.8	76.3 ± 2.0	44.1 ± 0.1	08.3 ± 2.4
0.9	75.6 ± 0.1	38.9 ± 1.0	0.76 ± 1.6
1.0	75.1 ± 0.7	33.2 ± 2.6	0.77 ± 1.3

(-) denotes no flocculating activity

Table 5: Screening of bioflocculant produced by *Streptomyces platensis, Arthrobacter humicola* and *Terrabacter* sp. in brewery wastewater treatment

	(%) flocculating activity		
Bioflocculant dosage (mg/ml)	***Streptomyces platensis***	***Arthrobacter humicola***	***Terrabacter* sp**
0.1	-	-	-
0.2	-	-	-
0.3	-	-	-
0.4	-	-	-
0.5	-	-	-
0.6	-	-	-
0.7	-	-	-
0.8	-	-	-
0.9	-	-	-
1.0	-	-	-

(-) denotes no flocculating activity

4.4.3. Comparison of efficiency of bioflocculant produced by *Streptomyces platensis* and chemical flocculants

The flocculation efficiency of conventional flocculants (polyaluminium chloride, polyethylemine, ferric chloride and alum) and the purified bioflocculant against kaolin clay suspension at a varying concentrations (0.1 – 1.0 mg/mL) is presented in Table 6. The results revealed that the purified bioflocculant was significantly efficient at the optimum concentration (0.2 mg/mL) when compared with 0.3, 0.8 and 1.0 mg/mL for polyethylemine, alum and ferric chloride, respectively. Polyacrylamide as an inorganic flocculant was able to flocculate kaolin clay suspension at an optimum concentration of

0.1 mg/mL and there was no significant difference ($p>0.05$) in its flocculating activity when compared to the test bioflocculant Table 6. The relatively good and significant flocculating efficiency of the purified bioflocculant against kaolin clay suspension and waste water treatment in this study may be ascribed to its inherent constituents and thermally stable nature. Consequent upon this, it may be inferred that the *Streptomyces platensis* bioflocculant may serve as a good replacement for conventional flocculants in wastewater treatment and fermentation industries.

Table 6: **Comparison of efficiency of bioflocculant produced by *Streptomyces platensis* and chemical flocculants on kaolin clay suspension**

Flocculant	Dosage (mg/mL)	Flocculating activity (%)
Bioflocculant	0.2	94.88 ± 0.24^{a}
Polyethylimine	0.3	86.95 ± 0.28^{b}
Alum	0.8	77.13 ± 2.40^{c}
Polyacrylamide	0.1	95.02 ± 0.21^{a}
Ferric chloride	1.0	41.89 ± 0.10^{d}

Percentage flocculating activities with different alphabetic letters are significantly different ($P<0.05$).

4.4.4. Application of bioflocculant produced by *Streptomyces platensis* in the treatment of river water and wastewater

The physico-chemical properties of untreated river and meat processing wastewater are shown in Table 7. Table 8 presents the flocculating efficiency of the purified bioflocculant on river and wastewater. It was observed that the bioflocculant could flocculate the river water better than the wastewater with efficiency, COD removal, and

turbidity removal values of 91.4%, 63.1% and 84%, respectively when compared with 82%, 46.6%, and 75.6% for the wastewater at 0.2 mg/mL. However, the bioflocculant could remove suspended solids in meat processing wastewater better than river water at percentage removal of 72.8% and 60.2% respectively. The overall significant bioflocculating efficiencies exhibited in this study have demonstrated that the bioflocculant from *S. platensis* possesses high flocculating activity in kaolin suspension. However, to be applied in treatment of water, the performance of the bioflocculant should also be assessed in river water and wastewater. The river water is a representative of surface water with less COD and turbidity and the results of our evaluations on the river water in this study was quite interesting and of significant importance judging by previous reports. (Gong *et al.* 2008). It has been established that the flocculant of *Serratia ficaria* flocculated river water at an efficiency of 90.4% with COD and turbidity removal efficiencies of 87.1% and 84.2%, respectively (Gong *et al.,* 2008). In another study, maximum COD and turbidity removal of 61.2% and 95.6% were achieved when a bioflocculant produced by *Bacillus licheniformis* X14 was used to treat low-temperature drinking water (Li *et al.,* 2009). In addition, the industrial application of bioflocculant produced by *Streptomyces platensis* was also evaluated on meat processing wastewater and its turbidity removal efficiency may be attributed to polymer-floc interaction of the bioflocculant, which leads to increase in agglomeration of particles. On the other hand, bioflocculant produced by *Azotobacter indicus* was able to reduce BOD, COD and suspended solids in wastewater samples in the range of 38-80% 37-79% and 41-68% respectively at a bioflocculant dosage of 500 mgL^{-1} (Desouky

