PRODUCTION AND CHARACTERISATION OF BIOFLOCCULANT PRODUCED BY BACTERIAL ISOLATES FROM RICHARDS BAY HARBOUR, KWAZULU-NATAL

BY

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(201445532)

A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

(MICROBIOLOGY)

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

FACULTY OF SCIENCE AND AGRICULTURE

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DECLARATION

I, Charles Sewanu Oluwaseun Akapo, declare that this dissertation entitled "Production and characterisation of bioflocculant produced by bacterial isolates from Richards Bay harbour, Kwazulu Natal" is submitted to the University of Zululand for the degree of Master of Science in Microbiology in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture. The reported research in this dissertation, except otherwise stated or indicated, is my original research with exemptions to the citations and that this dissertation has not been submitted, either in whole or in part, for a degree or examination at this or any other university.

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Date: ……..

DEDICATION

This thesis is dedicated to God Almighty for His love, knowledge, wisdom and protection throughout this study.

*But God chose the foolish things of the world to shame the wise; **...**so that no one may boast in His presence.… 1 Corinthians 1:27 & 29 (BSB).*

ACKNOWLEDGEMENTS

My utmost appreciation goes to the Almighty God, the loving Jesus Christ and wonderful counsellor, Holy Spirit who makes all things possible.

I appreciate Prof Albert K. Basson for insisting on my starting and completion of this degree having shown me the way to this field of bioflocculants research and encouraging me to stay on. Mr Z.G. Ntombela was close enough to correct; your criticism and encouragement blended together helped me throughout this project. I will always be grateful to my former supervisors Prof Jean Simonis who was a guide to the Richards Bay Harbour habitat and my boss Dr Anthony Ugbenyen

I want to appreciate the National Research Foundation, South Africa and the University of Zululand Postgraduate Research Department for supporting my study.

My special appreciation goes to the Laboratory Technologists and their assistants for helping with consumables and glassware as the need arose.

To all my close and distant colleagues, I say thank you for your understanding, encouragement and for responding to my greetings every morning.

I wish also to appreciate the Bioflocculation project group members. Thank you all for your adjustment and support. Special thanks to my colleagues that offered the necessary assistance during the course of this study especially in the inconvenient hours of the day..

My deepest thought goes to my immediate family; Olufunmilayo, Oluwapelumi, God’s Delight and Deborah Akapo. You were a great inspiration and stabilising factor during the period of my study. Special thanks to my members of the body of Christ who gave their necessary assistance and prayer support during this period of study.

To all who will read this project, may it add good knowledge to your research.

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LIST OF ACRONYMS

%	Percent
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Celsius
µl	Microlitre
µm	Micrometer
16S-rRNA	16S-ribosomal Ribonucleic Acid
ANOVA	Analysis of Variance
BA-CGB	Bioflocculant from *Bacillus atrophaeus*
BLAST	Basic Local Alignment Search Tools
BOD	Biological oxygen demand
BSA	Bovine serum albumin
C	Carbon
Ca	Calcium
CFU	Colony forming units
Cl	Chlorine
COD	Chemical oxygen demand
DLVO	Derjaguin, Landau, Verwery and Overbeek
DNA	Deoxyribonucleic Acid
EPS	Exopolysaccharides
EDX	Energy Dispersive X-ray spectroscopy
FA	Flocculating activity
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram

L	Litre
LB-EPS	Loosely Bound-Exopolysaccharides
Mg	Magnesium
mg	Miligram
ml	Millilitre
N	Nitrogen
Na	Sodium
NCBI	National Centre for Biotechnology Information
NRF	National Research Foundation
nm	Nanometer
OD	Optical density
PAA	Polyacrylamide
PAC	Polyaluminium Chlorides
PCR	Polymerase Chain Reaction
PFS	Polyferric Sulphate
rRNA	Ribosomal Ribonucleic Acid
Rpm	Revolutions per minute
RSA	Republic of South Africa
S	Sulfur
SD	Standard deviation
SEM	Scanning Electron Microscopy
TB-EPS	Tightly Bound-Exopolysaccharides
TGA	Thermo gravimetric analyzer
UN-Water	United Nations Water
UV	Ultraviolet

v	Volume
w	Weight
WHO	World Health Organisation

ABSTRACT

Bioflocculants are safe and biodegradable extracellular polymers produced by microorganisms during their growth. In comparison with conventionally used flocculants, bioflocculants have the advantage of being safe (no known toxic effects), biodegradable and harmless to the environment. In this study, halophile novel bacteria was isolated from Richards Bay Harbour, Kwazulu-Natal, Republic of South Africa and screened for bioflocculant producing potential using a production medium at 30 ^{o}C for 72 hours with a shaking speed of 160 rpm. Kaolin clay suspension was used as test material. Microorganisms with bioflocculant-producing potential were further identified using 16S rRNA molecular sequencing method. Among the identified isolates, one with flocculating activity above 70% was used for bioflocculant production. *Bacillus atrophaeus* culture conditions were optimised for improved flocculating activity as well as the production yield. To obtain an optimum culture medium condition, the parameters such as carbon and nitrogen (energy) sources, inoculum size, metal ions, cultivation time, initial pH of the medium, incubation temperature, shaking speed and fermentation time were investigated. The bioflocculant was extracted using ice-cold ethanol and purified with the mixture of chloroform and butanol (5:7 v/v). The purified bioflocculant was characterized using FTIR, scanning electron microscopy (SEM) equipped with element analyser and TG analyser. The bioflocculant was further analysed for its chemical composition. The purified bioflocculant BA-CGB was also assessed for antimicrobial activity properties and further applied on Vulindlela domestic wastewater and Mzingazi river water. Among the screened isolates, three of them with more than 70% flocculating activities were identified through 16S rRNA as *Bacillus safensis*, *Bacillus pumilus*

and B*acillus atrophaeus*. A bioflocculant yield of 3.165 g was recovered for *B. atrophaeus* after 96 hours of incubation from 1 liter of fermentation broth in the presence of 4% inoculum size, glucose, ammonium chloride, at pH 9 and temperature of 35 ^{o}C as well as shaking speed of 110 rpm stimulated by Ba^{2+}. The bioflocculant BA-CGB produced, showed a strong thermal stability character as managed to attain more than 60% flocculating activity at 121 °C for 15 min, with 0.4 mg/ml dosage size and Ca^{2+} as a stimulating agent. The flocculating activity of purified BA-CGB was greatly influenced by a wide pH range (3-12). BA-CGB is amorphous in structure, composed of carbohydrates (65%), uronic acid (25%) and trace proteins (10%). Functional groups include hydroxyl, carboxyl, amine, amide and elements such as carbon and oxygen among others, were present in the molecular chain of bioflocculant BA-CGB. No antimicrobial activity was observed with bioflocculant BA-CGB. 0.4 mg/ml dosage size of a bioflocculant was more effective in flocculating wastewater, improving COD, BOD and removing nitrates, phosphates as well as sulphate from Mzingazi riverwater and Vulindlela domestic wastewater. This bioflocculant holds a promising future to replace in-use chemical flocculants in wastewater treatment.

Keywords: Bioflocculants, kaolin clay suspension, flocculating activity, *Bacillus atrophaeus*

CHAPTER ONE

1.0 BACKGROUND OF THE STUDY

1.1 Introduction

One of the environmental problems that have adverse universal effect on economic developments in many communities worldwide is water pollution (Pathak *et al*., 2014). Water is an important natural resource that is continually being polluted due to various processes leading to contamination of water with agricultural, industrial and domestic wastes (Cosa *et al.*, 2013a). The process of urbanization and growth developments has contributed to these sources of pollution. Majority of this pollution comes from the discharge of untreated agricultural and household wastes coupled with industrial waste into the environments (Liz *et al.,* 2013). This has brought untold hardship to many people who are majorly suffering from lack of safe water for drinking (United Nations Water, 2017).

The consequence of these pernicious and life threatening situations demands a pragmatic approach. This is because it affects aquatic life and renders potable water unavailable for both household and industrial use (Yang *et al.,* 2012). Many countries have put in place strict regulations to ensure and control the level or presence of domestic and industrial contaminants in water discharged into the environment (Willmott *et al*., 1998). One of these regulations is to enforce that it has been properly treated using different available water treatment technologies before being discharged into water bodies (Liz *et al.,* 2013). Examples of such water treatment methods include flocculation, ion exchange, filtration, adsorption, oxidation processes, electrolysis and solvent extraction (Low *et al.,* 2011; Ong *et al.,* 2012).

Flocculation is one of the methods of choice for removing contaminants and organic matter from water. This is done by forming flocs with synthetic or naturally occurring flocculants. The flocculants become attached to the surface of the particles in suspension after overcoming the electrostatic repulsion forces to aid settling rate. Meanwhile the settling rate of larger particles is more rapid (Lee *et al.,* 2012). Chemical flocculants have been the preferred flocculating agents because they are less expensive, efficient, and require less skilled personnel (Li *et al.,* 2013). However, due to the observed health complications in the course of their use, the search for biodegradable, economical and sustainable extracellular biopolymers has been on the rise globally (Subudhi *et al.,* 2015) including the Republic of South Africa. In recent years, there has been various researches aimed at screening for novel microorganisms, which could produce bioflocculants with excellent flocculating activity and cost-effective production process with high polymer yield (Ugbenyen *et al.,* 2012). Marine sediments have been reported to contain a wide array of unique, diversified and novel organisms than those found in terrestrial environments (Cosa and Okoh, 2012).

Several strategies have been developed on how to optimize culture medium conditions for better bioflocculant yields as well as improved flocculating activity (Li *et al.,* 2009; Ugbenyen *et al.,* 2012). This has been one of the focus of many research groups worldwide (Abu-Elreesh and Abd El-Haleem, 2014). However, one of the greatest limitations to the effective large-scale application of these biopolymers to wastewater treatment is due to its low flocculating capability compared to the chemical polymers and the high cost of production due to expensive media composition. The scientific research progress has been limited by these

setbacks. This is due to restrictive financial costs to produce bioflocculants (Okaiyeto *et al.,* 2013; Li *et al.,* 2015).

Reports have indicated the increase of the use of axenic cultures (Ugbenyen *et al.,* 2012) and mixed cultures (consortium) for bioflocculants production (Li *et al.,* 2015). More *et al.* (2015), reported the use of more than 13 microorganisms in production of a compound bioflocculant. It has also been reported that since microbes exist axenic or impure state in nature, growing them in consortium could also increase their rate of substrate utilisation and the yield of extracellular polymer substances (EPS). The alternative use of low cost media, rather than expensive media could as well favour researches seeking to bridge the cost-gap between biopolymers and chemical coagulants (Vijayaraghavan *et al.,* 2011).

Presently, there is a continuous need to search for alternative polymers to replace the chemical polymers used in the treatment of wastewater, brewery and coal mine in South Africa. However, most findings have been limited to the research institution due to non-implementation with regards to application. The major limitations to the use of these new alternative biopolymers are seemingly due to its huge cost of production, metal dependency and unclear future successes (Okoh *et al*., 2012). Several reports on prokaryotes being responsible for over 50% of microbial and bioactive metabolites from soil and marine water sediments (Alain and Querellou 2009, Ugbenyen *et al.* 2017). Therefore, the alternative use of low cost media and the production of bioflocculants by isolates from the natural environment will be of immense benefits. This is because bioflocculants are biodegradable, non-toxic and possess antimicrobial activity (More *et al.,* 2012). This study was focused mainly on

isolation of novel bacterial isolates from marine environments with high flocculating activity and high polymer yield through optimisation.

1.2 Rationale for the study

Various flocculants including inorganic flocculants, organic high-polymer flocculants and naturally occurring flocculants have been used in wastewater treatment, dredging, and industrial downstream processes (Yokoi *et al*., 1995). Polyacrylamides, as a high-polymer organic flocculants have been in use over time because they are inexpensive and highly effective. However, some of them are not easily degraded in nature. Similarly, some of the monomers derivatives from these synthetic polymers are harmful to the health of humans. In recent years, one of the solutions to these environmental problems is the use of microbial flocculants due to their harmless degradable intermediates in the environment (Shih *et al*., 2001). There has been an ongoing global investigative research to isolate novel bacteria from the marine or aquatic environment and test for their bioflocculation efficiency.

1.3 Problem statement

Commercial chemical flocculants such as aluminium sulfate, ferric chloride and polyacrylamide that have been widely used and been reported to have a negative effect on the environment (). Thus, based on the concerns, it has been projected that the use of bioflocculants will increase (Zhang *et al.*, 2007). Bioflocculants in comparison with the frequently used chemical flocculants, show more advantages based on their safety and biodegradability (Piyo *et al.,* 2011). However, production costs and yield are limiting factors to their large scale use.

1.4 Hypothesis

It is hypothesized that the marine environment of Richards Bay Harbour possess novel organisms with high flocculating activity.

1.5 Aim(s) of the study

To screen and identify bacteria isolated from Richards Bay Harbour, optimise for improved bioflocculant production, purify and characterise the purified bioflocculant as well as the application of purified bioflocculant in water purification and wastewater treatment.

1.6 Objectives of the study

To isolate and screen for the bioflocculant-producing microorganisms from soil and water samples.

To identify selected microorganisms for bioflocculant-production by 16S rRNA gene sequence analysis;

To optimize culture conditions for bioflocculant production, using selected bacterial isolates;

To extract, purify and chemically analyse the produced bioflocculant;

To assess antimicrobial activity of a purified bioflocculant;

To apply the purified bioflocculant in wastewater treatment and comparison with synthetic flocculants.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

This section outlines the problem of water shortage as a renewable resource and life threat due to contaminants such as heavy metals, chemical flocculants residues and pathogens from developmental processes leading to the need for bioflocculants. The section also describes comparative advantages of synthetic flocculants compared to naturally occurring flocculants, the bioflocculant producing organism and their habitats. It also describes the growth phase as a factor that affects bioflocculant production and optimisation of such factors that determine bioflocculant constituents. The application of bioflocculant in wastewater treatment and identification of bioflocculant-producing potential is subsequently discussed.

2.2 Water as a renewable resource

Water is the most abundant resource natural to humans. More than seventy-one percent (71%) of the world's environment is aquatic, however, over ninety nine percent (99%) of the hydrosphere is situated in oceans (Boyd, 2015). Water is for both industrial and domestic beneficial use, which makes the demand for water to be more compared to what the environment provides. According to UNO (2007), it is projected that by year 2025, sixty-seven percent (67%) of nations will be experiencing water shortage. The major cause being the groundwater and surface water depletion due to excessive use. South Africa is presently experiencing drought in few areas and water restriction in many areas (Masante *et al.,* 2018). The

wastewater from domestic and industrial use that could have been re-used has become life threatening due to contaminants such as heavy metals, chemical, flocculants residues and pathogens. It is therefore necessary that more biodegradable flocculants be applied greatly in wastewater treatment, downstream processing and fermentation processes (Sanghi *et al*., 2006). Notwithstanding, the effective performance of these chemical flocculants in the flocculation process, their low cost, readily availability and their usage have resulted into some forms of threat to the health of humans and the ecosystem. Thus, it is imperative that naturally occurring bioflocculants research will be useful as an alternative to the menace of the chemical flocculants. There is however, the need to obtain a low cost media with high yield of biopolymers instead of the high maintenance media. This could as well favour researches seeking to bridge the cost-gap between biopolymers and chemical coagulants used in the process of flocculation.

Flocculation is an integral and important phenomenon in wastewater treatment (Lee *et al.,* 2017). Flocculation is one of the stages for removing contaminants and organic matter in wastewater. Flocculating agents such as synthetic chemical flocculants, naturally occurring bioflocculants and grafted synthetic flocculants form aggregate flocs with the suspended matters in water (Zhang *et al.,* 2007; Pathak *et al.,* 2014; Lee *et al.,* 2014).

2.3 Classification of flocculants

Flocculants are charged compounds used widely in the flocculation of solids, cells, and colloidal suspensions in water (Subudhi *et al.,* 2015). Flocculants are classified as anionic or cationic substances (Fleer *et al.,* 2005), mainly used in industrial processes including wastewater treatment, purification of drinking water and food

processing to form aggregated flocs. Bigger flocs increase the settlement rate. These flocs help to speed up settling rate to remove colloids in wastewater and clear the system on formation (Cosa *et al.,* 2013b).

Based on their origin, Lee *et al.* (2014) classified flocculants into three main groups as shown in Figure 2.1. These include (i) chemical coagulants namely (a) inorganic flocculants (such as polyaluminium chloride (PAC), (b) synthetic organic flocculants (such as polyacrylamide derivatives). (ii) naturally occurring flocculants (made up of cellulose, starch, chitosan, natural gums, mucilage and microbial by-products) and (iii) grafted flocculants or copolymers which involves the grafting of synthetic and naturally occurring polymers such as glycogen-g-polyacrylamide (Gly-g-PAM) (Adhikary *et al.,* 2007 ; Lee *et al.,* 2014).

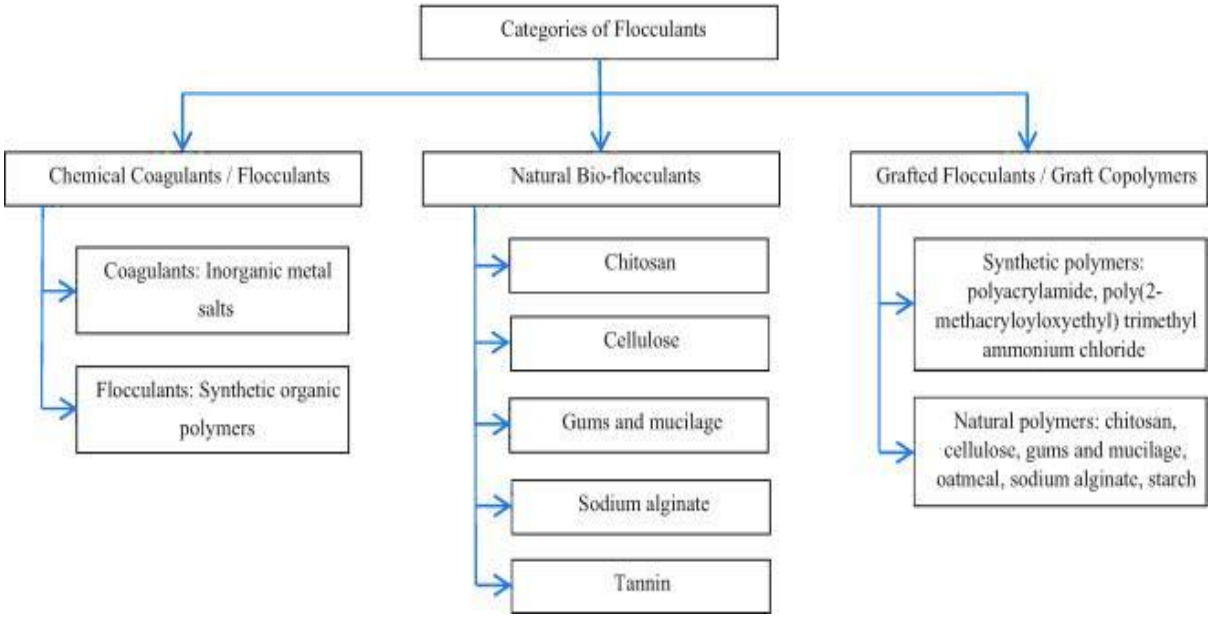

Figure 2. 1: Classification of flocculants. (i) Chemical coagulants, (ii) Natural bio-flocculants and (iii) Grafted flocculants. Image adapted from Lee *et al.,* 2014.

2.3.1 Inorganic flocculants

Inorganic and synthetic organic flocculants are widely used in flocculation in waste water purification due to cost effectiveness, high flocculation property and efficiency in flocculating suspended solids in solution or wastewater. The commercial inorganic and multivalent metal flocculants that are readily used for wastewater treatment is the polyaluminium silicate sulphate and polyaluminium chloride (PAC) (Kurane *et al.,* 1986; Lachhwani, 2005). In spite of overall advantages of inorganic flocculants, large amounts are required to cause a settling rate of solid-liquid dispersion thereby resulting in large amounts of sludge wastes, highly sensitive to pH and unable to coagulate fine particles (Sharma *et al*., 2006). Therefore, these disadvantages require great reconsideration in terms of the alternatives especially in water purification field.

2.3.2 Organic flocculants

The use of organic synthetic flocculants has been on the rise because of their high performance in flocculation at a low cost (Salehizadeh and Shojaosadati, 2001). They are readily available in dry powder and suitable for flocculating suspended solids, and clarifying of potable water (Lachhwani, 2005). Kurane *et al. (1986)* reported the frequent use of these flocculating agents such as polyacrylamide and polyacrylic acid in dredging fields and water treatment plants because of their economic advantages (Kurane *et al*., 1996; Cosa *et al.,* 2013a). However, these polymers are non-biodegradable and toxic to the environment. Therefore an alternative replacement that is biodegradable, non-toxic and environmentally friendly is needed.

2.3.3 Naturally occurring flocculants

Naturally occurring flocculants are made up of natural polymers such as: cellulose, starch, chitosan, natural gums, mucilage and bioflocculants (Li *et al.,* 2009). Bioflocculants, as an example of naturally occurring flocculants are secreted by microorganisms during their growth phase and exist in forms such as polysaccharides, proteins, uronic acids, and glycoproteins (Salehizadeh and Yan, 2014). Bioflocculants are products of natural origin and can be described therefore as, (1) an entire organism (e.g. a plant, an animal or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g. drying) and (2) a pure compound (e.g. glycosides, sugars, proteins etc) isolated from plants, animals or microorganisms (Sarker *et al.,* 2006). Their products could be a way of reducing the overhead costs and making the process more profitable (Abu-Elreesh and Abd El-Haleem, 2014). Bioflocculants that have been produced by microbes are subjected to further screening for ecotoxicity and antimicrobial activity (Aljuboori *et al.,* 2015). In terms of their action, bioflocculants can work with or without a cation. Aljuboori *et al.* (2015), reported that cation-independent bioflocculant that functions without the addition of cation have positive impacts in terms of cost reduction. Gong *et al.* (2008) reported cation-dependent bioflocculants produced by *Serratia ficaria*. *Corynebacterium glutamicum* and *Halomonas* sp. were also reported to flocculate effectively without the addition of cation (He *et al.,* 2004; He *et al.,* 2010). Cation-independent bioflocculants require no addition of cation such as the bioflocculant produced by *Bacillus mucilaginosus* and *Klebsiella pneumoniae* that requires no addition of cations (Deng *et al.,* 2003 ; Zhao *et al.,* 2013). However, the bioflocculant BA-CGB produced by *Bacillus*

atrophaeus is an example of cation-dependent bioflocculant, since it can not work efficiently without the addition of cation.

2.3.4 Grafted Flocculants

Grafted flocculants are a combination of the properties of synthetic polymers and that of natural polymers backbone to obtain a tailor-made grafted flocculant (Mishra *et al.,* 2011). This is used in order to overcome the shorter shelf life and utilise the moderate flocculate efficiency of the naturally occurring flocculants (Lee *et al.,* 2014). These emerging new grafted bioflocculants have shown tremendous wastewater treatment potential, as they combine with the naturally occurring flocculants properties to obtain a unique and superior performance. Such grafted polymer seeks to overcome any polymer disadvantage posed by natural flocculants. In recent years, polyacrylamide has been grafted to agar, chitosan and starch (natural polymers) to form St-g-PAM (Wang *et al.,* 2007; Mishra *et al.,* 2011; Rani *et al.,* 2012). It has been reported in a study that a composite flocculant was chemically bonded to a novel amphoteric carboxymethyl chitosan-graftpolyacrylamide (CMC-g-PAM). The grafted flocculants was made of chitosan and polyacrylamide which showed different mechanism of flocculation in acidic, neutral and alkaline medium (Yang *et al.,* 2012).

2.3.5 Bioflocculants advantages over chemical flocculants in flocculation

Bioflocculants, as alternatives, possess several promising advantages over the chemical flocculants (such as polyacrylamide, aluminium sulphate and ferric chloride) that have been widely used due to their safety and biodegradability in nature. Bioflocculants have less side effects compared to chemically synthesised flocculants (Xiong *et al.,* 2010). The use of non-degradable chemical flocculants

produces residues of aluminium from PAC and polyacrylamide derivatives which have been implicated in the development of Alzheimer disease and cancer (More *et al.,* 2014; Pu *et al.,* 2018). Polyacrylamide, a synthetic polymer containing acrylamide monomers which results to neurotoxic, senile dementia and carcinogenic effects in human beings (Zhuang *et al*., 2012). Chemical flocculants are recalcitrant in the environment (Banks *et al*., 2006; Xia *et al.,* 2008; Hierrezuelo *et al.,* 2010). Li *et al.* (2008) have reported that ferrite flocculants cause bad metallic taste, malodour and are a potential risk to end industrial processes products due to their corrosiveness. The solution to these above listed problems and the detrimental effects of synthetic flocculants is to replace them with biodegradable, non toxic flocculants in waste water purification (Bank *et al.,* 2006).

Bioflocculants are biodegradable, non-carcinogenic, non-toxic polymer, possessing high flocculating ability. They require low dosage use in diverse applications (Ugbenyen *et al.,* 2012). Okaiyeto *et al.* (2015a) stated the disadvantages of naturally occurring bioflocculants such as: expensive cost of production, low product yield, pH dependence, shorter shelf-life, requirement of large dosage for flocculating efficiency in applications and low temperature use range. The molecular weight and the functional groups in the molecular chains of bioflocculants are responsible for their flocculating activity (More *et al.,* 2012). Bioflocculants help to overcome the electrostatic repulsion forces and form bonds to adsorb to the surface of the particles (Salehizadeh *et al.,* 2000; Lee *et al.,* 2012; Nouha *et al.,* 2017). Recent research with industrial potential seeks for alternative flocculants that are biodegradable, eco-friendly and possess high flocculating activity (Bezawada *et al.,* 2013). Bioflocculants could be produced from agricultural and biological wastes (Aljuboori *et al.,* 2013; Aljuboori *et al.,* 2014; Aljuboori *et al.,* 2015; Ma *et al.,* 2018). A summary of some

flocculants, their advantages and disadvantages are tabulated in Table 2.1 below, adapted from Okaiyeto *et al.* (2016)

Table 2.1: A summary of some advantages and disadvantages of flocculants in flocculation .

Flocculant(s)	Advantage(s)	Disadvantage(s)	References
Inorganic flocculants such as Aluminium chloride, Alum, Ferric chloride	• High flocculating activity • Cost effective and easily accessible • Requires no special skill to use	• Inefficient in cold water • PAC generates slugde and it is difficult to dispose • Highly sensitive to pH • Do not coagulate very fine particles • Aluminium salts have neurotoxic effect while ferrite flocculants causes unpleasant metallic odour	• Wei *et al.,* 2003 • Sharma *et al.,* 2006 • Banks *et al.,* 2006
Organic Flocculants such as Polyacrylamide, sulfonated polystyrene	• High flocculating efficiency • Not sensitive to pH • Coagulate fine particles • Effective in cold and warm water	• Not biodegradable • Toxic to the environment • Monomers derivateives of polyacrylamide are neurotoxic and carcinogenic • It cause environmental pollution	• Lee *et al.,* 2014 • Li *et al.,* 2008
Naturally occuring flocculants such as Chitosan, guar gum, polysaccharide, starch and bioflocculants	• Harmless & biodegradable flocculant • Coagulates very fine particles • They are effective in both cold and warm water • They produces lesser sludge compared to PAC • Not sensitive to pH	• Shorter shelf-life due to biodegradability • Flocs loose stability and strength with time • Flocculation requires high flocculant-particles ratio in systems • Production cost is high and low yield is a problem	• Okaiyeto *et al.,* 2015 • Sheng *et al.,* 2010

2.4 Bioflocculant producing microorganisms and their habitats

Microorganisms that have the potential to secrete bioflocculants (flocculation biopolymer) have been studied and however are steadily receiving more scientific attention (Xiong *et al.,* 2010). Over 100 species of microorganism that have the ability to produce bioflocculants have been isolated and reported over the past decades (Zhang *et al.,* 2010). Microorganisms, ranging from filamentous bacteria of the genus Streptomyces (Actinomycetes) and fungi. are sources of many useful primary and secondary metabolites These microorganisms such as fungi, actinomycetes, algae and bacteria have been isolated (Gong *et al.,* 2008; Xia *et al.,* 2008; Xiong *et al.*, 2010) from extreme environments that were once thought to be unfavourable to support their growth and production of secondary metabolites for useful tools in biotechnology (Wang *et al*., 2011). Varying samples are collected from sampling sites and grown on different selective and enriched media. These media are incorporated with addition of antibiotics under appropriate incubation period that could favour the growth of fastidious and non-fastidious microorganisms. Liquid broths enable cell growth and metabolite production. Pure colonies obtained are stored in liquid nitrogen or freeze-dried in the presence of a cryopreservative (Seidel, 2006).

Rhodoccocus erythropolis was reported by Kurane *et al.* (1994) to produce a proteinous bioflocculant but loses its activity to flocculate when exposed to enzyme digestion (Subudhi *et al.,* 2015). Some bioflocculants have been reported to be produced by a number of bacteria including *Alcaligenes cupidus* KT-201 (Kurane *et al.,* 1991), *Bacillus sp.* Gilbert (Piyo *et al*., 2011), *Cobetia* sp. OAUIFE (Ugbeyen *et*

al., 2012), *Bacillus sp*. AS-101 and *Bacillus firmus* from the soil (Salehizadeh and Yan, 2014). *Bacillus mucilaginosus* was reported by Deng *et al. (*2003) to have a flocculating rate of 99.65% for kaolin suspension at a dosage size of only 0.1 ml/l. Ugbenyen *et al*. (2012) reported a thermal stable crude bioflocculant produced by *Cobetia* sp. OAUIFE which shows a residual flocculating activity of about 78% after heating for 25 minutes at 100 ^{o}C. Ugbeyen *et al.* (2017) reported a novel bioflocculant producing *Pantoea* sp. isolated from the Mtunzini beach, KwaZulu Natal with a flocculating activity of 92.4% and inoculum size of 3% (v/v). Most of these reported microorganisms producing bioflocculants were obtained from marine water and sediments (Lam, 2006).

2.4.1 Halophilic microorganism with bioflocculant-producing potential

Halophiles (salt loving) organisms find their most adaptable habitat in the marine environment. Halophiles are distinguished by their characteristics of adapting to different concentrations of salt for their growth and have evolved physiological and genetic features to survive in this halophilic environment. Halophiles live in water, soil and salt deposits or salted products. They have various other coping strategies to combat the osmotic stress exerted by this halophilic environments (Edbeib *et al.,* 2016). Halophiles could be classified as being extreme, slight or moderate. They maintain their cytoplasmic osmotic balance by either accumulating high concentrations of inorganic ions or organic osmotic solutes (Ventosa *et al.,* 1998). Halophilic microorganisms have been reported to produce various types of biomolecules such as extracellular polysaccharide, pigments and intracellular polyester polyhydroxyalkanoates due to their hypersaline deleterious nature (Poli *et al.,* 2011). This has been the focus of various scientists and biotechnologists across

the globe (Biswas & Paul, 2017). Some microorganisms have been reported as halophilic microorganisms. Examples include *Bacillus safensis* and *Bacillus pumilus,* which grows in halophilic environment and are members of the firmicutes (Edbeib *et al.,* 2016).

