ASSESSMENT OF THE FLOCCULATING EFFICIENCY OF BIOFLOCCULANT PRODUCED BY *BACILLUS SP.* AEMREG4 ISOLATED FROM TYHUME RIVER, EASTERN CAPE, SOUTH AFRICA

By

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DECLARATION

I, Nozipho Ntsangani solemnly declare that this dissertation, submitted to the Department of Biochemistry and Microbiology, University of Fort Hare, for a Master of Science in Biochemistry, is my original work, unless otherwise where indicated and has not been submitted to any University for any degree award or examination purposes.

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Date: ……

Supervisor's signature: …….

DEDICATION

I dedicate this dissertation to the wonderful God for His grace upon my life and my lovely daughter, Lwandle Ntsangani.

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GENERAL ABSTRACT

Bioflocculants are flocculating substances produced by microorganisms during growth and have recently received considerable attention from researchers; due to their biodegradability, non-toxicity and lack of secondary pollution from degradation intermediates. This study evaluated the efficiency of bioflocculant produced by *Bacillus* sp. AEMREG4 isolated from Tyhume River. The bacterial identification was through 16S rDNA sequencing; nucleotide sequences were deposited in GenBank as *Bacillus* sp. AEMREG4 with an Accession number KP406729. The optimum culture conditions for bioflocculant production were an inoculum size of 4% (v/v) and starch as well as yeast extract as sole carbon and nitrogen sources respectively. The addition of $CaCl_2$ enhanced the flocculating activity, at a wide range of pH 4-10 and the highest flocculating activity was reached at an initial pH 8 (80%). A bioflocculant yield of 0.78 g was recovered from 1 L of culture broth. The optimum flocculating activity of 78% was reached at the lowest bioflocculant dosage of 0.1 mg/ml and the presence of divalent cations (Ca^{2+}, Mn^{2+} and Mg^{2+}) as well as a trivalent cation (Al^{3+}) enhanced flocculating activity. The purified bioflocculant retained more than 70% flocculating activity when subjected to heating at 100 °C for 1 h and maximum flocculating activity of 83% was achieved at both acidic and basic pH values of 3 and 10 respectively. Chemical analysis showed that the bioflocculant is predominantly polysaccharide. The Fourier transform infrared (FTIR) spectrum revealed the presence of carboxyl, hydroxyl and methoxyl groups as the functional moieties and the scanning electron microscopy (SEM) imaging of the purified bioflocculant showed its morphological structure as rod-shaped which contributes to its high flocculating efficiency. The high flocculation activity displayed by this bioflocculant indicates its potential suitability for industrial application.

Keywords: Bioflocculant, *Bacillus* sp. AEMREG4, flocculating activity

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CHAPTER 1

1. INTRODUCTION

1.1 Study background

Various industrial processes have been applying flocculants in treating starch wastewater (Deng *et al*., 2003), for drinking water purification (Li *et al*., 2009), in dyeing wastewater treatment (Liu *et al*., 2009) and in downstream processes (Hao *et al*., 2005). Flocculants are classified into three major groups: (a) organic flocculants (polyacrylic acid and polyacrylamide derivatives), (b) inorganic flocculants (aluminium sulphate and polyaluminum chloride) and, (c) bioflocculants (Salehizadeh and Shojaosadati, 2001; Li *et al*. 2010). The first two groups are widely used industrially due to their low cost and effectiveness, however, the major problem is that they are detrimental to human health and are not environmentally friendly (Mabinya *et al*., 2011; Piyo *et al*., 2011). For example, acrylamide monomers of polyacrylamide were found to be both neurotoxic and carcinogenic to humans (He *et al*., 2010; Nwodo *et al*., 2014). It has also been reported that aluminium, the main constituent of polyaluminum may lead to the development of Alzheimers disease (Li *et al*., 2007; Okaiyeto *et al*., 2015). As a consequence thereof, research focus by many scientists has been directed towards finding an alternative and safer substitute to these chemical flocculants.

Bioflocculation is a process that involves the removal of colloidal particles from a suspension by living cells. The mechanism of microbial flocculation was first reported by Louis Pasteur in 1876 and till to date, it has been the focus of interest by a number of researchers, culminating in the establishment of the relationship between the increase of extracellular bioflocculant and cell aggregation (Gao *et al*., 2006). Bioflocculants, also known as microbial

flocculants, are defined as polymers produced by microorganisms during their growth (Deng *et al*., 2003). Several microorganisms have been reported in literature to produce bioflocculants, including among others; *Rhodococcus erythropolis* (Takeda *et al*., 1991; Kurane *et al*., 1994), *Paecilomyces* sp. (Hiroaki and Kiyoshi, 1985), *Klebsiella pneumonia* (Nakata and Kurane 1999), *Citrobacter* sp. TKF04 (Ike *et al*., 2000), *Brachybacterium* sp. (Nwodo *et al*., 2013), *Cellulomonas sp*. Okoh (Nwodo and Okoh, 2013) and *Bacillus subtilis* F9 (Giri *et al*., 2015). To date, several bioflocculant-producing microorganisms have been isolated and investigated (Deng *et al*., 2005; Cosa and Okoh, 2014; Salehizadeh and Yan; 2014) the majority of which produced either polysaccharides or proteins as predominant components (Mabinya *et al*., 2012; Sathiyanarayanan *et al*., 2013; Giri *et al*., 2015). Glycoproteins have also been reported for some (Feng and Xu 2008). Examples of microorganisms producing proteinaceous bioflocculants include the following: *Bacillus subtillis* (Yokoi *et al*., 1996), *Bacillus licheniforms* (Shih *et al*., 2001) *Paecilomyces* sp. (Hiroaki and Kiyoshi, 1985) and *Norcardia amarae* YKI (Takeda *et al*., 1992). *Alcaligenes latus* KT201 (Toeda and Kurane, 1991) and *Enterobacte*r sp. (Yokoi *et al*., 1997) produce polysaccharide bioflocculants, while *Arcuadendron* sp. TS-4 has been reported to produce a glycoprotein bioflocculant (Lee *et al*., 1995).

Bioflocculants have attracted considerable interest scientifically and biotechnologically in the main because they have advantages of being biodegradable, non-toxic and lack secondary pollution (Cosa *et al*., 2011). Several experiments carried out have confirmed bioflocculants effectiveness in the removal of humic acids, treatment of dye solutions, wastewater treatment and the removal of metal ions from polluted effluents (Li *et al*., 2000; Salehizadeh and Shojaosadati 2001; Zhang *et al*., 2002; Zouboulis *et al*., 2004). However, the main drawback has been low flocculating capability thus leading to large dosages, with obvious treatment

costs implications, being required for effective treatment (Deng *et al*., 2003; Okaiyeto *et al*., 2013).

The *Bacillus* genus belongs to a member of the phylum Firmicutes. This genus is omnipresent in nature and includes pathogenic and free living species which have been noted for their potential to grow very well on different low cost carbon sources (Bunk *et al*., 2010). The members of the Bacillus genus have been considered as the most important industrial species with the advantage of being safe to use in both food and drinking water processes (Desouky *et al*., 2008). Several studies have documented that the most isolated microorganisms that can produce microbial flocculants belong to the *Bacillus* genus (Shi *et al*., 2001; Deng *et al*., 2003; Zheng *et al*., 2008; Piyo *et al*., 2011; Zulkeflee *et al*., 2012). Examples include polyglutamic acid (PGA) bioflocculant produced by *Bacillus subtilis* which was reported to have high flocculating activity (Yokoi *et al*., 1995) and bioflocculant produced by *Bacillus musilaginosus* reported by Deng *et al*. (2003) to exhibit 99.9% flocculating efficiency against Kaolin suspension. Piyo *et al*. (2011) reported a bioflocculant produced by *Bacillus* sp. Gilbert is a promising source of polysaccharide bioflocculant. A bioflocculant producing isolate from wastewater sludge, identified as *Bacillus subtilis* F9, was reported to produce bioflocculant at highest flocculating rate (Giri *et al*., 2015). In a study carried by Sathiyanarayanan *et al*. (2013), *Bacillus subtilis* MSBN17 was reported to be an effective bioflocculant-producing organism.

Although several bioflocculant-producing microorganisms have been screened and characterized, no wide scale industrial application has been achieved to-date due mainly to high production costs. Hence there's a need to screen and characterize more microorganisms with potentials for bioflocculant production, optimize culture conditions for enhanced bioflocculant yields as well as containing production costs by utilizing alternative cost-effective substrates.

1.2 Statement of Research Problem

Flocculants are widely used in different industrial processes such as drinking and wastewater treatment, downstream processing and in food and fermentation processes (Okaiyeto *et al*., 2013; Zhang *et al*., 2013; Ugbenyen and Okoh, 2014). However, usage of conventional synthesized flocculants has resulted in detrimental health effects such as Alzheimer's disease, cancer and neurotoxicity in addition to having detrimental effects to the environment (Campbell, 2002; Ruden, 2004; Nwodo *et al*., 2014). As a consequence, bioflocculants have gained a lot of attention because it lacks secondary pollution and non-toxicity for both ecosystems and humans (Gao *et al*., 2009; Zhuang *et al*., 2012). However, high production costs accompanied by low flocculant production have been the major drawbacks to large-scale bioflocculant production for industrial applications thus prompting the need to screen new microorganisms with enhanced bioflocculant-producing capability and optimize the flocculating efficiency of the bioflocculant to justify the production costs.

1.3 Aim and Objectives

The aim of this study is to assess the flocculating efficiency of bioflocculant produced by *Bacillus* sp. AEMREG4 and optimize culture conditions to enhance bioflocculant production

The specific objectives shall include the following:

- To isolate and screen the freshwater bacterial strain from Tyhume River for bioflocculant production.
- To optimize culture conditions for bioflocculant production by *Bacillus* sp.
- To determine the kinetics involved in bioflocculant production by this microorganism.
- To produce, extract, purify and characterize the produced bioflocculant.

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CHAPTER 2

LITERATURE REVIEW

2.1 Historical background of bioflocculation

Microbial flocculation was first reported in a yeast strain, *Levur casseeuse* by Louis Pasteur in 1876. Later, a similar phenomenon was observed by Bordet (1899), in bacterial cultures, extensive studies of flocculation mechanism using Zoogloea-forming bacteria isolated by Butterfield (1935) from activated sludge showed a direct correlation between the accumulation of extracellular bioflocculants and cell aggregation. The involvement of extracellular produced compounds in microbial flocculation was further confirmed when Tenny and Stunn (1965) proposed that flocculation by microorganisms was mediated by polymers they excreted extracellularly. Using cell-free supernatant, these extracellular polymers were extracted and examined for their ability to destabilize dispersions of bacteria. To date, over 100 bioflocculant-producing microorganisms have been reported in literature with the produced bioflocculants extensively characterized (Aljuboori *et al*., 2015).

“Flocculants are substances with a synthetic or natural origin used as sedimentation aids to bring about the solid liquid separations by the process of flocculation in industrial plants” (Lacchwani, 2005). They are used in different industrial processes such as water treatment, downstream processing in fermentation processes and mineral ore treatment in metallurgical industries (Nwodo *et al*., 2013). Flocculants can either be cationic or anionic charged and they exhibit a wide range of the moleculer weights (Salehizadeh and Shojaosadati, 2001).

2.2 Categories of flocculants

In general, flocculants are divided into three major groups depending on their chemical composition: (a) inorganic flocculants (aluminium sulphate and polyaluminum chloride), (b) organic synthetic flocculants (polyacrylamide derivatives and polyethylene amine) and (c) bioflocculants (Salehizadeh and Shojaosadati, 2001; Xia *et al*., 2007; Yim *et al*., 2007).

2.2.1 Inorganic flocculants

This class of flocculants has been commonly used in water treatment and fermentation industries due to their excellent flocculating rate and low cost (Piyo *et al*., 2011). The available salts of multivalent metals such as aluminium sulphate and ferric chloride are classified under this class and evidence of their capability as efficient flocculating agents has been reported (Sharma *et al*., 2006). However, these flocculants have inherent utilization limitations which include the following:

- Large amounts are required
- They are highly sensitive to pH variations (Brostow *et al*., 2009)
- They are not applicable to all kinds of disperse systems
- They are not effective at coagulating very fine particles

2.2.2 Organic synthetic flocculants

Organic flocculants such as polyacrylic acid, polydiallylmethyl ammonium chloride and polyacrylamide (PAM) have been generally used in different industrial processes due to their high effectiveness and low cost, although they are very harmful to both humans and

environment (Kwon *et al*., 1996). The monomers of acrylamide are known to be neurotoxic as well as carcinogenic to humans whereas the aluminium salts have been reported to cause Alzheimer's disease (Shadia *et al*., 2011)

2.2.3 Natural occurring flocculants

This group includes chitosan, sodium alginate and microbial flocculants (Xia *et al*., 2007; Yim *et al*., 2007). Within this group, bioflocculants have gained a lot of interest because of the following advantages:

- They are nontoxic, harmless and lack secondary pollution (Xia *et al*., 2007).
- They are biodegradable (Lachhawani, 2005)
- They form strong and larger flocs without affecting the pH of the medium thus effecting faster settling process than those of simple coagulating electrolytes.
- They are cost-effective and require need lesser quantities (1-5 ppm).

