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EVALUATION OF FLOCCULATING POTENTIALS AND CHARACTERIZATION OF BIOFLOCCULANTS PRODUCED BY THREE BACTERIAL ISOLATES FROM ALGOA BAY, SOUTH AFRICA

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BY

KUNLE OKAIYETO

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SUPERVISOR: PROF L.V. MABINYA

CO-SUPERVISOR: PROF A.I. OKOH

2016

DECLARATION

I, the undersigned, declare that this thesis entitled "Evaluation of flocculating potentials and characterization of bioflocculants produced by three bacterial isolates from Algoa Bay, South Africa" submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences. The work contained herein is my original work with exemption to the citations and that the work has not been submitted to any other University in partial or entirely for the award of any degree or examination purposes.

Name: Kunle Okaiyeto

Signature: …….....

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DECLARATION OF PLAGIARISM

I, **Kunle Okaiyeto**, student number: 201103152 hereby declare that I am fully aware of the University of Fort Hare's policy on plagiarism and I have taken every precaution to comply with the regulations.

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CERTIFICATION

This thesis titled "Evaluation of flocculating potentials and characterization of bioflocculants produced by some bacterial isolates from Algoa Bay, South Africa" meets the regulation governing the award of degree of Doctor of Philosophy of the University of Fort Hare and is approved for its contribution to scientific knowledgment.

…….. ……

Prof. L.V. Mabinya (Major Supervisor) Date

……. ……

Prof A.I. Okoh (Co-supervisor and Head of Department) Date

DEDICATION

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

"In his heart a man plans his course, but the LORD determines his steps"

Proverbs 16:9 (NIV)

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"*A teacher affects eternity; he can never tell where his influence stops*"

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LIST OF ACRONYMS

AEMREG:	Applied and Environmental Microbiology Research Group
ANOVA:	Analysis of Variance
BA:	Bioflocculant Alone
BLAST:	Basic Local Alignment Search Tools
BOD:	Biochemical Oxygen Demand
BSA:	Bovine Serum Albumin
BPMs:	Bioflocculant-producing microorganisms
CMCNa:	Sodium CarboxyMethyl-Cellulose
COD:	Chemical Oxygen Demand
DADMAC:	Polyethylene Amine Poly (Diallyl Dimethyl Ammonium Chloride
DCC:	Dicarboxylic Acid
EC:	European Commission
EDX:	Energy Dispersive X-ray spectroscopy
EPS:	Exopolysaccharides
FA:	Flocculating Activity
FTIR:	Fourier Transform Infrared Spectroscopy
GMRDC:	Goven Mbeki Research and Development Centre
LB-EPS:	Loosely Bound-Exopolysaccharides
NCBI:	National Centre for Biotechnology Information
NRF:	National Research Foundation
NTU:	Nephelometric Turbidity Units
PAA:	Polyacrylamide
PAC:	Polyaluminium Chlorides
PCR:	Polymerase Chain Reaction

PDMDAAC:	Poly-Dimethyl-Diallyl-Ammonium Chloride
PFC:	Polyferric Chloride
PFC-PDMDAAC:	Polyferric Chloride-Poly-Dimethyl-Diallyl-Ammonium Chloride
PFS:	Polyferric Sulphate
rDNA:	Ribosomal Deoxyribonucleic Acid
SAMRC:	South Africa Medical Research Council
SEM:	Scanning Electron Microscopy
SS:	Suspended Solid
TB-EPS:	Tightly Bound-Exopolysaccharides
TGA:	Thermogravimetric Analyses
UNICEF:	United Nations International Children's Emergency Fund
UK:	United Kingdom
USA:	United State of America
WHO:	World Health Organizations

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GENERAL ABSTRACT

GENERAL ABSTRACT

Flocculation has been widely adopted as one of the most effective methods to remove colloidal particles in water or wastewater treatment. Synthetic flocculants are conventionally used because of their high flocculating efficiency and cost-effectiveness. However, they have been reported to have hazardous properties and implicated in some serious health problems including senile dementia and neuro-toxicity, as well as being recalcitrant in the environment. Consequently, efforts are being geared away from the use of synthetic flocculants in water and wastewater treatment. Hence, the need for safe and eco-friendly flocculants has become imperative. Compared with synthetic flocculants, bioflocculants have special advantages such as safety, biodegradability and harmlessness to the environment and humans; attributes which make them potential alternatives in water treatment, downstream as well as fermentation processes. In the current study, the potentials of bacterial isolates recovered from Algoa Bay in the Eastern Cape Province of South Africa for bioflocculant production were investigated. The bacterial isolates were identified by polymerase chain reaction (PCR) as belonging to the *Bacillus* genus. The analysis of 16S ribosomal deoxyribonucleic acid (rDNA) nucleotide sequence of isolate M72 showed 99% similarity to *Bacillus toyonensis* strain BCT-7112 and was deposited in the GenBank as *Bacillus toyonensis* strain AEMREG6 with accession number KP406731. Likewise, the 16S rDNA nucleotide sequences of isolates M69 and M67 showed 98% sequence similarity to *Bacillus licheniformis* strain W7 and *Bacillus algicola* strain QD43 respectively; and M67 isolate was subsequently deposited in the GenBank as *Bacillus* sp. AEMREG7 with accession number KF933697.1. The results of the nutritional requirements and fermentation conditions revealed that optimum inoculum size for REG-6 production was 4% (v/v), while 5% (v/v) and 3% (v/v) were most favourable for MBF-W7 and MBF-UFH production respectively. Glucose was the best carbon source for the production of bioflocculants (REG-6 and MBF-UFH) by *Bacillus toyonensis* AEMREG6 and

Bacillus sp. AEMREG7 respectively, while maltose supported optimum bioflocculant (MBF-W7) production by *Bacillus* specie. Inorganic nitrogen (NH_4NO_3) was the favoured nitrogen source for both REG-6 and MBF-W7 production, while mixed nitrogen sources [yeast extract + urea + $(NH_4)_2SO_4$] supported the maximum production of MBF-UFH. The initial medium pH for REG-6 was 5, while MBF-W7 and MBF-UFH were both maximally produced at the initial pH of 6. After a 96 h cultivation period under optimal culture conditions, 3.2 g of purified REG-6 with a maximum flocculating activity of 77% was recovered from 1 L fermented broth of *Bacillus toyonensis* AEMREG6. Yields of 3.8 g and 1.6 g pure bioflocculants with the respective highest flocculating activities of 94.9% and 83.2% were also obtained from 1 L, 72 h-fermented broths of *Bacillus licheniformis* and *Bacillus* sp. AEMREG7 respectively. Furthermore, all the three bioflocculants (REG-6, MBF-W7 and MBF-UFH), displayed thermal stability within the temperature range of 50 to 100 ^{o}C, with strong flocculating activities of over 80% against kaolin suspension over a wide range of pH range (3–11) and relatively low dosage requirements of 0.1-03 mg/ml in the presence of divalent cations in the treatment of kaolin clay suspension and Thyme River waters. Chemical composition analyses of the bioflocculants showed them to be glycoproteins with a predominantly polysaccharide backbones as shown by the following carbohydrate/protein (w/w) ratios: 77.8%:11.5% (REG-6); 73.7%:6.2% (MBF-W7) and 76%:14% (MBF-UFH). Fourier transform infrared spectroscopy (FTIR) revealed the presence of hydroxyl, carboxyl and amide groups which are preferred for effective flocculation. Scanning electron microscopy (SEM) images of the purified bioflocculants showed that they have an irregular, coarse-grained structure connected in netted textures; it also revealed how the bioflocculants connected the scattered kaolin particles firmly together to form bigger flocs which subsequently precipitated out of suspension as a result of gravity. MBF-W7 showed good turbidity removal potential (86.9%) and chemical oxygen demand (COD) reduction

efficiency (75.3%) of Thyume River waters. MBF-UFH showed higher flocculating activity for kaolin clay suspension compared to synthetic flocculants (aluminium chloride and iron chloride). The results obtained from this study suggested that the bioflocculants (REG-6, MBF-W7 and MBF-UFH) produced by these bacterial isolates have great potentials to serve as an alternatives to hazardous synthetic flocculants conventionally utilized in various industrial processes including water/wastewater treatment, and stand as attractive candidates for further research and development for industrial-scale application.

CHAPTER ONE

GENERAL INTRODUCTION

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1.0 INTRODUCTION

Environmental pollution is a major universal predicament with its cognate debilitating effects on economic development (Pathak *et al.*, 2014). Water pollution is one of the most challenging environmental issues and has become a global stumbling block for improving the quality of life in many communities (Prasertsan *et al.*, 2006). Water is a source of life and energy, even though millions of people worldwide are suffering with the paucity of safe water for drinking purposes (Bhatnagara and Sillanpaa, 2010). Unwitting urbanization and expeditious growths in population have immensely contributed to the parlous state of water pollution and the prevailing unhealthy environment (Prasertsan *et al.*, 2006). The major source of these pollutions comes from the discharge of domestic and agricultural wastes, untreated sanitary and toxic industrial effluents (Li *et al.*, 2013). The presence of these pollutants in water-bodies can be pernicious to aquatic life as well as render it unsuitable as potable water sources for domestic usage; and a consequence of pollution of freshwater environment is a life-threatening effect on man's healthy living (Yang *et al.*, 2012).

According to the report of WHO/UNICEF (2000), about 70-80% of all illnesses in developing countries are linked to consumption of contaminated water especially among the vulnerable population groups (Bhatnagar and Sillanpaa, 2010). Pollutants from wastewaters, when discharged into the natural water bodies, becomes toxic to aquatic life and render the waters unfit for consumption. As a result of these alarming water-borne diseases as well as an increase in demand for safe water for both municipal and industrial purposes, much attention has been focused on water treatment; thus making it imperative to appraise water quality on a perpetual basis (Yang *et al.*, 2012). Various stringent regulations have been inaugurated by many countries with respect to the presence of contaminants in water to ensure proper treatment of domestic and agricultural wastewater as well as industrial effluents prior to their discharge into different water-bodies (Bhatnagar and Sillanpaa, 2010; Li *et al.*, 2013). Quite a lot of water

treatment technologies are available and these include coagulation/flocculation, filtration, ion exchange, oxidation processes, adsorption, solvent extraction and electrolysis (Low *et al.*, 2011; Ong *et al.*, 2012; Radiou *et al.*, 2004).

Nevertheless, the major impediment with some of these techniques is restrictive financial costs in their operation which tend to reduce their efficient and effective utilization (Li *et al.*, 2013). Coagulation/flocculation on the other hand, is the preferred process because it is less expensive, efficient, not labour-intensive, required less skilled personnel (Li *et al.*, 2013; Renault *et al.*, 2009). Furthermore, one of the major advantages of coagulation/flocculation process is the decolourization of wastewater due to the removal of dye molecules from the final effluents thus eliminating possible decomposition of dyes which can release noxious chemicals which contain aromatic compounds (Aboulhassan *et al.*, 2006).

Flocculation is a process whereby colloids, cells and suspended solids come out of suspension in the form of floc or flakes as a consequence of aggregation (Bhunia *et al.*, 2012). Flocculants are commonly used in the various industrial processes such as wastewater treatment, drinking water purification and downstream processes in fermentation industries (Shih *et al.*, 2001). Shih and Van (2001) found that flocculation could be used as an alternative to centrifugation and filtration for the separation of microbial cells from broth in food, beverage and pharmaceutical industries. In addition, Deng *et al.* (2003) observed that flocculation is an effective technique that is commonly used in wastewater treatment for removing not only suspended solids but also metal ions.

Flocculants are classified into three categories: inorganic flocculants such as polyaluminium chloride, ferric chloride, organic synthetic flocculants such as polyacrylamide derivatives and naturally occurring flocculants such as chitosan, sodium alginate and bioflocculant (Aljuboori *et al.*, 2013). Both inorganic and organic flocculants are referred to as chemical flocculants.

These chemical/synthetic flocculants are commonly used to remove particulate matter from a variety of waters because of their high flocculating efficiency and low cost (Zheng *et al.*, 2008). However, several neuropathological studies have showed a linkage between residual aluminium in water and the pathogenesis of Alzheimer's disease and dementia (Banks *et al.*, 2006). Ferrite flocculants are also good flocculating agents except for causing unpleasant metallic taste, malodour and being highly corrosive in nature (Li *et al.*, 2008). Polyacrylamides on the other hand are non-biodegradable, and the carcinogenicity property of the residual acrylamide monomers restricts their industrial applicability (Zhuang *et al.*, 2012). Various cationic polymers are recognised to pose a potentially significant hazard to aquatic life particularly fishes and other aquatic lives. The surface of fish gills carries a negative charge to which cationic polyelectrolytes will readily bind due to electrostatic attraction (Murgatroyd *et al.*, 1996). This effect leads to suffocation and reduction in oxygen transfer and mucous production on the gill surfaces thereby causing death of the fishes which consequently reduces the supply of healthy fish for human consumption (Narkis and Rebhun, 1975). Due to the numerous problems associated with the usage of chemical flocculants, more attention has been diverted to microbial flocculants (bioflocculants).

Microorganisms such as bacteria, algae, fungi and actinomycetes can utilize the nutrients in the culture medium to synthesize intracellular high molecular weight polymers which can be excreted into the medium or on the surface of the bacteria as capsules possessing flocculating properties (Deng *et al.*, 2003; Gao *et al.*, 2006). Recently, the use of microbial flocculants has been instigated as a panacea to environmental problems emanating from the use of chemical flocculants (Li *et al.*, 2009). Bioflocculants are safe and eco-friendly because they possess no secondary pollution (Zhang *et al.*, 1999; Yim *et al.*, 2007). The major chemical components of these bioflocculants include proteins and polysaccharides with nucleic acids, humic acids, lectins, lipids and other polymers usually present in lower concentrations (Badireddy *et al.*,

2010). The flocculating activity of a bioflocculant depends solely on its chemical structure which can be related to the molecular weight and the functional groups present in the molecular chain of such a biopolymer (Gao *et al.*, 2006). For example, Li *et al.* (2010) and Nwodo *et al.* (2014) reported that the biopolymers produced by both *Agrobacterium* sp. M-503, and a consortium of *Streptomyces and Cellulomonas* species were predominantly composed of glycoprotein while the bioflocculants produced by *Bacillus* sp. Gilbert (Ugbenyen *et al.*, 2014) and *Halomonas* sp. AAD6 (Sam *et al.*, 2011) were composed of polysaccharide.

However, the production of bioflocculants is usually influenced by culture media constituents such as carbon, nitrogen sources, salts as well as factors such as the pH and temperature of the medium, and aeration level (Lopez *et al.*, 2003; Salehizadeh and Shojaosadati, 2001). Several researchers have reported on the production of bioflocculants from different microorganisms utilizing complex media for growth (Gao *et al.*, 2006; Li *et al.*, 2009; Piyo *et al.*, 2011). For example, Cosa *et al.* (2011) found that glucose and peptone were utilized as carbon and nitrogen sources respectively with Fe^{2+} as a salt of choice for production of bioflocculant by *Virgibacillus* sp. Rob. *Serratia ficaria* utilises beef extract, urea, lactose and Mg^{2+} or Ca^{2+} for bioflocculant production (Gong *et al.*, 2008), whereas *Enterobacter cloacae* WD7 preferred sucrose, yeast extract and Ca^{2+} as the salt for bioflocculant production (Prasertsan *et al.*, 2006). The complex media utilization renders the fermentation process to be economically extortionate due to high cost of nutrients such as glucose, sucrose, peptone, yeast extract and salts. There is a need to reduce fermentation costs whilst increasing bioflocculant yields thus necessitating the need for alternative, but cost-effective, substrates (Fujita *et al.*, 2000).

Agro-industrial wastes such as sugarcane molasses, fishmeal wastewater and corn-steep liquor could be utilised as alternative substrates for cost-effective production of bioflocculants (Zhuang *et al.*, 2012). The utilization of organic wastes as substrates in production media is ecologically auspicious, sustainable and economically plausible. Hauang *et al.* (2001) and

Zhou *et al.* (2003) both reported on the utilization of some cost-effective substrates such as soybean juice and fishmeal wastewater for bioflocculant production. For example, when yeast extract in the growth medium of microorganism producing NOC-1 was replaced by bean cake, aquafarm wastewater, or cattle blood, two-thirds of the cultivation cost could be saved (Kurane *et al.*, 1994). The presence of nutrient substances in brewery wastewater makes it a potential substrate for bioflocculant production by certain microorganisms (Chen *et al.*, 2003). For example, Zhang *et al.* (2007) investigated the utilization of brewery wastewater as carbon source for bioflocculant production by multiple-microorganism consortia. High flocculating activity and bioflocculant yields were obtained when *Serratia ficaria* and *Klebsiella mobilis* were cultivated in the presence of dairy wastewater (Gong *et al.*, 2008; Wang *et al.*, 2007). The production of a novel polygalacturonic acid bioflocculant REA-11 by *Corynebacterium glutamicum* was achieved optimally when sucrose and corn steep liquor were used as substrates (He *et al.*, 2004). In contrast, the production of an exopolysaccharide bioflocculant by *Sorangium cellulosum* was realized in the presence of soluble starch, a cheaper substrate than glucose (Zhang *et al.*, 2002). The utilization of these cost-effective substrates for production of bioflocculants makes logical sense if production costs are to be contained.

Bioflocculants have been recognized to have applications in wastewater treatment, for example in the decolourization of dyes in solution (Deng *et al.*, 2005), inorganic solid suspensions e.g. bentonite, solid clay, aluminium oxide and activated carbon (Levy *et al.*, 1992; Yim *et al.*, 2007).

1.1 RESEARCH PROBLEMS

Increasing industrialization has been recognised as an enviable choice due to its contribution to economic growth. However, it has considerably raised the rate of water pollution especially

from industrial sources and this has become a major environmental concern (Sarkar *et al.*, 2006). Water pollution is one of the most challenging issues globally affecting the good quality of life for many communities. The major source of water pollution is the discharge of domestic and agricultural wastes, and untreated sanitary and toxic industrial effluents (Li *et al.*, 2013). The presence of pollutants in water bodies can be detrimental to aquatic life as well as rendering it unsuitable for domestic usage. As a consequence thereof, an increase in waterborne diseases becomes inevitable, thus putting more for safe water for both municipal and industrial purposes. Hence, much attention has been focused on water treatment; thus making it compulsory to evaluate water quality on a continuous basis (Yang *et al.*, 2012).

Chemical flocculants are widely employed in wastewater and drinking water treatment, food and fermentation industries and also for downstream processing due to their high flocculating efficiency and cost-effectiveness (Salehizadeh and Shojaosadati, 2001; You *et al.*, 2008). However, their extensive usages have raised serious environmental and health concerns (Mabinya *et al.*, 2012). The utilization of aluminium as the coagulant in water treatment may lead to a higher level of aluminium in the treated effluent than in raw water. In addition, residual aluminium in excessive sludge produced during coagulation tends to accumulate in the environment (Ma *et al.*, 2008). For example, several studies have shown that aluminium salts are associated with Alzheimer's disease (Arezoo, 2002; Bank *et al.*, 2006).

In addition, although polyacrylamide has high flocculating efficiency, the acrylamide monomer residues are neurotoxic and carcinogenic to humans (Polizzi *et al.*, 2002; Ruden, 2004). Besides, acrylamides are not biodegradable and consequently constitute an environmental nuisance (Lofrano *et al.*, 2013). These inevitable drawbacks associated with chemical flocculants necessitate a search for alternative flocculants that are eco-friendly and safe (Nwodo *et al.*, 2013).

On the other hand, bioflocculants are harmless, biodegradable, neither neurotoxic nor carcinogenic when compared to the synthetic flocculants used in various industrial processes (Li *et al.*, 2009; Liu *et al.*, 2010). However, the high costs associated with their production as well as corresponding low yields are still the major problems limiting their industrial usage (Gao *et al.*, 2006; He *et al.*, 2004; Wang *et al.*, 2007). Hence, there is need to screen new microogranisms with high bioflocculant production potentials and also develop several strategies on how to optimize culture conditions for better bioflocculant yields as well as with improved flocculating activity (Li *et al.*, 2009; Ugbenyen *et al.*, 2012).

Several reports on bioflocculant-producing microorganisms isolated from freshwaters have been documented in previous studies but only few studies on bioflocculant-producing microorganisms from marine environment were documented in the literature. The rate of discovery of new compounds from terrestrial bacteria has decreased, thus the need for exploration of underexploited habitats such as the marine environment as sources of novel bioactive compounds including bioflocculants is highly desirable (Ugbenyen and Okoh, 2013).

1.2 HYPOTHESIS

This study was based on the hypothesis that Algoa Bay in the Eastern Cape Province of South Africa is a potential reservoir of bioflocculant-producing microorganisms of industrial importance.

1.4 AIM AND OBJECTIVES

This study broadly aimed at evaluating the flocculating potentials and characterizing the bioflocculants produced by some bacterial isolates belonging to the Bacillus genus from Algoa Bay in the Eastern Cape Province of South Africa.

The specific objectives include:

- To isolate and screen bioflocculant-producing microorganisms from sediment samples of Algoa Bay in the Eastern Cape Province of South Africa
- To identify the bioflocculant-producing microorganisms using molecular approaches
- To optimize culture conditions for bioflocculant production by selected bacterial isolates
- To determine the kinetics (time course conditions) of bioflocculant production
- To extract, purify and characterize the bioflocculants in details
- To compare the flocculating activities and yields of the produced bioflocculants from the bacterial isolates
- To determine the efficiency of the bioflocculant in the treatment of river waters
- To compare the efficiency of the bioflocculant with the conventionally (synthetic) flocculants

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CHAPTER TWO

LITERATURE REVIEW

Implications for public health demands alternatives to inorganic and synthetic flocculants: Bioflocculants as important candidates

(Published in Microbiology Open)

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Abstract

Chemical flocculants are generally used in drinking water and wastewater treatment due to their efficacy and cost effectiveness. However, the question of their toxicity to human health and environmental pollution has been a major concern. In this paper, we review the application of some chemical flocculants utilized in water treatment and bioflocculants as a potential alternative to these chemical flocculants. To the best of our knowledge, there is no report in the literature that provides an up-to-date review of the relevant literature on both chemical flocculants and bioflocculants in one paper. As a result, this review paper comprehensively discussed the various chemical flocculants used in water treatment, including their advantages and disadvantages. It also gave insights into bioflocculants production, challenges and their industrial applications, as well as future research directions including improvement of bioflocculant yields and flocculating activity, production of cation-independent bioflocculants. The molecular biology and synthesis of bioflocculant was also discussed.

Key words: Chemical flocculants, Environmental pollution, Bioflocculants, Industrial applications, Molecular biology.

2.1 Introduction

Water is one of the key constituents required for extant and thriving of carbon based life form (Rani *et al.*, 2013). The bounteous quantity of water on earth is one unique factor that differentiates this planet from others. The importance of water to the survival of life is so crucial that the search for water on other planets has become the key factor to suggest the presence of life (Bhatnagara and Sillanpaa, 2010; Rani *et al.*, 2013). Water occupies about 78% of the earth's surface; it is a source of life and energy, nonetheless, millions of people worldwide lack access to safe water for drinking purposes and human utilization (Rout and Sharma, 2011). The quality of water consumed by people in a particular community can be taken as a key indicator of the quality of individual's life within that environment. Water is exceptionally important for domestic, agricultural, industrial and environmental purposes (Kumar *et al.*, 2005). However, impurities in the water reduce its effective usage as the negative impact of water pollution has minacious effects on man and his environment.

Water pollution is one of the most challenging environmental issues and has become a global impediment to a good quality of life for many communities. Unplanned urbanization and expeditious growths in populations have immensely contributed to the parlous state of water pollution and the prevailing unhealthy environment (Prasertsan *et al.*, 2006). The major source of water pollution is the discharge of domestic and agricultural wastes, and untreated sanitary and toxic industrial effluents (Li *et al.*, 2013). The presence of pollutants in water bodies can be pernicious to aquatic life as well as render it unsuitable as potable water sources for domestic usage. The pollution of the freshwater environment has a life-threatening effect on man's healthy living (Yang *et al.*, 2012).

According to WHO/UNICEF (2000), about 70-80% of all illnesses in developing countries are linked to the consumption of contaminated water especially among vulnerable population groups (Bhatnagar and Sillanpaa, 2010). Pollutants from wastewaters, when discharged into

natural water bodies, becomes toxic to aquatic life and render the waters unfit for consumption. The result of this is the alarming increase in waterborne diseases, as well as an increase in the demand for safe water for both municipal and industrial purposes. Much attention has been focused on water treatment; thus making it imperative to appraise water quality on a perpetual basis (Yang *et al.*, 2012). In order to provide these services adequately to meet consumers' demands, it is incumbent upon governments and societies at large to develop, among other things, appropriate scientific strategies in wastewater treatment technology that are not only environmentally friendly but also cost-effective. Of utmost importance is the development of a novel strategy in the wastewater treatment technology to encompass a stricter environmental policy on the quality of the final effluents released into water bodies (Wong *et al.*, 2006). Many countries have inaugurated several stringent regulations with respect to the presence of contaminants in water, to ensure proper treatment of domestic and agricultural wastewater as well as industrial effluents prior to their discharge into different waterbodies (Bhatnagara and Sillanpaa, 2010; Li *et al.*, 2013).

2.2 Flocculation process in water treatment

In most water treatment plants, water from reservoir passes through the first compartment into which flocculants are added. The water then moves to the sedimentation tank where the flocculation process occurs and suspended particles settle at the bottom of the tank. The clarified water from this stage goes through a filtration process prior to being disinfected for distribution to end users. The main reaction stage where natural organic matter and other contaminants are removed is the flocculation stage (Jarvis *et al.* 2012; Rong *et al.*, 2013). Flocculation is a process whereby colloids, cells and suspended solids are removed from suspension. The solids simply look like flocs or flakes as a consequence of aggregation (Bhunia *et al.*, 2012). Flocculants are substances that are used in the separation of solid-liquid by the process of flocculation in various industrial processes (Hu *et al.*, 2006); they could be of

natural or synthetic origin. The larger the size of the particle, the faster the sedimentation rate, resulting in an efficient and rapid flocculation process that produces a clearer upper phase (Lacchwani, 2005).

Flocculants are commonly used in the various industrial processes, for example, drinking water purification, wastewater treatment and downstream processes in the fermentation industries (Shih *et al*., 2001). Shih and Van (2001) found that flocculation could be exploited as a substitute for filtration and centrifugation in the separation of microbial cells from broth in food, beverage and pharmaceutical industries. In addition, Deng *et al*. (2003) observed that flocculation is an effective technique that is commonly used in wastewater treatment for removing various suspended particles as well as metal ions.

According to the flocculation mechanism proposed by Wang *et al*. (2011), for the flocculants to adsorb onto the surface of the suspended particles, it must not only be in close proximity to the suspended particles, but must also exert a strong enough attractive force to overcome the electrostatic repulsion force. In addition, an efficient and rapid flocculation process depends, among other things, on the suspended particle size, which implies that the larger the size the faster the settling rate (Lee *et al*., 2012). The choice of flocculant has a major influence on the performance of the flocculation process, the strength of the aggregated particles and the number and strength of the bonds formed as a result of flocculation (Zhang *et al*., 2014). For example, the flocculation efficiency and strength of the bonds of polyelectrolytes is greater than that of ferric chloride.

However, despite the high efficiency of the flocculation process in water treatment, the major disadvantage of flocculation is that it generates small flocs when flocculation occurs at low temperatures or generates fragile flocs that can disperse on the application of physical force

(Lee *et al.*, 2014). Consequently, it is crucial to surmount these problems and improve the flocculation processes in order to optimize its effective utilization.

2.3 Classification of flocculants

Flocculants have been used for various wastewater treatments, drinking water purification and dredging/downstream processes in a variety of industrial fields (Salehizadeh and Shojaosadati, 2001). Flocculants are generally categorized as inorganic flocculants, organic flocculants and naturally occurring flocculants (Fig. 1).

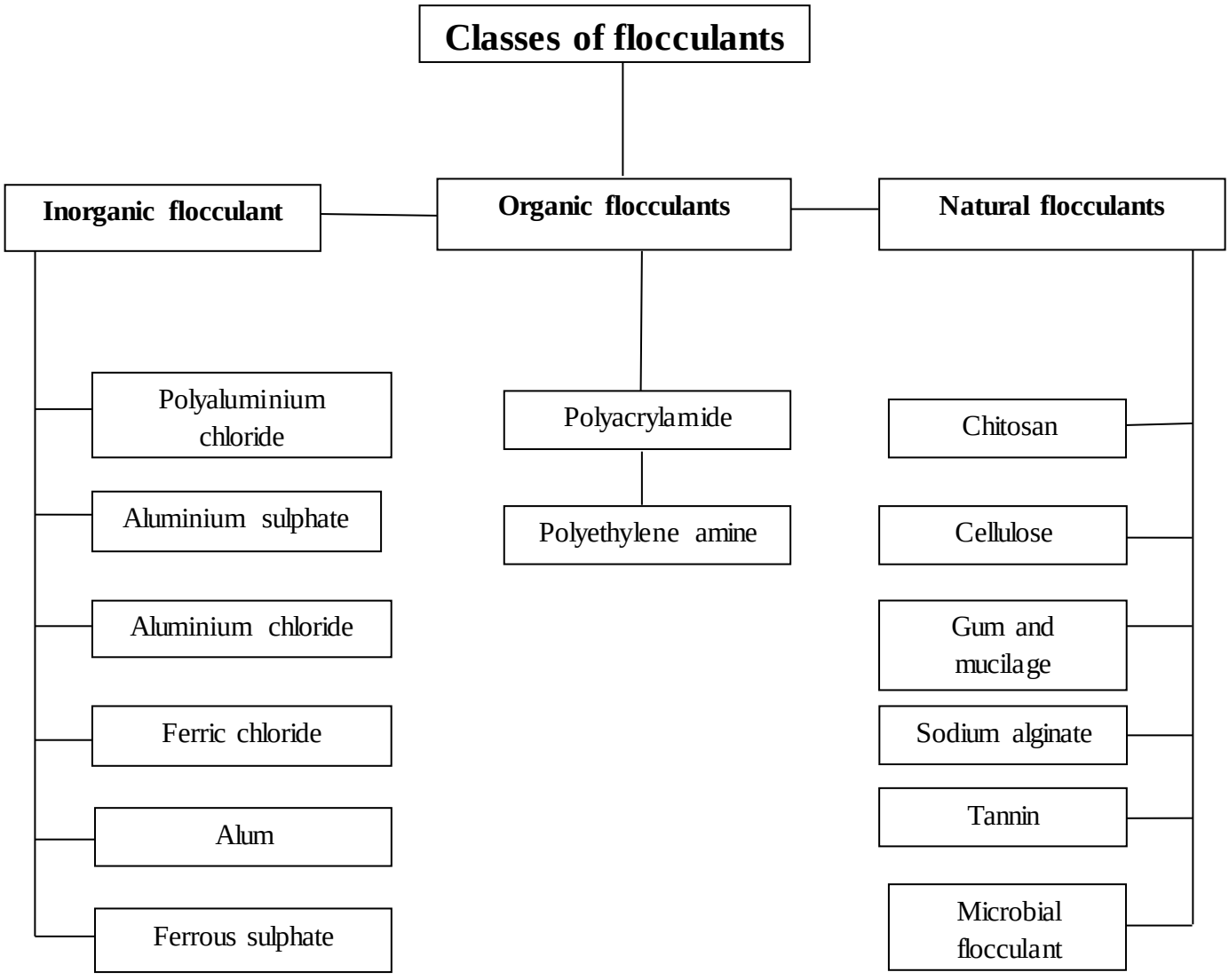

Figure 2.1. Classes of flocculants.

2.3.1 Inorganic flocculants

Inorganic flocculants include, alum, polyaluminium chloride, aluminium chloride, aluminium sulphate, ferric chloride and ferrous sulphates. Since most of the suspended particles in wastewater usually exhibit a negative charge (Lee *et al.*, 2014), the salt of these metals will be ionized when they are added to wastewater to form cationic charges which can bind to the negatively charged suspended particles. This interaction leads to a reduction in surface charge and the formation of microfloc which in turn aggregates to form larger flocs that can easily settle out of solution (Suopajarvi *et al.*, 2013). Among these inorganic flocculants, polyaluminium chlorides (PAC) are widely used in drinking water and wastewater treatment. However, they are very sensitive to pH, inefficient at low temperatures, limited to only a few disperse systems and large amounts are required for effective flocculation; thus generating a large volume of sludge which is challenging in wastewater treatment plant systems (Wei *et al.*, 2003; Bratby, 2006; Sharma *et al.*, 2006). Consequently, it is essential to investigate effectual technologies that will be logical and effective in the recycling of excess sludge. Furthermore, several studies have reported that PAC contains aluminium which could contaminate drinking water, and lead to serious health problems for consumers (Banks *et al.*, 2006).

Recently, inorganic polymeric flocculants such as ferricpolysilicates have been discovered, although they have a lower molecular weight and flocculating efficiency compared to organic polymeric flocculants (Shi and Tang, 2006; Moussas and Zouboulis, 2008). In addition, regardless of the flocculating capability of modified ferricpolysilicates over ferric sulfate, the addition of polysilicic acid, which is negatively charged, will upset the destabilization ability of the modified flocculant, since the positive charges on iron species will be compromised (Moussas and Zouboulis, 2009). Subsequently, it is vital to subdue these aforesaid challenges in order to increase flocculating efficiency.

Composite inorganic-organic coagulants such as poly-dimethyl-diallyl-ammonium chloride (PDMDAAC) are normally made by grafting a cationic inorganic coagulant on organic polymers in order to derive a holistic flocculating efficiency from the attributes of both molecules (Moussas and Zouboulis, 2009). In recent times, the idea of utilizing composite flocculants in wastewater treatment has attracted more attention and several workers have reported on them (Shi and Tang, 2006; Wang *et al*., 2007; Gao *et al*., 2008). For instance, Gao *et al*. (2008) observed that, on treating kaolin suspension or dye solution with polyferric chloride-poly-dimethyl-diallyl-ammonium chloride (PFC-PDMDAAC), a higher flocculating efficiency than that of the individual reactive components (PFC and PDMDAAC) was observed. Furthermore, the addition of this composite flocculant to dye solutions for textile industries generated a high flocculating efficiently. The flocculating competence depends on the percentage of organic polymer used, since PFC-PDMDAAC carries a higher cationic charge when compared to PFC (Gao *et al*., 2007; Wang *et al*., 2007). Nonetheless, the application of these composite flocculants is narrow because they can only be efficient in treating specific samples as a result of the high cationic charge they possess (Moussas and Zouboulis, 2009).

2.3.2 Organic flocculants

Organic flocculants are conventionally utilized in different industrial processes as flocculation agents (Kang *et al*., 2007). They include polyacrylamide (PAA), polyethylene amine, poly(diallyl dimethyl ammonium chloride) (DADMAC) (Singh *et al*., 2000). Moussas and Zouboulis (2009) documented that acrylamide derivatives are major groups of organic synthetic polymers that are widely used as flocculating agents because of their effectiveness and cost-efficiency. According to Suopajarvi *et al*. (2013), these organic polymers are commonly derived from oil-based or non-renewable raw materials. They usually have a high molecular weight and possess numerous charges (polyelectrolytes) in their molecular chain

which enhance their flocculating effectiveness (Lee *et al.*, 2014). The amount of sludge generated in wastewater treatment can be reduced by using synthetic polymers such as PAA, which are also not sensitive to pH (Huang *et al.*, 2014). Furthermore, the use of non-ionic organic polymers such as polyacrylamide can overcome some of the previously mentioned problems encountered with inorganic flocculants. A combination of polyacrylamide (PAA) and polyferric sulphate (PFS) should give an increased flocculating efficiency since polyacrylamide, on its own, is a good flocculating agent. Combining PAA with PFS should give rise to a higher molecular weight polymer; thus enhancing its flocculating efficiency when compared to an inorganic flocculants. In addition, PAA is a non-ionic polymer, which does not contribute additional charges to the flocculation process and thus has no effect on the destabilization capacity of the inorganic cationic coagulant (Moussas and Zouboulis, 2009).

Acrylamide is crystalline in nature. It is a moderately stable monomer that is extremely soluble in water and many organic solvents (Wong *et al.*, 2006). It is a polyfunctional molecule that comprises of both a vinylic carbon-carbon double bond and an amide group with a deficient double bond that is prone to a broad scope of chemical reactions (Girma *et al.*, 2005). However, the monomers of the PAA are not easily biodegradable, thereby constituting an environmental nuisance. Besides, these monomers have been reported to have both neurotoxic and carcinogenic properties (Li *et al.*, 2008). Consequently, these demerits associated with them have discouraged their use in most countries.

2.3.3 Naturally occurring flocculants

2.3.3.1 Chitosan

Chitosan is a partially deacetylated polymer derived from alkaline deacetylation of chitin, a biopolymer obtained from shellfish sources (Lee *et al.*, 2014). It is a cationic polysaccharide which serves as a synthetic polymeric flocculant that can be applied in the coagulation of suspended particles in the water treatment process because of its safety, non-corrosiveness and

biodegradability (Defang *et al.*, 2008). It is a linear hydrophilic amino-polysaccharide which has several amino groups ($–NH_2$) and hydroxyl groups (–OH) on its structure. These –OH and $–NH_2$ groups, both have lone-pair electrons that can donate an electron pair to empty d-trajectories of metal ions; thereby chelating into a complex compound (–N–M–O–). Chitosan is insoluble in water as well as in concentrated organic solvents. However, it is soluble in dilute organic solvents (Szygula *et al.*, 2009). At low pH, chitosan exists as a soluble cationic polymer possessing a high charge density (Rinaudo, 2006). When Chitosan dissolves in acids, it produces protonated amine groups that can remove various unwanted metal ions such as Ag^{+}, Pb^{2+}, Ca^{2+}, Cu^{2+}, Al^{3+}, Zn^{2+}, Cr^{2+}, Hg^{2+} and Cd^{2+} present in the wastewater through electrostatic attraction (Defang *et al.*, 2008). Jaafari *et al.* (2004) observed that chitosan has a strong electrostatic and adsorption power owing to the fact that the amino groups ($–NH_2$) in the molecular chain could be protonated with H^{+} in water to form cationic NH_3^{+}. Consequently, it can flocculate smaller particles into bigger flocs which can easily be precipitated out of solution. Chitosan has been reported to be effective in the removal of the chemical oxygen demand (COD) of water contaminated with organic solutes and suspended solid substances (SS) in water treatment (Bolto, 1995; Ishii *et al.*, 1995). It has numerous advantages over the traditional chemical flocculants that are widely used in water treatment. These advantages include: a lesser dosage requirement, a faster sediment ingrate, a high COD reduction capability, suspended solids (SS) and metal ions. In addition, it is used to reduce the production of the large quantity of sludge usually generated by inorganic flocculants and it does not cause any secondary pollution. Due to the high density of chitosan, it increases the floc size, which in turn increases the floc settling rate and decreases the sedimentation period (Renault *et al.*, 2009). Although chitosan is effective in water treatment, it is expensive; therefore, its usage might inflate overall treatment costs.

2.3.3.2 Sodium alginate

Sodium alginate is a linear water-soluble anionic polymer that is derived from the sodium salt of alginic acid and has a molecular weight of about 500,000 (Wu *et al.*, 2012). Recently, Wu *et al.* (2012) examined its flocculating capability in combination with aluminium sulphate as the coagulant in the treatment of industrial textile wastewater contaminated with synthetic dyes and found that it exhibited strong flocculating rates of about 90% and 80% for colour removal and COD reduction respectively.

2.3.3.3 Tannin

Tannin is an anionic polymer that has been reported to be a safer flocculant which can conveniently be used as a substitute for the commonly used polymers in water treatment due to its biodegradability and safety to humans and the environment (Ozacar and Sengil, 2000). Tannin is obtained from the secondary metabolites of vegetables such as fruits, leaves and others (Beltran-Herediaa and Sanchez-Martin, 2009). Lately, several researchers have experimentally confirmed the flocculating capability of tannin in eliminating suspended and colloidal particles found in drinking water after treatment, as well as in the removal of suspended particles from synthetic raw water and the removal of dyes, pigments and inks from ink-containing wastewater (Ozacar and Sengil, 2003; Roussy *et al.*, 2005). In these studies, and because tannin is anionic in nature, a coagulant such as aluminium sulphate was added in order to stabilise the negatively charged colloidal particles prior to the addition of tannin (Lee *et al.* 2014).

2.3.3.4 Cellulose

Cellulose is one of the most abundant natural polysaccharides obtained from agricultural wastes (Lee *et al.*, 2014). In recent years, cellulose has been the subject of research because of its numerous industrial applications (Das *et al.*, 2012). Anionic sodium carboxymethyl-cellulose (CMCNa) is a typical example of flocculant prepared from cellulose, which has been

shown to be eco-friendly and has been used, complexed to aluminium sulphate, as a coagulant for the removal of turbidity in drinking water (Khiari *et al.*, 2010). Suopajarvi *et al.* (2013) reported that anionized dicarboxylic acid nanocellulose (DCC) flocculant derived from cellulose had a strong flocculating property in the presence of ferric sulphate in municipal wastewater treatment.

2.3.3.5 Exopolymeric substances (EPS)

Lately, demand for biopolymers for diverse industrial applications has resulted in an interest in the production of exopolysaccharides (EPS). They are usually complex long-chain, high molecular weight mixtures of polymers comprising branched repeating units of sugars or sugar derivatives such as fructose, galactose, glucose and mannose which are produced and released during the growth of microorganisms (Ismail and Nampoothiri, 2010; Sheng *et al.*, 2010).

2.3.3.5.1 Bioflocculant

Among the numerous EPS reported in the literature, those that have flocculating properties are particularly interested in the field of bioflocculation and this suggests their candidature for application in water treatment and other industrial processes. Owing to the limitations of these inorganic and organic flocculants, biopolymers produced by microorganisms during growth, called bioflocculants, have gained huge scientific attention because they are biodegradable, produce no secondary pollution and their degraded intermediates are safe for humans and their immediate environment (Buthelezi *et al.*, 2010; Mabinya *et al.*, 2012). They are pH independent and generate less sludge, which is easily degraded by microorganisms.

However, the major limiting factors that hinder their large scale production and industrial application are low flocculating efficiency, low yields and high cost of production (He *et al.*, 2010; Zhao *et al.*, 2012). Consequently, it has become imperative to identify and screen new bioflocculant-producing microorganisms and investigate strategies for the optimization of

fermentation conditions to improve on bioflocculant yields or on utilizing microbes in a consortium to increase bioflocculant yields (Yang *et al.*, 2007; Okaiyeto *et al.*, 2013).

Abdel-Aziz *et al.* (2011) observed that certain enzymes that subsist in clusters regulate the utilization of nutrients in the medium by microorganisms to produce polymers that have a high molecular weight, and which when released, can exist in the medium or form a capsule on the surface of the bacteria. EPSs are often called exopolysaccharides due to their location. This is to differentiate them from other forms of polysaccharides that may be found within the cell (Nwodo *et al.*, 2012). They usually exist as a layer on the surface of the organism, thereby protecting the cell wall against adverse environmental conditions such as high osmotic pressure, oxygen tension, temperature, and toxic compounds. Furthermore, they may contribute to the uptake of metal ions as well as prevent dehydration under certain environmental conditions (Nichols *et al.*, 2005). The capsular polysaccharides are normally extremely immunogenic, and may have changed their unusual diversity as a way of evading antibody responses as is the case in their use in the development/design of vaccines. In addition, they play a vital function in the adhesion and penetration of the host (Morris and Harding, 2009). According to Wingender *et al.* (1999), the release of enzymes by microorganisms into their external environment forms the center of contact between the exogenous substrate and the cells.

Exopolysaccharides are the most important constituent of biological aggregates responsible for the degradation of organic matter in wastewater treatment, which also includes biofilms and activated sludge (Martin-Cereceda *et al.*, 2001). Exopolysaccharides promote the development of bioflocs by amending the relationship among microbial aggregates, different bacterial strains, as well as both organic and inorganic particles. In addition, their fundamental role is to hold the cells firmly together (Li and Yang, 2007; Sheng *et al.*, 2010). They can be classified into two types: sheath and slime. Sheath EPSs are tightly bounded to the cell wall, and are

commonly called tightly bound-exopolysaccharides (TB-EPS). Slime EPSs have no directed contact with the cell. They loosely/weakly attach to the cell wall and they are usually called loosely bound-exopolysaccharides (LB-EPS). Centrifugation is the basis for the separation of these two fractions (Anna *et al.*, 2006; Sheng *et al.*, 2010). Exopolysaccharides are usually complex in nature with heterogeneous substances, whose constituents and location can be contingent on several metabolic processes such as active secretion, changes in a growth phase, cell breakage due to cell death, release of cell surface macromolecules (outer membrane proteins and lipopolysaccharides) and their interaction with the immediate environment (Cristina *et al.*, 2011).

Recently, several microorganisms such as algae, bacteria, actinomycetes and fungi have been implicated in the production of bioflocculants (Gong *et al.*, 2008; Xia *et al.*, 2008; Ugbenyen *et al.*, 2012; Ntsaluba *et al.*, 2013; Cosa and Okoh, 2014; Nwodo *et al.*, 2014; Okaiyeto *et al.*, 2014). Although a number of reports are available for EPSs produced by different bacteria found in different habitats, the marine environment, which supports a rich biodiversity of bacteria, remains largely unexplored (Kumari *et al.*, 2014). Li *et al.* (2008) observed that the majority of the bioflocculants documented in the literature are exopolysaccharides (EPS) which are secreted by microorganisms which were isolated either from soil or wastewater sludge.

2.4 Composition analysis and flocculating potential of some bioflocculants

Several studies have shown that most of the bioflocculants produced are either functional proteins (Zhang *et al.*, 1999) or functional polysaccharides (He *et al.*, 2004; Huang *et al.*, 2005). Deng *et al.* (2003) documented that the bioflocculant MBFA9 secreted by *Bacillus mucilaginosus* was a polysaccharide composed mainly of amino sugar (2.7% w/w), uronic acid (19.1% w/w) and neutral sugar (47.4% w/w). The infrared spectrum analysis revealed the presence of carboxyl and hydroxyl as the major functional moieties. The flocculating efficiency of the biopolymer produced by *Bacillus mucilaginosus* for kaolin suspension was about 99.6%

at a dosage of 0.1 mg/l (Deng *et al.*, 2003). Feng and Xu (2008) observed that the acidic bioflocculant produced by *Bacillus* sp. BF3-3 is composed of polysaccharide (66.1% w/w) and protein (29.3% w/w).