2.4.2.1 *Bacillus safensis*

Bacillus safensis (*B. safensis*) is a Gram-positive, mesophilic, spore-forming, aerobic and chemo-heterotrophic bacterium (Kothari *et al.*, 2013). It is a rod-shaped and motile bacterium with high tolerance for salt, heavy metals, and ultraviolet and gamma radiations (Satomi *et al.,* 2006; Raja & Omine 2012). *B. safensis* was originally isolated from a spacecraft in Florida and was supposed to have been transported to planet Mars on a spacecraft. The prefix SAF in the name "safensis" was arbitrarily derived from "Spacecraft-Assembly Facility" in the USA from where the organism was first isolated (Satomi *et al.,* 2006; Lateef *et al.,* 2015). *B.safensis* has been reported to colonize various habitats including terrestrial and marine environments (Liu *et al.,* 2013). *Bacillus safensis* has been reported to produce flocculants in consortium with other Bacillus species (Harun *et al.,* 2017).

2.4.2.2 *Bacillus pumilus*

Bacillus pumilus is a Gram-positive bacteria. It has been found to be the second most dominant species among the aerobic spore-forming bacteria (La Duc *et al.,* 2004). *B. pumilus* has been placed using multiple genome comparison in microbial taxonomy as strains that are likely to belong to the *B. safensis* group (Tirumalai *et al.,* 2018). Coli *et al.* (2016) has reported that endospores of a strain of *B. pumilus* has been found to grow better on International Space Station (ISS) than here on

earth. This is a concern for forward and backward contamination in aerospace and this level of resistance in such closed environments could affect living conditions (Moissl-Eichinger *et al.,* 2016). *Bacillus pumilus* is an example of halophilic organism. There has been few reports on *Bacillus pumilus* in the production of bioflocculants (Makapela *et al.,* 2016) and also in consortium with other microorganisms (Maliehe *et al.,* 2016). Makapela *et al. (*2016) reported a thermostable bioflocculant with low dosage concentration of 0,1 mg/ml and a working pH range of 3-11 while Maliehe *et al.* (2016) observed a 3.0 g/l yield of bioflocculant TPT from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis*.

2.5. Bioflocculation

Bioflocculation is a dynamic process by which living cells synthesize extracellular polymer to clarify solutions of suspended particle and form flocs. This is an important and effective process of separation of microorganism in a treated wastewater effluent (Laspidou and Rittman, 2002 ; Lachhwani, 2005 ; Salehizadeh and Yan, 2014). Louis Pasteur (1876) described the process for the first time in a microbial system in *Saccharomyces cerevisiae.* The process of bioflocculation was later observed in bacterial cultures. It is established that there is a correlation between the aggregation of particles and the secretion of extracellular polymeric substance such as bioflocculants (Salehizadeh and Shojaosadati, 2001; Cosa, 2010). Many microorganisms including yeasts, fungi, bacteria, actinomycetes and algae isolated from soil and wastewater and screened for bioflocculants production have been reported (Desouky *et al*., 2008; Cosa, 2010). During their growth they excrete macromolecules or extrapolymers called bioflocculants (Salehizadeh and Shojaosadati, 2001). The composition of the media affects the expression of genes

and therefore, determines the compounds produced by the cell. This implies that bioflocculant production is influenced by three factors such as: genotypic, physiologically and environmental aspects (Salehizadeh and Shojaosadati, 2001). Bioflocculant can be classified as glycoprotein, polysaccharides and nucleic acids in nature (Ugbenyen *et al.*, 2012). These naturally produced extracellular polymeric substances (EPS) form an inter-bridge mineral surfaces. The molecular weight and the functional groups in the bioflocculants molecular chains are responsible for their flocculating activity (More *et al.,* 2012).

2.6 The roles and mechanism of Extracellular Polymeric Substances (EPS) in bioflocculation

Polysaccharides are biomolecules synthesized via different biosynthesis pathways by microbes. These wide range of exopolysaccharides are classified according to their functions into three namely, intracellular storage polysaccharides, capsular polysaccharides (closely linked to the cell surface) and extracellular polysaccharides (such as xanthan, alginate, cellulose, etc) (Jochen *et al.,* 2015). Extracellular polymeric substances (EPS) are polymers of high molecular weight secreted into the surrounding environment by microorganisms as they maturate utilising the organic matters in their environments or due to cell lysis (Gomoiu and Catley, 1996; More *et al.,* 2014; Ghashoghchi *et al.,* 2017). These EPS contain various functional groups including hydroxyl, carboxyl, amide, amino and phosphoryl group obtained from synthesising organic carbon and nitrogen sources (Sheng *et al.,* 2010; Poorni and Natarajan, 2014 ;Manivasagan *et al.,* 2015; Gupta & Thakur, 2016). The biochemical composition of EPS is influenced by various factors such as carbon source, microbial species, the downstream extraction methods and nutrient supplementation (Nouha

et al., 2017). These chemical composition and physical properties usually determine the capability and mechanisms of bioflocculants. The bio-formation of aggregated particles involves four main mechanisms namely; (i) polymer bridging (ii) charge neutralization (iii) colloid entrapment and (iv) double layer compression (Salehizadeh and Shojaosadati, 2001).

2.6.1 Polymer bridging mechanism

Polymer bridging occurs when threads or fibres formed by the bioflocculants adsorb itself to the particles and become aggregated. The threads capture and bind the particles together (Lachhwani, 2005; Laspidou and Rittman, 2002). This assists to overcome the electrostatic repulsion forces and form bonds that adsorb to the surface of the particles (Salehizadeh *et al.,* 2000; Lee *et al.,* 2012; Nouha *et al.,* 2017). A three-dimensional aggregate is formed with the flocs that consequently increases the mineral particles rate of settling (Karthiga and Natarajan, 2015; Ghashoghchi *et al.,* 2017). The cations help in this process by increasing the rate of adsorption of the bioflocculants through a process of decreasing or reducing negative charges on the aggregated particles and the bioflocculant chain (Abd El-Salam *et al.,* 2017). Figure 2.2 adapted from Feng *et al.* (2015) illustrates the role of the non-ionic polymer or cations on addition to the dispersed colloid suspension, the polymer with affinity for the particles starts to adsorb to the surface, forming a bridge where the polymer adsorbs to one or two more particles. This leads to the flocculation and stabilisation role of non-ionic polymer or polyelectrolyte (Feng *et al.,* 2015).

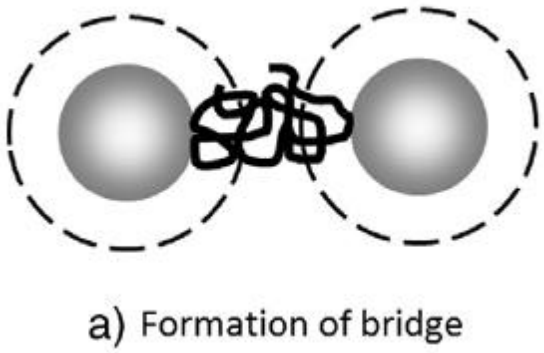

Figure 2. 2: Formation of colloids in bridging method adapted from Feng *et al.,* 2015. The threadlike polymer produced adsorb to the surface of the particles forming a bridge between two particles.

2.6.2 Charge neutralization mechanism

The charge neutralization mechanism are related to the opposite charges on the bioflocculants and the particles. The positive charge on the bioflocculant neutralises the negative charge on the particles thereby reducing the electrostatic repulsion between the colloid and the bioflocculants (Figure 2.3). This leads to formation of flocs and decreases repulsion between them (Feng *et al.,* 2013; Aljuboori *et al.,* 2015). Charges that are repelling in (A), are neutralised and free to aggregate and form flocs in (B) (Lachhwani, 2005)

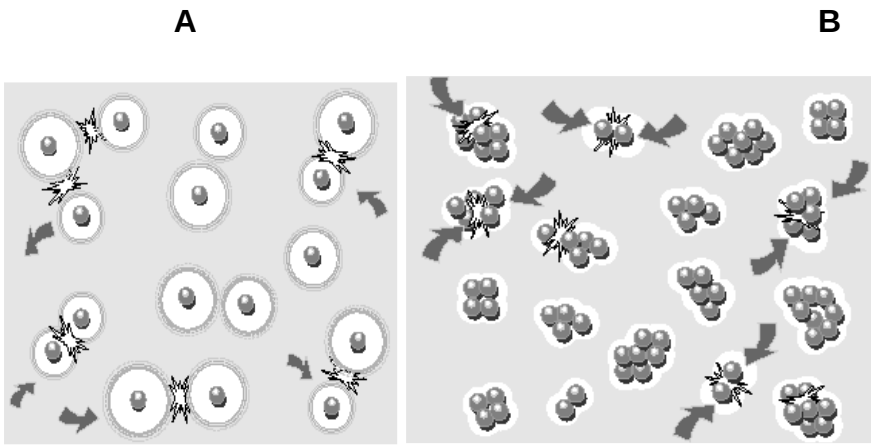

Figure 2.3: Charges neutralisation mechanism adapted from Lachhwani, 2005. Repelling charges between colloids and particles in figure 2.3(A) overcomed by the bioflocculants to allow formation flocs in figure 2.3 (B)

2.6.3 Colloid entrapment mechanism

The colloid entrapment mechanism make use of the principle of destabilisation and transportation (Sharma *et al.,* 2006). When there is excess dosage of chemical flocculants (such as inorganic salts of iron or aluminium) more than the amount required for charge neutralisation, precipitates of hydrous metal oxides is formed. These precipitates of hydrous metals oxides entrap the particles (Figure 2.4) in the suspension to form colloids and aids in sedimentation (Lachhwani, 2005). Figure 2.4 adapted from Lachhwani *(*2005).

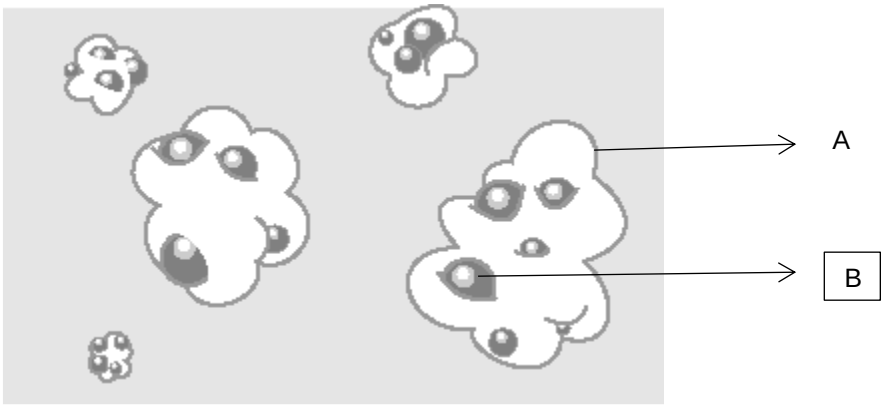

Figure 2. 4: Colloid entrapment flocculation mechanism adapted from Lachhwani, 2005. Precipitate of metal oxides (A) form colloids that traps the particles (B) and aids sedimentation.

2.6.4 Double layer (DLVO) Mechanism

The double layer mechanism also known as Derjaguin, Landau, Verwey and Overbeek theory (DLVO), describes the colloid particles which have counter ions of double layer around them namely, stern (with tightly associated counter ions) and diffuse layers (made up of counter ions that are less closely associated in proximity to the surface of the particles (Ntsangani, 2016). These two layers (Figure 2.5) work in reverse action, to first inhibit aggregation and as the repulsion of one of the layers decreases repulsion, there is an increase in aggregation of particles. In the process of addition of cations, there is an improvement of bioflocculation due to reduction in the size of the double layers and repulsive forces between the particles (Ntsangani, 2016). The reduction in the repulsive forces (electrostatic repulsion and van der Waals) between the particles in the two layer facilitates bioflocculation attraction. The

DLVO theory combines the van der Waals attraction with the double-layer repulsion as shown and adapted from Tadros (2005) in Figure 2.5.

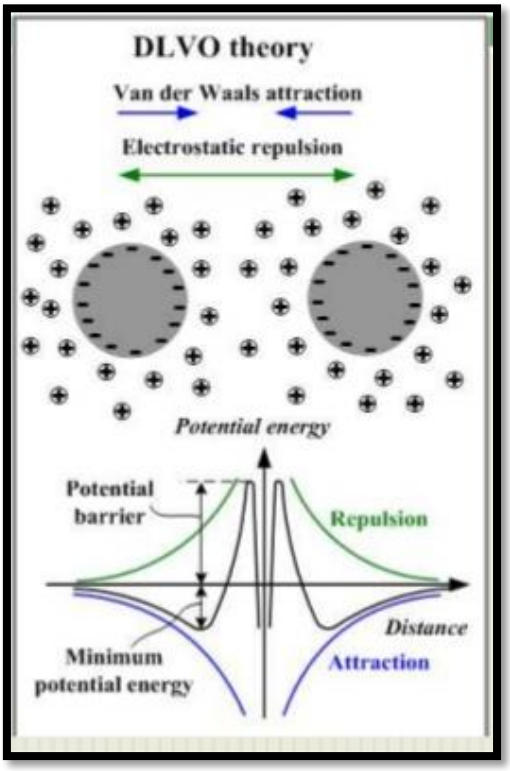

Figure 2. 5: DLVO theory mechanisms adapted from Tadros, 2005

2.7. Role and determinants of the constituents of EPS

The role of EPS that is secreted outside the cells in the interaction of bacteria cells and their environment is related to constraint effects based on the amount of available carbon or energy sources (Laspidou and Rittman, 2002). EPS is a source of renewable resources (Wang *et al.*, 2010). Sheng *et al.* (2010) have reported that bacterial secreted EPS are usually in the form of capsule, loosely or tightly bound slime based on their interaction with other cells and the method of extraction used. The role of EPS have been reported in common wastewater treatment systems in the sludge-flocs formation (Zhang *et al.,* 2012), flocculation dewatering (Houghton *et*

al., 2001) and biofilms (Sheng *et al.,* 2010). This role is not only in accelerating the aggregation of particles by bridging in close proximity to the cells but it has also been found to influence the shape, surface charge adsorption (Zheng *et al.,* 2008) and flocculation of the particles. The aggregation of particles and surface charge adsorption work together to bring about removal of contaminants (Liu *et al.,* 2004). Many researches have been focused on the various applications of EPS in recent years (Gupta & Thakur, 2016). These extracellular polysacharride play different roles and functions such as preventing dessication and protection from environmental stress especially to halophilic microorganisms that are being studied in this research. EPS also helps in the adherence to surfaces, sequestering nutrient in the environment, symbiosis, bioflm formation and protection of the intergrity of the bioflm (Biswas and Paul, 2017).

The EPS molecules are regarded as the major factor influencing the microbial adhesion process. EPS molecules strengthen the interactions between the microorganisms, as a result they determine the cell aggregate formation process on the solid surface (Biswas and Paul, 2017). Parker *et al. (*1996), established that the removal of exopolysaccharides from the cells of *Bacillus sp.* decreased their attachment to stainless steel surfaces. Different processes determine the composition of EPS which include active secretion, shedding of cell surface material, lysis of cells and other processes (Laspidou and Rittman, 2002). In modern concept, for bacteria to stay in close proximity and in microbial aggregates at a high density in a mixed population community, they need to produce these polymers to stay together (Laspidou and Rittman, 2002). Organisms produce natural products like bioflocculants commonly to display or show certain degrees of physiological and metabolic stabilities (Martin *et al.*, 2006). EPS can be tighthly bound to the cells as

capsular/sheath EPS or loosely bound to the cells as soluble/colloidal slime EPS (Sheng *et al.,* 2010). EPS can be referred to as capsular (CPS) or slime (EPS) exopolysaccharides (De Vuyst *et al*., 2001). Centrifugation is required to separate the microbial cells in sediment and the produced biopolymer in the supernatant (Castro *et al.,* 2014). This implies that in order to ensure reproducibility, the closest similar conditions must be ensured during isolation of organisms in the laboratory and in the scale-up fermentation (Martin *et al.*, 2006). The culture fermentation occurs under controlled conditions initially in a flask fermentation set-up. This should initiate the production of metabolites. The selection of extraction method is based on the understanding that the metabolites is partially or completely excreted by the cells into the (extracellular) medium or they are present within the cells (intracellular) and the cells need to be harvested and broken up (Seidel, 2006).

2.8. Growth phase of microorganism

Exopolymeric substances (EPS) are produced based on the costituents of the medium and on various metabolic processes involved changes during the growth phases. The production of these naturally occurring bioflocculant metabolites may be due to breakage of cells due to autolysis, release of outer membrane protein of the cell or environmental interaction with the cell (Cristina *et al.,* 2011). The bacterial growth curve of an organism is determined by the kind of nutrients present in the medium with respect to time and other factors. The growth phase is broadly divided into four phases typically: lag phase, log/exponential phase, stationary phase and death phase (More *et al.*, 2014). A study observed that *Bacillus subtilis* exhibits four phases and one clear transition phase (Figure 2.6). These phases were namely: lag phase, an exponential growth phase, a transition point, an early and late stationary

phase. There are different stress response between these growth phases (Prescott *et al.,* 2003; Blom *et al.,* 2011)

2.8.1 Lag phase

This is the first growth phase where the microorganisms are just new in the environments. Depending on the method of inoculation of the pre-culture inoculum, the optical density (OD) at the lag phase of the growth phase could affect be increased or decreased in the controlled media (Bisaria, 2007; Blom *et al.,* 2011). The rate of multiplication of bacteria is usually low as they tend to adjust to the new medium. Bioflocculant production has been reported by More *et al.* (2014) to be either growth related, growth synonymous or could be growth independent. The lag phase flocculating activity is usually low with respect to time as in most documented studies as the microorganisms are still adapting to the new environments (Okaiyeto *et al.,* 2015). One type of microorganism grow faster than others depending on their adaptability to the culture medium and conditions.

2.8.2 Logarithmic or exponential phase

The second phase of growth continues with incubation time as there are abundance of nutrients compared to the number of cells and the multiplication rate increases due to nutrients availability. Nwodo and Okoh (2013) reported that growth in the logarithmic rate and flocculating activity that is corresponding to the cell growth at this point has been observed in many studies. Kurane and Nohata (1991) reported maximum flocculating activity at the middle and late logarithmic growth phase spanning 2-3 days for *Alcaligenes latus.*

2.8.3 Transition phase

Transition phase has been reported to occur at a period when the culture cease from exponential phase and enters an early stationary phase. The phase represents metabolic switch from glycolysis to gluconeogenesis utilising different carbon sources (Blom *et al.,* 2011). A slight transitional period was observed between the logarithmic phase and the third phase, and early stationary phase, which is characterised by a slowly increasing microbial growth. Various investigations such as those by Li *et al.,* 2009 and Cosa, 2010 have reported that the maximum flocculating activity is reached between late logarithmic and the early stationary phase (Salehizadeh and Yan, 2014).

2.8.4 Stationary phase

In this phase, oxygen level decreases as the nutrients get depleted. This results in toxic waste production by microorganism, number of viable cells reduced due to death, and to survive the bacteria may produce bioflocculant degrading enzymes. The flocculating activity depending on the amount of produced bioflocculant remain stationary at this phase (Okaiyeto *et al.,* 2015a). Most bioflocculant production is associated with growth and reached maximum flocculating activity by the early stationary phase (Tawila *et al.,* 2018).

2.8.5 Death phase

Flocculating activity decreases as bioflocculant degrading enzymes are produced due to nutrients limitation (Lu *et al.,* 2005; Elkady *et al.,* 2011). The rate of cells death is higher than the rate of multiplication thereby reducing population. Cell lysis

occurs in addition to intracellular materials introduced into the environment. *Corynebacterium daeguense* maximum flocculating activity (>90%) was attained sharply at the death phase (Liu *et al.,* 2010). This is due to complete depletion of nutrients in this phase of growth. Only spore formers can survive at this stage and reproduce a population, if conditions become favourable again. Blom *et al.* (2011) used visualization of a 40 points experiment on the growth phase of *Bacillus* sp. to identified the phases namely I: lag phase, II: exponential growth phase, III:early

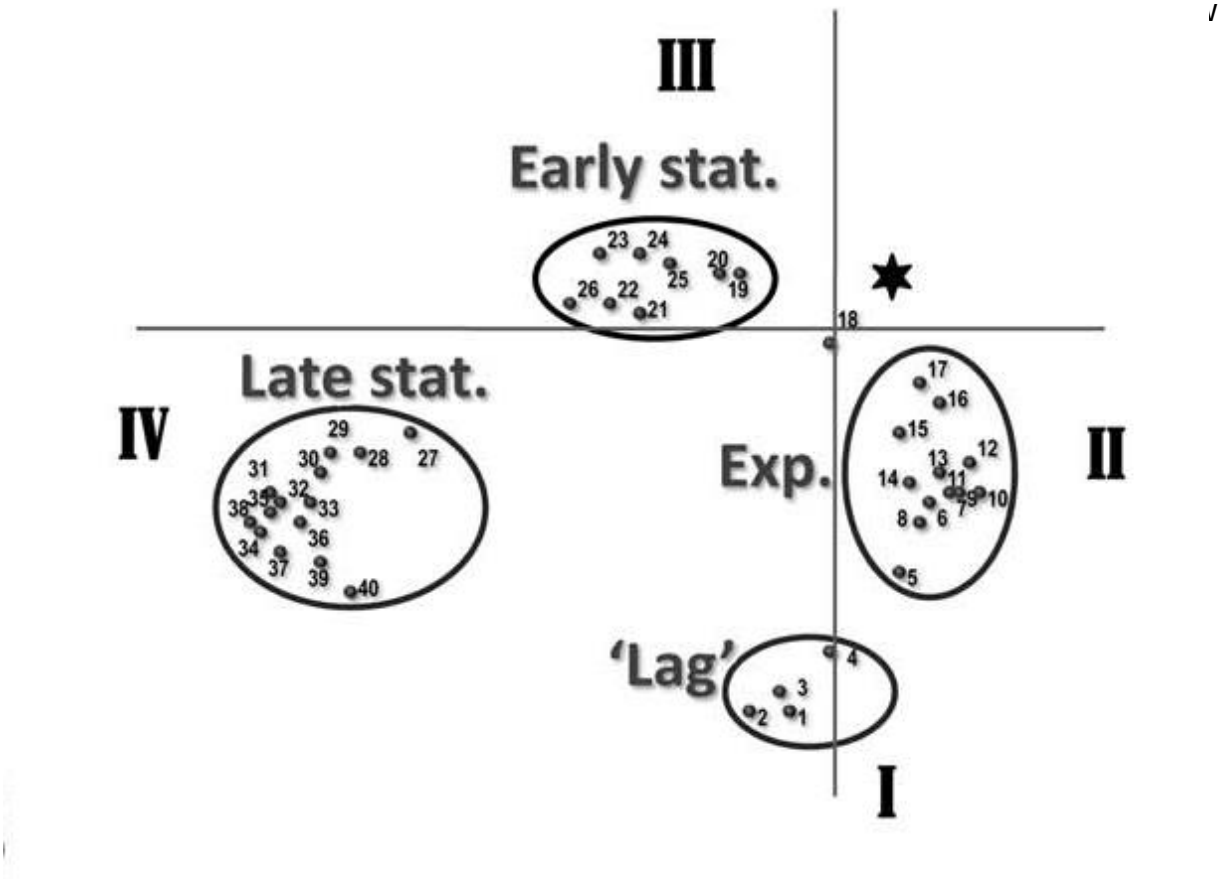

Figure 2. 6: The growth phase of *Bacillus* sp. Image adapted from Blom *et al.* (2011). (i) Lag phase (1-4) (ii) Exponential phase (5-17), A transitional phase (18*) (iii) Early stationary phase (19-26) (iv) Late stationary phase or death phase (27-40)

2.9 Optimisation of culture conditions for bioflocculant production and extraction of microbial natural products

Bacteria utilize available nutrients in the culture media to synthesize high molecular weight polymers with the action of specific enzymes. These polymers can be excreted into the external environment which may serve varied purposes.

2.9.1 Optimisation of culture conditions for bioflocculants production

Optimisation of culture conditions is done as a matter of improving both bioflocculant production and flocculating activity. Therefore, It is to optimize culture conditions when aiming to manipulate bacterial cells to synthesize these high molecular weight polymers, especially for industrial application and enhanced product-yield (Liu and Cheng, 2010). In this process of optimising the production of bioflocculants, there is a required need for effective design of specific set of culture parameters through optimal adjustments (Gupta & Thakur, 2016). This is achieved by running experiments involving changes made to independent variables (input variables), while keeping the other parameters constant in order to obtain output responses (Ghosh *et al.,* 2014). This is one of the goals of minimising the cost and maximising the output in the production of bioflocculants (Montgomery, 2005). These optimised conditions are those at which the microorganism produce at the desired level with limited inhibition. These conditions during a scale-up bioreactor experiment will generate the desired yield and results. Optimisation of culture parameters will minimise cost of production for bioflocculants using a scale up in a large bioreactor (Gupta & Thakur, 2016).

To increase and obtain high product yield is to optimise the culture medium conditions (Abd El-Salam *et al.,* 2017). Several bacteria strains are well adapted to glucose, including *Bacillus* sp. (MBFF19) which produced 0.8 g L^{-1} yield of bioflocculants in the presence of glucose as a carbon source (Zheng *et al.,* 2008). *Klebsiella terrigena* (R2) produced 27.7 g L^{-1} while utilising glycerol and ethanol for bioflocculant production (Gupta & Thakur, 2016). Several physiological factors largely affect the process of microbial flocculant production which includes culture composition medium and the physicochemical conditions of included parameters (Wang *et al.,* 2010; Fang *et al.,* 2013). Optimisation of the constituents of these culture medium affects productivity and distribution of bioflocculant (Salehizadeh & Shojaosadati, 2001; Abdel-Aziz *et al.,* 2011). The impact of various conditions and culture parameters if optimised does not only enhance and increase the yield, it also influences the bioflocculant production (He *et al.,* 2004). Culture parameters affect the productivity and flocculating efficiency of the produced bioflocculant (Salehizadeh and Yan, 2014). Growth conditions and media constituents also affect the production of bioflocculants.

2.9.2 Effect of various factors affecting bioflocculant production

Several factors have been reported to affect bioflocculant production. These factors range from inoculum size, temperature, pH of the medium, presence of metal ions, salinity, colloid types, mixing speed, aeration, cultivation time and bioflocculant concentration (Aljuboori *et al.,* 2015).

2.9.2.1 Effects of carbon and nitrogen sources

Carbon and nitrogen sources (Table 2.2) have been acknowledged in a number of studies to be a significant part of medium composition for bioflocculant production. The growth of organism being cultured is impacted by the availability of these carbon and nitrogen sources (Salehizadeh and Yan, 2014). These nutrients have been emphasized in the production of bioflocculants (Nwodo *et al.,* 2012 ; Ugbenyen and Okoh, 2013). More *et al.* (2015) postulated that the more carbon or nitrogen sources available or utilised during the period of growth will determine the high content composition of the biomolecule produced. Nitrogen sources are as imperative as carbon sources in the medium composition as they provide the appropriate biochemical and biophysical environment. Therefore, its optimization is crucial in bioflocculant production (Cosa *et al.,* 2013b). The composition and structure of the flocs formed by these bioflocculants also affect the preferred carbon and nitrogen sources utilised by the various microorganisms (Bura *et al*., 1998; Okaiyeto *et al.,* 2015). Suffice therefore to state that it is important to investigate the optimum carbon and nitrogen source for each microorganism during optimisation for high bioflocculant yield and high flocculation efficiency. Table 2.2 shows the various carbon and nitrogen sources reported to be utilised by different microrganisms.

Table 2.2 : Different energy sources utilised by different microorganisms (Table adapted from Okaiyeto *et al.,* 2015).

Microorganism	Carbon source	Nitrogen source	Citations	Flocculating Activity (%)	Yield
Klebsiella mobilis	Ethanol	Dairy wastewater	Wang *et al.,* 2007	95.4	2.58
Bacillus lichemiformis X14	Glucose	NH_4Cl	Li *et al.,* 2009	99.2	NA
Cobetia sp. OAUIFE	Glucose	Urea, Yeast extract and $(NH4)_2SO_4$	Ugbenyen *et al.,* 2012	92.78	NA
Virgibacillus sp. Rob	Glucose	Urea, Yeast extract and $(NH4)_2SO_4$	Cosa *et al.,* 2013b	91.8	2.43
Bacillus subtilis	Cane molasses	Yeast extract	Abdul-Rasack *et al.,* 2014	NA	4.92
Bacillus agaradhaerens C9	Glucose	Yeast extract	Wang *et al.,* 2015	NA	4.65
Bacillus atrophaeus	Glucose, Sucrose, Fructose	Ammonium chloride, Yeast extract and Complex nitrogen	This study	99	3.17
Bacillus sp. Gilbert	Sucrose	Ammonium chloride	Piyo *et al.,* 2011	91	NA
Paenibacillus mucilaginosus	Sucrose	Yeast extract	Tang *et al.,* 2014	97	NA
Aspergillus parasiticus	Starch	Peptone and Sodium nitrate	Deng *et al.,* 2005	98.1	NA
Enterobacter cloacae WD7	Glucose/Sucrose	$(NH_4)_2SO_4$	Prasertsan *et al.,* 2011	105	2.27

2.9.2.2 Effects of inoculum size on bioflocculant production

One of the most important parameters in the optimisation of culture conditions for bioflocculant production is the inoculum size. Inoculum size is the cell percent that is

required to inoculate the bioflocculant production medium. The inoculum size is pivotal to the other parameter optimisation experiments as it does not only affect the growth pattern of the organism but also the final product produced by the organism (Ntozonke, 2015). Ugbenyen and Okoh (2014), reported that 3% inoculum size was preferred by *Bacillus* sp. Gilbert for optimum bioflocculation production. *Klebsiella pneumoniae* was reported to prefer 1% inoculum size (Luo *et al.*, 2014) while Lie *et al. (*2009), reported similar inoculum size (1%) for *Bacillus licheniformis* X14 as well. The inhibition of bioflocculation process caused by the strain on the habitat of the microganisms is caused by large inoculum size, whereas small inoculum delay the onset of lag phase during bacterial growth (Zhang *et al.,* 2007). The reduction in the flocculating activity associated with large inoculum size is largely attributed to incomplete dispersion of excess bioflocculants (Suh *et al.,* 1997; Abd-El-Haleem *et al.,* 2008). Optimization of inoculum sizes is reported necessary and crucial, as it gives optimum bioflocculant activity and also differs with regards to different organisms (Ntozonke, 2015). Table 2.3 shows some of the reported optimum inoculum size by different microorganisms for their optimal bioflocculant production.