2.3 Mechanisms of flocculation

The understanding of the exact mechanism of microbial flocculation, except for the central role played by cations in enhancing bioflocculation, is yet to be fully elucidated (Sobeck and Higgins, 2002; Lian *et al*., 2008). Varying flocculation mechanisms have been proposed and reported in literature (Novak and Higgins, 1999). Unravelling the mechanisms of action of microbial flocculants is vital not only for determining their suitability but also for their effective applications in various industrial processes (Lian *et al*., 2008). Bridging and charge neutralization were proposed as the two main mechanisms of flocculation in biological systems (Li *et al*., 2009). Some of the theories proposed include the following:

2.3.1 Bridging mechanism

The bridging mechanism occurs if the biopolymer causes aggregation of particles and cells and extends from the particles surfaces into the solution for a distance greater than the distance over which the inter-particle repulsion acts as illustrated in Figure 2.1. This can then result in the adsorption of the biopolymer to other particles to form flocs (Salehizadeh and Shojaosadati, 2001). The efficacy of the bridging mechanism depends on the molecular weight of the microbial flocculant, its charge and that of the suspended particle, the ionic strength of the suspension and the nature of mixing (Zhang *et al*. 2010). This mechanism is often used in conjunction with charge neutralization to grow fast settling and/or shear resistance flocs (Lachhwani, 2005). For example, flocculation of yeast cells by a flocculant produced by *Aspergillus sojae* had been described by the bridging between cells and linearly extended biopolymer chains, resulting to the formation of a 3-dimensional matrix that had an ability of settling under inactive conditions (Nakamura *et al*., 1976).

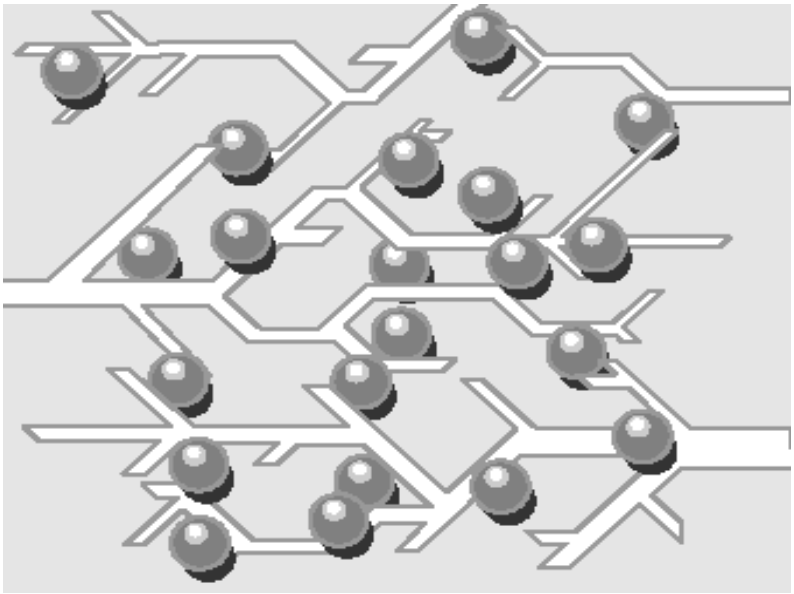

Figure 2.1: Schematic diagram showing polymer chain attaching many particles (Lachhwani, 2005)

2.3.2 Charge neutralization

Charge neutraliztion occurs when the flocculant is positively charged as compared to the particles. Most bioflocculants and particles are negatively charged, therefore, charge neutralization seldom take place in the bioflocculation process (Deng *et al*., 2005). This type of mechanism is caused by the reduction in the electric double layer repulsion between particles due to adsorption of highly charged polyelectrolytes on positively charged particles as shown in Figure 2.2. It is believed that low molecular weight polymers tend to adsorb and neutralize the opposite charges on the particles. “In this case the particle surface charge density is reduced by adsorption of the bioflocculant and the particles can approach sufficiently close to each other so that attractive forces become effective” (Levy *et al*., 1992; Hantula and Bamford, 1991).

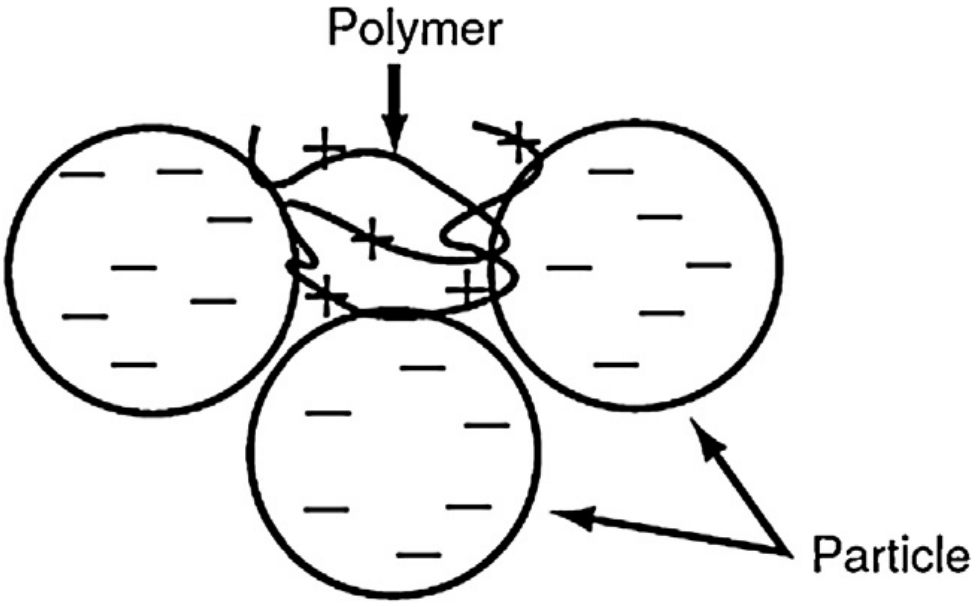

Figure 2.2: Schematic diagram showing the charge neutralization mechanism (Dobias, 2005)

2.3.3 Double layer Theory

Double layer theory also known as DLVO was named after its developers, Derjaguin, Landau, Verwey, and Overbeek (Lacchawani, 2005). This mechanism is a classical colloidal theory that involves a charged particle as having a double layer of counterions surrounding the particle. The first layer is known as Stem layer, and is made up of a hard linked layer and the second layer is known as the diffuse layer which is comprised of less tightly associated counterions. In the diffuse layer, the concentration of ions decreases with distance from the particle surface until the concentration of ions is equal to the bulk solution. This results in an electrical potential that develops around the particle. The cloud ions surrounding the particle result in repulsion of adjacent particles and inhibit aggregation. An increase in the ionic strength causes a decrease on the size of double layer which results in a decrease of the repulsion between particles, allowing short-range attractive forces to promote aggregation as shown in Figure 2.3 (Schryver *et al*., 2008).

Several studies have been conducted to support the DLVO theory for the role of cations in bioflocculation. Examples include the study that was done by Cousin and Ganczarczyk (1998) which reported that additional of sodium to a biological suspension caused an increased in floc size and improved floc porosity. The suggested changes in floc properties are explained by DLVO theory. Zita and Hermansson (1994) also reported that the ionic strength of a solution affected the floc stability and these effects could be explained by DLVO theory. They also reported that K^+ and Ca^{2+} produced similar effects on particle stability.

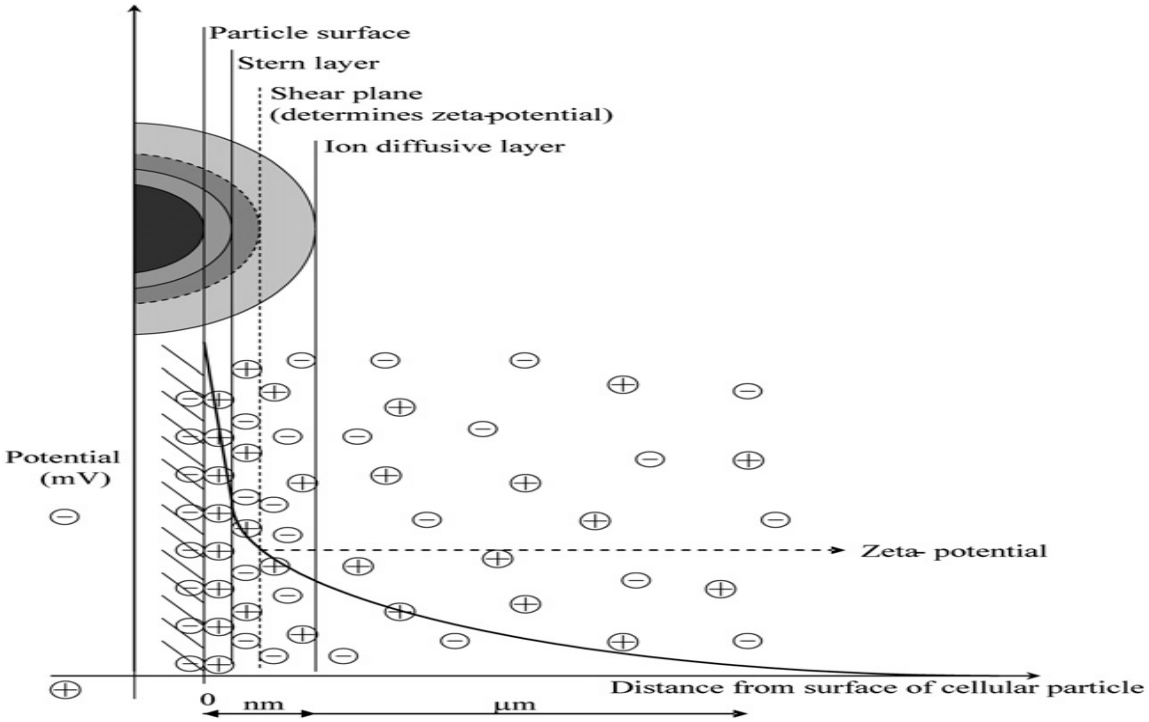

Figure 2.3: Schematic diagram showing a charged cellular particle with its counter charges and the potential in the area of a particle surface (Schryver *et al*., 2008).

2.3.4 Colloidal entrapment

Colloid entrapment, also called the sweep mechanism, involves the use of large doses of flocculants (aluminium or iron salts) which precipitate as hydrous metal oxides. The dose of flocculant used is much more in excess of the amount needed to neutralize the charge on the colloid as shown in Figure 2.4 (Lacchwani, 2005). This mechanism is mostly used for the treatment of water of variable turbidity and dissolved organic carbon in conjunction with rapid mixing, flocculation, sedimentation and filtration facilities (Sharma *et al*., 2006).

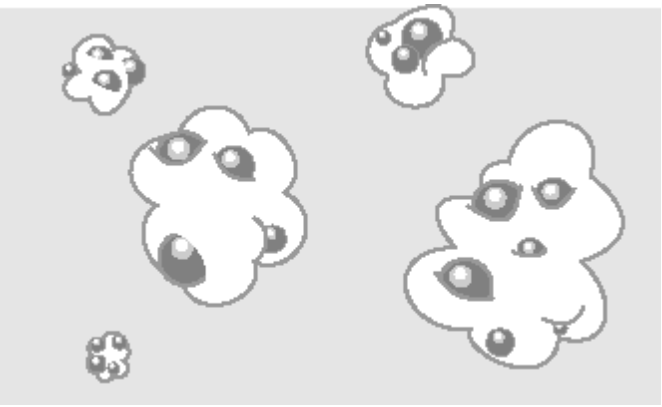

Figure 2.4: Diagram depicting colloids enmeshed in the growing precipitate (Lachhwani, 2005)

2.4 Composition and chemical structure of bioflocculants

Bioflocculants are extracellular biopolymers that are produced by several microorganisms such as algae, yeast and bacteria (Cui *et al*., 2014). They are mostly composed of macromolecular compounds such as polysaccharides, proteins, glycoproteins, lipids, glycolipids and nucleic acids (Salehizadeh and Shojaosadati, 2001; Cosa *et al*., 2011; He *et al*., 2010). The infrared spectra of several bioflocculants show the presence of the carboxyl, hydroxyl, amino and phosphate groups in the structure (Zheng *et al*., 2008). A study conducted by Zajic and Knetting (1971), concluded that these functional groups play an effective role in enhancing flocculation though some researchers emphasize the presence of amino and phosphate groups as critical to the flocculation process (Sousa *et al*., 1992).