The hydroxyl and carboxyl groups play a fundamental role in the flocculation of suspended particles because these functional groups provide adsorption sites where the suspended particles can be attached. Deng *et al.* (2005) documented the bioflocculant produced by *Aspergillus parasiticus* utilizing corn starch as a carbon and peptone supplemented in the medium as a nitrogen source. This bioflocculant showed a high flocculating efficiency of 98.1% for kaolin suspension. The bioflocculant was able to flocculate dye from a suspension. The purified bioflocculant was composed mainly of sugar (76.3% w/w) and protein (21.6% w/w) and the average molecular weight was 3.2×10^5 Da. The existence of amino and amide groups in the molecular chain might also influence the flocculation process (Deng *et al.*, 2005). The extracellular bioflocculants produced by a bacterium, a member of *Bacillus* genus, isolated from a Qatari ecosystem was able to flocculate kaolin suspension at a rate of over 85% at a dosage of 20 mg/l (Desouky *et al.*, 2008). Also, Gao *et al.* (2006) found that the bioflocculant produced by *Vagococcus* sp. W31 was thermostable exhibiting a high flocculating efficiency in a wide pH range of 7-11 with a dosage requirement of 25 mg/l. The bioflocculant was mainly composed of polysaccharides with a molecular weight over 2×10^6 Da and composed of neutral sugar (71.5% w/w) and uronic acid (15.4% w/w). The infrared spectra revealed the existence of hydroxyl, carboxyl and methoxyl groups as major functional groups in the molecular chain of the bioflocculant. He *et al.* (2004) found that the bioflocculant produced by *Corynebacterium glutamicum* was composed of polysaccharides and exhibited thermo-stability in an acidic pH range of 3.0-6.5. The flocculating activity of the bioflocculant was relatively high compared to synthetic flocculants. These attributes indicate its potential utilization in the decolourization of molasses wastewater. The novel bioflocculant HBF-3 produced by a deep-

sea bacterium mutant *Halomonas* sp. V3a' was composed of a polysaccharide containing neutral sugar (20.6 % w/w), uronic acid (7.6 % w/w), amino sugar (1.6 % w/w) and sulphate (5.3 % w/w). The infrared spectra showed the existence of both hydroxyl and carboxyl groups in the molecular chain (He *et al.*, 2010). Gao *et al.* (2006) found that the characteristics of the bioflocculant produced by any microbes are a pre-determining factor that influences its flocculating efficiency. Also, in our research group, we recovered several bacterial isolates that produce bioflocculants which are polysaccharides (Mabinya *et al.*, 2011; Piyo *et al.*, 2011; Ntsaluba *et al.*, 2013; Nwodo and Okoh, 2014) and glycoproteins (Cosa *et al.*, 2011, 2012; Mabinya *et al.*, 2012; Ugbenyen *et al.*, 2012; Cosa *et al.*, 2013a; Nwodo *et al.*, 2013; Nwodo and Okoh, 2013; Okaiyeto *et al.*, 2014, 2015a).

2.5 Factors affecting bioflocculant production

According to the available literature, the production of microbial flocculants is highly influenced by the culture medium composition and several other physicochemical parameters (Sheng *et al.*, 2006; Wang *et al.*, 2010; Fang *et al.*, 2013). In addition to these findings, He *et al.* (2004) reported that the production of bioflocculants is influenced by numerous factors that include the media constituents as well as growth conditions. The impacts of the nutritional constituents of the production of bioflocculants have been widely investigated (Abdel-Aziz *et al.*, 2011). The impact of the main factors, such as carbon source, culture time, metal ion, initial pH of the production medium, shaking speed, nitrogen source, ionic strength, incubation temperature and inoculum size greatly influence bioflocculant production (He *et al.*, 2004). Commonly, an appropriate medium for bioflocculant production consist of glucose or fructose as the sole carbon source. However, lactose and yeast extract have been used as the carbon and nitrogen sources respectively (Kanmani *et al.*, 2011). In addition, small amounts of phosphates and ions are essential (Fujita *et al.*, 2000).

2.6 Optimization of culture conditions for bioflocculant production

2.6.1 Effect of carbon and nitrogen sources on bioflocculant production

Carbon sources play a substantial role in enhancing the secretion of bioflocculants by microorganisms (Goo *et al*., 2013). Salehizadeh and Yan (2014) have referred to a number of studies that have documented the significance of carbon and nitrogen sources in the production of bioflocculants. Lee *et al*. (2001) reported that *Bacillus licheniformis* X14 favoured ethanol, sucrose and starch as appropriate carbon sources for the secretion of ZS-7 bioflocculant, whereas ammonium chloride was preferred as a nitrogen source of choice.

In the case of another study carried out by Sheng *et al*. (2006) on *Klebsiella* sp. in the production of bioflocculant, it was documented that maltose and urea were the preferred carbon and nitrogen sources respectively. Cosa *et al*. (2013b) observed that sodium carbonate and tryptone were most favourable for bioflocculant production by *Oceanobacillus* sp. pinky, while tryptone was a preferred organic nitrogen source for bioflocculant production by *Chryseobacterium daeguense* W6 (Liu *et al*., 2010a). Gong *et al*. (2003) reported that sucrose, maltose, xylitol, lactose and glucose are all suitable substrates for the production of bioflocculant by *Paenibacillus polymyxa* BY-28. He *et al*. (2004) documented a novel polygalacturonic acid bioflocculant REA-11 produced by *Corynebacteria glutamicum* from sucrose as the carbon source and complex nitrogen sources comprising of urea and corn steep liquor. The ability of the microorganism to utilize sucrose as a carbon source for the production of bioflocculant points to the possibility of using molasses in large scale industrial bioflocculant production. Cosa *et al*. (2013a) found that glucose was the preferred carbon source among other sources investigated for bioflocculant production by *Virgibacillus* sp. while Deng *et al*. (2005) showed sucrose, corn starch, glycerol and glucose as appropriate substrates for bioflocculant production by *Apergillus parasiticus*, exhibiting a high flocculating activity above 80% at 72 h of fermentation. The production of bioflocculants was optimal when

maltose was utilized as a carbon source in the medium for the cultivation of *Solibacillus silvestris* W01 (Wan *et al.*, 2013). For example, soluble starch was the carbon source that supported optimal bioflocculant production by *Sorangium cellulosum*, while the inclusion of glucose as a supplement at 3 g/l entirely repressed cell growth and production of the bioflocculant (Zhang *et al*. 2002).

In another study reported by Shih *et al.* (2001), glucose, fructose and lactose were not suitable for bioflocculant production by *B. licheniformis* whereas the concurrent presence of multiple carbon sources (glycerol, citric acid and glutamic acid) in the cultivation medium improved cell growth and the production of bioflocculants. Liu and Cheng (2010) recorded an increase in bioflocculant production by *Penicillium* sp. HHE-P7 in the medium containing glucose and yeast extract. Glucose was the most favourable carbon source for bioflocculant secretion (95% flocculating activity) by microorganisms, but the high cost of glucose inflates the production cost. However, when molasses was substituted for glucose, flocculating activity for kaolin suspension was more than 90%, a clear indication of cost saving when cheaper substrates are used.

Substantial evidence has shown that some bacterial strains can utilize either organic nitrogen source, inorganic nitrogen or their combination sources produce bioflocculant (Deng *et al.*, 2005; Gong *et al.*, 2008; Xia *et al.*, 2008). For instance, Deng *et al*. (2005) reported that peptone combined with sodium nitrate was the most suitable nitrogen source for *Aspergillus parasiticus* for bioflocculant production. On the other hand, when combined with $(NH_4)_2SO_4$, no bioflocculant was produced. Nevertheless, organic nitrogen sources, improved bioflocculant production in some microorganisms. For example, beef extract, and urea used together as a nitrogen source were more favourable for production of bioflocculant by the S-14 strain. Xia *et al*. (2008) found that strain TJ-1 was able to effectively utilize peptone, yeast and beef extracts as a nitrogen source, but peptone alone (organic nitrogen source) was the most cost-effective

with high bioflocculant production. Cosa *et al*. (2013a) reported that a complex nitrogen source consisting of urea, yeast extract and $(NH_4)_2SO_4$ supported optimal bioflocculant production by *Virgibacillus* sp. Similarly, Gong *et al*. (2008) indicated that a mixed nitrogen source comprises of urea and beef extract showed a substantial improvement on bioflocculant production by *Serratia ficaria* among others investigated. Also, Kurane and Matsuyama (1994) reported on a bioflocculant produced from a mixed culture of *Acinetobacter*, *Agrobacterium* and *Enterobacter* species in which the medium incorporated a combination of ammonium sulphate and yeast extract as the nitrogen source. Deng *et al*. (2005) documented that peptone and sodium nitrate were the best nitrogen sources among other sources tested for bioflocculant production by *A. parasiticus*. Li *et al*. (2013) noticed that peptone was more favourable for the production of bioflocculant by *Paenibacillus elgii* B69 among other nitrogen sources evaluated.

Table 2.1. Optimum culture conditions, chemical compositions, flocculating activity and yields of some bioflocculants.

Microorganism	Source	Carbon source	Nitrogen source	Chemical composition	Flocculating activity (%)	Yield	Reference
Paenibacillus mucilaginosus	Soil	Sucrose	Yeast extract	Polysaccharide	97	NA	Tang *et al.*, 2014
Enterobacter aerogenes	Soil	Fructose + glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Glycoprotein	80	1.3	Lu *et al.*, 2005
Oceanobacillus sp. Pinky	Marine	Sodium carbonate	Tryptone	Glycoprotein	84.5	2.44	Cosa *et al.*, 2013b
Chryseobacterium daeguense W6	Backwashing sludge	Glucose	Tryptone	Glycoprotein	96.9	NA	Liu *et al.*, 2010a
Paenibacillus polymxya BY-28	Soil	Sucrose	Bean cake powder	Glycoprotein	99	NA	Gong *et al.*, 2003
Coryneobacillus glutamicum	NA	Corn steep liquor	Urea +	NA	520 U/ml	NA	He *et al.*, 2004

			Corn steep liquor				
Virgibacillus sp.	Marine	Glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Polysaccharide	91.8	2.43	Cosa *et al.*, 2013a
Aspergillus parasiticus	NA	Starch	Peptone + sodium nitrate	Glycoprotein	98.1	NA	Deng *et al.*, 2005
Solibacillus silvestris	Marine	Maltose	Yeast extract	Glycoprotein	90	1.7	Wan *et al.*, 2013
Sorangium cellulosum	NA	Soluble starch	$NaNO_3$	Glycoprotein	96.6	17.5	Zhang *et al.*, 2002
Klebsiella sp.	Activated sludge	Glucose	Yeast extract + urea	Polysaccharide	86.5	1.8	Yang *et al.*, 2012
Klebsiella mobilis	Soil	Dairy wastewater + Ethanol		Polysaccharide	95.4	2.58	Wang *et al.*, 2007
Penicillium purpurogenum	NA	Glucose	Yeast extract	Polysaccharide	96	6.4	Liu and Cheng, 2010
Aeromonas sp.	Activated sludge	Glucose	Peptone	Polysaccharide	92.4	2.25	Li *et al.*, 2007
Serratia ficaria	Soil	Lactose	Yeast extract	Polysaccharide	95.4	NA	Gong *et al.*, 2008
Paenibacillus elgii B69	Soil	Sucrose	Peptone + yeast ext.	Polysaccharide	87	25.63	Li *et al.*, 2013
Rhodococcus erythropolis	Activated sludge	Livestock waste water	NA	Glycoprotein	87.6	1.6	Peng *et al.*, 2014
Bacillus licheniformis	Contaminated LB medium	Sucrose	Yeast extract + urea	Glycoprotein	700 U/ml	2.94	Xiong *et al.*, 2010
Halomonas sp.	Marine sediment	Glucose	Urea	Polysaccharide	95	NA	Mabinya *et al.*, 2011
Klebsiella sp. TG-1	Wastewater	Sucrose	Beef extract	Glycoprotein	86.9	NA	Liu *et al.*, 2013

Klebsiella pneumoniae	Human saliva	Glucose	Peptone	Glycoprotein	96.5	4.7	Luo *et al*., 2014
Methylobacterium sp.	Freshwater	Glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Glycoprotein	95	8.203	Ntsaluba *et al.*, 2013
Bacillus licheniformis X14	Soil	Glucose	NH_4Cl	Glycoprotein	99.2	NA	Li *et al.*, 2009
Aeromonas sp.	Activated sludge	Corn flour	Soyabean flour	Polysaccharide	49.34	NA	Li *et al.*, 2008
Brachybacterium sp.	Freshwater	Maltose	Urea	Glycoprotein	87.8	NA	Nwodo *et al.*, 2013
Klebsiella sp. ZZ-3	Sludge	Glucose	$NaNO_3$	Glycoprotein	92.6	0.126	Yin *et al.*, 2014
Bacillus clautti	Brewery wastewater	Glucose	NA	Glycoprotein	88.67	NA	Adebayo-Tayo and Adebami, 2014
Vagococcus sp.	Wastewater	Glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Polysaccharide	86.5	2.3	Gao *et al.*, 2006
Klebsiella pneumoniae	Sputum	Glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Glycoprotein	98	NA	Zhao *et al.*, 2013
Citrobacter sp. TKF04	Soil	Propionic acid and acetic acetic acid	Yeast extract	Glycoprotein	85	0.2	Fujita *et al.*, 2000
Aureobasidium pullulans	NA	Sucrose	$NaNO_3$	Polysaccharide	NA	12.5	Ravella *et al.*, 2010
Klebsiella sp.	Activated sludge	Glucose	Yeast extract + urea	Polysaccharide	86.5	3.52	Yang *et al.*, 2012
Funalia trogii	Laboratory	Maltose	Tryptone	Polysaccharide	NA	8.68	He *et al.*, 2012
Enterobacter cloacae WD7	Activated sludge	Glucose or sucrose	$(NH_4)_2SO_4$	Polysaccharide	105	2.27	Prasertsan *et al.*, 2006

Bacillus velezensis 40B	Brackish water	Glucose	Yeast extract	Glycoprotein	99.9	3.54	Zaki *et al.*, 2013
Bacillus alvei NRC-14	Soil	Chitosan	Yeast extract	Polysaccharide	98	10	Abdel-Aziz *et al.*, 2011
Halobacillus sp. Mvuyo	Marine water	Glucose	Ammonium chloride	Glycoprotein	93	0.34	Cosa *et al.*, 2012
Bacillus sp. Maya	Marine	Glucose	Ammonium nitrate	Glycoprotein	95.6%	NA	Ugbenyen and Okoh, 2013
Cobetia sp. OAUIFE	Marine	Glucose	Urea + Yeast extract + $(NH_4)_2SO_4$	Glycoprotein	92.78	NA	Ugbenyen *et al.*, 2012
Bacillus sp. Gilbert	Marine	Sucrose	Ammonium chloride	Polysaccharide	91	NA	Piyo *et al.*, 2011
Halomonas sp. Okoh	Marine	Glucose	Urea	Polysaccharide	95	NA	Mabinya *et al.*, 2011
Arthrobacter sp. Raats	Freshwater	Lactose	Urea	Glycoprotein	87.5	NA	Mabinya *et al.*, 2012
Methylobacterium sp.	Freshwater	Glucose	Peptone	Polysaccharide	72	NA	Ntsaluba *et al.*, 2011
Micrococcus sp. Leo	Marine	Glucose	Urea + yeast extract + urea	Glycoprotein	87.5	0.738	Okaiyeto *et al.*, 2014
Bacillus toyonesis strain AEMREG6	Marine	Glucose	NH_4NO_3	Glycoprotein	89.5	3.2	Okaiyeto *et al.*, 2015a
Bacillus sp. AEMREG7	Marine	Glucose	Urea + yeast extract + $(NH_4)_2SO_4$	Glycoprotein		1.6	Okaiyeto *et al.*, 2015b
Cellulomonas sp. Okoh	Freshwater	Glucose	$(NH_4)_2SO_4$	Glycoprotein	86.3	4.47	Nwodo and Okoh, 2013
Streptomyces and Brachybacterium species	Freshwater	Glucose	NH_4NO_3	Polysaccharide	63.7	3.02	Nwodo and

							Okoh, 2014
Brachybacterium sp.	Freshwater	Maltose	Urea	Glycoprotein	91.2	NA	Nwodo *et al.*, 2013
Bacillus subtilis	Soil	Cane molasses	Yeast extract	Polysaccharide	NA	4.92	Abdul-Razack *et al.*, 2014

*NA- Not applicable

2.6.2 Effect of metal ions on the production of bioflocculants

Cations play a vital role in bioflocculation, in that they enhance the flocculating rate by neutralizing and stabilizing the residual negative charge/net surface charge of the functional groups on the bioflocculant; and thus, encouraging the formation of bridges between particles and the bioflocculant (Wu and Ye, 2007). Cation plays a vital role in stimulating the adsorption of flocculants on suspended particles by lessening the distance between them and increasing the electrostatic attraction between the bioflocculant molecules and the suspended particles (Wang *et al.*, 2011). Cosa *et al.* (2013b) found that calcium chloride and aluminium chloride were the most stimulating cations on the flocculation rate of the bioflocculant secreted by marine bacteria, *Oceanobacillus* sp. Pinky. The cations were effective due to the protein nature of the bioflocculant which is rich in amino acids containing carboxyl groups that contribute to the negative charges of the particles; this produces a neutralization effect and bridge-forming between the particles thus allowing for improved bioflocculation. More carboxylate groups on the bioflocculant served as binding sites for the cations (Li *et al.*, 2007). The addition of these cations to a suspension increased the floc size, resulting in enhanced sedimentation (Li *et al.*, 2007). Banks *et al.* (2006) observed that the flocculating activity of a proteinous bioflocculant produced by *Rhodococcus erythropolis* and *Alcaligenes cupidus* was enhanced by the addition of Ca^{2+} and Al^{3+} respectively. Zheng *et al.* (2008) reported that the flocculating activity of the bioflocculant, MBFF19 was increased in the presence of calcium ions while Feng and Xu (2008) reported that the flocculating rate of the bioflocculant, MBF3-3 produced by *Bacillus*

sp. was enhanced in the presence of the following metals: Mg^{2+}, Al^{3+}, Ca^{2+}, K^{+} and Na^{+} ions, but inhibited in the presence of Fe^{3+} ions. A similar finding in which Fe^{3+} completely inhibited the flocculating efficiency of the biopolymer secreted by *Bacillus* sp. F19 was also reported by Zheng *et al*. (2008). Contrary to the above observations, Wu and Ye (2007) reported that the flocculating rate of the bioflocculant p-KG03 produced by *Gyrodinium impudicum* KG03 was improved in the presence of Fe^{3+} with a similar observation reported on the bioflocculant produced by *Enterobacter* sp. BY-29 (Yokoi *et al*., 1997). Prasertsan *et al*. (2006) found that the bioflocculant produced by *Enterobacter cloacae* WD7 was stimulated in the presence of Cu^{2+}. The novel biopolymers produced by *Citrobacter* sp. TKF04, *Gyrodinium impudicum* KG30 and *Bacillus* sp. F19 required no cations for their flocculating activity (Fujita *et al*., 2000; Yim *et al*., 2007; Zheng *et al*., 2008). The flocculating efficiency of the bioflocculant produced by a haloalkalophilic *Bacillus* specie was drastically improved in the presence of divalent cations such as Ca^{2+}, Cu^{2+}, and Zn^{2+} (Kumar *et al*., 2004). Also, He *et al*. (2010) observed that the flocculation efficiency of the bioflocculant extracted from *Halomonas* sp. V3a' was mediated by Ca^{2+} over a wide pH range of 3-11 resulting in flocculating activity of over 80% against kaolin suspension at a dosage of 4 mg/l.

The production of bioflocculants was influenced by the chemical nature of metal ions present in the culture medium (Li *et al*., 2009), with the bioflocculant produced by *Flavobacterium* sp. stimulated by Ca^{2+}, Ba^{2+}, and Mn^{2+} but subdued by the presence of Mg^{2+} (Gonzalez and Hu, 1991). Li *et al*. (2009) reported that for strain X14, cations which included: Na^{+}, Ca^{2+}, Fe^{2+} and Mg^{2+} had no effect on bioflocculant ZS-7 production, while Cu^{2+} drastically inhibited cell growth. Cations can cause the neutralization of both the negative charges of the bioflocculant and suspended particles, thereby increasing the initial adsorption of a bioflocculant onto suspended particles (Wu and Ye, 2007). The carboxylic functional groups of the bioflocculant provide the adsorption sites for cations (Prasertsan *et al*., 2006); thereby making the

bioflocculant and kaolin clay particles form complexes. Lu *et al.* (2005) observed that the bioflocculant produced by *Enterobacter aerogenes* required Zn^{2+} for its flocculating activity whilst Feng and Xu (2008) reported a synergic stimulation by Al^{3+}, K^{+}, Ca^{2+}, Mg^{2+} and Na^{+} of the flocculating activity of the bioflocculant MBF3-3 produced by *Bacillus* sp. Under optimized culture conditions, the flocculating efficiency of the biopolymer extracted from *Serratia ficaria* reached a maximum of 95.4% for kaolin suspension within a pH range of 5-7 with Ca^{2+} and Mg^{2+} serving as stimulants.

2.6.3 Effect of temperature and agitation on the production of bioflocculants

Cultivation temperature has a great impact on bioflocculant production in microorganisms (Li *et al.*, 2009). Enzymes responsible for bioflocculant production are activated at an optimum temperature (Zhang *et al.*, 2007). From the literature search, the optimal temperature range for bioflocculant production varies between 25-37 ^{o}C (Salehizadeh and Shojaosadati, 2001). The bioflocculant secreted by *Citrobacter* sp. TKF04 was cultivated at 30 ^{o}C. Temperature has great influence on bioflocculant production, since optimum enzymatic reactions are usually attained at optimum temperature for microbial growth (Nakata and Kurane, 1999). Shaker speed determines the concentration of dissolved oxygen that influences nutrient absorption and enzymatic reaction (Lopez *et al.*, 2003). Li *et al.* (2009) reported that shaker speed of 140-160 rpm was optimal for the bioflocculant produced by *Bacillus licheniformis* X14. Nonetheless, the disparity in shaking speed requirement for different microorganisms could be the result of the different oxygen requirements at different growth phases (Li *et al.*, 2009).

2.6.4 Effect of inoculum size on production of bioflocculants

Both Jang *et al.* (2001) and Gong *et al.* (2008) observed that the inoculum size among various physiological properties plays a substantial role in metabolic processes, in that it has a significant effect on cell growth and the production of secondary metabolites. A small inoculum size prolongs the stagnant growth phase; nevertheless, a large inoculum size causes

the niche of the microorganism to overlap excessively, thereby suppressing bioflocculant production (Li *et al.*, 2009). Cosa *et al.* (2013b) found that 2% inoculum size was optimal for bioflocculant production by *Oceanobacillus* sp. Pinky. Li *et al.* (2009) reported that 1% (v/v) inoculum size for X14 allowed the adaptation of strain X14 to the cultivation medium, decreasing the lag phase and promoting the production of bioflocculant ZS-7. Studies by our group showed that the production of bioflocculant by *Micrococcus* sp. Leo was more propitious at 2% (v/v) inoculums size (Okaiyeto *et al.*, 2014), while 3% (v/v) inoculums size was preferred for the production of bioflocculant by *Bacillus* sp. Gilbert (Ugbenyen *et al.*, 2014).

2.6.5 Effect of initial pH of production medium on bioflocculants production

The initial pH of the fermentation medium is one of the factors that play a major role in the production of bioflocculant and also its flocculating efficiency (Zheng *et al.*, 2008). It determines the electrification of the cells and oxidation-reduction potential which could influence the absorption of nutrients in the production medium and enzymatic reaction (Salehizadeh and Shojaosadati, 2001). Mabinya *et al.* (2011) reported optimum bioflocculant production by *Halomonas* sp. OKOH at pH 7. Deng *et al.* (2003) reported that *Aspergillus parasiticus* preferred acidic conditions for synthesis, secretion as well as bioflocculant production in the fermentation medium while *Klebsiella* sp. TG-1 preferred alkaline conditions of pH 8 for bioflocculant production (Liu *et al.*, 2013). The bioflocculant secreted by *Halobacillus* sp. Mvuyo was more favourable at pH 7 (Cosa *et al.*, 2012).

2.6.6 Effect of pH on the flocculating activity of produced bioflocculants

When the pH of the medium is alkaline, the hydroxide ion (OH^-) may obstruct the complex formed between the bioflocculant and the suspended particles mediated by metal ions and, consequently, lead to the suspension of suspended particles in the reaction mixture (Prasertsan *et al.*, 2006). On the other hand, when the pH of the reaction mixture is adjusted to an acidic

condition, the bioflocculant and the kaolin particles adsorb the H^+ that weakens the complex formed between the bioflocculant and kaolin particles mediated by the metal ion, resulting in lower flocculating efficiency of the bioflocculant. The negative charge of the bioflocculant is believed to have resulted from the carbohydrate content, and the relevance of the proportion of protein to carbohydrates in determining the surface charge could be allied to the distinctive charge properties of proteins. Proteins consist of many amino acids which contain both carboxyl and amino groups and according to the observation of Liao *et al.* (2001), the amino groups from proteins possess positive charges which can neutralize some of the negative charges from both carboxyl and phosphate groups which in turn reduces the surface net charge (negative charge).

As with other organic acids, the carboxyl and amino groups ionize in aqueous solution. The molecule exists as a dipolar ion at a certain pH value, where both the acidic (acetic) and basic groups are ionized as zwitterions or hybrid ions (Liao *et al.*, 2001). Prasertsan *et al.* (2006) found that the flocculating efficiency of the bioflocculant extracted from *Enterobacter cloacae* WD7 was optimal at pH 6.0 whereas Wang *et al.* (2011) noticed that the flocculating activity of the bioflocculant CBF-F26 secreted by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 was maximal at neutral and weak alkaline circumstances. Deng *et al.* (2005) reported on the bioflocculant secreted by *Aspergillus parasiticus* at a pH range of 5-6. In addition, higher pH lowers biomass production as well as the flocculating activity of the produced bioflocculant. However, lower pH greatly improved fungal synthesis, secretion, as well as the accumulation of the bioflocculant in the fermentation medium (Deng *et al.*, 2005). The bioflocculant produced by *Agrobacterium* sp. M-503 maintained high flocculating activity at a pH range of 7-12 (Li *et al.*, 2010). The flocculating efficiency of the biopolymer produced by *Gyrodinium impudicum* KG03 was observed to be optimum within a pH range of 3-6 with optimum activity recorded at pH 4 (Yim *et al.*, 2007).

2.7 Cost-effective substrates for bioflocculants production

In recent years, bioflocculants have gained huge scientific and biotechnological interest because of their degradability, the harmless nature of their breakdown products and future application prospects (Nwodo *et al.*, 2013). However, they have not been industrially applied because of their low flocculation ability in real wastewaters treatment, low yield and high cost of production (Mabinya *et al.*, 2012). The comparatively high cost of the commonly used substrates such as fructose, sucrose, galactose, maltose and glucose has negative influence on production costs and this consequently restrict the market potential of these bioflocculants. One major measure to reduce the cost associated with the production of bioflocculants on an industrial scale was to employ low-cost substrates (Fujita *et al.*, 2000). Cheap substrates have been utilized for bioflocculant production (He *et al.*, 2004; Xiong *et al.*, 2010; Zhuang *et al.*, 2012). Zhang *et al.* (2007) documented the utilization of brewery wastewater as a carbon source for bioflocculant production by a mixed culture of microorganisms.

Furthermore, efforts have also focused on the isolation of bioflocculant producing microbes competent in exploiting cost-effective substrates and optimizing the media constituents and fermentation conditions in order to increase bioflocculant yield (Sathiyanarayanan *et al.*, 2013). Currently, response surface methodology (RSM), a statistical modelling is a promising tool that has been effectively applied to optimize bioflocculant production and this has provided consistent information that can be adduced for the optimization of bioflocculant production on a large-scale (He *et al.*, 2009; Li *et al.*, 2013; Nwodo *et al.*, 2014; Nwodo and Okoh, 2014; Peng *et al.*, 2014).

2.7.1 Molasses as a substrate

Molasses is a by-product of the sugarcane industry that comprises of approximately 50% (w/w) total sugars, vitamins and nitrogenous compounds (Moosavi-Nasab *et al.*, 2010). The sugarcane molasses is a strong liquid with some distinctive features such as a high biochemical

oxygen demand (BOD) concentration range (40000-60000 mg/l) and chemical oxygen demand (COD) concentrations range (80000-120000 mg/l), and this liquid requires treatment before disposal in order to prevent environmental pollution. Zhuang *et al.* (2012) reported that the abundance of carbohydrates, amino acids and proteins confers molasses with excellent properties for use as a possible substrate for bacterial growth culture and bioflocculant production.

Several bioflocculant-producing microbes investigated hitherto utilize carbohydrate-rich compounds as the sole source of carbon and energy (Li *et al.*, 2009; Piyo *et al.*, 2011; Ugbenyen *et al.*, 2012). According to He *et al.* (2004), molasses is a cost-effective nutrient that could be used as a high-quality substrate by many microorganisms for the production of exopolymeric substances. Liu *et al.* (2010b) found that *Penicillium* sp. HHE-P7 grows on molasses and that flocculating activity could reach 85% after 3 days of cultivation. *Pseudomonas alcaligenes* PS-25 (Mao *et al.*, 2010) and *Pseudomonas fluorescens* C-2 (Mao *et al.*, 2008) produced bioflocculants after 3 days of cultivation in molasses. He *et al.* (2004) reported that the bioflocculant REA-11 production by *Corynebacterium glutamicum* CCTCC M201005 was supported by the presence of glucose, fructose and sucrose. Sucrose was preferred as the carbon source due to the lower cost and higher production rate of the bioflocculant (He *et al.*, 2004). The ability to exploit sucrose makes it possible to utilize molasses as a carbon source for large-scale production, thus making it possible to produce bioflocculants commercially. Sam *et al.* (2011) reported on the production of exopolysaccharides by halophilic bacteria which grew on a pre-treated molasses as fermentation substrate.

2.7.2 Brewery wastewater as a substrate

In breweries, the cleaning of tanks, bottles, machinery and floors generates high quantities of contaminated water (Doubla *et al.*, 2007). During brewing, large quantities of water are usually

used and discharged into water bodies (Parawira *et al*., 2005; Simate *et al*., 2011). The discharge of untreated brewery wastewater may have a direct impact on water bodies (e.g. Oceans, rivers, streams, or lakes) because the effluents are composed of organic compounds that need oxygen for degradation. For instance, when water of high organic matter, content runs into a river, the microbes flora in the river tend to oxidize the organic matter, utilizing the available oxygen in the water quicker than the amount of oxygen dissolves back into the river from the air, thereby reducing the availability of oxygen for aquatic organisms (Simate *et al*., 2011). However, Chen *et al*. (2003) reported that, due to the availability of nutrient substances, brewery wastewater can perhaps be used as a good substrate for some microorganisms. Zhang *et al*. (2007) documented a novel bioflocculant produced by multiple-microorganism consortia utilizing brewery wastewater as the sole carbon source. About 15 g of purified bioflocculant was recovered from 1 L of fermented culture. Under optimized culture conditions, the flocculating activity of the bioflocculant was about 96.8%. In addition, Roukas (1999) reported the production of pollulan from brewery wastes by *Aureobasidium pollulans*.

2.7.3 Diary wastewater as a substrate

Dairy industries produce various products such as ice-cream, butter, milk, yoghurt, desserts of different kinds, cheeses which vary greatly in their characteristics which rely on the kind of system and methods of operation employed (Vidal *et al*., 2000). The dairy wastewaters usually have high biological oxygen demand (BOD) and chemical oxygen demand (COD) concentrations, a representative of high organic content (Orhon *et al*. 1993). Perle *et al*. (1995) and Kasapgil *et al*. (1994) documented that dairy wastewaters are rich in nature, because of their high organic load which are supplied to these effluents from fats, proteins and carbohydrates derived from the milk. Nonetheless, dairy wastewater is composed of a high concentration of organic matters which makes the effluents a serious threat to the local municipal sewage treatment systems (Perle *et al*. 1995). Most of the wastewater volume

obtained from the dairy industry comes from the cleaning of equipment in the production cycles, tank trucks, rinsing of milk silos and equipment malfunctions or operational errors (Danalewich *et al.*, 1998). According to Fang and Yu (2000), dairy wastewater is mainly composed of simple degradable carbohydrates, mainly lactose, with fewer biodegradable proteins and lipids. It can simply be described as a complex kind of substrate (Fang and Yu, 2000). Demirel *et al.* (2006) revealed that lactose is the major carbohydrate in dairy wastewater and is a readily accessible substrate for the consumption of anaerobic bacteria. The high carbohydrate contents in dairy wastewater were found to reduce the amount of proteolytic enzymes synthesized, resulting in low levels of protein degradation (Fang and Yu, 2000). However, McInerney (1988) observed that carbohydrates may perhaps restrain the synthesis of exopeptidases, a cluster of enzymes assisting protein hydrolysis. Wang *et al.* (2007) documented the production of a novel bioflocculant from a culture of *Klebsiella mobilis* using dairy wastewater supplemented with 2% (v/v) ethanol. By using the optimized culture conditions, 2.58 g of crude bioflocculant was extracted from 1 L fermentation broth and the flocculating activity was about 95.4%.

2.8 Application of extracellular polymeric substances (EPS)

Recently, the exploration of potential EPS utilization has increased tremendously because of its numerous unique properties that suggest its potential applications in various industrial processes (Vu *et al.*, 2009; Elkady *et al.*, 2011). Due to their unique bio-physicochemical properties, EPS are used in various industrial processes, for example, in the production of cosmetics, textiles, adhesives, detergents, pharmaceuticals, food additives, as well as brewing (Liu *et al.*, 2010b; Mishra and Jha, 2013). Furthermore, EPS could serve as bioflocculants, antioxidant, heavy metal removal, a natural immunomodulator, drug delivery agents in wastewater treatment, oil recovery, dredging and in diverse downstream processes (Wang *et al.*, 2008). Some of these biopolymers have also been reported to have anti-viral,

anti-inflammatory, anti-tumor properties, serve as inducers for interferons, and colony stimulating systems (Lin and Zhang, 2004). Among biopolymers identified, polysaccharides draw the attention of researchers in the field of flocculation predominantly in water treatment (Crini, 2005; Raza *et al*., 2011).

The production of polysaccharide-rich bioflocculants is not species specific and it is possible that each strain of the same species secretes diverse kinds of polysaccharides in the cultivation medium with different biological functions (Sathiyanarayanan *et al*., 2013). Polysaccharides possess hydroxyl groups, with a hemiacetal reducing end, as well as other functionalities that play essential roles in reduction reactions (Mata *et al*., 2009). Kunmani *et al*. (2011) found that exopolymeric substances can be used in the food industry as viscosifying, stabilizing and emulsifying agents. These compounds have been of interest as anti-tumor, anti-viral, and anti-inflammatory agents and as inducers of interferon, platelet aggregation inhibitors and in colony stimulating factor synthesis utilized in various medical and pharmaceutical industries. Bioflocculants have been extensively used in wastewater treatment, for example, in the treatment of dye solutions (Zhang *et al*., 2002; Deng *et al*., 2005), inorganic solid suspensions (bentonite, solid clay, alumimum oxide, $Ca(OH)_2$ and activated carbon) (Levy *et al*., 1992; Shih *et al*., 2001; Yim *et al*., 2007).

2.9 Purification of wastewater, COD and suspended solids removal

The bioflocculant produced by *P. elgii* B69 showed high flocculating ability in purifying different wastewaters which included COD removal (68%), turbidity reduction (83%) and colour removal (88%) (Li *et al*., 2013). Gong *et al*. (2008) also reported on the bioflocculant produced by *Serratia ficaria* with the high flocculating activity of kaolin suspensions as well as showing good flocculating efficiency in different wastewaters. River water is surface water characterized with low COD and turbidity. Compared with chemical flocculants in the clarification of river water, bioflocculant SF-1 produced by *Serratia ficaria* had a better

flocculating activity, with the removal efficiency of 87.1% for COD, 84.2% for turbidity and 90.4% for colour (Gong *et al.*, 2008). Furthermore, bioflocculant SF-1 produced by *Serratia ficari*a was also used to flocculate different wastewaters. The COD removal for brewery wastewater was 80.7%, turbidity removal was 91.8%, whereas for meat processing wastewater, the COD removal was 76.3%, turbidity removal was 93.7% and soy sauce brewery wastewater colour removal of 64.1% (Gong *et al.*, 2008). The bioflocculant MBFA9 produced by *Bacillus mucilaginosus* had a strong flocculating activity for suspended solids with a removal rate of 85.5% and the COD removal rate of 68.5% (Deng *et al.*, 2003). Zhengshan *et al.* (2014) also reported on bioflocculant MBF-6 produced by *Klebsiella pneumoniae* YZ-6 isolated from human saliva with the ability to flocculate several wastewaters from the textile, dairy, brewery and the sugar industries. The maximum flocculating efficiency observed with wastewater from the sugar industry showed the highest reduction of COD (77.8%) and BOD (80.7%). In addition, the bioflocculant reduced the suspended solids by up to 78.6% (Zhengshan *et al.*, 2014). The bioflocculant produced by *Bacillus mucilaginosus* had a COD removal rate of 74.6% and 42.3% for BOD for domestic wastewater with the removal rates for brewery wastewater recorded at 70.5% and 77.4% for COD and BOD respectively. Furthermore, its removal rates of COD and BOD for pharmaceutical wastewater was 66.2% and 41.7% respectively (Lian *et al.*, 2008). Gong *et al.* (2008) found that the bioflocculant produced by *Serratia ficaria* had a good COD removal capability and decolourization of pulp effluent than traditional chemical flocculants.

In addition, a number of studies have demonstrated the efficiencies of bioflocculants in the removal of suspended solids, latex particles, microorganisms, COD, humic acids, heavy metals from waste streams, as well as separation of oil from oil-water emulsions and fine coal processes (Ma *et al.*, 2008; Zemmouri *et al.*, 2011). Most bioflocculants produced in the literature have demonstrated good flocculating activity for kaolin suspension. Nevertheless,

they show different flocculating ability for other suspended particles in aqueous solution. Deng *et al*. (2003) found that a polysaccharide-rich bioflocculant exhibited an excellent flocculating potential in the recovery of the organic solids from starch wastewater while Kurane *et al*. (1991) reported on the bioflocculant produced by *Aspergillus latus* that was able to flocculate oil emulsion.

2.9.1 Heavy metal removal

Quite a number of industrial processes resulted in the discharge of heavy metals into aquatic ecosystems. This has necessitated appropriate attention because of the negative impacts of these heavy metals on the environment (Salehizadeh and Shojaosadati, 2003). The problem associated with heavy metals in wastewater entering natural waters has been well documented (Florence and Morrison, 1992). Inorganic effluents from the industrial processes comprise of harmful metals such as Cd, Zn, Cr, Ni and Cu (Kuniawan *et al*., 2002), which tend to build up in the food chain, and their pollution of fresh water is a cause for great concern (Cobbing, 2008). The metals tend to sediment to the bottom of the freshwater where they concentrate and are capable of accumulating in the tissues of aquatic biota. However, their high solubility in aquatic environments means that they can be assimilated by living organisms and once they enter the food chain, they are bound to also manifest in humans. If the metals accumulate in the body above their limit, they can pose severe health problems. Hence, it is essential to treat metal-contaminated wastewaters before their release to the environment (Kuniawan *et al*., 2002). The escalating crisis of heavy metal pollution of soil, water and some other sediment has made seeking for alternatives to eliminate these contaminants a priority. Recently, studies on heavy metal removal from wastewater and coal have spotlighted the development of materials that have increased affinity, capacity and selectivity for target metals (Parirandeh *et al*., 1998). The removal of toxic heavy metals from industrial wastewaters is essential from the standpoint of environmental pollution control (Guangyu and Thiruvenkatachari, 2003). The use

of bacteria capable of producing compounds such as extracellular polysaccharide (EPS) and cell wall components that can take up heavy metal was reported elsewhere (Geddie and Sutherland, 1993), and EPS have been documented to play a significant function in controlling heavy metal contamination in the sewage treatment processes (Kaewchai and Prasertsan, 2002).

The EPS produced by most microorganisms reported in the literature is usually acidic polysaccharides with numerous carboxylate functional groups that carry negative charges that bind the metal ions (Ozdemir *et al.*, 2003). The bioflocculant produced by *Bacillus firmus* could remove 98.3% of Pb^{2+}, 74.9% of Cu^{2+} and 61.8% of Zn^{2+} from aqueous solution (Salehizadeh and Shojaosadati, 2003). Rawat and Rai (2012) found that the bioflocculant produced by *Paenibacillus validus* MP5 demonstrated maximum adsorption values of 27%, 16%, 15%, 9%, 7.5% for Zn^{2+}, Ni^{2+} and Cd^{2+}, Cr^{2+}, Co^{2+}, Pb^{2+} respectively. The bioflocculant produced by *P. elgii* exhibited maximum adsorption activity for Al^{3+} at 72% with significant removal rates for Pb^{2+} (60%), Cu^{2+} (53%) and Co^{2+} (49%). Aluminium (Al^{3+}) has a higher ionic valency thus increasing affinity to the EPS. Lin and Harichund (2012) reported that since bioflocculants have an extensive capacity for interacting with metals, they are recommended as surface-active agents for the removal of heavy metals. A number of studies have also demonstrated the potential utilization of bioflocculants in heavy metal removal (Salehizadeh and Shojaosadati, 2003; Wu and Ye, 2007; Quintelas *et al.*, 2008). Lin and Harichund (2011) reported the ability of bacterial bioflocculants in the removal of bacterial populations, heavy metals and turbidity from three industrial effluents.

The pollution caused by heavy metal deposition in aquatic systems is detrimental to both animal and human health and deserves special attention (Cristina *et al.*, 2011). Therefore, the exploration of new technologies for the treatment of industrial wastewaters becomes essential. Gao *et al.* (2009) reported that the bioflocculant MBF4-13 had a stronger removal efficiency of

69.3% for $Cr_2O_7^{2-}$ than Ni^{2+} (19.2%). The bioflocculant MBF4-13 mainly composed of polysaccharides that have hydroxyl groups in the molecular chain which can easily form hydrogen bonds with $Cr_2O_7^{2-}$ thereby resulting in higher removal efficiency than for Ni^{2+}. Lin and Harichund (2011) found that the bioflocculant produced by *Paenibacillus* sp. CH11 had over 90% removal rate for Cd^{2+}. Also, a novel glycoprotein bioflocculant MBF-TG-1 secreted by a strain of *Klebsiella* TG-1 had a flocculation efficiency of about 86.9% for trona suspension. Several reported studies have shown that the bioflocculant produced by *Paenibacillus* had the ability to remove heavy metals from water (Morillo *et al.*, 2006; Mokaddem *et al.*, 2009; Rawat and Rai, 2012).

2.9.2 Decolourization of dyeing wastewater

The bioflocculant produced by *P. elgii* possesses functional groups that have the ability to decolourize cationic dyes in wastewater. This bioflocculant has a high removal rate of 65% for methylene blue and 72% for Red X-GRL (Li *et al.*, 2013). Lower removal efficiencies that were below 50% were obtained when it was used to treat anionic and neutral dyes (Li *et al.*, 2013). According to Li *et al.* (2013), there are two mechanisms of adsorption of cationic dyes by the bioflocculant. The acidic bioflocculant has a negatively charged COO^- group in the molecular chain which provides an adsorption site for the positively charged cationic dye molecules; thereby making electrostatic attraction possible (Verma *et al.*, 2012). Furthermore, an analysis of the sugar content revealed that the bioflocculant possesses high levels of mannose which makes van der Waal forces electrostatic interactions and hydrogen bonding possible (Blackburn, 2004). Li *et al.* (2003) also documented a higher flocculating efficiency for REA-11 secreted by *Corynebacterium glutamicum* in comparison with chemically synthetic flocculants and found very effectual in the decolourization of molasses in wastewater. He *et al.* (2004) found that REA-11 decolourizes molasses wastewater and proposed its potential for industrial application.

Bioflocculants have been applied in various industrial processes which included the flocculation of inorganic solid suspensions (Natarajan and Das, 2003; Lu *et al.*, 2005; Yim *et al.*, 2007; Zhang *et al.*, 2007). Dye removal in wastewater poses a serious challenge since largely all the dyes are absolutely soluble in aqueous solutions. The utilization of synthetic chemical dyes in different industrial processes such as the dyeing of cloth, paper and pulp manufacturing, leather treatment, printing and plastics has increased significantly recently, and this has led to the discharge of industrial effluents contaminated with dyes into the ecosystem (Aksu, 2005). However, since some of these dyes are lethal in nature, their occurrence in industrial effluents will be a threat to the environment because they are usually not easily degraded by microorganisms (Pagga and Brown, 1986).

In most cases, the partly degraded dyes from anaerobic degradation by some microorganisms generate potentially carcinogenic compounds that find their ways in the food chain and are later consumed by humans (Banat *et al.*, 1996). Heavily coloured wastewaters can block the access of sunlight and oxygen necessary for the survival of various aquatic forms (Crini, 2006). The bioflocculant secreted by *Nannocystics* sp. Nu-2 was recorded to be a glycoprotein which showed high efficiency in bleaching acid red and direct emerald blue (Zhang *et al.*, 2002). Gao *et al.* (2009) reported that the bioflocculant MBF4-13 produced by a novel bacterium strain ZHT4-13 isolated from *Ruditapes philippinarum* conglutination mud, was found to decolorize different dyes, with removal efficiencies of 86.11% for methylene blue, 97.84% for crystal violet and 99.49% for malachite green. In addition, it was observed that this bioflocculant MBF4-13 had a strong decolourizing efficiency for blue and violet series of dyes and possesses low decolourizing capability for red, pink and orange series of dyes. Conversely, an observation by Deng *et al.* (2005) revealed that the bioflocculant secreted by *Aspergillus parasiticus* was more effective in removing Reactive Blue 4 and Acid Yellow 25 than Basic Blue B.