et al., 2008). Our findings confirmed the possibility of the use of the bioflocculant in flocculation processes.

Table 7: Physicochemical properties of untreated river and meat processing wastewater

Parameters	River water	Meat wastewater
pH	7.1± 2.3	8.9± 0.7
SS (mgL^{-1})	186± 2.6	324± 1.1
Turbidity (NTU)	123± 1.8	238± 0.3
COD (mgL^{-1})	171± 0.5	356± 2.2

The values are means of triplicate determinations

NTU: Nephelometric turbidity units

COD: Chemical oxygen demand

SS : Suspended solids

Table 8: Flocculation of river water and wastewater

		%			
Water type	**Dosage (mg/mL)**	**COD removal**	**Turbidity removal**	**SS**	**FE**
RW	0.2	63.1± 1.1	84.3±1.4	60.2±2.4	91.4±1.3
MPWM	0.2	46.6±0.8	75.6±0.3	72.8± 0.9	82±0.1

RW: River water

MPWW: Meat processing wastewater

FE: Flocculating efficiency

SS: Suspended solids

The Values are expressed as means ± SD of triplicate determinations.

4.4.5. Application of the purified bioflocculant produced by *Arthrobacter humicola* and other flocculants in the treatment of sewage wastewater

The discharge of untreated or partially treated sewage water containing non-biodegradable materials, heavy metals and other toxicants complicates the degree of contamination of the receiving water bodies. This challenge has necessitated the need for proper treatment of various effluents prior to discharge into receiving water body. In addition, sewage carries an array of pathogenic microbes of clinical importance. During the course of decomposing the organic materials in water bodies, the availability of dissolved oxygen gets depleted and can lead to the death of many species of fish and other aquatic life forms (Mbewele, 2006). Having validated the flocculating efficiency of our test bioflocculant against kaolin clay suspension, its potential in treating sewage effluent wastewater was examined and the physicochemical properties are presented in Table 9. From the results (Table 10), it is evident that the bioflocculant produced by *Arthrobacter humicola* significantly reduced the degree of turbidity, removed nitrate, COD, BOD and the total suspended solids at removal efficiencies of 81.3%, 71.4%, 65.7%, 63.5% and 55.7% respectively (Table 10). In addition, the data from this study ascertained that the purified bioflocculant produced by *Arthrobacter humicola* competes very well with polyacrylamide in flocculating efficiency and removal of COD, turbidity and nitrate. However, weaker efficiencies were observed with alum and polyethylenime with respect to flocculation and other parameters removals (Table 10). The use of microbial flocculants in the treatment of wastewater has been well documented. For

instance, the bioflocculant produced by *Bacillus mucilaginosus* was able to remarkably remove COD, BOD and suspended solids in domestic, brewery and pharmaceutical wastewaters and was a suitable alternative to the synthetic flocculants (Lian *et al.,* 2008). Similarly, the bioflocculant produced from *A indicus* CAN was able to reduce BOD, COD and suspended in wastewater samples in the range of 38-80%, 37-79% and 41-68% respectively at a bioflocculant dosage of 500 mg/L (Patil *et al.,* 2011). Hence, the observations on the purified bioflocculant used in this study are confirmative that the test bioflocculant could serve as an alternative coagulant to chemical flocculants and can be employed in wastewater treatment prior to discharge into water bodies.