Bioflocculants produced by *Alcaligenes latus* KT201 (Toeda and Kurane, 1991), *Enterobacter* sp. (Yokoi *et al*., 1997), *Bacillus* sp. Gilbert (Piyo *et al*., 2011), *Methylbacterium* sp. Obi (Ntsaluba *et al*., 2011), *Virgibacillus* sp. Rob (Cosa *et al*., 2011), *Klebsiella pneumoniae* (Zhao *et al*., 2013), *Paenibacillus elgii* B69 (Li *et al*., 2013) were

found to be polysaccharides, while *Bacillus subtillis* (Yokoi *et al*., 1996), *Bacillus licheniformis* (Shi *et al*., 2001), *Pacilomyces* sp. (Hiroaki and Kiyoshi, 1985), *Nocardia amarae* YK1 (Takeda *et al*., 1992) produced proteinaceous bioflocculants and *Arcuadendron* sp. TS-4 (Lee *et al*., 1995), *Arthrobacter* sp. Raats (Mabinya *et al*., 2012), *Bacillus* sp. AEMREG7 (Okaiyeto *et al*., 2015) produced a glycoprotein bioflocculant. Proteinaceous biofloccuants usually have low molecular weights and their amino and carboxyl groups contribute to flocculation. In contrast, polysaccharide bioflocculants have high molecular weights and consist of several functional groups (Deng *et al*., 2005). Several bioflocculants reported in literature showed that the molecular weight range is between 10^5 and 2.5×10^6 Da (Salehizadeh and Shojaosadati, 2001). Flocculants with higher molecular weights tend to produce more, large and loosely packed flocs (Ebeling *et al*., 2005).

2.5 Bioflocculant producing microorganisms

Bioflocculants are known as polymers which are produced by microorganisms during their growth (Deng *et al*., 2003). Several kinds of microorganisms, which secreted biopolymer flocculants have been screened and isolated from activated sludge, soil and sediments, river and deep sea water samples (Salehizadeh and Shojaosadati, 2001; Cosa and Okoh, 2014; Nwodo *et al*., 2014; Salehizadeh and Yan, 2014). However, bioflocculant's utilization in industrial applications has been limited due mainly to high production costs as well as low yields (Kurane *et al*., 1994). Therefore, screening of new microorganisms with the ability to produce flocculants with the highest flocculating activity is of great importance (Zhang *et al*., 2002; Deng *et al*., 2003; He *et al*., 2004). In general, the microorganisms that have been screened and isolated include bacteria, fungi, actinomycetes and algae (Li *et al*., 2010; Li *et al*., 2013).

2.5.1 Bacteria

Several bacterial strains have been reported to produce flocculants. A bacterium, *Paenibacillus elgii* B69 was found to produce an exopolysaccharide composed of glucose, glucuronic acid, mannose and xylose. The exopolysaccharide was effective at removing pollutants from Kaolin suspension, dyeing wastewater, heavy metal ion solution and real wastewater. These findings showed its great potential for pre-treatment of water used in industry (Lu *et al*., 2013). A bioflocculant-producing bacterium was isolated from contaminated LB medium and was identified as *Bacillus licheniformis*. Chemical analyses of the bioflocculant revealed a novel extracellular proteoglycan composed of carbohydrate (89%) and protein (11%). The bioflocculant showed stability at a wide range of temperature and pH and other advantages over chemical synthesized flocculants which include a dosing rate of 5.8 mg/L to flocculate suspended solids and flocculation efficiency in the sugar refinery process. These properties suggest its potential industrial applications (Xiong *et al*., 2010).

In a study by Nwodo and Okoh (2013), *Cellulomonas* sp. Okoh, a bacterial strain isolated from a freshwater environment, was reported to produce a bioflocculant with high flocculating activity that is mainly composed of glycosaminoglycan polysaccharides. The presence of this unique property indicates higher potential for industrial applications as an effective yet safer alternative for replacing the hazardous flocculants used in several water treatment processes. *Chryseobacterium daeguense* W6 produced a bioflocculant named MBF-W6 and the bacterium was isolated from backwashing sludge. MBF-W6 showed maximum flocculation rate of more than 90% for Kaolin suspension. The addition of cations could not enhance flocculating activity of MBF-W6 produced by *Chryseobacterium*

daeguense, thus indicating its cation-independence resulting in the avoidance of secondary pollution as well as costs reduction (Liu *et al*., 2010).

Zheng *et al*. (2008) reported on bioflocculant MBFF19 produced by *Bacillus* sp. F19 that was composed of both sugar and protein albeit with the carbohydrate as the main effective flocculating component at a ratio of 66.4% to 16.4% of protein. Fourier transform infrared spectroscopy showed the presence of carboxyl, hydroxyl and methoxyl groups as major functional groups which have been reported to contribute towards enhancing flocculating efficiency (Deng *et al*., 2003). The flocculation process of MBFF19 relied on the bridging type mechanism rather than the charge neutralization mechanism (Zheng *et al*., 2008). Deng *et al*. (2003) also reported that bioflocculant MBFF19 was able to flocculate Kaolin suspension independent of cations (Deng *et al*., 2003).

2.5.2 Fungi

Aspergillus parasiticus was found to produce a bioflocculant with high flocculating activity for Kaolin suspension and water-soluble dyes. The bioflocculant mainly consisted of sugar (76.3%) and protein (21.6%), with an average molecular weight of 3.2×10^5 Da (Salehizadeh and Shojaosadati, 2001). X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared (FTIR) spectra showed the presence of amino, amide and hydroxyl groups in the bioflocculant molecule (Deng *et al*., 2005). The bioflocculant was shown to be more effective in flocculating soluble anionic dyes in aqueous solution, particularly Reactive Blue and Acid Yellow 25 with a decolorization efficiency of 92.4 and 92.9%, respectively (Deng *et al*., 2005). The amino and amide groups present in the bioflocculant molecule are known to play

an important role in the flocculation process with respect to electrostatic interactions (Deng *et al*., 2005).

2.5.3 Actinomycetes

Nocardia amarae YK-1 was found by Desouky *et al*. (2008) to be a bioflocculant-producing actinomycete which produced a bioflocculant named, FIX. Chemical analyses of this bioflocculant showed the presence of a protein as a predominant component. Flocculating activity of FIX was enhanced by the addition of both divalent cations (NaCl, $CaCl_2$, $Al_2(SO_4)_3$ and a trivalent cation ($FeCl_3$). However, the flocculation activity was inhibited by excess addition of $FeCl_3$ (Takeda *et al*., 1991). The other strains in the *Norcadia* family that are found to produce bioflocculants include *Norcadia restricta*, *Norcadia calcarea* and *Norcadia rhodnii* (Desouky *et al*., 2008).

2.5.4 Algae

The p-KG03 is an exopolysaccharide bioflocculant produced by *Gyrodinium impudicum* p-KG03 is isolated from the marine environment and has been reported to possess a strong antiviral activity against encephalomyocarditis virus (EMCV) and immunostimulating activity by NK cell activation (Yim *et al*., 2007). The p-KG03 flocculating activity is not dependent on the presence of any cations, including Ca^{2+} , similar observation was also noted in a study conducted by Fujita *et al*. (2000) for bioflocculant TKF04 produced by *Citrobacter* sp. These observations suggest that p- KG03 could be utilized successfully in the clarification of a broad range of wastewaters under various environmental conditions (Yim *et al*., 2007).

2.6 Factors affecting bioflocculant production and flocculating activity

2.6.1 Effect of carbon and nitrogen sources on bioflocculant production

Different carbon and nitrogen sources vary in the extent to which they affect bioflocculant production by different microogarnisms (Deng *et al*., 2005). In the case of a bioflocculant produced by *Aspergillus parasiticus*, it was evident that corn starch, sucrose, glucose and glycerol all enhanced its production with flocculating efficiencies above 80% after 72 h of cultivation with starch the most favoured carbon source at 96% flocculating activity. When corn starch was used as the carbon source, peptone and sodium nitrate provided the best nitrogen sources for bioflocculant production by *A. parasiticus*. In the presence of ammonium sulphate, no flocculant was produced (Deng *et al.,* 2005).

The effect of a variety of carbon sources on the production of a bioflocculant produced by *Bacillus* sp. showed that sucrose was the best carbon source for both productions of bioflocculant and cell growth but in the presence of maltose and ethanol relatively low bioflocculant yield was achieved. When organic nitrogen sources were used, bioflocculant production was enhanced compared to inorganic nitrogen sources with yeast extract as the preferred nitrogen source supporting the highest flocculating rate of 78% (Zheng *et al*., 2008).

A study carried by Mabinya *et al.* (2012) showed that a bioflocculant produced by *Arthrobacter* sp. Raats produced the highest flocculating activities of 75.4% and 73.4% when lactose and sucrose were utilized as the carbon sources respectively, no bioflocculating activity was detected in the presence of both fructose and starch. *Arthrobacter* sp. Raats was

able to utilize both organic (urea) and inorganic (ammonium sulphate) nitrogen sources with urea being more effectively used and resulting in the highest flocculating activity.

The highest flocculating activity (97.15%) was achieved by a bioflocculant produced by *Serratia ficaria* when lactose was the carbon source of choice. Glucose and ethanol were also found to be favourable carbon sources. Bioflocculant production by *S. ficaria* was not favoured in the presence of urea, peptone and ammonium sulphate as nitrogen sources. Although both beef and yeast extracts were individually supportive of bioflocculant production, the highest flocculating activity was achieved when a mixture of the two nitrogen sources (beef extract and urea) was utilized (Gong *et al*., 2008).

The new carbon source, palm jaggery and maltose were found to be both favourable for bioflocculant production by *Bacillus subtilis* MSBN17 as well as for cell growth. Palm jaggery was the most preferred and cheapest carbon source than commercial sugar and it resulted in the highest flocculating activity of up to 92.07%. From the various nitrogen sources investigated, inorganic nitrogen source (ammonium sulphate) was a preferred source for both production and cell growth, while all organic nitrogen sources tested led to poor bioflocculant production and cell growth (Sathiyanarayanan *et al.*, 2013).

2.6.2 Effect of cations on flocculating activity

Cations are important components in the culture medium for the production of bioflocculants. They are added to enhance the flocculating activity by neutralizing the negatively charged functional groups of both the bioflocculant molecules and the suspended particles and hence weaken the static repulsive forces thus achieving higher flocculating effect (Zheng *et al.,* 2008). Bioflocculant produced by *Bacillus* sp. UPMB13 was greatly stimulated by the presence of Na+, Ca^{2+}, and Mg^{2+} while the addition of Al^{3+} and Fe^{2+} inhibited flocculating

activity both in terms of optical density and observations of floc formation (Zulkeflee *et al.*, 2012. A bioflocculant produced by *Halomonas* sp. OKOH, had enhanced flocculating activity in the presence of $CaCl_2$ when compared to $MgCl_2$ and FeSO4·7H2O (Mabinya *et al.*, 2011). On the other hand, a bioflocculant produced by *Virgibacillus* sp. Rob was reported to have its flocculating activity stimulated by the presence of iron sulphate (Cosa *et al*., 2011).

2.6.3 Effect of initial pH of growth medium on bioflocculant production

The pH of the culture medium determines the electric charge of the cells together with the oxidation potential which can affect the nutrient absorption and enzymatic reactions (Xia *et al*., 2008). A bioflocculant TJ-1 produced by *Proteus mirabilis* reached the optimum pH when the initial pH was set to neutral pH 7, resulting in savings of large quantities of acids or alkali used to adjust the pH, therefore reducing the production cost of this bioflocculant. Microorganisms have different initial pH for bioflocculant production. In the case of *Aspergillus parasiticus*, lower pH was shown to be better for fungal growth and bioflocculant production (Deng *et al*., 2005). The optimal pH was in the range of 5-6 and growth and bioflocculant production were decreased above pH 7 (Deng *et al*., 2005). The optimal pH for the bioflocculant production produced by *Penicillium purpurogenum* was reached at an initial medium pH adjusted at 5.5. When the initial pH was set at 5-8, this bioflocculant was able to produce some substances to regulate pH because pH change of 5.5 was occurred after 1 day of cultivation and kept steady until the fourth day (Liu and Cheng, 2010).

2.6.4 Effect of incubation period on bioflocculant production

According to findings by Deng *et al*. (2005), flocculation increases with the time of incubation. Between the incubation period of 24-60 h, *Aspergillus parasiticus* was in its exponential growth phase and the flocculating activity increased from 12 to 95% respectively. Beyond a cultivation period of 72 h, the fungus reached its stationary phase in which the highest flocculating rate for Kaolin suspension was 98.1% (Deng *et al*., 2005). *P. mirabilis* TJ1 showed a growth curve in which biofflocculant production was almost parallel with cell growth during the logarithm phase and the pH of culture medium decreased sharply. This bioflocculant reached its highest flocculating activity of 93.13% at stationery phase (Xia *et al*., 2008). A study carried out by Liu *et al*. (2010) on bioflocculant production by *Chryseobacterium daeguense* W6, showed a sharp increase in cell growth for the first 8 h of incubation while the culture pH decreased slightly. However the flocculating activity was very low. During the death phase of the growth curve, the flocculating rate increased to 90% after 54 h when the OD_{600} was below 0.3 and it can be concluded that MBF-W6 was an intracellular bioflocculant.