Table 2.3 : Different inoculum size for bioflocculant production reported for some microorganisms

Microorganisms	**Optimum inoculum size**	**Citations**
Klebsiella pneumoniae YZ-6	1 %	Luo *et al.,* 2014
Bacillus sp. Gilbert	3 %	Ugbenyen *et al.,* 2014
Oceanobacillus sp. Pinky	2 %	Cosa *et al.,* 2013a
Bacillus licherniformis	4 %	Xiong *et al.,* 2010
Bacillus pumilus	4 %	Makapela *et al.,* 2016

2.9.2.3 Effects of temperature and shaking speed

Different strains of microorganisms prefer different cultivation temperatures as well as agitation speeds (Table 2.4). The cultivation temperature is a crucial factor in any microbial growth as the metabolism of microorganisms is directly influenced by the cultivation temperature. The attainment of maximal growth, enzymatic and microbial activity is highly influenced (directly) by the cultivation temperature (Li *et al.,* 2008). At an optimal temperature, maximum growth activity and enzymatic activity is observed (Li *et al.,* 2008). In literature search, the optimal temperature for bioflocculant production varies in the range of 25 ^{o}C and 37 ^{o}C (Salehizadeh and Shojasadati, 2001), however this is also dependent on the optimal temperature of the strain. *Bacillus* sp. Gilbert isolated from Algoa Bay of South Africa preferred an optimal cultivation temperature of 28 ^{o}C (Piyo *et al.,* 2011).

Shaking or agitation is applied to aerobic fermentation so as to allow dissolved oxygen which is needed for nutrient absorption and enzymatic reactions. Different bioflocculant strains have different optimal shaking speed which is used as a determinant for the concentration of dissolved oxygen (Lie *et al.,* 2009). The shaking speed requirement is dependent on the oxygen requirement for each phase of the organisms. This accounts for the disparity between different organisms (Li *et al.,* 2009). *Bacillus* sp. Gilbert produced optimally at an agitation speed of 160 rpm (Piyo *et al.,* 2011).

Table 2.4 Different temperature and agitation (speed) effect on the production of bioflocculant as reported for few microorganisms.

Microorganism	Temperature (^oC)	Agitation speed (rpm)	Citation
Aspergillus flavus	40	180	Aljuboori *et al.* (2013)
Aspergillus parasiticus	28	150	Deng *et al.* (2005)
Paenibacillus elgii	30	220	Li *et al.* (2013)
Proteus mirabilis	25	130	Xia *et al.* (2008)
Bacillus cereus and *Pichia membranifaciens*	30	120	Zhang *et al.* (2008)
Bacillus atrophaeus	35	110	This study

2.9.2.4 Effects of metal ions

A bioflocculant obtained from *Klebsiella* sp. strain S11, a bacteria isolated from activated sludge was reported to flocculate effectively in the presence of Ca^{2+} with a flocculating activity of 69% (Dermlim *et al.,* 1999). Cations are major factors in the neutralization of surface charge of bioflocculant and the stabilizing of residual net charges formed due to the charges of the functional group on the bioflocculant. This aids in forming a bridge between the particles and the bioflocculant to form flocs; which improves bioflocculation (Wu and Ye, 2007). Biofloculant due to the carboxyl groups of amino acids in their structure are mostly negatively charged; thus exist an electrostatic attraction distances that needs to be reduced between bioflocculant and suspended particles (Wang *et al.,* 2011). When cation binds to the carboxylate group sites, it increases the floc in suspension and enhanced sedimentation (Li *et al.,* 2007). Table 2.5 shows some microorganisms with their preferred cations.

Table 2.5: Different cations used for bioflocculant process, reported for some microorganisms

Name of Organism	**Preferred cation**	**Citation**
Rhodococcus erythropolis & *Alcaligenes cupidus (consortium)*	Ca^{2+}, Al^{3+}	Banks *et al.,* 2006
Oceanobacillus sp. Pinky	Ca^{2+}, Al^{3+}	Cosa *et al.,* 2013a
Gyrodinium impudicum KG03	Fe^{3+}	Wu & Ye, 2007
Enterobacter sp. BY-29	Fe^{3+}	Yokoi *et al.,* 1997
Enterobacter cloacae WD7	Cu^{2+}	Prasertsan *et al.,* 2006
Citrobacter sp. TKF04	No cation required	Nakata & Kurane, 1999
Flavobacterium sp.	Ca^{2+}, Ba^{2+}, Mn^{2+}, but not Mg^{2+}	Gonzalez & Hu, 1991
Enterobacter sp. ETH-2	No cation	Tang *et al.,* 2014

2.9.2.5 Effects of initial pH of a production medium on bioflocculant production

The initial pH of a production medium determines the electrification of cells creating reduced oxidation potential that enables the absorption of nutrients by cells from the environment (Salehizadeh and Shojaosadati, 2001). It is one of the major factors in the production of bioflocculant which also affects its flocculating efficiency (Zheng *et al.,* 2008). Table 2.6 shows different intial pH effects on the production medium reported for some microorganisms.

Table 2.6: Different initial pH of production medium reported for few microorganisms

Organism	Initial pH	Citation
Halomonas sp. OKOH	Neutral (pH 7)	Mabinya *et al.* (2011)
Aspergillus parasiticus	Acidic range (pH 5 - 6)	Deng *et al.* (2005)
Klebsiellus sp. TG-1	Alkaline (pH 8)	Liu *et al.* (2013)
Halobacillus sp. Mvuyo	Neutral (pH 7)	Cosa *et al. (*2012)
Bacillus atrophaeus	Alkaline (pH 9)	This study

2.9.3 Fermentation time for bioflocculant production

Bioflocculant production occurs during different phases of microbial growth of microorganisms (Salehizadeh & Yan, 2014). The production of bioflocculant is mostly due to synthesis through nutrients assimilation during the growth of the microorganism. The production fluctuates with different periods of growth such as biosynthesis, cell autolysis, complexing of metals and enzymatic action in the cell medium (Lu *et al.,* 2005; Vatansever, 2005; Li *et al.,* 2009; Cosa, 2010). Different microorganism prefer different fermentation time at which the production of the highest amount of bioflocculant is produced (Table 2.7).

Table 2.7 Different fermentation time and culture conditions reported for few microorganisms.

Microorganism	Initial pH	Fermentation time (hours)	Citation
Aspergillus flavus	7	60	Aljuboori *et al.* (2013)
Aspergillus parasiticus	6	72	Deng *et al.* (2005)
Paenibacillus elgii	7.2	96	Li *et al.* (2013)
Proteus mirabilis	7.0	48	Xia *et al.* (2008)
Bacillus cereus and *Pichia membranifaciens*	3.6	24	Zhang *et al.* (2008)
Bacillus atrophaeus	9	96	This study

2.10 Optimisation of different factors using Plackett-Burman Design

In recent years, the classical method of changing one factor while making others constant has become ineffective since it does not clarify the process that entails in the interactions between factors and consequently their influence on the fermentation process or system (Rodriguez *et al.,* 2006). In order to reduce the large number of experiments requiring time and efforts of changing these factors, a simple statistical approach called Plackett-Burman design (Placket and Burman, 1946) can be used to observe the most significant factors that need further optimisation. This is required where large number of factors are to be investigated (Abd El-Salam *et al.,* 2017). Less numbers of experiments are used in the process. The Plackett-Burman design is a design for two levels experimental designs that allows the investigation of n+1 factors while using *n* experiments (Placket and Burman, 1946). The main effects being investigated is measured at their high concentrations and low level concentrations and their mean measurements are obtained. By using the formula,

the difference of the mean of the high level and low-level results of the experiment are calculated. Below is the matrix equation of Plackett-Burman designed to calculate each variable main effect :

$$E_{xi} = \left(\sum M_{i+} - \sum M_{i-}\right) / N$$

Where E_{xi} is the main effect of the variable, M_{i-} is flocculating activity in trials of the independent factors present in low concentrations; M_{i+} is the flocculating activity in trials of the independent factors present in high concentrations and N represents the number of trials divided by two. Elucidated positive sign results will indicate that the use of the factor at high concentration will be close to the optimum while the negative sign will show that the use of the factor at low concentration will be near to the optimum. Microsoft Excel is used to calculate the t-values in order to determine the variable significance for the equal unpaired samples.

2.11. Mollases in bioflocculant production

Molasses is a by-product of many industrial processes including the sugarcane industry and it is usually generated in large quantities beyond the current usage capacity. It is a strong liquid with a Chemical Oxygen Demand range of 80,000-120,000 mg/l and Biochemical Oxygen Demand (40,000 – 60,000 mg/l) that comprises approximately 50% (w/w) total sugars, vitamins and nitrogenous compounds (Moosavi-Nasab *et al*., 2010, Zhuang *et al.,* 2012). Molasses is a carbon source for bacterial growth and bioflocculant production. It requires treatment before

it is introduced into the environment because of its high biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) concentrations. There are different methods employed in treatment of the effluent containing molasses ranging from anaerobic, aerobic and physicochemical method. However, anaerobic method of treatment has been effective in removing 80% BOD while energy is recovered in the form of biogas. There is a further combination of the use of microorganism such as fungi, algae to reduce the organic load as well as physicochemical method such as adsorption, oxidation and membrane filtration before the effluent is discharged (Satawali & Balakrishnan, 2008). This is treatment is required to prevent environmental pollution. It is an excellent alternative substrate that can be used as a carbon source by many microorganisms for the production of exopolymeric substances (He *et al*., 2004). It was reported that pre treatment molasses and dilutions in range of 2% to 10% favours the growth of *Gordonia polyisoprenivorans* CCT 7 137 (Okaiyeto, 2016). It is also reported that organisms with bioflocculant producing potemial grown on media which utilises sucrose can possibly utilise molasses on a large scale (Okaiyeto *et al.,* 2015a)

Another industrial waste that has been used in bioflocculant production includes brewery and diary wastewater (Perle *et al.,* 1998; Doubla *et al.,* 2007). This waste contains large volumes of water composed of organic compounds. The presence of nutrient substances in brewery wastewater makes it a potential substrate for bioflocculant production by certain microorganisms (Chen *et al*., 2003). Diary wastewater contains high organic contents from the carbohydrates and proteins content of the milk (Perle *et al.,* 1998).

2.12 Chemical composition of bioflocculants

The characteristics of the bioflocculant produced are the pre-determining factor that influences its flocculating efficiency by microbes (Gao *et al*., 2006). The constituent in their structure, either as functional proteins (Zhang *et al.,* 1999) or functional polysaccharide (Huang *et al.,* 2005) also determines some bioflocculants functions. However, different bioflocculants possess different functional groups such as hydroxyl and carboxyl. These functional groups act as binding sites for suspended particles (Deng *et al.,* 2003). The infrared analysis reveals these functional moieties and the different derivate in a polysaccharide, glycoprotein or functional protein. TGA decomposition over time is employed to assess composition and thermal stability of organic compounds, including bioflocculant. Characterisation reveals the flocculating activity and the effect of pH and dosage size on the mechanism of action by the bioflocculant. He *et al.* (2004), reported that bioflocculants with a polysaccharide backbone tends to be thermostable in a pH range of 3 - 6.5. The composition of the protein, sugar or uronic acid content is carried out for proper naming of the compound. In a reported study by Feng and Xu (2008), bioflocculant BF3-3 was observed to be composed of 66.1% (polysaccharide) and 29.3% (protein). *Aspergillus parasiticus* bioflocculant is composed of carbohydrate (76.3%) and protein (21.6%) (Deng *et al.,* 2005) however, on the contrary, the bioflocculant produced by *Halomonas* sp. V3a consist of majorly polysaccharide (29%), neutral sugar component (20.6%), uronic acid (7.6%), amino acids (1.6%) and a sulfate group (5.3%) (He *et al.,* 2010).

2.13 Application of a bioflocculants

2.13.1 Rivers water and wastewater treatment processes

The exploration of EPS has been based on their unique potential utilisation in the industrial processes (Vu *et al*., 2009; Elkady *et al*., 2011), in wastewater treatment, oil recovery, drug delivery agents, and dredging (Wang *et al*., 2008). Drinking water sources can be classified as, ground water such as springs, infiltration galleries, wells and surface water (rivers, lakes, ponds, streams, impounded reservoirs, and stored water (Navin *et al.,* 2006): River water are referred to as surface water and do have a low chemical oxygen demand (COD) and turbidity. Studies on removal of BOD, COD and turbidity removal from wastewater using *Serratia ficaria* has been 87% for COD, 84.2% for turbidity and or 90.4% for colour (Gong *et al.,* 2008) whereas for *B. mucilaginous,* the BOD and COD removal efficiency for domestic waste water were 42.3% and 74.6%. However, a slight difference was obtained with the removal efficiency of 66.2% (COD) and 41.7% (BOD) from pharmaceutical wastewater, respectively (Lian *et al.,* 2008).

2.13.2 The removal of pathogenic microorganisms in water

One major cause for high morbidity and mortality rate globally is the failure to treat wastewater and fresh water properly (Madigan *et al*., 2012). Waterborne diseases result in more than 3000 deaths per day worldwide (WHO, 2011). The major aim of wastewater treatment is to remove suspended solids as much as possible and then re-introduce the effluent back into the environment. As this suspended solid material decays, it uses up oxygen, which is needed by the plants and animals living in the

water (Prescott *et al.,* 1996; Nester *et al.,* 2001). Ghosh *et al.* (2009) reports *Klebsiella terrigena* to be able to remove *Salmonella* sp. cells while the bioflocculant produced by *Paenibacillus* was used to harvest *Chlorella vulgaris* from a culture broth (Oh *et al.,* 2001).

2.13.3 Heavy metal removal

There is rapid expansion of industry due to manufactured development such as metallurgy, mining and metal surfacing industries (Sun *et al.,* 2018). There have been high levels of introduction of metallic toxic wastes into the environment. This also has detrimental effects on the biological functions and of health concerns to humans. This is due to their non-biodegradable component being recalcitrant in the environments (Xiong *et al.,* 2010). Cadmium, mercury and lead have been reported to be the most dangerous metals even at low concentration, especially in South Africa where mining contributes immensely to the economy of the nation (Benavides *et al.,* 2005). Different methods of remediation such as incineration and precipitation of metals residues using ion exchange technologies are in use but the methods are expensive and not eco-friendly (Subudhi *et al.,* 2015). In the treatment of wastewater, excess heavy metals are been removed by using flocculating activity of flocculants (Xia *et al.,* 2008). The EPS is likely to play a critical role in bio sorption of metals (Shi-Jie *et al.,* 2011).

Cationic polymers used in metal residue removal from water are hazardous to aquatic animals as they bind to the surfaces of organs of animals such as gills in fishes leading to suffocation and death. This is because of reduction in available oxygen and could lead to food insecurity (Mohamed, 2008; Abalaka *et al.,* 2015). Due to the various problems associated with the use of chemical flocculants, more

attention has been drawn to the use of biodegradable microbial flocculants that could easily clean up environments in recent times. This is carried out under strict environmental standards (Bank *et al.,* 2006; Wang and Chen, 2014). Bioflocculants produced by bacteria which are resistant to metals in wastewater treatment plants have been reported in some studies (Subudhi *et al.*, 2015). These biopolymers produced act as adsorbents of particulars based on physicochemical properties, as they possess various chemical functional groups such as: carboxyl, hydroxyl, amino or acetamido group in their polymer chains (Dobrowolski *et al.,* 2017). Salehizadeh and Shojaosadati (2003) reported the removal of Pb^{2+}, Zn^{2+}, Hg^{2+} and Cd^{2+} by *Bacillus firmus* bioflocculant.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Introduction

This section presents the different parameters investigated and carried out towards determining the bioflocculant producing potential of different marine strains; sample collection, isolation from different marine water and soil sediment using different cultivation media. Also, flocculating activity using production media was determined as well as identification of selected microbial strains in which the use of 16S rRNA method was adopted. Other methods adopted in the study include: optimisation of different culture conditions for bioflocculation, extraction and purification methods of bioflocculant, characterisation of purified bioflocculant, assessment of a bioflocculant for antimicrobial activity, optimisation of purified bioflocculant, application of purified bioflocculant in comparison with conventional flocculants using wastewater from Vulindlela wastewater treatment plant.

3.2 Sample collection, bacterial isolation and identification

3.2.1 Sample collection

Soil, water and sediment samples were collected from Richards Bay harbour, KwaZulu Natal, Republic of South Africa. Samples were collected aseptically in sterile plastic bottles of 250 ml capacity and McCartney bottles (25 ml), transported for analysis in a cooler box with ice to the Microbiology Laboratory of the University of Zululand, KwaDlangezwa, South Africa, and analysed within six hours after collection.

3.2.2 Cultivation Media

Nutrient agar, yeast extract agar (YEA) and M1 medium (YEA + starch) were used for the cultivation of microorganisms. In the cultivation of microorganism, non-selective media such as Nutrient media is used for general cultivation and maintenance. However, there are also fastidious organism that grows on enriched media such as yeast extract agar and M1 medium (YEA + starch). M1 agar and YEA agar were used for the isolation of actinomycetes with modifications (Ogunmwonyi *et al.,* 2010). The basal ingredient in nutrient agar per litre consists of meat extract (1.0 g), peptone (5.0 g), yeast extract (2.0 g), sodium chloride (8.0 g), and bacteriological agar (15 g) as solidifying agent. All ingredients were dissolved in 1 litre of filtered seawater. M1 consist of 10 g starch, 4 g yeast extract, 2 g peptone and 18 g bacteriological agar were dissolved in 1 litre of filtered seawater as described by Mincer *et al*. (2002) and Ogunmwonyi *et al.* (2010). Yeast extract agar composed of yeast extract (3 g), peptone (5 g) and bacteriological agar (15 g) supplemented with 10% starch (2.3 g) in I litre of filtered marine water.

3.2.3 Isolation of Microorganisms

Tenfold serial dilution of the water samples was prepared with sterile saline solution. One hundred microliter (100 µl) of the diluted water samples was inoculated to nutrient agar, yeast extract agar and M1 agar plates using spread plate techniques. To isolate microorganisms from the sediment samples, the dry/dilute and dry/stamp method slightly modified were used (Jensen *et al.,* 2005).

3.2.3.1 Dry/stamp Method

Sediment samples were dried in a laminar flow hood overnight. An autoclaved foam plug (2 cm in diameter) was pressed unto the sediment first and repeatedly pressed onto the surface of the nutrient agar, yeast extract agar and M1 agar plates in clockwise direction creating a serial dilution effect (Jensen *et al.,* 2005).

3.2.3.2 Dry/dilute Method

Dried sediment sample (0.5 g) was diluted with 5 ml of saline solution. The diluted samples were shaken vigorously and allowed to settle for a few minutes. One hundred microliter (100 µl) of the resulting solution was inoculated onto the surface of nutrient agar, yeast extract agar and M1 agar plates using spread plate techniques (Ugbenyen *et al.,* 2017).

3.2.3.3 Incubation

All plates were incubated at 37 ^{o}C for 24-72 hours after which the plates were observed for growth.

3.2.3.4 Subculturing

Single colonies were selected randomly based on their colour, size, structure, morphology, subcultured onto nutrient agar plates and incubated at 37 ^{o}C for 24-72 hours to obtain pure cultures.

3.2.4 Purification and identification of bacterial organism

Pure colonies obtained after the incubation (or sub culturing) for 24-72 hours at 37 ^{o}C were used for screening for bioflocculant producing bacteria and identification. Identification was done based on their morphology, cultural characteristics using Bergey's manual of systematic bacteriology (Christen, 2008).

3.3 Screening for bioflocculant production

3.3.1. Production medium and bacterial growth

The production medium composed of ingredients listed in Table 3.1 was prepared as described by Zhang *et al.* (2007) in marine water. The production medium was prepared by dissolving the ingredients (Table 3.1) in marine water and autoclaved for 15 minutes at 121 ^{o}C. One loopful of bacterial colony was inoculated into 50 ml of sterile production medium in a 100 ml conical flask. This was the initial method used for the production medium and was carried out according to Zhang *et al.* (2007). A standardised inoculum was hereafter used after the inoculum size for the selected bacterium was obtained. The inoculated medium was incubated at 30 ^{o}C for 72 hours in a shaking incubator with a rotating speed of 160 rpm. After the incubation period, 2 ml of the fermentation broth was then centrifuged (8000 xg, for 30 minutes at 4 ^{o}C) to remove the cells, while the cell-free supernatant was used as crude bioflocculant to determine the flocculating activity (Xia *et al.,* 2008).

Table 3.1 Production medium for screening microorganism for production of bioflocculant

Bioflocculant pre-culture composition	Ingredients
Glucose	20.0 g
Yeast Extract powder	0.5 g
Urea	0.5 g
$(NH_4)_2SO4$	0.2 g
K_2HPO_4	5.0 g
KH_2PO4	2.0 g
NaCl	0.1 g
$MgSO_4.7H_20$	0.2 g
Marine water	1 litre

3.3.2 Determination of flocculating activity.

The flocculating activity tests were carried out according to the method previously described by Kurane *et al.* (1994) with slight modifications as described by Ugbenyen and Okoh (2013). Four gramme per litre of kaolin clay suspension was used as a test material. Three millilitres (3 ml) of 1% $CaCl_2$ and 2 ml of cell-free supernatant were added to 100 ml of kaolin clay suspension (4.0 g/l) contained in a 250 ml conical flask. The mixture was agitated vigorously, poured into a 100 ml measuring cylinder and allowed to stand for 5 minutess at room temperature for sedimentation. A control experiment was prepared using the same method but the bioflocculant was replaced by sterile culture medium. The top clear solution (1cm)

was used to determine the optical density at 550 nm with a spectrophotometer (Pharo 100, Merck KGaA, Germany) (Xia *et al.,* 2008).

Flocculating activity was calculated using the following formular equation:

Flocculating Activity = {(A - B)/A} x 100%

Where *A* = optical density at 550 nm (OD_{550}) of control and *B* = optical density at 550 nm (OD_{550}) of a sample.

3.4 Molecular identification using the organism 16S rRNA gene

The bacterial isolate was cultured in 50 ml fresh Luria Broth (LB) and incubated at 37 ^{o}C on rotatory shaker speed of 200 rpm for 16 hours. The bacterium was further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacterium, the universal primers 27F and 1492R were used to amplify the 16S target region of this bacterium (Lane *et al.,* 1991; Turner *et al.,* 1999). The bacterial strain was analysed using the 16S rRNA and the phylogenetic tree was constructed with similar sequences found in Gen Bank. Nucleotide sequence analysis based on gyraseA gene using the Basic Local Alignment Search Tool (BLAST) of the 16S rRNA. The amplified and purified PCR products were used to determine the sequence of the bacteria. The findings were compared with the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree was thereafter constructed as described by Okaiyeto *et al.* (2013). These isolates were stored in the 20% glycerol broth at -80 ^{o}C freezer in the Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, KwaZulu-Natal, South Africa.

3.5 Optimisation of culture conditions for bioflocculation production

To enhance and increase the yield and flocculating efficiency of a bioflocculant, the conditions in the culture broth must be optimised by varying factors such as carbon and nitrogen sources, metal ions, aeration ratio, initial pH effect, inoculum size, culture incubation time and temperature effect (Salehizadeh and Yan, 2014).

3.5.1 Effect of inoculum size on bioflocculant production

To determine the optimum inoculum size, the stock culture ranging from 1% (0.5 ml), 2% (1.0 ml), 3% (1.5 ml), 4% (2.0 ml) to 5% (2.5 ml) was prepared and inoculated into 50ml of production medium. Flasks were incubated at 30 ^{o}C for 72 hours at 160 rpm. To measure the flocculating activity, three millilitres of 1% $CaCl_2$ (1% w/v) and 2 ml of supernatant from the centrifuged production medium were added to 250 ml conical flasks with 100 ml kaolin clay suspension (4 g/l). The solution was agitated and poured into a graduated 100 ml measuring cylinder and allowed to stand for 5 minutes at room temperature for sedimentation. One millilitre of clear supernatant was withdrawn and the flocculating activity was determined at 550 nm using spectrophotometer (Ntozonke *et al.,* 2017).

3.5.2 Effect of different carbon, nitrogen sources on bioflocculating production

Different carbon sources such as glucose, lactose, fructose, sucrose, carbonate, starch (20 g/l) were used and flocculating activities were measured. The impact of various organic and inorganic nitrogen sources using yeast extract powder, peptone, urea, $(NH4)_2SO_4$ and NH_4Cl were also monitored. The multiple nitrogen sources in

the original pre culture medium was replaced with equivalent amount (1.2 g/l) contained in the basal production medium as described by Lachhwani (2005).

3.5.3 Effect of initial pH of the production medium

The effect of initial pH was assessed by adjusting the pH of the production medium with 0.1 N NaOH and 0.1 N HCl to pH values ranging from 3 -12 and the flocculating activity was determined as previously described (Ntozonke *et al.,* 2017).

3.5.4 Effect of shaking speed on flocculating activity for bioflocculant production

The effect of different ranges of shaking speeds on the bioflocculating activity was assessed according to the method described by Zhang *et al.* (2007). Different conical flask containing 50 ml of production medium was inoculated with the 4% (v/v) inoculum size of the bioflocculant producing isolates and incubated at 30 ^{o}C for 72 hours in different speeds, ranging from 0-220 rpm. The flocculating activity was measured at 550 nm wavelength using a spectrophotometer.

3.5.5 Effect of metal ions on flocculating activity for bioflocculant production

The effect of metal ions on the flocculating activity was assessed using a method described by Nie *et al.* (2011). To obtain the flocculating activity, three millilitres of $CaCl_2$ (1% w/v) and 2 ml of bioflocculant solution was added to 100 ml kaolin suspension (4 g/l) in 250 ml conical flasks. From the standard method, the 1% $CaCl_2$ was replaced by various metal salt solutions (1%) (LiCl, $BaCl_2$, $FeCl_3$, $MnCl_2$, $MgCl_2$ and KCl). A control experiment was also prepared without cation added on 100 ml

Kaolin solution and 2 ml supernatant. The flocculating activity was determined as described by Ugbenyen and Okoh (2013).

3.5.6 Time course Assay

For the fermentation time, the method described by Gao *et al*. (2006) was used. The optimum medium composition obtained was used. The pH of the medium was adjusted with either 1N NaOH or 1N HCl to pH 9.0. The production medium made up of glucose (20 g/l), K_2HPO_4 (5 g/l), KH_2PO_4 (2 g/l), NH_4Cl (1.2 g/l) and $MgSO_4$-$7H_2O$ (0.2 g/l) was dissolved in 1 litre of marine water and autoclaved at 121 ^oC for 15 minutes. Saline solution (50 ml) was used to prepare the suspension of the bacterial isolate as the standard inoculum size. The optical density of the suspension (100 µl) in distilled water (1 ml) was first measured at OD_{660} and then gradually adjusted to 0.1. The optimum volume of the bacterial suspension obtained was inoculated into 50 ml production medium in a 250ml flask and incubated in a rotary shaking speed at 110 rpm, 35 ^oC for 120 hours. At every 12 hours up to 120 hours, 2 millilitres of the broth were drawn and assessed for flocculating activity in accordance with the method of Kurane *et al.* (1994). The bacterial growth was measured at OD_{660} in a spectrophotometer and the pH of the culture broth was also monitored for 120 hours.

3.6 Extraction and purification of bioflocculants

The method of extraction and purification of the bioflocculant was carried out as described by Chang *et al.* (1998) and Cosa *et al.* (2011). After 96 hours of fermentation, the bioflocculant from the production medium was harvested by centrifugation for 15 minutes at 5000 g at 4 ^oC. For isolation of EPS, one volume of distilled water was added to the supernatant phase and further centrifuged for 15

minutes at 5000 g at 4 ^{o}C to remove insoluble substances. Two (2) volumes of ice-cold ethanol was added to the culture supernatant, agitated properly and stored for 12 hours at 4 ^{o}C. The precipitate was vacuum-dried to obtain the crude bioflocculant. The crude bioflocculant obtained was re-dissolved in 100 ml of distilled water and one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v) was added. The mixture was shaken vigorously and left to stand at room temperature for 12 hours. The supernatant was centrifuged at 5000 g for 15 minutes at 4 ^{o}C and vacuum dried to obtain a pure bioflocculant. The weight of the dried bioflocculant was expressed in g l^{-1} culture (Poli *et al.,* 2009; Okaiyeto *et al.,* 2013, Gupta *et al.,* 2017).

3.7. Physicochemical composition of the purified bioflocculants

3.7.1. Chemical compositional analysis of the purified bioflocculants

The total sugar content analysis was done using the phenol-sulfuric acid method with glucose being used as a standard (Chaplin and Kennedy, 1994). Bradford assay with Bovine serum Albumin (BSA) as standard was used to measure the total protein content of the bioflocculant produced (Bradford, 1976). The carbazole–sulfuric acid method as described by Bitter and Muir (1962) was used to measure the content of uronic acid.

3.7 Chemical Analysis of a purified bioflocculant

3.7.1 SEM Analysis of bioflocculant

The morphological structure of the bioflocculant was investigated using the scanning electron microscope (SEM) equipped with elemental analyser (Oxford Instruments X-MaxN). Five milligram (5 mg) of purified bioflocculant was added to a silicon-coated

slide. The silicon-coated slide was fixed with a spin coater at 1000 rpm for 60 seconds. The SEM image of the purified bioflocculant, kaolin clay particles and flocculated kaolin clay particles were obtained (Xia *et al.,* 2008). The elements present in the purified bioflocculants were also determined.

3.7.2 Fourier transform infrared spectrophotometer (FTIR) analysis

The functional groups analysis of the purified bioflocculants was done using the Fourier transform infrared spectrophotometer to obtain the infrared spectrum of the dried purified bioflocculant sample. This was recorded at room temperature (25 ^{o}C) at wavenumber ranges of 4000 - 400 cm $^{-1}$ using a FTIR spectrophotometer (Xia *et al.,* 2008; Cosa *et al.,* 2013b).

3.7.3 Thermo-gravimetric analysis (TGA)

Ten milligrams of the bioflocculant was analysed using a TG analyser (Perkin Elmen Pyris 6 TGA) to obtain the pyrolysis of the purified bioflocculant at a temperature range of 30 – 900 ^{o}C and heating rate of 10 ^{o}C per minute. This rate was kept constant under flow of nitrogen gas (Yim *et al.,* 2007).