Table 9: Physical chemical properties of untreated sewage wastewater

pH	Turbidity (NTU)	COD (mg/l)	Nitrate (mg/l)	BOD (mg/l)	SS(mg/l)
7.73± 0.1	128± 0.8	1360± 0.4	8.40± 1.7	49.2± 0.4	201± 1.9

NTU: Nephelometric turbidity units

COD: Chemical oxygen demand

BOD: Biological oxygen demand

SS: Suspended solids

Table 10: Comparism of the flocculent activity of *Arthrobacter humicola* with chemically synthesized and naturally occurring flocculants

		(%)					
Flocculant	**Dosage (mg/ml)**	**BOD removal**	**COD removal**	**Turbidity removal**	**SS(mg/l)**	**F/A**	**Nitrate (mg/l)**
AH	0.8	63.5 ± 0.9	65.7. ± 1.2	81.3 ± 0.2	55.7 ±0.7	89.4 ± 1.3	71.4 ± 0.9
PAC	0.1	72.9 ± 1.2	65.2 ± 0.3	87.8 ± 0.4	75.6 ± 0.2	91.0 ± 1.0	73.3 ± 1.3
PEI	0.4	38.0 ± 2.1	29.0 ± 1.7	64.0 ± 1.9	38.0 ±1.2	58.0 ± 2.3	66.1 ± 2.4
Alum	0.9	41.2 ± 0.8	44.2 ± 1.1	57.8 ± 2.1	46 ± 0.4	66.1 ± 1.0	53.8 ± 1.7

The Values are expressed as means ± SD of triplicate determinations.

SD: Standard deviation

PAC: Polyaluminium chloride

PEI: Polyethylenime

F/A: Flocculating activity

COD: Chemical oxygen demand

BOD: Biological oxygen demand

AH: *Arthrobacter humicola*

4.4.6. Application of bioflocculant produced by *Terrabacter* sp. and other flocculants in the treatment of dairy wastewater

Water has been a key processing medium in dairy industries and it has been used in cleaning, heating, cooling and floor washing. Hence, there is a great demand of water in dairy industries and the need for suitable technology to recycle the wastewater which are being used in the plant. The concept of coagulation-flocculation technology is the best reliable treatment method of reducing suspended and colloidal particles that are responsible for turbidity of the wastewater. It is also employed in reducing organic matter that is responsible for the BOD and COD content of wastewater (Song *et al.,* 2004). Dairy wastewater is free of most toxic chemicals that are listed under Environmental Protection Agency's (EPA) toxic release inventory. However, it contains high concentration of dissolved organic materials like whey proteins, lactose, fats and minerals (Daloun & Shinhong, 2008). The physical chemical properties of the raw dairy wastewater are shown in Table 11. The comparison of experimental results obtained when microbial flocculant SFD 11, PACL, PEI and alum were used in treatment of dairy wastewater are shown in Table 12. The performance of flocculants are highly dependent on dosage concentration (Gomaa, 2012). In this study, the dosage concentration for each flocculant used in the treatment of dairy wastewater was evaluated at different concentration range of 0.1 – 1.0 mg/ml. Each flocculants attained its maximum efficiency with respect to BOD removal, COD reduction, turbidity removal and flocculation efficiency at different dosage concentration (Table 12). After the interaction of the flocculants with the wastewater, turbidity reduction for SFD 11, PACl, PEI and alum was 89.7, 87.2, 52.1 and 43% respectively with BOD and COD removal