2.7 Applications of bioflocculants

Recently, various studies have been conducted about replacing synthetic flocculants for the treatment of various industrial wastes (Gao *et al*., 2009; Liu *et al*., 2009; Yim *et al*., 2007) including heavy metal removal (Wu and Ye, 2007; Gong *et al*., 2008; Lin and Harichund, 2012) with natural by occurring flocculants such as microbial flocculants. Bioflocculants have been recommended for their effectiveness in the removal of heavy metals (Morillo *et*

al., 2006), but the drawback has always been the high costs of bioflocculant production compared to conventional approaches (Lian *et al*., 2008).

2.7.1 Removal of heavy metals

Various industries release tons of heavy metals into aquatic environments during production processes (Baby *et al*., 2010). This has led to an increase of toxic heavy metals that result in environmental pollution. Different methods have been developed for the removal of these toxic chemicals before they are released into the environment and these include precipitation, coagulation, ion exchange, electrochemical and membrane processes (Salehizadeh and Shojaosadati, 2003). Bioflocculants have attracted the attention of many researchers due to their economical value, effectiveness and safety for the removal of heavy metals from wastewater. A bioflocculant produced by *Bacillus firmus* resulted in the removal of lead (98.3%), copper (74.9%) and zinc (61.8%) from aqueous solution at optimum pH values of 4.5, 4 and 6, respectively (Salehizadeh and Shojaosadati, 2003).

2.7.2 Removal of steroid estrogen

Estrogens are released into the aquatic environment through the effluents from wastewater treatment plants (WTPs). Although many studies have reported on the occurrence and removal of estrogens in WTPs (Andersen *et al*., 2005), conventional processes in municipal wastewater treatment plants have been shown to be ineffective at removing estrogens (Braga *et al*., 2005), hence there's a great need for advanced treatment processes to eliminate the presence of these estrogens. According to a study by Zhong *et al*. (2014), the efficiencies of

flocculants produced by *Sphingomonas yabuuchiae* SW-2 for the removal of target estrogens increased as the flocculant dosage increased from 20 to 50 mg/L beyond which a decrease in removal efficiencies was observed. The removal efficiencies of estrogens increased as the culture pH dropped from 8 to 3 but slowed down when the culture pH fell below 3. According to Zhong *et al*. (2014), this observation suggests that the bioflocculant produced by *S. yabuuchiae* has the potential of removing estrogens under acidic conditions than alkali conditions.

2.7.3 Wastewater treatment

Bioflocculants have been used in wastewater treatment for the removal of dyes (Deng *et al*., 2005), inorganic solid suspensions and aluminun oxide (Levy *et al.,* 1992; Shih *et al*., 2001; Yim *et al*., 2007), humic acids (Zouboulis *et al*., 2004) and other synthetic suspensions (Salehizadeh and Shojaosadati, 2002; Lu *et al*., 2005). For example, during the treatment of wastewater, the bioflocculant decolorization efficiencies for methylene blue at an initial concentration of 10 mg/L and for fast blue at 50 mg/L were found to be 82.9% and 77.8%, respectively. It was concluded that the bioflocculant, referred to as M-1 showed good flocculating activity and could thus be used in the pre-treatment of dyed wastewater (Liu *et al*., 2009). A study carried out by Gong *et al*. (2008), indicated that a bioflocculant produced by *Serratia ficaria* was successfully used to treat numerous samples of wastewater, including river water, brewery wastewater, meat processing wastewater and soy sauce brewing wastewater. The treatment of river water using *S. ficaria,* showed the removal efficiencies of COD, turbidity and colour to be 87.1%, 84.2% and 90.4%, respectively. The results were then compared with commonly used chemical synthetic flocculants; bioflocculant produced by *S. ficaria* showed better flocculating efficiency in the clarification of river water (Gong *et*

al., 2008). The results obtained at treating brewery wastewater, meat processing wastewater and soy sauce brewing wastewater were better when compared to those obtained using conventional chemical synthetic flocculants (Gong *et al*., 2008). Also, the bioflocculant produced by a consortium of *Corbetia* sp. and *Bacillus* sp. showed flocculating activities for river water, brewery and dairy wastewater of 99%, 90.2% and 78.8%, respectively, thus indicating the potential applicability of this bioflocculant for wastewater treatment (Ugbenyen and Okoh, 2014).

2.7.4 Removal of humic acids

Humic acids are regarded as capable of forming a complex with metal ions and consequently are involved in the transportation of metals and their subsequent discharge in soil and waters (Calase *et al*., 2001). The formation of these complexes tends to limit the removal efficiency of metals by sorption, since there's a competition for the availability of sorption sites (Calase *et al*., 2001). A study done by Zouboulis *et al*. (2004) revealed that the bioflocculant produced by *Rhizomonas* sp. was efficient in removing humic acids from synthetic solutions. An optimal pH of between 7 and 7.5 and a bioflocculant dosage of 20 mg/L were effective in removing more than 85% of humic acid.

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CHAPTER 3

Characterization of a bioflocculant produced by *Bacillus* sp. AEMREG4

ABSTRACT

A fresh water bacterium isolated from Tyhume River, Eastern Cape was investigated for its bioflocculant production potentials; also the effect of optimizing culture conditions to enhance bioflocculant production was evaluated. Analysis of the 16S rDNA sequence of nucleotide revealed it to have 99% similarity to *Bacillus* sp. BCT-7112 and it was deposited in GenBank as *Bacillus* sp. AEMREG4 with an accession number KP406729. The optimum culture conditions included the following: 4% (v/v) inoculum size; starch and yeast extract as preferred carbon and nitrogen sources respectively. Bioflocculant production was greatly enhanced by the addition of Ca^{2+} at a wide pH range of 4 - 10 and the produced bioflocculant exhibited thermal stability when heated at 100 °C for 1 h. A yield of 0.78 g of purified bioflocculant was recovered from 1 L of culture broth. Chemical analysis of the purified bioflocculant indicated the presence of carbohydrate as a predominant component at a concentration of 79% (w/w) with protein at 5% (w/w) and 15% (w/w) of uronic acid. Scanning electron microscopy (SEM) image of the purified bioflocculant revealed that it had a rod shaped structure which contributes to its high flocculating efficiency. The Fourier transform infrared (FTIR) spectrum analysis showed the presence of carboxyl, hydroxyl and methoxyl groups in its structure which have been shown to be essential for the flocculation process

Keywords: *Bacillus* sp, Bioflocculant, Flocculating activity

1. INTRODUCTION

Flocculating agents are utilised abroad in various industrial processes such as drinking water, wastewater treatment, downstream processes, dredging, textile, and in various fermentation fields (Nwodo *et al*., 2013; Okaiyeto *et al*., 2013; Salehizadeh and Yan, 2014). Flocculants can be generally classified into three major groups: inorganic flocculants (aluminium sulphate and polyaluminium chloride), organic flocculants (polyacrylamide and polyethylene imines), and natural polymer flocculants or bioflocculants (chitosan, gelatin, lignin and microbial flocculants) (Nwodo and Okoh, 2013; Zhao *et al*., 2013; Li *et al*., 2014; Pu *et al*., 2014). The first two groups of conventional flocculants have been commonly used due to their cost effectiveness and strong flocculation efficiency (Mabinya *et al*., 2011). However, the major drawbacks of some of these synthetic flocculants have been their detrimental effects to public health and environmental pollution. For example, the acrylamide monomers were found to be both neurotoxic and carcinogenic to humans and furthermore, the residual aluminium has also been implicated as a causative agent of Alzheimer's disease (Zheng *et al*., 2008; Zhao *et al*., 2009; Wang *et al*., 2011; Ugbenyen *et al*., 2012).

Due to the above concerns, bioflocculants have recently attracted wide attention and have also received considerable scientific and biotechnological attention, mainly because they possess, amongst other advantages, environment-friendly, harmlessness and lack of secondary pollution risks (Gong *et al*., 2008; Liu *et al*., 2009). However, low productivity and cultivation costs are still major drawbacks for applying these bioflocculants industrially (Guo *et al*., 2013). To date, several bioflocculant-producing microorganisms have been reported (Deng *et al*., 2005) and they have been used in various processes such as in the removal of humic acids (Zouboulis *et al*., 2004), dye removal (Deng *et al*., 2005; Gao *et al*., 2009; Liu *et*

al., 2009; Huang *et al*., 2014), steroid estrogen removal (Zhong *et al*., 2014) and in heavy metal removal (Salehizadeh and Shojaodati, 2003; Wu and Ye, 2007; Lin and Harichund, 2012). The purpose of this study is to characterize and assess the flocculating efficiency of a bioflocculant produced by *Bacillus* sp. AEMREG4.

2. MATERIALS AND METHODS

2.1 Isolation of the microorganisms and culture conditions

The rock scraping, water and sediment samples were collected from different points of the Tyhume River in the Eastern Cape, South Africa. Samples were transported using ice buckets and analysed in the laboratory within 2-3 h of collection. Serial dilutions of up to 10^{-3} were made in distilled water. Screening of all the isolates was done in according to the method described by Jensen *et al*. (1991) and Jensen *et al*. (2005) with minor modifications.

Water samples: One millilitre of the water sample was added to 9 ml of distilled water in the test tubes. The diluted solution was agitated for 30 s and further diluted up to 10^{-3}-fold. The diluted solution (100 µl) was spread using a glass spreader on both M1 and R2A agar plates prepared according to the manufacturer's instructions.

Sediment samples: Wet sediment samples (2 ml) were transferred into 8 ml of sterilized distilled water and vortexed for 30 s. Serial dilutions of up to 10^{-3}- fold were made using test tubes. The diluted sample (100 µl) was spread on both M1 and R2A agar plates using a sterilized glass spreader as previously described.

Rock scraping: Rock scraping samples (0.5 g) were weighed and dissolved in 5 ml of sterilized distilled water. Serial dilutions of the samples were made up by mixing 1 ml of the sample solution with 9 ml of distilled water and further diluted up to 10^{-3}- fold in distilled water. As described previously, 100 µl of the diluted solution was plated on both M1 and R2A agar.

All plates were incubated at 28 °C with R2A and M1 agar plates incubated for 4 and 8 days, respectively. The isolates on both R2A and M1 agar plates were purified by re-streaking on fresh agar plates and further incubated at 28°C for 2 and 5 days, respectively.

2.2 Initial screening of the microorganism for bioflocculant production

Activation medium was prepared by dissolving the following components in 1 L of sterile distilled water: 3 g of beef extract 10 g of tryptone, 5 g of NaCl. Activation medium (5 ml) was dispensed into separate test tubes prior to autoclaving at 121 °C for 15 min. For inoculum preparation, the isolates from M1 and R2A agar plates were cultured in 5 ml activation medium and incubated at 28 ºC for 24 h. This was used as a seed culture for screening of the microorganisms for bioflocculant production.

Screening of the microorganisms for bioflocculant production was done using the growth medium described by Zhang *et al*. (2007). The medium contained the following: 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.1 g NaCl and 0.2 g $MgSO_4.7H_2O$ in 1 L of distilled water. After sterilizing by autoclaving at 121 ºC for 15 minutes and cooling to room temperature, the seed culture (1 ml) was inoculated into 50 ml of growth medium in 250 ml flasks. The culture was incubated in a rotary shaker at 28 °C, 160 rpm for 5 days. After 72 h of incubation, 2 ml of culture suspension was

withdrawn and centrifuged at 4000 × g, 4 °C at 30 min. The cell free culture supernatant was used to determine flocculating activity and the strain with a high and stable flocculating activity for Kaolin suspension was selected for this study.

2.3 Determination of flocculating activity

A suspension of Kaolin (4 g/L) was used as test material for estimating flocculating activity of the produced bioflocculant as described by Kurane *et al*. (1994). Clay suspension (100 ml), 3 ml of 1% $CaCl_2$ and 2 ml of the culture supernatant were all mixed into a 250 ml conical flask. The solution was mixed by agitation for 60 s, and was then transferred into a 100 ml graduated cylinder and allowed to sediment for 5 min at room temperature. The control was also prepared similarly except for the bioflocculant which was replaced with fresh broth. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Thermo Spectronic, USA) and the flocculating activity was calculated using the following formula:

FA = [(A-B/A)] × 100%, where

FA = Flocculating activity, A and B are optical densities of control and sample measured at 550 nm respectively. All experiments were performed in triplicates and the mean was used for calculating activity.

2.4 Optimization of culture conditions for bioflocculant production

2.4.1 Effect of carbon and nitrogen sources on bioflocculant production

Carbon and nitrogen sources are considered as playing a huge and influential role in the production of bioflocculants by microorganisms (Salehizadeh and Yan, 2014). The effect of various carbon and nitrogen sources on bioflocculant production was assessed according to the method described by Ugbenyen *et al*. (2012). A bioflocculant-producing bacterial strain was inoculated into growth medium contained in separate flasks supplemented with different carbon and nitrogen sources, and incubated in a rotary shaker at 160 rpm, 28°C for 5 days. The carbon source candidates included glucose, maltose, fructose, lactose, sucrose and starch with tryptone, peptone, urea, yeast extract (organic nitrogen sources) and ammonium sulphate (inorganic nitrogen source) as nitrogen source candidates. The flocculating activity was determined according to the method previously described.