3.8 Flocculation characteristics of a purified bioflocculants

3.8.1 Effect of dosage concentration on flocculating activity (Jar test)

To assess the effect of dosage concentration of the purified bioflocculant, the method described by Makapela *et al.* (2016) was followed. A range of concentration of the purified bioflocculant between 0.2 and 1.0 mg/ml (w/v) were prepared. Two millilitres from each solution was mixed with 100 ml of kaolin clay suspension together with 3 ml of 1% (w/v) $CaCl_2$ in a 250 ml conical flask. The solution was

vigorously agitated, transferred into 100 ml measuring cylinder and allowed to stand for sedimentation for 5 minutes. One millilitre of the supernatant was drawn and used to determine flocculating activity in a spectrophotometer at 550 nm. The obtained optimum concentration dosage was used for subsequent experiments.

3.8.2 Effect of temperature on bioflocculant activity

The purified bioflocculant was dissolved in distilled water to give an optimum concentration of 0.4 mg/ml. Two millilitres of the bioflocculant solution was heated at various cultivation temperatures such as: 50, 60, 70, 80, 90, and 100 ^{o}C for a period of 30 minutes. Another tube (2 ml) was autoclaved with steam under pressure at 121 ^{o}C for 15 minutes. The residual flocculating activity of the purified bioflocculant for kaolin suspension (4 g/l) at room temperature was determined in order to obtain the temperature dependence of the purified bioflocculant (Ahmad *et al.,* 2015).

3.8.3 Effect of pH on the flocculating activity of purified bioflocculant

Optimum dosage concentration (0, 4 mg/ml) of purified bioflocculant was prepared and used to measured pH stability of a bioflocculant. The pH of individual 100 ml of kaolin solutions (4 g/l) were adjusted in separate flasks, ranging from 3 –12 prior to determination of flocculating activity. The flocculating activity values for each experiment were obtained at each pH values.

3.8.4 Effect of cations on flocculating activity

The effect of cations on the flocculating activity was assessed with the method described by Zulkeflee *et al.* (2012). Different metal ions such as Fe^{2+}, Ba^{2+}, Li^{+}, K+, Mg^{2+}, Fe^{3+} and Mn^{2+} were used to replace 1% $CaCl_2$ in the experiment. A solution of

bioflocculant optimum concentration was prepared. The effects of these metal ions on the flocculating activity of the purified bioflocculant were determined.

3.8.5. Antimicrobial activity of purified bioflocculant

Different Gram-positive and Gram-negative bacteria were first resuscitated by inoculation into sterile nutrient broth and incubated for 37 ^{o}C for 18-24 hours. 1 ml from each culture was inoculated into seperate test tubes containing 9 ml of sterile nutrient. The test bacteria of choice labelled on the test tubes were *E.coli* MHA 25922, *Bacillus cereus* and *Klebsiella pneumoniae* 1706. The cultures were then incubated overnight at 37 ^{o}C in Mueller-Hinton agar. The absorbance of each test tube was determined at 600 nm using ultraviolet–visible spectrophotometer to check and adjust the turbidity of each test tube to an absorbance range between 0.1- 0.5 which is within McFarland accepted standard. The antimicrobial activity of purified bioflocculant from *Bacillus atrophaeus* (hereafter called BA-CGB) was quantitatively determined by using the 96 well plates according to a method described by Eloff (1998). All the 96 well plates were inoculated with 50 µl of sterile nutrient broth. 0.2 g of Bioflocculant BA-CGB were dissolved in 2 ml of distilled water. The solution of BA-CGB poured into the first row and a 3 - fold dilution was then carried out whereby 50 µl from row A was taken to row B, mixed again and then from row B to subsequent rows until all the wells have been filled with different concentrations of BA-CGB. The last 50 µl was discarded to ensure each well remains 50 µl. 50µl of test organisms of choice were then added to each of the corresponding wells. Ciprofloxacin (20 µg / ml) was used as a positive control while sterile media was used as negative control. After incubation at 37 ^{o}C overnight in covered microplates, 40 microliter of 0. 2 mg/ml P-iodonitrotetrazodium violet (INT) solution was added to

each well as an indicator and the plate was further incubated at 37 ^{o}C for a further 0 - 30 minutes until there was no colour change observed. Positive results showed absence of pink colour due to inactivity or inhibition of the test bacteria from reducing the INT to formazan while a negative result will showed a pink colour due to the test bacteria reducing the yellow dye to pink colour (Wiengand *et al.,* 2007; Mfegwana and Mashele, 2016).

3.9 Treatment of waste water using a purified bioflocculant

Two litres of wastewater samples were freshly collected from Vulindlela domestic waste water treatment outlet and Mzingazi River from Richards Bay. Different parameters such as Biochemical Oxygen Demand (BOD), ammonia, nitrate, phosphate, pH, sulphate content, and Chemical Oxygen Demand (COD) were determined prior to and after flocculation with a bioflocculant. This was determined using a pH meter and spectrophotometer (Pharo 100, Merck KGaA, Germany) (Li *et al.,* 2013; Okaiyeto *et al.,* 2016). One hundred millilitres (100 ml) of wastewater sample was adjusted to the optimum pH of 11 in a 250 ml beaker together with 2 ml of 0.4 mg/ml bioflocculant solution and 3 ml of 1% (w/v) Calcium chloride ions. The mixture was vigorously agitated at 200 rpm for 3 minutes and then the speed was reduced to 45 rpm for 5 minutes. After 5 minutes of agitation, the flask was left to stand for 5 minutes at room temperature for sedimentation. The clear supernatant just 3 cm below the surface was used to determine the residual parameters of phosphate, sulphate content, nitrate, BOD and COD of the treated sample. The flocculating activity was also measured using a spectrophotometer at OD_{550}. In comparison, the bioflocculant was replaced with the chemical flocculants known as

polyacrylamide in the same concentration of 0,4 mg/ml. To calculate the removal efficiency of flocculant, the following formula was used;

Removal Efficiency (RE) (%) = $[C_0 – C/C_0] \times 100$ %

Where C_0 and C are the removal efficiency values before and after the flocculation process.

3.10. Statistical analysis

All data were conducted and collected in triplicates with results expressed as mean and standard deviation values. These data were subjected to one-way analysis of variance (ANOVA) using Graph Pad Prism ™ 6.1. Differences were considered at a significant level of $p<0,05$.

CHAPTER FOUR

4.0 Results and Discussion

4.1 Isolation, screening and identification of bioflocculant producing bacteria

Richards Bay Harbour was the habitat for the isolation of the preferred microorganisms. These microorganisms have evolved physiological and genetic features to survive in hypersaline environments (Edbeib *et al.,* 2016). Halophilic microorganisms are considered to be potential source of various types of biomolecules (Poli *et al.,* 2011). Research of naturally occurring flocculants has been the focus of scientists and biotechnologists worldwide due to lots of problems arising from the use of chemical coagulants (Saritha *et al.,* 2017; Biswas & Paul, 2017).

In this study, twenty-two (22) bacterial isolates were obtained from marine sediments, water and soil samples of Richard Bay Harbour in the Kwazulu Natal, South Africa. They were classified according to their colony colour, size and structural morphologies. The bacterial isolates were screened for bioflocculant production using kaolin clay suspension. Among the screened isolates, three isolates showed significant flocculating activities greater than 70% in kaolin clay suspension. The selected three isolates with their corresponding flocculating activities were named as S1(94%), S13(97%) and S15(74%), respectively. Identification using 16S rRNA revealed that S1, S13 and S15 were identified as *Bacillus safensis*, *Bacillus pumilus* and *Bacillus atrophaeus*, respectively. S15 isolate *(Bacillus atrophaeus)* with an initial flocculating activity of 74% was selected for the entire study on the basis that its flocculating activity was the least out of the three isolates that have flocculating activity above 70%.

The selected bioflocculant-producing bacterium (S15) was isolated from the soil sample of Tuzi Gazi Harbour beach line, Richards Bay. The Tuzi Gazi harbour beach is an example of a marine environment that has been reported to be a habitat to moderately halophilic microorganisms (Biswas & Paul, 2017). *Bacillus atrophaeus* on nutrient agar plate, appeared to be circular and possess smooth and milky white colonies, with irregular edges, mostly flat. It also possesses mucoid and ropy colony morphology which has been reported to be the basic identification properties for bioflocculant producing potential such as *Bacillus* sp. UPMB13 (Amir *et al.,* 2003; Zulkeflee *et al.,* 2012).

The bacterium was further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacterium, the universal primers 27F and 1492R were used to amplify the 16S target region of this bacterium (Lane *et al.,* 1991; Turner *et al.,* 1999). The bacterial strain was analysed using the 16S rRNA and Nucleotide sequence analysis based on gyraseA gene using the Basic Local Alignment Search Tool (BLAST) of the 16S rRNA revealed that the S15 bacterium has 98.64% similarity to *Bacillus atrophaeus* (*B. atrophaeus*) strain SRCM191359 with accession number CP021500.1. The selected organism was simply referred to as *Bacillus atrophaeus.(picture of colonies on culture plate in appendix section)*

Bacillus atrophaeus (formerly known and misclassified as *Bacillus subtilis var niger* or *Bacillus subtilis* subsp. globigii (simply called BG) belongs to the great diversity of industrially important *Bacillus* genus strains (Sella *et al.,* 2015). Nakamura (1989) first proposed the species *B*. *atrophaeus* after examining a number of pigmented and non-pigmented strains of *B*. *subtilis.* Burke *et al.* (2004) reported that a cluster of

strains ATCC 9372 was designated as a new sub-species, *Bacillus globigii*. *Bacillus atrophaeus* has been identified to belong to a group of useful bacteria with known production of biomolecules (Ma *et al.,* 2018).

Bacillus atrophaeus (atrophaeus – black, phalis – brown, atrophaelis, dark brown) is a Gram-positive rod shaped bacterium, motile, facultative anaerobic, produces ellipsoidal endospores with characteristics virtually closer to that of *Bacillus subtilis,* however on a medium containing organic nitrogen source they produce a brown pigment after 2 to 6 days (Nakamura, 1989; Burke *et al.,* 2004). *B. atrophaeus* has been used as an indicator organism in drinking water treatment as well as a biological indicator in assurance of sterilization (Sella *et al.,* 2009). Reports show that multiple environmental signals trigger the onset of production of spores such as nutrient starvation and high cell densities where a set of cells diverts into a genetic programme (Veening, 2007).

4.2 Optimisation of pre-culture medium conditions of *Bacillus atrophaeus*

Optimisation is aimed at exploring different growth medium compositions and culture conditions in order to improve bioflocculant production and flocculating activity under laboratory conditions (Korsten and Cook, 1996; Chen *et al.,* 2011). Different parameters known to have effect on bioflocculant production were investigated including inoculum size, carbon and nitrogen (energy) sources, cations effect on flocculating activity, pH, temperature, shaking speed, fermentation time in order to improve flocculating activity and increase in yield of bioflocculant production of *B. atrophaeus*. This is achieved by running experiments involving changes are made to independent variables (input variables), while keeping other parameters constant in

order to obtain output responses (Ghosh *et al.,* 2014)

4.2.1 Effect of inoculum size on bioflocculant production

The effect of inoculum size on bioflocculant production was investigated for *Bacillus atrophaeus* ranging from 1- 5% (v/v). Previous reports from Salehizadeh *et al.* (2001) and Ugbenyen *et al.* (2012) have shown that inoculum size has an effect on the production of bioflocculant and flocculating activity. Small inoculum size prolongs the log phase of microbial growth and large inoculum size limits and inhibits the bioflocculant production due to nutrient limitation (Salehizadeh and Yan, 2014).

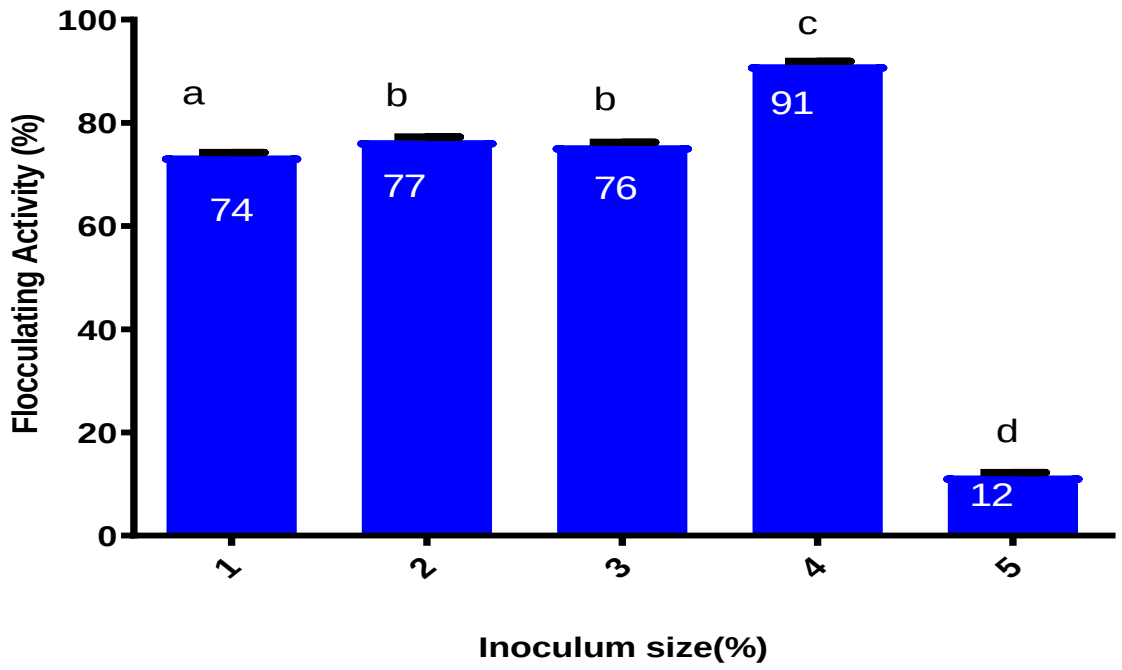

Figure 4. 1:The effect of inoculum size on bioflocculant production. Percentage flocculating activities with different alphabets (a, b, c and d) are significantly different ($p < 0.05$) from each other

In Figure 4.1, the flocculating activity increased as the inoculum size increases from 1% until it reached the highest flocculating activity of 91% at 4% (v/v). The flocculating activity thereafter decreased drastically to 12% at 5% (v/v) inoculum

size. This decrease in the flocculating activity is an indication of less bioflocculant being produced. The inoculum size of 4% (v/v) was used in the subsequent experiments. Similarly, Ntozonke *et al.* (2017) reported 4% (v/v) inoculum size for bioflocculant production by *Bacillus sp*. *Bacillus licheniformis* and *Bacillus pumilus* were also reported to optimally produce bioflocculant at an inoculum size of 4% (v/v) (Xiong *et al*., 2010; Makapela *et al.*, 2016). Contrary to the findings of this study, Wang *et al.* (2007) reported highest flocculating activity by *Klebsiella mobilis* at an inoculum size of 5% while Luvuyo *et al.* (2013) reported the highest flocculating activity of 92% with an inoculum size of 1% obtained from a mixed culture of *Methylobacterium* sp. and *Actinobacterium* sp.

4.2.2. Effect of carbon and nitrogen sources on bioflocculant production

In a number of studies, carbon and nitrogen sources are important nutrient factors, which have significant effects on the production medium of bioflocculant (Ugbenyen & Okoh, 2013; Salehizadeh and Yan, 2014). Bacterial growth and bioflocculant production is largely affected by varying carbon and nitrogen sources (Cosa *et al.,* 2011). *Bacillus atrophaeus* in this study, preferably utilised glucose (91%) as the best carbon sources (Figure 4.2). The bacterium also utilised sucrose (85%), fructose (86%), maltose (68%), galactose (6%) and starch (1%), meanwhile, glucose was used in all subsequent tests as it was statistically significant to other sugar sources. Similarly, a strain of *Bacillus* sp. was reported to utilise glucose as the best carbon source (85.65%) for bioflocculant production and sucrose as alternative (60.6%) (Ntozonke *et al*., 2017). In a study with *Serratia ficaria*, glucose was the suitable preferred carbon source for the organism to produce a bioflocculant (Cosa *et al.,* 2011). Studies on *Proteus mirabilis* TJ-1 (Xia *et al.,* 2008); strain RDL-1

(Lachhwanni, 2005) and *Streptomyces* sp. Gansen (Nwodo *et al.,* 2012) showed that the microorganisms effectively or optimally utilized glucose as the sole carbon source. In contrast, *Bacillus licheniformis* X14 was reported to utilise ethanol, sucrose and starch for the production of ZS-7 bioflocculant (Lee *et al.,* 2001). Piyo *et al.* (2011) reported *Bacillus sp*. Gilbert a marine bacterium isolate from Algoa Bay utilised sucrose as sole carbon source and ammonium chloride as nitrogen source with a flocculating activity of 72% and 91%, respectively. It is also noted that Liu and Cheng (2010) substituted molasses as carbon source in the production of bioflocculant by *Penicillium* sp. HHE-P7 in place of glucose. Molasses is a by–product of sugarcane processing. The bioflocculant produced using molasses was above 90% compared to that of glucose (95%). Similar result to this study where the *Bacillus atrophaeus* could utilise sucrose, showed that there is an indicated feasibility that *the tested organism* could utilise the by-product of sugarcane processing industry such as sugarcane molasses or bagasse which are cheaper substances than glucose (Okaiyeto *et al.,* 2016).

The most preferred nitrogen for the tested bacterium was ammonium chloride (97%), yeast extract as well as complex nitrogen (91%). Flocculating activity with other array of nitrogen sources were reported as peptone (79%), urea (72%) and ammonium sulphate (77%) (Figure 4.3). Ugbenyen *et al.* (2012) reported that *Cobetia* sp. OAUIFE poorly utilised tested nitrogen sources (peptone, tryptone, urea, casein) ranging from 12-52% for bioflocculant production while Cosa *et al.* (2013b) reported that complex nitrogen supported the bioflocculant production by *Virgibacillus* sp. *Proteus mirabilis* TJ-1 was reported to have utilised peptone (Xia *et al.,* 2008) while *Bacillus licheniformis* X14 (Lee *et al.,* 2001) utilised ammonium chloride as nitrogen

source for bioflocculant production. Piyo *et al.* (2011) also reported *Bacillus sp*. Gilbert utilised ammonium chloride (91%) as nitrogen source.

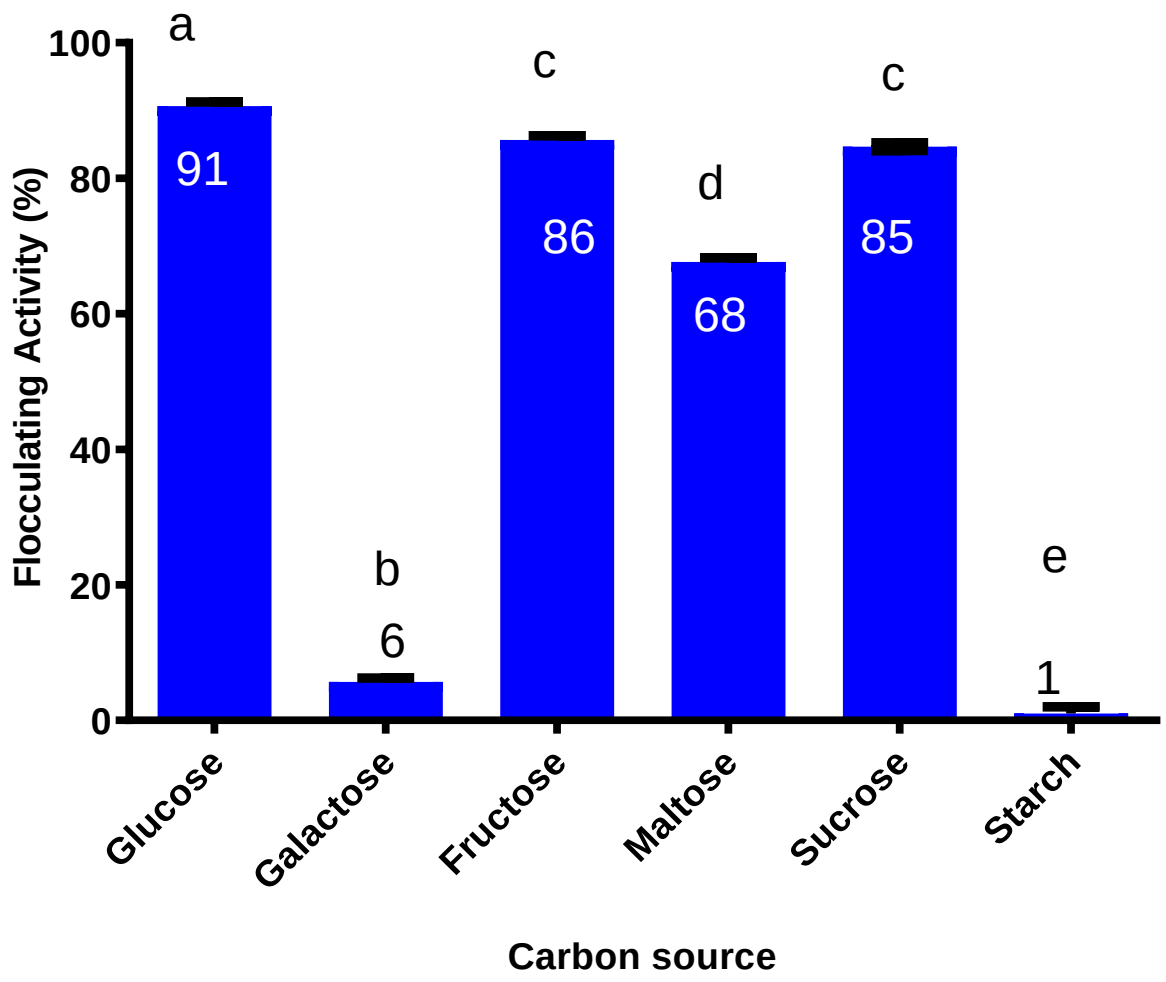

Figure 4. 2: The effect of carbon sources on bioflocculant production. Percentage flocculating activities with different alphabets (a, b, c, d, e) are significantly different ($p < 0.05$) from each other

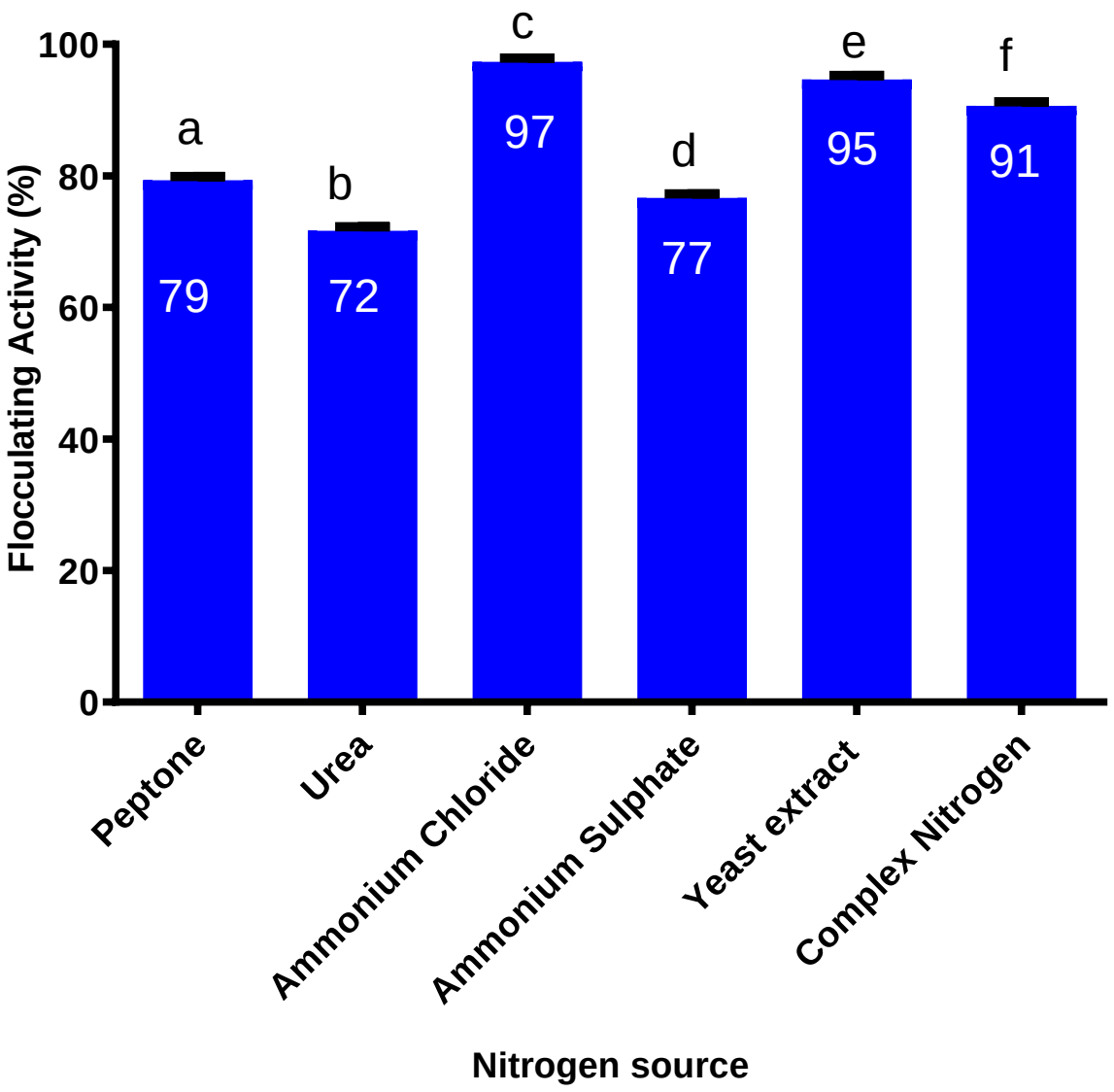

Figure 4. 3: The effect of nitrogen sources on bioflocculant production. Percentage flocculating activities with different alphabets (a,b,c,d,e,f) are significantly different ($p < 0.05$) from each other

4.2.3. Effect of cations on flocculating activity

The presence of cations in the bioflocculation process enhances the rate of flocculating activity (Li *et al.,* 2007). The net result is the neutralisation of the residual negative charge on the functional groups of the bioflocculant (Wu & Ye, 2007). This resulted into the increase of electrostatic attraction between bioflocculant and the

suspended particles for the formation of bridges between the bioflocculant and suspended particles (Wang *et al.,* 2011; Okaiyeto *et al.,* 2015). In this study, the flocculating rate was highly stimulated by Ba^{2+} (92%) followed by a comparable non-significant stimulation by Li^{+} (90%) and Ca^{2+} (88%) cations (Figure 4.4). This implies that there is a significance difference existing between the flocculating activities obtained using Ba^{2+} (92%) and Li^{+} (90%) as cations on the activity of the bioflocculant. This is judged as such, since the p- value obtained is less than 0.05. It therefore implies that Ba^{2+} can not be substituted for by Lithium cation.It is noteworthy that all cations tested except ferrous chloride (7%) stimulated flocculation process with flocculating activity above 80%. It has been reported that calcium chloride and aluminium chloride stimulated the flocculation rate of bioflocculant produced by marine bacteria (Cosa *et al.,* 2013a; Okaiyeto *et al.,* 2015). In a study on MBF-6 produced by *Klebsiella pneumoniae*, the bioflocculation process was inhibited by Al^{3+}, but stimulated by Na^{+}, K^{+}. Ca^{2+}, Mg^{2+} and Zn^{2+} (Luo *et al.,* 2014). The carboxylic functional group of the bioflocculant provide the adsorption sites for cations (Prasertsan *et al.,* 2006), thereby making the bioflocculant and kaolin clay particles to form complexes (Li *et al.,* 2007). The flocculating activity of the bioflocculant without the addition of any cation (control) as in Fig 4.4 was barely 50% indicating that the bioflocculant cannot yield a better flocculant without the use of cations and it is therefore cation dependent.

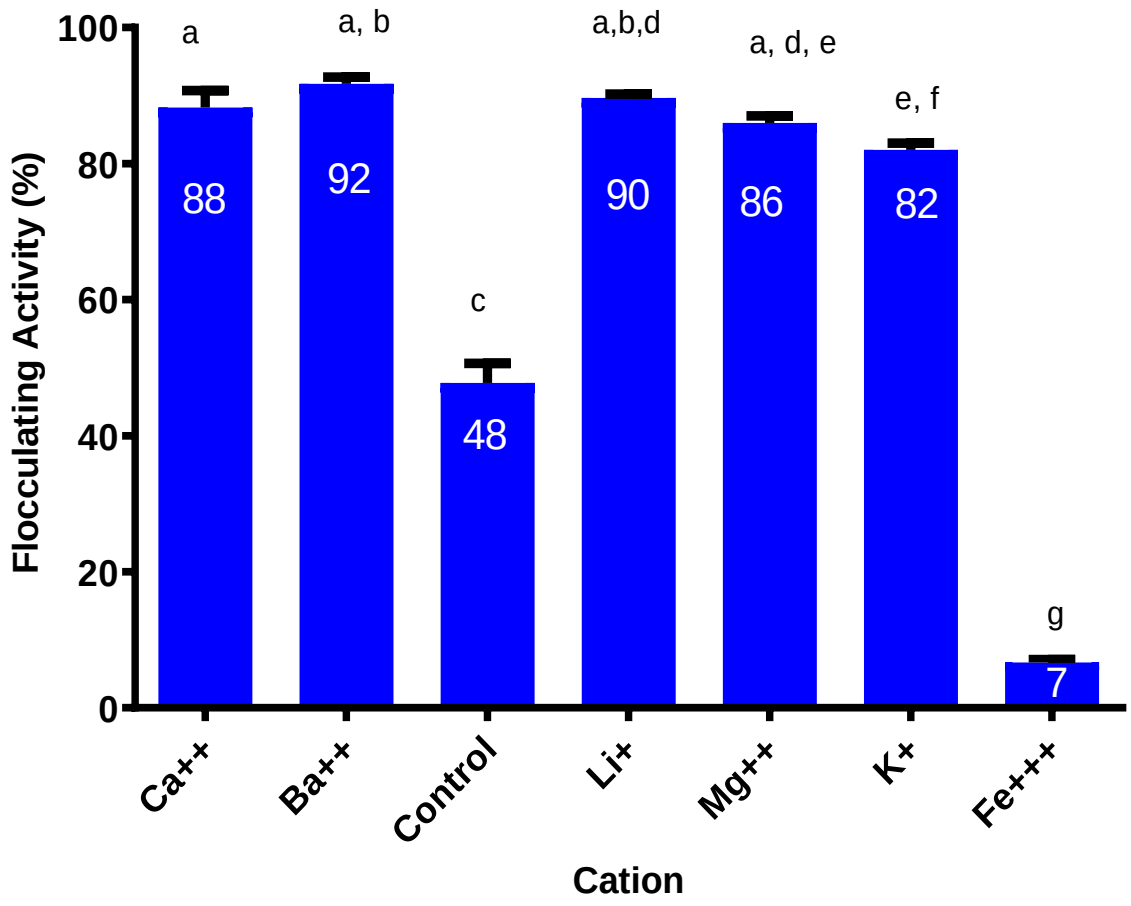

Figure 4. 4: The effect of cations on flocculating activity. Percentage flocculating activities with different alphabets (a,b,c,d,e,f and g) are significantly different ($p < 0.05$) from each other

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4.2.4. Effect of initial pH on bioflocculant production

The initial pH of a cell culture medium affects the redox potential and influence electrification state of the bacterial cells. This can affect enzyme reaction as it affects the biomolecules production (Luo *et al.,* 2014). Bioflocculant production depends on nutrient absorption and enzymatic reaction, which in turn affect the flocculating activity (Wang *et al.,* 2010). Figure 4.5 shows the effect of initial pH on the flocculating activity where the flocculating activity is steadily increasing from pH 3

(75%) to pH 8 (81%) and reached the optimum flocculating activity of 92% at pH 9. A remarkable decrease thereafter was observed at pH 10 (41%) to pH 12 (43%). The reason for the significant decline could be as a result of re-stabilization of the kaolin particles which further prevents agglomeration and bridging by the bioflocculant (Wang *et al.,* 2011).The bioflocculant production from the test bacterium was favoured by acidic pH (pH 3), neutral and slightly alkaline pH (pH 9). This was similar to the observed results from *Bacillus* sp. MAYA as reported by Ugbenyen and Okoh (2013) whereby the bacterium produced bioflocculant within the range of pH 3 - 9. The culture condition and production medium for *Bacillus* sp. MAYA was not favourable at alkaline pH 10-12, as occurred to the test bacterium of this study. Piyo *et al.* (2011) reported that the pH 8.42 for the habitat of *Bacillus* sp. Gilbert did not have any bearing on its bioflocculant production. However, Deng *et al.* (2005) and Liu *et al.* (2013) reported *Aspergillus parasiticus* and *Klebsiella* sp. TG-1 preferred acidic and alkaline conditions, respectively, for bioflocculant production.