efficiencies of (63.3 and 54.1%), (60.9 and 43.3%), (46.0 and 36.0%) and (33.4 and 20.9%) respectively. Interestingly, the bioflocculant exhibited better flocculating activity by significantly removing suspended solids and nitrate at efficiencies of 66.6 and 75.6% respectively. In addition, this bioflocculant performed better than the other tested flocculants in BOD and COD removal efficiencies. The outstanding turbidity removal could be as a result of polymer-floc interaction of the microbial flocculant that led to increase in the conglomeration of suspended particles. Moreover, the significant flocculating efficiency exhibited could be attributed to the presence of carboxyl and hydroxyl group in the functional group of the bioflocculant. The presence of carboxyl and hydroxyl group has higher adsorption forces that facilitates the process of aggregation in floc formation. Hence they may be the preferred group for floc formation (Wang *et al.,* 2013). Our finding is consistent with results on the bioflocculant produced by *Serratia ficaria, Bacillus lichenifromis* and mixed culture of *Halobacillus* sp. and *Oceanabacillus* sp. when compared with other conventional flocculants (Cosa & Okoh, 2014; Gong *et al.,* 2008).

Table 11. Physical chemical properties of untreated dairy wastewater

pH	Turbidity (NTU)	COD (mg/l)	Nitrate (mg/l)	BOD (mg/l)	SS(mg/l)
7.21± 1.4	907± 0.9	1758± 2.1	5.24± 1.3		6.238± 0.3
528±2.4					

NTU: Nephelometric turbidity units

COD: Chemical oxygen demand

BOD: Biological oxygen demand

Table 12. Comparism of the flocculent activity of *Terrabacter* sp. with chemical synthesized and naturally occurring flocculants

		(%)					
Flocculant	**Dosage (mg/ml)**	**BOD removal**	**COD removal**	**Turbidity removal**	**SS (mg/l)**	**F/A**	**Nitrate (mg/l)**
SFD 11	0.5	63.3 ± 0.4	54.1 ± 0.5	89.7 ± 0.6	66.6 ±1.2	85.1 ± 0.1	75.6 ± 0.4
PAC	0.3	60.9 ± 0.7	43.3 ± 0.8	87.2 ± 1.1	71.2 ± 0.5	79.0 ± 0.6	68.1 ± 2.9
PEI	0.7	46.0 ± 1.4	36.0 ± 0.4	52.1 ± 0.5	43.6 ±1.4	56.0 ± 1.0	49.3 ± 1.2
Alum	1.0	33.4 ± 1.3	20.9 ± 0.6	43.0 ± 1.5	50.2 ± .9	38.1 ± 0.5	51.8 ± 0.8

The Values are expressed as means ± SD of triplicate determinations.

SD: Standard deviation

PAC: Polyaluminium chloride

PEI: Polyethylenime

F/A: Flocculating activity

COD: Chemical oxygen demand

BOD: Biological oxygen demand

SFD11: *Terrabacter* sp.

Table 13. Heavy metal ion adsorption analysis.

Metals	Treated sample	Untreated sample	(%) Removal
Aluminium	0.029	0.115	77.7
Manganese	0.008	0.021	74.8
Zinc	0.042	0.099	61.9
Iron	0.029	0.130	57.6

4.4.7. Heavy metal ion adsorption by *Terrabacter* sp.

Bioflocculant can be an effective biotechnological tool for the removal of toxic metals from water bodies and polluted industrial effluents. The rate and mechanisms of metal uptake by microbial flocculants rely on initial metal concentration, bioflocculant dosage, pH, temperature and the conformational polymer type with adsorbed ions (Morillo *et al.,* 2006; Rawat & Rai, 2012; Ugbenyen *et al.,* 2014). As shown in Table 13, the bioflocculant could remove 77.7% Fe 74.8% Al, 61.9% Mn and 57.6% Zn respectively at a bioflocculant dosage of 0.5 mg/ml. The highest adsorption activity exhibited by Fe and Al could be attributed to higher ionic valency which allows easy binding of the metals to the bioflocculant. The adsorption of heavy metal ions by bioflocculants producing acidic polysaccharides as their main backbone has been reported for bioflocculants (Salehizadeh & Shojaosadati, 2003; Wan *et al.,* 2013; Morillo *et al.,* 2006).