2.9.3 Removal of pathogens in water

Oh *et al.* (2001) documented that the bioflocculant secreted by *Paenibacillus* sp. was effectively utilized in harvesting *Chlorella vulgaris* from a culture broth whilst another bioflocculant produced by *Paenibacillus polymyxa* AM49 was shown to be successful in the harvesting of a high density *Scenedesmus* sp. culture (Kim *et al.*, 2011). Similarly, the bioflocculant produced from a culture broth of *Solibacillus silvestris* W01 demonstrated high flocculating activity of 90% on marine microalgae *Nannochloropsis oceanica* and as such has great prospects for harvesting marine microalgae for the commercial production of microalgal bioproducts. A similar report was observed for a bioflocculant produced by *Solibacillus silvestris* W01 (Wan *et al.*, 2013). Also, another study by Zhao *et al.* (2013) suggests that the bioflocculant MBF-5 produced by *Klebsiella pneumoniae* isolated from sputum samples showed a high flocculating rate of 84% in the removal of *Acanthamoeba* cysts, a potent pathogen in water and soil.

Table 2.2. Some bioflocculants reported in the literature and their respective applications

Application	Microorganism	Remarks	Reference
Removal of pathogens	*Klebsiella terrigena*	Remove *Salmonella* sp.	Ghosh *et al.*, 2009
	Solibacillus silvestris W01	Harvest *Nannochloropsis oceania*	Wan *et al.*, 2013
	Klebsiella pneumonia	Remove *Acanthamoeba* cysts	Zhao *et al.*, 2013
	Paenibacillus polymyxa AM49	Remove *Scenedesmus* sp.	Kim *et al.*, 2011
	Bacillus agaradhaerens C9	Harvest *Clorella minutissima* UTEX2341	Liu *et al.*, 2015
Dye decolourization	*Ruditapes Philippinarum*	Remove methylene blue, crystal violet, malachite	Wei *et al.*, 2011
	Klebsiella sp.	Remove sulfamethoxaazole	Xing *et al.*, 2013
	Serratia ficaria	Decolourize pulp effluent	Gong *et al.*, 2008
	Corynebacterium glutamicum	Decolourize molasses wastewater	He *et al.*, 2004

	Aspergillus parasiticus	Decolourize Reactive Blue 4, Acid Yellow 25. Basic Blue B	Deng *et al.*, 2005
	Staphylococcus and Pseudomonas species	Decolourize indigotin printing and dyeing wastewater	Zhang *et al.*, 2007
	Klebsiella mobilis	Remove disperse yellow, disperse violet, reactive light-yellow, and reactive turquoise blue	Wang *et al.*, 2007
	Paenibacillus polymyxa BY-28	Reactive brilliant blue and Reactive brilliant yellow	Gong *et al.*, 2003
Water purification	*Paenibacillus elgii* B69	Real wastewater treatment	Li *et al.*, 2013
	Bacillus mucilaginosus	Treat domestic, brewery and pharmaceutical wastewater	Lian *et al.*, 2008
	Paenibacillus mucilaginosus G1M16	Treat paper mill waterwater	Tang *et al.*, 2014
	Bacillus licheniformis	Treat sugar industry wastewater	Zhuang *et al.*, 2012
	Oceanobacillus and Halobacillus species	Treat brewery, dairy wastewater and river water	Cosa and Okoh, 2014
	Cobetia and Bacillus species	Treat brewery, dairy wastewater and river water	Ugbenyen and Okoh, 2014
	Chlorella sp. and *Micratinium* sp.	Industrial wastewater	Wang *et al.*, 2014
	Arthrobacter sp. B4	Treat alkaline wastewater	Li *et al.*, 2014
	Azotobacter indicus	Treat dairy, woollen, starch and sugar wastewater	Patil *et al.*, 2011
	Aspergillus niger	Treat river water	Aljuboori *et al.*, 2014
	Bacillus sp.	Brewery waste water	Feng and Xu, 2008
Heavy metal removal	*Herbaspirillus* sp. CH7, *Paenibacillus* sp. CH 11, *Bacillus* sp. CH15 and *Halomonas* sp.	Pb^{2+}, Zn^{2+}, Hg^{2+}, Cd^{2+}	Lin and Harichund, 2012
	Bacillus firmus	Pb^{2+}, Cu^{2+} and Zn^{2+}	Salehizadeh and Shojaosadati, 2003
	Paenibacillus validus strain MP5	Zn^{2+}, Ni^{2+}, Cd^{2+}, Cr^{2+}, Co^{2+} and Pb^{2+}	Rawat and Rai, 2012
	Pseudomonas aeruginosa strain IASST201	Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Fe^{2+}, Cr^{2+} and Mn^{2+}	Pathak *et al.*, 2014
	Achromobacter sp.	Pb^{2+}	Batta *et al.*, 2013
	Klebsiella sp. TG-1	Defecating the trona suspension	Liu *et al.*, 2013
	Enterobacter aerogenes	Defecating the trona suspension	Lu *et al.*, 2005
	Pseudomonas fluorescens BM07	Hg^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}	Noghabi *et al.*, 2007

Rhodococcus erythropolis	Pb^{2+}	Guo and Yu, 2014	

2.10 Molecular biology and synthesis of bioflocculant

Genes involved in the synthesis of bioflocculants in different microbes are highly conserved and organized in clusters (Bai *et al.*, 2008) and have been identified in some bioflocculant-producing microbes such as in *Streptomyces* species, *Bacillus licheniformis*, *Rhizobium radiobacter* (Stingele *et al.*, 1996; Tang *et al.*, 1996; Li *et al.*, 2003; Bai *et al.*, 2008; Yan *et al.*, 2013). The gene products, mostly enzymes, are involved in the formation of polysaccharides by sequential addition of sugars to membrane anchored repeating units which are then exported (Cerning, 1990).

Biosynthetic processes can be controlled at three different levels: synthesis of sugar nucleotide precursors; assembly of the repeating unit; and polymerization and export (Bai *et al.*, 2008). The modification of the expression of single genes or groups of genes can be used to increase the conversion efficiency of the chemical entities involved, and therefore, enhance bioflocculant yield. However, it might also provide a means of altering the polymer composition (Bajaj *et al.*, 2007). Most bioflocculants are synthesized intracellularly and exported to the extracellular environment as macromolecules (Rehm, 2009; Ullrich, 2009). Bacterial biosynthetic pathways comprise of a substrate uptake, a central metabolite pathway and a polysaccharide. Depending on the substrate type, it can be taken up by the cell either through a passive or an active transport system, following which, it is catabolized by intracellular phosphorylation; or it can be transported and oxidized through a direct oxidative periplasmic pathway. The periplasmic oxidative pathway exists only in certain bacteria, whereas the intracellular phosphorylative pathway is ubiquitous amongst bacteria. Both these systems have been reported in several bioflocculant-producing microbes and they can function simultaneously if there is substrate availability (Schaechter and Lederberg, 2004). In the

cytoplasm, the substrate is catabolized through glycolysis and the primary metabolites formed are used as precursors for the synthesis of small biomolecules (e.g. amino acids or monosaccharides). Polysaccharide synthesis requires the biosynthesis of activated precursors that are energy-rich monosaccharides, mainly nucleoside diphosphate sugars (NDP-sugars), which are derived from phosphorylated sugars.

Even though bioflocculant production is a process that entails a perceptible energy cost, owing to the need for carbon as substrate for the growth of microorganisms, the gain together with their existence is significantly higher compared to the costs (taking into account the growth enhancement and the survival of the microbial producers) (Wolfaardt *et al.*, 1999). As bioflocculant production is associated with a precise gene cluster, the information of the genome sequence of bioflocculant microbes is certainly the essential point for the optimization of their biosynthesis through the molecular biology approach (Ates *et al.*, 2011, 2013). The production of bioflocculants is a genetically determined process and metabolic engineering is a powerful tool to improve metabolite productivity (Delbarre-Ladrat *et al.*, 2014).

Several gene clusters have been identified in both Gram-positive and Gram-negative bacteria that are involved in the biosynthesis of bioflocculant (Stingele *et al.*, 1996). The enzymes encoded by these gene clusters can be divided into four groups: enzymes responsible for the initial metabolism of carbohydrates, enzymes involved in sugar nucleotide synthesis and interconversion, glycosyltranferases that form the repeating unit attached to the glycosyl carrier lipid and translocases and polymerases that form the polymer (Looijesteijn *et al.*, 1999). Therefore, in order to improve on the production, a precise approach is to identify all the genes responsible for bioflocculant synthesis and then attempt to understand the mechanisms involved. This is actually the research focus in biotechnology, paying attention to studies relating to the genomic level of bioflocculant-producing microorganisms (Finore *et al.*, 2014). Once the whole genome of these microorganisms has been sequenced, it will be suitable to

select an appropriate tactic to improve the bioflocculant produced by manipulating those genes encoding the enzymes implicated in the bioflocculant synthesis (Yang *et al.*, 2007). In addition, regulating the pathways that influence gene expression and enzyme activity, as well as the choice of the most appropriate substrate that will be supplemented with the media for cultivation of bioflocculant-producing microbes ought to be considered (Yang *et al.*, 2007). This could interfere with the physicochemical characteristics of the bioflocculants and may eventually have a great impact on bioflocculant properties and potential applications in industry. Nevertheless, the regulation of bioflocculant synthesis in marine microorganisms is still poorly understood (Bajaj *et al.*, 2007; Rehm, 2009) and it will be vital to explore the advances in genetic engineering of bioflocculant-producing microbes in order to improve yields.

2.11 Conclusion and future prospects

Chemical flocculants are effective at aggregating colloids and have been widely used in different industrial processes. Because of their negative health impacts and the environmental hazards associated with chemical flocculants, microbial flocculants have gained huge scientific and biotechnology consideration because of their safety and eco-friendly attributes.

Marine habitats which support a rich biodiversity of marine bacteria remain underexplored for this purpose and yet hold tremendous promise as reservoirs of novel bioflocculant producing organisms. Although many bioflocculants have been reported in the literature, their large scale production is still limited by low yields, high production costs and low flocculating activity. Optimization of media constituents and fermentation conditions is also one of the strategies to improve on bioflocculants yields and flocculating activity. However, the high cost of media constituents, will make it highly propitious to utilize cost effective substrates for large scale bioflocculant production in industries. Furthermore, the utilization of microorganisms in

consortia for bioflocculants production that will possess better flocculating activity and higher bioflocculant yield than pure strains is essential.

In addition, only a few cation-independent bioflocculants have been identified and documented in the literature. Therefore, further studies are needed to produce cation-independent bioflocculants with high flocculating efficiencies and consequently reduce the environmental pollution caused by the cations used in the flocculation processes.

Additional knowledge of the genetics and biochemistry of bioflocculant biosynthesis is imperative before their production processes are modified for better yield and increased activities which are subjects of ongoing investigations in our group.

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CHAPTER THREE

Bacillus toyonensis strain AEMREG6, a bacterium isolated from South African marine environment sediment samples produces a glycoprotein bioflocculant

(Published in Molecules)

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Abstract

A bioflocculant-producing bacterium, isolated from sediment samples of a marine environment in the Eastern Cape Province of South Africa demonstrated a flocculating activity above 60% for kaolin clay suspension. Analysis of the 16S ribosomal deoxyribonucleic acid (rDNA) nucleotide sequence of the isolate M72 in the GenBank database showed 99% similarity to *Bacillus toyonensis* strain BCT-7112 and it was deposited in the GenBank as *Bacillus toyonensis* strain AEMREG6 with accession number KP406731. The bacteria produced a bioflocculant (REG-6) optimally in the presence of glucose and NH_4NO_3 as the sole carbon and nitrogen source, respectively, initial medium pH of 5 and Ca^{2+} as the cation of choice. Chemical analysis showed that purified REG-6 was a glycoprotein mainly composed of polysaccharide (77.8% w/w) and protein (11.5% w/w). It was thermally stable and had strong flocculating activity against kaolin suspension over a wide range of pH values (3–11) with a relatively low dosage requirement of 0.1 mg/mL in the presence of Mn^{2+}. Fourier transform infrared spectroscopy (FTIR) revealed the presence of hydroxyl, carboxyl and amide groups preferred for flocculation. Scanning electron microscopy (SEM) revealed that bridging was the main flocculation mechanism of REG-6. The outstanding flocculating performance of REG-6 holds great potential to replace the hazardous chemical flocculants currently used in water treatment.

Keywords: marine environment; *Bacillus toyonensis* strain AEMREG6; REG-6; flocculating activity; glycoprotein; thermostable

3.1 Introduction

Industrial wastewater treatment is a hot research topic globally; flocculation has been recognised to be an excellent approach for removing pollutants from wastewater (Tang *et al.*, 2014). Although, many inorganic and organic synthetic flocculants exhibit good flocculation efficiency but their environmental and health problems cannot be neglected (Li *et al.*, 2013). Alternatively, bioflocculants could be substitutes due to their innocuousness and biodegradability (Liu *et al.*, 2013). Bioflocculants are secondary metabolites produced during the growth of microorganisms, which are predominantly composed of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Amongst these macromolecules, the polysaccharide-based bioflocculants have attracted wide attention because of their high rates of flocculation in removing different kinds of heavy metals, cell removal and biomass recovery, and waste/drinking water treatment (Lin and Harichund, 2011; Patil *et al.*, 2011; Zhao *et al.*, 2013).

Several reports have shown the production of bioflocculants by bacteria, fungi, and algae isolated from activated sludge, soil and water (Gao *et al.*, 2006; Piyo *et al.*, 2011; Okaiyeto *et al.*, 2013). Fermentation conditions and nutritive components of the cultivation medium have been reported to have a great influence on bioflocculant production (He *et al.*, 2004). Due to the low flocculating capability and high cost of production, industrial production of bioflocculants is not well established. Consequently, there is a need to seek microorganisms with greater bioflocculant production capability to reduce the production cost (Gao *et al.*, 2006). Furthermore, it will be economically auspicious to investigate technology for improving the flocculating activity of the purified bioflocculants.

From previous studies, species of the genus *Bacillus* isolated from freshwater sources are well documented to produce bioflocculants (Vijayalakshmi and Raichur, 2003; Elkady *et al.*, 2011; Zaki *et al.*, 2013 Adebayo-Tayo and Adebami, 2014), however, the production of

bioflocculants by *Bacillus* species isolated from the marine environment is still scarce (Ugbenyen *et al*., 2014). The marine environment is the largest habitat on the Earth, accounting for more than 90% of the total biosphere volume (Lauro *et al*., 2009). It is one of the most adverse environments due to the varying temperature, salinity and pH conditions. Besides, due to the constant variation of environmental conditions, the microorganisms present in that environment are suitably adapted to these adverse conditions since they exhibit complex adaptation features (Dash *et al*., 2013).

Bacillus toyonensis is a Gram-positive, spore-forming bacterium that forms a homogeneous independent branch within the *Bacillus* genus. It has a prodigious economic importance, for example the spores of *Bacillus toyonensis* BCT–7112 have been used in animal nutrition in some parts of the world (Jimeneza *et al*., 2013). In this paper, we report on bioflocculant production by *Bacillus toyonensis* strain AEMREG6 isolated from sediment samples from Algoa Bay in the Eastern Cape Province of South Africa. To the best of our knowledge, this is the first study to implicate the species *Bacillus toyonensis* in bioflocculant production.

3.2 Results and Discussion

3.2.1 Identification of the bioflocculant-producing bacteria

The bacteria used in this study were isolated from sediment samples from Algoa Bay (a marine environment) in the Eastern Cape of Province South Africa and screened for the ability to flocculate a kaolin clay suspension. The bacteria (isolate M72) showed good bioflocculant production potential, having flocculating activity of over 60% for kaolin clay suspension. The bacterial colonies appear to be creamy, smooth and viscous on nutrient agar plate. The identity of the isolate was confirmed by 16S rDNA analysis. The BLAST analyses of the nucleotide sequence of the 16S rDNA of the bacterium showed a 99% similarity to *Bacillus toyonensis* strain BCT-7112, and the nucleotide sequence was deposited in the GenBank as *Bacillus*

toyonensis strain AEMREG6 with accession number KP406731. Many *Bacillus* species have been implicated in bioflocculant production in previous studies (Zheng *et al.*, 2008; Li *et al.*, 2009; Abdel-Aziz *et al.*, 2011; Elkady *et al.*, 2011; Piyo *et al.*, 2011; Sathiyanarayanana *et al.*, 2013; Adebayo-Tayo and Adebami, 2014; Tang *et al.*, 2014), but nowhere in the literature is *Bacillus toyonensis* reported as a bioflocculant producer.

3.2.2 Optimization of culture conditions for REG-6 production

3.2.2.1 Effect of carbon and nitrogen sources on REG-6 production

One of the most critical factors influencing bioflocculant production is the carbon source in the growth medium (Bereka *et al.*, 2014). Carbon sources generally used in culture media for bioflocculant production have a direct impact on the production cost of bioflocculants, which limits the market potential of these products (Cosa *et al.*, 2013a). In this respect, the effect of various carbon and nitrogen sources on REG-6 production by the bacterium was assessed. Among these carbon sources, glucose and maltose containing media showed flocculating activity of over 60%, which is a measure of REG-6 production (Figure 3.1).

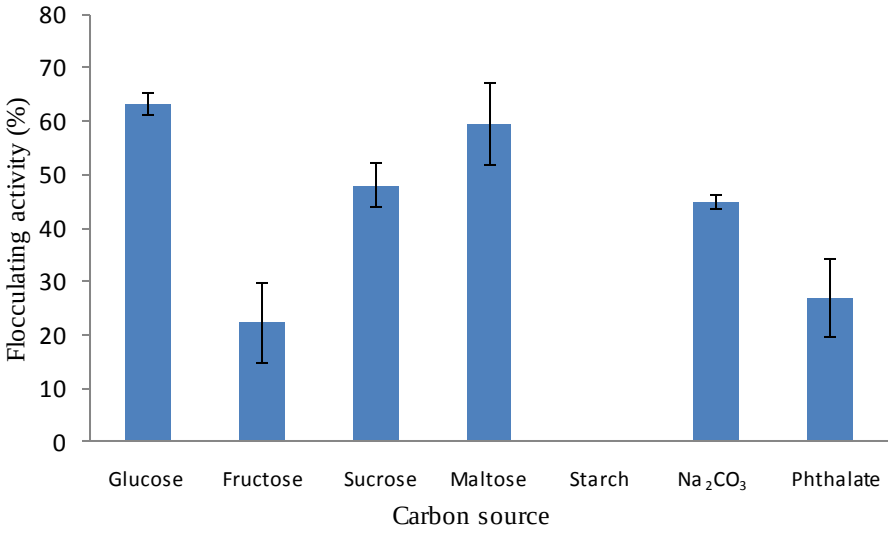

Figure 3.1. Effect of carbon source on REG-6 production by *Bacillus toyonensis* strain AEMREG6.

Several studies in the literature reporting on bioflocculant production were in accordance with our present study. Most microorganisms utilized for bioflocculant production in the literature preferred glucose as the sole carbon source (Cosa *et al.*, 2013a; Nwodo *et al.*, 2013). On the other hand, fructose- and phthalate-containing media resulted in poor flocculating activity. Only a few studies have documented the use of Na_2CO_3 as a carbon source for bioflocculant production (Cosa *et al.*, 2013b). Maltose was the carbon source of choice for *Solibacillus silvestris* (Wan *et al.*, 2013), although starch inhibited the production of bioflocculant by the bacterium. On the contrary, *Aspergillus parasiticus* and *Bacillus licheniformis* preferred starch as the carbon source for bioflocculant production (Deng *et al.*, 2005; He *et al.*, 2009).

The effect of different nitrogen sources on REG-6 production was examined and is presented in Figure 3.2. All the organic nitrogen sources tested were poorly utilized by this strain and resulted into low bioflocculant production. Comparatively, inorganic nitrogen sources greatly enhanced REG-6 production with the maximum flocculating activity of 73% being achieved with NH_4NO_3. Different inorganic nitrogen sources have been reported to be suitable for bioflocculant production in previous studies. For example, in a study documented by Nwodo *et al.* (2013), $(NH_4)_2SO_4$ was the most preferred nitrogen source for bioflocculant production among others.

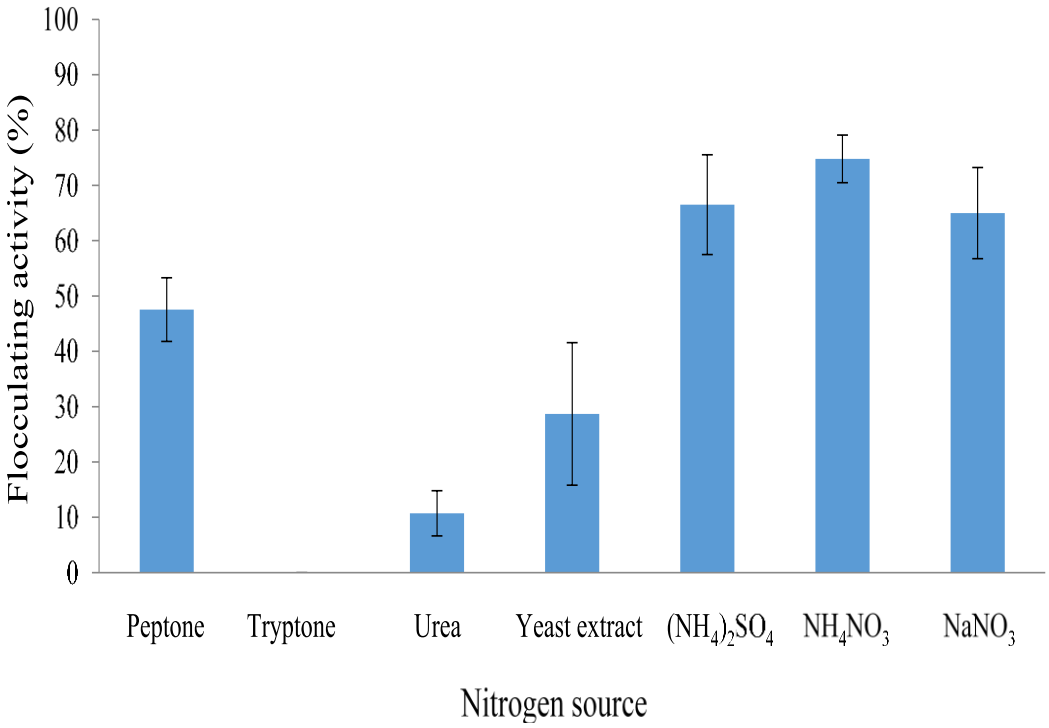

Figure 3.2. Effect of Nitrogen Source on REG-6 production by *Bacillus toyonensis* strain AEMREG6.

In the same way, NH_4Cl, $(NH_4)_2SO_4$ and $NaNO_3$ were the most favourable nitrogen sources for bioflocculant production by *Gyrodinium impudicum*, *Halomonas* sp. and *Candida anglica* (Yim *et al.*, 2007; He *et al.*, 2009; Yan *et al.*, 2013). On the contrary, Li *et al.* (2013) and Aijuboori *et al.* (2013) found that peptone was most suitable for bioflocculants production by *Paenibacillus elgii* and *Aspergillus flavus*. Yeast extract was utilized for bioflocculants production by *Penicillium purpurogenum*, *Kloeckera* sp. and *Solibacillus silvestris* (Liu *et al.*, 2010; Abu-Elreesh *et al.*, 2011; Wan *et al.*, 2013).

3.2.2.2 Effect of initial pH of growth medium on REG-6 production

It has been well documented in previous studies that the initial pH of the growth medium required for bioflocculant production differs between microorganisms (Liu *et al.*, 2010; Ugbenyen *et al.*, 2012). According to Xia *et al.* (2008), the initial pH of the growth medium affects the electric charge of the cells and redox reactions which in turn affect nutrient assimilation and enzymatic reaction. The effect of initial pH of the growth medium on REG-6 production was investigated in the pH range from 4 to 10 (Figure 3.3). It was observed that the

bioflocculant production was better under acidic conditions, with the highest flocculating activity being 65% at pH 5. Further increases in pH either to neutral or alkaline conditions poorly supported the production of REG-6, with the lowest flocculating activity being observed at pH 10.

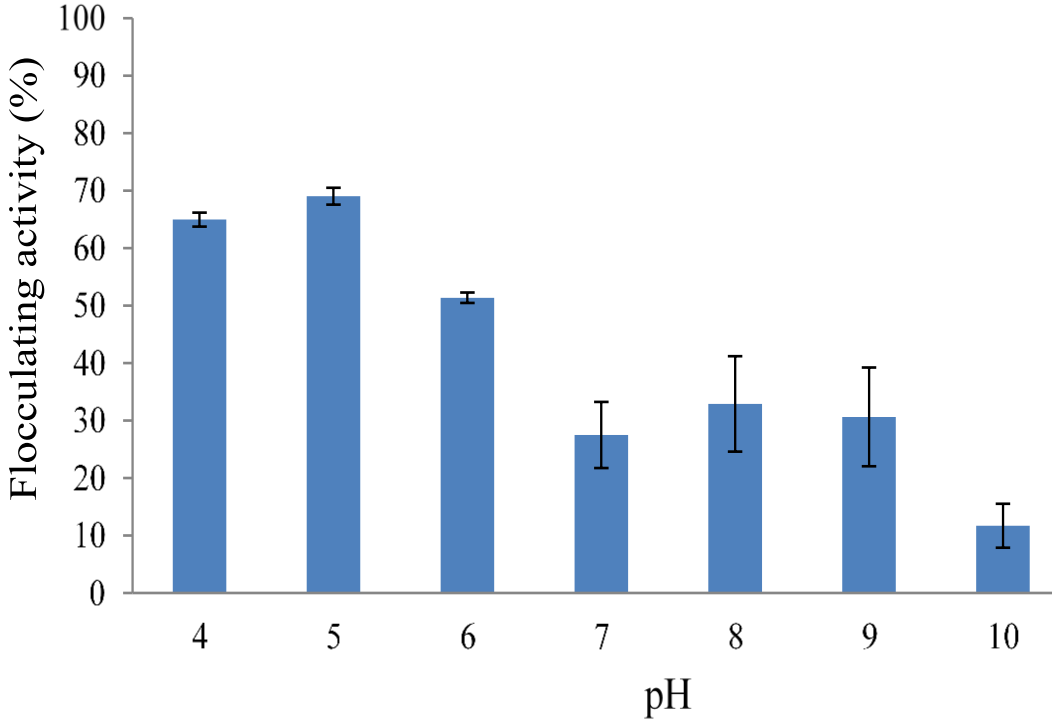

Figure 3.3. Effect of initial pH of growth medium on REG-6 production by *Bacillus toyonensis* strain AEMREG6.

Similar findings were documented by Liu *et al.* (2010) and Zufarzaana *et al.* (2012) for bioflocculant production by *Chryseobacteria daeguense* W6 and *Bacillus* sp. UPMB13 under weak acidic condition to alkaline conditions. In the case of the bioflocculant produced by *Cobetia* sp., the maximum production was obtained at pH 6 (Ugbenyen *et al.*, 2012). On the contrary, the optimum pH for bioflocculant production by *Klebsiella* sp. TG-1 was pH 8 (Liu *et al.*, 2013). Likewise, an alkaline pH range of 7–12 was more suitable for bioflocculant production by *Bacillus* sp. F19, with the maximum flocculating activity being observed at pH 9, whereas acidic condition completely inhibited bioflocculant production (Zheng *et al.*, 2008).

3.2.2.3. Effect of inoculum size on REG-6 production

Effect of inoculum size on REG-6 production by *Bacillus toyonensis* strain AEMREG6 was examined (Figure 3.4). Inoculum size play important role in cell growth and bioflocculant production. Small inoculum sizes tend to prolong the lag phase, while a large inoculum will make niches of the strain overlap excessively and consequently inhibit bioflocculant production (Salehizadeh and Shojaosadati, 2001). In case of *Serratia ficaria* and *Aspergillus flavus*, optimum bioflocculant production was observed at 1 and 2% inoculum size, respectively (Zheng *et al.*, 2008; Aljuboori *et al.*, 2013). In this present study, REG-6 production by the bacterium was increased with the increase in inoculum size, with the optimum production being observed at 4% inoculum size (Figure 3.4). Our findings were comparable to the report by Wang *et al.* (2007) for bioflocculant production by *Klebsiella mobilis*, where the optimal production was observed at 5% inoculum size.

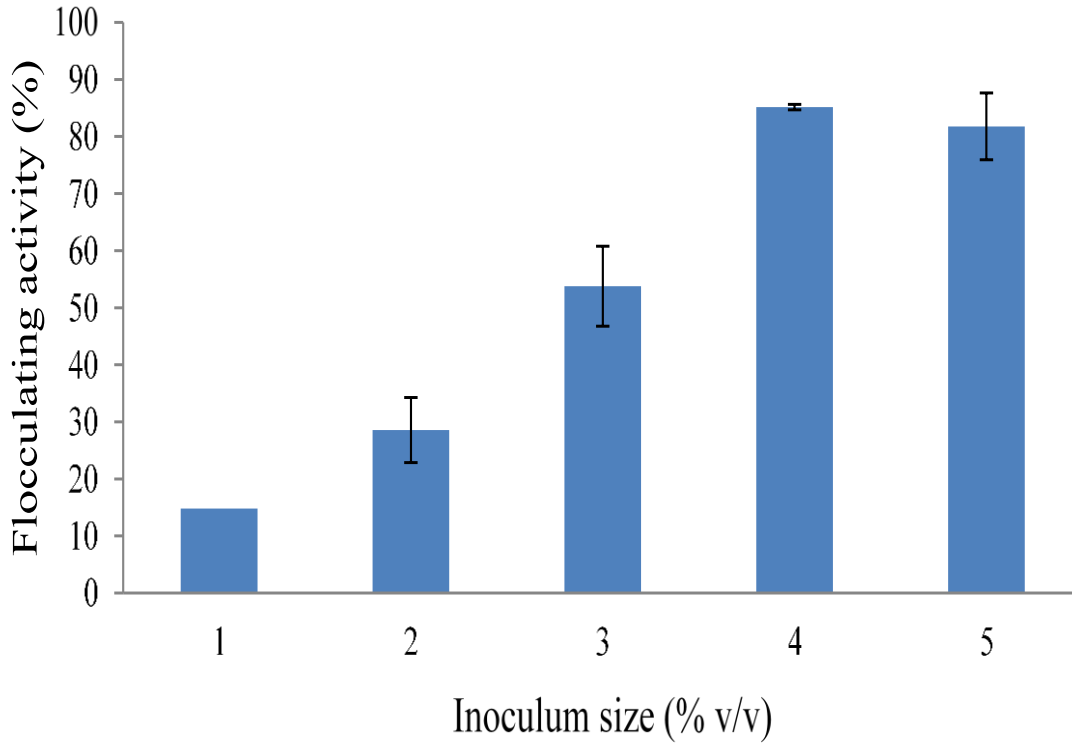

Figure 3.4. Effect of inoculum size on REG-6 production by *Bacillus toyonensis* strain AEMREG6.

3.2.3 Optimization of the flocculating conditions of crude REG-6

3.2.3.1. Effect of cations on the flocculating activity of crude REG-6

Cations can neutralize the negative charges of both bioflocculant and suspended particles, and subsequently increase the adsorption of bioflocculant onto suspended particles (Wu and Ye, 2007). As shown in Table 3.1, both monovalent and divalent cations enhanced the flocculating activity of produced REG-6 by more than 80%. The highest flocculating activity of 86.35% was observed with Ca^{2+}, followed by K^{+} (85.05%), Mn^{2+} (84.02%), Mg^{2+} (82.21%), Na^{+} (81.29%) and Al^{3+} (72.27%). The flocculating activity of REG-6 was completely inhibited in the presence of Fe^{3+} due to excessive adsorption of the ions (Wu and Ye, 2007). In another study reported by Nwodo *et al.* (2013), the flocculating activity of the bioflocculant produced by *Brachybacteria* sp. was synergistically enhanced in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}.

Table 3.1. Effect of cations on the flocculating activity of crude REG-6 produced by *Bacillus toyonensis* strain AEMREG6.

Cations (w/v)	Flocculating activity (%)
K^{+}	85.05
Na^{+}	81.29
Mg^{2+}	82.21
Ca^{2+}	86.35
Mn^{2+}	84.02
Fe^{3+}	-
Al^{3+}	72.27

Likewise, Ca^{2+}, Na^{+} and K^{+} were more effective in stimulating the flocculating activity of the bioflocculant produced by *Bacillus mojavensis* (Elkady *et al.*, 2011). On the other hand, the

addition of cations in a flocculation process usually increases treatment cost and besides, cations can also constitute environmental pollution. Hence, it will be propitious to produce a bioflocculant that is cation-independent. Some microorganisms such as *Citrobacter* sp., *Coryneobacteria daeguense* and *Solibacillus silvestris* have been reported to produce such cation-independent bioflocculants (Fujita *et al.*, 2000; Liu *et al.*, 2010; Wan *et al.*, 2013).

3.2.3.2. Effect of pH on the flocculating activity of crude REG-6

The pH of the environment is one of the most important external factors influencing the flocculating activity of a bioflocculant (Tang *et al.*, 2014). Figure 3.5 shows the effect of pH on the flocculating activity of REG-6 as its exhibited different electrical charges at different pH value which in turn affect the bridging mechanism required for optimal flocculation (Yong *et al.*, 2009). REG-6 had high flocculating activity above 85% over a wide range of pH values from 3-11, with the maximum flocculating activity of 94% occurring at pH 3. Wang *et al.* (2011) stated that pH determines the formation of flocs and also affects the stability of the suspended particles. The high flocculating activity shown over a wide pH range suggests that REG-6 can be applied under extreme environmental conditions which portends its good industrially applicability. Correspondingly, Zheng *et al.* (2008) found that the best pH that supported flocculation of the bioflocculant produced by *Bacillus* sp. F19 was pH 2. In the case of the bioflocculant produced by *Ruditapes philippinarum*, it showed strong flocculating activity for kaolin clay suspensions over a wide pH range (1–13) with the optimum flocculating activity being observed in the range of 7–9 (Gao *et al.*, 2009). The bioflocculant produced by *Ochrobactrum ciceri* exhibited a good flocculating activity above 94% over the pH 1–10 range (Wan *et al.*, 2013), whereas the bioflocculant produced by *Bacillus licheniformis* was relatively pH stable in the range of 2–11 (Ji *et al.*, 2010). When a bioflocculant only flocculates well or is stable in a narrow pH range, the possibility of its application under extreme conditions will be limited and the treatment cost will be high since there will be a need to adjust the pH of the

water or wastewater to be treated to the desired pH range of the bioflocculant and consequently this inflates treatment costs.

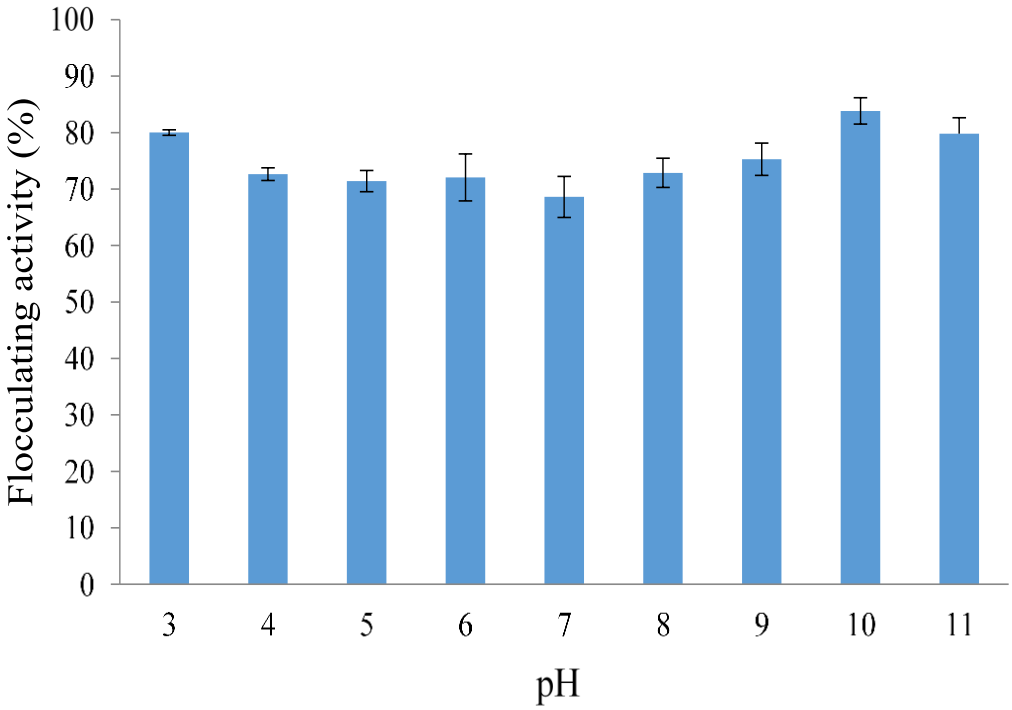

Figure 3.5. Effect of pH on the flocculating activity of crude REG-6 produced by *Bacillus toyonensis* strain AEMREG6.

3.2.4 Time course of REG-6 production by Bacillus toyonensis strain AEMREG6

The time course of REG-6 production by *Bacillus toyonensis* strain AEMREG6 was investigated and the results are presented in Figure 3.6. In previous studies, many researchers have reported that the production of bioflocculant was congruent with cell growth and maximum flocculating activity was achieved in the early stationary phase (Salehizadeh and Yan, 2014). In Figure 6, the flocculating activity of the produced REG-6 increased progressively with the increase in cell growth, with the maximum flocculating activity of 77% being achieved after 96 h of cultivation. The cell growth reached a stationary growth phase between 48 and 96 h of cultivation after which the cell growth declined with a corresponding decrease in flocculating activity. This is an indication that bioflocculant production was associated with cell growth and not cell autolysis (Gao *et al.*, 2006).

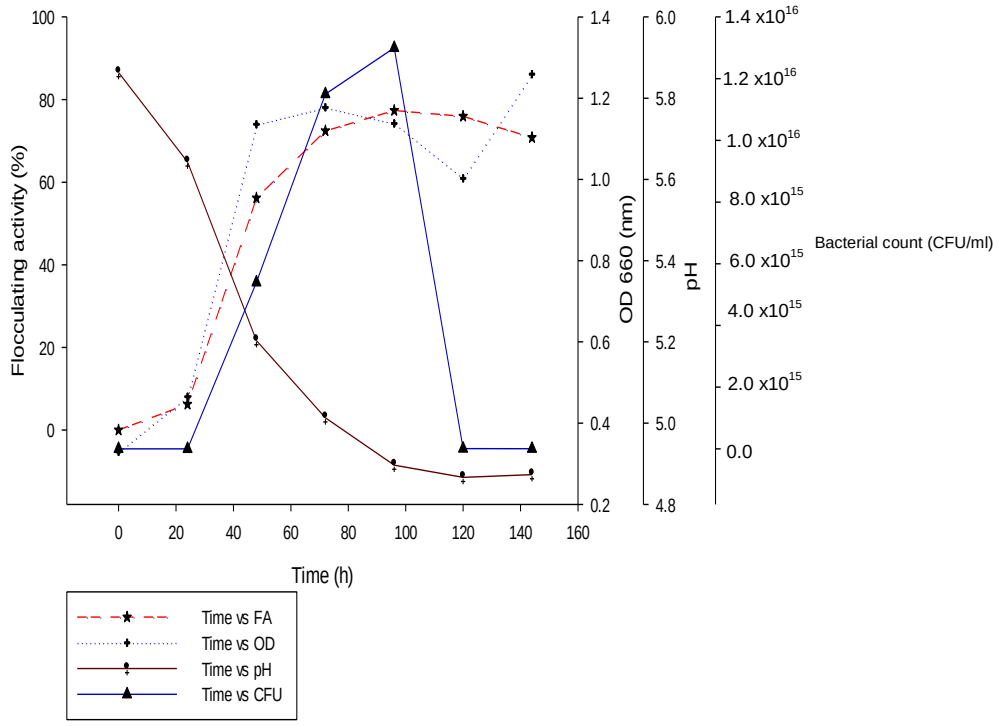

Figure 3.6. Time course of REG-6 production by *Bacillus toyonensis* strain AEMREG6.

However, the decrease in flocculating activity observed after 96 h of cultivation could be due to the release of bioflocculant-degrading enzymes by the microorganism in the death phase of growth or the utilization of the produced bioflocculant as a carbon source for further cell growth (Li *et al.*, 2009; Zaki *et al.*, 2013). Consequently, 96 h of cultivation was chosen for the subsequent experiments. We also observed a decrease in pH of the culture broth from 5.86 to 4.87 (Figure 3.6), and this decline in pH might be due to the production of organic acids from glucose or the presence of an organic acid as a component of REG-6 (Lu *et al.*, 2005). The growth pattern of *Aspergillus flavus* was observed to be closely-related to the flocculating activity of the produced bioflocculant (Aljuboori *et al.*, 2013).

Also, in the case of the bioflocculant produced by *Proteus mirabilis*, the flocculating activity also matched the cell growth pattern with the highest flocculating activity being observed in the stationary phase (Xia *et al.*, 2008). The bioflocculant production by *Bacillus licheniformis* X-14, *Bacillus mojavensis* and *Klebsiella* sp. TG-1 reached a maximum after shorter cultivation

times of 20, 24 and 36 h, respectively (Li *et al.*, 2009; Elkady *et al.*, 2011; Cosa *et al.*, 2013b). This indicated that these strains were able to produce bioflocculants at a lesser cultivation time than some other reported strains and consequently reduce the production cost.

3.2.5 Characterization of the purified REG-6

3.2.5.1. REG-6 dosage

The effect of REG-6 dosage was investigated in the range of 0.05–0.5 mg/mL. The flocculating activity of the bioflocculant was highest at 0.1 mg/mL and further dosage increases resulted in a lower flocculating activity (Table 3.2). Lower dosages also induced lower flocculation efficiency of REG-6. The lower dosage effect implies that there were not enough bioflocculant molecules to adsorb the suspended kaolin clay particles to make the bridge process effective (Zhang *et al.*, 2010).

Table 3.2. Effect of dosage, pH, cations and temperature on the flocculating activity of purified REG-6.

Dosage (mg/mL)	FA (%)	pH	FA (%)	Cations	FA (%)	Temp (^{o}C)	FA (%)
0.05	48.23	3	80	Li^{+}	-	50	81.79
0.1	64.03	4	72.64	K^{+}	-	60	81.87
0.2	54.29	5	71.41	Na^{+}	-	70	82.74
0.3	55.37	6	72.05	Mg^{2+}	86.82	80	81.94
0.4	46.79	7	68.61	Ca^{2+}	84.96	-	-
0.5	45.51	8	72.86	Mn^{2+}	89.51	-	-
-	-	9	75.29	Fe^{3+}	-	-	-
-	-	10	83.84	Al^{3+}	-	-	-
-	-	11	79.80	-	-	-	-

*FA-Flocculating activity

On the other hand, a higher dosage means the addition of the negatively charged REG-6 will increase the electrostatic repulsion forces between the kaolin clay particles; and consequently

increase the distance between the kaolin clay particles which in turn inhibits floc formation and precipitation (Luo *et al.*, 2014). The relationship between bioflocculant dosage and flocculating activity of the purified bioflocculant was similar to that of the bioflocculants produced by other pure bacterial strains (Yim *et al.*, 2007; Liu *et al.*, 2010; Elkady *et al.*, 2011).

3.2.5.2. Effect of pH on the flocculating activity of REG-6

The pH of reaction mixtures is a key factor influencing the flocculation activity of bioflocculants (Zaki *et al.*, 2013). The effect of pH of the reaction mixture on the flocculating activity was investigated (Table 3.2). It was observed that REG-6 was tolerant to extreme pH values and showed excellent flocculating activity either under strongly acidic conditions below pH 5 or strongly alkaline conditions above pH 9. This may be due to the fact that REG-6 has different electric states at different pH values and this affects its flocculating efficiency for kaolin clay suspensions (Pan *et al.*, 2009). It was observed that the flocculating activity of REG-6 was highest under alkaline conditions (pH 10). Over 70% flocculating activity of the bioflocculant was observed at both strong acidic and alkaline conditions. In agreement with our findings, the bioflocculant produced by *Chlamydomonas reinhardtii* had its highest flocculating activity for kaolin clay suspensions at pH 10 (Zhu *et al.*, 2012). Similarly, the bioflocculant MBF-5 showed an excellent flocculating activity (over 90%) under both acidic and alkaline conditions. The purified bioflocculant 40B produced by *Bacillus velezensis* 40B had its optimal flocculating activity under acidic conditions, with the peak flocculation occurring at pH 7 (Zaki *et al.*, 2013).

3.2.5.3. Effect of cations on the flocculating activity of REG-6

It is well-established that cations are necessary to induce effective flocculation by increasing the initial adsorption of the bioflocculant on the kaolin clay suspension (Yim *et al.*, 2007). The synergistic effects of cations on the flocculating activity of REG-6 are different from those of most other bioflocculants documented in the literature. In the present study, a synergistic effect

of cations was only observed with the divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+},with the maximum flocculating activity of 89.51% being observed with Mn^{2+}(Table 3.2). These cations stimulated flocculation by accelerating bridge formation between the suspended particles and REG-6. The flocculating activity of REG-6 was completely inhibited by both the monovalent and trivalent cations tested.