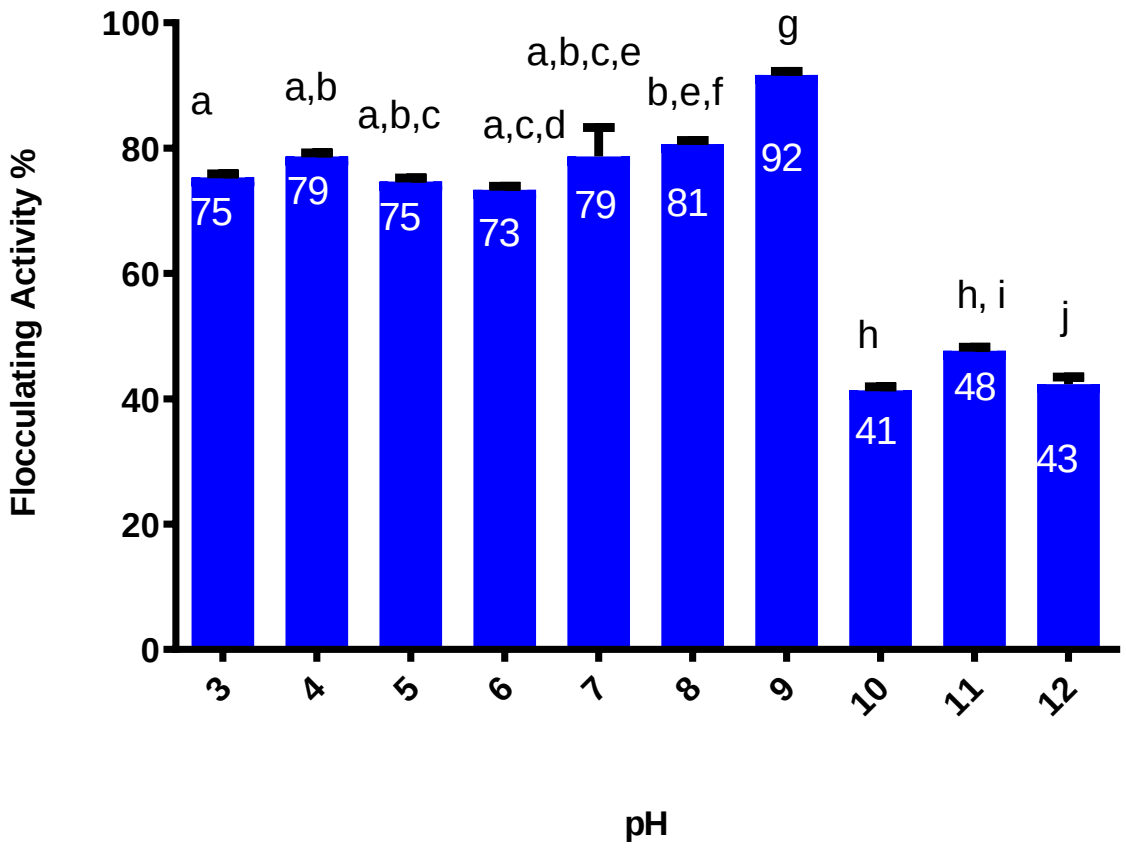


Figure 4. 5: The effect of initial pH on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.2.5. Effect of cultivation temperature and shaking speed on bioflocculant production

Different strains of microorganisms prefer different cultivation temperatures as well as agitation speeds. A review of literature indicates that the optimal temperature for bioflocculant production varies in the range of 25 ^{o}C and 37 ^{o}C (Salehizadeh and Shojasadati, 2001). *Bacillus atrophaeus* strain tested in this study showed low flocculating activity at 20 ^{o}C (52%) and steadily increasing from 25 ^{o}C (75%) until it reached optimum cultivation temperature at 35 ^{o}C (91%). This implies that the tested organism's (*Bacillus atrophaeus*) optimum temperature falls within the relative

optimal temperature for Bacillus, which is between 25 ^{o}C and 35 ^{o}C. Thereafter, a slight reduction in flocculating activities were observed at 45 ^{o}C (78%) and 50 ^{o}C (75%) which differences are non-significant (Figure 4.6). Bacillus species have been reported to have evolved various structural adaptations to higher temperature range by producing resistant spores (Nicholson *et al.,* 2000). Contrary to this finding, Luo *et al.* (2017) reported a high flocculating activity of 85.5% for *Klebsiella pneumoniae* YZ-6 at 30 ^{o}C while *Bacillus* sp. Gilbert preferred 28 ^{o}C as optimum temperature for bioflocculant cultivation and production. A consortium of *Staphylococcus* sp. and strain *Pseudomonas* sp. CYGSI optimum cultivation temperature was reported as 30 ^{o}C for bioflocculant production (Zhang *et al.,* 2007

Through continuous agitation, the sparingly soluble oxygen stimulates aerobic growth in the aqueous production medium (Lopez *et al.,* 2003). In terms of agitation speed, the flocculating activities at 0 rpm was 78% and this steadily increased from 91% at 55 rpm until it reached its significant optimum agitation speed of 110 rpm (99%). There was a slight non significant decrease to 98% (165 rpm) and further significant decrease to 83% (220 rpm) respectively (Figure 4.7). The differences in the shaking speed could be attributed to the oxygen requirement during the different growth phases of the organisms (Li *et al.,* 2009). *Bacillus licheniformis* X14 was reported to produce a bioflocculant at a shaking speed ranging from 140-160 rpm (Li *et al.,* 2009).

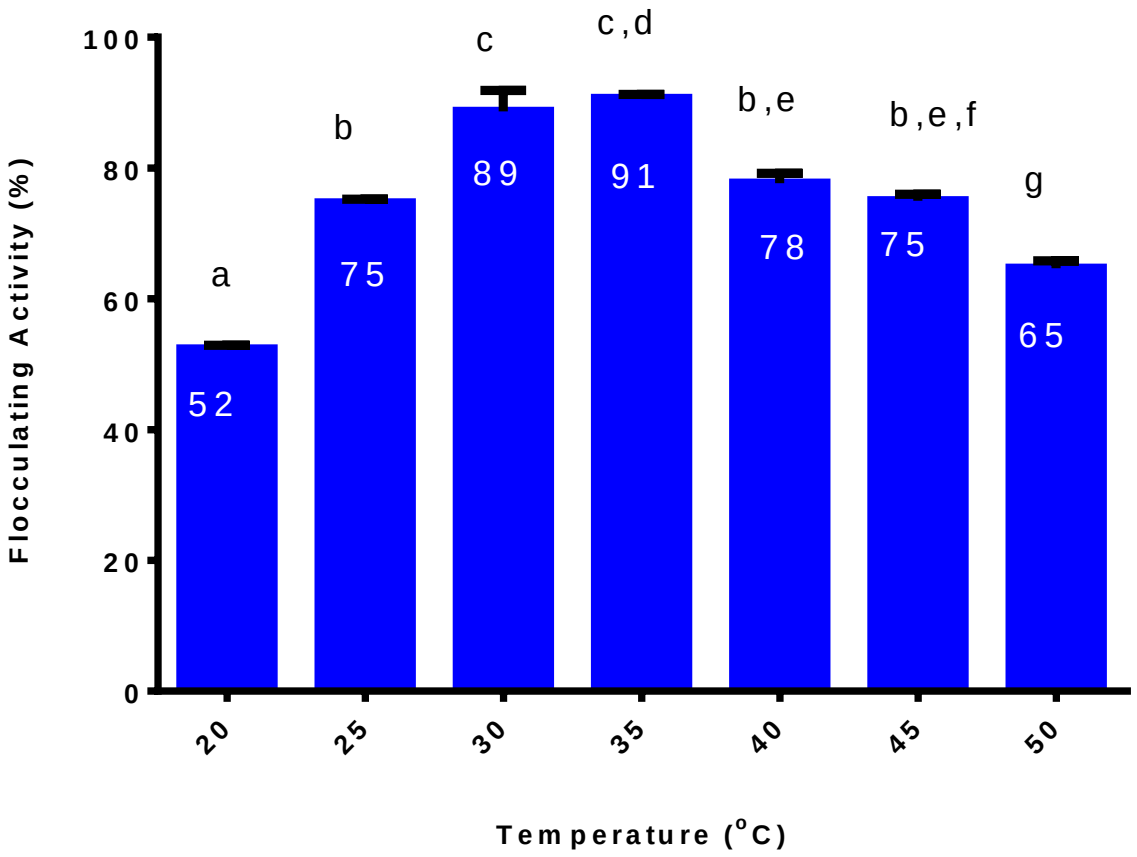


Figure 4. 6: The effect of cultivation temperature on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

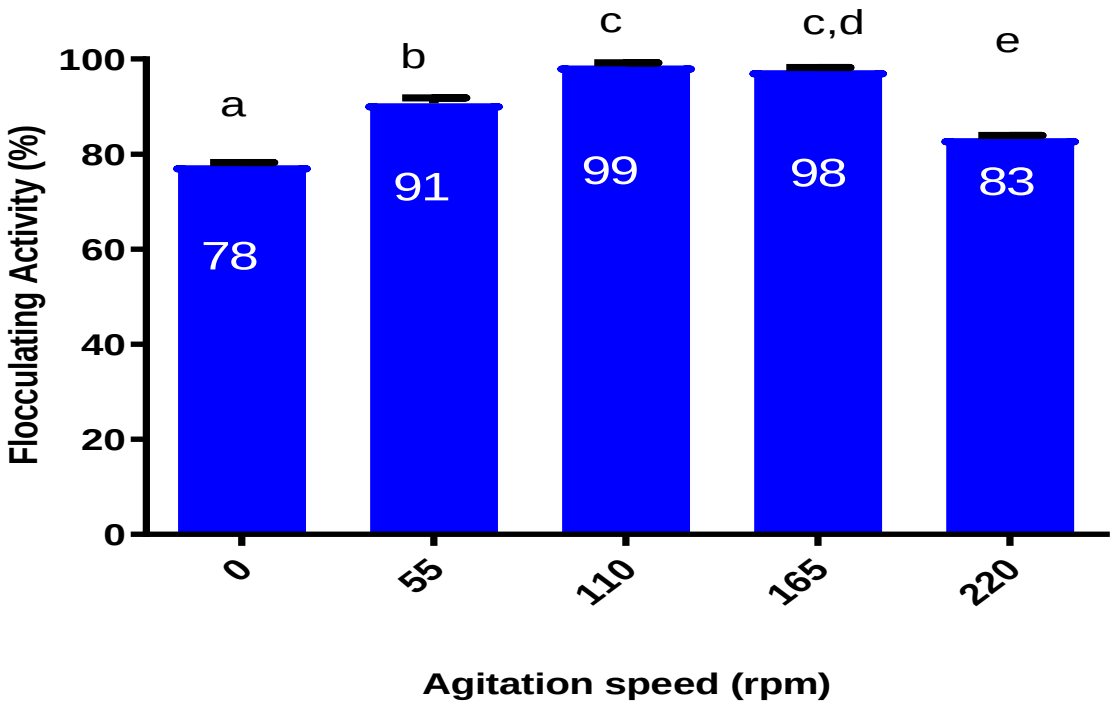


Figure 4. 7: The effect of shaking speed on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.3 Time course assay on bioflocculant production

The pH of production medium, as observed in the time course assay experiment (Figure 4.8) with *Bacillus atrophaeus* decreased from 9.00 to 5.84 during the 5 days of the experiment. The test organism has a minimal flocculating activity of 41% after first 12 hours of incubation and the cell growth represented by an OD_{550} was observed to be 0.382. Studies have reported that at this stage of growth (lag phase), the production of bioflocculant is not majorly associated with the cell growth (Li *et al.,* 2007), indicating that the bioflocculant was produced extracelularly by biosynthesis during its growth as a secondary metabolites (Lu *et al.,* 2005) to keep the cells close together. There was a remarkable increase in flocculating activity to 88% after 24 hours of incubation observed with OD_{550} of 1.020. The highest flocculating activity

was observed after 96 hours (99%) of growth and the cell growth increased to an OD_{550} of 2.622. After 96 hours of growth, there was a decrease in flocculating activity to 82% at 108 hours and an observed cell growth with an OD_{550} of 2,611. There was however an increase in flocculating activity observed at 120 hours to 95% but with an observed reduced cell growth with OD_{550} of 2.609. The decrease in flocculating activity from 99% to 82% at this period (108 hours) has been reported to be due to autolysis of bacterial cell and or in combination with bioflocculant degrading enzymes (Ugbenyen *et al.,* 2012). A similar finding was reported on a study of *Pantoea* sp., a novel organism isolated from Mtunzini Beach which reached the maximum flocculating activity after 96 hours (Ugbenyen *et al.*, 2017). Change in pH of the medium may also be attributed to the production of organic acids in the production medium during the process of formation of the bioflocculant components or from the glucose metabolism (Ntsaluba *et al*., 2011). Culture time could influence the production of bioflocculant and flocculating activity. Cosa *et al.* (2011) reported a study where the pH of the medium remained between (6.2 – 6.4) throughout the incubation period. Shimofuruya *et al.* (1995) reported similar findings in which flocculating activity of *Streptomycetes griseus* increased over time until it reached maximum flocculating activity after 84 hours. Flocculating activity of different microorganism and the time of release of bioflocculant into the culture medium is variedly dependent on the different culture time. Piyo *et al.* (2011) also reported that *Bacillus* sp. Gilbert produced bioflocculant because of biosynthesis with increasing cultivation time. This growth pattern of microorganism can be manipulated in order to minimise cost (Ntozonke *et al.,* 2017). The highest flocculating activity of the test bacterium was reached at 96 hours with the organism in the stationary phase. This is also similar to the reported findings on *Streptomyces* sp. which reached its highest

flocculating activity during the exponential growth phase indicating that bioflocculant production process is in synonymous with biosynthesis (Nwodo *et al*., 2012). *Bacillus licheniformis* cell growth and bioflocculant production *was* reported to reach the highest peak during the stationary phase at 96 hours simultaneously (Shih *et al*., 2001). This is in tandem to the test bacterium in this study. On the contrary, Liu & Cheng (2010) observed that the secretion of bioflocculant is not correlated with higher biomass as reported with *Penicilium* strain HHE-P7 cultivation on beef extract where a flocculating activity of 80% was produced with a growth biomass of below 3 g/l while a yeast extract yielded a flocculating activity of above 90% with a biomass growth of 9 g/l.

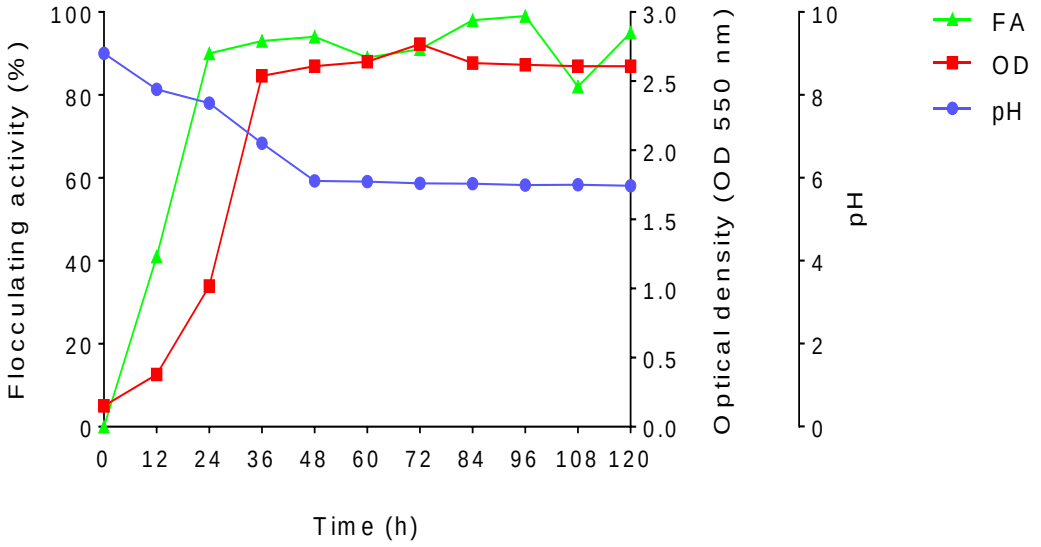


Figure 4. 8: The time course assay on bioflocculant production for *Bacillus atrophaeus.*

4.4 Extraction and purification of bioflocculant

After extraction, the crude bioflocculant (BA-CGB) from *Bacillus atrophaeus* strain yielded 5,916 g/l, a milky white colour substance. The purified bioflocculant, BA-CGB was a white powdered substance with a yield of 3,165 g recovered from 1l fermentation broth. The yield of BA-CGB in this study is higher than the 1, 6 g/l yield obtained from *Bacillus* sp. AEMREG7 (Okaiyeto, 2016). However, the produced bioflocculant was smaller in yield compared to a yield of 4.52 g/l reported for *Halomonas* sp. V3a by He *et al.* (2009) and a yield of 3.8 g/l reported by *Ochrobactium cicero* W2 yield of 3.8 g/l reported by Wang *et al.* (2013). A polysaccharide yield of 25.63 g/l produced from a culture of *Paenibacillus elgii* B69 using sucrose as carbon source was reported (Li *et al.,* 2013), which is 8 times higher than the tested bacterium produced. The yield of bioflocculant by microorganism is an important factor when considering its use in industry (Cosa and Okoh, 2014), for the tested bacterium, the observations of yield at the diferent phase can be further manipulated to increase the yield or with the use of bioreactor or genetic engineering (Liu *et al.,* 2017).

4.5 Characterization of the purified bioflocculant BA-CGB

4.5.1 FTIR analysis of the purified bioflocculant BA-CGB

The FTIR spectrum indicates important peaks, which correlates with the presence of various functional groups in the molecular chain of the bioflocculant (Okaiyeto, 2016). The FTIR analysis for bioflocculant BA-CGB showed a broad stretching peak observed at 3389 cm $^{-1}$ which indicated the presence of amine groups. The infrared spectrum revealed the presence of hydroxyl (3384 cm^{-1}), strong amide (1654 cm^{-1})

and amino (1090 cm^{-1}) groups (Figure 4.10). The presence of sugar derivatives was revealed at the weak peaks of 859 cm^{-1} whereas, the peak at 1460.62 cm^{-1} indicated the presence of uronic acid in the polysaccharide. The IR vibration peak at 1020 cm^{-1} revealed strong C-O stretching vibration for alcohols and this further predicts the presence of OH group and carboxylate ion in the bioflocculant (BA-CGB). The peak at 1654 cm^{-1} can be attributed to C-O stretching in NH_2 bending, CO-NH group from the polymeric and dimeric OH stretches of phenol or tertiary alcohol bends with the presence of carboxyl and hydroxyl groups. The strong absorption peak observed at 528 cm^{-1} is for halo-alkanes C-X bending vibrations and a typical characteristic of sugar derivatives. The presence of amino groups was reported by Abd El Haleem *et al*. (2014) in bioflocculants produced by tested strains QUST2, QUST6 and QUST9. The presence of hydroxyl, amide and amino groups may also be because of vibration of OH^- or NH_2 groups present in the sugar ring (Ntozonke *et al.,* 2017). Abd El Haleem *et al.* (2014) reported that peaks between 1304 cm^{-1} and 1654 cm^{-1} being consistent with the presence of carboxylate. The indicated functional groups of carboxyl, hydroxyl and amino groups revealed in the infrared spectrum of the reported bioflocculant MBF-6 (Piyo *et al.,* 2014) and in BA-CGB could serve as a confirmation that the bioflocculant is with a polysaccharide backbone; accounting for its thermal stability (Okaiyeto, 2016). This could explain the rationale behind the observed high flocculating efficiency of BA-CGB observed in this study with the various functional groups as enhancement of the flocculating process and binding capacity to the cations and the particles (Freitas *et al.,* 2009; Aljuboori *et al.,* 2014).

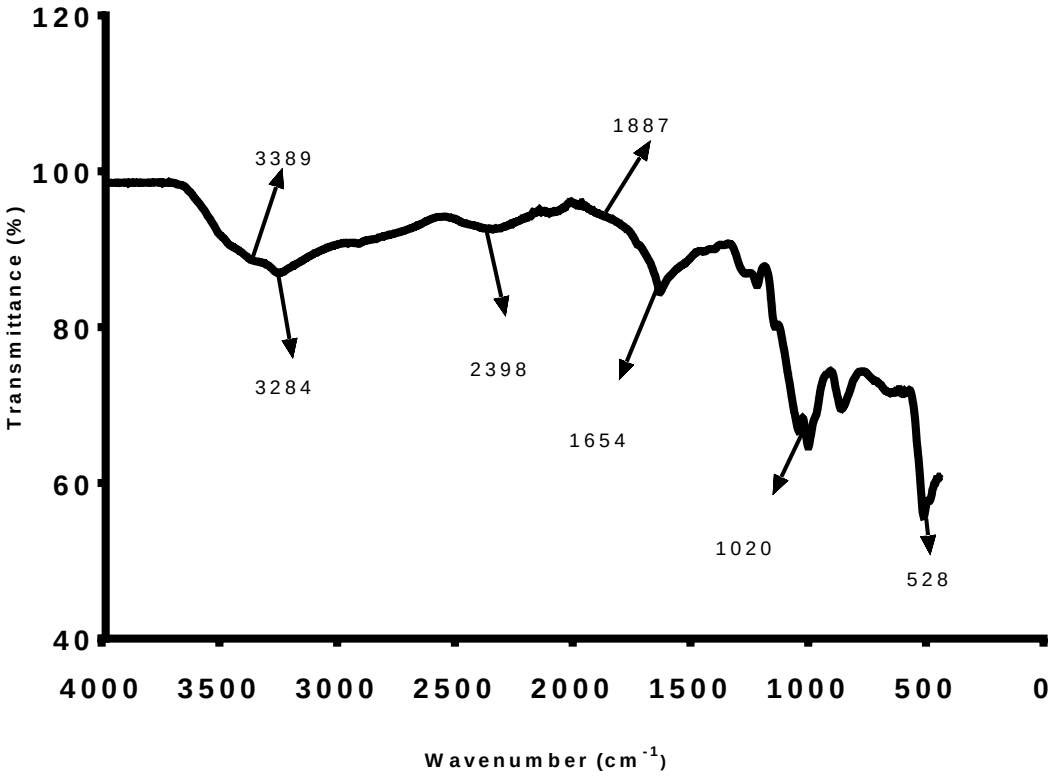


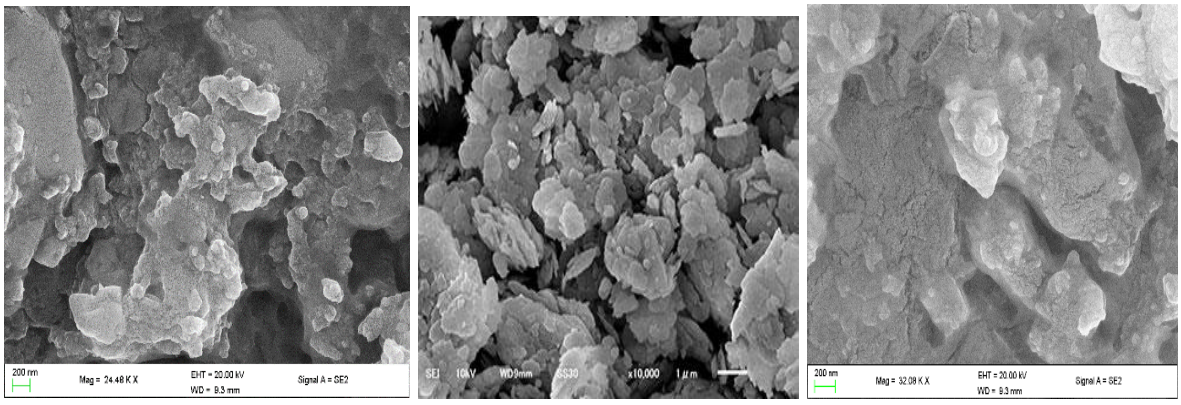
Figure 4. 9: FTIR spectrum of the purified bioflocculant BA-CGB

4.5.2 SEM and EDX analysis of bioflocculant BA-CGB

4.5.2.1 SEM Analysis

SEM analysis shows the surface images of sample as high-energy beam of electrons scan in a faster scan pattern. The signal containing information about the sample surface is revealed as the electrons interact with the atoms of the sample (He *et al.,* 2010; Selepe, 2017). The surface morphology of purified BA-CGB was examined with its flocculation of kaolin clay powder by SEM (Figure 4:10). Figure 4.10 shows the images of purified bioflocculant (A) kaolin clay particles before

flocculation (B) and flocculated kaolin clay particle (C). Figure 4:10(A) shows the coarse and amorphous structure of the bioflocculant (BA-CGB). The SEM image of the bioflocculant BA-CGB showed that it is an amorphous and irregularly shaped biopolymer. Figure 4:11(B) shows the fine and white scattered uniform-sized kaolin clay particles before flocculation. Figure 4:10(A) is comparable to Figure 4.10(B) and Figure 4.10(C) in terms of structure and sizes. It was observed that larger flocs were easily formed and precipitated due to gravity as the kaolin clay particles connects to the large size particles of the bioflocculant (Figure 4.10). These observations were consistent with the findings of Okaiyeto *et al.* (2015a) on glycoprotein REG-6 bioflocculant produced from *Bacillus toyonensis* strain AEMREG6 and glycoprotein PRKF5 bioflocculant produced from *Providencia rettgeri* KF534469 (Selepe, 2017).



A **B** **C**

Figure 4. 10: SEM Analysis. SEM surface images of the bioflocculant (A), kaolin particles (B) and flocculated kaolin particles (C).

4.5.2.2 SEM EDX for BA-CGB

The SEM-EDX analysis of the purified bioflocculant depicted in Table 4.1 shows its elemental composition (% wt) to comprise C, O, Na, Mg, P, Cl, K, Ca in the

percentage of 3.46%, 44.14%, 5.41%, 4.61%, 19.97%, 2.33%, 19.34%, and 0.75%, respectively. The elemental composition indicates the presence of polysaccharide as a backbone in the compound bioflocculant. FTIR analysis spectrum also revealed the presence of amino, hydroxyl and carboxyl groups in the bioflocculant. Pathak *et al.* (2017) reported the elemental analysis of a novel bacterial bioflocculant revealed the presence of C, N, O, Na, P, and Cl in this macromolecule as 31.20, 6.12, 46.39, 5.67, 10.40, and 0.23 %, respectively

Table 4.1: Percentage composition of elements in the purified (BA-CGB) bioflocculant

Element	**% Weight**
C	3.46
O	44.14
Na	5.41
Mg	4.61
P	19.97
Cl	2.33
K	19.34
Ca	0.75
Total	100.00

4.5.2.3 Chemical composition of bioflocculant BA-CGB

The chemical composition of bioflocculant BA-CGB was found to contain approximately 65% of sugar, 25% of uronic acid and is approximately 10% of trace protein. In a reported study by Feng and Xu (2008), bioflocculant BF3-3 was composed of 66.1% (polysaccharide) and 29.3% (protein) while Deng *et al.* (2005)

reported *Aspergillus parasiticus* bioflocculant was composed of carbohydrate (76.3%) and protein (21.6%). However, on the contrary, the bioflocculant produced by *Halomonas* sp. V3a consist of majorly polysaccharide (29%), neutral sugar component (20.6%), uronic acid (7.6%), amino acids (1.6%) and a sulfate group (5.3%) (He *et al.,* 2010). The bioflocculant BA-CGB could then be described as mainly polysaccharides, hence, its thermo stability.

4.6 Characteristics of a purified bioflocculant BA-CGB

4.6.1 Effect of dosage size on flocculating activity of BA-CGB

Dosage concentration is a critical factor in determining bioflocculant performance. Insufficient or over dosage may lead to inhibition or reduced performance (Cosa and Okoh, 2014). Appropriate dosage size helps in minimising cost and to obtain better flocculating performance in industrial processes and treatments (Okaiyeto *et al.,* 2016). A bioflocculant solution (0.2 mg/ml) showed flocculating activity of 72%, 0.4 mg/ml (85%), 0.6 mg/ml (83%), 0.6 mg/ml (82%) and 1.0 mg/ml (87%), respectively (Figure 4.11) with addition of 1ml (3%) Calcium chloride as cation. No significant difference was observed between the dosage concentrations values from 0.4 mg/ml and 1.0 mg/ml; therefore, further experiments were carried out using 0.4 mg/ml dosage concentration as the preferable dosage size since low dosage is preferable and beneficial in industrial scale application in order to reduce cost. Similar to the study, 0.4 mg/ml dosage concentration was reported to produce optimum flocculating activity (Maliehe *et al.,* 2016).

Contrary to this study, the maximum dosage size of 1.2 mg/ml for *C. daeguense* resulted to a flocculating activity of 96.9%, was reported by Liu and Cheng (2010).