2.4.2 Effect of inoculum size on bioflocculant production

The effect of inoculum size on bioflocculant production was assessed by inoculating the production medium in separate flasks with varying amounts of seed culture broth ranging from 1%-5% (v/v) prior to incubating in a rotary shaker at 160 rpm, 28°C for 5 days (Zhang *et al*., 2007). The flocculating activity assay was done as previously described.

2.4.3 Effect of initial pH of the medium

Carbon and nitrogen sources that supported optimum flocculating activity were used to supplement the production medium contained in separate flasks with the pH ranging between 4 and 10. Either NaOH (0.1 M) or HCl (0.1 M) was used to adjust the pH. The medium was incubated in a rotary shaker at 160 rpm, 28ºC for 5 days after which flocculating activity was determined as previously described (Yim *et al*., 2007).

2.4.4 Effect of various cations on flocculating activity

The effect of metal ions on flocculating activity was assessed following the same procedure as described above for determining flocculating activity but the 1% $CaCl_2$ solution was replaced by various metal ion solutions prior to measuring flocculating activity. Solutions of NaCl, KCl (monovalents), $MgCl_2$, $MnCl_2.4H_20$, $CaCl_2$ (divalents), $AlCl_3$ and $FeCl_3 \cdot 6H_2O$ (trivalents) were used as cation sources (Liu *et al*., 2010) and flocculating activity was measured as previously described.

2.4.5 Effect of temperature on flocculating activity

The effect of temperature on the bioflocculant was tested whereby, 2 ml portions of the seed culture broth were transferred into separate tubes and centrifuged at 4000 × g, 4 °C for 30 min. Aliquotes cell free culture supernatant were transferred into clean 2 ml sterile Eppendorf tubes which were then each incubated at different temperatures ranging from 50 °C to100 °C for 1 h. Flocculating activity was determined at room temperature as previously elaborated (He *et al*., 2004).

2.5 Time course assay of bioflocculant production

Composition of culture medium: The composition of the medium for bioflocculant production was prepared according to the method described by Zhang *et al*. (2007), and contained the following in 1 L of distilled water: 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.1 g NaCl and 0.2 g $MgSO_4 . 7H_2O$. The pH was adjusted to pH 8 with either NaOH or HCl.

Standardization of inocula: For inoculum preparation, the isolate was cultured in 50 ml activation medium (3 g L^{-1} of beef extract 10 g L^{-1} of tryptone, 5 g L^{-1} of NaCl) in a 250 ml flask on a rotary shaker at 28 ºC, 160 rpm for 24 h. After 24 h of incubation, distilled water was used to adjust the turbidity of the fermented culture to give an optical density (OD_{600}) of 0.1.

Time course assays: The seed culture (4% v/v) was inoculated into 50 ml of production medium in 250 ml flasks (prepared in triplicates) on a rotary shaker at 28 °C, 160 rpm. Samples were drawn at 24 h intervals to determine pH, cell count, cell growth and flocculating activity. Flocculating activity and the pH of the sample broth were determined as previously described. Bacterial growth was determined by measuring the OD_{600} and bacterial counts were determined by the standard spread plate technique on nutrient agar plates. All plates were incubated at 37 °C for 24 h (Okaiyeto *et al*., 2013).

2.6 Extraction and purification of the bioflocculant compound

After 5 days of fermentation, the fermentation broth was centrifuged at 4000 × g, 4 °C for 30 min to remove cell debris, 1 L of distilled water was added to the supernatant and the mixture centrifuged at 4000 × g, 4 °C for 15 min to remove all the insoluble substances. Two volumes of cold ethanol was added to the supernatant and the mixture was stirred and left standing at 4 °C for 12 h to precipitate. The supernatant was discarded and the precipitate was vacuum-dried to obtain the crude biopolymer, according to the method described by Zhang *et al*. (2010). The obtained precipitate was re-dissolved in distilled water (1% v/v) followed by the addition of one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v) with stirring. The mixture was then left standing at room temperature for 12 h and the upper phase was centrifuged at 4000 × g, 4 °C for 15 min, and the supernatant was dialyzed overnight against distilled water. The dialysate was then vacuum-dried to obtain a purified bioflocculant.

2.7 Flocculating characteristics of purified bioflocculant

2.7.1 Determination of bioflocculant dosage using Jar Test

Bioflocculant dosage for optimum activity was determined using the method of Wang *et al*. (2010), with minor modifications. Different concentrations of the purified bioflocculant (0.1-0.5 mg/ml) were added to 100 ml Kaolin suspension (4 g/L) containing 3 ml of 1% $CaCl_2$ in 250 ml flasks. The solution was agitated rapidly at 180 rpm for 3 min during the addition of the bioflocculant, followed by slow stirring at 40 rpm for 5 min after which the mixture was

allowed to settle for 10 min. The upper phase of the solution was used to determine the flocculating activity as previously elaborated.

2.7.2 Thermal stability of purified bioflocculant

To examine thermal stability of the bioflocculant, the purified bioflocculant was dissolved in 10 ml distilled water to achieve a concentration of 0.1 mg/ml. Aliquots of the solution were incubated at various temperatures ranging from 50 °C to100 °C for a period of 1 h prior to measuring residual flocculating activity at room temperature (Sun *et al*., 2012).

2.7.3 Effect of pH and cations on purified bioflocculant activity

The effect of pH on flocculating activity was determined by adjusting the pH of Kaolin solution in separate test tubes between the range of 3 to 10 using either HCl or NaOH after which flocculating activity of each solution was determined as previously described (He *et al*., 2010).

The effect of cations on flocculating activity of the purified bioflocculant was determined by replacing 1% $CaCl_2$ with each of the following salt solutions: NaCl, KCl (monovalents), $MgCl_2$, $MnCl_2.4H_2O$, $CaCl_2$ (divalents), $AlCl_3$ and $FeCl_3 \cdot 6H_2O$ (trivalents) as cation sources (He *et al*., 2010; Liu *et al*., 2010). Flocculating activity was determined as previously described.

2.8 Characterization of the purified bioflocculant

2.8.1 Protein content

The total protein content was measured by the Folin-Lowry method as elaborated by Lowry *et al*. (1951) using bovine serum albumin (BSA) as the standard solution.

2.8.2 Sugar content

The total sugar content of the purified bioflocculant was determined using the Phenol-Sulfuric acid method with glucose as the standard solution as described by Chaplin and Kennedy (1994).

2.8.3 Fourier Transform Infrared (FTIR) Spectroscopy

Potassium-bromide (KBr) powder was used to grind the dried bioflocculant before pressing it into pellets for Fourier transform infrared (FTIR) spectroscopy analysis over a wavelength range of 4000 to 370 cm^{-1} (He *et al*., 2010).

2.8.4 Scanning electron microscopy (SEM)

The scanning electron microscopy (SEM) images of the bioflocculant were also evaluated (Xiong *et al*., 2010). They were viewed on a Japanese Electron Optical Lab (JEOL) 6390 LV SEM at 15 KV. Scanned images of the purified bioflocculant, Kaolin clay powder and flocculated dried Kaolin clay were analysed.

3. RESULTS AND DISCUSSION

3.1 Isolation of bioflocculant-producing microorganisms

A total of 144 bacterial strains were isolated from freshwater of the Tyhume River and 13 strains displayed positive bioflocculant activity when tested against Kaolin suspension. The strain that was selected for this study produced a bioflocculant that showed the highest flocculating activity of 78% for Kaolin clay suspension (4 g/L). Based on the morphological and physiological characteristics, the selected strain was found to be a Gram-positive with circularly-shaped cream coloured colonies which is characteristic of the *Bacillus* genus. The bacterial isolate was sent for sequencing and analysis of the 16S rDNA sequence revealed it to have 99% similarity to *Bacillus* sp. BCT-7112. The nucleotide sequence was then deposited in GenBank as *Bacillus* sp. AEMREG4 with an accession number KP406729.

The Bacillus genus belongs to a member of the division Firmicutes. They are gram positive, spore forming bacteria used commercially as probiotics (Turnbull, 1996). They are found everywhere in the environment and include free living and microorganisms. They grow well in different and low cost carbon sources (Bunk *et al*., 2010). The members of the genus have been considered as important industrial species with the advantage of being safe for utilization even in food processing industries (Desouky *et al*., 2008).

A wide range of Bacillus species has been utilized for a variety of different applications. For example, *Bacillus subtilis* has been used as a food source in Japan (Djien and Hesseltine, 1979). Many studies with *Bacillus toyonensis* have proven to be beneficial in animals, such as improvement in digestive health and weight gain (Casanovas-Massana *et al*., 2014). The spores of *Bacillus toyonensis* BCT-7112 have been used in animal nutrition for swine,

poultry, cattle, rabbits and aquaculture for more than 30 years in a number of countries around the world (Jimenez *et al*., 2013). A number of Bacillus species have been reported to produce bioflocculants and these include *Bacillus mucilaginosus* (Deng *et al*., 2003), *Bacillus licheniformis* X14 (Li *et al*., 2009), *Bacillus* sp. Gilbert (Piyo *et al*., 2011), *Bacillus alvei* NRC-14 (Abdel-Aziz *et al*., 2011), and *Bacillus subtilis* MSBN17 (Sathiyanarayanan *et al*., 2013).

3.2 Factors affecting bioflocculant production and flocculating activity

There are several factors affecting bioflocculant production and flocculating activity (Zhang *et al*., 2007). The major key factors include inoculum size, carbon and nitrogen sources, cations, pH and temperature. Hence, there's a need to optimize these factors to enhance both bioflocculant yields and flocculating activity.

3.2.1 Effect of inoculum size on bioflocculant production

Inoculum size is of great significance factor in bioflocculant production and cell growth (Salehizadeh and Yan, 2014). Salehizadeh and Shajoasadati (2001) reported that a small size of inoculum can prolong the lag phase, while the large inoculum size will create niches of the strain to overlap excessively, thus inhibiting bioflocculant production. The effect of inoculum size on bioflocculant production of *Bacillus* sp. AEMREG4 was investigated as shown in Figure 3.1. An inoculum size of 4% (v/v) supported an optimum flocculating activity of 80% beyond which a decrease in activity was observed, thus making 4% an inoculum size of choice for all subsequent culture experiments. Xiong *et al*. (2010) reported a similar finding

for bioflocculant production by *Bacillus licheniformis* where it was observed that an inoculum size of 4% (v/v) was optimum. In contrast, studies carried out on bioflocculant production by the following bacterial strains, *Serratia ficaria* (Gong *et al*., 2008), *Klebsiella pnumoniae* YZ-6 (Luo *et al*., 2014) and by multiple-microorganism consortia of *Staphylococcus* sp. and *Pseudomonas* sp. (Zhang *et al*., 2007) showed maximum flocculating activity being attained at 1% inoculum size.

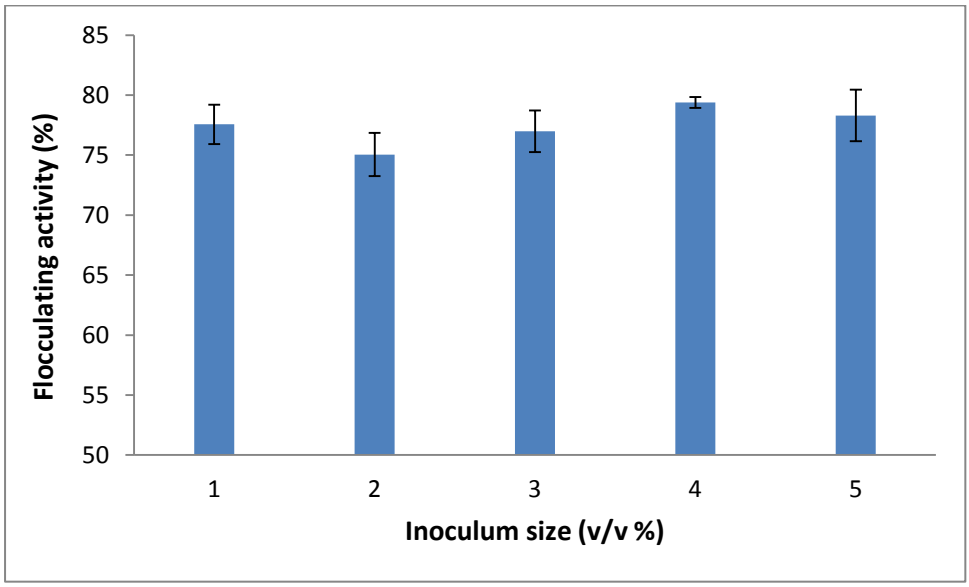

Figure 3.1: Effect of inoculum size on bioflocculant production by *Bacillus* sp. AEMREG4

3.2.2 Effect of carbon source on bioflocculant production

Carbon source play an important role in enhancing bioflocculant production and different microorganisms prefer different carbon sources for bioflocculant production (Piyo *et al*., 2011). The effects of various carbon sources on bioflocculant production by *Bacillus* sp. AEMREG4 were investigated (Figure 3.2). Among the different carbon sources studied,

starch was the most favourable carbon source supporting a flocculating activity of 81% and maltose being the least favourable carbon source with flocculating activity of 29%. Starch was then chosen as the sole carbon source for all subsequent experiments. Similar findings were reported by Li *et al*. (2009) on bioflocculant production by *Bacillus licheniforms* X14, in which starch, sucrose and ethanol were the favourable carbon sources. Deng *et al*. (2005) also reported similar findings from a study carried out on bioflocculant production by *Aspergillus parasiticus* in which 96% flocculating efficiency was achieved with starch as a sole carbon source. Contrary to these findings, starch showed the least flocculating activity of 5% for the production of bioflocculant produced by *Virgibaccilus* sp. Rob (Cosa *et al*., 2011) and completely inhibited bioflocculant production by *Halomonas* sp. OKOH (Mabinya *et al*., 2011). These findings support the observations that preferences for carbon source vary among bioflocculant-producing microorganisms (Salehizadeh and Yan, 2014).