On the contrary, the flocculating activity of the bioflocculant produced by *Aeromonas* sp. was greatly improved in the presence of K^{+}, Na^{+} and Ca^{2+} (Li *et al.*, 2007). In case of the bioflocculant produced by *Serratia ficaria*, the flocculating activity was greatly enhanced in the presence of Ca^{2+} and Mg^{2+}, whereas Fe^{3+} and Al^{3+} inhibited the flocculating activity of the bioflocculant (Gong *et al.*, 2008). Bioflocculants reported by some researchers in previous studies are cation-independent. For example, the bioflocculants produced by *Klebsiella pneumoniae* and *Aspergillus flavus* showed outstanding flocculating activities for kaolin suspension in the absence of cations (Zhoa *et al.*, 2013; Aljuboori *et al.*, 2013).

3.2.5.4. Thermal stability of REG-6

The thermal stability of the bioflocculant was tested at different temperatures for 60 min. It was observed that the bioflocculant was heat stable as it retained and maintained a high flocculating activity of 81.94% at 80 °C (Table 3.2). The heat stability of REG-6 is consistent with the general understanding that bioflocculants rich in polysaccharides are more resistant to heat than those that are mainly composed of proteins or have a lesser polysaccharide content (Li *et al.*, 2008). Similar findings were documented by Liu *et al.* (2010) and Gong *et al.* (2008) for the bioflocculants produced by *Corynebacterium daeguense* and *Serratia ficaria* respectively.

3.2.6. REG-6 yield and chemical analysis

About 3.2 g of purified REG-6 was obtained from 1 L of fermentation broth of *Bacillus toyonensis* strain AEMREG6 and this was higher than the bioflocculant yields reported

elsewhere (Li *et al.*, 2007; Li *et al.*, 2008; Gong *et al.*, 2008; Cosa *et al.*, 2011). The chemical composition analysis revealed that purified REG-6 was a glycoprotein composed of polysaccharide (77.8%) and protein (11.5%). This shows that REG-6 has a polysaccharide as the main backbone in its molecular chain and this account for its high thermal stability. These results concur with previous studies on the bioflocculants produced by *Solibacillus silvestris*, *Klebsiella pneumoniae* and *Bacillus agaradhaeren* C9 which are also thermally stable (Wan *et al.*, 2013; Zhao *et al.*, 2013; Liu *et al.*, 2015).

3.2.7. Functional group analysis by FTIR

The analysis of the functional groups of purified REG-6 produced by *Bacillus toyonensis* strain AEMREG6 showed a broad stretching peak at 3474 cm^{-1} (Figure 3.7), which is a common characteristic of hydroxyl and amino groups (Li *et al.*, 2009). The spectrum also displays an asymmetrical stretching band at 1643 cm^{-1} which is consistent with the presence of carboxylates. Furthermore, the peak at 1465 cm^{-1} indicated the presence of carboxylic acid groups, and polysaccharide C-O and C-O-C groups. The strong absorption peak present in the range from 1000–1200 cm^{-1} is characteristic of all sugar moieties (Li *et al.*, 2008). The small absorption band at 878 cm^{-1} could be associated with β-glycosidic linkages between the sugar monomers (Gomaa, 2012). The peaks at 653 and 527 cm^{-1} are the absorption peaks for the aromatic CH bending vibration (Zhang *et al.*, 2013). The FTIR analysis thus revealed the presence of carboxyl, hydroxyl and amide functional groups which might be responsible for the flocculation in polyelectrolytes (Luo *et al.*, 2014). The infrared spectra of REG-6 produced by *Bacillus toyonensis* strain AEMREG6 were similar to those of most bioflocculants produced by many microorganisms in previous studies (Gomaa, 2012; Kavita *et al.*, 2013; Nwodo *et al.*, 2012; Zhang *et al.*, 2013).

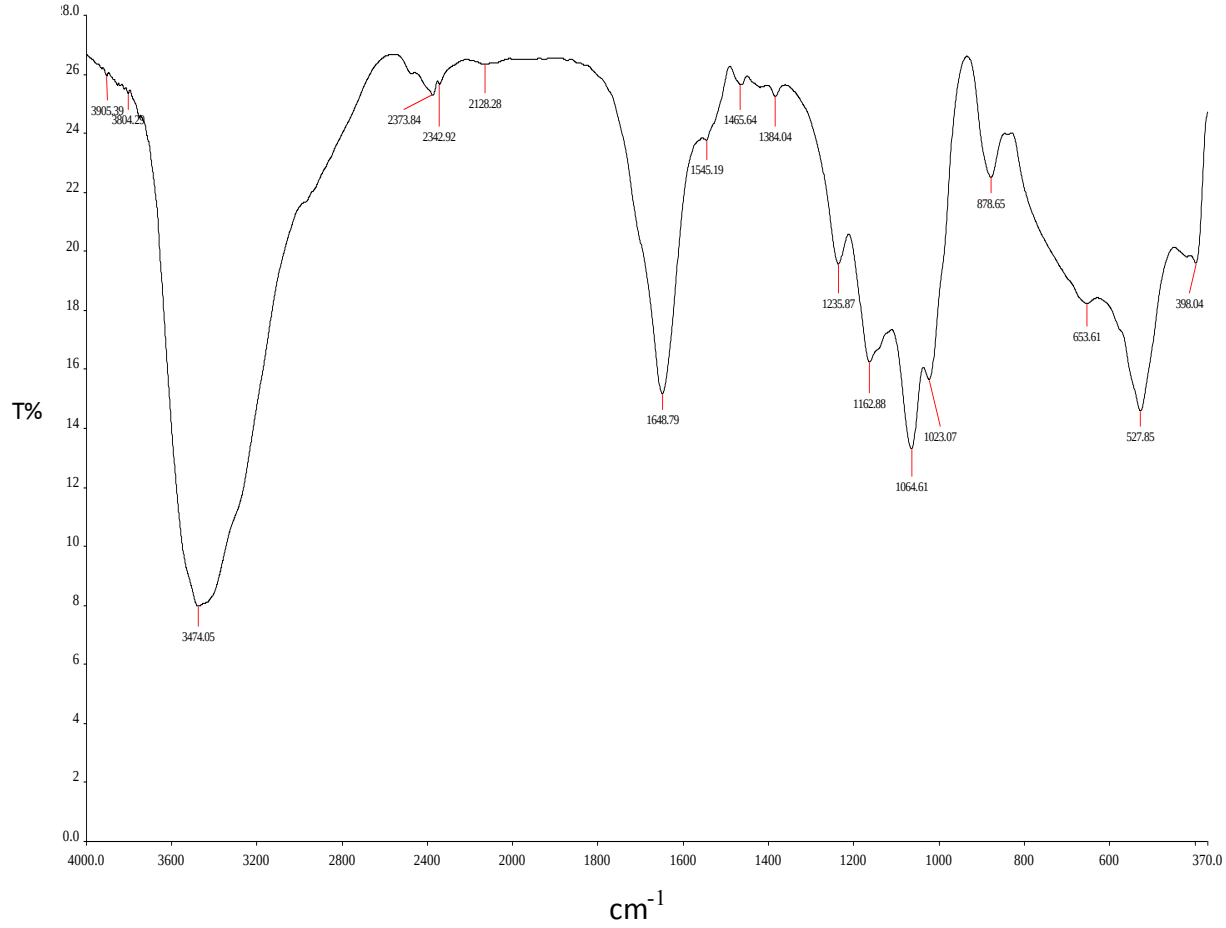

Figure 3.7. Functional groups identification by FTIR of REG-6 produced by *Bacillus toyonensis* strain AEMREG6.

3.2.8. Scanning electron microscopy (SEM) images

The surface morphology of purified REG-6 and its flocculation to kaolin clay suspension were examined by SEM. Figure 3.8A shows the compact nature of the REG-6 structure and Figure 8B shows the fine and scattered kaolin clay particles before flocculation. It was also observed that the sizes of the kaolin particles are uniform. Figure 3.8C can easily be compared with Figures 3.8A and 3.8B in terms of structure and sizes. It was observed that REG-6 flocculated the kaolin clay particles by connecting the scattered kaolin particles firmly together to form bigger flocs which easily precipitated as a result of gravity. These observations were consistent with the findings of Zhang *et al*. (2010) and Mabrouk (2014) on the bioflocculants produced by *Proteus mirabilis* and *Nocardiopsis aegyptia* sp. nov. respectively.

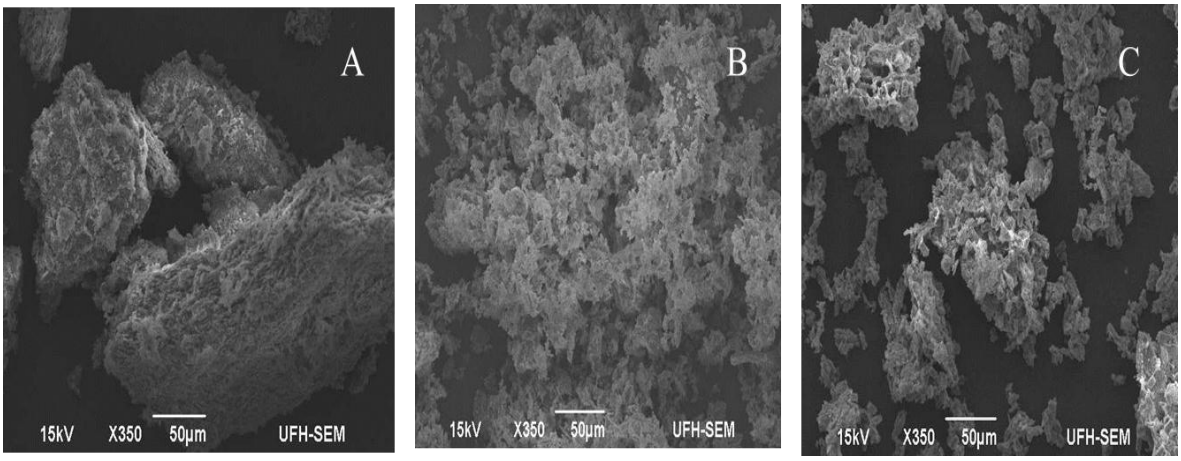

Figure 3.8. SEM images of REG-6 (**A**); kaolin clay particles (**B**) and kaolin clay suspension flocculated with REG-6 (**C**).

3.3 Experimental section

3.3.1 Sample collection and isolation of bioflocculant-producing bacteria

A sediment sample from Algoa Bay (a marine environment) in the Eastern Cape Province of South Africa was collected and processed according to Jensen *et al.* (2005) with some modifications. A wet sample (0.5 g) was diluted with sterile seawater (5 mL). The suspension was stirred and allowed to sediment for 60 s, out of which 100 µL of the suspension was inoculated onto the surface of R2A agar plates, spread with a sterile glass rod and incubated for 96 h at 30 ^{o}C. The distinct isolates were picked and streaked onto nutrient agar plates to ascertain their purity and separate from mixed populations.

3.3.2 Media and cultivation conditions

The composition of activation medium included (per litre): Beef extract 3 g, tryptone 10 g, NaCl 5 g (Ntsaluba *et al.*, 2013). Screening medium contained (per litre): glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ 0.3 g, NaCl 0.1 g (Zhang *et al.*, 2007). The medium for a slant included (per litre): glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.1 g and agar 20 g (Li *et al.*, 2008). Production medium composition was (per litre):

glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, NH_4NO_3 1.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.1 g. All media were prepared using filtered seawater and sterilized by autoclaving at 121 °C for 15 min. In addition, all experiments were performed using a rotary shaker at 160 rpm, 28 °C.

3.3.3 Screening of bioflocculant-producing bacteria

About 48 bacterial isolates were obtained from the Algoa Bay sediment sample and screened for bioflocculant production as follows. Two loopfulls of bacterial isolates from a nutrient agar plate were inoculated into activation medium and incubated for 24 h. One millilitre of the seed culture was inoculated into a 250 mL flask containing 50 mL of production medium and incubated at 28 °C in a rotary shaker at 160 rpm for 96 h. Two millilitres of the fermented broth were carefully withdrawn and centrifuged at 4000 rpm for 30 min, and the cell-free supernatant was next used to determine the flocculating activity according to the description of Kurane *et al.* (1994). The isolate with the highest flocculating activity (REG-6) was preserved in 20% glycerol stock and stored at -80 °C for future studies.

3.3.4 Determination of flocculating activity of REG-6

The flocculating activity of REG-6 was determined according to the description of Kurane *et al.* (1994). Kaolin clay was used as a test material in preparing a kaolin clay suspension which is a simulation of surface water turbidity. One hundred millilitres of the kaolin suspension (4 g/L) were measured into a 250 mL conical flask and 3 mL of $CaCl_2$ (1% w/v) were added, followed by 2 mL of cell-free supernatant obtained by centrifuging a fermented culture after 96 h of cultivation. The solution was agitated for 60 s, transferred into a graduated measuring cylinder and allowed to sediment for 5 min. A control was prepared in a similar way, but the bioflocculant was replaced with un-inoculated production medium. The flocculating activity was calculated using the formula:

$$Flocculating\ activity\ (\%) = [A - B/A] \times 100$$

where A = optical density of the control at 550 nm and B = optical density of a sample at 550 nm.

3.3.5 Identification of the bioflocculant-producing bacteria

The pure culture of the isolate was streaked on nutrient agar and incubated for 24 h. The purity of the isolate was ascertained and the isolate was then identified by 16S ribosomal deoxyribonucleic acid (rDNA) sequence analysis. DNA extraction was conducted using the boiling method described by Cosa *et al.* (2011) whereby two to three colonies were suspended in 70 µL of sterile double distilled water. The samples were heated in a water bath at 100 °C for 10 min, cooled for 5 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4 °C. This serves as the template in the polymerase chain reaction (PCR) assay. PCR of the 16S rDNA was conducted according to the description of Zheng *et al.* (2008) with some modifications using the universal primers (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGA CTT-39) and 2 µL template DNA. Gel electrophoresis of PCR products was conducted on 1% agarose gels to confirm that a fragment of the correct size had been amplified. The PCR product was sequenced at University of KwaZulu-Natal Province (Durban, South Africa) and the results obtained were aligned with published 16S rDNA sequences in the GenBank through a BLAST sequence tool from the National Centre for Biotechnology Information (NCBI) database (Bethesda, MD, USA).

3.3.6. Optimization of culture conditions for REG-6 production

3.3.6.1 Effect of carbon and nitrogen nources on REG-6 production

The experiment to investigate the effect of carbon source on REG-6 production was done according to the description of Luo *et al.* (2014), where glucose in the screening medium was replaced with 20 g/L of each of the following carbon sources: fructose, sucrose, maltose, Na_2CO_3 and phthalate. Similarly, the mixed nitrogen source [urea + yeast extract + $(NH_4)_2SO_4$]

in the screening medium was also replaced with 1.3 g/L of one of the following nitrogen sources: peptone, tryptone, urea, yeast extract, $(NH_4)_2SO_4$, NH_4NO_3, and $NaNO_3$ in order to examine the effect of nitrogen source on REG-6 production (Abdel-Aziz *et al.*, 2012).

3.3.6.2 Effect of Initial pH of Growth Medium on REG-6 Production

To evaluate the effect of initial pH of growth medium on REG-6 production, the pH of the media were adjusted to 3, 4, 5, 6, 7, 8, 9, 10 and 11 with 0.1 M HCl and NaOH accordingly. The seed culture (24 h old) was inoculated into production media at different pH values and incubated in a rotary shaker at 28 °C for 96 h at 160 rpm (He *et al.*, 2012).

3.3.6.3 Effect of inoculum size on REG-6 production

To examine the effect of inoculum size on bioflocculant production, the seed culture (24 h old) was standardized to 0.1 at an optical density 660 nm, and then different inoculum sizes ranging from 1% to 5% were inoculated into different flasks containing the production medium and incubated for 96 h (Zhang *et al.*, 2007).

3.3.7 Optimization of flocculating conditions of crude REG-6

3.3.7.1 Effect of cations on flocculating activity of crude REG-6

To assess the synergistic effect of various cations on the flocculating activity of REG-6, the $CaCl_2$ in the flocculation assay described in Section 3.3.4 was replaced with the metals of the following salts: KCl, NaCl, $MgCl_2$, $MnCl_2$, $FeCl_3$ and $AlCl_3$ (Zaki *et al.*, 2013).

3.3.7.2 Effect of pH on the flocculating activity of crude REG-6

To investigate the effect of pH on the flocculating activity of crude REG-6, 100 mL of kaolin clay suspension was measured into a 250 mL conical flask and the pH was adjusted from 3-11 with 0.1 M HCl and NaOH (Xia *et al.*, 2008). The flocculating activity of REG-6 at different pH values was determined by the flocculation assay described above.

3.3.8 Time course of REG-6 production by Bacillus toyonensis strain AEMREG6

The time course of REG-6 production was conducted according to the description of Yang *et al.* (2012) with some modifications. The seed culture (24 h old) was standardized to OD_{660} 0.1, then 4% v/v of standardized culture was inoculated into 1 L of production medium and incubated in a rotary shaker at 28 °C, 160 rpm for 96 h. The fermentation broth was withdrawn periodically and monitored at 24 h intervals, while the bacterial counts were determined by the culture technique to determine the colony forming units. Likewise, the optical density of the fermentation medium was measured spectrophotometrically (Helios Epsilon, Madison, WI, USA) at 660 nm wavelength and pH of the fermented broth was measured with pH meter.

3.3.9 Extraction and purification of REG-6

The extraction and purification of REG-6 was done according to the Wong *et al.* (2012). After 96 h of fermentation, the viscous culture broth was diluted with two volumes of distilled water and centrifuged at 4000 rpm for 30 min at 15 °C in order to remove the bacterial cells. Two volumes of ethanol were added to the supernatant to precipitate REG-6. The resulting precipitate was collected by centrifugation at 4000 rpm for 30 min, dissolved in water and lyophilised to obtain the crude REG-6. The bioflocculant was dissolved in 100 mL of distilled water to which one volume of a mixture of chloroform and *n*-butyl alcohol (5:2 v/v) was added, stirred for 60 sec and kept overnight at room temperature. The upper phase was collected by centrifugation at 4,000 rpm for 30 min at 15 °C, re-dissolved in 50 mL of distilled water and lyophilised.

3.3.10. Characterization of the purified REG-6

3.3.10.1 Chemical composition analysis of REG-6

The total sugar content of REG-6 was determined according to phenol-sulphuric acid method described by Chaplin and Kennedy (Chaplin and Kennedy, 1994) using glucose as the

standard. The total protein contain was determined by the Bradford method described by Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

3.3.10.2 Effect of REG-6 dosage on flocculating activity

To find a suitable dosage for the flocculating activity of purified REG-6, different dosages ranging from 0.1 to 0.5 mg/mL were prepared in distilled water and their flocculating activities were examined subsequently.

3.3.10.3 Effect of pH on the flocculating activity of REG-6

The experiment concerning the effect of pH on the flocculating activity of purified REG-6 was carried out by adjusting the pH of the kaolin clay suspension from pH 3–11 with 0.1 M HCl and NaOH as needed (Liu *et al.*, 2010). The purpose of using a wide range of pH values is to determine the optimal pH range at which the flocculating activity of REG-6 will be optimum.

3.3.10.4 Effect of cations on the flocculating activity of REG-6

The synergistic effect of various cations on the flocculating activity of REG-6 was examined by replacing $CaCl_2$ in the flocculation assay with metal of the following salts; KCl, NaCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$ and $AlCl_3$ (Zhao *et al.*, 2013). The pH of the kaolin clay suspension was adjusted to pH 10 prior to flocculation assay.

3.3.10.5 Thermal stability of REG-6

The thermal stability was investigated by preparing REG-6 solution in distilled water at 0.1 mg/mL. The solution was divided into four groups and treated at different temperatures ranging from 50 to 80 °C (Gomaa, 2012).

3.3.11 FTIR analysis of REG-6

The functional groups in the molecular chain of REG-6 were identified using a Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, Buckinghamshire, UK).

REG-6 was ground with KBr salt at 25 °C and pressed into a pellet for FTIR spectroscopy analysis over a wavenumber range of 4000–370 cm^{-1} (Adebayo-Tayo and Adebami, 2014).

3.3.12. Scanning electron microscopy (SEM) images

The surface morphology of REG-6 and kaolin clay particles was observed and elucidated using scanning electron microscopy (SEM) (JSM-6390LV, JEOL, Tokyo, Japan). Five milligrams of REG-6, kaolin clay and dried flocculated kaolin suspension were added on slide separately and fixed by air-drying. The fixed specimens were coated with gold and examined under SEM (Wan *et al*., 2014).

3.3.13 Statistical analysis

All data were obtained in triplicate experimentation and subjected to one-way analysis of variance (ANOVA) using the MINITAB Student Release 12 statistical package. A significance level of $p < 0.05$ was used.

3.4 Conclusions

Owing to the stupendous advantages such as innocuousness and biodegradability of bioflocculants over chemical flocculants, the study of microbial flocculants has attracted wide attention in the water treatment field. In this present study, the bioflocculant REG-6 was optimally produced when glucose, NH_4NO_3, calcium chloride and pH 5 as favourable carbon, nitrogen source, cation of choice and initial growth medium pH were used, respectively. The high flocculating efficiency achieved at low dosage over a wide pH range and its thermal stability properties show that REG-6 has enormous potential for becoming a new member of the bioflocculants isolated and studied by our research group so far from Algoa Bay in the Eastern Cape Province of South Africa. Consequently, it is anticipated that the REG-6 produced by *Bacillus toyonensis* strain AEMREG6 could be a good substitute for the hazardous chemical flocculants currently used in drinking/wastewater treatment.

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CHAPTER FOUR

Evaluation of flocculating performance of a thermostable bioflocculant produced by a marine *Bacillus* sp. and its potential for river water treatment

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Abstract

This study assessed the bioflocculant (named MBF-W7) production potential of a bacterial isolate M69 obtained from Algoa Bay, Eastern Cape Province of South Africa. The 16S ribosomal deoxyribonucleic acids (rDNA) gene sequence analysis showed 98% sequence similarity to *Bacillus licheniformis* strain W7. Optimum culture conditions for MBF-W7 production includes 5% (v/v) inoculum size, maltose and NH_4NO_3 as carbon and nitrogen sources of choice in medium pH of 6 as the initial pH of the growth medium. Under these conditions, maximum flocculating activity of 94.9% was attained after 72 h of cultivation. Chemical composition analyses showed that the purified MBF-W7 was a glycoprotein composed predominantly of polysaccharides 73.7% (w/w) and protein 6.2% (w/w). Fourier transform infrared spectroscopy (FTIR) revealed the presence of hydroxyl, carboxyl and amino groups as the main functional groups identified in the bioflocculant molecules. Thermogravimetric analyses (TGA) showed the thermal decomposition profile of the MBF-W7. Scanning electron microscopy (SEM) imaging revealed that bridging played important role in flocculation. MBF-W7 exhibited excellent flocculating activity for kaolin clay suspension at 0.2 mg/ml over a wide pH range 3-11; with the maximal flocculation rate of 85.8% observed at pH 3 in the presence of Mn^{2+}. It maintained and retained high flocculating activity over 70% after heating at 100 ^{o}C for 60 min. MBF-W7 showed good turbidity removal potential (86.9%) and chemical oxygen demand (COD) reduction efficiency (75.3%) in Thyume River. The high flocculating rate of MBF-W7 makes it an attractive candidate to replace chemical flocculants utilized in water treatment.

Keywords: Marine environment; *Bacillus* sp.; MBF-W7; flocculating activity; Thyume River.

4.1 Introduction

Chemical flocculants are widely employed in wastewater and drinking water treatment, food and fermentation industries and also for downstream processing due to their high flocculating efficiency and cost-effectiveness (Salehizadeh and Shojaosadati, 2001; You *et al.*, 2008). Nevertheless, their extensive usages have raised serious environmental and health concerns (Mabinya *et al.*, 2012). The utilization of aluminium as the coagulant in water treatment may lead to a higher level of aluminum in the treated effluent than in raw water. In addition, residual aluminum in excessive sludge produced during coagulation tends to accumulate in the environment (Ma *et al.*, 2008). For example, several studies have shown that aluminium salts are associated with Alzheimer's disease (Arezoo, 2002; Banks *et al.*, 2006). Recently, the European Commission regulated that the maximum contaminant level of aluminum in drinking water cannot exceed 200 µg/L. Consequently, in order to reduce the genetic risks posed by the use of aluminum coagulants, more environment-friendly and safe coagulants for pollutant removal in water treatment remain necessary to be developed (Ma *et al.*, 2008). Furthermore, although polyacrylamide has high flocculating efficiency, the acrylamide monomer residues are neurotoxic and carcinogenic to humans (Polizzi *et al.*, 2002; Ruden, 2004). Besides, acrylamides are not biodegradable and hence, they constitute environmental nuisance (Lofrano *et al.*, 2013). These inevitable drawbacks associated with chemical flocculants necessitate seeking for an alternative flocculants that are eco-friendly and safe (Nwodo and Okoh, 2013).

Conversely, bioflocculants have gained globally attention in biotechnology because they hold an immense potential in replacing chemical flocculants (Gao *et al.*, 2009). Compared with chemical flocculants, bioflocculants have nonpareil advantages such as environmentally friendly due to their biodegradability, free of secondary pollution and harmless to both human and animals (Yang *et al.*, 2012). They are natural product metabolites produced during growth of microorganisms such as bacteria, yeast, and fungi which are composed of polysaccharides,

proteins, lipids, glycoproteins and glycolipids (Liu *et al.*, 2009; More *et al.*, 2014; Okaiyeto *et al.*, 2015). However, low flocculating activity and high production costs are the major hindrances for their large-scale production and industrial applications (Wang *et al.*, 2007; Yang *et al.*, 2007). Consequently, to exploit utilization of bioflocculants extensively in industrial fields, continual exploration for microbes with high bioflocculant yield and improved flocculating efficiency has, therefore, become a subject of intensive investigations globally (Ugbenyen *et al.*, 2012).

Certainly, novel efficient bioflocculants from microorganisms inhabiting unusual environments such as the marine environments are beginning to be of prodigious interest (Mabrouk, 2014). Marine microorganisms have become recognized as an important and untapped resource for secondary metabolites (Fang *et al.*, 2013). Marine microorganisms developed unique metabolic patterns, which led them to produce different kind of metabolites including bioflocculants with distinct features which are different from those produced by terrestrial microorganisms (Zhang *et al.*, 2002).

In this study, we reported on bioflocculant production potential of *Bacillus* sp. isolated from a marine environment in South Africa, the bioflocculant was purified and characterized. In addition, the physicochemical properties of the purified bioflocculant were determined afterwards and it's potentials for Thyume River treatment was evaluated.

4.2 Materials and methods

4.2.1 Sample collection and isolation of bioflocculant-producing bacteria

The sediment was collected and processed according to Jensen *et al.* (2005) with some modifications. Wet sample (0.5 g) of was diluted with 5 mL of sterile seawater. The suspension was agitated and allowed to sediment for 60 s, out of which 100 µL of the upper phase was inoculated onto the surface of R2A agar plates, spread with a sterile glass rod and incubated for

96 h at 28 ^{o}C. The distinct isolates were selectively picked and streaked onto nutrient agar plates to purify them.

4.2.2 Media and cultivation conditions

The composition of activation medium included the following (per liter): Beef extract 3 g, tryptone 10 g, NaCl 5 g (Ntsaluba *et al.*, 2013). Production medium composed of (per litre): glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.1 g (Zhang *et al.*, 2007). The medium for a slant included (per liter): Glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, NaCl 0.1 g and agar 20 g (Gong *et al.*, 2008). All media were prepared using filtered seawater and sterilized by autoclaving at 121 ^{o}C for 15 min. For all experiments, the cultivations were done at 28 ^{o}C and 160 rpm.

4.2.3 Screening of bioflocculant-producing bacteria

About 48 bacterial isolates were obtained from Algoa Bay (a marine environment) in the Eastern Cape and screened for bioflocculant production. Two loops of bacterial colonies were inoculated into 10 ml of the activation medium and incubated in a rotary shaker at 160 rpm for 48 h at 28 ^{o}C. The seed culture was inoculated into production medium incubated in a rotary shaker at 160 rpm, 28 ^{o}C for 72 h. Two millilitres of the fermented broth were carefully withdrawn and centrifuged at 4 000 rpm for 30 min and the cell-free supernatant (MBF-W7) was used to determine the flocculating activity.

4.2.4 Determination of flocculating activity of MBF-W7

The flocculating activity of MBF-W7 was determined according to the method described by Kurane *et al.* (1994). Kaolin clay was used as a test material in preparing the suspension at a concentration of 4 g/L in distilled water. In brief, 3 mL of 1% $CaCl_2$ and 2 mL of cell free supernatant were added to 100 mL kaolin clay suspension contained in 250 mL flask. The

mixture was shaken vigorously for 60 sec and transferred into a graduated measuring cylinder and allowed to stand for 5 min. A control was prepared in a similar way, but MBF-W7 was replaced with un-inoculated culture medium. The flocculating activity was calculated using the formula below:

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

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

4.2.5 Identification of the MBF-W7-producing bacteria

The pure culture of the isolate was streaked on nutrient agar and incubated for 24 h at 30 ^{o}C. The purity of the isolate was ascertained and the isolate was then identified by 16S ribosomal deoxyribonucleic acids (rDNA) sequence analysis. DNA extraction was conducted using the boiling method described by Ugbenyen *et al*. (2012), whereby two to three colonies were suspended in 70 µL in sterile double distilled water. The samples were heated in a water bath at 100 °C for 10 min, cooled for 5 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4 °C. This serves as the template in the Polymerase chain reaction (PCR) assay. PCR of the 16S rDNA was conducted according to the description of Mabinya *et al*. (2012) with some modifications using the universal primers (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 µL template DNA. Gel electrophoresis of PCR products were conducted on 1% agarose gels to confirm that a fragment of the correct size had been amplified. The PCR product was sequenced at University of KwaZulu-Natal Durban (South Africa) and the results obtained were aligned with published 16S rDNA sequences in the GenBank through a BLAST sequence tool from the National Centre for Biotechnology Information (NCBI) Database.

4.2.6 Optimization of culture conditions for MBF-W7 production

Different inoculum sizes ranging from (1-5% v/v) of the seed culture were used to inoculate the production medium and the effect of each on MBF-W7 production was assessed. The effects of medium compositions and fermentation conditions on MBF-W7 production by *Bacillus* sp. were also investigated. Different carbon sources used include: glucose, fructose, starch, sucrose, maltose and lactose (Zheng *et al*., 2008). The effect of both inorganic and organic nitrogen sources was examined on MBF-W7 production. The nitrogen sources included: yeast extracts, urea, $(NH_4)_2SO_4$, NH_4NO_3, peptone and tryptone (Li *et al*., 2009). The effect of metal on MBF-W7 production was also assessed and the metal ions used were the following: K^+, Li^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}. The effect of initial pH of the production medium was also examined. The pH of the medium was adjusted with HCI and NaOH with a range of 3-11 (Xiong *et al*., 2010). The thermal stability of crude MBF-W7 was also investigated according to Gong *et al*. (2008) with some modifications. Five day old cultures was centrifuged at 4 000 rpm for 30 min to obtain cell-free supernatant. Two millilitres of cell-free supernatant was heated in a water bath at different temperatures range from 50-100 ^oC for 1 h. The flocculating activities of residual MBF-W7 were determined.

4.2.7 Time course for MBF-W7 production

Optimum medium compositions were used to produce MBF-W7 in accordance with the description of Okaiyeto *et al*. (2013). The seed culture was prepared by inoculating two loops of the bacterial colonies into 10 ml of activation medium and incubated overnight in a rotary shaker at 28 ^oC. The fermented broth was adjusted with sterile saline water to optical density of 0.1 at OD_{660} nm (Cosa *et al*., 2012). The standardized bacterial suspension was inoculated into 200 mL of the production medium in 500 ml flasks and incubated on a rotary shaker at 160 rpm, 28 ^oC. Ten millilitres of the samples were withdrawn periodically at intervals of 24 h over 168 h of cutivation. Two milliliters of the fermented broth were used to determine the

flocculating activity in accordance with the method of Kurane *et al*. (1994). The growth of the bacteria was monitored by measuring the optical density at OD_{660} and the bacterial counts were determined by a standard spread plate technique (Okaiyeto *et al*., 2013). The pH of the culture broth was measured by a pH meter.

4.2.8 Extraction and purification of MBF-W7

The extraction and purification of MBF-W7 were done in accordance with Gao *et al*. (2006) and Ugbenyen *et al*. (2012) with some modifications. The optimal culture conditions were used for MBF-W7 production over a growth period of 72 h, after which the fermented broth was centrifuged at 4000 rpm for 30 min. One volume of sterile distilled water was added to the supernatant and centrifuged at 4 000 rpm for 30 min. Two volumes of ethanol were added to the supernatant and the mixture allowed to stand at 4 ^{o}C overnight. The precipitate was collected by centrifugation and vacuum dried. The crude extract was weighed and dissolved in 100 mL of distilled water and one volume of a mixed solution of chloroform and n-butylalcohol (5:2, v/v) was added and the mixture agitated for 60 sec. The solution was left standing for 12 h at room temperature and later dialyzed against distilled water overnight. Two volumes of ethanol were added to the dialyzed solution (100 mL) and the precipitate (MBF-W7) recovered was dissolved in 50 mL of distilled water and vacuum dried.

4.2.9 Characterization of the purified MBF-W7

The protein content of the purified MBF-W7 was determined by the Bradford method with bovine serum albumin (BSA) used as the standard (Bradford, 1976). The total sugar content was determined by phenol-sulphuric acid method with glucose as the standard solution (Chaplin and Kennedy, 1994). FTIR analysis of purified MBF-W7 was done using Fourier transforms infrared spectrophotometer (Perkin Elmer System 2000, England). Ten milligram of the dried MBF-W7 were ground with potassium bromide (KBr) and pressed into pellets for FTIR spectral measurement in the frequency range of 4000-370 cm^{-1}. The pyrolysis pattern of

the MBF-W7 was monitored with Thermogravimetric analyzer (STA 449/C Jupiter Netz, Germany; Perkin Elmer TGA7 Thermogravimetric Analyzer, USA). The surface morphology structure of MBF-W7 before and after flocculation with kaolin clay was elucidated using Scanning electron microscopy (JEOL-JSM-6390LV, Japan). Different MBF-W7 dosages ranging from 0.1-0.5 mg/mL were used to determine the optimum dose for effective flocculation (Okaiyeto *et al.*, 2015; Yim *et al.*, 2007). To investigate the synergetic effect of cations on the flocculating activity of MBF-W7, the $CaCl_2$ solution that was previously used in flocculation assay was replaced with various salt solutions which included, NaCl, LiCl, KCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $AlCl_3$ and $FeCl_3$ (Zhao *et al.*, 2013). To examine the effect of pH on flocculating activity of purified MBF-W7, the pH of the kaolin suspensions was adjusted to pH values ranging from 3-11 using HCl and NaOH (Wang *et al.*, 2013). The effect of temperature on the flocculating activity was assessed by heating MBF-W7 solution at different temperatures from 50-100 ^{o}C (Luo *et al.*, 2014). The flocculating activities of the residual MBF-W7 solutions were determined afterwards.

4.2.10 Application of MBF-W7 in the treatment of Thyume River

The turbidity and COD (chemical oxygen demand) of Thyume River were measured with a 2100P turbidimeter (HACH Company, Germany) and spectro-quant (Pharo 300, Merck KGaA, Germany) respectively before and after treatment with MBF-W7. The Jar test experiment was carried out in accordance with the description of Wang *et al.* (2010). Three millilitres of 1% (w/v) $MnCl_2$ and 2 mL of MBF-W7 solution were both added to 100 mL Thyume River contained in 500 mL beakers, the pH of the mixture was adjusted to 3 (optimum pH for MBF-W7 activity). The mixture was agitated at 200 rpm, at room temperature for 3 min; the speed reduced to 45 rpm and then allowed to agitate for a further 10 min. The turbidity removal and COD reduction efficiencies of MBF-W7 were calculated as follows:

$$Removal\ efficiency\ (\%) = [A_o\text{-}A/A_o] \times 100\%$$

Where A_o and A are the initial and final values obtained before and after treatment with MBF-W7 respectively.

4.2.11 Statistical analysis

All data were treated in replicates and the mean values were taken. Data were subjected to one-way analysis of variance (ANOVA) using MINITAB Student Release 12 statistical package. A significance level of $p<0.05$ was used.

4.3 Results and discussion

4.3.1 Screening of bioflocculant-producing bacteria

In this study, forty-eight promising bacterial isolates obtained from the sediment samples of Algoa Bay were assessed for bioflocculant production. This bacterial isolate designated as M69 with the highest flocculating activity of 72% for kaolin clay suspension was selected for further studies. The morphological characteristics of the bacterial isolate on nutrient agar were cream, rough, tough and dry, with vegetative hyphae that are highly branched like filaments. The BLAST program analysis of the 1198-bp sequence against the GenBank database showed 98% identity at the nucleotide level with 16S rDNA genes from *Bacillus licheniformis* strain W7 with accession no GU945228.1. Based on the morphological characteristics and BLAST program results, the pure isolate was classified as *Bacillus* sp. strain. Despite several studies on microbial flocculants have been documented in literatures, low flocculating activity and high cost of production are the major barriers with regards to their industrial applications (Zhang *et al.*, 2007). Consequently, continual identification of microorganisms with high bioflocculant production potential and improve upon the flocculation efficiency forms the basis of research in the recent decades (Zaki *et al.*, 2011).

4.3.2 Optimization of culture conditions for MBF-W7 production

4.3.2.1 Effect of inoculum size on MBF-W7 production

Inoculums size is a critical factor that influences the growth of microorganisms as well as bioflocculant production (Wang *et al.*, 2011). The effect of inoculum size on MBF-W7 production by *Bacillus* sp. was investigated and the results are depicted in Table 4.1. It was observed that all the inoculum sizes of the seed culture examined were favourable for MBF-W7 production resulting in flocculating activity above 70%. However, the highest MBF-W7 production was observed with 5% inoculum size with flocculating activity of 87%. Similarly, 5% inoculum size greatly enhanced bioflocculant production by *Klebsiella mobilis* (Wang *et al.*, 2007). According to Li *et al.* (2009), optimal inoculum size allows for the adaption of the microorganisms to the production medium and consequently promotes the production of bioflocculant. On the contrary to our findings, Aljuboori *et al.* (2013) and Cosa *et al.* (2013a) reported an optimal bioflocculant production by *Aspergillus flavus* and *Virgibacillus* sp. Rob at 2% (v/v) inoculums size, whereas higher inoculums size of 8% (v/v) was most suitable for bioflocculant production by *Firmicutes* sp (Zhoa *et al.*, 2012).

4.3.2.2 Effect of carbon source on MBF-W7 production

Culture conditions optimization is a powerful approach to increase the production of by-products from microorganism cultivation (Nwodo *et al.*, 2013; Pathak *et al.*, 2014). It has been well enunciated in previous studies that carbon sources have great impact on microorganism's growth and bioflocculant production (Sheng *et al.*, 2006). The effect of different carbon sources on MBF-W7 production by *Bacillus* sp. was examined (Table 4.1). It was observed that all the carbon sources assessed greatly improved MBF-W7 production with resultant flocculating activities above 75%. Disaccharide sugars, maltose and sucrose supported the highest flocculating activities at 95.96% and 95.67% respectively with the lowest flocculating activity of 73.96% among disaccharides recorded for lactose. Starch, a polysaccharide

significantly buttressed MBF-W7 production with the flocculating activity of 81.71% (Table 4.1). Hence, maltose was chosen as a carbon source for the subsequent experiments. Most reported strains prefer organic over inorganic carbon sources for bioflocculant production (Ugbenyen *et al*., 2014). Some other investigators have also found maltose to be favourable for bioflocculant production by *Bacillus thuringiensis* 27, *Funalia trogii* and *Solibacillus silvestris* W01 (Wang *et al*., 2011; He *et al*., 2012; Wan *et al*., 2013). Conversely, *Stemphylium* sp. preferred sucrose as the sole carbon source for bioflocculant production (Banerjee *et al*., 2009), while in the findings of Aljuboori *et al*. (2013) on bioflocculant production by *Aspergillus flavus*, sucrose and starch were most suitable carbon sources. Besides, Zhuang *et al*. (2012) found that molasses was the most appropriate for bioflocculant by *Bacillus licheniformis* among others carbon sources tested.

4.3.2.3 Effect of nitrogen source on MBF-W7 production

Nitrogen source is one of the major nutrients in the medium for both biomass and bioflocculant production (Salehizadeh and Yan, 2014). Different nitrogen sources were appraised on their effect on bioflocculant production (Table 4.1). Among the tested nitrogen sources used to supplement the medium, ammonium nitrate (NH_4NO_3) was the best for MBF-W7 production, followed by ammonium sulphate $(NH_4)_2SO_4$ while other nitrogen sources led to poor MBF-W7 production as shown in Table 4.1. The lowest production was recorded with urea with flocculating activity of 3.71%, although it supported the cell growth. On the other hand, in comparison with organic nitrogen source, it was observed that the inorganic nitrogen sources resulted into higher MBF-W7 production. These findings concur with the reports of Nwodo and Okoh (2012) on the bioflocculant produced by *Cellulomonas* sp. Okoh utilising NH_4NO_3 as the sole nitrogen source with flocculating activity of about 82.74%. Similarly, Li *et al*. (2009) established that the production of bioflocculant by *Bacillus licheniformis* X14 was optimal with inorganic nitrogen source, ammonium chloride as the sole nitrogen source in the

basal medium. Our results were not in agreement with Deng *et al*. (2005), who reported that highest bioflocculant production by *Aspergillus parasiticus* was observed in the medium supplemented with peptone and sodium nitrate, whereas Aljuboori *et al*. (2013) found that peptone and yeast extract were the most preferred nitrogen sources for bioflocculant production by *Aspergillus flavus*.

Table 4.1. Effect of inoculum size, carbon and nitrogen sources on MBF-W7 production.

Inoculum size (% v/v)	**FA(%) ± SD**	**Carbon source**	**FA(%) ± SD**	**Nitrogen source**	**FA(%) ± SD**
1	78.96 ± 4.43	Glucose	80.84 ± 7.14	Tryptone	7.77 ± 3.81
2	82.76 ± 2.71	Fructose	75.43 ± 2.11	Urea	3.71 ± 6.43
3	83.84 ± 5.13	Maltose	95.96 ± 0.85	NH_4NO_3	92.80 ± 1.47
4	77.62 ± 2.45	Lactose	73.97 ±11.28	Yeast extract	11.25 ± 13.08
5	87.01 ± 2.26	Sucrose	95.68 ± 0.27	$(NH4)_2SO_4$	79.87 ± 6.65
6	-	Starch	81.71 ± 14.34	Peptone	31.48 ± 6.04

*The results are represented as mean value of triplicates ± SD, FA – Flocculating activity, SD- standard deviation.

4.3.2.4 Effect of initial medium pH on MBF-W7 production

The effect of initial pH of medium ranging from (pH 4-9) on the production of MBF-W7 by *Bacillus* sp. was examined. From Table 4.2, the flocculating activity of MBF-W7 increased from pH 4-6 with the maximum flocculating activity of 76.6% observed at pH 6. Moreover, increasing the pH beyond 6 resulted in a decrease in the flocculating activity. It was observed that cultivating the microorganism at pH medium below or above 6 led into lower flocculating activity. However, the pH requirement for bioflocculant production by microorganisms differs from each other (Ugbenyen *et al.*, 2014). In addition, according to Salehizadeh and Shojosadati (2001) investigations, the initial pH of the production medium determines the electric charge of the cell, oxidation-reduction potential which in turn influences nutrient absorption and

enzymatic reaction. The results of this present study were in agreement with the observations of Wang *et al.* (2007) in which production of bioflocculant by *Klebsiella mobilis* was recorded over a wide pH range of 3-10 with the highest flocculating activity of 95% observed at pH 6. The bioflocculant produced by *Pseudomonas aeruginosa* strain IASST201 was optimal at pH 6.5 with flocculating activity of 83.4% (Pathak *et al.*, 2014), whereas Ugbenyen *et al.* (2014) found that *Bacillus* sp. Gilbert produced polysaccharide-bioflocculant optimally at neutral pH 7 with flocculating activity of 91.62%. Similarly, the highest production of bioflocculant by *Klebsiella pneumoniae* YZ-6 isolated from human saliva was observed within a pH range of 6-8 with the highest flocculating activity of 80% noticed at pH 7 (Luo *et al.*, 2014). On the other hand, Cosa *et al.* (2013a) reported that pH 10 was most suitable for bioflocculant production by *Virgibacillus* sp. with flocculating activity of 85.8%.

4.3.3 Effect of cations on the flocculating activity of crude MBF-W7

The effect of cations on the flocculating activity of crude MBF-W7 was investigated and the results depicted in Table 4.2. It was observed that only Ca^{2+} (83.63%) and Al^{3+} (75.4%) that reasonably enhanced the flocculating activity of crude MBF-W7 among all the cations tested (Table 4.2). The addition of cations allows effective flocculation as a result of complexes formed between bioflocculant molecules and the kaolin particles (Cosa *et al.*, 2013a). Similar reports have been documented in the previous studies (Gao *et al.*, 2006; Gong *et al.*, 2008; Xiong *et al.*, 2010; Mabinya *et al.*, 2012; Ntsaluba *et al.*, 2013).

4.3.4 Thermal stability of crude MBF-W7

The effect of temperature on the flocculating activity of crude MBF-W7 was investigated (Table 4.2). The flocculating activity of MBF-W7 increased from 50-60 ^{o}C with flocculating activity of 84.19% and 87.46%, respectively, after which a gradual but low decrease was observed with the increase in temperature to 100 ^{o}C. The highest flocculating activity of 87.46% was observed at 60 ^{o}C with MBF-W7 still maintained excellent flocculating activity of

about 80.29% at 100 ^{o}C. This attribute possessed by MBF-W7 affords the possibility of its industrial application under different climatic regions. Similar findings were observed by Gomaa (2012) on the bioflocculant produced by *Pseudomonas aeruginosa* with a residual flocculating activity of 60.16% at 100 ^{o}C thus indicating bioflocculant thermostability. In addition, the thermal behaviour of MBF-W7 is also similar to the thermal property of some bioflocculants underscored by other researchers in previous studies (Li *et al.*, 2009; Abdel-Aziz *et al.*, 2012; Liu *et al.*, 2015).