Ugbenyen and Okoh (2014) as well as Agunbiade *et al. (*2017) reported an optimum dosage size of 0.8 mg/ml for Arthrobacter *humicola* and a consortium of *Cobetia* spp and *Bacillus* sp., respectively. However, a dosage concentration size of 0.1 mg/ml was reported for bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. LEO (Okaiyeto *et al.,* 2013) while Ntsaluba *et al.* (2011) reported 2.0 mg/ml for a consortium of *Methylobacterium* sp. Obi and *Actinobacterium* sp. Mayor. Wang *et al.* (2011) reported a maximum flocculating activity (96. 21%) of a bioflocculant at 12 mg/ml produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6.

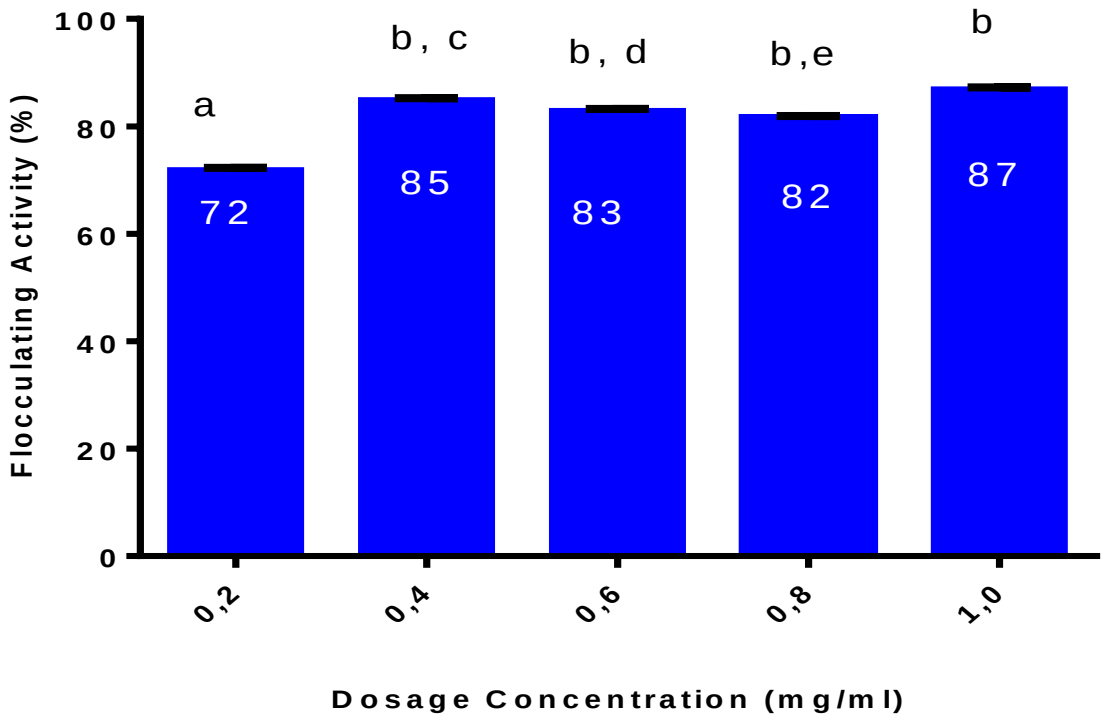


Figure 4. 11: The effect of dosage size on flocculating activity of (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

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4.6.2. Effect of cation on bioflocculant activity

Divalent cations (Figure 4.12) largely supported the flocculating activity of BA-CGB with Ca^{2+} at 85%, Ba^{2+} (73%), Mn^{2+} (74%), Mg^{2+} (81%) while the effect of monovalent cations were moderate with Li^{+} (50%), K^{+} (58%) and trivalent cations Fe^{3+} sitting at 37%. Therefore, Ca^{2+} was used for all subsequent experiments. Similar results were reported by Subudhi *et al.* (2014) on *Achromobacter* sp. TERI-IASST N which produced a bioflocculant that preferred Ca^{2+} as cation source, and urea as nitrogen source. These findings were also similar to the results obtained using a bioflocculants from *Enterococcus hirae* (Subudhi *et al.,* 2014). The bioflocculant BA-CGB is a cation-dependent as less than 5% flocculating activity was observed during the experiment.

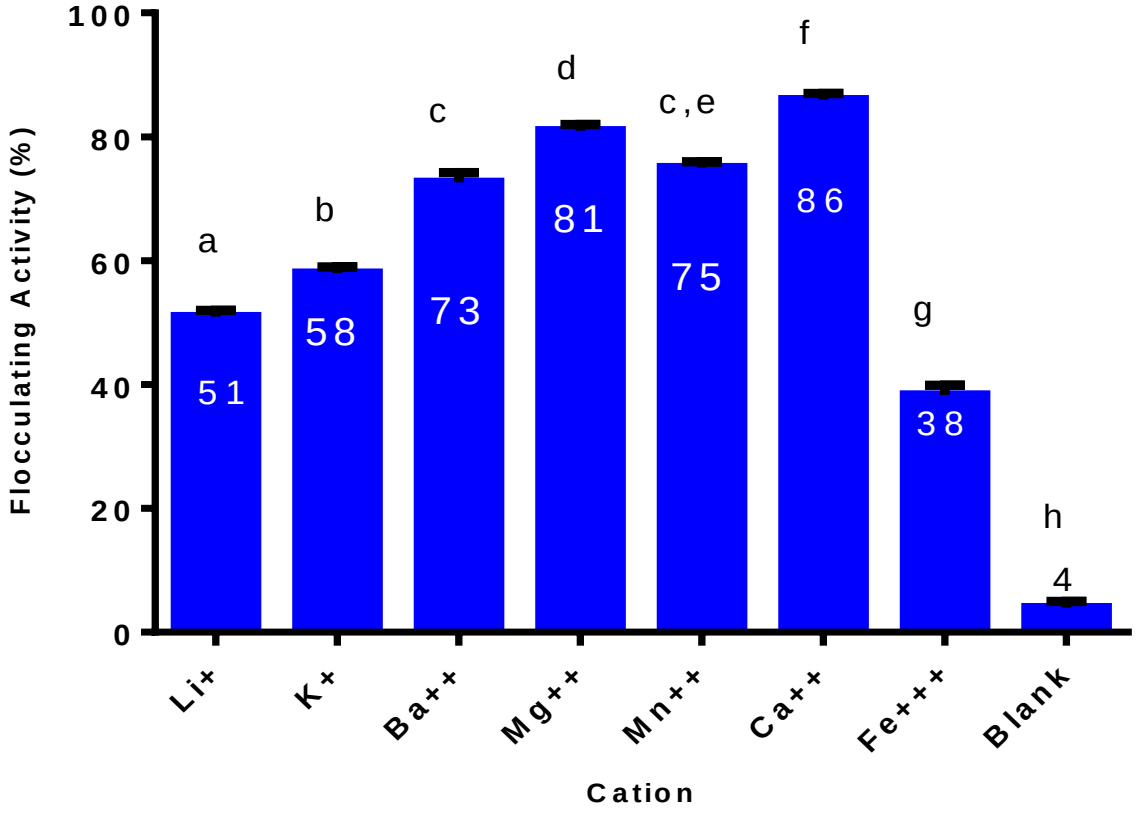

Figure 4. 12: The effect of cations on the flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.6.3 Effect of heat on flocculating activity of bioflocculant BA-CGB

The bioflocculant BA-CGB showed a strong thermal stability within the range of 50 - 90 ^{o}C, and the flocculating efficiency was 76% at 50 ^{o}C, increased slightly to 77% at 60 ^{o}C (Figure 4.13). A slight decrease in flocculating activity from 77% at 90 °C to 69% at 100 ^{o}C was observed. The bioflocculant is thermally stable as it retained above 60% flocculating activity at 100 ^{o}C. Although the bioflocculants have a strong thermal stability, increase in heat above 90 ^{o}C had a significant effect on the flocculating activity. Okaiyeto (2016) reported similar findings where all the three thermal stable bioflocculants (REG-6, MBF-W7 and MBF-UFH) retained above 80% flocculating activity against kaolin clay suspension in the presence of divalent cations

at 100 ^{o}C. The bioflocculant BA-CGB solution was also autoclaved at a temperature of 121 ^{o}C and pressure of 103, 4 kpa for 15 minutes and retained over 60% flocculating activity. Autoclaving is one of the methods of sterilisation using steam under high-pressure method to sterilize objects used in various fields. Sterilisation utilises many different methods including steam or dry heat, chemicals and radiation (Tao, 2012). Thermostability is one of the factor that a bioflocculant need to possess to be usable in water and wastewater treatment in industry (Giri *et al.,* 2015). This bioflocculant (BA-CGB) high flocculating activity can be due to its polysaccharide backbone, which has been reported to become extended on exposure to high temperature; thereby exposing more sites for binding to form flocs and increase flocculating activity (Li Giri *et al.,* 2015). Bioflocculants rich in polysaccharide has been reported to have better thermal resistance than those of proteins and nucleic acids; owing to possessing of heat resistance properties (Zhang *et al.*, 2012). Bioflocculants containing sugars as main flocculation component have been reported to be heat stable as they can retain up to 50% of their flocculating activity when heated in water (Lu *et al.,* 2005).

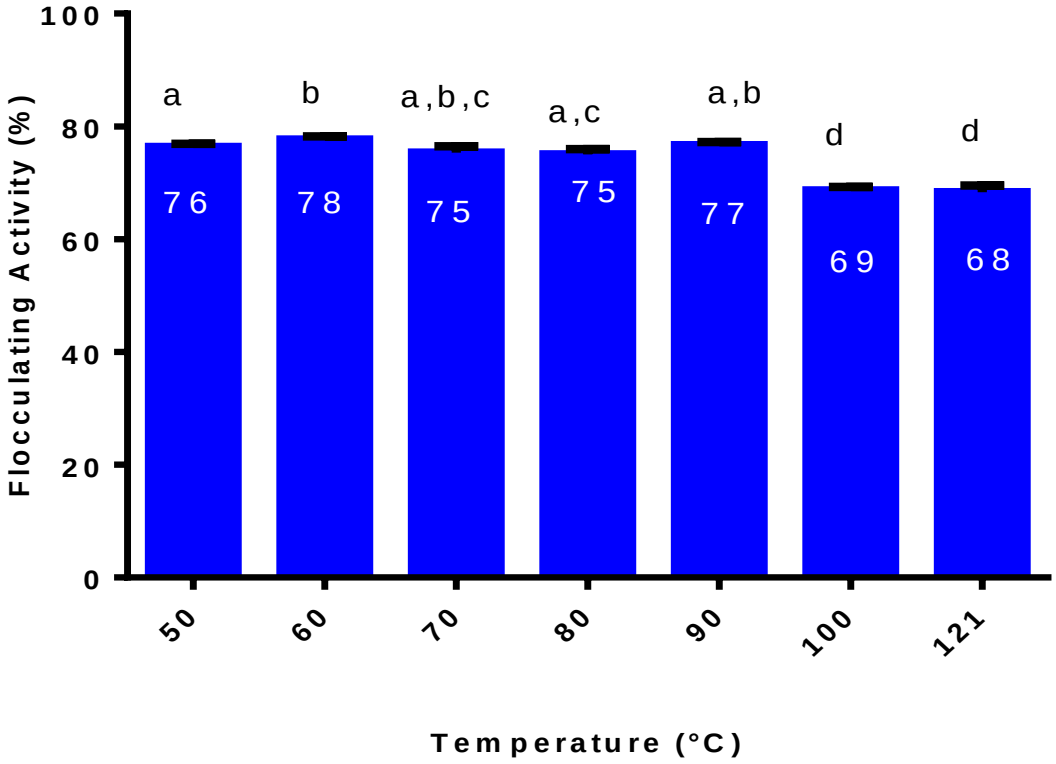

Figure 4. 13: The effect of temperature on flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.6.4 Effect of pH on the flocculating activity of pure BA-CGB

One of the factors that contribute to the flocculation efficiency is the increased surface area that is initiated by different pH regimes during optimisation (Ntozonke *et al.,* 2017). The flocculating activity of purified BA-CGB was greatly influenced by all pH tested, ranging from 3 - 12. Figure 4.14 shows the maximum flocculating activity that was observed at the extreme alkaline pH of 11 (94%). The flocculating activity for BA-CGB was above 70% for all pH range from pH 3 to pH 12 except pH 5 (65%). This implies that the bioflocculant can be used at various pH ranges, especially at acidic pH 3 (86%), pH 4 (80%) and alkaline conditions at a pH 10 (82%), pH 11 (94%) and pH 12 (90%). The decrease in flocculating activity around pH 5 (65%),

may be due to adsorption of the hydroxyl ions (OH^-) close to neutral pH 7. Okaiyeto *et al.* (2015b) reported a similar result where a bioflocculant MBF-UFH showed a good flocculating activity from pH 3 to pH 8 but had a sharp reduction in flocculating activity at pH 9. The reason advanced for this sharp reduction is that MBF-UFH exhibited different spatial arrangement at different pH as a result of different electric states (Okaiyeto *et al.,* 2015b). This may interfere with the cation-mediated neutralisation of charges between Ca^{2+} and kaolin clay suspension (Ntozonke *et al.,* 2017). Aljuboori *et al.* (2014) reported the pH stability of the purified bioflocculant promises good industrial applicability. Ugbenyen *et al.* (2014) reported similar results to this study, where a bioflocculant produced by a consortium of *Cobetia* and *Bacillus* species demonstrated a flocculating activity of over 70% across a wide pH range of 3 - 11 but at variance to this study, the highest flocculating activity was attained at pH 8.

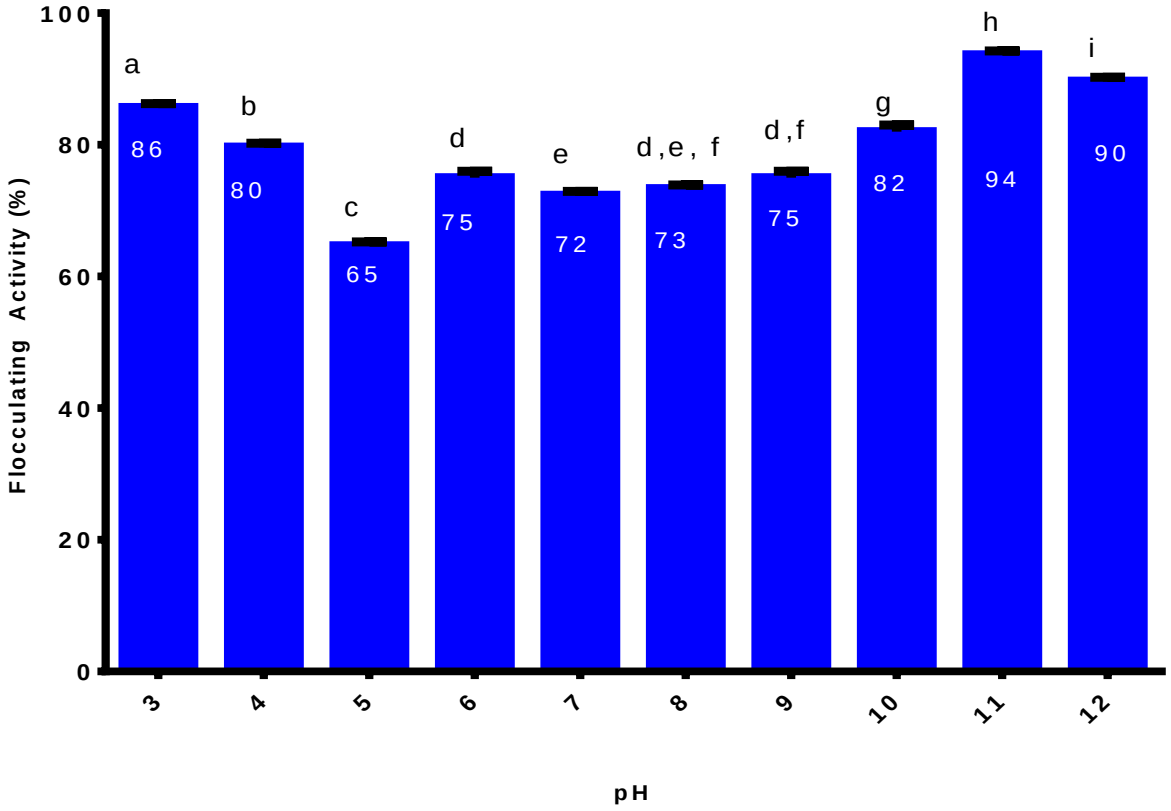

Figure 4. 14: The effect of pH stability on flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.6.5 Antimicrobial effect of pure BA-CGB on tested organism

The test result using the 96 well method showed a negative result as the concentration of bioflocculant BA-CGB was not active against the 3 tested organisms namely *E.coli* MHA 25922, *Bacillus cereus* and *Klebisiella pneumoniae* 1706, however, the control using cloxacillin was postive. Biofloculant with antibacterial propreties are excellent natural disinfectatnt for use in water treatment, this is because of their dual properties of being a flocculant and a disinfectatnt at the same time (Khaira *et al.,* 2013). This is cost effective in industrial applications.

4.7 Thermogravimetric analysis of the purified bioflocculant

The Thermogravimetric analysis (TGA) was used to study the pyrolysis property of the bioflocculant BA-CGB. The results are depicted in Figure 4.15. An initial weight loss of about 19% was observed between 0 - 200 ^{o}C. This downward trend continued with corresponding increase in temperature resulting in 40% weight loss being recorded at 792 ^{o}C with a final residual weight of 60%. This first weight loss under 200 ^{0}C has been reported to be due to moisture loss (Kumar and Anand, 1998). It has also been reported to be due to protein part associated with protein content molecules (Pathak *et al.,* 2017)

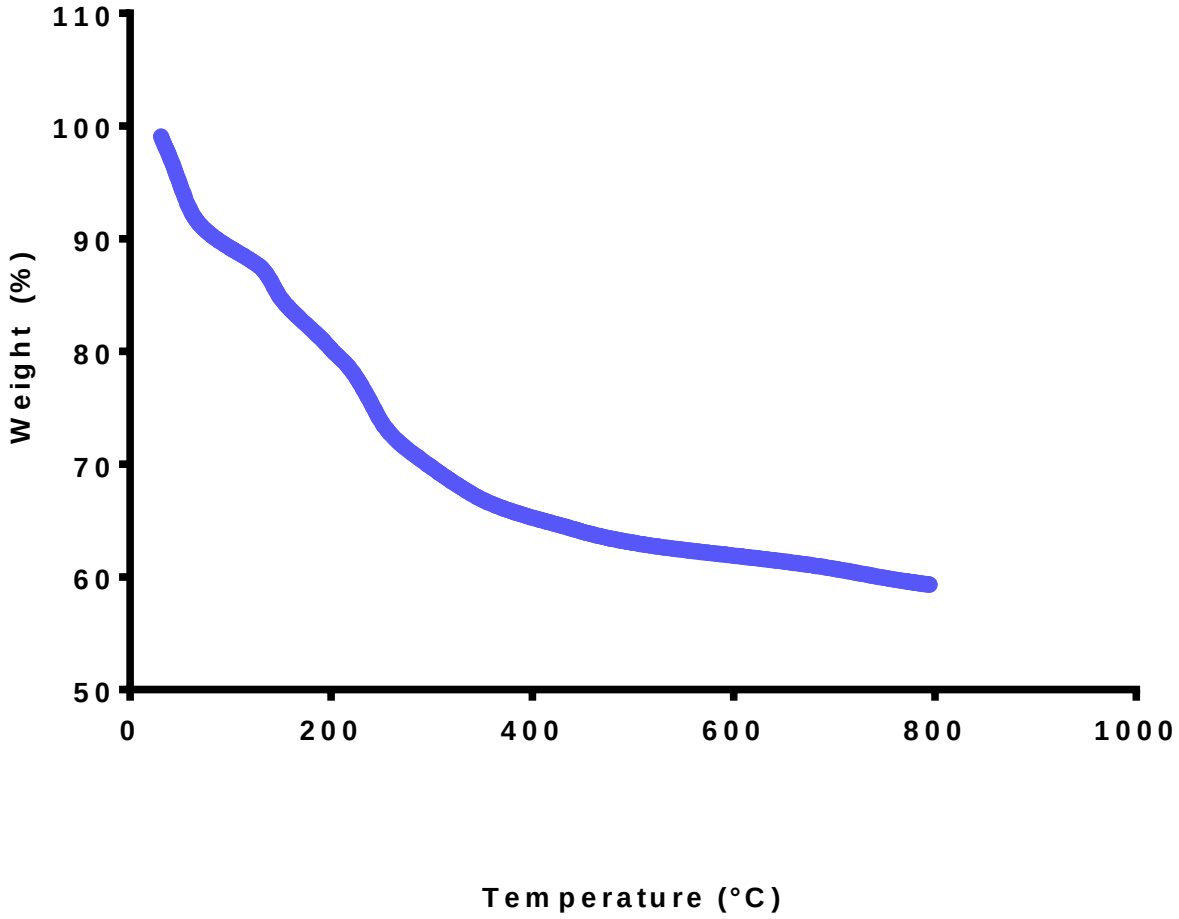

Figure 4. 15: TGA of purified bioflocculant BA-CGB from *Bacillus atrophaeus*

The decomposition of BA-CGB started between 30 ^{o}C and 67 ^{o}C. The thermogravimetry for BA-CGB exhibited about four decomposition steps. These steps were at 67 °C, 121 °C, 208 °C, and 258 °C. The four decomposition steps of BA-CGB showed corresponding weight losses of 7.51, 5.57, 8.40 and 6.88 %, respectively. The corresponding weight loss of the bioflocculant as observed in 30-100 ^{o}C (10%), 100-200 ^{o}C (19%), 200-500 ^{o}C (36%) and 500-791 ^{o}C (40%) (Figure 4.17) The thermogravimetry for BA-CGB exhibited distinct decomposition steps which represented mass loss at every stage. The various decomposition steps reflect the number of distinctive compounds present in the bioflocculant BA-CGB which further affirms the results obtained with FTIR and EDX elemental analysis. There was a complete decomposition of bioflocculant BA-CGB at a temperature of 791 ^{o}C, this indicates a good thermal stability as more than 60 % weight retained. Nwodo *et al*. (2013) reported similar findings with four decomposition steps and complete decomposition above 600 ^{o}C. The complete thermal decomposition of the bioflocculant was achieved at a temperature above 600 ^{o}C, thus implying a good thermal stability. Wang *et al.* (2011) reported a slight different initial loss of 10% between 20 ^{o}C and 150 ^{o}C for a bioflocculant produced using a consortium of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6 , then a further decrease at 400 ^{0}C by 40% and total weight loss at 1000 ^{0}C.

4.8 Application of bioflocculant treatment of wastewater

4.8.1 Comparison of flocculating efficiencies of BA-CGB and conventional flocculants

Okaiyeto *et al.* (2015) compared the flocculating efficiency of BA-CGB with other

conventionally used chemical flocculants in wastewater treatment using the method described by Ugbenyen et al. (2014) with a modified method. The optimum dosage (0.4 mg/ml) for BA-CGB was also compared with same dosage concentration for the other flocculants using kaolin clay suspension (Table 4. 2) The results showed that polyacrylamide has the highest flocculating activity of 93%, followed by BA-CGB and ferric chloride at 91% respectively. All the three values obtained are non statistically significant from each other. However, Alum, a locally used flocculant has a flocculating activity of 83% (Figure 4.16) without the addition of cation. The results show that BA-CGB was comparably significant to both polyacrylamide as well as ferric chloride and was more efficient than alum. Okaiyeto *et al.* (2015b) reported a similar result where the bioflocculant MBF-UFH was comparable to polyacrylamide. However, in that study, the reported flocculating activity of aluminium chloride (67.99%) and $FeCl_3$ (42.78%) was much lower than the results obtained in comparison with BA-CGB in this study. The results suggest that BA-CGB could possess great potential in the treatment of wastewater or river water.

Table 4.2: Comparison of flocculating activity of BA-CGB with conventional flocculants.

Flocculants	Conc (mg/ml)	Flocculant activity (%)
Polyacrylamide	0.4	93
BA-CGB	0.4	91
Alum	0.4	83
Ferric Chloride	0.4	91

Note: All values obtained in triplicate

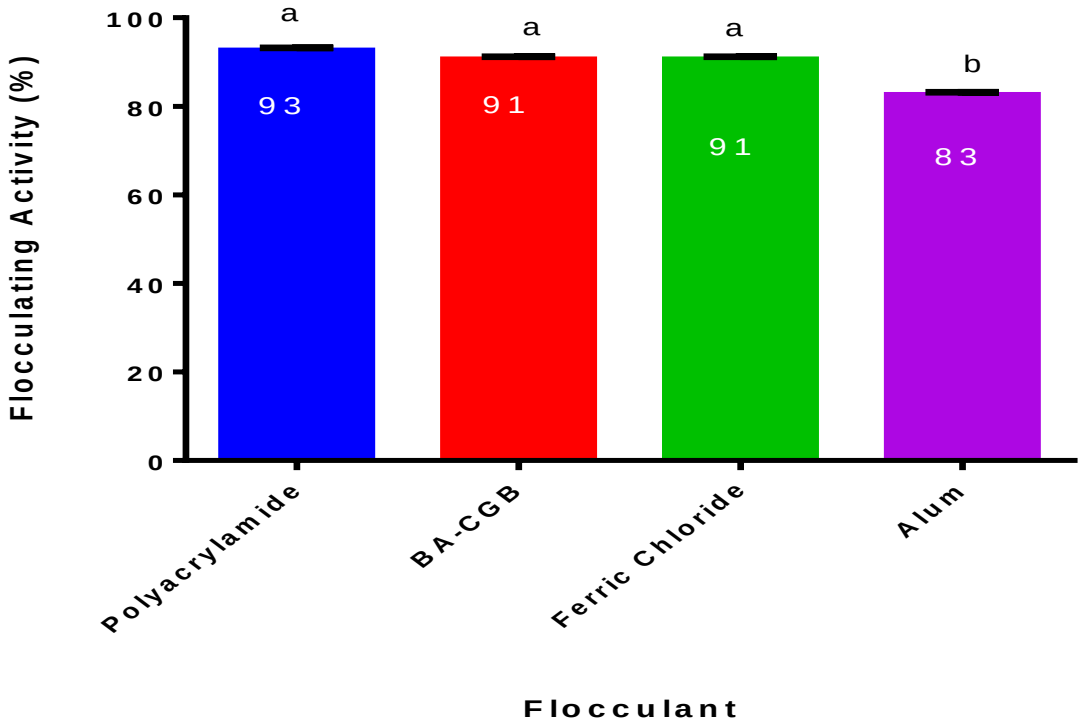

Figure 4. 16: Comparison of flocculating activity of purified bioflocculants BA-CGB with conventional flocculants. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other

4.8.2 Application of bioflocculant BA-CGB in treatment of Vulindlela wastewater and Mzingazi river water

Bad odours and anaerobic conditions are results of high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in stagnant waters that do not support aquatic life (Mihelcic and Zimmerman, 2010). Ma *et al. (*2008) have reported efficient removal of suspended solids, microorganisms, COD, BOD and heavy metals using bioflocculants. Waste water from Vulindlela domestic and Mzingazi river were treated with bioflocculant BA-CGB, thereafter compared with the conventional flocculant (polyacrylamide) and the removal efficiency were tabulated in Table 4.3 and Table 4.4.

Table 4.3 showed the physical properties of BA-CGB application in wastewater and river water treatment. Removal efficiency of COD for polyacrylamide (84%) was comparable to that of BA-CGB (80%). The removal efficiency for BOD on Vulindlela wastewater was 87% for BA-CGB and 89% for polyacrylamide. The removal efficiency of sulphate 60% compared to that of polyacrylamide (82%). Bioflocculant BA-CGB showed lower efficacy in the removal of phosphate (52%) and nitrate (88%) when compared with polyacrylamide (phosphate 96%, nitrate 93%), respectively.

Table 4.4 also showed that both bioflocculant BA-CGB and polyacrylamide had removal efficiency above 75% for all the parameters assessed in this study on Mzingazi river water. Li *et al.* (2013) reported a bioflocculant produced by *P. elgii* B69 with a 68% COD removal, 83% turbidity reduction with a dye colour removal efficiency of 88%. Contrary to the findings in this study, bioflocculant MBFA9 showed a COD removal of 68.5% (Deng *et al.,* 2003) while the bioflocculant produced from *Bacillus mucilaginous* had 74.6% COD and 42.3% BOD removal, respectively. Selepe (2017) conducted a comparative study on bioflocculant ORY84 with other conventional flocculants on Tendele coal mine and Erwat wastewater treatment plants. The findings showed that the COD removal efficiency value of 94% showed no significance difference in comparison ferric chloride (94%) and alum (93%), respectively. The removal of COD, BOD and other consistuents of wastewater plays an important factor in the re-use of water effluents.

Table 4.3: Removal efficiency of bioflocculant BA-CGB in the treatment of Vulindlela wastewater

Flocculants		**COD (mg/L)**	**BOD (mg/L)**	**Sulphate (mg/L)**	**Phosphate (mg/L)**	**Nitrate (mg/L)**
Polyacrylamide	Before	422±0.0	335±0.0	245±0.0	6,7±0.0	0,8±0.0
	After	67± 0.58	38±0.58	45±0.58	0,25± 0.06	<0,1±0.00
	Removal efficiency (%)	84	89	82	96	93
BA-CGB	Before	422±0.0	335±0.0	245±0.0	6,7±0.0	0,8±0.00
	After	85±1.00	43±0.58	99±0.58	3,2±0.06	0,1±1.00
	Removal efficiency (%)	80	87	60	52	88

Table 4. 4: Removal efficiency of bioflocculant BA-CGB in the treatment of Mzingazi river

Flocculants		**COD (mg/L)**	**BOD (mg/L)**	**Sulphate (mg/L)**	**Phosphate (mg/L)**	**Nitrate (mg/L)**
Polyacrylamide	Before	50±0.0	32±0.0	210±0.0	1,5±0.0	0,5±0.0
	After	3,5±0.03	5±0.06	32± 0.58	<0.1±0.01	<0.1± 0.01
	Removal efficiency (%)	93	85	85	95	90
BA-CGB	Before	50±0.0	32±0.0	210±0.0	1,5±0.0	0,5±0.0
	After	6.8±0.01	8.0±0.01	44±0.58	0,3±0.01	<0.1±0.01
	Removal efficiency (%)	86	76	78	80	85

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Introduction

This chapter includes the conclusion based on the findings of this study and the recommendation for further research work.

5.2 Conclusion

Bioflocculants have shown great potential to improve quality of waste water treatment, productivities and are safe for both human and the environment compared to the in-use chemical flocculants, which have been implicated in undesirable health conditions. However, high production cost and complicated production process are limiting the use of bioflocculants. Optimization of culture medium conditions has been observed as a way of improving both production yield and flocculating efficiency.