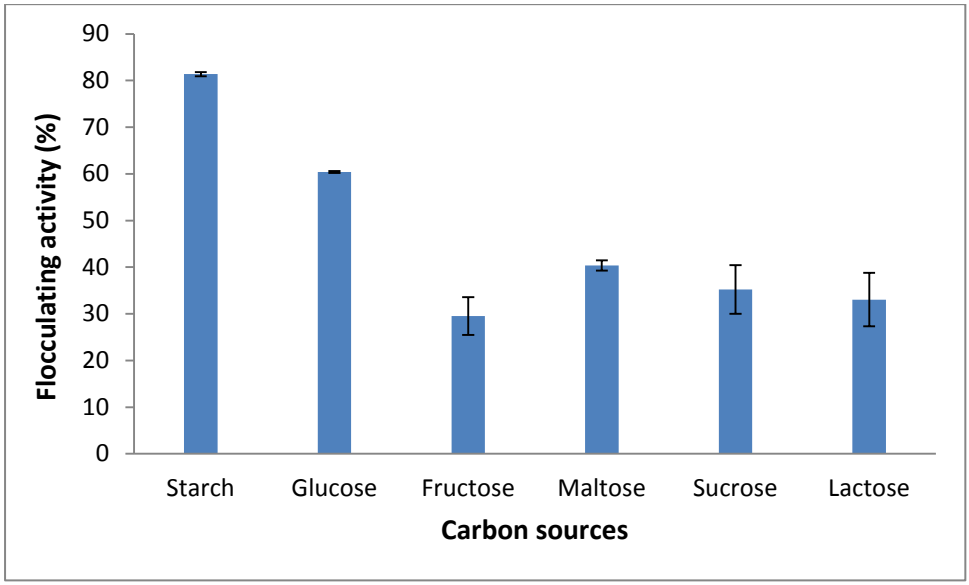

Figure 3.2: Effect of carbon sources on bioflocculant production by *Bacillus* sp. AEMREG4

3.2.3 Effect of nitrogen source on bioflocculant production

Various microorganisms require the presence of either organic or inorganic nitrogen sources for bioflocculant production (Ugbenyen *et al*., 2012). The effect of organic (yeast extract, tryptone, peptone, urea) and inorganic nitrogen sources (ammonium sulphate) on bioflocculant production by *Bacillus* sp. AEMREG4 was investigated. As shown in Figure 3.3, among the various nitrogen sources examined, yeast extract proved to be the best nitrogen source with the highest flocculating activity of 82% and $(NH_4)_2SO_4$ was the least favourable nitrogen source with a flocculating activity of 57%. Similarly, the presence of yeast extract as sole nitrogen source showed maximum flocculating activity on a bioflocculant produced by *Penicillium* sp. (Liu *et al*., 2010). In another study done by Zheng *et al.* (2008), the production of bioflocculant by *Bacillus* sp. F19 showed maximum flocculating activity of 78% when yeast extract was used as a sole nitrogen source. Shadia *et al*. (2011) investigated the effect of various nitrogen sources on bioflocculant production by *Bacillus alvei* NRC-14 and reported both $(NH_4)_2SO_4$ and yeast extract to be the most effective. It has been reported in literature that organic nitrogen sources are easily absorbed by the cells; hence they are most favourable for bioflocculant production when compared to inorganic nitrogen sources (Cosa *et al*., 2013)

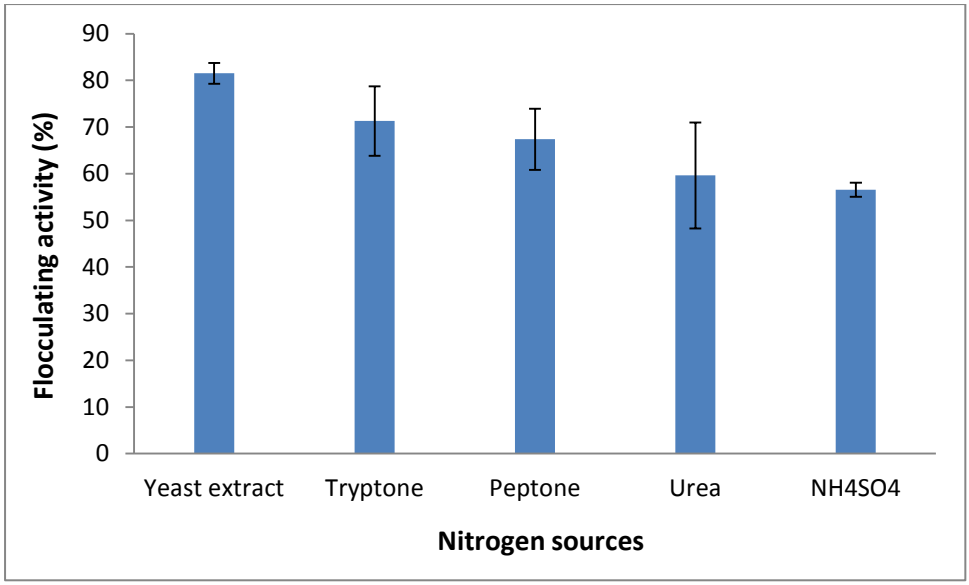

Figure 3.3: Effect of nitrogen sources on bioflocculant production by *Bacillus* sp. AEMREG4

3.2.4 Effect of the initial pH of the medium

Initial pH for bioflocculant production varies with different microorganisms (Salehizadeh and Yan, 2014). The effect of initial pH of the culture medium on bioflocculant production was investigated at a pH range of 4-10 and the results are depicted in Figure 3.4. In this study, bioflocculant production by *Bacillus* sp. AEMREG4 remained relatively constant at a wide range of pH 4 to10 with flocculating activity between 74% and 80% as shown in Figure 3.4. Optimum flocculating activity was reached at pH 8 (80%). According to He *et al*. (2010), the flocculating activity of a bioflocculant produced by *Halomonas* sp. V3a' was above 80% in the pH range of 3 to 11 and the highest flocculating activity of 97% was recorded at pH 7. The optimum initial pH for the production of bioflocculants by *Bacillus* xn12 and *Streptomyces* xn17 strains was in the range of 3-10 and 3-9 respectively, with the highest flocculating activity (97%) being observed at pH 5 for both strains (Zhang *et al*., 2013).

Bioflocculant production by *Aspergillus flavus* was investigated at a pH range of 5 to 9 and the highest flocculating activity was reached at pH 7 (Aljuboori *et al*., 2013), whereas Zheng *et al*. (2008) noted maximum flocculating activity at pH 9 when culturing *Bacillus megaterium* within a wide pH range ranging from acidic to an alkaline pH of 12. Contrary to the above observations, *Anabaera* sp. produced a bioflocculant utilizing a very acidic medium of pH 2 (Choi *et al*., 1998). It was stated in literature that at low pH both the bioflocculant and the Kaolin particles are likely to absorb the hydrogen ions (H^+), consequently weakening the bioflocculant-Kaolin complex mediated by Ca^{2+} and a similar effect was also observed at high pH values due to OH- ions (He *et al*., 2010).

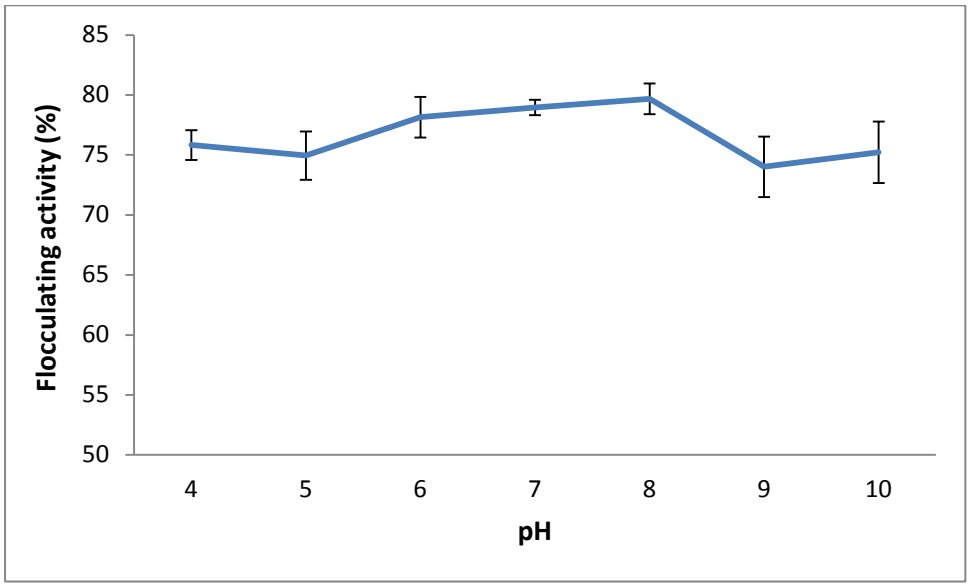

Figure 3.4: Effect of initial pH on bioflocculant production by *Bacillus* sp. AEMREG4

3.2.5 Effect of various cations on flocculating activity of the crude bioflocculant

Addition of cations in the culture medium has in the majority of studies, been reported to enhance flocculating efficiency by charge neutralization and by forming bridges between particles (Salehizadeh and Shojaosadati, 2001; Salehizadeh and Yan, 2014). Bioflocculant

efficiency, in the majority of cases reported in literature, is stimulated not only by the presence and concentration of cations but is also greatly influenced by the valency of ions (Zulkeflee *et al*., 2012). The effects of various cations on flocculating activity of a bioflocculant produced by *Bacillus* sp. AEMREG4 were examined and the results are illustrated in Figure 3.5. The effect of cations with various valence ions (monovalent, divalent and trivalent) on flocculating activity was studied. Amongst the cations tested, Ca^{2+} stimulated the highest activity at 76%, followed by Mn^{2+} , Al^{3+} and Mg^{2+} at 73%, 71% and 57% respectively with observed significant inhibition by Fe^{+3} and complete inhibition by Na^{+} and K^{+} (Figure 3.5) . Similar results were observed in a study conducted by Mabinya *et al*. (2011), whereby the flocculating activity of a bioflocculant produced by *Halomonas* sp. OKOH was increased by the addition of divalent cations with Ca^{2+} being highly effective. Ugbenyen and Okoh (2014) also reported that the divalent cations tested (Ca^{2+}, Mg^{2+}, Mn^{2+}) were the best metal ions for the activity of the bioflocculant produced by the consortium of the *Cobetia* sp. and *Bacillus* sp. which in turn, was completely inhibited by Li^{+} and K^{+}. Contrary to these observations, the flocculating activity of the bioflocculant produced by the S-4K strain was inhibited by the presence of Ca^{2+} and Fe^{2+} ions (Huang, 1990) whereas that of *Citrobacter* sp. TKF04 bioflocculant was not stimulated by the addition of any cations including Ca^{2+} (Fujita *et al*., 2000). In this study, monovalent cations (Na^{+} and K^{+}) had no effect at all on flocculating activity which can be attributed to the loose bonds in its structure hence result in a decrease in floc density, size and floc resistance to shear as compared to divalent cations (Zulkeflee *et al*., 2012).