Table 4.2. Effect of initial pH of production medium on MBF-W7 production, cations effect and temperature on flocculating activity of crude MBF-W7.

pH	FA ± SD	Cations	FA ± SD	Temp (^{o}C)	FA ± SD
4	28.92 ± 11.50	Li^{+}	6.28 ± 2.18	50	84.19 ± 2.50
5	46.10 ± 6.05	Na^{+}	11.04 ± 3.89	60	87.46 ± 0.96
6	76.60 ± 3.34	K^{+}	14.28 ± 0.25	70	85.44 ± 4.84
7	56.08 ± 2.79	Ca^{2+}	83.63 ± 1.53	80	83.69 ± 2.73
8	51.0 ± 5.95	Mg^{2+}	21.85 ± 1.71	90	82.60 ± 0.75
9	6.92 ± 4.66	Mn^{2+}	20.72 ± 3.09	100	80.29 ± 3.76
-	-	Fe^{3+}	-	-	-
-	-	Al^{3+}	75.40 ± 1.09	-	-

*The results are represented as mean value of triplicates ± SD, FA – Flocculating activity, SD- standard deviation.

4.3.5 Time course of MBF-W7 production

Most bioflocculants are usually produced during the exponential growth phase of microorganisms (Shih *et al.*, 2001). As shown in Figure 4.1, the production of MBF-W7 was observed to be almost in parallel with the cell growth (bacterial counts) as the flocculating activity increased with increasing cultivation time; an indication that MBF-W7 production was associated with cell growth (Lu *et al.*, 2005). The flocculating rate of MBF-W7 increased to 78.5% after 48 h of cultivation with the maximum flocculation of 94.9% at pH 6.17 attained after 72 h with a corresponding exponential increase in the bacterial counts. This observation

indicated that the microorganism produced MBF-W7 optimally at the logarithmic phase of growth in the presence of abundant nutrient (Nwodo and Okoh, 2013). Further increase in the cultivation time beyond 72 h drastically decreases the bacterial counts as the nutrients get depleted from the culture, the oxygen level available for the microorganisms become reduced, the toxic waste products of metabolic activity increased (Nie *et al*., 2011). Nonetheless, the optical density of the culture broth increases with cultivation time which might be due to the metabolic wastes and death cells contributing to the increase in turbidity of the fermented broth (Figure 4.1). The flocculating activity of MBF-W7 was 73.1% at pH 6.1 after 216 h and according to Li *et al*. (2009), this decrease in flocculating activity might either be ascribed to cell autolysis or production of MBF-W7-degrading enzymes by the bacteria. Our findings were consistent with the results of Raza *et al*. (2012), who reported that maximum bioflocculant production by *Pseudomonas* sp. was attained after 72 h of fermentation. Likewise, the flocculating activity of a bioflocculant MBF-6 produced by *Klebsiella pneumoniae* YZ-6 was in parallel with cell growth and the highest flocculating activity of 91.5% was attained at the early stationary growth phase at 60 h whereas Yang *et al*. (2012) documented that the bioflocculant produced by *Klebsiella* sp. reached its maximum flocculation rate of 86.5% at 60 h of cultivation.

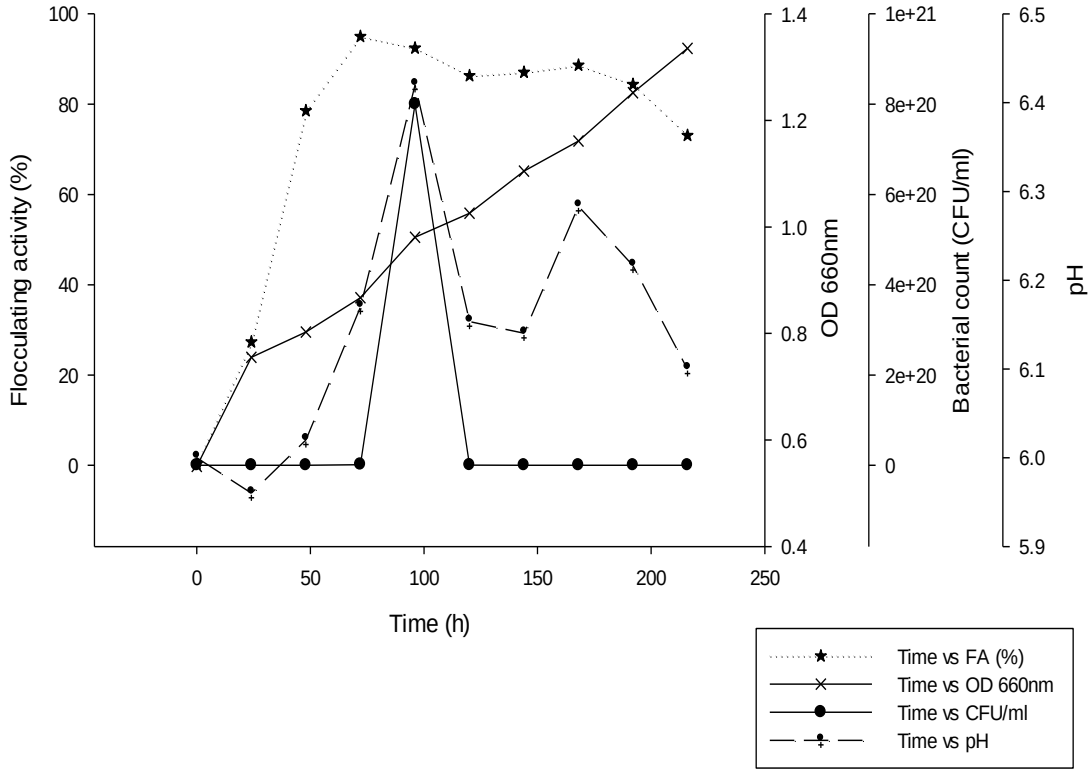

Figure 4.1. Time course for MBF-W7 production by *Bacillus* sp.

4.3.6 Chemical analysis of the purified of MBF-W7

Determination of the active ingredients of bioflocculant is essential to elucidate their flocculation mechanisms, which would be suitable for optimizing the flocculating parameters and consequently improve their efficiency in practical applications (Li *et al.*, 2014). In this study, the chemical analysis of purified MBF-W7 revealed that it was a glycoprotein with a predominant polysaccharide component at 73.7% (w/w) and 6.2% (w/w) protein. The elemental analysis of MBF-W7 revealed the mass ratio of C:N:O:P to be 5.25%, 5.55%, 23.73%, 10.23% (w/w) respectively. Chemical analysis revealed that purified MBF-W7 be a glycoprotein predominantly composed of polysaccharides. Flocculation with high-molecular-weight polysaccharide bioflocculant involves more functional groups which serve as adsorption sites for the suspended particles resulting in strong bridging and higher flocculating efficiency (Kumar *et al.*, 2004). Comparatively, bioflocculants with a predominant protein

component have lower molecular weights with lesser functional groups resulting in lower flocculation rates (Zhang *et al.*, 2007; Xia *et al.*, 2008). Similar trends were also documented on polysaccharide-rich bioflocculants in several studies reported in the literature (Cosa *et al.*, 2011; Zhao *et al.*, 2012; Nwodo *et al.*, 2014).

4.3.7 Fourier transform infrared spectroscopy (FTIR)

To examine the characteristics of purified MBF-W7, the FTIR spectrum was used to analyze the functional groups in the molecular chain of MBF-W7 as shown in Figure 2. The FTIR spectrum displayed a broad stretching peak at 3422 cm^{-1} for hydroxyl and amine groups, a weak stretching band at 2962 cm^{-1} for carbohydrates. An asymmetrical stretching peak was observed at 1646 cm^{-1} for carbonyl group stretching vibration in peptide, a peak at 1463 cm^{-1} might be for carbonyl symmetrical and asymmetrical stretching of a carboxylate group. Furthermore, the absorption peaks observed within 1000-1200 cm^{-1} showed asymmetrical stretching vibration of an ester linkage, a characteristic of all sugar derivatives. Several functional groups such as hydroxyl, carboxyl and amine groups have been identified as the preferred groups that enhanced the flocculation process (Kumar *et al.*, 2004; Kanmani *et al.*, 2011). The obtained results were in accordance with some observations for some bioflocculants documented in literature (Cosa *et al.*, 2013b; Adebayo-Tayo and Adebami, 2014; Peng *et al.*, 2014).

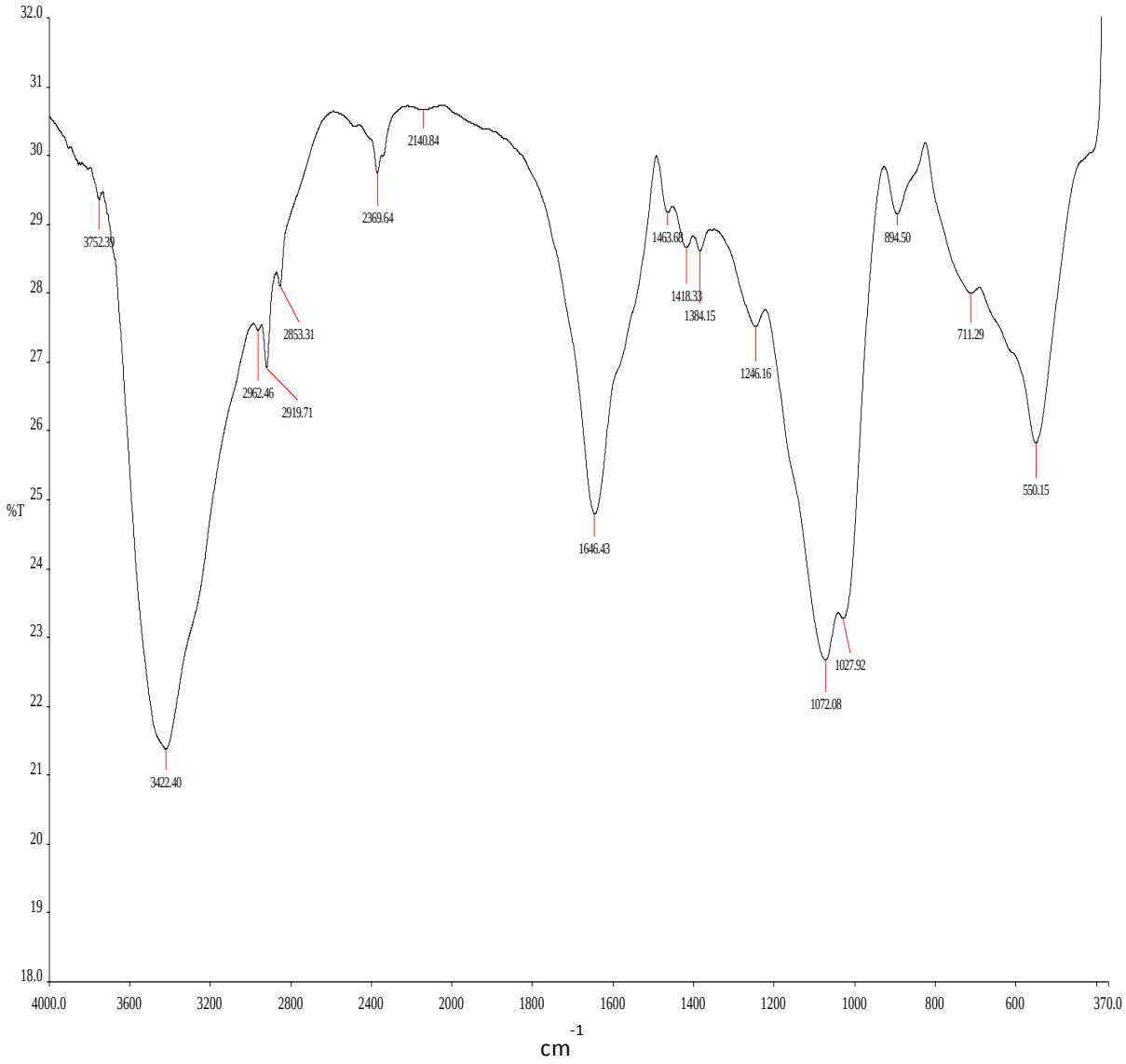

Figure 4.2. FTIR spectra of purified MBF-W7 produced by *Bacillus* sp.

4.3.8 Thermogravimetric analysis of MBF-W7

The pyrolysis profile of purified MBF-W7 was examined with a thermogravimetric analyzer and the results are depicted in Figure 4.3. At a temperature of 23.7 ^{o}C, the initial weight of purified MBF-W7 was 99.9% (w/w). With an increase in temperature from 23.7-100 ^{o}C, about 5% weight loss was observed with the resultant residual weight of 94.86% (w/w). Furthermore, about 16.3% reduction in weight was noticed when the temperature was increased to 150 ^{o}C and this initial weight loss might be due to loss of moisture content (Ugbenyen *et al.*, 2014). The moisture content was as a result of the presence of carboxyl and hydroxyl groups in the

molecular chain of purified MBF-W7 (Kumar *et al.*, 2004). The higher the carboxyl content the greater the affinity of the polysaccharide for water molecules (Kumar and Anand, 1998). A reduction in weight between 150-400 ^{o}C, was an indication of the decomposition of the main chain of purified MBF-W7 which resulted into about a 33% weight lost. Nonetheless, about 45% (w/w) of the initial weight of MBF-W7 was lost at 500 ^{o}C (Figure 4.3).

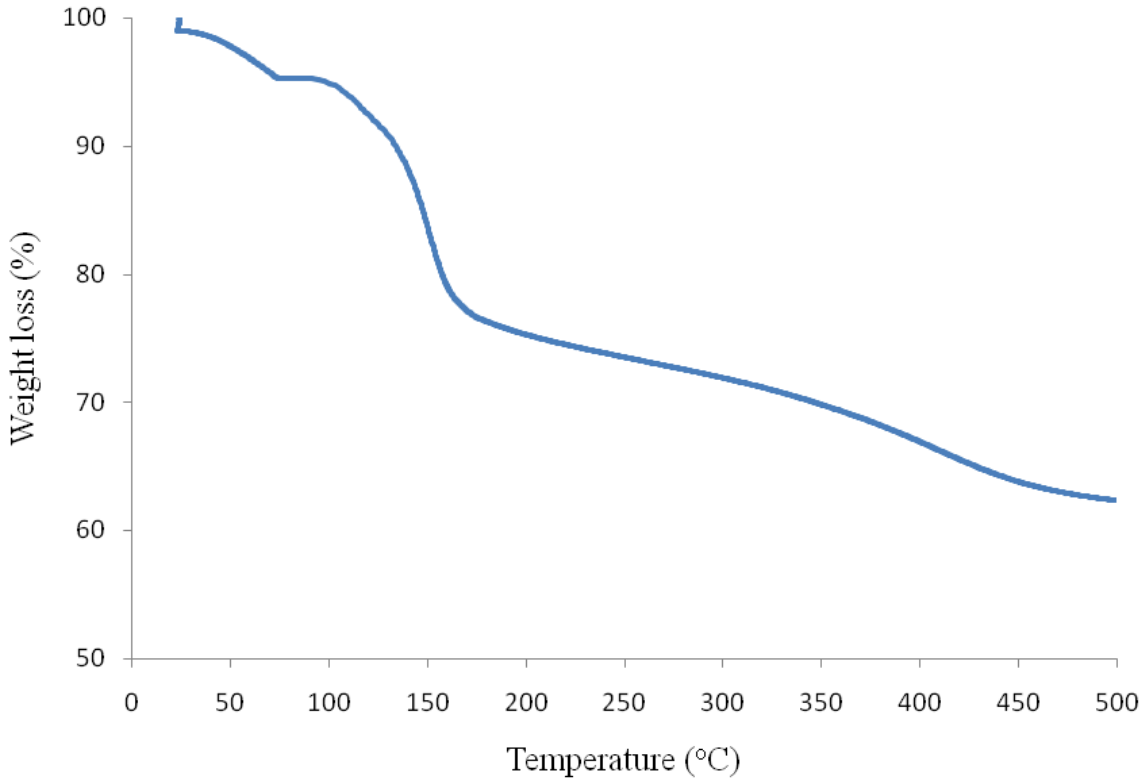

Figure 4.3. Thermal pyrolysis profile of purified MBF-W7 produced by *Bacillus* sp.

4.3.9 Scanning electron microscopy (SEM) imaging

The surface morphology structures of MBF-W7 before and after flocculation as well as kaolin clay particles were elucidated (Figure 4.4). Scanning electron microscopy imaging (Figure 4.4A) revealed the whitish colour of MBF-W7 and its compact nature with filamentous branches which were used as attachment sites for cations and suspended particles (Salehizadeh and Shojaosadati, 2001). Figure 4.4B shows the fine and scattered kaolin particles before flocculation and Figure 4.4C depicted how the bioflocculant flocculated the kaolin particles

forming larger flocs which sediment easily. Scanning electron microscopy images of MBF-W7 and the flocculated kaolin suspension revealed that bridging might be responsible for the flocculation process of MBF-W7. In agreement with our observations, several researchers have reported similar phenomenon with some bioflocculants investigated in the previous studies (Gao *et al*., 2006; Yim *et al*., 2007; Lian *et al*., 2008; He *et al*., 2010).

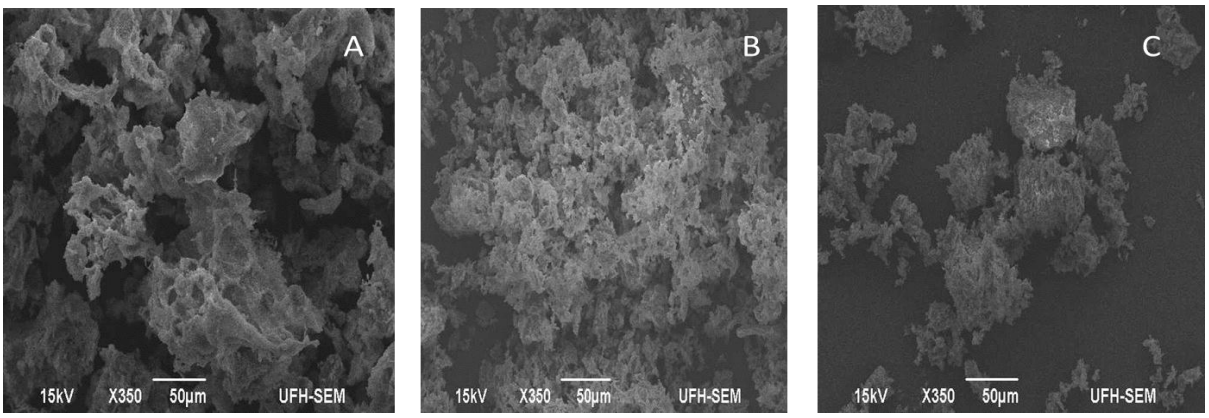

Figure 4.4. SEM images of MBF-W7 (A), Kaolin clay particles (B) and kaolin clay suspension flocculated with MBF-W7 (C).

4.3.10 Optimization of conditions for flocculating activity of MBF-W7

4.3.10.1 Effect of MBF-W7 concentration on the flocculating activity of the purified MBF-W7

The relationship between MBF-W7 concentration/dosage and flocculating activity was investigated and the results are presented in Figure 4.5A. MBF-W7 concentartion/dosage range was between 0.1-0.5 mg/mL. The highest flocculating activity was observed at 0.2 mg/mL with the flocculation rate decreasing below or above this dosage value (Figure 4.5A). In accordance with Salehizadeh and Yan (2014) reports, the maximum flocculating activity was attained at optimal concentration of MBF-W7 under ideal conditions. Lower or higher dosage declined the flocculating activity of MBF-W7. Lower MBF-W7 dosage indicates insufficient bioflocculant molecules to adsorb the suspended kaolin particles and form a bridge between them (Luo *et al*., 2014). On the other hand, when MBF-W7 is in excess, it enwrapped and blocked the binding's sites of one dispersive kaolin particle by one or few bioflocculant molecules in a higher MBF-

W7 dosage instead of forming strong bridging among different MBF-W7 molecules and diverse particles in a proper MBF-W7 dosage (He *et al.*, 2010). Our investigation was consistent with the results of Okaiyeto *et al.* (2013) who reported that highest flocculation rate was achieved at lower bioflocculant concentrations of 0.2 mg/mL for the purified bioflocculant produced by a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. On the contrary, Zhao *et al.* (2013) found that flocculating rate of the bioflocculant γ-PGA produced by *Bacillus licheniformis* decreases at concentrations below or above 1.5 mg/L whereas, in the case of the bioflocculant produced by *Corynebacterium daeguense*, the optimal concentration that was favourable for the flocculating activity of the bioflocculant was 1.2 mg/L (Liu *et al.*, 2010).

4.3.10.2 Effect of cations on the flocculating activity of the purified MBF-W7

The concentration and valence of metal ions play important roles in destabilizing of colloids system (Salehizadeh and Yan, 2014). Figure 4.5B shows the effect of monovalent, divalent and trivalent cations on the flocculating activity of purified MBF-W7. It was observed that monovalent cations Na^+, Li^+ and K^+ have less pronounced effects on the flocculating activity of purified MBF-W7. On the other hand, all the divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}) tested greatly enhanced the flocculating activity of MBF-W7 with the maximum flocculation rate of 71.3% achieved with Mn^{2+}. For trivalent cations, only Al^{3+} enhanced the flocculating activity of MBF-W7 with Fe^{3+} far less effective in stimulating activity (Figure 4.5B). Aluminium salts have been frequently used in drinking water and wastewater treatment due to its high flocculating efficiency and cost-effectiveness. Nevertheless, residual aluminium concentrations in treated water impose health problems (Wong *et al.*, 2012). The addition of cations aid aggregation of the charged particles in a suspension by decreasing the size of the double layer and the repulsive force between Mn^{2+} increases the initial adsorption of MBF-W7 on suspended particles by decreasing the negatives charges of both MBF-W7 and the suspended

particles thus improving the flocculation process (Yin *et al.*, 2014). Several studies in the literature concur with our findings. For example, the bioflocculant produced by *Brachybacterium* sp. required Ca^{2+}, Mg^{2+} and Mn^{2+} for effective flocculation (Nwodo *et al.*, 2012), whereas the flocculating activity of the bioflocculant produced by *Bacillus velezensis* was stimulated in the presence of Ca^{2+}, Zn^{2+}, Na^{+} and inhibited in the presence of Al^{3+}, Fe^{3+}, and Mg^{2+} (Zaki *et al.*, 2012). Contrary to these findings, very few bioflocculants have been reported in the literature to have high flocculation capability in the absence of a cation. Examples of such bioflocculants include those produced by *Chryseobacterium daeguense*, *Solibacillus silvestris* and *Klebsiella pneumonia* (Liu *et al.*, 2010; Wan *et al.*, 2013; Zhao *et al.*, 2013).

4.3.10.3 Effect of pH on the flocculating activity of purified MBF-W7

The effect of pH on the flocculating activity of MBF-W7 was examined and the outcome is illustrated in Figure 4.5C. It was shown that MBF-W7 was quite effective exhibiting flocculating activities above 70% over a wide pH range from 3-11 except at pH 7 with a flocculating activity below 50%. However, MBF-W7 flocculated well at both strong acidic and alkaline pH conditions with the maximum flocculation rate of 85.6% observed at pH 3. This shows that **t**he ionization of the functional groups in the molecular chain of MBF-W7 is pH-dependent which completely ionize at strong acidic and alkaline conditions. Thus, this is an indication that MBF-W7 exhibits different electric states at different pH conditions and consequently affected the flocculating efficiency of MBF-W7 for kaolin particles (Yong *et al.*, 2009). These functional groups serve as attachment sites for the suspended particles. In addition, the $[H^{+}]$ and $[OH^{-}]$ adsorbed at both acidic and alkaline conditions increases the stability of the complex formed between MBF-W7 and kaolin particles thus resulting into higher flocculating efficiency. At pH 7, the low flocculating rate observed was supposedly due to the inability of the functional groups to ionize completely; hence, the flocculating rate of

MBF-W7 decreases dramatically above pH 7 (Figure 4.5C). Similarly, Nie *et al.* (2011) documented an analogous observations with a novel bioflocculant MNXY1 produced by *Klebsiella pneumonia* strain NY1 with high flocculating activity at both strong acidic and alkaline conditions. Conversely, lower flocculating activity was observed at neutral pH. Likewise, the purified bioflocculant MBF-5 produced by *Klebsiella pneumoniae* maintained higher flocculating activity (90-95%) under acidic condition (pH 2-5) and alkaline condition pH 8-11 (Zaki *et al.*, 2012). Besides, many kinds of bioflocculants performed well only in acidic condition, such as the bioflocculants produced by *Streptomyces griseus* and *Bacillus mojavesis* (Yim *et al.*, 2007; Elkady *et al.*, 2011). On the contrary, Aljuboori *et al.* (2013) observed that the bioflocculant IH-7 produced by *Aspergillus flavus* showed over 90% flocculating rate at a wide pH range of 3 to 7. The bioflocculant was only suitable at acidic and neutral conditions. The pH ranges for optimal flocculating activity of the bioflocculant produced by *Pseudomonas aeruginosa* and *Ochrobactium cicero* were 3-11 and 1-10 respectively (Gomaa, 2012; Wang *et al.*, 2013).

4.3.10.4 Thermal property of the purified MBF-W7

The thermal property of MBF-W7 was investigated over 50, 80 and 100 ^{o}C for 1 h. The initial flocculating activity of unheated sample was 85.8%, and on heating MBF-W7 at 50 ^{o}C, 82% flocculating rate was observed (Figure 4.5D). Furthermore, when the temperature was increased to 80 ^{o}C, about 13% of the residual flocculating activity was lost. MBF-W7 exhibited thermal stability at 100 ^{o}C with a flocculating activity of about 70%. This property portends its application potential in the treatment of industrial wastewaters with high temperature. It thermal stability property might be because the backbone of MBF-W7 was found to be mainly composed of polysaccharide. The decrease in flocculating activity observed at 100 ^{o}C might be due to denaturation of the proteins content of MBF-W7 (Wang *et al.*, 2011). The bioflocculant produced by *Klebsiella pneumoniae* YZ-6 exhibited good flocculating rate over a wide range of

temperature 0-70 °C (Luo *et al.*, 2014). The thermal stability of bioflocculant MBF-TG-1 produced by *Klebsiella* sp. TG-1 showed a flocculating rate range of 86%-75.9% at a temperature range of 25-100 °C (Liu *et al.*, 2013). Likewise, in another study described by Lian *et al.* (2008), the bioflocculant produced by *Bacillus mucilaginosus* was thermally stable over 23-70 °C, whereas the physicochemical properties of the bioflocculant was affected by increasing the temperature above 70 °C.

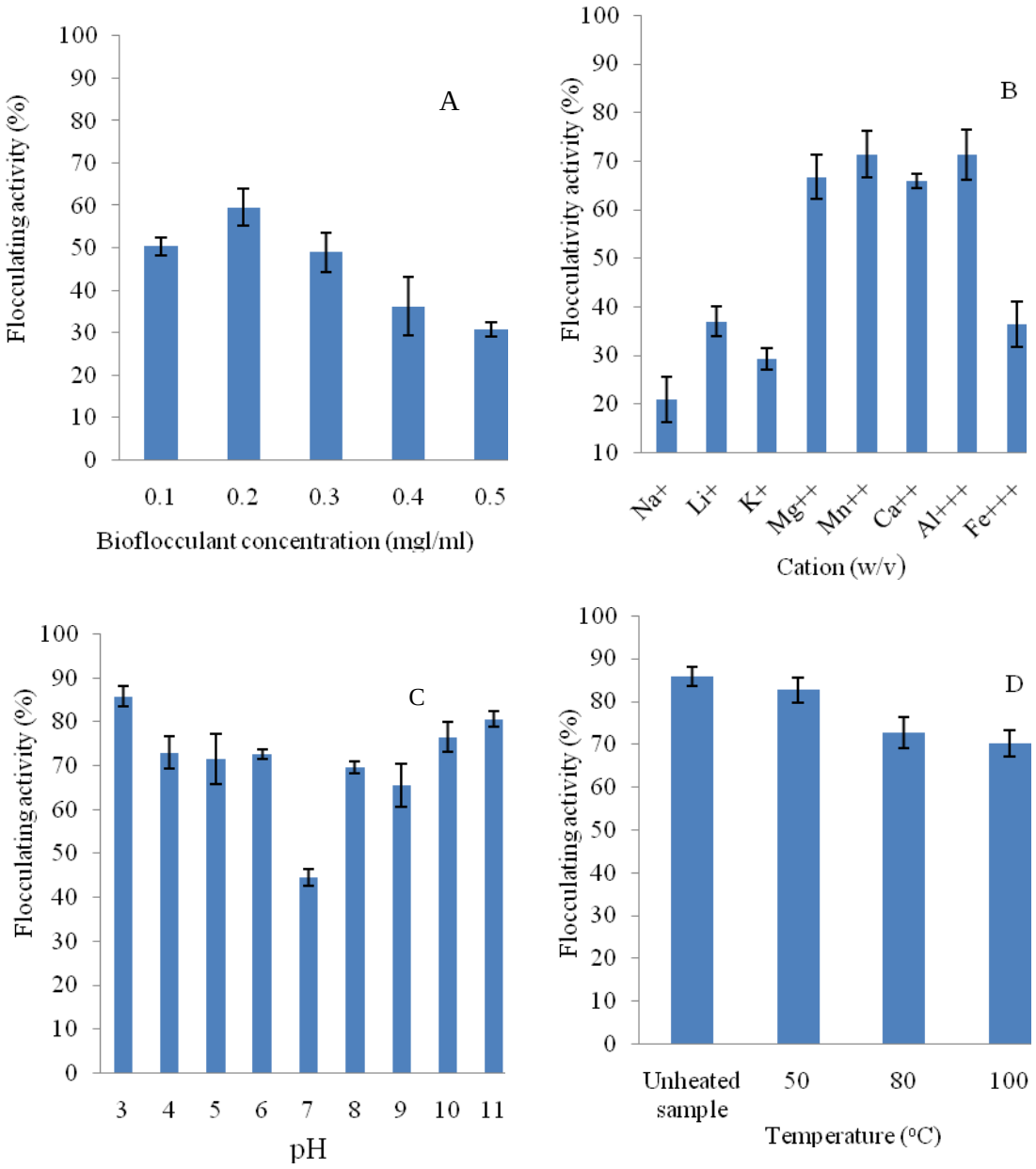

Figure 4.5. Effect of dosage (A), cations (B), pH (C) and temperature (D) on flocculating activity of MBF-W7 produced by *Bacillus* sp.

4.3.11 Application of MBF-W7 in the treatment of Thyume River

The ability of MBF-W7 to flocculate various suspended particles in raw water (Thyume River) was investigated and the result is depicted in Table 4.3. River water is one of the typical surface water with low turbidity and COD (Gong *et al.*, 2008). The removal efficiency of turbidity and COD of MBF-W7 in Thyume River were 86.9% and 75.3% respectively. Our results concur with some findings documented in the previous studies. In the case of bioflocculant (SF-1) produced by *Serratia ficaria* in the treatment of river water (Gong *et al.* 2008), the turbidity removal efficiency (86.9%) of MBF-W7 in this study of raw water was higher than SF-1 (84.2%). However, the COD reduction ability of MBF-W7 (75.3%) was lower than that of SF-1 (87.1%). In another study carried out by Auhim and Odaa (2013), the bioflocculant produced by *Azotobacter chrococcum* showed 81% turbidity removal potential in river water. The polyglutamic acid based-bioflocculant (PG.a21 Ca) had a 93.4% turbidity removal and 72% COD reduction in river water (Pan *et al.*, 2009), Ugbenyen and Okoh (2014) reported that the bioflocculant produced by a consortium of *Cobetia* and *Bacillus* species had 98.9% turbidity removal and 73.9% COD reduction for river water.

Table 4.3. Treatment of Thyume River using MBF-W7

Bioflocculant	**Turbidity removal**		
MBF-W7	Before treatment (NTU)	After treatment (NTU)	Turbidity removal (%)
	65	8	86.9
	68	11	
	59	6	
	COD reduction		
	Before treatment (mg/l)	After treatment (mg/l)	COD reduction (%)
	152	38	75.3
	131	33	
	146	35	

*NTU-Nephelometric Turbidity Units, COD-Chemical oxygen demand

4.4 Conclusions

In view of environmental and health concerns consequent to the adverse effects of conventionally used flocculants, hence the need for microbial flocculants which has been adduced with merits including innocuousness, biodegradability and sensitivity under extreme environmental conditions. These salient features possessed by microbial flocculants imply an imperative alternative. In this present study, the potentials of *Bacillus* sp. for bioflocculant (MBF-W7) were investigated and the culture conditions required for its optimal bioflocculant production were assessed. MBF-W7 production was observed to be associated with cell growth, optimally produced in the presence of excess nutrient with maltose as the carbon source, NH_4NO_3 as the nitrogen source of choice at initial growth medium pH of 6. Purified MBF-W7 was a glycoprotein composed of polysaccharide (73.7% w/w) and protein (6.2% w/w). The flocculating activity for kaolin suspension reached maximal level at 0.2 mg/mL over a wide pH range of 3-11 with Mn^{2+} as a flocculating aid. The thermostable MBF-W7 exhibited excellent flocculating activities of 80% at both strong acidic and alkaline conditions; thus indicating that it could be used under extreme environmental conditions. MBF-W7 showed high flocculating activity 86.9% (turbidity removal) and COD reduction (75.3%) in Thyume River, MBF-W7 can be used in the treatment of River water.

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CHAPTER FIVE

Studies on a bioflocculant production by *Bacillus* sp. AEMREG7

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Abstract

The detrimental effects of inorganic and organic synthetic flocculants on humans and their recalcitrance in the environment have necessitated the search for safe, eco-friendly alternatives including microbial flocculants. A bioflocculant-producing bacteria (M67) was isolated from sediment samples of Algoa Bay in the Eastern Cape Province of South Africa. The bacteria was identified through partial 16S ribosomal deoxyribonucleic acids (rDNA) nucleotide sequencing and BLAST analysis of the gene sequence showed the bacteria to have 98% similarity to *Bacillus algicola* strain QD43 and deposited in the GenBank as *Bacillus* sp. AEMREG7 with accession number KF933697.1. The effect of culture conditions on MBF-UFH production revealed optimally at inoculum size of 3% (v/v), glucose, a mixed nitrogen source [NH_4 $(SO_4)_2$ + urea + yeast extract] and pH 6 for inoculums size, carbon and nitrogen sources, and initial medium pH for the fermentation process respectively. The spent culture broth-rich bioflocculant attained the highest flocculating activity of 85.8% after 72 h of fermentation during the exponential phase of bacterial growth. It showed a high flocculating activity for kaolin clay suspension over a wide pH range of 4-10 with maximum flocculating activity observed at pH 6; and also retained 70.5% flocculating activity after heating at 100 ^{o}C for 60 min. These findings suggest that MBF-UFH has a great potential to substitute hazardous chemical flocculants commonly used in water treatment and hence, reducing deformities among individuals in the societies.

Keywords: *Bacillus* sp. AEMREG7, MBF-UFH, biodegradable, glycoprotein, flocculating activity

5.1 Introduction

Flocculants are used in waste/drinking water treatment processes as well as in downstream processing (Zaki *et al.*, 2013). They are usually grouped as synthetic organic, inorganic and naturally occurring flocculants (Yu *et al.*, 2009), and owing to the efficacy and cost effectiveness of synthetic organic and inorganic flocculants, they find important uses in industries (Chen *et al.*, 2010; Lin and Harichund, 2012). Aluminium salts are amongst the most widely utilized inorganic flocculants due to the high efficiency of flocculation and cost effectiveness relative to other flocculants (Verma *et al.*, 2012); nevertheless, the problem of residual aluminium in water has raised serious public health concerns (Levesque *et al.*, 2000). Besides, large aluminium salts dosage is required for effective flocculation which always resulted in large volumes of sludge (Sharma *et al.*, 2006). The challenge of sludge generated by aluminium salts has been abolished by the use of polyacrylamide. Nevertheless, major consequences have been associated with polyacrylamide including poor biodegradability and production of carcinogenic monomer (Ahluwalia and Goyal, 2007).

As a result of these demerit allied with chemical flocculants, is imperative to assess water quality on a perpetual basis (Yang *et al.*, 2012). In order to adequately provide these services to meet people's demands, it is incumbent upon governments and society at large to develop among other things, appropriate scientific strategies in wastewater treatment technology that are not only environmentally friendly but also cost-effective. Therefore, the development of safe and biodegradable flocculants cannot be overemphasized (Yang *et al.*, 2012).

Bioflocculants, on the other hand, have enormous advantages such as biodegradability, lack of secondary pollution from degradation intermediate and being innocuous to humans, hence, their relevance in water treatment technology has gained much popularity (Gao *et al.*, 2009). Bioflocculants are secondary metabolites secreted by microorganisms during growth (Sun *et al.*, 2012). Over the decades, many microorganisms, including bacteria, fungi and algae have

been reported to produce bioflocculants with different chemical compositions such as polysaccharide, protein, glycoprotein and nucleic acids (Gao *et al*., 2006; Feng and Xu, 2008). Though several studies have been reported on bioflocculant production from different microbes (Liu *et al*., 2010), high cost of production has been the major limiting factor to the large scale production for industrial application (Yang *et al*., 2007; Peng *et al*., 2014). Therefore, it would be economically favourable, as a cost-cutting measure to utilize cost-effective substrates for bioflocculant production on an industrial scale (Wang *et al*., 2007).

In this paper, bioflocculant-producing bacteria were isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa. The bacterial isolate was identified by 16S rDNA sequence analysis and the various factors influencing MBF-UFH production were investigated to determine the cost-optimal culture conditions favourable for the bacteria.

5.2 Materials and methods

5.2.1 Sample collection and isolation of bacterial strain

The sediment samples of Algoa Bay (a marine environment) were collected and processed according to Jensen *et al*. (2005) with some modifications. Half gram (0.5 g) of wet sample was diluted with 5 ml of sterile seawater. The suspension was vortexed and allowed to sediment for 60 s, out of which 100 µl of the suspension was inoculated onto the surface of R2A agar plates, spread with a sterile glass rod and incubated for 96 h. The distinct isolates were picked and streaked onto nutrient agar plates to obtain their purity and separate from mixed populations.

5.2.2 Media and cultivation conditions

The composition of activation medium included (per litre): Beef extract 3 g, Tryptone 10 g, NaCl 5 g (Ntsaluba *et al*., 2013). Production medium composed (per litre): Glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $NH_4(SO_4)_2$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ 0.3

g, NaCl 0.1 g (Zhang *et al.*, 2007). The medium for a slant included (per litre): Glucose 20 g, K_2HPO_4 5 g, KH_2PO_4 2 g, $NH_4(SO_4)_2$ 0.3 g, urea 0.5 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ 0.3 g, NaCl 0.1 g and agar 20 g (Gong *et al.*, 2008). All media were prepared using filtered seawater and sterilized by autoclaving at 121 ^oC for 15 min. For all experiments, the cultivations were carried out at 28 ^oC and 160 rpm.

5.2.3 Screening of bioflocculant-producing bacteria

About 48 bacterial isolates were obtained from sediment samples of Algoa Bay and screened for bioflocculant production as follows. Two loopfulls of the isolate from a nutrient agar plate were inoculated into 50 mL of activation medium and incubated for 24 h. One millilitre of the activation medium was inoculated into 250 ml flask containing 50 mL of production medium and incubated at 28 ^oC in a rotary shaker at 160 rpm for 72 h. Two millilitres of the fermented broth were carefully withdrawn and centrifuged at 4 000 rpm for 30 min, the cell-free supernatant was used to determine the flocculating activity according to the description of Kurane *et al.* (1994). The isolate with the highest flocculating activity (MBF-UFH) was preserved in 20% glycerol stock and stored at -80 ^oC for future studies.

5.2.4 Determination of flocculating activity

The flocculating activity of MBF-UFH was determined according to the description of Kurane *et al.* (1994). Kaolin clay was used as test material in preparing the water suspension at a concentration of 4 g/L. One hundred millilitres of the kaolin suspension were measured into 250 ml conical flask. Three millilitres of 1% CaCl were added, followed by 2 mL of MBF-UFH. The solution was agitated for 60 s, transferred into a graduated measuring cylinder and allowed to sediment for 5 min. A control was prepared in a similar way, but MBF-UFH was replaced with un-inoculated production medium. The flocculating activity was calculated using the formula:

Flocculating activity (%) = [A-B/A] × 100%

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

5.2.5 16S rDNA sequence identification of bacteria

DNA extraction was conducted using the boiling method described by Cosa *et al*. (2011) whereby two to three colonies were suspended in 70 µL of sterile double distilled water. The samples were heated in a water bath at 100 °C for 10 min, cooled for 5 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4 °C. This serves as the template in the PCR assay. PCR was carried out in 50 µL reaction volume containing 2 mM $MgCl_2$, 2U Supertherm Taq polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 µL template DNA. The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 ^{o}C for 2 min), 30 cycles of denaturation (96 ^{o}C for 45 s), annealing (56 ^{o}C for 30 s), extension (72 ^{o}C for 2 min), and a final extension (72 ^{o}C for 5 min). Gel electrophoresis of PCR products was conducted on 1% agarose gels to confirm that a fragment of the correct size had been amplied.

5.2.6 Optimization of culture conditions for MBF-UFH production

Different inoculum sizes ranging from (1-5%) of the seed culture were used to inoculate the production medium and the effect of each on MBF-UFH production was assessed according to the description of Zhang *et al*. (2007). The effects of the medium compositions on MBF-UFH produced were also investigated. We examined the effect of different carbon sources on MBF-UFH production by replacing the glucose in the production medium by one of the following carbon sources (20 g/L): fructose, starch, sucrose, maltose, lactose and Na_2CO_3. Both inorganic and organic nitrogen sources were also evaluated for their effects on bioflocculant production.

The nitrogen sources (1.3 g/L) included: yeast extract, urea, NH_4SO_4, NH_4NO_3, peptone, tryptone and mixed nitrogen source [$(NH_4)_2SO_4$ + urea + yeast extract]. The impact of initial pH of the production medium on MBF-UFH production and flocculating activity were also examined by adjusting the pH from 4-10 with 0.1 M HCI and NaOH (Liu *et al.*, 2010). The effect of cations on the flocculating activity of MBF-UFH was appraised by replacing the $CaCl_2$ solution in the flocculation assay with various metal solutions which included: Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} (Okaiyeto *et al.*, 2014). The thermal stability of the crude MBF-UFH was investigated as described by Gong *et al.* (2008) with some modifications: A 72 h old culture was centrifuged at 4 000 rpm for 30 min to obtain cell-free supernatant. Two millilitres of MBF-UFH was heated in a water bath for 1 h at different temperatures range of 50-100 ^oC.

5.2.7 Time course of MBF-UFH production

Optimum culture conditions were used for time course of MBF-UFH production in accordance with the method described by Liu *et al.* (2010) and Nwodo *et al.* (2013) with some modifications. The seed culture was prepared by inoculating two loopfulls of bacterial colonies into 50 ml of activation medium and incubated overnight in a rotary shaker at 28 ^oC. The fermented broth was diluted with sterile saline water to an optical density of 0.1 at OD_{660} (Cosa *et al.*, 2012). The standardized bacterial suspension was inoculated into 200 ml of the production medium in 500 ml flasks and incubated in a rotary shaker at 160 rpm, 28 ^oC for 192 h of cultivation. Ten millilitres of the samples were withdrawn periodically at time intervals of 24 h; two millilitres of the fermented broth were centrifuged and the supernatant used to determine MBF-UFH in accordance with the method of Kurane *et al.* (1994). The growth of the bacteria was monitored by bacterial counts using a standard spread plate technique and optical density OD_{660}, pH and flocculating activity of MBF-UFH with time.

5.2.8 Extraction and purification of MBF-UFH

The extraction and purification of the bioflocculant were carried out in accordance with Cosa *et al.* (2013) and Li *et al.* (2013). Optimal culture conditions were used to produce MBF-UFH over a growth period of 72 h and the fermented broth was centrifuged at 4000 rpm, for 30 min. One volume of sterile distilled water was added to the supernatant and centrifuged at 4 000 rpm for 30 min. Two volumes of ethanol were added to the supernatant and allowed to stand overnight at 4 ^{o}C. The precipitate was collected by centrifugation and vacuum dried. The crude MBF-UFH was weighed and further purified by dissolving in 100 mL of distilled water and one volume of a mixture of chloroform and n-butyl alcohol (5:2, v/v) was added and agitated for 60 s. The solution was left standing at room temperature for 12 h prior to dialysing against distilled water overnight. Two volumes of ethanol were added to the dialysed solution and the precipitate recovered was dissolved in distilled water and vacuum dried.

5.2.9 Statistical analysis

Triplicate values were obtained, averaged and statistically analysed using SPSS version 8. The error bars represent the standard deviation (SD) of the data.

5.3 Results

5.3.1 Identification of bioflocculant-producing microorganisms

A total of 48 bacterial isolates were obtained from the sediment samples of Algoa Bay, this isolate showed good bioflocculant production potential with flocculating activity of over 60% for kaolin clay suspension. It appeared yellowish, filamentous with branching, and spore-forming on the nutrient agar. Amplification of its 16S rDNA gene yielded the expected amplicon size of appropriately 1.5 kb. The partial sequence (1073 nucleotides) 16S rDNA gene was aligned with all related sequences in NCBI database by the BLASTN program and showed about 98% similar to *Bacillus algicola* strain QD43 and deposited as *Bacillus* sp. AEMREG7

in the GenBank with accession number KF933697.1. A phylogenetic tree was constructed according to the neighbour-joining algorithm as shown in Figure 5.1.