4.5. Conclusion

In conclusion, the bioflocculant produced by *Streptomyces platensis*, *Arthrobacter humicola* and *Terrabacter* sp. confirmed their potentials as a replacement with chemical and natural flocculants in biotechnological process. They were able to significantly

remove COD, BOD, nitrate, turbidity and suspended solids at a relative lesser dosage compared to some of the chemical flocculant investigated. Having established their flocculating potentials, their toxicological evaluation will be ascertained in future studies prior the production in a large scale.

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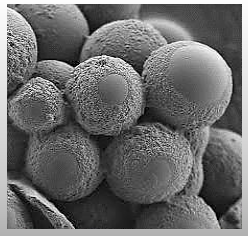

CHAPTER FIVE

General Discussion and Conclusion

5.0. General discussion and conclusion

The rapid increase in population, climatic variation, environmental pollution, urbanization and industrialization are currently responsible for global water crisis and some health related problems. Subsequently, the discharge of poorly or untreated wastewaters from industries and agricultural sector has drastically affected the quality of fresh water in rivers and streams. In addition, anthropogenic activities which include mining operations and the release of industrial wastes water have led to accumulation of metals in the environment which has resulted into serious environmental pollution and also poses serious threat to ecosystem and human health (Chisti, 2004). Consequently, this has gravely impacted on both aquatic and human life forms and has necessitated urgent attention in wastewater treatment prior to discharge into the receiving water bodies or environment. Although, chemical flocculants have been widely employed in wastewater treatment due to their high flocculating efficiency and cost effectiveness (You *et al*., 2009), their usage have resulted into environmental challenges (Mabinya *et al*., 2012). For instance, aluminum as a coagulant in wastewater treatment could lead to higher level of aluminum precipitate in treated effluent than in raw water which also poses a detrimental effects on humans, liquefaction pipes and water quality (Piyo *et al.,* 2011). Therefore, to minimize the risks posed by the usage of chemical flocculants in wastewater treatment and industries, an immediate replacement of eco-friendly flocculants is imperative. Little wonder, microbial flocculants have gained

global acceptance in biotechnology as suitable and viable alternatives to the chemical flocculants (Gao *et al*., 2009). Studies have shown algae, bacteria, yeast and actinomycetes as good microbial flocculants and have been explored in flocculation (Salehizadeh and Shojoasadati, 2001). However, there is a dearth of information on the usage of actinobacteria in wastewater treatment.

In this study, actinomycetes strains from seven different sites were isolated, screened and tested against kaolin clay prior to evaluating their potentials in wastewater treatment. This study has proved that *Streptomyces platensis and Terrabacter sp*. represented the positive bioflocculants that were isolated from Sterkfontein dam while *Arthrobacter humicola* was isolated from Monontsha river. Of the strains screened for flocculating activity, three strains (*Streptomyces platensis*, *Terrabacter* sp, and *Arthrobacter humicola* exhibited significant flocculating activity with respect to chemical oxygen demand (COD), biological oxygen demand (BOD), suspended solids (SS) and nitrate removals. Furthermore, the potential of the positive bioflocculants were also investigated in heavy metals removal in the wastewaters evaluated and the result revealed that genus *Terrabacter* sp. significantly removed Al, Mn, Zn and Fe in dairy wastewater. The identities of the positive strains were also validated using 16S rRNA gene sequencing and their accession numbers have been deposited in the Genbank. Reports have also affirmed that microbial flocculants productions are greatly influenced by the composition of the culture medium and some physicochemical properties (Fang *et al*., 2013). In addition, carbon source, culture time, cations, initial pH of the production medium, nitrogen source, incubation temperature, inoculum size and dosage concentration are very important factors in bioflocculant production. It has been