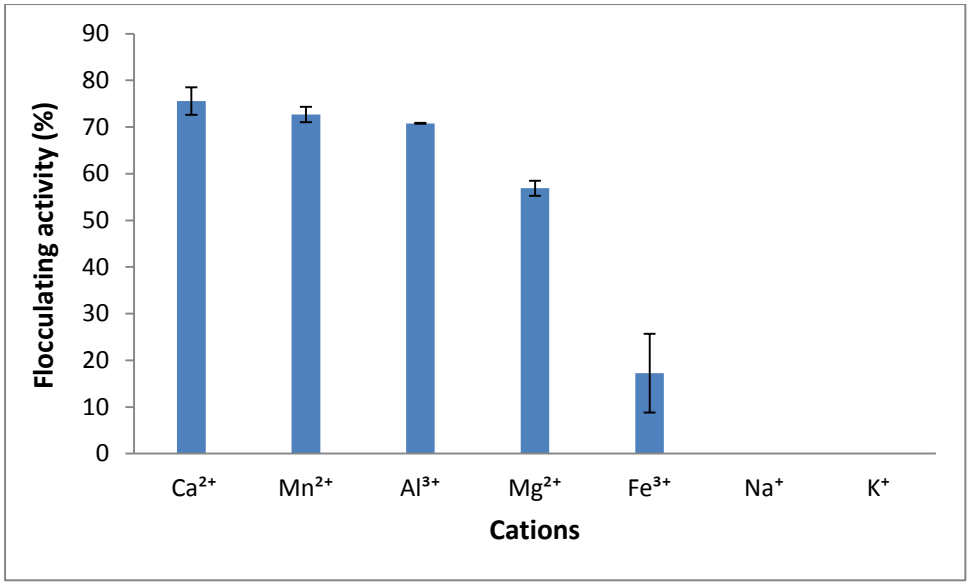

Figure 3.5: Effect of various cations on the flocculating activity of the bioflocculant

3.2.6 Effect of temperature on flocculating activity of the bioflocculant

The effect of heat on the bioflocculant activity was investigated over a heating period of 1 h at a temperature range of 50-100 °C for 1 h. Optimum flocculating activity above 70% was recorded over this temperature range thus indicating relative thermostability characteristics of the bioflocculant. Similar findings have been reported for a bioflocculant produced by *Cobertia* sp. whereby heating up to 100 °C for 25 min had no effect on the flocculating activity of the bioflocculant (Ugbenyen *et al*., 2012). Gao *et al*. (2006) reported that a bioflocculant produced by *Vagococcus* sp. W31 retained its stability when heated at 100 °C with the residual flocculating activity of culture broth and purified flocculant being 86.5% and 87.2%, respectively. Also, a bioflocculant from *Agrobacterium* sp. M-503 showed heat-stability in the alkaline pH range 7-12 with no decrease of flocculating activity when subjected to elevated temperature of 121 °C for 20 min (Qiang *et al*., 2010). However, a bioflocculant produced by *Chryseobacterium daeguense* contained a protein backbone with

the optimum flocculating activity at 96.9% achieved at 15 °C and a decrease in flocculating activity at temperatures above 45 °C (Liu *et al*., 2010).

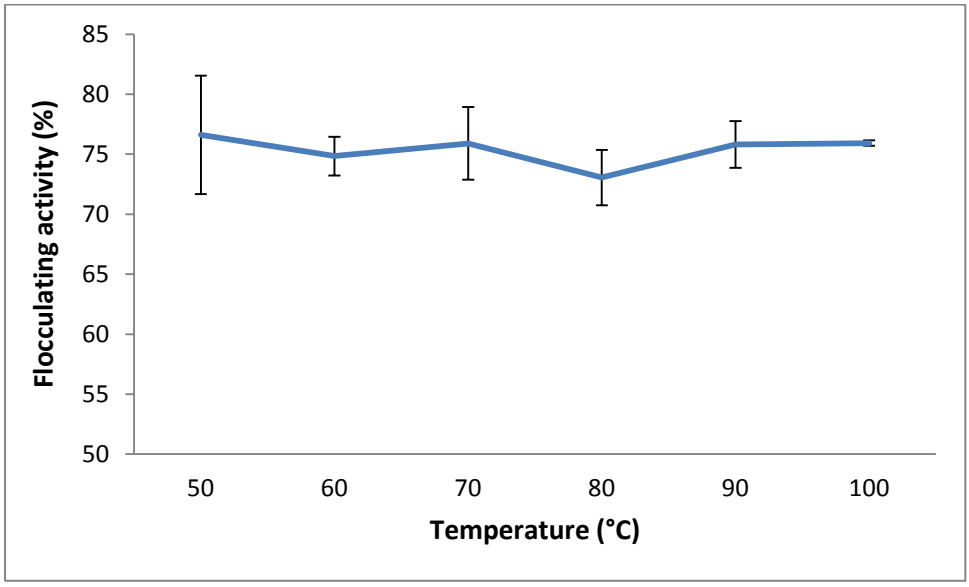

Figure 3.6: Effect of temperature on flocculating activity of the bioflocculant

3.3 Time course assay of bioflocculant production

Previously determined optimal culture conditions were used for the time course assay and Figure 3.7 depicts the time course assay of bioflocculant production by *Bacillus* sp. AEMREG4 over a cultivation period of 96 h. Optimal bioflocculant production as indicated by flocculating activity (65%) was observed at 72 h of cultivation beyond which flocculating activity started to decline. A decline in cell growth and flocculating activity at late growth phase may be attributed to the depletion of nutrients in the production medium as well as to the production and release of bioflocculant-degrading enzymes which utilize the produced bioflocculant as a carbon source (Li *et al*., 2009; Zaki *et al*., 2011). Similarly, a bioflocculant produced by *Serratia ficaria* reached its maximum flocculating activity in the early stationary

phase (72 h) before an observed a slow decrease after 84 h which was attributed to autolysis and enzymatic activity (Gong *et al*., 2008). It was also observed that at the early stationary phase there was a rapid increase in bacterial cell growth up to 48 h of incubation, as expressed in CFU/ml, after which a significant decrease in viable cells was observed eventually levelling off at 72 h with an observed gradual decrease in flocculating activity (Figure 3.7). These findings are an indication that the production of the bioflocculant is associated with cell growth rather than with cell autolysis (Lu *et al*., 2005; Gao *et al*., 2006). The majority of reported studies in the literature indicate that bioflocculants are produced during active growth phase of microorganisms (Salehizadeh and Yan, 2014; Lian *et al*., 2008; Prasertsan *et al*., 2006; Nwodo *et al*., 2013). The present study seems to show a similar phenomenon. On the contrary, the bioflocculant produced by *Chryseobacterium daeguense* W6 was associated with cell autolysis and not cell growth (Liu *et al*., 2010). Running parallel to the profile of flocculating activity, the pH profile showed that the pH increased from 7- 9.5 within an incubation period of 96 h.

Bioflocculant production takes place at various phases of growth of microorganisms. At various periods, production drops due to cell autolysis, metal complexing or decreased enzymatic activity depending nevertheless on the microbial cell culture (Lu *et al*., 2005, Vatansever, 2005; Cosa *et al*., 2010). Bioflocculants can be distinguished into either primary or secondary metabolites depending on the period of secretion in the culture broth (Salehizadeh and Yan 2014). A bioflocculant produced by *Streptomyces* sp. reached its highest flocculating activity during the logarithmic growth phase, suggesting that biosynthetic processes were responsible for the bioflocculant production process (Nwodo *et al*., 2012). Shih *et al*. (2001) reported that cell both cell growth and bioflocculant production

by *Bacillus licheniformis* simultaneously reached the highest peak during the stationary phase at 96 h.

Based on a study by Cosa *et al*. (2011), a bioflocculant produced by *Virgibacillus* sp. Rob was reported to produce a bioflocculant with maximum flocculating activity reached at 96 h of cultivation. The flocculating activity of the bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo reached its maximum flocculating activity of 63.2% at late stationary phase of 120 h and a further increase in cultivation period resulted in a decrease in both flocculating activity and cell growth (Okaiyeto *et al*., 2013). *Proteus mirabilis* production of a bioflocculant named TJ-1 was almost parallel to cell growth and the highest flocculating activity was observed at the stationary phase (Xia *et al*., 2008). Production of a bioflocculant produced by *Enterobacter aerogenes* was in parallel with cell growth and reached its maximum flocculating activity in early stationary phase (60 h), indicating that the bioflocculant was produced by biosynthesis during its growth (Lu *et al*., 2005). Maximum flocculating activity of a bioflocculant produced by *Citrobacter* sp. TKF04 was obtained in 24 h of cultivation and thereafter the activity decreased (Fujita *et al*., 2001).

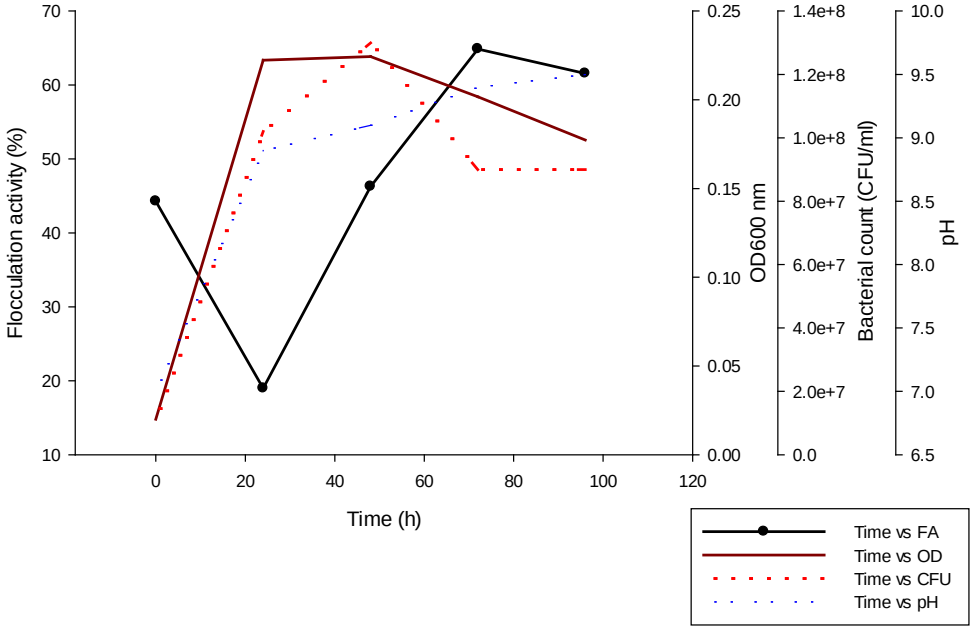

Figure 3.7: Time course for bioflocculant production by *Bacillus* sp. AEMREG4

3.4 Flocculating properties of bioflocculant

3.4.1 Effect of the bioflocculant dosage

The effect of bioflocculant dosage on flocculating efficiency was tested in the dosage range of 0.1-0.5 mg/ml as depicted in Figure 3.8. It was noted that an increase in dosage above 0.1 mg/ml resulted in a decrease in flocculating activity. The maximum flocculating activity (78%) was reached at the lowest dosage of 0.1 mg/ml. A similar observation was observed by Okaiyeto *et al*. (2013) on the bioflocculant produced by a consortium of *Halomonas* sp. OKOH and *Micrococcus* sp. Leo which required a dosage of 0.1 mg/ml for effective flocculating activity. The maximum flocculating rate of a bioflocculant produced by

Enterobacter aerogenes was achieved at an optimum bioflocculant dosage of 0.09 mg/ml (Lu *et al*., 2005). Also, a bioflocculant produced by *Bacillus musilaginous* required a dosage of 0.1 mg/ml for maximum flocculating efficiency (Deng *et al*., 2003). According to the study done by Zulkeflee *et al*. (2012), bridging flocculation mechanism of the bioflocculant will not be affected at low dosage while at high dosage it will result in inhibition of the restabilization of Kaolin particles. Findings documented by Wang *et al*. (2011) of a bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus* F6 showed optimal flocculating activity being attained only at a maximum dosage of 0.012 mg/ml.