Three halophilic bacteria were isolated from marine environment of Richards Bay Habour, KwaZulu-Natal province of RSA. These halophilic isolates have shown a strong potential of bioflocculant production with flocculating activities greater than 70% in kaolin clay suspension. They were identified through 16S rRNA nucleotide sequence and BLAST analyses as *Bacillus safensis*, *Bacillus pumilus* and *Bacillus atrophaeus*. Optimization of culture medium conditions to obtain an optimum bioflocculant production from *Bacillus atrophaeus* was done. A bioflocculant was observed to be produced parallel to the cells multiplication of *Bacillus atrophaeus*. A bioflocculant yield of 3.165 g was recovered from one litre growth medium using 4% inoculum size, glucose and ammonium chloride as energy sources. The production medium adjusted to pH 9 and incubated at 35 ^{o}C with a shaking speed of 110 rpm

produced the optimal bioflocculant after 96 hours of fermentation with Ba^{2+} cation as a stimulating agent.

The purified bioflocculant BA-CGB was characterized. Bioflocculant BA-CGB with an amorphous structure has been observed to consist of elements in major proportion such such as carbon, oxygen, phosphorus and potassium. BA-CGB was revealed to be composed of carbohydrates (mainly), proteins and uronic acid. FTIR analysis revealed that the molecular chain of BA-CGB possess hydroxyl, amine, carboxyl and amide functional groups, characteristics favouring the flocculation mechanism. The bioflocculant BA-CGB is thermal stable within the range of 50 - 100 ^{o}C and retained more than 60% flocculating activity at 121 ^{o}C after 15 minutes of exposure. An effective performance of BA-CGB was observed at a wide range of pH (3-12) with an optimum dosage concentration of 0.4 mg/ml and Ca^{2+} cation stimulating the process. This is a great benefit as it is contemplated for the use in industrial processes.

The microbial flocculant showed the potential in reducing COD and BOD and at the same time aid in the removal of efficiencies of certain parameters such as sulphate, nitrate as well as phosphorous in both Mzingazi river water and Vulindlela domestic wastewater. The bioflocculant BA-CGB could be a great substitute to in-use chemical flocculants.

5.3 Recommendations

Sequel to the findings of this study, the following recommendations are made

(i) Large scale production conditions should be developed.

(ii) Further characterization of the BA-CGB bioflocculant and its mechanisms. Also to explore the contribution of a molecular weight to the flocculation process and charge density of the bioflocculant.

(iii) Exploration of the possibility of using agricultural waste such as sugarcane, bargasse supplemented with agricultural nitrogen (plant) source waste.

(iv) Shelf-life increase of the produced bioflocculant by optimally grafting into a synthetic polymer.

(v) Biosynthesis of nanoparticles using a bioflocculant to improve both water quality and remove antibiotic-resistant microorganisms in water, simultaneously by using a very small amount of the product.

REFERENCES

Abalaka SE (2015). Heavy metals bioaccumulation and histopathological changes in Auchenoglanis occidentalis fish from Tiga dam, Nigeria. *Journal of Environmental Health Science and Engineering*, 13: 67.

Abdel-Aziz SM, Hoda AH, Foukia EM, Nayera A, Abdelwahed M (2011). Extracellular metabolites produced by a novel strain, *Bacillus alvei* NRC-14:3. Synthesis of a bioflocculant that has chitosan-like structure. *Life Sciences Journal*, 8: 883-890.

Abdul-Razack S, Velayutham V, Thangavelu T (2014). Medium optimization and in vitro antioxidant activity of exopolysaccharide produced by *Bacillus subtilis*. *Korean Journal of Chemical Engineering,* 31: 296-303.

Abd –El – Haleem DAM, Al Thani RF, Thourya M, Sidra M, Fatima H (2008). Isolation and charactezation of extracellular bioflocculants produced by bacteria isolated from Qatari Ecosystems. *Polish Journal of Microbiology,* 57: 231-239.

Abd El-Salam AE, Abd-El-Haleem D, Youssef AS, Zaki S, Abu-Elreesh G, El-Assar SA (2017). Isolation, characterization, optimization, immobilization and batch fermentation of bioflocculant produced by *Bacillus aryabhattai* strain PSK1 *Journal of Genetic Engineering and Biotechnology,* 15*: 335-344.*

Abu-Elreesh GM, Abd-El-Haleem DA (2014). Biodiesel, bioflocculant and biosorbent from the fungal *Curvularia sp.* strain DFH1. *European Journal of Experimental Biology,* 4: 218-226*.*

Adhikary P, Tiwari NK, Singh R (2007). Synthesis, characterization, and flocculation characteristics of polyacrylamide-grafted glycogen. *Journal of Applied Polymer Science,* 103: 773-778.

Agunbiade MO, Van Heerden E, Pohl CH, Ashafa AT (2017). Flocculating performance of a bioflocculant produced by Arthrobacter humicola in sewage waste water treatment. *BMC biotechnology*, *17*: 51. doi:10.1186/s12896-017-0375.

Ahmed HRA, Azni I, Hamid HRA, Yoshimitsu U, Abubakar BSUI (2015). Flocculation behaviour and mechanism of bioflocculant produced by *Aspergillus flavus*. *Journal of Environmental Management,* 150: 466-471.

Alain K, Querellou J (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles,* 13: 583-594.

Aljuboori AHR, Idris A, Abdullah N, Mohamad R (2013). Production and characterization of a 1359 bioflocculant produced by *Aspergillus flavus*. *Bioresource Technology*, 127: 489-493.

Aljuboori AHR, Uemura Y, Osman NB, Yusup S (2014). Production of a bioflocculant from *Aspergillus niger* using palm oil mill effluent as carbon source. *Bioresource Technology,* 171: 66-70.

Aljuboori AHR, Idris A, Al-joubory HHR, Uemura Y, Ibn Abubakar BSU (2015). Flocculation behavior and mechanism of bioflocculant produced by *Aspergillus flavus*. *Journal of Environmental Management,* 150: 466-471.

Amir HG, Shamsuddin ZH, Halimi MS, Ramlan MF, Marziah M (2003). "N2 fixation, nutrient accumulation and plant growth promotion by rhizobacteria in association with oil palm seedlings," *Pakistan Journal of Biological Sciences*, 14: 1269-1272.

Banks WA, Niehoff ML, Drago D, Zatta P (2006). Aluminum complexing enhances amyloid (protein penetration of blood–brain barrier). *Brain Resource,* 1116: 215-221.

Benavides MP, Gallego SM, Tomaro ML (2005). Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology,* 17: 21-34.

Bezawada J, Hoang NV, More TT, Yan S, Tyagi N, Tyagi RD, Surampalli RY (2013). Production of extracellular polymeric substances (EPS) by *Serratia* sp.1 using wastewater sludge as raw material and flocculation activity of the EPS produced. *Journal of Environmental Management,* 128: 83-91.

Bisaria VS (2007). Growth and Transport. Microbial physiology and Biochemistry. Department of Biochemical Engineering & Biotechnology, Indian Institute of Technology – Delhi Hauz Khas, New Delhi 110016, India.

Biswas J, Paul AK (2017). Diversity and production of extracellular polysaccharide by halophilic microorganisms. *Biodiversity International Journal,* 2: 32-39.

Bitter T, Muir HM (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, 4: 330-334.

Blom E, Ridder ANJA, Lulko AT, Roerdink JBTM, Kuipers OP (2011). Time-Resolved transcriptomics and bioinformatic analyses reveal intrinsic stress

responses during batch culture of *Bacillus subtilis*. *Public Library of Science One*, 11: 27160.

Boyd CC (2015). Water quality - An introduction. 2nd ed. *Springer,* 21 - 40, 189-200, 243-260, 263-277, 313-324.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry,* 72: 248-254.

Bura R, Cheung M, Liao B, Finlayson J, Lee BC, Droppo IG, Leppard GG, Liss SN (1998). Composition of extracelular polymeric substances in the activated sludge floc matrix. *Water Science Technology,* 37: 325-333.

Burke SA, Wright JD, Robinson MK, Bronk BV, Warren RL (2004). Detection of Molecular Diversity in *Bacillus atrophaeus* by Amplified Fragment Length Polymorphism Analysis. *American Society for Applied and Environmental Microbiology,* 70: 2786-2790.

Buthelezi SP (2008). Application of bacterial bioflocculants for wastewater and river water treatment. University of Durban, KwaZulu-Natal, South Africa (MSc Thesis).

Castro L, Zhang R, Muñoz JA, González F, Blázquez ML, Sand W, Ballester A (2014). Characterization of exopolymeric substances (EPS) produced by *Aeromonas hydrophila* under reducing conditions, Biofouling: *The Journal of Bioadhesion and Biofilm Research, 30: 501-511*.

Chang WC, Soon AY, In HO, Sang HP (1998). Characterisaion of an extracellular

flocculating substance produced by planktonic cyanobacterium, *Anaebaena* sp. *Biotechnology Letters,* 20: 643-646.

Chaplin MF, Kennedy JF (1994). Carbohydrate Analysis, 2nd ed.; *Oxford University Press*: New York, NY, USA.

Chen YP, Fu YS, Li XM (2003). Characteristics and treatment of brewery wastewater. *Journal Pollution Control Technology,* 16: 146-149.

Chen YG, Zhang YQ, Chen QH, Klenk HP, He JW, Tang SK, Cui XL, Li WJ (2011). *Bacillus xiaoxiensis* sp. nov., a slightly halophilic bacterium isolated from non-saline forest soil. *International Journal Systematic Evolutionary Microbiology,* 61: 2095-2100.

Christen R (2008). Global sequencing: a review of current molecular data and new methods available to assess microbial diversity. *Microbes and Environment,* 23: 253-268

Coil DA, Neches RY, Lang JM, Brown WE, Severance M, Cavalier D, Eisen JA (2016). Growth of 48 built environment bacterial isolates on board the international Space Station (ISS). *Peer Journal,* 4: 1842.

Cosa S, Okoh A (2014). Bioflocculant production by consortium of two bacterial species and its potential application in industrial wastewater and water treatment. *Polish Journal Environmental Study*, *23*: 689-696.

Cosa S, Mabinya LV, Olaniran AO, Okoh OO, Okoh AI (2011). Bioflocculant production by *Virgibacillus* sp. Rob isolated from the bottom sediment of Algoa Bay in the Eastern Cape, South Africa. *Molecules,* 16: 2431-2442.

Cosa S, Okoh AI (2012). Prevalence of culturable marine *Actinomycetes* genera in near-shore sediments of Algoa Bay in the Eastern Cape Province of South Africa. *Journal of Pure and Applied Microbiology,* 6: 1111-1118.

Cosa S, Ugbenyen AM, Mabinya LV, Rumbold K, Okoh AI (2013a). Characterization and flocculation efficiency of a bioflocculant produced by a marine *Halobacillus. Environmental Technology,* 18: 2671-2679.

Cosa S, Ugbenyen MA , Mabinya LV, Okoh A.I. (2013b). Characterization of a thermostable polysaccharide bioflocculant produced by *Virgibacillus* species isolated from Algoa Bay. *African Journal of Microbiology Research,* 7: 2925-2938.

Cosa S, Ugbenyen MA, Mabinya LA, Rumbold K, Olaniran AO, Aghdasi F, (2013c). A marine bacterium, *Oceanobacillus* sp. Pinky, isolated from Algoa Bay sediment produces a thermostable glycoprotein flocculant. *African Journal Biotechnology,* 12: 4135-4146.

Cosa S. (2010). Assesment of bioflocculant production by some marine bacteria isolated from the bottom sediment of Algoa Bay [M.Sc. thesis] Alice, South Africa: Faculty of Science and Agriculture, University of Fort Hare.

Cristina Q, Vitor B, Silva D, Bruna S, Hugo F, Teresa T (2011). Optimization of production of extracellular polymeric substances by *Arthrobacter viscosus* and their interaction with a 13X zeolite for the biosorption of Cr(VI). *Environmental Technology,* 32: 1541-1549.

Deng SB, Bai RB, Hu XM, Luo Q (2003). Characteristic of bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. *Applied Microbial Biotechnology,* 60: 588-593.

Deng SB, Yu G, Ting YP (2005). Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. *Colloids Surfactant Biointerface*, 44: 179-186.

Dermlim W, Prasertsan P, Doelle H (1999). Screening and characterization of bioflocculant produced by isolated *Klebsiella* sp. *Applied Microbiological Biotechnology,* 52: 698-703.

Desouky AM, Haleem AE, Roda FT, Thourya M, Sidra M, Fatima H (2008). Isolation and characterization of extracellular bioflocculants produced by bacteria isolated from Quatari Ecosystems. *Polish Journal of Microbiology,* 57: 231-239.

De Vuyst L, De Vin F, Vaningelgem F, Degeest B (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *International Dairy Journal,* 11: 687-707.

Dobrowolski R, Szczes A, Czemierska M, Jarosz-Wikołazka A (2017). Studies of cadmium(II), lead(II), nickel(II), cobalt(II) and chromium(VI) sorption on extracellular polymeric substances produced by *Rhodococcus opacus* and *Rhodococcus rhodochrous*. *Bioresource Technology,* 225: 113-120.

Doubla A, Laminsi A, Nzali A, Njoyim E, Kamsu-Kom J, Brisset JL (2007). Organic pollutants abatement and biodecontamination of brewery effluents by

a non-thermal quenched plasma at atmospheric pressure. *Chemosphere*, 69: 332-337.

Edbeib MF, Wahab RA, Huyop F (2016). Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments. *World Journal of Microbiological Biotechnology*, 32: 135.

Elkady MF, Farag S, Zaki S, Abu-Elreesh G, Abd-El-Haleem D (2011). *Bacillus mojavensis* strain 32A, a bioflocculant-producing bacterium isolated from an Egyptian salt production pond. *Bioresource Technology*, 102: 8143-8151.

Eloff JN (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica,* 64: 711-713.

Feng J, Yang Z, Zeng G, Huang J, Xu H, Zhang Y, Wei S, Wang L (2013). The adsorption behavior and mechanism investigation of Pb(II) removal by flocculation using microbial flocculant GA1. *Bioresource Technology, 148: 414-421.*

Feng DL, Xu SH (2008). *Characterization of bioflocculant MBF3-3 produced by an isolated Bacillus sp. World Journal of Microbiology Biotechnology, 24:* 1627-1632.

Feng L, Stuart MC, Adachi Y (2015). Dynamics of polyelectrolyte adsorption and colloidal flocculation upon mixing studied using mono-dispersed polystyrene latex particles. *Advances in Colloid and Interface Science,* 226: 101-114.

Fleer G, Stuart MC, Leermakers F (2005). Effect of polymers on the interaction between colloidal particles. Fundamentals of Interface and Colloid Science. *Soft Colloids*, 5: 10.

Freitas F, Alves VD, Pais J, Costa N, Oliveira C, Mafra L, Reis MA (2009) Characterisation of an extracellular polysaccharide produced by a *Pseudomonas* strain grown on glycer. *Bioresource technology*, 100: 859-865

Fugita M, Ike M, Jang JH, Kim SM, Hirao T (2001). Bioflocculant production from lower molecular fatty acids as a novel strategy for utilization of sludge digestion liquor. *World Science of Technology, 44*: 237-243.

Gao J, Bao H, Xin M, Liu Y, Li Q, Zhang Y (2006). Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31. *Journal Zhejiang Universal Science B,* 7: 186-192.

Ghashoghchi RA, Hosseini MR, Ahmadi A (2017). Effects of microbial cells and their associated extracellular polymeric substances on the bio-flocculation of kaolin and quartz. *Applied Clay Science, 138: 81-88.*

Ghosh M, Pathak S, Ganguli A (2009). Application of a novel biopolymer for removal of *Salmonella* from poultry wastewater. *Environmental Technology,* 30: 337-344.

Giri SS, Harshiny M, Sen SS , Sukumaran V , Park SC (2015). Production and characterization of a thermostable bioflocculant from Bacillus subtilis F9, isolated from wastewater sludge, *Ecotoxicology and Environmental Safety,* http://dx.doi.org/10.1016/j.ecoenv.2015.06.010

Gomoiu I, Catley BJ (1996). Properties of a kaolin-flocculating polymer elaborated by *Byssochlamys nivea*. *Enzymology and Microbial Technology,* 19: 45-49.

Gong W, Wang S, Sun X, Liu X, Yue Q, Gao B (2008). Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. *Bioresource Technology,* 99: 4668-4674.

Gonzalez JF, Hu W (1991). Effect of glutamate on the degradation of pentachlorophenol by *Flavobacterium* sp. *Applied Microbiology and Biotechnology*, 35: 100-104**.**

Gupta A, Thakur IS (2016). Study of optimization of wastewater contaminant removal along with extracellular polymeric substances (EPS) production by a thermotolerant *Bacillus* sp. ISTVK1 isolated from heat shocked sewage sludge. *Bioresource Technology*, 213: 21-30.

Harun AAC, Mohammad NAH, Ikhwanuddin M, Ismail N, Ibrahim Z, Kasan NA (2017). Consortium of bioflocculant-producing bacteria as inoculum on flocculation process for sustainable production of Pacific whiteleg shrimp, *Penaeus vannamei*. *Journal of Fisheries and Aquatic Science,* 12: 197-206.

He N, Li Y, Chen J (2004). Production of a novel polygalacturoic acid bioflocculant REA-11 by *Corynebacterium glutamicum*. *Bioresource Technology,* 94: 99-105.

He J, Zou J, Shao Z, Zhang J (2010). Characteristics and flocculating mechanism of a novel bioflocculant HBF-3 produced by deep-sea bacterium mutant *Halomonas* sp. V3a. *World Journal of Microbiology and Biotechnology*, 26: 1135-1141.

He J, Zhen Q, Qiu N, Liu Z, Wang B, Shao Z, Yu Z (2009). Medium optimization for the production of a novel bioflocculant from *Halomonas* sp. V3a using response surface methodology. *Bioresource Technology,* 100: 5922-5927.

Hierrezuelo J, Vaccaro A, Borkovec M (2010). Stability of negatively charged latex particles in the presence of a strong cationic polyelectrolyte at elevated ionic strengths. *Journal of Colloid and Interface Science, 347: 202-208.*

Houghton JI, Quarmby J, Stephenson T (2001). Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer. *Water Science Technology*, 44: 373-379.

Huang XW, Cheng W, Hu YY (2005). Screening of flocculant-producing strains by NTG mutagenesis. *Journal of Environmental Science,* 17: 494-498.

Jensen PR, Gontang E, Mafnas C, Mincer TJ, Fenical W (2005). Culturable marine *Actinomycetes* diversity from tropical Pacific Ocean sediments**.** *Environmental Microbiology,* 7: 1039-1048.

Jochen S, Volker S, Bernd R (2015). Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Frontier Microbiology,* 6: 1-24.

Karthiga K, Natarajan KA (2015). Production and characterization of bioflocculants for mineral processing applications. *International Journal of Mineral Processing,* 137: 15-25.

Khaira GK, Ganguli A, Ghosh M (2013). Synthesis and evaluation of antibacterial activity of quaternized biopolymer from Klebsiella terrigena. *Journal of Applied Microbiology, 116: 511-518*

Korsten L, Cook N (1996). Optimizing Culturing Conditions for *Bacillus Subtilis*. *South African Avocado Growers' Association*, 19: 54-58.

Kothari VV, Kothari RK, Kothari CR, Bhatt VD, Nathani NM, Koringa PG, Joshi CG, Vyas BRM (2013). Genome sequence of salt-tolerant *Bacillus safensis* strain VK, isolated from saline desert area of Gujarat, India. *Genome Announcement,* 5: 00671-13.

Kumar CG, Anand SK (1998*).* Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology,* 42: 9-27.

Kurane R, Hatamochi K, Kiyohara T, Hirao M, Taniguchi Y (1994). Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols. *Bioscience, Biotechnology and Biochemistry, 58: 428-429.*

Kurane R, Nohata Y (1991). Microbial flocculation of waste liquids and oil emulsions by a bioflocculant from *Alcaligenes latus*. *Agricultural and Biological Chemistry,* 55: 1127-1129.

Kurane R, Matsuyama H (1994). Production of a bioflocculant by mixed culture. *Bioscience, Biotechnology and Biochemistry,* 58: 1589-1594.

Kurane R, Takeda K, Suzuki T (1986). Screening for characteristics of microbial flocculants. *Agricultural and Biological Chemistry,* 50: 2301-2307.

La Duc MT, Kern R, Venkateswaran K (2004). Microbial monitoring of spacecraft and associated environments. *Microbial Ecology,* 47: 150-158.

Lachhwani P (2005). Studies on polymeric bioflocculant producing microorganisms. Master of Science dissertation thesis. Thapar Institute of Engineering & Technology, Patiala.

Lam KS (2006). Discovery of novel metabolites from marine actinomycetes. *Industrial Microbiology,* 9: 245-251.

Lane DJ (1991). 16S/23S rRNA sequencing, 115-175. In: Stackbrandt E, Goodfellow M (Eds.). Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

Laspidou CS, Rittman BE (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Resources,* 36: 2711-2720.

Lateef A, Adelere I, Bosco Gueguim-Kana E (2015). The biology and potential biotechnological applications of *Bacillus safensis*. *Biologia*, 70: 411-419.

Lee CS (2017). Extraction of bio-flocculant from okra using hydrothermal and microwave extraction methods combined with a techno-economic assessment. PhD thesis, University of Nottingham.

Lee SC, Robinson J, Chong FM (2014). A review on application of flocculants in wastewater treatments. *Process Safety and Environmental Protection,* 92: 6.

Lee KE, Morad N, Teng TT, Poh BT (2012). Development, characterization and the application of hybrid materials in coagulation/flocculation of wastewater: a review. *Chemical Engineering Journal, 1: 370-386.*

Lee SH, Shin WS, Shin MC, Choi SJ, Park LS (2001). Improvement of water treatment performance by using polyamine flocculants. *Environmental Technol*ogy, 22: 274-278.

Li Y, Li Q, Hao D, Hu Z, Song D, Yang M (2014). Characterization and flocculation mechanism of an alkali-activated polysaccharide flocculant from *Arthrobacter* sp. B4. *Bioresource Technology, 170: 574-577.*

Li F, Huang J, Zeng G, Yuan X, Li X, Liang J, Wang X, Tang X, Bai B (2013). Spatial risk assessment and sources identification of heavy metals in surface sediments from Dongting Lake, Middle China. *Journal of Geochemistry Exploration,* 132: 75-83.

Li L, Xing J, Ma F, Pan T (2015). Introduction of compound bioflocculant and its application in water treatment. *Advance Journal of Food Science and Technology,* 9: 695-700.

Li Z, Zhong S, Lei H, Chen R, Yu Q, Li H (2009). Production of a novel bioflocculant by *Bacillus licheniformis* X14 and its application to low temperature drinking water treatment. *Bioresource Technology,* 100: 3650-3656.

Li WW, Zhou WZ, Zhang YZ, Wang J, Zhu XB (2008). Flocculation behavior and mechanism of an exopolysaccharide from the deep-sea psychrophilic

bacterium *Pseudoalteromonas* sp. SM9913. *Bioresource Technology,* 99: 6893-6899.

Li XY, Yang SF (2007). Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Research,* 41: 1022-1030.

Li X, Yang Q, Huang K, Zeng G, Liao D, Liu J (2007). Screening and characterization of a bioflocculant produced by *Aeromonas* sp. *Biomedical Environmental Science*, 20: 274-278**.**

Lian B, Ye C, Jin Z, Teng HH, Zhu L, Yua S (2008). Microbial flocculation by *Bacillus mucilaginosus*: applications and mechanisms. *Bioresource Technology,* 99: 4825-4831.

Liu P, Chen Z, Yang L, Li Q, Ning He N (2017). The bioflocculant production and identifying the effect of overexpressing *epsB* on the synthesis of polysaccharide and γ-PGA in *Bacillus licheniformis. Microbial Cell Factories, 16:163 DOI 10.1186/s12934-017-0775-9.*

Liu Z, Zhi-quan H, Tao W, Yan-ying C, Jianbin Z, Jing-ran Y (2013). Production of novel microbial flocculants by *Klebsiella* sp. TG-1 using waste residue from the food industry and its use in defecating the trona suspension. *Bioresource Technology,* 139: 265-271.

Liu F, Sun W, Su F, Zhou K, Li Z (2012). "Draft genome sequence of the sponge-associated strain *Bacillus atrophaeus* C89, a potential producer of marine drugs," *Journal of Bacteriology*, 16: 4454-4454.

Liu LF, Cheng W (2010). Characteristics and culture conditions of a bioflocculant produced by *Penicillium sp. Biomedical and Environmental Sciences,* 23: 213-218.

Liu W, Yuan H, Yang J, Li B (2009). Characterization of bioflocculants from biologically aerated filter backwashed sludge and its application in dying wastewater treatment. *Bioresource Technology,* 100: 2629-2632.

Liu YQ, Liu Y, Tay JH (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Applied Microbiology and Biotechnology, 65: 143-148.*

Liz MRH, Luis MPS, Yailen BY (2013). Development of a Technology for Treating Wastewater Contaminated with Nitric Acid, *Journal of Waste Management,* Article ID 278618:7. https://doi.org/10.1155/2013/278618.

Lopez E, Ramos I, Sanroman MA (2003). Extracellular polysaccharides production by *Arthrobacter viscosus*. *Journal of Food Engineering,* 60: 463-467.

Low LW, Teng TT, Anees A, Morad N, Wong YS (2011). A novel pretreatment method of lignocellulosic material as adsorbent and kinetic study of dye waste adsorption. *Journal of Water, Air Soil Pollution, 218: 293-306.*

Lu WY, Zhang T, Zhang DY, Li CH, Wen JP, Du LX (2005). A novel bioflocculant produced by *Enterobacter aerogenes* and its use in defaecating the trona suspension. *Biochemical Engineering Journal,* 27: 1-7.

Luo Z, Chen L, Chen C, Zhang W, Liu M, Han Y, Zhou J (2014). Production and characteristics of a bioflocculant by *Klebsiella pneumoniae* YZ-6 isolated from human saliva. *Applied Biochemistry and Biotechnology,* 172: 1282-1292.

Luvuyo N, Nwodo UU, Mabinya LV, Okoh AI (2013). Studies on bioflocculant production by a mixed culture of *Methylobacterium* sp. Obi and *Actinobacterium* sp. Mayor. *BioMed Central Biotechnology,* 3 : 1-7.

Ma J, Wang C, Wang H, Liu K, Zhang T, Yao L, Zhou Z, Binghai D, Yanqin D (2018). Analysis of the complete genome sequence of *Bacillus atrophaeus* GQJK17 reveals its biocontrol characteristics as a plant growth-promoting Rhizobacterium. *Hindawi BioMed Research International,* 9473542.

Ma F, Zheng LN, Chi Y (2008). Applications of biological bioflocculants (BFs) for coagulation treatment in water purification: Turbidity elimination. *Chemical Biochemical Engineering Quarterly,* 22: 321-326.

Mabinya LV, Cosa S, Mkwetshana N, Okoh AI (2011). *Halomonas* sp. OKOH - A marine bacterium isolated from the bottom sediment of Algoa Bay-produces a polysaccharide bioflocculant: partial characterization and biochemical analysis of its properties. *Molecules,* 22: 4358-4370.

Makapela B, Okaiyeto K, Ntozonke N, Nwodo UU, Green E, Mabinya LV, Okoh AI (2016). Assessment of *Bacillus pumilus* isolated from fresh water milieu for bioflocculant production. *Applied Science, 6: 211.*

Maliehe ST, Selepe N, Ntombela G, Simonis J, Basson AK, Ngema S, Xaba PS, Mpanza F (2016). Production and characteristics of bioflocculant TPT-1 from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2. *African Journal of Microbiology Research,* 37: 1561-1575.

Manivasagan P, Kang KH, Kim DG, Kim SK (2015). Production of polysaccharide based bioflocculant for the synthesis of silver nanoparticles by *Streptomyces* sp. *International Journal of Biological Macromolecule,* 77: 159-167.

Martin SM, Kau DA, Wrigley SK, Sarker SD (Ed) (2006). Scale-up of natural product isolation in methods in biotechnology. 2nd Edition. Vol 20 Humana Press Inc. Totowa, New Jersey.

Masante D, McCormick N, Vogt J, Carmona-Moreno C, Cordano E, Ameztoy I (2018). Drought and water crisis in Southern Africa, European Commission, Ispra, ISBN 978-92-79- 85851-2, doi:10.2760/81873, JRC111596.

Mfengwana PH, Mashele SS (2016). Antimicrobial activity screening of *Philenoptera violacea* (Klotzsch) schrire and *Xanthocercis zambesiaca* (Baker) Dumaz-Le-Grand. *Journal of Pharmacological Science & Research,* 8: 1132-1135.

Mihelcic JR, Zimmerman JB (2010). Environmental Engineering –Fundamentals, sustainability, Design. Wiley, 397-417.

Mincer TJ, Jensen PR, Kauffman CA, Fenical W (2002). Widespread and persistent populations of a major new marine *Actinomycete* taxon in ocean sediments. *Applied Environmental Microbiology, 68: 5005-5011.*

Mishra S, Mukul A, Sen G, Jha U (2011). Microwave assisted synthesis of polyacrylamide-grafted starch (St-g-PAM) and its applicability as flocculant for water treatment. *International Journal of Biological macromolecules,* 48: 106-111.

Mohamed FAS (2008). Bioaccumulation of selected metals and histopathological alterations in tissues of *Oreochromis niloticus* and *Lates niloticus* from Lake Nasser, Egypt. *Global Veterinary*, 2: 205-218.

Moissl-Eichinger C, Cockell C, Rettberg P (2016). Venturing into new realms? Microorganisms in space. *Federation of European Microbiological Societies Microbiological Review,* 40: 722-737.

Moosavi-Nasab M, Pashangeh S, Rafsanjani M (2010). Effect of fermentation time on xanthan gum production from sugar beet molasses. *World Academy Science Engineering Technology,* 44: 1244-1247.

Montgomery DC (2005). Design and Analysis of Experiments: Response Surface Method and Designs. John Wiley and Sons Inc, New Jersey.

More TT, Yan S, Hoang NV, Tyagi RD, Surampalli RY (2012). Bacterial polymer production using pre-treated sludge as raw material and its flocculation and dewatering potential. *Bioresource Technology,* 121: 425-431.

More TT, Yadav JSS, Yan S, Hoang NV, Tyagi RD, Surampalli RY (2014). Extracellular polymeric substances of bacteria and their potential environmental applications. *Journal of Environmental Management,* 144: 1-25.

More TT, Yan S, Tyagi RD, Surampalli RY (2015). Biopolymers production by mixed culture and their applications in water and wastewater treatment. *Water Environment Research,* 87: 533-546.

Nakamura LK (1989). Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. *International Journal Systemic Bacteriology,* 39: 295-300.