established that addition of metal ions to suspension plays an important role in inducing effective flocculation of microbial flocculants (Deng *et al*., 2005; He *et al*., 2009). In this study, flocculating activities were enhanced in the presence of Mg^{2+} and Ca^{2+} with the bioflocculant produced by *Streptomyces platensis* and *Arthrobacter humicola* while Ca^{2+} supported bioflocculant produced by *Terrabacter* sp. On the other hand, when flocculating activities were evaluated in the absence of cations in all positive bioflocculants, it was confirmed there were no activity and thus depicts that this bioflocculant were cation-dependent. Initial pH of the culture medium determines the electric charge of the cells and the oxidation-reduction potential which might affect absorption of nutrients and enzymatic reaction (Salehizadeh and Shojoasadati, 2001; He *et al*., 2009). The pH requirement for different strains differs and this was ascertained in this study. For instance, bioflocculant produced by *Streptomyces platensis* preferred neutral pH (7) whereas, *Arthrobacter humicola* and *Terrabacter* sp. attained their maximum flocculating activity at basic medium. The variation in the pH requirement of reaction mixture may be as a result of bioflocculants exhibiting different electric states at different pH media which could affect the flocculating activity of bioflocculants for kaolin particles (Pan *et al*., 2009). The heat stability displayed by the positive bioflocculants confirmed the inherent composition of their backbone as polysaccharides. Also, the thermal stability of the bioflocculant indicated that bioflocculants that are rich in carbohydrate exhibited better thermal resistance than those composed of mainly protein and nucleic acid in their backbone. Another important factor that influences bioflocculant production is the dosage concentration. Dosage is an important tool in validating the efficiency of flocculants in the process of

coagulation and flocculation. As earlier reported, over dosage or insufficient dosage concentration might affect flocculating efficiency or limit the performance of the flocculant in flocculation (Hassan *et al*., 2009). Therefore, it becomes imperative to establish the optimum bioflocculant dosage as this may help to reduce the cost to be spent in treatment processes. This study reaffirmed that each positive strains attained optimum flocculating activity and removed different parameters (COD, BOD, SS, turbidity and nitrate) at different bioflocculant dosage concentrations in wastewater treatment. The effectiveness of the positive strains were compared with some natural and conventional synthetic flocculant at different concentrations under similar conditions. It was validated from this study that the purified bioflocculants were more efficient than the inorganic flocculant. Thus, suggesting the application of the positive bioflocculants as a biotechnological tool in wastewater treatment. Furthermore, the micrographs of the morphological structure of the positive bioflocculants interacting with kaolin clay suspension using scanning electron microscope were elucidated. Interestingly, the interaction of kaolin clay suspension with the purified bioflocculant resulted to a rapid aggregation of kaolin clay forming larger flocs which confirmed that flocculation was achieved through bridging. Similarly, the Fourier Transformer Infrared Spectroscopy and Thermogravimetric analyzer were used to identify the functional groups and pyrolysis profile of the purified bioflocculants. The characterization investigated on the purified bioflocculant revealed the stability of the bioflocculant and confirm the presence of carboxyl, hydroxyl and amino groups as the major functional groups which were responsible for facilitating the process of flocculation.

5.1. Conclusion

Conclusively, this study has shown that fresh water environment could be an important reservoir of bioflocculant producing actinomycetes capable of treating different wastewaters. The cost of production, reproducibility and downstream processing would be taking into consideration prior their usage in the treatment of sludge and as a substitute for chemical flocculants. Going forward, the oral toxicological assessment of the positive bioflocculants will be evaluated on key metabolic and systemic markers of animals and humans prior to their large scale production.

5.2. References

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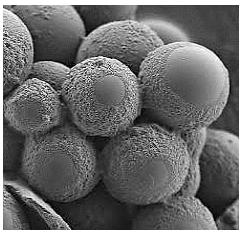

APPENDIX

Published/Accepted Manuscript

1. A review of the application of bioflocculants in wastewater treatment.

2. Flocculating performance of a bioflocculant produced by *Arthrobacter humicola* in sewage wastewater treatment.

3. Bioflocculant production from *Streptomyces platensis* and its potential for river and wastewater treatment **(In Press).**