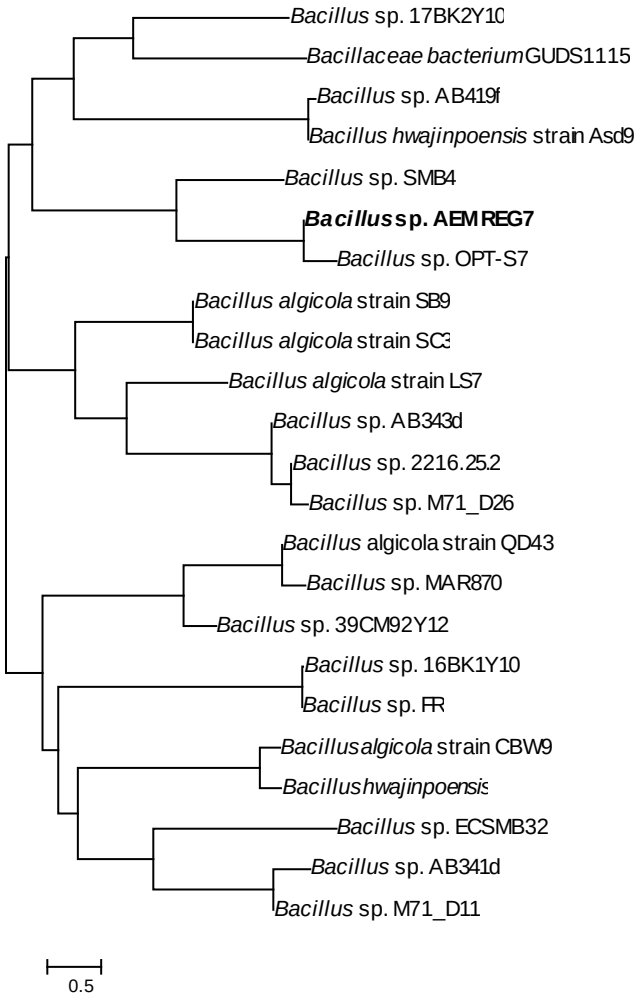

Figure 5.1. Phylogenetic tree showing the relationship among *Bacillus* sp. AEMREG7 and published 16S rDNA sequence of some *Bacillus* species.

5.3.2 Optimization of culture conditions for MBF-UFH production

5.3.2.1 Effect of inoculum size on MBF-UFH production

The relationship between inoculum size and MBF-UFH production was investigated and the results are presented in Figure 5.2. It was observed that the increase in inoculum size of the seed culture from 1 to 3 (%v/v) resulted in an increase in flocculating activity (83.7-88.2%) which is an indication of MBF-UFH production. However, further increase in inoculum size led to a steady decrease in flocculating activity culminating in 85.8% observed at 5% v/v.

Inoculum size of 3% (v/v) resulted in optimum MBF-UFH production and was used in all subsequent experiments.

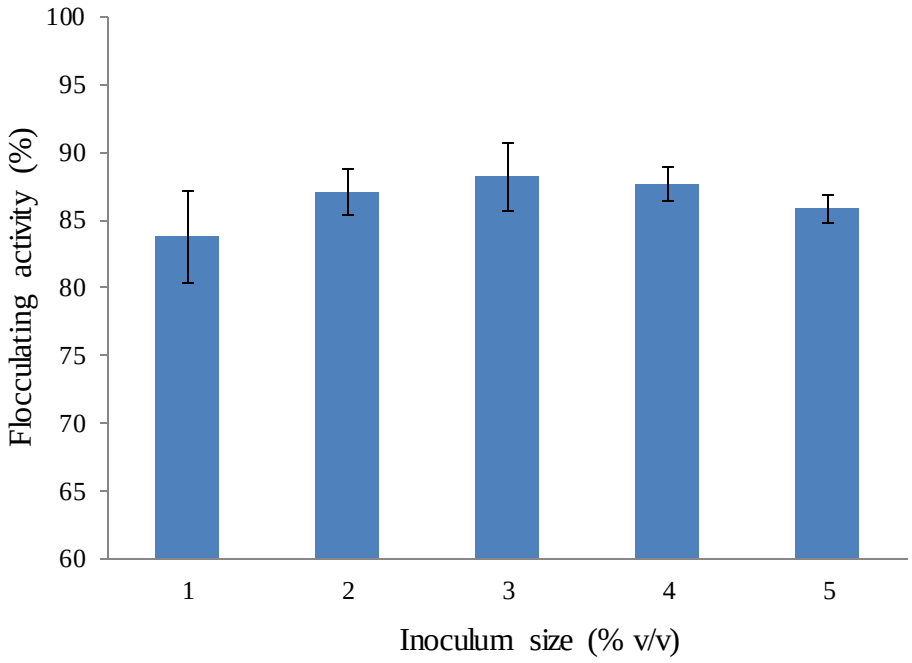

Figure 5.2. Effect of inoculums size on the production of MBF-UFH.

5.3.2.2 Effect of carbon and nitrogen source on MBF-UFH production

The effect of different carbon sources on MBF-UFH production was evaluated and represented in Table 5.1. Culture media were separately supplemented with different carbon sources cultivated at agitation speed of 160 rpm, at 28 ^{o}C for 72 h. The microorganism showed preference for glucose as effective carbon source for MBF-UFH production with the highest flocculating activity of 88.03%, followed by sucrose (70.34%) and lactose (67.03%). The medium containing inorganic carbon source Na_2CO_3 supported MBF-UFH production with flocculating activity of 55.62% compared to fructose and maltose. The lowest flocculating activity of 6.1% was observed with starch as a carbon source.

The effect of different nitrogen sources was investigated to find the suitable nitrogen supplement for MBF-UFH production and the results are presented in Table 5.1. Apart from tryptone that had the lowest flocculating activity (37.85%), other organic nitrogen sources such as peptone (71.5%), urea (72.12%), and yeast extract (79.02%) favoured the production of MBF-UFH. Both inorganic nitrogen sources tested moderately buttressed MBF-UFH production with flocculating activity of 53.58% and 54.36% for $NH_4(SO_4)_2$ and NH_4NO_3 respectively. The highest flocculating activity of 86.3% was observed when the mixed nitrogen source [$NH_4(SO_4)_2$ 0.3 g, + urea 0.5 g, + yeast extract 0.5 g] was used as a supplement.

Table 5.1. Effect of carbon and nitrogen sources on the production of MBF-UFH.

Carbon source	FA ± SD	Nitrogen source	FA ± SD
Glucose	88.03 ± 2.37	Peptone	71.50 ± 2.98
Fructose	46.64 ± 8.34	Tryptone	37.85 ± 5.74
Maltose	34.16 ± 3.37	Urea	72.12 ± 8.50
Sucrose	70.34 ± 5.27	Yeast extract	79.02 ± 3.92
Lactose	67.03 ± 2.27	NH_4SO_4	53.58 ± 2.86
Starch	6.10 ± 0.92	NH_4NO_3	54.36 ± 5.85
Na_2CO_3	55.62 ± 2.65	Mixed nitrogen	86.35 ± 1.72

* FA = Flocculating activity, SD = Standard deviation, mixed nitrogen = [$NH_4(SO_4)_2$ + urea + yeast extract]

5.3.2.3 Effect of initial pH of growth medium on MBF-UFH production

The pH of growth medium has a great influence on bioflocculant production (Ugbenyen *et al.* 2012). The effect of initial pH of growth medium was assessed (Figure 5.3). It was noticed that the production of MBF-UFH was optimal at pH 6 with the flocculating activity of about 82.2%, followed by pH 7 with flocculating activity of 79.5%. The production of MBF-UFH was completely inhibited at pH 10 and as a result, pH 6 was selected for the subsequent experiments.

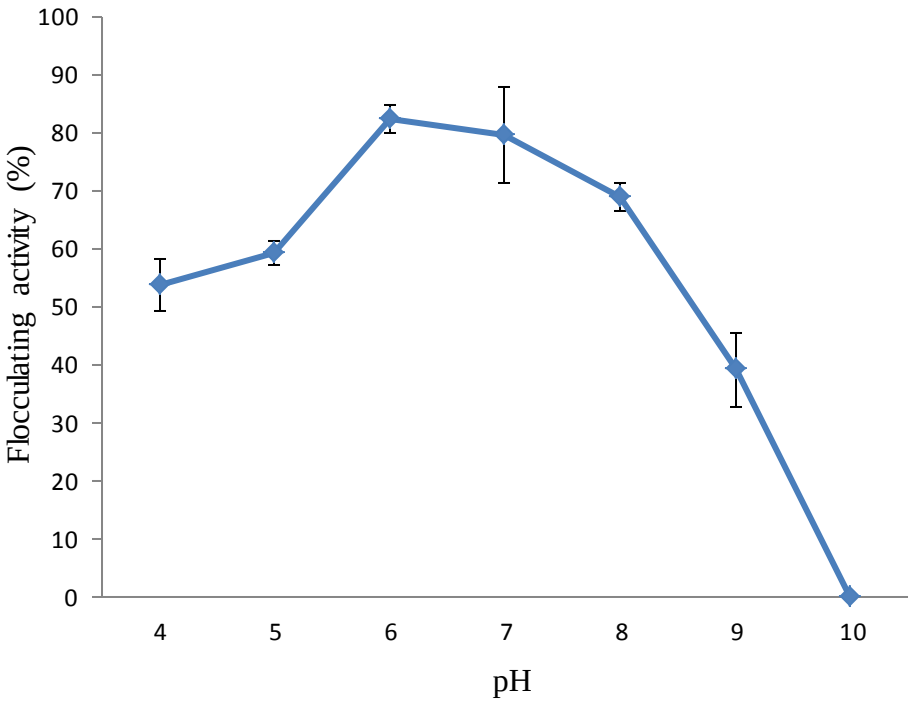

Figure 5.3. Effect of initial medium pH on the flocculating activity of MBF-UFH.

5.3.2.4 Effect of cations and pH on the flocculating activity of MBF-UFH

The synergistic effect of various cations on MBF-UFH produced by *Bacillus* sp. AEMREG7 is depicted in Table 5.2. All the cations evaluated enhanced flocculating activity above 80%, except for Fe^{3+} which inhibited the flocculating activity of MBF-UFH. The highest flocculating activity of 92.2% was observed with Al^{3+} followed by Ca^{2+} at 87.2%.

Evaluation of the effect of pH on the flocculating activity of MBF-UFH is represented in Table 2. MBF-UFH exhibited a wide pH range from acidic (pH 4) to alkaline (pH 9) with the flocculation rate marginally better at weak acidic condition. The highest and lowest flocculating activities of 87.8% and 48.8% were achieved at pH 6 and 10 respectively. There was no significant difference in flocculating activity from pH 4-6 as against a noticeable sharp decline between pH 9 and 10.

Table 5.2. Effect of cations and pH on the flocculating activity of MBF-UFH.

Cations	FA(%) ± SD	pH	FA(%) ± SD
Na^{+}	85.03 ± 3.96	4	53.78 ± 4.41
K^{+}	83.76 ± 1.99	5	59.57 ± 2.06
Li^{+}	86.57 ± 1.74	6	82.20 ± 2.39
Mg^{2+}	80.59 ± 3.51	7	79.49 ± 8.23
Mn^{2+}	86.27 ± 1.01	8	68.84 ± 2.36
Ca^{2+}	87.20 ± 2.11	9	39.12 ± 6.46
Fe^{3+}	-	10	-
Al^{3+}	92.19 ± 3.03		

*FA = Flocculating activity, SD = Standard deviation

5.3.3 Time course of MBF-UFH production

Figure 5.4 shows the time course of MBF-UFH by *Bacillus* sp. AEMREG7. The bacterial growth was monitored over a period of 192 h. Initially, a corresponding increase in flocculating activity with cultivation time was observed with a maximum activity of 85.8% attained at 72 h during the active phase of cell growth. It was observed that further increase in cultivation time led to a decrease in flocculating activity as well as the number of viable cells expressed as CFU/mL; and at the same time, the optical density of the culture increased progressively with increase in flocculating activity of the produced MBF-UFH. The pH of the culture broth decreased gradually throughout the cultivation time from 6.0 to 4.65. A yield of about 1.6 g of crude bioflocculant was obtained under optimum culture conditions from 1 L fermented broth of *Bacillus* sp. AEMREG7.

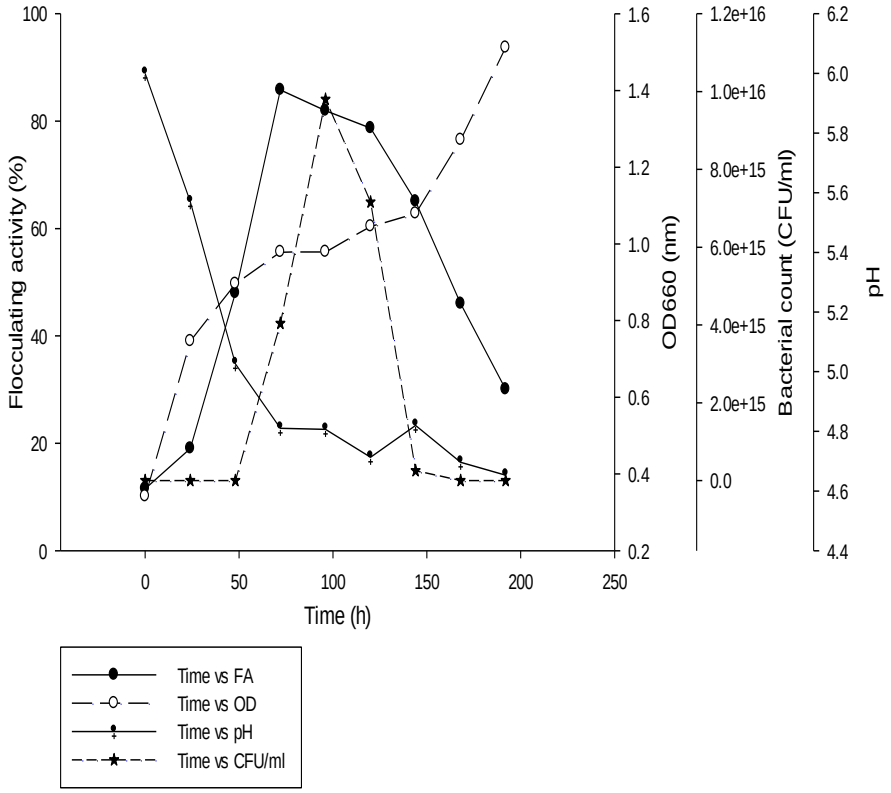

Figure 5.4. Time course of MBF-UFH produced by *Bacillus* sp. AEMREG7 showing the relationship between cell growth (monitored with bacterial counts and optical density) and flocculating activity as well as the pH changes over time.

5.3.4 Thermal stability of crude MBF-UFH

Results of thermal stability of crude MBF-UFH are represented in Table 5.3. It was found that the increase in temperature from (50-70 ^{o}C) resulted in an increase in flocculating activity of MBF-UFH from 79.7-85.5%. Further increase in temperature resulted in a decline in activity with a residual flocculating activity of about 70.5% recorded at 100 ^{o}C.

Table 5.3. Effect of temperature on the flocculating activity of MBF-UFH produced by *Bacillus* sp. AEMREG7.

Temperature (^{o}C)	FA (%) ± SD
50	79.7 + 4.23
60	85.1 + 4.54
70	85.4 + 3.47
80	83.1 + 2.72
90	82.9 + 0.50
100	70.5 + 5.32

* FA = Flocculating activity, SD = Standard deviation

5.4 Discussion

Water is one of the most precious resources without which life on earth is not possible (Sharma *et al.*, 2006). It is a basic need connected with the very survival of human beings. Acess to safe drinking water is a basic human right and essential for achieving gender equality, sustainable development and poverty alleviation. Lack of safe water and basic sanition is acute problem for women and girls who live in poor overcrowded urban slums, as women are usually the one that face the burden of water collection in the homes and consequently lessening the burden on women who are usually the caregivers to crippled person resulted from drinking chemically-treated water.

Every industrial process is a potential source of pollution and requires a specific treatment of the wastes produced (Elkady *et al.*, 2011). The introduction of waste treatment(s) processes increase the plant running costs thus making any effort to improve their efficiency difficult to achieve. Cost-effective treatment such as flocculation by microbial flocculants has gained huge biotechnological attention in recent years (Zaki *et al.*, 2013). In view of this, isolation of bioflocculant-producing microorganisms from diverse environments has become imperative.

Water milieu remains a good source of microbes with novel metabolites (Nwodo *et al.*, 2013). Depending on specific environmental conditions, microbes can be manipulated to produce exopolymeric substances of a particular composition and physiochemical properties which promote the survival of microbial population (Abdel-Aziz *et al.*, 2012). Nonetheless, marine environments have been documented as supply of interesting bacteria producing unique bioactive compounds such as bioflocculants that have various industrial applications (He *et al.*, 2010).

5.4.1 Identification of MBF-UFH producing microorganisms

In this study, a marine bacterial isolate obtained from sediment samples of Algoa Bay was identified based on 16S rDNA sequence analysis, the BLAST results showed about 98% similar to *Bacillus algicola* strain QD43 and deposited as *Bacillus* sp. AEMREG7 in the GenBank with accession number KF933697.1. The results obtained from the blasted nucleotide sequence of the bacterial gene were compared with those in the Genebank as shown in Figure 5.1. Our findings was in accordance with many previous studies which reported several bacterial strains closely related to the genus Bacillus which have been explored in bioflocculant production (Feng and Xu, 2008; Zheng *et al.*, 2008; Elkady *et al.*, 2011; Bhunia *et al.*, 2012; Cosa *et al.*, 2013; Zaki *et al.*, 2013; Ugbenyen *et al.*, 2014). Nonetheless, no report in the literature where *Bacillus* sp. AEMREG7 has been explored in bioflocculant production.

5.4.2 Optimization of culture conditions for MBF-UFH production

Bacteria can utilize the nutrients in the culture medium to synthesize high molecular-weight polymers intracellularly under the action of specific enzymes, which can be excreted into the medium or on the surface of the bacteria as a capsule (Abdel-Aziz *et al.*, 2012). The bacteria produce a wide range of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, lipids and other biological macromolecules (Noghabi *et al.*, 2007). Hence, optimization of media compositions and fermentation conditions, discovering new

bioflocculant-producing bacterial strains and utilization of cost-effective substrates have become logical approaches of improving the yields and production cost of these exopolymeric substances from different microbes (Rehm, 2010).

5.4.2.1 Effect of inoculum size on MBF-UFH production

It has been articulated in previous studies that small inoculum sizes prolong stagnant phase of bacteria growth and a large inoculum size forms niche and inhibits bioflocculant production (Zhang *et al.*, 2007; Ugbenyen *et al.*, 2014). In Figure 5.2, the production of MBF-UFH was significantly enhanced when 3% inoculum size was used. A slight decrease in MBF-UFH production was noticed with further increase in inoculum sizes. Gong *et al.* (2008) found that 1% inoculum size was favourable for bioflocculant production by *Serratia ficaria*. On the other hand, Wang *et al.* (2007) observed that 5% inoculum size was found to be preferable for bioflocculant production by *Klebsiella mobilis.*

5.4.2.2 Effect of carbon source on MBF-UFH production

The structure and composition of bioflocculants depend on a number of factors such as the type of carbon source used in the production medium and also fermentation conditions (Li *et al.*, 2009). Optimization of fermentation conditions influences not only the yield of bioflocculants and physicochemical properties of bioflocculants, but also their chemical composition (Liu *et al.*, 2010; Abdel-Aziz *et al.*, 2012). In Table 5.1, glucose was the preferred carbon for MBF-UFH production by *Bacillus* sp. AEMREG7 followed by sucrose and lactose. Our findings were in accordance with some of the investigations reported by other researchers. For example, the production of bioflocculants by *Azotobacter* sp. SSB81, *Chryseobacterium daeguense* W6, *Cordyceps ophioglossoides* L2 and a facultative oligotroph *Klebsiella* sp. PB12 reached optimal in the medium containing glucose as a sole carbon source (Liu *et al.*, Gauri *et al.*, 2009; 2010; Qinqin *et al.*, 2012 Amit *et al.*, 2013). On the contrary, Aljuboori *et al.* (2013) found that sucrose was more preferable to other carbon sources assessed for bioflocculant

production by *Aspergillus flavus*. The production of bioflocculant was optimal in the medium where ethanol was used as a carbon source by *Pseudomonas aeruginosa* (Gomaa, 2012). Furthermore, among other carbon sources evaluated, lactose was most favourable for the production of exopolysaccharide by lactic acid bacteria *Streptococcus phocae* PI80 (Kanmani *et al*., 2011).

5.4.2.3 Effect of nitrogen source on MBF-UFH production

It has been well documented that the presence of nitrogen sources in the cultivation medium greatly influences the bioflocculant production and cell growth (Salehizadeh and Yan, 2014). In Table 5.1, MBF-UFH production by *Bacillus* sp. AEMREG7 was optimal in the presence of a mixed nitrogen sources followed by yeast extract. These results were in agreement with the observations made by Liu *et al*. (2010), in which organic nitrogen sources were better sources for bioflocculant production compared to inorganic nitrogen sources. The highest flocculating activity was observed with yeast extract followed by casein hydrolysate, tryptone and beef extract. However, Ugbenyen *et al*. (2014) found all the nitrogen sources tested supporting bioflocculant production by *Bacillus* sp. Gilbert, the highest flocculating activity was observed in the medium with potassium nitrogen (76.6%) as the nitrogen source. Ismail and Nampoothiri (2010) documented that yeast extract was a more favourable nitrogen source that enhanced EPS production by *L. plantarum* MTCC 9510. Cosa *et al*. (2013) reported that tryptone was the nitrogen source of choice for bioflocculant production by *Oceanobacillus* sp. Pinky whereas, in the findings of Aljuboori *et al*. (2013), peptone was the best nitrogen source that supported the bioflocculant production by *Apergillus flavus* among other nitrogen sources investigated. The production of extracellular polysaccharide by *Aureobasidium pullulans* was greatly supported in the medium containing $NaNO_3$ (Ravellaa *et al*., 2010).

5.4.2.4 Effect of initial medium pH on MBF-UFH production

Figure 5.3 shows that pH 6 and 7 were more favourable for MBF-UFH production with the highest flocculating activity observed at pH 6. It has been well established in several studies that the initial pH of the production medium greatly influences bioflocculant production as it determines the electric charge of the cells and redox potential which affects nutrient absorption as well as influencing the rate of enzymatic reaction (Xia *et al.*, 2008; Zheng *et al.*, 2008). Similarly, Noghabi *et al.* (2007) found that the production of extracellular biopolymer by *Pseudomonas fluorescens* BM07 was favourable between pH 6-8. Also, He *et al.* (2012) found that pH 6 was more favourable for both cell growth and bioflocculant production by *Funalia trogii*. The optimum pH for cell growth and bioflocculant production by *Citrobacter* sp. TKF04 and *Aspergillus sojae* were at pH 7.2-10 and 8 respectively (Fujita *et al.*, 2000; Lu *et al.*, 2005).

5.4.2.5 Effect of cations on the flocculating activity of MBF-UFH

As shown in Table 5.2, the flocculating activity of MBF-UFH was stimulated in the presence of the cations evaluated except Fe^{3+} which inhibited the flocculation of MBF-UFH. Cations play a vital role in flocculation in neutralizing and stabilising the residual negative charge of both functional groups of MBF-UFH and the surface charge of the suspended particles and consequently weaken electrostatic repulsion between particles thus enhancing flocculation (Li *et al.*, 2008; Cosa *et al.*, 2013). From our findings, the highest flocculating activity was observed with Al^{3+} and followed by Ca^{2+}. However, many previous studies have reported on the human health being implicated in alumium residual water (Banks *et al.*, 2006). As a result, calcium chloride was chosen as a cation of choice in this study.

5.4.2.6 Effect of pH on the flocculating activity of MBF-UFH

The flocculating activity of bioflocculant is highly influenced by the pH of the medium (Bhunia *et al.*, 2012). The relationship between pH of the kaolin suspension and the

flocculating activity of MBF-UFH is represented in Table 5.2. MBF-UFH flocculated better at acidic conditions compared to neutral and strong alkaline conditions with the maximum flocculating activity at pH 6. At high pH, the flocculating activity of MBF-UFH was inhibited, thereby resulting into lower flocculation. The hydroxyl ions (OH^-) adsorbed at high alkaline medium may interfere with the complex formed between MBF-UFH and the kaolin particles mediated by Ca^{2+} thereby resulting in destabilization of the suspended particles (Prasertsan *et al.*, 2006). A similar trend was equally observed by Okaiyeto *et al.* (2014) where the bioflocculant produced by *Micrococcus* sp. Leo flocculated well in acidic condition from pH 2-6. On the contrary, the flocculating activity of the exopolysaccharide produced by the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913 was optimal at pH 7 (Prasertsan *et al.*, 2006). Gong *et al.* (2008) reported that the crude bioflocculant produced by *Serratia ficaris* flocculated well at pH 5-7 whereas, Gao *et al.* (2009) and Zhu *et al.* (2012) found that the flocculating activity of bioflocculants produced by *Rothia* sp. and *Chlamydomonas reinhardtii* were maximal at pH 9 and 10 respectively.

5.4.3 Time course of MBF-UFH production

The time course of MBF-UFH production by *Bacillus* sp. AEMREG7 is depicted in Figure 5.4. The flocculating activity curve was parallel to a typical bacteria growth pattern, an indication that the production of MBF-UFH was associated with cell growth and not by cell autolysis (Deng *et al.*, 2005). Some reports showed that most bioflocculants are produced by microorganisms during growth (Shih *et al.*, 2001). Figure 5.4 also shows that further increase in cultivation time to 192 h resulted in a decrease in flocculating activity with concomitant decrease in the viable cells and optical density (OD) which was observed as the nutrient got depleted. As the metabolic activity changes, cell death as well as the accumulation of toxic metabolic wastes negatively affect the production rate of MBF-UFH. Furthermore, the deflocculation enzymes released as a result of cell autolysis may have degraded MBF-UFH in

the medium, thereby reducing their flocculating activity (Gong *et al*., 2003; Lu *et al*., 2005). Similar trends were observed by Gong *et al*. (2008), Elkady *et al*. (2011) and Ugbenyen *et al*. (2012) with bioflocculants produced by *Serratia ficaria, Bacillus mojavensis* strain 32A, *Cobetia* sp. that attained maximum flocculating activity at 72 h after which flocculating activity declined with cultivation time. Contrary to the above, Zheng *et al*. (2008) found that the production of bioflocculant by *Bacillus* sp. F19 increases with an increase in cultivation time with the maximum flocculating activity reached after 36 h. In the findings of Gao *et al*. (2006), the flocculating activity of the bioflocculant produced by *Vagococcus* sp. W31 increased with cultivation time and reached maximum at 60 h.

5.4.4 Effect of temperature on the flocculating activity of MBF-UFH

Flocculation rate has been reported to increase with an increase in reaction temperature (Zhao *et al*., 2013). Table 5.3 shows the stability of MBF-UFH increased with increase in temperature. Chemical analyses of purified MBF-UFH showed that it is a glycoprotein composed of polysaccharide (76%) and protein (14%). The observed thermal stability is an indication of MBF-UFH predominantly composed of a polysaccharide. The decrease in flocculating activity noticed at 100 ^{o}C, might be due to the denaturation of the protein component of MBF-UFH (Sun *et al*., 2012). Similar findings were reported for a thermostable bioflocculant produced by *Klebsiellae pneumonia* YZ-6 which retained more than 70% of its residual flocculating activity at 70 ^{o}C (Zhengshan *et al*., 2014).

5.5 Conclusions

The high flocculating activity possessed by the studied bioflocculant (MBF-UFH) under different physicochemical conditions indicates that this microorganism has the potential to be utilized on an industrial scale for bioflocculant production which could serves as a possible substitute for harzardous chemical flocculants commonly utilized in water treatment processes.

Furthermore, frequent usage of microbial flocculants will reduce water borne diseases caused by chemically-treated water and consequently reducing deformities among infants, elderly and immunocompromised persons; and hence ensuring proper growth and development of individual in the society.

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CHAPTER SIX

Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7

(Published in International Journal of Molecular Sciences)

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Abstract

A bioflocculant named MBF-UFH produced by *Bacillus* specie isolated from sediment samples of Algoa Bay of the Eastern Cape Province of South Africa was characterized. The bacterial identification was through 16S rDNA sequencing, nucleotide sequences were deposited in GenBank as *Bacillus* sp. AEMREG7 with accession number KP659187. The production of the bioflocculant was observed to be closely associated with cell growth. The bioflocculant had highest flocculating activity of 85.8% after 72 h of cultivation and approximately 1.6 g of purified MBF-UFH was recovered from 1 L of fermented broth of *Bacillus* sp. AEMREG7. Its chemical analyses indicated that it is a glycoprotein composed of polysaccharide (76%) and protein (14%). Fourier transform infrared spectroscopy (FTIR) revealed that it consisted of hydroxyl, amide, carboxyl and methoxyl as the functional moieties. Scanning electron microscopy (SEM) revealed the amorphous structure of MBF-UFH and flocculated kaolin clay particles. The maximum flocculating activity of 92.6% against kaolin clay suspension was achieved at 0.3 mg/mL over pH ranges of 3-11 with the peak flocculating rate at pH 8 in the presence of Mg^{2+}. The bioflocculant retained high flocculating activity of 90% after heating at 100 °C for 1 h. MBF-UFH appears to have immense potential as alternative to conventional chemical flocculants.

Keywords: *Bacillus* sp. AEMREG7; MBF-UFH; flocculating activity; glycoprotein; thermostable

6.1 Introduction

The flocculation stage is a major phase in water treatment technology for the exclusion of both organic and inorganic pollutants (Jarvis *et al*., 2012). Flocculants are substances used in the clumping of colloids, cells and suspended solids into larger sizeable flocs that can be removed effectively from solution by sedimentation (Hu *et al*., 2006). Applications of flocculants have included downstream processes in the fermentation industries, as well as drinking and waste-water treatment facilities (Shih *et al*., 2001). Flocculants may be categorized into three groups: organic flocculants, such as polyacrylamide derivatives; inorganic flocculants, such as polyaluminum chloride and ferric chloride; naturally-occurring flocculants, such as chitosan, sodium alginate and bioflocculants. However, the choice of flocculants has a major influence on the performance of the flocculation process (Zhang *et al*., 2014).

Bioflocculants stands out among others, as they have the advantages of innocuousness, biocompatibility, biodegradability and environmentally friendliness, unlike organic and inorganic flocculants, which are toxic and whose degradation intermediates are difficult to remove from the environment (Yang *et al*., 2012). Besides, organic flocculants, such as polyacrylamide and polyethylene imine derivatives, have been implicated in adverse human health effects (Nwodo *et al*., 2014). An outstanding example is aluminum salts, which have been demonstrated to cause Alzheimer's disease in humans (Banks *et al*., 2006). Conversely, the enormous advantages associated with bioflocculants motivate its consideration as an alternative, hence the vast interest in the scientific and industrial community worldwide (Nwodo *et al*., 2012). Bioflocculants are mostly composed of macromolecular substances, such as polysaccharides, protein, lipids and nucleic acids (Zheng *et al*., 2008). The chemical composition and flocculating efficiency of bioflocculants depends on some factors, including the nature of the environment in which bioflocculant-producing microorganisms are isolated, the media compositions in which the microorganisms are cultivated, the functional groups and

molecular weight of the bioflocculant (Liu *et al.*, 2010). Several studies have demonstrated the efficiencies of bioflocculants in the treatment of drinking/waste waters and other downstream processing (Nwodo *et al.*, 2014). Nevertheless, low flocculating efficiency, low yields and high cost of production compared with the conventional flocculants are major limitations to large-scale production and application of bioflocculants (Zhang *et al.*, 2010; Abdel-Aziz *et al.*, 2011; Mao *et al.*, 2011). Consequently, there is a need for continual exploration for novel microbes with efficient bioflocculant-producing capability. Thus, efficient bioflocculant-producing microbes should possess bioflocculants with high flocculating efficiency and yields (He *et al.*, 2002; Yang *et al.*, 2007).

Marine environment have remained a potential reservoir of microbes with novel metabolites, and the exploitation of this ecosystem through prospecting for microbes with novel metabolites is still very nascent (Li *et al.*, 2008; Zhang *et al.*, 2010; Nwodo *et al.*, 2013; Mabrouk, 2014; Manivasagan *et al.*, 2014). Consequently, the current study aimed at characterizing the bioflocculant produced by *Bacillus* sp. AEMREG7 isolated from a sediment sample of Algoa Bay in the Eastern Cape Province of South Africa. In addition, the flocculating efficiency of the produced bioflocculant was later compared with the synthetic flocculants.

6.2 Results and Discussion

6.2.1 Time course of bioflocculant production

Figure 6.1 shows the relationship between MBF-UFH production and cell growth over a cultivation time of 192 h. Most microorganisms produced bioflocculant during the exponential growth phase (Abdel-Aziz *et al.*, 2011). The flocculating activity of the bioflocculant increased gradually with an increase in cultivation time. Generally, the production of MBF-UFH by *Bacillus* sp. AEMREG7 was observed to correspond with cell growth. Nonetheless, the highest flocculating activity of 85.8% was attained at 72 h in the late exponential growth phase, thus

indicating that the production of MBF-UFH was closely associated with cell growth (Ugbenyen *et al.*, 2012). However, the decrease in the flocculating activity observed after 72 h may be due to the deflocculating enzyme released by the microorganism during the death phase of the cells. These results were similar to the findings of Wu and Ye (2007) in which the bioflocculant production was synchronous with cell growth and reached maximum flocculating activity in the late logarithmic growth phase and early stationary phase. Furthermore, an increase in cultivation time led to a decrease in bioflocculant production as the flocculating activity decreased steadily. This decrease in flocculating activity of MBF-UFH might be due to deflocculating enzymatic activities or accumulation of toxic metabolic wastes affecting the produced bioflocculant (Zheng *et al.*, 2008). Therefore, a cultivation time of 72 h was chosen for the subsequent experiments. It is apparent that MBF-UFH biosynthesis occurred during different microbial growth phases for different organisms (Mabinya *et al.*, 2012). Additionally, under optimum culture conditions, about 1.6 g of purified MBF-UFH was recovered from 1 L of fermentation broth of *Bacillus* sp. AEMREG7, which is higher than 0.4 g of the purified bioflocculant produced by *Aspergillus flavus* (Aljuboori *et al.*, 2013). On the contrary, the bioflocculant production by *Chryseobacterium daeguense* W6 was released into the culture broth in the death phase of cell growth, when the nutrient in the medium had been depleted (Liu *et al.*, 2010).

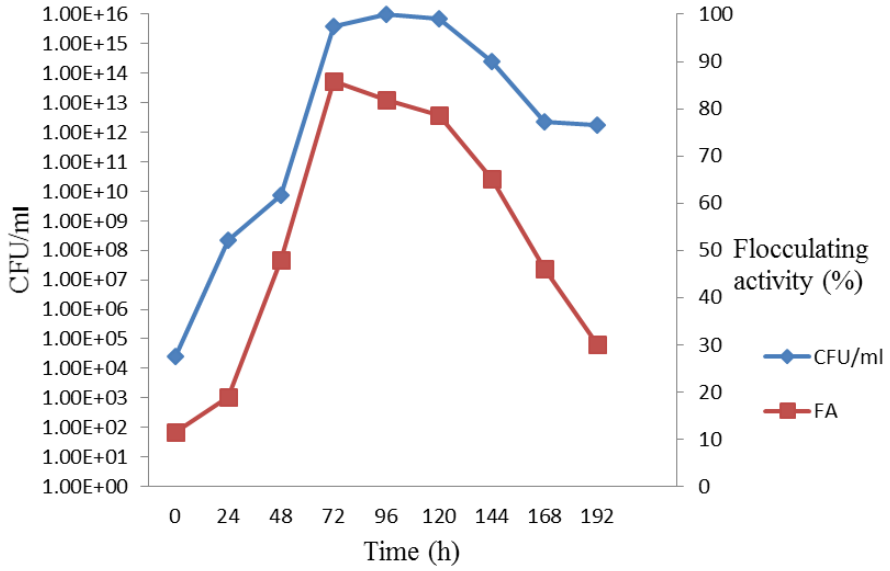

Figure 6.1. Time course of bioflocculant production by *Bacillus* sp. AEMREG7. Shows the relationship between bacteria growth (bacterial counts expressed in CFU/ml) and bioflocculant (MBF-UFH) production.

6.2.2 Fourier transform infrared (FTIR) analysis

The functional moieties in the molecular chain of MBF-UFH were identified with FTIR spectrophotometry (Figure 6.2). The spectrum displayed an intense broad stretching peak at 3440 cm^{-1}, which indicated the presence of a hydroxyl or amide group (He *et al.*, 2010; Kavita *et al.*, 2013). The water solubility of the bioflocculant was attributed to the presence of hydroxyl group forming a hydrogen bond with a water molecule. A weak peak observed at 2367 cm^{-1} was either CO_2 adsorption or may be from the amine group (Ahluwalia *et al.*, 2005). Furthermore, an asymmetric stretching peak was at 1638 cm^{-1}, which showed the presence of carbonyl group stretching vibration in the peptide (Yin *et al.*, 2014). The peak detected at 1412 cm^{-1} could be ascribed to the symmetric stretching of the –COO− group (Nwodo *et al.*, 2013). The presence of carboxyl groups provides more adsorption sites for particle attachment; so many particles can be adsorbed to the long molecular chain of the bioflocculant (Luo *et al.*, 2014). The absorption peaks ranging from 1000–1200 cm^{-1} were designated to C–O–C and C–O, which indicated the presence of polysaccharides (Freitas *et al.*, 2009; Nie *et al.*, 2011). The

peak at 1077 cm^{-1} indicated the presence of methoxyl groups (Zheng *et al.*, 2008). The characteristics of the FTIR spectrum showed the presence of hydroxyl, amide, carboxyl and methoxyl groups in the MBF-UFH structure as the main functional moieties preferred for flocculation (Wan *et al.*, 2013).

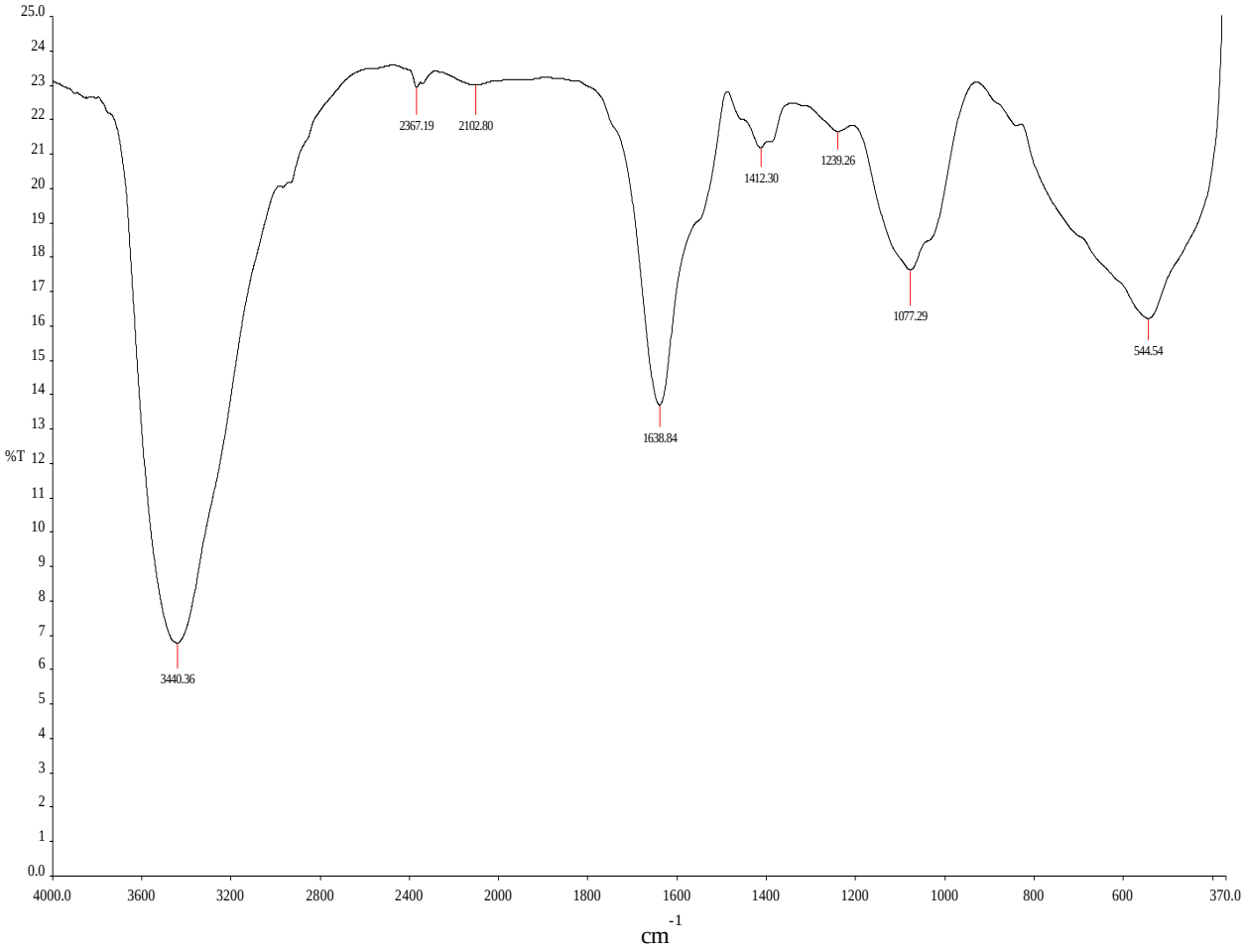

Figure 6.2. Fourier transform infrared (FTIR) spectra of purified MBF-UFH produced by *Bacillus* sp. AEMREG7.

6.2.3 Scanning electron microscopy (SEM) imaging

Surface morphology structure of the bioflocculant was explicated by scanning electron microscopy (SEM). Scanning electron microscopy is a type of electron microscope that divulges the image of a sample by scanning it with a high-energy beam of electrons in a raster scan pattern (He *et al.*, 2010). The electrons interrelate with the atoms that make up the sample,

producing signals that contain information about the sample's surface structure (Wan *et al.*, 2014). The SEM image showed that MBF-UFH has an amorphous structure of a compact nature (Figure 6.3A). The configuration of this bioflocculant may be accountable for its high flocculation efficiency. Before the flocculation process, the kaolin clay particles appeared to be fine and scattered (Figure 6.3B), and after the flocculation process, the functional moieties in the molecular chain of MBF-UFH were used for attachment on the kaolin clay particle. Consequently, the interaction between the bioflocculant and kaolin clay particle resulted in the formation of flocs that later aggregated to larger sized flocs, which precipitated out of the suspension as the result of gravity (Figure 6.3C). This observation showed that bridging played a vital role in the flocculation process (Ugbenyen *et al.*, 2014). These results concur with previous findings for the purified bioflocculants produced by *Nocardiopsis aegyptia* sp. nov. and a consortium of *Streptomyces* and *Cellulomonas* species and (Mabrouk, 2014; Nwodo *et al.*, 2014).

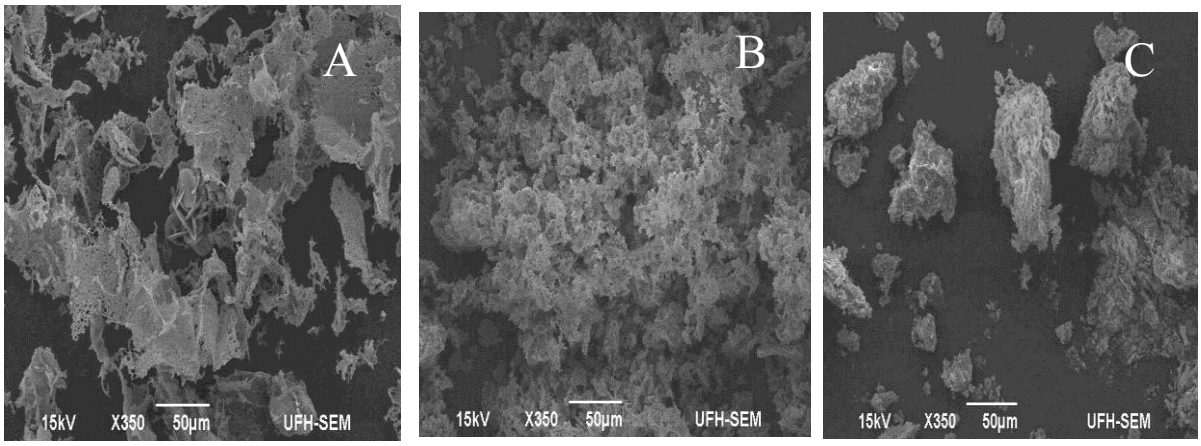

Figure 6.3. SEM imaging of purified MBF-UFH produced by *Bacillus* sp. AEMREG7 (A), Kaolin clay particles (B), Kaolin clay suspension flocculated with MBF-UFH (C).

6.2.4 Elemental analysis by Energy dispersive X-ray spectroscopy (EDX)

The EDX analysis of the purified MBF-UFH revealed its elemental compositions in mass proportion (% *w*/*w*): C (17.21), N (6.66), O (40.04), Na (5.21), Mg (5.02), P (7.90), S (0.60), Cl (6.11), K (1.63) and Ca (9.63). Likewise, the presence of non-sugar components is usually small; nevertheless, they may provide flexibility and stabilize MBF-UFH (Nwodo *et al*., 2014). In agreement with our observation, Singh *et al.* (2011) documented that the exopolysaccharide produced by *Bacillus licheniformis* was composed of the following elements in mass proportion (% *w*/*w*): C (38.48), O (55.71), P (0.50), S (1.47), Cl (1.24), Ca (0.25), Na (2.34). Furthermore, Mabrouk (2014) reported analogous cases with the bioflocculant produced by *Nocardiopsis aegyptia* sp. nov., which was composed of the following elements in mass proportion (% *w*/*w*): Na (7), P (6.9), S (1.9), Cl (66.3), K (16.9), Cu (0.5), Zn (0.6).