Nakata K, Kurane R (1999). Production of extracellular polysaccharide bioflocccculants by *Klebsiella pneumoniae. Journal of Bioscience Biotechnology and Biochemistry,* 63: 2064-2068.

Nester EW, Anderson DG, Roberts CE, Pearcal NN, Nester MT (2001). Microbiology: a human perspective third edition. The McGraw-Hill, Inc., New York, 787-799.

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Molecular Biology Reviews,* 64:548-572.

Nie M, Yin X, Jia J, Wang Y, Liu S, Shen Q (2011). Production of a novel bioflocculant MNXY1 by *Klebsiella pneumoniae* strain NY1 and application in precipitation of cyanobacteria and municipal wastewater treatment. *Journal of Applied Microbiology,* 111: 547-558.

Nouha K, Kumar RS, Balasubramanian S, Tyagi RD (2017). Critical review of EPS production, synthesis and composition for sludge flocculat*ion. Journal of Environmental Sciences, 66: 225-245.*

Ntsangani N (2016). Assessment of the flocculating efficiency of bioflocculant produced by *Bacillus* sp. AEMREG4 isolated from Tyhume River, Eastern

Cape, South Africa. [M.Sc. thesis]: Faculty of Science and Agriculture, University of Fort Hare.

Ntozonke N (2015). Assessment of bioflocculant production by two marine bacteria isolated from the bottom sediment of marine Algoa Bay. Master of Science dissertation. University of Fort Hare, South Africa.

Ntozonke N, Okaiyeto K, Okoli AS, Olaniran AO, Nwodo UU, Okoh AI (2017). A marine bacterium, *Bacillus* sp. isolated from the sediment samples of Algoa Bay in South Africa Produces a Polysaccharide-Bioflocculant. *International Journal of Environmental Research and Public Health, 14: 1149.*

Ntsaluba L, Agundiade O, Mabinya L, Okoh A (2011). Studies on bioflocculant production by *Methylobacterium* sp. Obi isolated from a freshwater environment in South Africa. *African Journal of Microbiology Research,* 5: 4533-4540.

Nwodo UU, Agunbiade MO, Green E, Mabinya LV, Okoh AI (2012). A freshwater Streptomyces, isolated from Tyume River, produces a predominantly extracellular glycoprotein bioflocculant. *International Journal of Molecular Science*, 13: 8679-8695.

Nwodo UU, Okoh AI (2013). Characterization and flocculation properties of biopolymeric flocculant (glycosaminoglycan) produced by *Cellulomonas* sp. Okoh. *Journal of Applied Microbiology,* 114: 1325-1337.

Nwodo UU, Agunbiade MO, Green E, Nwamadi M, Rumbold K, Okoh AI (2013). Characterization of an exopolymeric flocculant produced by a *Brachybacteria* sp. *Materials,* 6: 1237-1254.

Ogunmwonyi IH, Mazomba N, Mabinya L, Ngwenya E, Green E, Akinpelu DA, Olaniran AO, Bernard K, Okoh AI (2010). Studies on the culturable marine *Actinomycetes* isolated from the Nahoon beach in the Eastern Cape Province of South Africa. *African Journal of Microbiology Researches, 21: 2223-2230.*

Okaiyeto K, Nwodo UU, Mabinya LV, Okoh AI (2014). Evaluation of the flocculation potential and characterization of bioflocculant produced by *Micrococcus* sp. Leo. *Applied Biochemistry and Microbiology, 50: 601-608.*

Okaiyeto K (2016). Evaluation of the flocculation potential and characterization of bioflocculant produced by three bacterial isolates from Algoa Bay, South Africa*. A Doctor of Philosophy Thesis submitted to University of Forth Hare 308 pages.*

Okaiyeto K, Nwodo UU, Okoli SA, Mabinya LV, Okoh AI (2015a). Implications for public health demands alternatives to inorganic and synthetic flocculants: bioflocculants as important candidates. *Microbiology Open,* 5:177-211.

Okaiyeto K, Nwodo UU, Mabinya LV, Okoli AS, Okoh AI (2015b). Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7. *International Journal of Molecular Science,* 16: 12986-13003.

Okaiyeto K, Nwodo UU, Mabinya LV, Okoh AI (2013). Characterization of a bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. *International Journal of Environmental Research and Public Health*, 10: 5097-5110.

Okoh A, Ugbenyen A, Cosa S, Mabinya L, Babalola OO, Aghdasi F (2012). Thermostable bacterial bioflocculant produced by *Cobetia* Spp. isolated from Algoa Bay (South Africa). *International Journal of Environmental Research and Public Health*, 9: 2108-2120.

Oh HM, Lee SJ, Park MH (2001). Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49. *Biotechnology Letters,* 23: 1229-1234.

Ong SA, Ho LN, Wong YS, Raman K (2012). Performance and kinetics study on bioremediation of diazo dye (Reactive Black 5) in wastewater using spent GAC-biofilm sequencing batch reactor*. Journal of Water, Air and Soil Pollution*, 223: 1615-1623.

Parker DL, Schram BR, Plude JL, Moore RE (1996). Effect of metal cations on the viscosity of pectin-like capsular polysaccharide from the cyanobacterium *Microcystis xosaquae* C3-40. *Applied and Environmental Microbiology,* 62: 1208-1213.

Pasteur L (1876). Etudes Sur la Bierrer. Paris : Gauthier Villars, 229-249

Pathak M, Arundhuti D, Hridi KS, Banwari L (2014). Application of bioflocculating property of *Pseudomonas aeruginosa* strain IASST201 in treatment of oil-field formation water. *Journal of Basic Microbiology, 54: 1–12.*

Pathak M, Sarma HK, Bhattacharyya KG , Subudhi S, Bisht V, Lal B, Devi A (2017). Characterization of a novel polymeric bioflocculant produced from bacterial utilization of *n*-Hexadecane and Its application in removal of heavy metals. *Frontiers in Microbiology*, Doi.org/10.3389/fmicb.2017.00170.

Petzold G, Schwarz S (2014). Polyelectrolyte complexes in flocculation applications. *Advance Polymer Science,* 256: 25-66.

Piyo N, Cosa S, Mabinya VL, Okoh IA (2011). Assessment of bioflocculant production by *Bacillus sp*. Gilbert, a marine bacterium isolated from the bottom sediment of Algoa Bay. *Drugs, 9: 1232- 1242.*

Plackett RL, Burman JP (1946). The design of optimum multifactorial experiments. *Biometrika, 33: 305-325.*

Poorni S, Natarajan K (2014). Flocculation behaviour of hematite–kaolinite suspensions in presence of extracellular bacterial proteins and polysaccharides. *Colloidal Surfactants, 114: 186-192.*

Prasertsan P, Dermlim W, Doelle H, Kennedy JF (2006). Screening, characterization and flocculating property of carbohydrate polymer from newly isolated *Enterobacter cloacae* WD7. *Carbohydrate Polymer,* 66: 289-297.

Prescott LM, Harley JP, Klein DA (2003). Microbiology, 2nd ed.,WMC Brow Publishers, New York.

Prescott LM, Harley JP, and Klein DA (1996). Microbiology 3rd ed., WMC Brow Publishers, New York, 935.

Pu S, Ma H, Deng D, Xue S, Zhu R, Zhou Y, Xiong X (2018). Isolation, identification, and characterization of an *Aspergillus niger* bioflocculant-producing strain using potato starch wastewater as nutrilite and its application. *PLoS ONE, 13: 0190236.*

Raja CE, Omine K (2012). Characterization of boron resistant and accumulating bacteria *Lysinibacillus fusiformis* M1, *Bacillus cereus* M2, *Bacillus cereus* M3, *Bacillus pumilus* M4 isolated from former mining site, Hokkaido, Japan, *Journal of Environmental Science and Health*, 10: 1341-1349.

Rani GU, Mishra S, Sen G, Jha U (2012). Polyacrylamide grafted agar: synthesis, applications of conventional, and microwave assisted technique. *Carbohydrate Polymers,* 90: 784-791.

Rodriguez L, Teixeira J, Oliveira R, van der Mei H (2006). Response surface optimization of the medium components for the production of biosurfactants by Probiotic bacteria, *Process Biochemistry*, 41 : 1-10.

Salehizadeh H, Vossoughi M, Alemzadeh I (2000). Some investigations on bioflocculant producing bacteria. *Journal of Biochemical Engineering,* 5: 39-44.

Salehizadeh H, Shojaosadati SA (2001). Extracellular biopolymeric flocculants: recent trends and biotechnological importance. *Biotechnology Advances,* 19: 371-385.

Salehizadeh H, Shojaosadati SA (2003). Removal ofmetal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. *Water Research, 37: 4231-4235.*

Salehizadeh H, Yan N (2014) Recent advances in extracellular biopolymer flocculants. *Biotechnological Advances,* 32: 1506-22.

Sanghi R, Bhattacharya B, Dixit A, Singh V (2006). *Ipomoea dasysperma* seed gum: An effective natural coagulant for the decolourization of textile dye solutions. *Journal of Environmental Management*, 81: 36-41.

Saritha V, Srinivas N, Srikanth Vuppala NV (2017). Analysis and optimization of coagulation and flocculation process. *Applied Water Science,* 7: 451-460.

Sarker SD, Latif Z, Gray AI (2006). Natural Product Isolation: An Overview in methods in biotechnology, 2nd Edition. Humana Press Inc. Totowa, New Jersey.

Satomi M, La Duc MT, Venkateswaran K (2006). *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *International Journal of Systematic and Evolutionary Microbiology,* 56: 1735-1740.

Satyawali Y, and Balakrishnan M (2008). atomi M, La Duc MT, Venkateswaran K (2006). *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *Journal of Environmentsl Management,* 86: 481-497

Seidel V (2006). Initial and Bulk Extraction in Methods in Biotechnology, 20: 2nd Edition. Humana Press Inc. Totowa, New Jersey.

Selepe TN (2017). Characterisation of selected microbial secies for bioflocculant producing potential and comparison with traditional flocculants in industrial waste water treatment (PHD in Microbiology Thesis), University of Zululand.

Sella SRB, Guizelini BP, Vandenberghe LPS, Medeiros ABP, Soccol CR (2009). Lab-scale production of *Bacillus atrophaeus*' spores by solid state fermentation

in different types of bioreactors. *Brazillian Archives of Biology and Technology*, 52: 159-170.

Sella SRB, Vandenberghe LP, Soccol CR (2015). Bacillus atrophaeus: main characteristics and biotechnological applications - a review. *Critical Reviews in Biotechnology,* 35:533-545.

Shamsi H, Mardani K, Ownagh A (2017). Phylogenetic analysis of Escherichia coli isolated from broilers with colibacillosis based on gyrA gene sequences. *Cananadian Journal Veterinary Research,* 81:28-32.

Sharma BR, Dhuldhoya NC, Merchant UC (2006). Flocculants-an Ecofriendly Approach. *Journal of Polymer Environment,* 14: 195-202.

Sheng GP, Yu H, Li XY (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. *Biotechnology Advances*, 28: 882-894.

Shih IL, Van LC, Lin HG, Chang YN (2001). Production of a biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties. *Bioresource Technology,* 78: 267-272.

Shi-Jie Y, Min S, Guo-Ping S, Yin L, Wen-Wei L, Ri-Sheng Y (2011). Identification of key constituents and structure of the extracellular polymeric substances excreted by *Bacillus megaterium* TF10 for their flocculation capacity. *Environmental Science Technology,* 45: 1152-1157.

Shimofuruya H, Koide A, Shirota K, Tsuji T, Nakamura M, Suzuki J (1995). The production of flocculating substance (s) by *Streptomyces griseus*. *Biosci*ence, *Biotechnol*ogy and *Biochem*istry, *60*: 498-500.

Singh RP, Tripathy T, Karmaker GP, Rath SK, Karmaker NC, Pandey SR, Kannan K, Jain SK, Lan NT (2000). Novel biodegradable flocculants based on polysaccharides. *Current Science,* 78: 7-10.

Subudhi S, Bishta V, Batta N, Pathak M, Devib A, Lal B (2015). Purification and characterization of exopolysaccharide bioflocculant produced by heavy metal resistant *Achromobacter xylosoxidans*. *Carbohydrate Polymers,* 137: 441-451.

Subudhi S, Batta N, Pathak M, Bisht V, Devi A, Lal B (2014). Bioflocculant production and biosorption of zinc and lead by a novel bacterial species, Achromobacter sp TERI-IASST N, isolated from oil refinery waste, *Chemosphere,* 113: 116-124.

Suh HH, Kwon GS, Lee CH, Kim HS, Oh H.M (1997). Characterization of bioflocculant produced by *Bacillus* sp. Dp-152. *Journal of Fermentation Bioengineering,* 84: 108-112.

Sun DT, Peng L, Reeder WS, Moosavi SM, Tiana D, Britt DK, Ovelsi E, Queen WL (2018). Rapid, selective heavy metal removal from water by a metl-organic framework/polydopamine composite. *American Chemical Society Central Science,* 4: 349-356.

Tadros TF, (2007) General principles of colloid stability and the role of surface forces part 1. *Colloids and Interface Science Series*, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Volume 1.

Tang J, Suijian Q, Zhigang L, Qun A, Mingquan X, Bo Y (2014). Production, purification and application of polysaccharide-based bioflocculant by *Paenibacillus mucilaginosus*. *Carbohydrate Polymer,* 113: 463-470.

Tawila ZMA, Ismail S, Dadrasnia A, Usman MM (2018). Production and characterization of a bioflocculant produced by *Bacillus salmalaya* 139SI-7 and Its applications in wastewater treatment. *Molecules,* 23: 2689.

Tirumalai MR, Stepanov VG, Wünsche A, Montazari S, Gonzalez RO, Venkateswaran K and Fox GE. (2018). *Bacillus safensis* FO-36b and *Bacillus pumilus* SAFR-032: a whole genome comparison of two spacecraft assembly facility isolates. *BMC Microbiology,* 18: 57.

Turner S, Pryer KM, Miao VPW, Palmer JD (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology,* 4: 327-338.

Toeda K, Kurane R (1991). Microbial flocculant from *Alcaligenes cupidus* KT201. *Agriculture Biology Chemistry, 55: 2793-2799.*

Ugbeyen MA, Dafel J, Akapo CO, Mazibuko X, Simonis JJ, Basson AK (2017). Optimisation of the bioflocculant produced by *Pantoea* sp. a novel bacterium isolated from marine sediment. *Nigeria Journal of Pure and Applied Science, 30: 3066-3073.*

Ugbenyen AM, Cosa S, Mabinya LV, Okoh AI (2014). Bioflocculant production by *Bacillus* sp. Gilbert isolated from a marine environment in South Africa. *Applied Biochemical Microbiology,* 50: 49-54.

Ugbeyen MA, Okoh AI (2013). Flocculating properties of a bioflocculant produced by *Bacillus* sp. isolated from a marine environment in South Africa. *Chemical Biochemistry Engineering,* 27: 511-518.

Ugbenyen A, Cosa S, Mabinya L, Babalola OO, Aghdasi F, Okoh A (2012). Thermostable bacterial bioflocculant produced by *Cobetia* sp. isolated from Algoa Bay (South Africa). *International Journal of Environmental Research and Public Health, 9: 2108-2120.*

United Nations Water (2007*).* *https://www.un.org/waterforlifedecade/scarcity.shtml* (accessed on 16/04/2019).

United Nations Water (2017) Development Cooperation. First report on development cooperation. https://www.ohchr.org *(accessed on 16/04/2019).*

Veening J (2007). Phenotypic variation in *Bacillus subtilis*: bistability in the sporulation pathway. Enschede: Print Partners Ipskamp, Proefschrift, Rijksuniversiteit Groningen, Nederlands.

Vijayaraghavan G, Sivakumar T, Vimal Kumar A (2011). Application of plant based coagulants for wastewater treatment. *International Journal of Advanced Engineering Research and Studies:* Research Article, 88-92 .

Vu B, Chen M, Crawford R, Ivanova E (2009). Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules,* 14: 2535–2554.

Ventosa A, Nieto JJ, Oren A (1998). Biology of moderately halophilic aerobic bacteria**.** *Microbiology Molecular Biology Review,* 62: 504-544.

Wang JP, Chen YZ, Ge XW, Yu HQ (2007). Optimization of coagulation-flocculation process for paper recycling wastewater treatment using response surface methodology. *Colloids surfaces A: Physicochemistry Engineering Aspects,* 302: 204-210.

Wang JP, Chen YZ, Zhang SJ, Yu HQ (2008). A chitosan based flocculant prepared with gamma-irradiation-induced grafting. *Bioresource Technology*, 9: 3397- 3402.

Wang L, Maa F, Lee DJ, Wanga A, Ren N (2013). Bioflocculants from hydrolysates of corn stover using isolated strain *Ochrobactium ciceri* W2. Bioresource Technology, 145: 259-263.

Wang X, Wang X, Liu M, Zhou L, Gu Z, Zhao J (2015). Bioremediation of marine oil pollution by *Brevundimonas diminuta*: effect of salinity and nutrients. Desalin Water Treat. doi:10.1080/19443994.2015.1106984.

Wang L, Ma F, Qu Y, Sun D, Li A, Guo J, Yu B (2011). Characterization of a compound bioflocculant produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6. *World Journal of Microbiology Biotechnology,* 27: 2559-2565.

Wang ZR, Sheng JP, Tian XL, Wu TT, Liu WZ, Shen L (2010). Optimization of the production of exopolysaccharides by *Bacillus thuringiensis* 27 in sand biological soil crusts and its bioflocculant activity. *African Journal of Microbiological Research, 5: 2359–2366.*

Wiegand I, Hilpert K, Hancock REW (2007). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, 2: 163.

Willmott NJ, Guthrie JT, Nelson G (1998). The biotechnology approach to colour removal from textile effluent. *Journal of the Society of Dyers and Colourists,* 114:38-41.

Wu J, Ye HF (2007). Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtillis* DYU1 isolate. *Process Biochemistry,* 42: 1114-1123.

Xia S, Zhang Z, Wang X, Yang A, Chen L, Zhao J, Leonard D, Jaffrezic-Renault N (2008). Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. *Bioresource Technology,* 99: 6520-6527.

Xiong Y, Wang Y, Yu Y, Li Q, Wang H, Chen R, He N (2010). Production and characterization of a novel bioflocculant from *Bacillus licheniformis. Applied Environmental Microbiology, 76: 2778–2782.*

Yang Q, Luo K, Liao D, Li X, Wang D, Liu X, Zeng G, Li X (2012). A novel bioflocculant produced by *Klebsiella* sp. and its application to sludge dewatering. *Water Environment Journal, 26: 560-566.*

Yim JH, Kim SJ, Ahn SH, Lee HK (2007). Characterization of a novel bioflocculant, p-KG03, from a marine dinoflagellate, *Gyrodinium impudicum* KG03. *Bioresource Technology,* 98: 361-367.

Yokoi H, Yoshida T, Mori S, Hirose J, Hayashi S, Takasaki Y (1997). Biopolymer flocculant produced by an *Enterobacter* sp. *Biotechnology Letters*, 19: 569-573.

Yokoi H, Natsuda O, Hirose J, Hayashi S, Takasaki Y (1995). Characteristics of a biopolymer flocculant produced by *Bacillus* sp. PY-90. *Journal of Fermentation Bioengineering,* 4: 378-380.

Zhang T, Lin Z, Zhu HT (1999). Microbial flocculant and its application in environment protection. *Journal of Environmental Science,* 11: 1-2.

Zhang Z, Lin B, Xia S, Wang X, Yang A (2007). Production and application of a novel bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source. *Journal of Environmental Sciences, 19: 667-673.*

Zhang F, Jiang WJ, Wang XD, Wang YN, Zhang W, Chen J (2008). The preparation and activity comparison of bioflocculant from alcohol wastewater using different microorganisms. *Journal of Sichuan University,* 40: 94–98.

Zhang ZQ, Xia SQ, Zhao SQ, Zhang JF (2010). Characterization and flocculation mechanism of high efficiency microbioflocculant TJ-F1 from *Proteus mirabilis*. *Colloids Surfactant Biointerfaces,* 75 : 247-51.

Zhang C, Cui Y, Wang Y (2012). Bioflocculant produced from bacteria for decolorization, Cr removal and swine wastewater applications. *Sustainable Environmental Research*, 22: 129.

Zhang WX, Yang H, Dong L, Yan H, Li HJ, Jiang ZW, Kan XW, Li AM, Cheng RS (2012). Efficient removal of both cationic and anionic dyes from aqueous solutions using a novel amphoteric straw-based adsorbent. *Carbohydrate Polymer,* 90: 887-893.

Zhao H, Liu H, Zhou J (2013). Characterization of a bioflocculant MBF-5 by *Klebsiella pneumoniae* and its application in *Acanthamoeba* cysts removal. *Bioresource Technology*, 137: 226-232.

Zheng Y, Ye Z, Fang X, Li Y, Cia W (2008). Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19*. Biosource Technol*ogy, 99: 7686-7691.

Zhuang X, Wang Y, Li Q, Yan S, He N (2012). The production of bioflocculants by *Bacillus licheniformis* using molasses and its application in the sugarcane industry. *Biotechnology and Bioprocess Engineering,* 17: 1041-1047.

Zulkeflee Z, Ahmad ZA, Zulkifli HS, Mohd KY (2012). Cation dependence, pH tolerance, and dosage requirement of a bioflocculant produced by *Bacillus* spp. UPMB13: Flocculation performance optimization through kaolin assays. The *Sciencific World Journal,* 7. doi:10.1100/2012/495659.

APPENDIX

Appendix 1

Table 4.1 Effect of inoculum size on bioflocculant produced by *Bacillus atrophaeus*

Inoculum size (%) w/v	Flocculating activity Abs@550nm		
1%	0.42	0.421	0.427
2%	0.375	0.377	0.380
3%	0.389	0.391	0.395
4%	0.141	0.145	0.135
5%	1.416	1.425	1.421

Table 4.2 : Effect of carbon source on bioflocculant production by *Bacillus atrophaeus*

Carbon source	Flocculating activity Abs@550nm		
Maltose	0.508	0.507	0.525
Fructose	0.223	0.224	0.235
Galactose	1.509	1.513	1.51
Sucrose	0.238	0.234	0.255
Starch	1.584	1.607	1.579
Glucose	0.141	0.145	0.141

Table 4.3 : Effect of nitrogen source on bioflocculant production by *Bacillus atrophaeus*

Nitrogen source	Flocculating activity		Abs@550nm
Urea	0.696	0.750	0.685
Ammonium sulphate	0.600	0.578	0.565
Peptone	0.522	0.489	0.520
Yeast extract powder	0.12	0.122	0.140
Ammonium chloride	0.063	0.064	0.054

Table 4.4 : Effect of cation on flocculating activity

Cation	Flocculating Activity	Abs@550nm	
Ca++	0.182	0.186	0.150
Ba++	0.139	0.125	0.120
Li+	0.160	0.171	0.161
Mg++	0.221	0.241	0.210
K+	0.271	0.291	0.301
Fe+++	1.488	1.508	1.487
without cation	0.766	0.730	0.748

Table 4.5 : Effect initial pH on bioflocculant production by *Bacillus atrophaeus*

pH	Flocculating Activity		Abs@550nm
3	0.610	0.639	0.637
4	0.535	0.562	0.542
5	0.681	0.696	0.680
6	0.685	0.684	0.656
7	0,401	0.619	0.62
8	0.485	0.520	0.491
9	0.196	0.219	0.204
10	1.512	1.501	1.521
11	1.344	1.370	1.327
12	1.468	1.456	1.511

Table 4.6 : Effect of cultivation temperature on bioflocculant production by *Bacillus atrophaeus*

Temp (^oC)	Flocculating activity		Abs@550nm
20	1.200	1.192	1.201
25	0.632	0.642	0.634
30	0.238	0.234	0.235
35	0.241	0.234	0.216
40	1.178	1.062	1.016
45	0.626	0.646	0.635
50	0.89	0.894	0.881

Table 4.7 : Effect of speed on bioflocculant production by *Bacillus atrophaeus*

Speed (rpm)	Flocculating Activity Abs@550nm		
0	0.541	0.551	0.571
55	0.210	0.253	0.259
110	0.014	0.031	0.016
165	0.041	0.044	0.047
220	0.403	0.375	0.379

Table 4.8 : Time course on bioflocculant production by *Bacillus atrophaeus*

Time (hr)	pH			Flocculating Activity			Optical density		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	9.00	9.00	9.00	-	-	-	0.156	0.151	0.157
12	8.26	7.95	8.21	49	35	39	0.331	1.077	0.471
24	7.82	7.62	7.95	86	90	93	0.345	1.047	0.937
36	6.86	6.66	7.01	95	92	93	2.513	2.562	2.551
48	5.82	5.92	6.05	92	95	94	2.603	2.421	2.811
60	5.82	5.97	5.95	86	92	88	2.644	2.629	2.638
72	5.82	5.93	5.87	92	91	91	2.85	2.866	2.601
84	5.82	5.92	5.84	97	98	98	2.828	2.519	2.552
96	5.81	5.89	5.8	99	98	99	2.736	2.501	2.628
108	5.81	5.88	5.82	81	83	82	2.653	2.522	2.66
120	5.81	5.87	5.76	95	95	94	2.631	2.401	2.796

Table 4.9 Effect of dosage concentration (mg/ml) on bioflocculant BA-CGB

Dosage conc. (mg/ml)	Flocculating activity		
0.2	0.671	0.665	0.695
0.4	0.368	0.366	0.367
0.6	0.399	0.406	0.426
0.8	0.463	0.443	0.463
1.0	0.319	0.328	0.318

Table 4.10: Effect cation on flocculating activity of pure bioflocculant BA-CGB

Cation	Flocculating Activity		
Ba++	0.654	0.635	0.685
Mg++	0.467	0.472	0.440
Ca++	0.368	0.300	0.340
Li+	1.205	1.185	1.160
K+	1.011	1.031	0.988
Fe+++	1.517	1.438	1.481
Mn++	0.620	0.563	0.589
without cation	2.301	2.325	2.285

Table 4.11: Effect of temperature on flocculating activity of pure bioflocculant BA-CGB

Temp ^{o}C	Flocculating Activity		
50	0.572	0.565	0.572
60	0.533	0.532	0.760
70	0.575	0.628	0.575
80	0.608	0.596	0.615
90	0.556	0.558	0.566
100	0.758	0.749	0.770
Autoclaved at 121	0.749	0.752	0.785

Table 4.12 : Effect of pH on flocculating activity of pure bioflocculant BA-CGB

pH	Flocculating Activity		
3	0.349	0.355	0.347
4	0.495	0.501	0.475
5	0.849	0.870	0.844
6	0.624	0.614	0.590
7	0.683	0.663	0.682
8	0.656	0.644	0.650
9	0.908	0.905	0.894
10	0.430	0.421	0.445
11	0.142	0.148	0.141
12	0.235	0.237	0,242

Table 4.13: Determination of carbohydrates content of BA-CGB

Concentration (Mm)	Average Absorbance	Adjusted OD
Blank	0.133	0.000
0.05	0.145	0.012
0.1	0.185	0.052
0.2	0.271	0.138
0.4	0.285	0.152
0.6	0.290	0.157
0.8	0.301	0.168
1.0	0.308	0.175
1.5	0.314	0.181
2	0.350	0.217
BA-CGB 250µl	1.197	1.064

Table 4.14: Determination of uronic acid content of BA-CGB

Concentration (Mm)	Average Absorbance	Adjusted OD
Blank	0.373	0.000
0.001	0.393	0.020
0.002	0.412	0.039
0.003	0.414	0.041
0.004	0.423	0.050
0.005	0.462	0.089
0.010	0.547	0.174
0.050	0.633	0.260
BA-CGB	0.544	0.171

Table 4.15: Determination of protein content of BA-CGB

Concentration (Mm)	Average Absorbance	Adjusted OD
Blank	0.113	0.000
5µl	0.958	0.845
10µl	1.069	0.956
15µl	1.211	1.098
20µl	1.155	1.042
25µl	1.367	1.254
BA-CGB	0.176	0.063

Note: Values collected in triplicates

Table 4.16: Comparison of flocculating activity of pure bioflocculant BA-CGB with other flocculants

Flocculants	Flocculating Activity		
Polyacrylamide	0.176	0.201	0.175
BA-CGB	0.230	0.245	0.231
Ferric chloride	0.223	0.230	0.229
Alum	0.444	0.454	0.443

Appendix 2

(a) *Phenol-sulfuric acid assay* for the determination of carbohydrates

Sensitivity: glucose (0.05−2mM)

Final volume: 1.4 ml

Reagents:

(a) phenol dissolved in water (5% w/v).

(b) Concentrated sulphuric acid.

Method

(i) Mix samples, standards and control solutions (200 µl containing up to 100 µg carbohydrate) with 200 µl of reagent A.

(ii) Add 1.0 ml of reagent B rapidly and directly to the solution surface without touching the sides of the tube.

(iii) Leave the solutions undisturbed for 10 minutes before shaking vigorously.

(iv) Determine the absorbance at 490 nm after a further 30 minutes.

(b) *Bradford Method* for the determination of protein

The Bradford Protein Assay is the preferred colorimetric assay for quantifying total protein concentration. Bradford method is based upon the complex formation between basic and aromatic amino acid residues with Coomassie® Brilliant Blue G-250 dye; the microassay is used for measuring 1-10 µg of protein. For maximum convenience, Bradford Reagent and 0.5 mg/ml BSA standard solution are offered individually or as components of a kit.

Bradford Test Kit Includes, Bradford Reagent – 1 litre , Albumin, Bovine – 0.5 mg/ml Standard, 0,15N Sodium chloride

Method

1. Different concentrations of BSA was added to microfuge and sufficient 0,15N NaCl were added to make a final volume of 100µl
2. 1 ml of aliquot of Bradford reagents was added to each standard/sample and the absorbance was determined with a spectrophotometer at 595 nm after a 2 minutes incubation.

(c) *Carbazole assay* for the determination of uronic acid

Sensitivity: D-glucurono-6,3-lactone (0.001−0.050mM)

Final volume: 1.8 ml

Reagents:

(a) Dissolve 0.95 g of sodium tetraborate decahydrates in 2.0 ml of hot water and add 98 ml of ice-cold concentrated sulphuric acid carefully with stirring.

(b) Dissolve 125 mg of carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.

Method

(i) Cool the samples, standards and controls (250 µl) in an ice bath.

(ii) Add ice-cold reagent A (1.5 ml) with mixing and cooling in the ice bath.

(iii) Heat the mixtures at 100 ^{o}C for 10 minutes.

(iv) Cool rapidly in the ice-bath.

(v) Add 50 µl of reagent B and mix well.

(vi) Reheat at 100 ^{o}C for 15 minutes.

(vii) Cool rapidly at room temperature and determine the absorbance at 525 nm.

Brown pigmented colonies of Bacillus atrophaeus on Nutrient Agar media isolated form Marine Sandy soil Samples, Tuzi Gazi, Richards Bay Harbour