6.2.5 Physicochemical properties of purified bioflocculant

6.2.5.1 MBF-UFH dosage

The effect of MBF-UFH dosage (concentration) on flocculation efficiency for kaolin clay suspension was examined in an attempt to determine the most cost-effective dose for flocculation process. Under optimal conditions, the maximum flocculating activity is usually attained at the optimal bioflocculant dosage (Salehizadeh and Yan, 2014). The flocculating activity of purified MBF-UFH was investigated in a range of 0.01–0.5 mg/mL (Figure 6.4). The flocculating activity of 61.5% was achieved at 0.01 mg/mL, and a further increase in MBF-UFH dosage resulted into a gradual decrease in flocculating activity. Nevertheless, the optimum bioflocculant dosage range for effective flocculation efficiency of over 90% was observed between 0.1 and 0.3 mg/mL, with the highest flocculating activity of 92.6% attained at 0.3 mg/mL. However, there was no significant increase in the flocculating activity of MBF-UFH when the dosage was increased from 0.1 up to 0.3 mg/mL (Figure 6.4). Although, the flocculating activity of MBF-UFH was low at 0.01 mg/mL (61.5%) compared to the flocculating activity noticed in the optimum range of 0.1–0.3 mg/mL (above 90%). At a lower

dosage, MBF-UFH was relatively small to destabilize the negative charge of the kaolin clay particles, and the excess kaolin particles restabilized and increased the turbidity of the suspension; lower flocculating activity was noted in comparison to the flocculating rate observed at 0.1 mg/mL (Figure 6.4). This showed that the bridging effect of MBF-UFH was lower at 0.01 mg/mL compared to when it was at a higher dosage. On the contrary, the flocculating activity slightly decreased to 87.7% on increasing MBF-UFH dosage to 0.5 mg/mL compared with the flocculating activity of over 90% observed at an optimum dosage range between 0.1 and 0.3 mg/mL. This observation was in agreement with those reported elsewhere (Wang *et al.*, 2011; Zaki *et al.*, 2013; Zhao *et al.*, 2013). The decrease in flocculating activity of MBF-UFH observed at 0.5 mg/mL might be due to the over addition of the negatively-charged MBF-UFH, generating strong repulsive forces between the kaolin clay particles and the bioflocculant. These processes restabilized the suspended particles, increasing the viscosity of the suspension, blocking the adsorption sites and noticeably reduced floc formation (Yuan *et al.*, 2011). These findings are consistence with previous studies reported by Elkady *et al.* (2011) and Zheng *et al.* (2008). It has been extensively documented that a lower concentration of bioflocculants with a high flocculating efficiency will contribute to treatment cost reduction. Besides, information on the dosage requirement is substantial for future prospects in water treatment applications (Yin *et al.*, 2014).

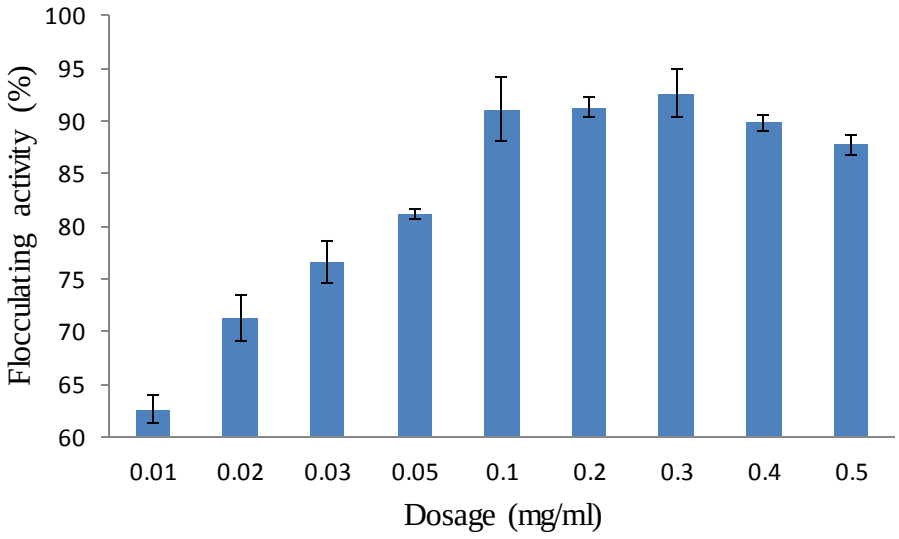

Figure 6.4. Effect of dosage on the flocculating activity of purified MBF-UFH produced by *Bacillus* sp. AEMREG7.

6.2.5.2 Effect of cations on the flocculating activity of MBF-UFH

In order to examine the effect of cations on the flocculating activity of MBF-UFH, a 4 g/L kaolin suspension in distilled water was used as the test material. Flocculation assays were set up in which $CaCl_2$ was replaced by other cations when necessary, and the synergistic effects of cations were found in all of the cations investigated, except for Fe^{3+} (Table 6.1). It was observed that oppositely-charged cations reduced the particle surface charge density, so that the MBF-UFH molecules and kaolin particles could draw closer to each other, and the attractive forces were capable of overcoming the electrostatic repulsion to become effective. This interaction brought about the flocculation process of MBF-UFH. Factors influencing the effects of cations on flocculation vary; this may include surface charge capacity and the pH of the reaction mixture (Shih *et al.*, 2001; Wang *et al.*, 2011). The studied bioflocculant alone flocculated kaolin clay suspension over 70% without the addition of cations. Consequently, high flocculating efficiency without cations implies reduction in treatment costs and also environmental pollution. Prior to this study, very few bioflocculants have been reported to have a high flocculation rate without cations' aid (Zhao *et al.*, 2013).

The synergistic effect of cations was observed most with monovalent and divalent cations, showing substantial enhancement on flocculating activity (with over 20%), with the highest flocculating rate observed with Mg^{2+} (Table 6.1). The flocculating activity of MBF-UFH was inhibited by Fe^{3+}. However, a variation in flocculating activity was observed with Al^{3+}, while Fe^{3+} may be accounted for by the surface charge availability of these cations in relation to the charge distributions of the bioflocculants. The trivalent cation could change the surface charge of kaolin particles and cover the adsorption sites, which led to a decrease in flocculating activity noticed in the presence of Al^{3+}. Nonetheless, another possible scenario may be due to the effect of cation on the ionic strength of the suspension, which might also affect the conformations of MBF-UFH chain. Since MBF-UFH showed a flocculation rate at a lower dosage, it showed that bridging was substantially involved in the flocculation process. However, bridging flocculation requires that the MBF-UFH chain remains in an extended conformation; a higher charge density or higher proportion of charged groups favored the presence of the extended conformation of MBF-UFH chain, provided that all of the groups are either anionic or cationic by means of the electrostatic repulsion.

Replacing divalent cations by trivalent cations increases notably the ionic strength of the suspension, and therefore, the electrostatic repulsive forces among charged groups of the bioflocculant chain decrease notably. This fact causes MBF-UFH chain conformation changes towards a more compact conformation, which is not able to form bridges. This might probably be the main cause of the poor flocculation ability of MBF-UFH in the presence of Al^{3+} and Fe^{3+}. In addition, the synergistic effect of Al^{3+} with MBF-UFH and the antagonistic effect of Fe^{3+} with MBF-UFH thus lead to high flocculating activity for the Al^{3+} and lower flocculating activity in the presence of Fe^{3+} when the synergistic effects of trivalent cations on the flocculating activity of MBF-UFH were compared. There have been several reports on divalent cations stimulating the flocculating activity of bioflocculants (Wu and Ye, 2007; Nwodo *et al.*,

2012). Ostensibly, it seems that the monovalent and divalent cations help to neutralize negative charges on MBF-UFH and the suspended kaolin particles, shortening the distance between them, increasing the initial adsorption of MBF-UFH onto the kaolin particle and thus leading to floc formation and sedimentation (Wu and Ye, 2007; Yim *et al.*, 2007). It has been well documented that the addition of cations to suspensions is necessary to induce the effective flocculation capability of a bioflocculant; the effects of cations on the flocculating activity of MBF-UFH are similar to the previous studies by Okaiyeto *et al.* (2014) on the bioflocculant produced by *Micrococcus* sp. Okoh. On the other hand, the bioflocculants produced by *Aspergillus flavus* and *Klebsiella pneumoniae* were cation independent, which showed an outstanding performance in kaolin clay suspension without the addition of metal ions (Aljuboori *et al.*, 2013; Zhao *et al.*, 2013).

Table 6.1. Effect of cations on the flocculating activity of purified MBF-UFH

Cation	Na^{+}	K^{+}	Li^{+}	Mg^{2+}	Ca^{2+}	Mn^{2+}	Fe^{3+}	Al^{3+}	BA
FA (%) ± SD	93.29	92.18	92.46	94.77	91.13	92.68	9.90	68.36	70.94
	1.47	1.49	1.54	0.71	2.35	1.12	5.14	2.51	3.12

*FA-Flocculating activity, BA- MBF-UFH alone.

6.2.5.3 Effect of pH on the flocculating activity of purified MBF-UFH

The pH of reaction mixtures is a key factor influencing the flocculation process (Zaki *et al.*, 2013). One of the ways in which pH influences flocculating activity is by affecting the stability of suspended particles and floc formation (Ugbenyen *et al.*, 2014); the effect of pH on the flocculating activity of purified MBF-UFH was evaluated, and the results are depicted in Figure 6.5. The bioflocculant had a strong flocculating activity over a wide range of pH, 3–11. There was no significant difference in the flocculating activity of MBF-UFH between the pH ranges 3–8. However, the peak flocculating activity of 95.4% was achieved at weak alkaline condition, pH 8, and a sharp decline in flocculating activity was noticed at pH 9, which might

be due to the fact that the surface charge spatial arrangement is both pH and temperature dependent. Thus, it would be safe to assume that the spatial charge arrangements for flocculation were not ambient at pH 9 and within the alkaline range.

MBF-UFH was tolerant to the extreme pH and showed excellent flocculating activity in a strong acidic than basic condition. Hence, this suggested that MBF-UFH could be applied under acidic, neutral and alkaline circumstances, as it shows different electric states at different pH, which, in turn, influences the bridging efficiency of MBF-UFH for kaolin clay particles (Gao *et al.*, 2009; Zaki *et al.*, 2013). These observations were similar to the findings reported by Ugbenyen *et al.* (2014) in which the flocculating activity of the bioflocculant produced by a consortium of *Cobetia* and *Bacillus* species was over 70% across a wide pH range of 3–11 with the highest flocculating activity attained at pH 8. The flocculating activity of the bioflocculant produced by *Bacillus* sp. F19 reached the maximum at pH 2 and maintained excellent flocculating activity within a range of pH 2–9. On the other hand, the bioflocculant produced by *Ruditapes philippinarum* flocculated over a wide pH range with the optimum flocculation rate between pH 7 and 9 (Gao *et al.*, 2009).

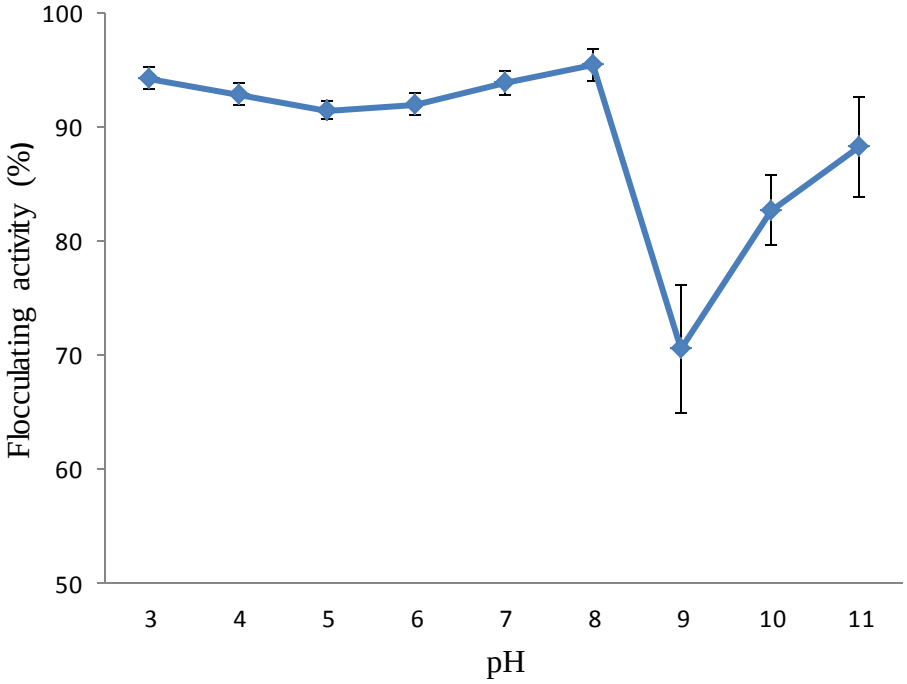

Figure 6.5. Effect of pH on the flocculating activity of purified MBF-UFH produced by *Bacillus* sp. AEMREG7.

6.2.5.4 Thermal stability of purified MBF-UFH

The thermal stability of the purified bioflocculant was examined at 50, 60, 70, 80, 90 and 100 °C for 1 h at each temperature. As depicted in Figure 6.6, MBF-UFH had an exceptional flocculating activity of 90% over all of the temperature regimes. The high flocculating efficiency showed by MBF-UFH at 100 °C indicated that it is thermally stable and consisted predominantly of polysaccharide (Lian *et al.*, 2008; Liu *et al.*, 2013). The thermal stability may be due to the presence of a hydroxyl group involved in the formation of hydrogen bonds in MBF-UFH structure (Ugbenyen *et al.*, 2014). The stability of MBF-UFH was similar to the bioflocculant produced by *Corynebacterium glutamicum*, which retained high flocculating activity of 96.9% at 80 °C, but the stability decreased slightly on increasing the temperature to 100 °C (Liu *et al.*, 2013). Besides, the bioflocculant produced by *Aspergillus flavus* was thermally stable, which retained high flocculating activity above 90% over a temperature range of 10–100 °C (Aljuboori *et al.*, 2013). Nevertheless, Salehizadel *et al.* (2000) reported a less

stable bioflocculant that lost about 50% of the flocculating activity after heating for 15 min at 100 °C.

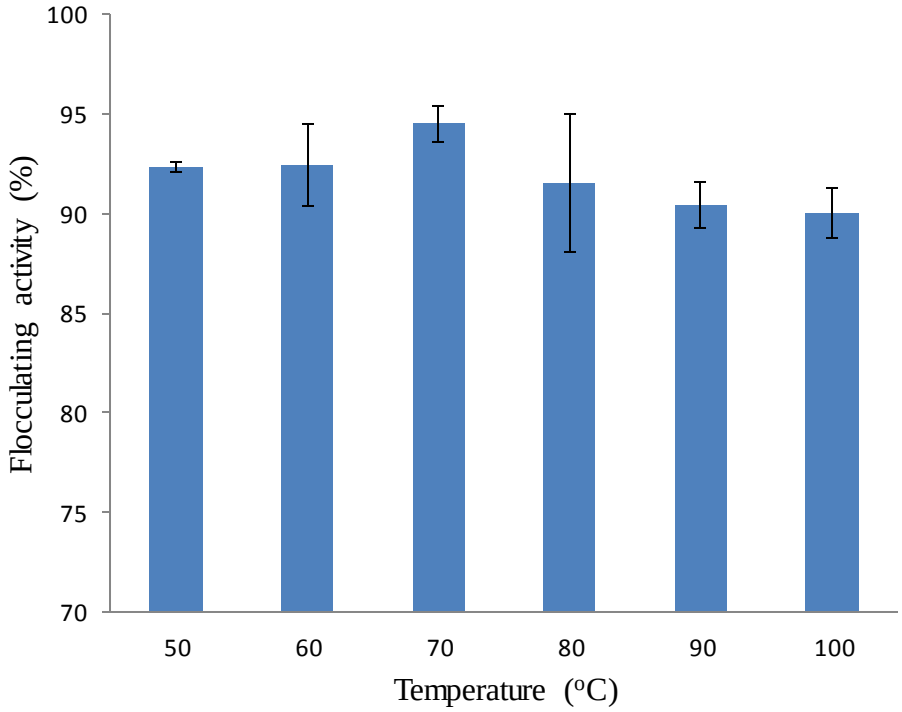

Figure 6.6. Thermal stability of purified MBF-UFH produced by *Bacillus* sp. AEMREG7.

6.2.6 Comparison of flocculating activity of MBF-UFH with conventional flocculants

The flocculating efficiency of MBF-UFH for kaolin clay suspension was compared with the conventional flocculants, such as aluminum chloride, ferric chloride and anionic polyacrylamide (Table 6.2). The maximum flocculation rate was achieved at the optimum concentration of these flocculants; and it was observed that polyacrylamide has the highest flocculating efficiency (94.30%), followed by MBF-UFH (91.1%), aluminum chloride (67.99%) and ferric chloride (42.78%). These findings showed that MBF-UFH was significantly more efficient than both aluminum chloride and $FeCl_3$, but slightly less efficient than polyacrylamide. However, since several studies have shown that polyacrylamide is neurotoxic, carcinogenic and also recalcitrant to degradation (Lian *et al.*, 2008), these results

suggest the great potential of MBF-UFH as an alternative to the commonly-used flocculants in waste/drinking water treatment.

Table 6.2. Comparison of flocculating activity of MBF-UFH with conventional flocculants

Flocculant	Conc. (mg/mL)	FA (%)
Aluminium chloride	1	67.99
$FeCl_3$	1	42.78
Polyacrylamide	0.1	94.30
Bioflocculant	0.1	91.1

*FA - Flocculating activity; Conc. - concentration

6.2.7 Proposed flocculation mechanism of MBF-UFH

The presence of a hydroxyl group in the molecular chain of MBF-UFH suggested that ionic bonds might be responsible for the interaction between MBF-UFH molecules and the kaolin clay particle, which led to the high flocculating efficiency observed in our study (Wan *et al.*, 2014). The force of adsorption may have likely come from hydrogen bonds that were formed between OH groups of MBF-UFH and the kaolin clay particles. Besides, the presence of the hydroxyl group might also be responsible for the high solubility property of the bioflocculant. In addition, its thermal stability could be linked to the presence of hydroxyl group involved in the formation of hydrogen bonds in the MBF-UFH structure (Ugbenyen *et al.*, 2014). Likewise, the presence of carboxyl groups in MBF-UFH indicated that there were chemical bonds existing between the COO^- groups and kaolin clay particles. The carboxyl groups in MBF-UFH molecules were used as binding sites for the kaolin particle mediated by Mg^{2+}. Therefore, we proposed that the flocculation process might be by bridging and charge neutralization. Bridging was proposed to be involved in the flocculation of MBF-UFH, since a high flocculating activity of 62.5% was observed even at a lower dosage of 0.01 mg/mL. Bridging occurred after the cation-kaolin complexes adsorbed onto the bioflocculant chains, leading to

the formation of three-dimensional flocs, which were capable of rapid setting (Wu and Ye, 2007). The MBF-UFH molecule extends the functional moieties beyond the particle's surface to the solution and attracts other particles in solution, thereby resulting in the aggregation of bigger flocs that can easily precipitate out of solution as a result of the bridging effect, and this phenomenon was confirmed by the SEM observations.

The role of cations is to increase the adsorption of MBF-UFH molecules on the surface of suspended particles by decreasing the negative charge on both MBF-UFH and the kaolin particle. When Mg^{2+} was added to the kaolin suspension, a decrease in charge density occurs, which eventually reversed the negatively-charged particles to positive and, hence, led to inter-particle bridging between kaolin particles and MBF-UFH. The divalent cation Mg^{2+} weakened the electrostatic repulsive forces between the bioflocculant and the kaolin clay particles; shortening the distance between them by compressing the double layer of kaolin particles and increasing the initial adsorption of MBF-UFH on the kaolin clay kaolin particles. Consequently, the functional moieties in the MBF-UFH molecule (hydroxyl, carboxyl and amine) adsorbed the kaolin clay particles and formed hydrogen bonds with them. Thus, the negatively-charged carboxyl group (COO^-) of MBF-UFH could react with the positively-charged site of the suspended kaolin particles. It can be assumed that cations stimulate flocculation by neutralization and stabilization of residual negative charges of the carboxyl group of the bioflocculant MBF-UFH forming a bridge that binds kaolin particles to each other (Wu and Ye, 2007).

Moreover, since the chemical composition analyses revealed that MBF-UFH is composed of both polysaccharide and protein, this showed that the flocculation process might involve multiple functional moieties from both polysaccharide and protein. Multiple functional moieties imply many adsorption sites for the kaolin particles, which led to the high flocculating efficiency observed with MBF-UFH (Verma *et al.*, 2012). Furthermore, it was observed that

MBF-UFH alone without cation flocculated kaolin clay suspension above 70%; this showed that the flocculation process could also be through charge neutralization as a result of electrostatic attraction between the positively-charged amino group of the protein part of MBF-UFH and the negative charge on the kaolin clay particle (Sun *et al*., 2012).

6.3 Experimental Section

6.3.1 Microorganism and culture conditions

Bacillus sp. AEMREG7 was previously isolated from a sediment sample of Algoa Bay in the Eastern Cape Province of South Africa, identified by 16S rDNA sequencing and preserved in 20% glycerol stock at -80 °C as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, South Africa. The activation medium was composed of 10 g tryptone, 3 g beef extract and 5 g NaCl in 1 L of filtered seawater (Ntsaluba *et al*., 2013). The medium was sterilized by autoclaving at 121 °C for 30 min and allowed to cool down at room temperature. The bacteria was activated by inoculating 5 µL of the glycerol stock into 5 mL of the activation medium and incubated overnight at 28 °C on a rotary shaker at 160 rpm. The growth medium was composed of 20 g glucose, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.3 g $(NH_4)_2SO_4$, 0.5 g urea, 0.5 g yeast extract, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl in 1 L of filtered seawater (Okaiyeto *et al*., 2013). After 24 h of fermentation, 2 mL of the fermented broth were inoculated into 50 mL of sterile growth medium incubated on rotary shaker at 160 rpm, 28 °C for 72 h. The flocculating activity of the cell-free supernatant was determined by flocculation assay after separating the bacterial cells from the culture broth by centrifugation at 4000 rpm for 30 min.

6.3.2 Determination of flocculating activity

A kaolin clay suspension was used as a model for the natural turbidity of raw surface water to determine the flocculating activity of the bioflocculant produced by *Bacillus* sp. AEMREG7.

Four grams of kaolin clay (Merck, Darmstadt, Germany) were suspended in 1 L of distilled water to make the kaolin suspension. Two milliliters of cell-free supernatant were added to 100 mL of kaolin suspension in 250-mL conical flask containing 3 mL of $CaCl_2$ (1% *w/v*). The mixture was agitated for 60 s, transferred to a 100 mL gradual measuring cylinder and allowed to stand for 5 min. The optical density (OD) of the supernatant was measured using a spectrophotometer (Helios Epsilon, USA) at 550 nm. The control experiment was set up by replacing the bioflocculant with 2 mL of freshly-prepared un-inoculated medium. The flocculating efficiency was calculated with the following equation:

$$Flocculating\ Efficiency\ (\%) = (A - B/A) \times 100\%$$

Where *A* and *B* are optical densities of the control and sample read at 550 nm, respectively.

6.3.3 Time course of MBF-UFH production

The time course of MBF-UFH production was done according the method described by Liu *et al.* (2010) and Nwodo *et al.* (2013) with some modifications. The culture broth (24 h old) was adjusted to OD_{660} 0.1; then, the standardized culture broth was inoculated into 200 mL of fermentation medium contained in 500 mL flasks. The flasks were placed in a rotary shaker at 160 rpm, 28 °C for 192 h. The production of MBF-UFH was monitored over time by withdrawing 10 mL of the culture broth at intervals of 24 h for flocculating activity determination and bacterial cell growth viable cell count (CFU/mL).

6.3.4 Bioflocculant purification

The purification of MBF-UFH was carried out according to the description of Cosa *et al.* (2013) and Li *et al.* (2013) and with some modifications. After 72 h of fermentation, the culture broth was centrifuged at 4000 rpm for 30 min at 15 °C. The viscosity of the culture broth was reduced by diluting with two volumes of distilled water and then centrifuged at 4000 rpm for 30 min in order to remove bacterial cells. The pellet was discarded, and two volumes of cold ethanol were added to the supernatant and kept at 4 °C overnight for ethanol precipitation. The

resulting precipitate was collected by centrifugation at 4000 rpm for 30 min at 15 °C and lyophilized to obtain crude MBF-UFH. The crude bioflocculant was dissolved in 100 mL of distilled water, to which one volume of a mixture of chloroform and *n*-butyl alcohol (5:2 *v*/*v*) was added, stirred for 60 s and kept overnight at room temperature. The upper phase was collected by centrifugation at 4000 rpm for 30 min at 15 °C, vacuum-dried and re-dissolved in distilled water. MBF-UFH solution was dialyzed against de-ionized water overnight and then lyophilized to obtain a purified MBF-UFH.

6.3.5 Chemical composition analysis of a purified MBF-UFH

The total sugar content of MBF-UFH was determined according to the phenol-sulfuric method described by Chaplin and Kennedy (1994) using glucose as the standard. The total protein contain was determined by the Bradford method described by Bradford (1976) using bovine serum albumin (BSA) as the standard. The functional moieties in the MBF-UFH molecule were identified using an FTIR analyzer (Perkin Elmer System 2000, Buckinghamshire, UK). Dried MBF-UFH sample was ground with KBr powder and pressed into pellets for FTIR spectra measurement in the frequency range of 4000−400 cm^{-1} (Wan *et al*., 2014). The surface morphology of MBF-UFH and kaolin clay particles was observed and elucidated using scanning electron microscopy (SEM) JEOL (JSM-6390LV, Tokyo, Japan). Five milligrams of MBF-UFH, kaolin clay and dried flocculated kaolin suspension were added on slides separately and fixed by air-drying. The fixed specimens were coated with gold and examined under SEM (Jain *et al*., 2013). The elemental analysis was carried out with SEM equipped with Noran Six 200 Energy Dispersive X-ray (JEOL Ltd., Tokyo, Japan) (Kavita *et al*., 2013).

6.3.6 Physicochemical properties of a partially-purified bioflocculant

6.3.6.1 MBF-UFH concentration

Different concentrations (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) of purified MBF-UFH were made with distilled water, and the flocculating activity of each was determined by the flocculation assay (Wang *et al*., 2011).

6.3.6.2 Effect of cations on flocculating activity

The synergistic effect of cations on the flocculating activity of purified MBF-UFH was investigated in accordance with the description of Zhao *et al.* (2013). The cation candidates included the metal of the following salts: NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$ and $AlCl_3$. The flocculation assays were determined as described above, but $CaCl_2$ was replaced by the solution of the aforementioned salts.

6.3.6.3 Effect of pH on the flocculating activity of MBF-UFH

The effect of pH on the flocculating activity was evaluated at a pH value ranging from 3–11; 4 g/L of the kaolin clay suspension was made and divided into 9 groups, and the pH of each group was adjusted accordingly with 0.1 M HCl and 0.1 M NaOH. The flocculating activity was determined while other conditions were kept constant (Wong *et al*., 2012).

6.3.6.4 Thermal stability of MBF-UFH

MBF-UFH was dissolved in 60 mL of distilled water; 10 mL each of the bioflocculant solution were heated at different temperatures 50, 60, 70, 80, 90 and 100 °C for 1 h (Sun *et al*., 2012). The bioflocculant solutions were allowed to cool, and their resulting flocculating activities were measured at room temperature, as described elsewhere (Nie *et al*., 2011).

6.3.7 Comparison of flocculating efficiencies of MBF-UFH and conventional flocculants

The flocculating efficiency of MBF-UFH and the conventionally-used chemical flocculants were compared by the flocculation assay in accordance with the description of Ugbenyen *et al.*

(2014) with some modifications. The chemical flocculants included: Alum, ferric chloride, anionic polyacrylamide (BDH chemicals Ltd.; Poole, UK, molecular weight ≥ 5000 KDa). Each of the flocculants was prepared by dissolving an appropriate concentration in distilled water, and jar tests were employed as described by Wang *et al.* (2010); the flocculating activities were measured separately afterwards.

6.3.8 Statistical analysis

All data were treated in replicates, and the mean values were taken. Data were subjected to one-way analysis of variance (ANOVA) using the MINITAB Student Release 12 statistical package. A significance level of $p < 0.05$ was used.

6.4. Conclusions

Bioflocculants are proposed to possess enormous industrial and biotechnological significance consequent to the advantages associated with them over conventionally-used flocculants. MBF-UFH produced by *Bacillus* sp. AEMREG7 under optimum fermentation conditions of 28 °C, 160 rpm and pH of 6 showed high flocculation activity of 83.2%. Bioflocculant production was, likewise, observed to be closely associated with cell growth. MBF-UFH's composition was predominantly polysaccharides and proteins. The FTIR spectrum indicated the presence of hydroxyl and carboxyl in its molecular chain, which is important for flocculation. The SEM imaging illustrated the morphology and structural arrangements of MBF-UFH. As compared to conventionally-used flocculants, MBF-UFH demonstrated a high flocculating activity at a low dosage; a characteristic feature that demonstrates its suitability for industrial application. Thus, MBF-UFH potentially stands as an alternative candidate to chemical flocculants, hence making it attractive for further research and possible development for industrial-scale application

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CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PROSPECTS

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Due to the detrimental effects of the synthetic flocculants commonly utilized in water treatment, microbial flocculants have in recent years been the focus of attention from a wide section of the scientific community (He *et al.*, 2004; Mabinya *et al.*, 2012). The use of the synthetic flocculants in water treatment is increasingly becoming questionable considering their adverse effects on human being and the environment (Li *et al.*, 2009). Hence, the usage of synthetic flocculants has been discouraged in most developed countries (Ho *et al.*, 2010). In addition, environmental considerations also dictated the development of strong, economically viable, and eco-friendly substitutes to conventional synthetic flocculants (Li *et al.*, 2009). In response to these challenges, various universities and research institutes have been directing more research focus towards the development of new bioflocculants with better prospects for application (Sun *et al.*, 2012).

Bioflocculants are metabolic products of microorganisms produced during their growth and can be composed of the following compounds: glycoprotein, polysaccharide, protein, cellulose, lipid, glycolipids and nucleic acids (Lu *et al.*, 2005; Lian *et al.*, 2008; Zheng *et al.*, 2008). Among these biomacromolecules, polysaccharides draw more attention especially with regard to wastewater treatment (Crini, 2005; Raza *et al.*, 2011). Activated sludge, soil and sediments, river and deep sea water samples have proven to be important sources for isolating bioflocculant-producing microorganisms (Salehizadeh and Shojaosadati, 2001; Subramanian *et al.*, 2010; Cosa *et al.*, 2011; Ugbenyen *et al.*, 2012; Ntsaluba *et al.*, 2013; Nwodo *et al.*, 2013; Okaiyeto *et al.*, 2015). Furthermore, these naturally occurring and environmentally-friendly flocculants can easily be produced through a fermentation processes (He *et al.*, 2010). The composition and properties of bioflocculants not only vary depending on the type of bioflocculant-producing microorganisms (BPMs), but are also influenced by the composition of media and environmental conditions, hence the differences observed in the nature of the charge carried by different bioflocculants (Wu and Ye, 2007).

In recent times, the enormous advantages associated with bioflocculants have attracted considerable scientific attention (Nwodo *et al.*, 2012). Bioflocculants are innocuous, environmentally friendly and attractive alternatives to existing chemical flocculants, because of their nontoxic, harmless characters, biodegradability; lack of secondary pollution from degradation intermediates and their environmentally inert nature (Li *et al.*, 2009; Yang *et al.*, 2012). They may potentially be applied in drinking and wastewater treatment, downstream processing as well as fermentation processes (Elkady *et al.*, 2011; Mao *et al.*, 2011).

Despite numerous studies in the past years, bioflocculant yield and low flocculating activity remain key factors limiting the wide-scale applications of these flocculants (Li *et al.*, 2003). New studies directed at discovering new bioflocculants with higher flocculating activity and improving flocculant yield have become the primary focus area recently (Li *et al.*, 2013). However, a major and persistent stumbling block for the commercial application of bioflocculants remains the high production costs compared with inorganic and synthetic flocculants (Liu *et al.*, 2010). In order to reduce production costs, several approaches have been adapted including the following: screening and generating mutant strains with high bioflocculant producing potentials, utilizing low-cost substrates and optimizing fermentation conditions for optimum bioflocculant production (He *et al.*, 2002; Wang *et al.*, 2007; Yang *et al.*, 2007). Continual exploration for microbes with high bioflocculant yield and high flocculation efficiency has therefore, become a subject of intensive investigations globally (Ugbenyen *et al.*, 2012). Consquently, bioflocculant-producing microorganisms were isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa. The potential of the bacterial isolates for bioflocculant production were investigated and those with high flocculating activity were used in this study.

7.1 Optimization of culture conditions

Optimization of the cultivation process is a rather powerful approach to improve the yield of by-products (Nwodo and Okoh 2013; Pathak *et al.*, 2014). The production of bioflocculant is highly influenced by many factors, such as the constituents of the culture medium and environmental conditions. Therefore, these factors need to be optimized in order to enhance yield, productivity and the flocculating efficiency of the bioflocculant (Salehizadeh and Yan, 2014). It is well documented that carbon and nitrogen sources significantly favours bacterial growth and bioflocculant production along with other constituents such as salt ions, most notably divalent cations (Sheng *et al.*, 2006). This current study also investigated the effects of various factors for the purpose of optimizing culture conditions in order to improve bioflocculant production as well as the flocculating efficiency.

7.1.1 Effect of inoculum size on bioflocculant production

Inoculums size is a critical factor that influences the growth of microorganisms as well as bioflocculant production (Wang *et al.*, 2011). In this current study, the inoculum size conducive for *Bacillus toyonesis* AEMREG6 production of bioflocculant REG-6 was 4% (v/v) (Figure 3.4), with 5% (v/v) inoculum size supporting maximum production of MBF-W7 by *Bacillus* sp. (Table 4.1), whereas, for the production of MBF-UFH by *Bacillus* sp. AEMREG7, 3% (v/v) inoculum size was the most appropriate (Figure 5.2). The plateauing in flocculating activity observed may be attributed to excess inoculum size, which causes an excessive niche overlap, resulting in inhibition of further bioflocculant production due to the limit of nutrient distribution (Salehizadeh and Shojaosadati, 2001). According to Li *et al.* (2009), optimal inoculum size allows for the adaptation of the microorganisms to the production medium and consequently promotes the production of bioflocculant. Ntsaluba *et al.* (2013) reported the highest flocculating activity of 92% by a flocculant produced by a mixed culture of *Methylobacterium* sp. and *Actinobacterium* sp. which was achieved with 1% (v/v) inoculum

size and that increasing inoculum size did not lead to a corresponding increase in flocculating activity. The use of 2% (v/v) inoculum size in the production medium recorded the highest flocculating rate of 86.6% for bioflocculant IH7 produced by *Aspergillus flavus*. The maximum yield of bioflocculant MBF-7 produced by *Klebsiella pneumonia* was obtained with an inoculum size of 10% (v/v) (Zhong *et al.*, 2014). In the case of *Bacillus licheniformis*, the maximum flocculating activity of the medium was 99.2% at the optimal inoculum size of 1% (v/v), allowing enough acclimatization of the strain to the medium to promote bioflocculant production, and shortening the stagnant phase period (Li *et al.*, 2009). The highest bioflocculant production by *Klebsiella mobilis* was achieved with inoculum size of 5%. A further increase in inoculum size did not affect the bioflocculant activity (Wang *et al.*, 2007).

7.1.2 Effect of carbon and nitrogen sources on bioflocculant production

Carbon and nitrogen sources are considered to influence bioflocculant production (Sheng *et al.*, 2006). The importance of carbon and nitrogen sources has been reported to have a crucial effect on the production of bioflocculant (Salehizadeh and Shojaosadati, 2001), which may differ with different bioflocculant-producing microorganisms (Cosa *et al.*, 2011). In the majority of reported studies, the production of such bioflocculants entailed the utilization of expensive substrates, thus leading to the problem of high production costs. Hence, there is need to seek for a suitable carbon source that can give the best bioflocculant production among these conventional carbon sources. Glucose had the most pronounced effect because it gets readily utilized by microorganisms compared to other sugars; hence it has been reported as a preferred carbon source in various studies for bioflocculant production by various microorganisms (Li *et al.*, 2009; Xia *et al.*, 2008). In this present study, the carbon sources for the *Bacillus* sp. were investigated to determine the most suitable one for optimal bioflocculant production. Glucose, maltose, sucrose and Na_2CO_3 were the favourable carbon sources for bioflocculant REG-6 production by *Bacillus toyonensis* AEMREG6 (Figure 3.1). With maltose as the most favoured,

all other carbon sources tested which included, glucose, fructose, lactose and starch significantly supported bioflocculant MBF-W7 production by *Bacillus* sp. (Table 4.1), thus indicating that the microorganism could utilize a range of sugars such as monosaccharides, disaccharides and polysaccharides for cell growth (He *et al.*, 2012). From an industrial application point of view, maltose seems a good carbon source supplement considering that it is cost-effective compared to other carbon sources (He *et al.*, 2012). On the other hand, MBF-UFH production was greatly improved when the medium was singly supplemented with glucose, sucrose and lactose while Na_2CO_3 supplemented medium partially supported MBF-UFH production (Table 5.1). Several studies reported in literature supported the results of this study. For example, sucrose, glucose and starch were favourable for IH-7 production by *Aspergillus flavus* while fructose and glycerol were least preferred. The highest production of IH-7 was achieved with sucrose as carbon source (Aljuboori *et al.*, 2013). Starch, sucrose and glucose were also reported as favourable carbon sources for *Aspergillus parasiticus* in the production of bioflocculant (Deng *et al.*, 2005). Li *et al.* (2009) reports indicated that sucrose, starch and ethanol were favourable carbon sources for bioflocculant production by *Bacillus licheniformis*, while lactose inhibited the production of bioflocculant.

The best nitrogen sources for the secretion of REG-6 by *Bacillus toyonensis* AEMREG6 were NH_4NO_3, $(NH_4)_2SO_4$ and $NaNO_3$ (Figure 3.2), a finding that is largely similar to the results obtained for MBF-W7 production by *Bacillus* sp. in which both NH_4NO_3 and $(NH_4)_2SO_4$ were found to be suitable nitrogen sources (Table 4.1). From these results, it showed that these bacterial strains prefer inorganic nitrogen sources for bioflocculant production compared to organic nitrogen sources tested. On the other hand, the production of MBF-UFH by *Bacillus* sp. AEMREG7 was favoured in the medium supplemented with both inorganic and organic nitrogen sources (Table 5.1). Several studies in the literature have been documented in which inorganic nitrogen source (Zhang *et al.*, 2002; Yin *et al.*, 2014; Okaiyeto *et al.*, 2015), organic

nitrogen source (Lu *et al.*, 2005; Nwodo *et al.*, 2013; Abdul-Razack *et al.*, 2014; Tang *et al.*, 2014) or the combination of both inorganic and organic nitrogen sources have been used for bioflocculant production (Gao *et al.*, 2006; Yang *et al.*, 2012; Zhao *et al.*, 2013).

Furthermore, some other studies have also revealed the variation in the level of utilization of nitrogen sources among different microorganisms. For example, organic nitrogen sources (peptone, yeast extract and urea) were effectively used in the production of IH-7 by *Aspergillus flavus*, while $(NH_4)_2SO_4$ and $NaNO_3$ poorly affected its production (Aljuboori *et al.*, 2013) whereas, for the fungus, *Mucorrouxii*, yeast extract or ammonium sulphate favoured the production of bioflocculant, with ammonium sulphate the most preferred, resulting in flocculating activity of more than 95%. Other organic or inorganic nitrogen sources were poorly utilized (Abdel-Aziz *et al.*, 2012). Many reported strains can either use organic nitrogen sources or a combination of organic and inorganic nitrogen sources to produce bioflocculants. For example, peptone (organic nitrogen source) and sodium nitrate (inorganic nitrogen source) provided the best nitrogen source for *Aspergillus parasiticus* for the production of the bioflocculant, whereas with $(NH_4)_2SO_4$ no flocculant was produced (Deng *et al.*, 2005). Compared with inorganic sources, beef or yeast extract were also more favourable for bioflocculant production by strain X-14 (Li *et al.*, 2009). However, a complex nitrogen source consisting of beef extract and urea was better than single inorganic or organic nitrogen sources (Gong *et al.*, 2008). Strain TJ-1 was able to use beef extract, yeast extract, or peptone as an organic nitrogen source for bioflocculant production (Xia *et al.*, 2008).

7.1.3 Effect of initial medium pH on bioflocculant production

It has been established from previous studies that the optimum pH for bioflocculant production varied with different organisms (Ugbenyen *et al.*, 2012). Initial pH of the production medium determines the electric charge of the cells and the oxidation-reduction potential, which can affect absorption of nutrients and enzymatic reactions (Salehizadeh and Shojaosadati, 2001;

Zhang *et al*., 2007). The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganisms. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity (Tandon and Sharma, 2014). If the cultivation of the microorganisms is carried out at an unfavourable pH, it may limit their growth as well as bioflocculant production. Different microorganisms achieve maximum bioflocculant production at specific pH optima, with higher or lower pH values of the fermentation medium lowers the flocculating activity because the pH of the culture medium affects the availability of certain metabolic ions and permeability of cell membranes (Tandon and Sharma, 2014). Hence, altering the pH may affect the medium composition thus resulting in varied bioflocculant activities. However, some previous studies have documented flocculant production by bacteria over a wide pH range encompassing both acidic and alkaline conditions of a fermentation media (Zhang *et al*., 2013). In this study, the production of REG-6 was maximal at pH 5 (Figure 3.3), whereas pH 6 was the most favourable for MBF-W7 (Table 3.2) and MBF-UFH (Figure 5.3). On the other hand, in the case of bioflocculant produced by *Bacillus megaterium*, alkaline pH in the 7–12 range was favoured with maximum yield obtained at pH 9.0, while it was inhibited in an acidic culture medium (Zheng *et al*., 2008). Furthermore, *S. silvestris* grew well at pH between 7 and 9, and the optimum flocculation efficiency was observed at pH 8 (Wan *et al*., 2013) whereas the suitable medium pH for the growth of *Cobetia* sp. was in the range of 3-7 with the highest bioflocculant production of 90.6% recorded at pH 6 (Ugbenyen *et al*., 2012).

7.2 Time course of bioflocculant production

As previously documented by Piyo *et al*. (2011), cultivation time for bioflocculant release into the medium varies with different microorganisms. The correlation between bioflocculant production and culturing time may differ among different organisms. It was observed that extension in cultivation period resulted in an increase in cell growth with concomitant increase

in flocculating activity (Mabrouk, 2014). According to Ugbenyen *et al*. (2012), the production of bioflocculant by *Cobetia* sp. was associated with cell growth, increased during the logarithmic growth and reached its maximum flocculating efficiency (89%) in stationary phase (4 days). Thus, the corresponding steady increase in cell growth is possibly an indication that the bioflocculant was produced by biosynthesis during growth and not by cell autolysis (Ugbenyen *et al.*, 2012). Further increase in cultivation period resulted in a slight decrease in both flocculating activity and cell growth, which could be attributed to cell autolysis and/or the presence of a bioflocculant degrading enzyme (Li *et al.*, 2009). On the contrary, bioflocculant production by *Streptomyces griseus* was not parallel to cell growth and was released into the culture in the death phase (Shimofuruya *et al.*, 1996). It is evident that the flocculant biosynthesis by different organisms occurred during different phases of the microbial growth (Mabinya *et al.*, 2012).

In this study, the flocculating activity of REG-6 was optimal after 96 h of cultivation (Figure 3.6) whereas, both MBF-W7 (Figure 4.1) and MBF-UFH (Figure 5.4) had the highest flocculating activities of 94.9% and 85.8% respectively after 72 h. The production of bioflocculant by *Bacillus licheniformis* reached maximum after 96 h of cultivation (Shih *et al.*, 2001) while a shorter cultivation time of 60 h was recorded for optimum bioflocculant production by *Klebsiella* sp. (Yang *et al.*, 2012).

In this study, both the cell growth and bioflocculants production decreased gradually during the death phase of growth, which might be due to cell autolysis and defloculating enzymes released into the culture broth (Yang *et al.*, 2012). Aljuboori *et al.* (2013) found that the flocculating activity of a bioflocculant produced by *Aspergillus flavus* was highest at early stationary phase with flocculating activity of 87.2% at 60 h. Consequently, a period of 60 h was chosen as the culture time for all subsequent experiments involving this particular organism. Additionally, using the optimal culture composition and conditions, about 0.4 g of pure IH-7 bioflocculant

could be obtained from 1 L of culture broth. The growth curve of *Enterobacter aerogenes* showed that bioflocculant secretion was parallel to cell growth, with the maximum flocculating activity observed in the early stationary phase (Lu *et al.*, 2005). *Bacillus licheniformis* CGMCC 2876 showed relatively short fermentation period of 40 h (Xiong *et al.*, 2010).

In the case of *Corynebacterium daeguense*, bioflocculant production was not parallel to the cell growth phase and it increased sharply during the death phase. It generated under low nutrient condition and included high contents of DNA in structure (Liu *et al.*, 2010). Cell growth of *Bacillus mojavensis* increased in the first 72 h and then remained constant. The bioflocculant was produced by biosynthesis during the growth phase, not during the autolysis phase; and the highest flocculating activity of the culture was achieved in the early stationary phase at 24 h, resulting in the shortest culture time for bioflocculant secretion compared with most other strains studied (Elkady *et al.*, 2011). The bioflocculant ZS-7 produced by *Bacillus licheniformis* X14 reached its maximum flocculating activity at stationary phase after 48 h of cultivation (Li *et al.*, 2009). In case of Prasertsan *et al.* (2006) reports, the production of bioflocculant by *Enterobacter cloacae* WD7 exhibited the highest flocculating activity after 72 h of cultivation.

7.3 Bioflocculant yields

Low yields and high production costs associated with relatively expensive substrates limit the practical application of bioflocculant (Li *et al.*, 2003). Thus, it is necessary to develop proper strategies to increase the bioflocculant yield in order to reduce the production costs. One of the ways in which bioflocculant yield can be improved upon is by optimizing the culture conditions so that the bioflocculant can be maximally produced with optimized conditions. In this present study, 3.2 g/l of REG-6 was produced by *Bacillus toyonensis* AEMREG6, 3.8 g/l of MBF-W7 was produced by *Bacillus* sp. while *Bacillus* sp. AEMREG7 produced 1.6 g/l of MBF-UFH. Although, the yields of these bioflocculants are still relatively small compared to

some reported studies (Elkady *et al.*, 2011; He *et al.*, 2009; Li *et al.*, 2013; Liu and Cheng, 2010), they are however significantly higher when compared to yields from other pure cultures (Aljuboori *et al.*, 2013; Adebayo-Tayo and Adebami, 2014; Xia *et al.*, 2008; Yan and Yun, 2013). Elucidation of pathways involved in bioflocculant biosynthesis may lead to possible better strategies being developed for enhancing bioflocculant yields. Developing several strategies on how those genes responsible for bioflocculant production can be manipulated so that they can be over-expressed and thereby increasing the yield are hot topics of research presently in biotechnology (Delbarre-Ladrat *et al.*, 2014).

7.4 Optimization of flocculating activity of purified bioflocculants

7.4.1 Effect of bioflocculant dosages

Dosage or concentration requirement is still one of the most critical factors to be considered when determining the optimum conditions for the performance of bioflocculant in the process of coagulation/flocculation, since an insufficient dosage or over-dosage may lead to reduced performance in flocculation (Hassan *et al.*, 2009). Therefore, it has becomes critical to establish the optimum bioflocculant dose, as this could help minimize costs and attain better performance in the treatment processes (Cosa and Okoh, 2014). The relationship between bioflocculant dosage and flocculating activity was investigated in this study. It was observed that 0.1, 0.2 and 0.3 mg/mL were optimal doses for REG-6, MBF-W7 and MBF-UFH (Table 3.2, Figure 4.5A, Figure 6.4) respectively. However, it has been stated in previous studies that an insufficient bioflocculant concentration might not be appropriate for the neutralization of the negative charges on kaolin particles (Li *et al.*, 2007). In addition, the settling of flocculated particles can be negatively affected due to the high viscosity from the excessive level of bioflocculant molecules in the solution (Wang *et al.*, 2011; Yim *et al.*, 2007). Higher or lower dosages induced lower flocculating efficiency. When the bioflocculant dosage is insufficient, the bridging phenomena cannot be effectively formed. On the other hand, excessive

concentration of bioflocculant may cause competition and repulsion of negatively charged particles, consequently blocking the sites available on the particle surfaces for the formation of inter-particle bridges and thereby leading to re-stabilization of the kaolin particles in suspension and hence, a decrease in the flocculating efficiency of the bioflocculant (Gong *et al.*, 2008; Guo *et al.*, 2014; Sun *et al.*, 2012).

According to Liang *et al.* (2010), the decrease in flocculation activity that occurred may be attributed to "flocculation deterioration" phenomenon whereby some colloidal particles were encased by the concentrated flocculant and a "colloid protection function" occurred, leading to reduced flocculating activity. The binding sites of the dispersive kaolin particles were blocked up by some bioflocculant molecules at high bioflocculant concentration instead of the formation of stronger bridging among the bioflocculant molecules and disperse particles in a proper flocculant concentration (He *et al.*, 2010). This hypothesis is premised on the assumption that a three dimensional matrix model is formed between disperse matters and extended polymer chains in terms of the bridging phenomena with the help of intermolecular force, such as van der Waals' force and hydrogen bond. It was difficult to coagulate and bridge when the bioflocculant was insufficient (Zhang *et al.*, 2010). On the contrary, super abundant bioflocculant would mask the disperse particles, and block the formation of bigger flocs (Lu *et al.*, 2005).

A similar phenomenon has been reported by Li *et al.* (2008) where the flocculating activities increased as the concentration of bioflocculant produced increased up to 0.8 mg/mL in concentration regimes of 0.2 mg/mL to 1.0 mg/mL. The optimum bioflocculant dose for the purified bioflocculant was 0.8 mg/mL with a resultant flocculating activity of 90%. Wang *et al.* (2011) similarly reported that the bioflocculant CBF-F26 produced from a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 at a bioflocculant dosage of 12 mg/L showed a maximum flocculating activity of 96%. Flocculating activity improved as the HBF-3

concentration increased from 0.25 to 4.0 mg/L. When HBF-3 concentration was 4.0 mg/L, flocculating activity reached a maximum value (96.9 ± 0.5%). However, the flocculating activity decreased with higher HBF-3 concentrations (Feng and Xu, 2008). The flocculating activity of biopolymer flocculant secreted by *Klockera* sp. was over 94% in the dosage range of (0.00425–0.013 mg/mL and attained its highest flocculating rate of 98.13% at 13 mg/mL (Abu-Elreesh *et al.*, 2011). In the case of *B. mojavensis*, the cost-effective bioflocculant dosage was 0.003 mg/mL, which resulted in flocculating activity of 89.7% at pH 7 (Elkady *et al.*, 2011).

7.4.2 Effect of cations on the flocculating activity of purified bioflocculant

Bioflocculant is essentially a kind of polymer which is usually negatively charged. However, this characteristic limits the application of bioflocculant in water treatment because most water pollutants are negatively charged as well (Huang *et al.*, 2014). Therefore, in order to extend the application of bioflocculant, researchers have used it in combination with conventional coagulants for water treatment. It has been well documented that to achieve high flocculating activity, metal ions are usually required to aid the flocculation process (Elkady *et al.*, 2011; Gong *et al.*, 2008; Salehizadel and Shojaosadati, 2002). Specifically, the cation is used as coagulant aid in achieving high flocculation activity by neutralizing the negatively charged functional groups on the bioflocculant and suspended particles thereby increasing the adsorption of bioflocculant to the suspended particles (He *et al.*, 2010; Mabinya *et al.*, 2011). In this present study, only divalent cations (Mg^{2+}, Ca^{2+} and Mn^{2+}) were found to synergistically enhance the flocculating activity of REG-6 (Table 3.2). The monovalent cation did not show any effect since they reduce the strength of the bonds and cause a loose structure of flocs, thus resulting in a floc density, size and the floc resistance to shear (Wu and Ye, 2007). In addition to the divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}), the trivalent cation Al^{3+} also enhanced the flocculating activity of MBF-W7 (Figure 4.5B). However, a monovalent cation such as Li^{+} Na^{+} and K^{+} could not stimulate the flocculating activity of MBF-W7 effectively which may be due

to the weak electro-static force between the monovalent cation and the bioflocculant (Li *et al*., 2008). MBF-UFH flocculated well (70.94% flocculation rate) without the addition of a cation and this showed that it can be used directly in flocculation process without the aid of cations. Consequently, high flocculating efficiency without cations implies reduction in treatment costs and also environmental pollution (Zhong *et al*., 2014). In addition, the flocculation rate of MBF-UFH was greatly improved in the presence of Na^+ (93.29%), K^+ (92.18%), Li^+ (92.46), Mg^{2+} (94.77%), Ca^{2+} (91.13%), and Mn^{2+} (92.68%) compared to Al^{3+} (68.36) where a decrease in flocculating activity was observed (Table 6.1).

The cations could stimulate the flocculation by neutralizing and destabilizing residual negative charges of carboxyl groups of uronic acid in an acidic polysaccharide, forming bridges which bind kaolin particles to each other (Liu *et al*., 2010). On the other hand, the addition of metal ions had no effects on flocculating activity of MBF-7, indicating that MBF-7 was cation-independent (Zhong *et al*., 2014). In the case of *Serratia ficaria*, flocculating activity was enhanced by the addition of Ca^{2+} and Mg^{2+}, whereas Al^{3+} and Fe^{3+} showed a negative effect (Gong *et al*., 2008). The bioflocculant produced by *Halomonas* sp. and *Micrococcus* sp. was cation-dependent with improved flocculating activity in the presence of Al^{3+}, Ca^{2+} and Mn^{2+} and inhibited by Ba^{2+}, Mg^{2+}, Fe^{3+}, Na^+, Li^+ and K^+ (Okaiyeto *et al*., 2013). In the case of a bioflocculant produced by *Virgibacillus* sp. Rob, monovalent cations (Na^+, Li^+, K^+) and the trivalent cation Fe^{3+}, showed little effect on flocculation activity whereas divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+}) and Al^{3+} greatly improved flocculating efficiency of the bioflocculant (Cosa *et al*., 2013). Salehizadeh and Shojaosadati (2002) and Elkady *et al*. (2011) reported similar findings where monovalent cations showed weak stimulation of flocculation by their respective bioflocculants.

The surfaces of kaolin particles were strongly negatively charged, divalent cation Ca^{2+} could compress the double layer of kaolin particles, weaken the static repulsive force, and promote

HBF-3 to form floc with kaolin particles (He *et al.*, 2010). Charge neutralization happened when suspended particles were oppositely charged against the bioflocculant. In this case, surface charge density of the suspended particles was reduced by the adsorption of the bioflocculant and the particles can approach sufficiently close to each other so that the attractive forces become more effective (Li *et al.*, 2009). As most bioflocculants and suspended particles are negatively charged, charge neutralization seldom occurs in the flocculating process (He *et al.*, 2010).

7.4.3 Effect of pH on the flocculating activity of the purified bioflocculants

The pH of reaction mixtures is a key factor influencing the flocculation process (Zaki *et al.*, 2013). The effects of the pH on flocculating activity in the reaction mixture were investigated. Literature suggests that the alteration of pH may ultimately alter the bioflocculant charge status and surface characteristics of suspended particles consequently changing the flocculating ability (Zhang *et al.*, 1999). In this study, these bioflocculants (REG-6, MBF-W7 and MBF-UFH) exhibited good flocculating efficiencies over a wide range of pH between 3-11 with the maximal flocculating activity for the individual bioflocculants attained at pH 10, 3 and 8 (Table 3.2, Figure 4.5C, Figure 6.5) respectively. This variation in the pH requirement of the reaction mixture may be due to the bioflocculants showing different electric states at different pH values and hence affecting the flocculation capability of the bioflocculants for the kaolin particles (Yong *et al.*, 2009). In addition, one of the ways that pH influences flocculating activity is by affecting the stability of suspended particles and the formation of floccules (Ugbenyen *et al.*, 2014). However, in this present study, a drop in flocculating activity of MBF-UFH was observed at pH 9-11 (Figure 6.5) which might be due to alkaline degradation of the polysaccharide bioflocculant which could have resulted in a number of changes i.e. molecular rearrangements of its residue or polysaccharide chain fragmentation (Prasertsan *et al.*, 2006).

However, it has been demonstrated that at very high pH, the OH^- ions may impede the formation of the complex between the bioflocculant and kaolin particles in the mixture.

The purified MBF-7 had an optimum pH of 5 with small noticeable differences in flocculating activity in the pH range of 3-6; whereas at higher pH of 7–12, flocculating activity decreased gradually (Zhong *et al*., 2014). In basic solutions (pH 9–12), the flocculating activity decreased gradually (from 74% down to 21%) due to alkaline degradation of the polysaccharide which could cause several changes such as molecular re-arrangement of its residue or fragmentation of the polysaccharide chain (Zhong *et al*. 2014). It might also be that the hydroxide ion (OH^-) absorbed at basic condition interferes with the complex formation of the polysaccharide and kaolin particles, consequently the kaolin particles were suspended in the mixture. The bioflocculant of *Gyrodinium impudicum* KG03 was active in acidic conditions ranging from pH 3 to 6, with the maximum activity observed at pH 4 (Zhang *et al*., 2002). On the other hand, He *et al*. (2010) reported that the flocculating activity of HBF-3 held more than 80% in the pH range and the peak flocculating activity (97.0 ± 1.0%) occurred at pH 7.0. At low pH, both HBF-3 and kaolin particles were likely to absorb hydrogen ions (H^+), which weakened the forming of complexes between HBF-3 molecules and kaolin particles mediated by Ca^{2+}. Similarly, hydroxide ions (OH^-) interfered with the combination of the bioflocculant molecules and kaolin particles at high pH, resulting in lower flocculating activity. The flocculating activity of bioflocculant produced by *C. daeguense* was recorded at more than 90% within the pH range of 4-8 with the highest flocculating activity of 96.8% at pH 5.6. A decrease in flocculating activity was observed out of this pH range of 4-8 (Liu *et al*., 2010). Bioflocculant produced by *Bacillus* sp. UPMB13 has a relatively wide pH tolerance ranging from slightly acidic to slightly alkaline condition. The result showed that the bioflocculant can perform at pH ranges from 4.0–8.0 (Zufarzaana *et al*., 2012). The negatively charged density of the bioflocculant rose with increasing pH, which further increased the electrostatic repulsion of the

negatively charged kaolin particles, and thus, poor flocculating activity was observed (Guo *et al.*, 2015).

7.5 Chemical compositions of the purified bioflocculants

The characteristics of bioflocculants from different microorganisms are different (Zhu *et al.*, 2012). Determination of the chemical composition of bioflocculants is crucial to elucidate their flocculation mechanisms, which would facilitate the optimization of flocculating parameters and consequently improve bioflocculant flocculating efficiency in practical applications (Li *et al.*, 2014). For polysaccharide bioflocculants, the molecular weight and functional groups are important for enhancing the flocculating activity (Kumar *et al.*, 2004). When analyses of the chemical composition of the purified bioflocculants were carried out to identify the main chemical constituents, the results showed that REG-6, MBF-W7 and MBF-UFH were glycoproteins. REG-6 composed of polysaccharide (77.8% w/w) and protein (11.5% w/w), MBF-W7 composed of polysaccharide (73.7% w/w) and protein (6.22% w/w) and MBF-UFH composed of polysaccharide (76% w/w) and protein (14% w/w). Since the main components of the bioflocculants under study were glycoproteins, it is assumed that both components will influence the bioflocculant flocculation efficiency (Zhu *et al.*, 2012). In the case of protein-based bioflocculants, the amino and carboxyl groups contribute effectively to flocculation (Kurane *et al.*, 1994). Several studies in the literature have reported extensively on glycoprotein bioflocculant production by a number of microorganisms. For example, *Pseudomonas aeruginosa* generated a sugar-protein derivative bioflocculant consisting of protein (27% w/w) and carbohydrate (89% w/w) (Gomma, 2012). *Bacillus velezensis* secreted a glycoprotein made up of protein (2% w/w) and polysaccharide (98% w/w) (Zaki *et al.*, 2013). The bioflocculant from *Arthrobacter* sp. was a glycoprotein made up of about 56% (w/w) protein and 25% (w/w) polysaccharide (Mabinya *et al.*, 2012). However, *Nocardia amarae* and *Pseudomonas* sp. A-99 produced predominantly protein based bioflocculants (Takeda *et al.*,

1992; Yokoi *et al.*, 1998). Several pure strains have been used to produce polysaccharide-rich bioflocculants in the literature (Gao *et al.*, 2006; Gong *et al.*, 2008; Li *et al.*, 2013; Mabinya *et al.*, 2011; Wang *et al.*, 2007).

7.6 Thermal stability of the purified bioflocculants

The thermal stability of bioflocculant is an important characteristic for its commercial exploitation (Marinho-Soriano and Bourret, 2005). In this study, REG-6, MBF-W7 and MBF-UFH (Table 3.2, Figure 4.5D, Figure 6.6) respectively showed good flocculating activities at high temperatures. The exhibition of thermal stability by these bioflocculants may be characteristic of their polysaccharide backbone (Lu *et al.*, 2005). Several studies on thermal stability of bioflocculants produced by different organisms have been documented in the literature (Gong *et al.*, 2008; Gao *et al.*, 2009; Wang *et al.*, 2013; Ugbenyen and Okoh, 2014). The bioflocculant produced by *O. ciceri* maintained flocculating activity of kaolin suspension at over 90% in the temperature range of 30-90 °C but sharply decreased at temperatures above 90 ^{o}C (Wang *et al.*, 2013). The bioflocculant produced by *A. flavus* was thermostable over acidic and neutral pH values, and over 90% of flocculating activity was maintained within the temperature range of 10–100 °C (Aljuboori *et al.*, 2013).

7.7 Fourier transform infrared analysis (FTIR)

Several functional groups such as hydroxyl, carboxyl and amine groups have been identified as the preferred groups that enhanced flocculation process in REG-6, MBF-W7 and MBF-UFH (Figure 3.6, Figure 4.2 and Figure 6.2) in this present study. The presence of diverse functional groups may impact on different applications in waste/drinking water treatment (Kumari *et al.*, 2014). Two major roles in flocculation are important attributes of these functional groups: hydrophilicity characteristics are utilized to extend the biopolymer chain (Michael and Morelos, 1955); and the groups span the gap in between in order to adsorb particles (Xia *et al.*, 2008; Yuan *et al.*, 2011). Water solubility of these bioflocculants is attributed to the presence

of hydroxyl groups in their molecular chains (Karbowiak *et al.*, 2011). The presence of hydroxyl and carboxyl groups within these bioflocculants molecules as indicated by the FTIR spectra enhances the formation of hydrogen bonds which might be responsible for thermal stability of these bioflocculants (Ugbenyen and Okoh, 2014). It is also possible that the attraction force (i.e van der Waals forces) overcome the electrostatic repulsion force when the bioflocculant approached the suspended particles, thereby the bioflocculant functional groups and H^+ and OH^- on the surface of particles resulted in the formation of hydrogen bonds (Deng *et al.*, 2003; Zheng *et al.*, 2008). These functional groups (OH^- and COO^-), together with amino groups, could serve as binding sites for metal ions which enhance flocculating activity of these bioflocculants by bridging between them and the suspended kaolin particles, and are likely to be preferred groups for the process of adsorption (Comte *et al.*, 2006). These groups were preferred for the adsorption process and may serve as binding sites for divalent cations and suspended particles (He *et al.*, 2010).

The carboxyl groups present on the molecular chain make the chain stretched-out because of electrostatic repulsion resulting in the stretched molecular chains providing more effective sites for particle attachment (Zhang *et al.*, 2002). Furthermore, carboxylate groups act as non-specific ion-exchange material which may impart chelating property on cations which aid the flocculation process (Lin and Harichund, 2011). The carboxyl groups may act as binding sites for the metal ions present in surface particles, hence forming chemical bonds (Deng *et al.*, 2003; Kumar *et al.*, 2004). The amino and carboxyl functional groups of bioflocculants can form a floc with heavy metals by neutralizing and stabilizing the residual charge as the binding distance is shortened (Yue *et al.*, 2006). The IR-spectra of these bioflocculants in this study are consistent with the results reported previously (Guo *et al.*, 2014; Patil *et al.*, 2010; Pathak *et al.*, 2014; Sathiyanarayanan *et al.*, 2013; Wan *et al.*, 2013).

7.8 Thermogravimetric analyses of the bioflocculants

According to Kumari *et al.* (2014) thermal degradation of the bioflocculant usually occurs in two steps. The first step involves an increase in temperature to about 150 ^{o}C resulting in the loss of moisture by the bioflocculant (Wang *et al.*, 2011). The second step entails depolymerisation of the bioflocculant structure at temperatures above 400 ^{o}C. In this present study, the pyrolysis patterns of these studied bioflocculants (REG-6, MBF-W7 and MBF-UFH) were similar to those reported in previous studies (Yim *et al.*, 2007; Desouky *et al.*, 2008; Nie *et al.*, 2011; Wang *et al.*, 2011; Luo *et al.*, 2014).

7.9 Scanning electron microscopy (SEM) analyses of bioflocculants

Scanning electron microscopy is widely used to elucidate the surface morphological features of the bioflocculants (Kumari *et al.*, 2014). It was observed that the bioflocculants (REG-6, MBF-W7 and MBF-UFH) in Figure 3.8, Figure 4.4 and Figure 6.2 are white in colour with compact structures (Wang *et al.*, 2010). Scanning electron microscopy (SEM) images of the interaction of the bioflocculants with kaolin clay showed that bridging might be possibly responsible for their flocculation process, a phenomenon also observed by Nwodo *et al.* (2013). The bridging mechanism is more often used to explain flocculation in biological systems (Zhang *et al.*, 2010). The effectiveness of the bridging mechanism depends on the molecular weight of the bioflocculant, the charge on the bioflocculant and the suspended particle, the ionic strength of suspension, and the nature of mixing (Zhang *et al.*, 2010). However, our findings were similar to that reported by Kumar *et al.* (2004). Prior to flocculation, the kaolin particles were dispersed, and during the process of coagulation-flocculation the scattered kaolin particles were probably joined and adsorbed onto the binding sites of the bioflocculants which thus aggregated, forming larger flocs and leading to rapid sedimentation due to gravity (Xiong *et al.*, 2010; Wang *et al.*, 2011).

7.10 Applications of bioflocculants in water treatment

River water is one of the typical surface water with low COD and turbidity (Gong *et al.*, 2008). The efficacy of MBF-W7 was investigated on its ability to remove turbidity and COD of Thyume River waters. The removal efficiency of turbidity and COD were 86.9% and 75.3% respectively (Table 4.3). These results indicated that this bioflocculant (MBF-W7) could possibly be successfully used in the clarification of a wide range of waters and/or wastewaters under various environmental conditions. Similar results were also reported for bioflocculants from previous studies (Lian *et al.*, 2008; Gong *et al.*, 2008; Li *et al.*, 2013; Cosa and Okoh, 2014).

The effectiveness of MBF-UFH in turbidity removal of kaolin clay suspension was compared with some conventional (synthetic) flocculants (Table 6.2). MBF-UFH showed 91.1% flocculating efficiency for kaolin clay suspension compared to polyacrylamide (94.3%), aluminium chloride (67.1%), $FeCl_3$ (42.78%). Although, the flocculating efficiency of polyacrylamide was higher than that of MBF-UFH, however, the chemical nature of MBF-UFH gives it an added advantage over and a viable alternative to polyacrylamide for water treatment. The effectiveness of turbidity removal may be attributed to the polymer-floc interaction of the bioflocculant, thereby leading to increased aggregation of particles (Cosa and Okoh, 2014). The flocculating activity of MBF-UFH was higher than MBF4-13 produced by *Rothia* sp. with flocculating activity of 86.01% reported in the literature (Gao *et al.*, 2009).

7.11 Conclusion and future prospects

The study entailed evaluation of bioflocculant production potentials of three bacterial species belonging to the *Bacillus* genus. Based on 16S rDNA nucleotide sequences and BLAST analyses, the isolates were identified as *Bacillus toyonesis* AEMREG6, *Bacillus* sp. and *Bacillus* sp. AEMREG7. Culture conditions for optimum bioflocculant production by the three isolates were determined and the characterization of the purified bioflocculants was also

undertaken. Bioflocculant production by all the three isolates was parallel to cell growth with optimum production achieved at inoculum sizes of 5% (v/v) or less. Glucose was the best sole carbon source for REG-6 and MBF-UFH production by *Bacillus toyonensis* AEMREG 6 and *Bacillus* sp. respectively, whereas maltose was the most suitable carbon source for MBF-W7 production by *Bacillus* sp. AEMREG7. Inorganic nitrogen source (NH_4NO_3) supported maximum bioflocculant production by *Bacillus toyonensis* AEMREG6 and *Bacillus* sp. at initial pH values of 5 and 6 with respective yields of 3.2 g/L and 3.8 g/L recovered after purification. Composition analyses revealed all bioflocculants to be of a glycoprotein nature with a predominant polysaccharide backbone and coupled with the presence of hydroxyl, carboxyl and amino functional groups which properly favours their flocculation processes. The presence of divalent cations was a requirement for enhanced flocculating activity of REG-6 and MBF-W7, in the case of bioflocculant (MFH-UFH) produced by *Bacillus* sp. AEMREG7, a high flocculating activity of 71% for kaolin clay suspension was achieved without the aid of any cations, an added advantage in terms of treatment costs implications. The comparatively low dosage requirements of $\leq$ 3 mg/ml for the studied bioflocculants coupled with the thermal stability displayed at high temperatures, augurs very well for their possibly future industrial applicability. The unique properties and remarkable flocculating activities demonstrated by all the three bioflocculants make them potential candidates for future industrial applications and thus a possible solution to health and environmental problems associated with the use of synthetic flocculants in water treatment.

The future development of microbial flocculants will depend on a number of factors, but the key question is whether they can be produced economically. Utilization of agricultural wastes or industrial wastewaters (possibly along with other substrates) is certainly a possibility for bioflocculant production. Only a limited number of microbial species show diverse enough substrate utilization for agricultural wastes to be suitable substrates. Considerable research will

be necessary to ensure that bioflocculant synthesized using agricultural waste substrates are of satisfactory quality and have acceptable properties. This will reasonably cut down production cost and encourage their large-scale production and industrial application.

The flocculation optimization practices in the industry are still scarce because of the highly complex nature of the flocculation process and the large variety of polyelectrolytes available. One of the ways to optimize the flocculation process is by selecting or controlling the range of the molecular weight and the charge density of the bioflocculant. Different molecular weights and charge densities produce different flocculation mechanisms (neutralization or bridging). Future research needs to look into how molecular weight and charge density distribution affect the flocculation performance to produce a better choice of bioflocculants for specific industrial applications. Optimization of these factors could significantly increase the treatment efficiency and reduce the chemical cost.

In addition, very limited work has been carried out on the industrial scale. Most reports have concentrated on laboratory studies. The complexity of the coagulation and flocculation systems justifies that a bioflocculant cannot be selected for a given application without experimental testing. Industrial trials or practices for confirming the dosage suitable and other physicochemical conditions for flocculation are still lacking. Furthermore, the applicability and effectiveness of these bioflocculants for wastewater treatment in large-scale is yet to be established. Further investigation on the industrial scale-up conditions is highly imperative.

The selection of high efficient bioflocculants that can remove all contaminants in wastewater is essential for a successful flocculation process. Environmental friendly bioflocculants that can be produced by simple and economically viable process which exhibits high removal efficiencies and considerably denser flocs is regarded as a promising material for application from the perspective of both performance and cost. In order to control and optimize the

flocculation process, it is very important to understand the flocculation mechanism during the whole process. However, the investigation and discovery of the underlying mechanism for removal of impurities or contaminants from wastewater with bioflocculants is still lacking and immature and so requires attention.

Development of suitable bioflocculant extraction methods is one of the factors that affect the property of the purified product. Suitable extraction methods with a high efficiency should be pursued. Such methods should be mild to avoid the lysis of cells and the disruption of bioflocculant characteristics.

Most bioflocculant-producing microorganisms are usually incubated at or near 30 °C although incubation at sub-optimal temperatures conventionally favours bioflocculant production. There would be obvious advantages in using thermophiles capable of growth at higher temperatures in order to avoid the necessity of expensive cooling systems for large-scale synthesis in the industries. Nevertheless, none of these bacteria have yet proved to be sources of bioflocculant with good rheological properties. Further research will be crucial to isolate these thermophilic microorganisms from different environments that will be utilized for bioflocculant production.

Lastly, it will be crucial to establish an appropriate fermentation (fed-batch versus continuous fermentation) conditions for scale-up process for bioflocculant production. Furthermore, to determine the shelve-live of the bioflocculant as well as establish appropriate packaging regimes. It will be imperative to carry out feasibility study on the marketability of the final bioflocculant product. For the bioflocculant to be of industrial benefit, all the aforementioned points must be put into consideration.

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APPENDIX

Table 1. Effect of carbon source on REG-6 production by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Carbon source (20 g/L)	Tube 1	Tube 2	Tube 3
Glucose	0.485	0.54	0.516
Fructose	0.985	1.196	1.075
Sucrose	0.761	0.758	0.66
Maltose	0.589	0.449	0.66
Starch	1.555	1.564	1.407
Na_2CO_3	0.758	0.758	0.79
Phthalate	0.973	1.137	0.951

Table 2. Effect of nitrogen source on REG-6 production by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Nitrogen source (1.3 g/L)	Tube 1	Tube 2	Tube 3
Peptone	0.618	0.687	0.628
Tryptone	1.774	1.832	1.512
Urea	0.456	1.062	0.861
Yeast extract	0.73	0.757	0.625
$(NH_4)_2SO_4$	0.492	0.262	0.493
NH_4NO_3	0.36	0.479	0.369
$NaNO_3$	0.667	0.436	0.936

Table 3. Effect of inoculums size on REG-6 production by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Inoculum size (% v/v)	Tube 1	Tube 2	Tube 3
1			
2	0.874	0.952	1.026
3	0.508	0.677	0.661
4	0.205	0.195	0.193
5	0.28	0.153	0.295

Table 4. Effect of initial pH of growth medium on REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
4	0.562	0.586	0.547
5	0.521	0.474	0.504
6	0.8	0.783	0.771
7	1.171	1.262	1.076
8	1.027	1.235	0.985
9	1.217	1.021	1.552
10	1.614	1.468	1.381

Table 5. Effect of cation on the flocculating activity of REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
K^+	0.225	0.184	0.188
Na^+	0.251	0.26	0.236
Mg^{2+}	0.287	0.196	0.227
Ca^{2+}	0.179	0.181	0.185
Mn^{2+}	0.214	0.238	0.186
Fe^{3+}	1.937	1.912	1.857
Al^{3+}	0.393	0.398	0.316

Table 6. Effect of temperature on the flocculating activity of crude REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Temperature (^oC)	Tube 1	Tube 2	Tube 3
Unheated sample	0.115	0.121	0.144
50	0.177	0.171	0.151
60	0.189	0.2	0.181
70	0.219	0.23	0.325
80	1.134	1.068	1.09

Table 7. Time course of REG-6 production by *Bacillus toyonensis* strain AEMREG6

Time (h)	Flocculating activity (Abs@550nm)			OD@660nm			pH			CFU/mL (No of colonies)		
0	1.552	1.563	1.598	0.325	0.352	0.312	5.9	5.89	5.8	49	45	78
24	1.514	1.469	1.316	0.466	0.423	0.506	5.65	5.63	5.65	191	191	168
48	0.792	0.693	0.523	1.195	1.11	1.103	5.37	5.09	5.15	97	46	19
72	0.451	0.418	0.394	1.127	1.149	1.257	5.19	4.9	4.95	84	206	54
96	0.371	0.326	0.341	1.112	1.181	1.121	5.03	4.85	4.81	282	49	58
120	0.455	0.311	0.336	1.003	1.003	1.003	4.94	4.81	4.85	110	129	15
144	0.479	0.461	0.398	1.145	1.167	1.466	4.9	4.81	4.91	54	48	45
168	0.865	0.889	0.925	1.794	1.105	0.981	4.75	4.72	4.82	50	39	41

Table 8. Effect of bioflocculant dosage on the flocculating activity of purified REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Dosage (mg/mL)	Tube 1	Tube 2	Tube 3
0.05	0.279	0.292	0.284
0.1	0.188	0.109	0.106
0.2	0.121	0.148	0.124
0.3	0.072	0.125	0.136
0.4	0.153	0.142	0.166
0.5	0.172	0.183	0.201

Table 9. Effect of cation on the flocculating activity of purified REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Cation (1% w/v)	Tube 1	Tube 2	Tube 3
Na^{+}	0.078	0.081	0.114
K^{+}	0.091	0.129	0.098
Li^{+}	0.095	0.126	0.086
Mg^{2+}	0.064	0.082	0.067
Ca^{2+}	0.149	0.126	0.086
Mn^{2+}	0.091	0.117	0.09
Fe^{3+}	1.302	1.195	1.171
Al^{3+}	0.397	0.465	0.426

Table 10. Effect of pH of the flocculating activity of purified REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
3	0.093	0.067	0.071
4	0.11	0.094	0.085
5	0.112	0.127	0.106
6	0.094	0.114	0.116
7	0.075	0.099	0.074
8	0.071	0.04	0.075
9	0.485	0.338	0.376
10	0.254	0.186	0.262
11	0.133	0.115	0.226

Table 11. Effect of temperature on the flocculating activity of purified REG-6 produced by *Bacillus toyonensis* strain AEMREG6

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
50	0.267	0.26	0.249
60	0.243	0.249	0.281
70	0.205	0.255	0.276
80	0.271	0.245	0.254

Table 12. Effect of carbon source on MBF-W7 production by *Bacillus* sp.

Abs@550nm

Carbon source (20g/L)	Tube 1	Tube 2	Tube 3
Starch	0.084	0.249	0.53
Glucose	0.393	0.335	0.176
Maltose	0.054	0.073	0.061
Fructose	0.363	0.41	0.316
Lactose	0.284	0.535	0.412
Sucrose	0.065	0.071	0.063

Table 13. Effect of nitrogen source on MBF-W7 production by *Bacillus* sp.

Abs@550nm

Nitrogen source (1.3 g/L)	Tube 1	Tube 2	Tube 3
Tryptone	1.413	1.301	1.359
Urea	1.51	1.516	1.308
NH_4NO_3	0.103	0.086	0.129
Yeast extract	1.352	1.485	1.095
$(NH_4)_2SO_4$	0.409	0.233	0.247
Peptone	0.62	1.312	1.094

Table 14. Effect of inoculum size on MBF-W7 production by *Bacillus* sp.

Abs@550nm

Inoculum size (% v/v)	Tube 1	Tube 2	Tube 3
1	0.27	0.179	0.21
2	0.16	0.124	0.20
3	0.181	0.11	0.215
4	0.26	0.209	0.232
5	0.108	0.149	0.15

Table 15. Effect of cations on MBF-W7 production by *Bacillus* sp.

Abs@550nm

Cations	Tube 1	Tube 2	Tube 3
K^{+}	1.551	1.59	1.437
Li^{+}	1.498	1.524	1.346
Ca^{2+}	1.664	1.589	1.548
Mn^{2+}	1.648	1.799	1.612
Mg^{2+}	0.12	0.138	0.148
Al^{3+}	1.641	1.614	1.652
Fe^{3+}	0.147	0.421	0.243

Table 16. Effect of pH on the flocculating activity of MBF-W7 produced by *Bacillus* sp.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
3	0.2	0.144	0.168
4	0.102	0.078	0.092
5	0.166	0.123	0.148
6	0.134	0.093	0.165
7	0.245	0.213	0.34
8	0.275	0.277	0.261
9	0.282	0.274	0.295
10	0.288	0.321	0.381

Table 17. Effect of temperature on the flocculating activity of crude MBF-W7 produced by *Bacillus* sp.

Abs@550nm

Temperature (^{0}C)	Tube 1	Tube 2	Tube 3
50	0.201	0.277	0.247
60	0.194	0.205	0.176
70	0.176	0.308	0.184
80	0.232	0.219	0.297
90	0.262	0.257	0.279
100	0.282	0.366	0.256

Table 18. Time course of MBF-W7 production by *Bacillus* sp.

Time (h)	Flocculating actrivity Abs@550nm)			Optical density (OD_{660})			pH			CFU/mL (No of colonies)		
0	1.639	1.664	1.51	0.574	0.521	0.557	6	6	6	113	79	136
24	1.112	1.088	1.117	0.716	0.818	0.732	5.93	5.98	5.97	229	205	187
48	0.36	0.395	0.225	0.756	0.861	0.791	5.99	6.06	6.01	213	231	274
72	0.072	0.082	0.075	0.826	0.852	0.924	6.26	6.15	6.1	123	100	131
96	0.129	0.122	0.095	0.959	0.972	1.011	6.4	6.52	6.34	77	79	85
120	0.264	0.163	0.198	1.035	0.965	1.078	6.27	6.17	6.02	44	32	36
144	0.177	0.181	0.232	1.11	1.071	1.134	6.26	6.18	5.98	42	29	43
168	0.211	0.159	0.149	1.205	1.103	1.176	6.5	6.25	6.1	32	41	59
192	0.419	0.141	0.154	1.331	1.155	1.27	6.4	6.24	6.01	85	56	71
216	0.674	0.248	0.306	1.528	1.118	1.36	6.14	6.21	5.95	178	156	143

Table 19. Effect of bioflocculant dosage on the flocculating activity of purified MBF-W7 produced by *Bacillus* sp.

Abs@550nm

Dosage (mg/mL)	Tube 1	Tube 2	Tube 3
0.1	0.607	0.665	0.683
0.2	0.594	0.534	0.481
0.3	0.745	0.624	0.664
0.4	0.895	0.742	0.901
0.5	0.927	0.936	0.895

Table 20. Effect of cation on the flocculating activity of MBF-W7 produced by *Bacillus* sp.

Abs@550nm

Cation (1% w/v)	Tube 1	Tube 2	Tube 3
Na^{+}	1.443	1.29	1.432
Li^{+}	1.103	1.161	1.056
K^{+}	1.271	1.2	1.258
Mg^{2+}	0.595	0.501	0.658
Mn^{2+}	0.541	0.563	0.407
Ca^{2+}	0.622	0.57	0.603
Al^{3+}	0.53	0.581	0.404
Fe^{3+}	1.202	1.109	1.042

Table 21. Effect of pH of the flocculating activity of MBF-W7 produced by *Bacillus* sp.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
3	0.234	0.191	0.172
4	0.408	0.407	0.318
5	0.49	0.373	0.335
6	0.379	0.401	0.374
7	0.779	0.804	0.751
8	0.41	0.447	0.423
9	0.561	0.44	0.449
10	0.334	0.28	0.373
11	0.297	0.267	0.249

Table 22. Effect of temperature on the flocculating activity of purified MBF-W7 produced by *Bacillus* sp.

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
Unheated sample	0.234	0.191	0.172
50	0.275	0.197	0.26
60	0.527	0.496	0.503
70	0.286	0.297	0.288
80	0.339	0.491	0.322

Table 23. Treatment of Thyume River using MBF-W7

Bioflocculant	Turbidity removal	
	Before treatment (NTU)	After treatment (NTU)
MBF-W7	65	8
	68	11
	59	6
	COD reduction	
	Before treatment (mg/lL)	After treatment (mg/L)
	152	38
	131	33
	146	35

Table 24. Effect of carbon source on MBF-UFH production by *Bacillus* sp. AEMREG7

Abs@550nm

Carbon source (20g/L)	Tube 1	Tube 2	Tube 3
Glucose	0.121	0.181	0.161
Fructose	0.668	0.863	0.687
Maltose	0.888	0.802	0.856
Sucrose	0.456	0.322	0.369
Lactose	0.456	0.398	0.421
Starch	1.418	1.238	1.154
Na_2CO_3	0.586	0.533	0.597

Table 25. Effect of nitrogen source on MBF-UFH production by *Bacillus* sp. AEMREG7

Abs@550nm

Nitrogen source (1.3 g/L)	Tube 1	Tube 2	Tube 3
Peptone	0.329	0.406	0.367
Tryptone	0.777	0.884	0.742
Urea	0.238	0.389	0.451
Yeast extract	0.221	0.322	0.268
$(NH_4)_2SO_4$	0.639	0.589	0.567
NH_4NO_3	0.502	0.622	0.641
Mixed nitrogen	0.159	0.168	0.201

Table 26. Effect of inoculum size on MBF-UFH production by *Bacillus* sp. AEMREG7

Abs@550nm

Inoculum size (% v/v)	Tube 1	Tube 2	Tube 3
1	0.163	0.214	0.25
2	0.192	0.151	0.156
3	0.187	0.124	0.146
4	0.176	0.159	0.143
5	0.18	0.171	0.197

Table 27. Effect of initial pH of growth medium on MBF-UFH produced by *Bacillus* sp. AEMREG7

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
4	0.575	0.66	0.552
5	0.549	0.496	0.526
6	0.194	0.244	0.25
7	0.369	0.157	0.267
8	0.435	0.394	0.376
9	0.998	0.638	0.718
10	1.607	1.674	1.52

Table 28. Effect of cations on the flocculating acivity of MBF-UFH produced by *Bacillus* sp. AEMREG7

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
Na^{+}	0.149	0.249	0.181
K^{+}	0.194	0.195	0.239
Li^{+}	0.195	0.174	0.15
Mg^{2+}	0.271	0.194	0.274
Mn^{2+}	0.175	0.165	0.191
Ca^{2+}	0.142	0.158	0.195
Fe^{3+}	1.762	1.731	1.613
Al^{3+}	0.071	0.086	0.145

Table 29. Effect of temperature on the flocculating activity of crude MBF-UFH produced by *Bacillus* sp. AEMREG7

Abs@550nm

Temperature (^{0}C)	Tube 1	Tube 2	Tube 3
50	0.264	0.206	0.315
60	0.148	0.259	0.171
70	0.147	0.177	0.235
80	0.225	0.18	0.249
90	0.227	0.214	0.22
100	0.446	0.309	0.385

Table 30. Time course of MBF-UFH production by *Bacillus* sp. AEMREG7

Time (h)	Flocculating activity (Abs@550nm)			Optical density (OD_{660})			pH			CFU/mL (No of colonies)		
0	1.429	1.509	1.417	0.34	0.408	0.277	6	6	6	258	218	292
24	1.424	1.225	1.337	0.642	0.857	0.74	5.59	5.58	5.54	258	223	198
48	0.856	0.873	0.831	0.914	0.887	0.887	4.99	5.15	4.94	61	73	85
72	0.229	0.251	0.218	0.989	0.961	0.987	4.89	4.75	4.79	38	52	41
96	0.242	0.369	0.278	1.02	0.97	0.948	4.84	4.78	4.8	99	73	122
120	0.314	0.392	0.342	1.064	0.98	1.094	4.71	4.71	4.72	241	210	197
144	0.564	0.612	0.543	1.106	1.012	1.12	4.85	4.76	4.85	165	179	120
168	0.889	0.827	0.942	1.289	1.167	1.358	4.7	4.71	4.68	276	196	234
192	1.035	1.134	1.275	1.543	1.405	1.587	4.61	4.68	4.67	179	150	188

Table 31. Effect of bioflocculant dosage on the flocculating activity of purified bioflocculant produced by *Bacillus* sp. AEMREG7

Abs@550nm

Dosage (mg/mL)	Tube 1	Tube 2	Tube 3
0.05	0.279	0.292	0.284
0.1	0.188	0.109	0.106
0.2	0.121	0.148	0.124
0.3	0.072	0.125	0.136
0.4	0.153	0.142	0.166
0.5	0.172	0.183	0.201

Table 32. Effect of cation on the flocculating activity of MBF-UFH produced by *Bacillus* sp. AEMREG7

Abs@550nm

Cation (w/v)	Tube 1	Tube 2	Tube 3
Na^{+}	0.078	0.081	0.114
K^{+}	0.091	0.129	0.098
Li^{+}	0.095	0.126	0.086
Mg^{2+}	0.064	0.082	0.067
Ca^{2+}	0.149	0.126	0.086
Mn^{2+}	0.091	0.117	0.09
Fe^{3+}	1.302	1.195	1.171
Al^{3+}	0.397	0.465	0.426

Table 33. Effect of pH on the flocculating activity of MBF-UFH produced by *Bacillus* sp. AEMREG7

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
3	0.093	0.067	0.071
4	0.11	0.094	0.085
5	0.112	0.127	0.106
6	0.094	0.114	0.116
7	0.075	0.099	0.074
8	0.071	0.04	0.075
9	0.485	0.338	0.376
10	0.254	0.186	0.262
11	0.133	0.115	0.226

Table 34. Effect of temperature on the flocculating activity of purified MBF-UFH produced by Bacillus sp. AEMREG7

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
50	0.104	0.107	0.101
60	0.127	0.072	0.107
70	0.066	0.088	0.069
80	0.063	0.129	0.153

Table 35. Comparison of flocculating activity of MBF-UFH with conventional flocculants

Abs@550nm

Flocculant	Tube 1	Tube 2	Tube 3
Aluminium chloride	0.521	0.425	0.501
$FeCl_3$	0.761	0.66	0.785
Polyacrylamide	0.075	0.099	0.074
MBF-UFH	0.063	0.109	0.090

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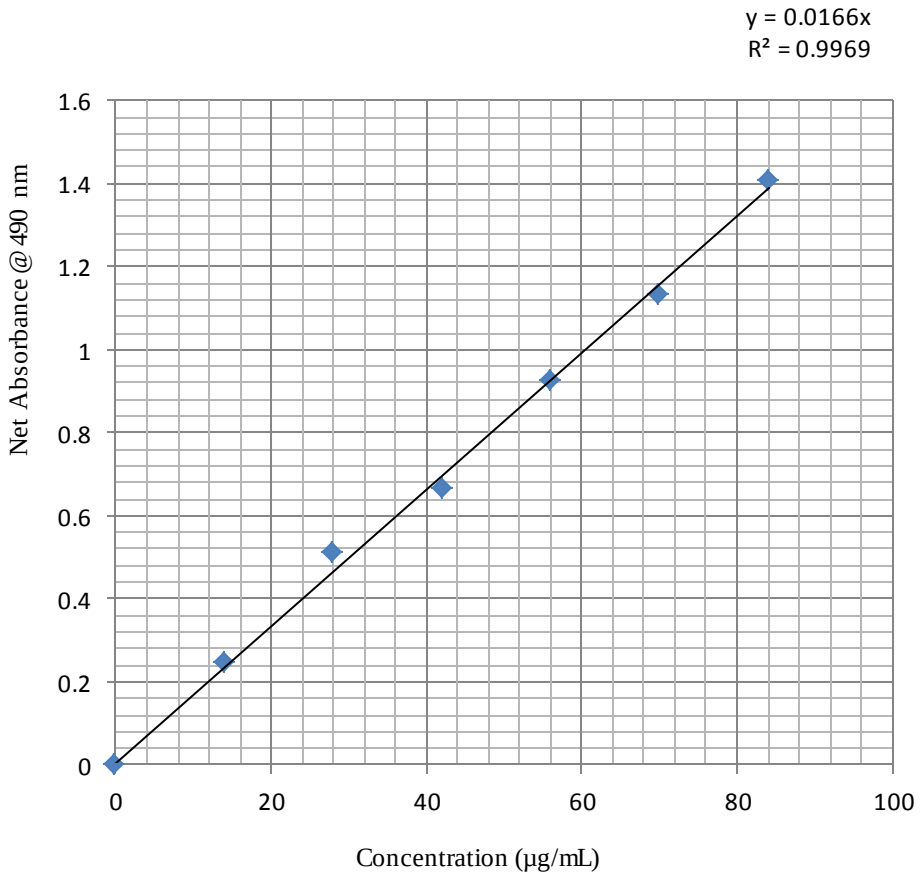
Graph 1. Glucose standard curve



Graph 2. Standard curve of protein estimation