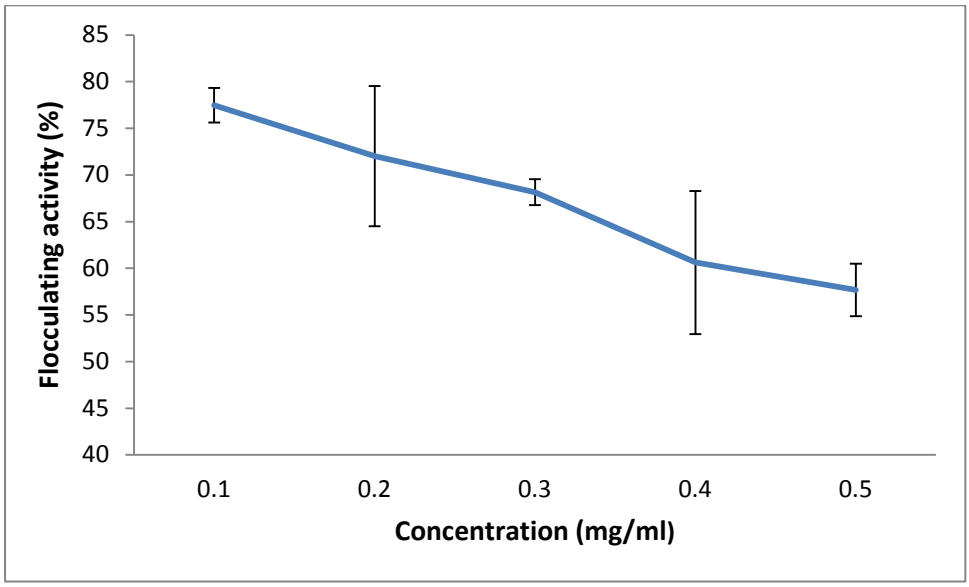

Figure 3.8: Effect of bioflocculant dosage on the flocculating activity of the purified bioflocculant

3.4.2 Effect of various cations on the flocculating activity of the purified bioflocculant

The effect of cations on the flocculating activity of the purified bioflocculant was investigated as shown in Figure 3.9. All tested cations enhanced the flocculating activity of

the bioflocculant produced by *Bacillus* sp. AEMREG4 albeit to varying degrees: Ca^{2+} (78%), Mn^{2+} (77%), Mg^{2+} (70%), Al^{3+} (80%) and K^{+} (60%) with Na^{+} (47%) and Fe^{3+} (48%) showing the least effect (Figure 3.9). The main cause of the poor flocculation of the bioflocculant produced by *Bacillus* sp. AEMREG4 in the presence of Fe^{3+} compared to Al^{3+} may be due to the synergistic effect of Al^{3+} with this bioflocculant and the antagonistic effect of Fe^{3+} with the bioflocculant; hence resulting to high flocculating activity of Al^{3+} and lower flocculating activity of Fe^{3+} (Okaiyeto *et al*., 2015). Divalent and trivalent cations are said to have the effect of stimulating the adsorption of bioflocculant on the suspended particles by decreasing the negative charge of both the polymer and the particle (Levy *et al*., 1992). The bioflocculant produced by a consortium of *Holomonas* sp. Okoh and *Micrococcus* sp. Leo, showed the maximum flocculating activity of 80% when Al^{3+} was the cation of choice (Okaiyeto *et al*., 2013). Stimulation of flocculating activity was observed in a bioflocculant produced by *Bacillus licheniforms* and *Bacillus circulans* when Al^{3+} and Ca^{2+} were used (Li *et al*., 2009).

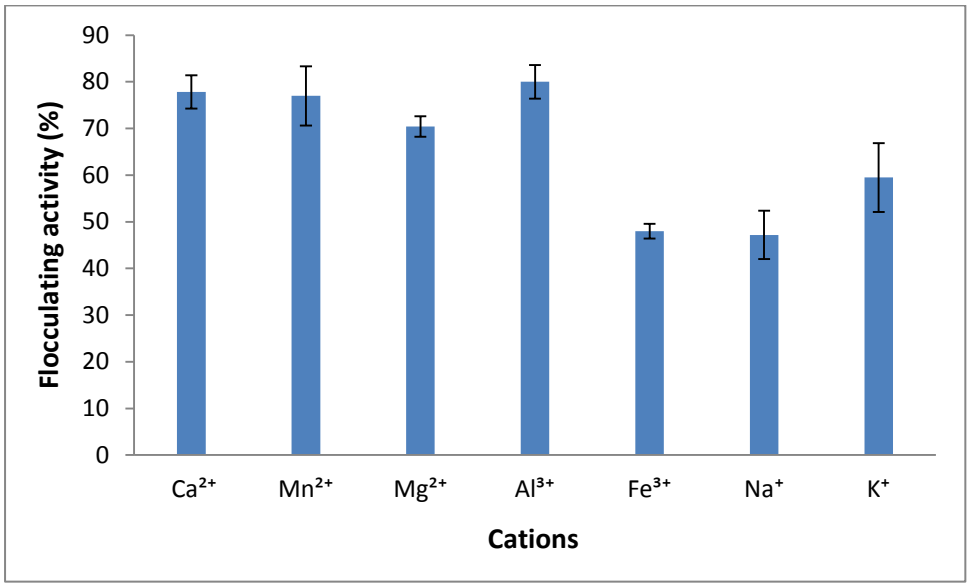

Figure 3.9: Effect of cations on the flocculating activity of the purified bioflocculant

3.4.3 Thermal stability test of the purified bioflocculant

The relationship between temperature and flocculating efficiency of the purified bioflocculant was examined at a temperature range of 50-100 °C for 1 h as depicted in Figure 3.10. The bioflocculant retained more than 74% flocculating rate when subjected to heating at 100 °C for 1 h. Therefore, it was deduced that the bioflocculant was thermo-stable and its flocculating activity was not affected when the temperature was elevated. Salehizadeh and Shojaosadati (2001) reported that the presence of protein or peptide in the structure of a bioflocculant is generally linked to its sensitivity to heat and those consisting of sugars are more heat-resistant, hence it can be concluded that the bioflocculant produced by *Bacillus* sp. AEMREG4 consisted predominantly of polysaccharide. The thermal stability of this bioflocculant may be due to the presence of a hydroxyl group that is responsible in the formation of hydrogen bonds in its structure (Ugbenyen *et al*., 2014). Cosa and Okoh, (2014) reported a residual flocculating activity of more than 80% for a purified bioflocculant produced by the consortium of *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky after heating at 100 °C for 1 h, thus indicating its thermo-stability.

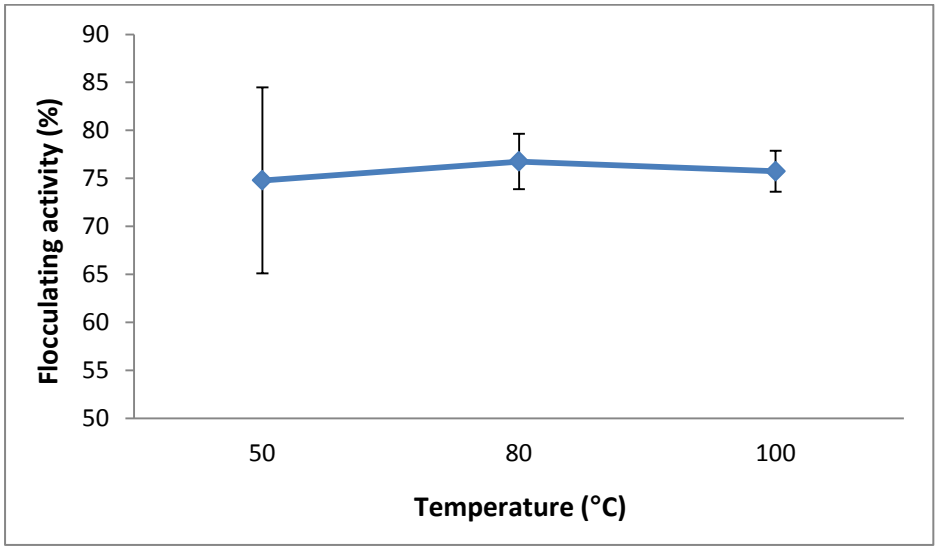

Figure 3.10: Thermal stability on flocculating activity of the purified bioflocculant

3.4.4 Effect of pH on the flocculating activity of purified bioflocculant

The pH has a great impact on flocculating activity (Cosa and Okoh, 2014). The flocculating activity of the purified bioflocculant was tested using bioflocculant dosage of 0.1 mg/ml within the pH range of 3-10 as illustrated in Figure 3.11. A strong flocculating activity was observed over a wide range of pH, 3-10. Maximum flocculating activity was achieved at very acidic pH 3 (83%) and basic pH 10 (83%). This may be due to the bioflocculant which shows different electric states at different pH, hence affecting the bridging efficiency of the bioflocculant for clay powder (Yong *et al*., 2009). A sharp decline in flocculating activity was observed at pH 5; this might be due to the arrangement of the surface charge which is both pH and temperature dependent. Hence, it can be deduced that the spatial charge arrangements for flocculation were not ambient at pH 5. This bioflocculant showed excellent flocculating activity in both strong acidic and basic conditions. Zaki *et al*. (2012) also reported similar results, whereby a bioflocculant produced by *Bacillus velezensis* 404 was stable at the pH range of 3-9 and reached its maximum stability at pH 7. Different microorganisms have been reported to produce bioflocculants with optimal activity at varying pH values (Wang *et al*., 2011); Liu *et al*. (2009) reported on a bioflocculant extracted from sludge, named as M-1 as showing high flocculating activity over 74% in the wider pH range of 3-11, whereby the maximum flocculating activity (93%) was achieved at pH 5.

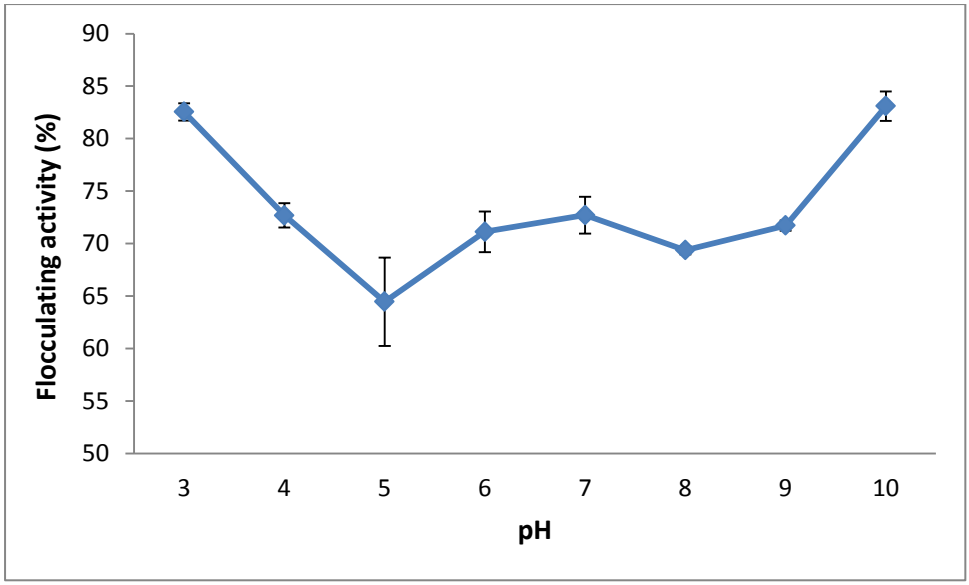

Figure 3.11: Effect of pH on flocculating activity of the purified bioflocculant produced by *Bacillus* sp. AEMREG4.

3.5 Characterization of bioflocculant

3.5.1 Yield and Composition analysis of the purified bioflocculant

The yield of bioflocculant recovered after purification was 0.78 g from a 1 L of culture broth. The freeze-dried purified polymer was white in colour and completely dissolved in distilled water. This yield is higher than the 0.264 g reported by Cosa *et al.* (2011) for a fermented culture of Virgibacillus sp. Rob. However, the yield of bioflocculant produced by *Myxobacterium nannocystics* sp. NU-2 was reported to be 14.8 g/L (Zhang *et al*., 2002). Low yields and the high costs pose major practical application limitations in bioflocculant production; hence the yield of bioflocculant production has been among critical factors considered for its application (Kurane *et al*., 1994). Chemical analysis showed that the bioflocculant is composed of total sugar (79%) and total protein (5%), suggesting that the bioflocculant is predominantly polysaccharide. Further analysis showed that the bioflocculant

consist of uronic acid (15%), which is an acidic polysaccharide. Several bioflocculants have been reported to have polysaccharide backbone as a major component, for example, the bioflocculant produced by *Enterobacter aerogenes* (Lu *et al*., 2005), the bioflocculant produced by *Virgibacillus* sp. Rob (Cosa *et al*., 2011), and the bioflocculant MBF-5 produced by *Klebsiela pneumonia* (Zhao *et al*., 2013).

Table 3.1: Chemical composition of the purified bioflocculant produced by *Bacillus* sp. AEMREG4

Dry weight (g/L)	**Total protein (%)**	**Total sugar (%)**	**Uronic acid (%)**
0.78	5	79	15

3.5.2 Functional group analysis of Bacillus sp. AEMREG4

FTIR analysis of the bioflocculant was undertaken in order to detect the presence of any functional groups that may contribute to its flocculating activity as shown in Figure 3.12. The spectrum displayed a broad stretching intense peak at 3423 cm^{-1} which is characteristics of hydroxyl groups. A weak C-H stretching vibration band was observed at 2934 cm^{-1}. The bands at 1646 $cm^{-1}$and 1455 $cm^{-1}$are characteristic of C=O asymmetrical and weak symmetrical stretching in the carboxylate, respectively (Deng *et al*., 2003), which represent the presence of carboxyl group in the structure of the bioflocculant produced by *Bacillus* sp. AEMREG4. The bands at 1027 cm^{-1} and 1151 cm^{-1} represent the methoxyl group (Zheng *et al*., 2008). The sorption peak at 1239 cm^{-1} indicates the presence of C-O stretching in ether or alcohol (Desouky *et al*., 2008). The absorption peaks around 1000-1100 cm^{-1} are known to be characteristic of all sugar derivatives (Zheng *et al*., 2008). The FTIR spectrum showed the

presence of carboxyl, hydroxyl and methoxyl groups, which are the preferred groups for flocculation (Zheng *et al*., 2008).

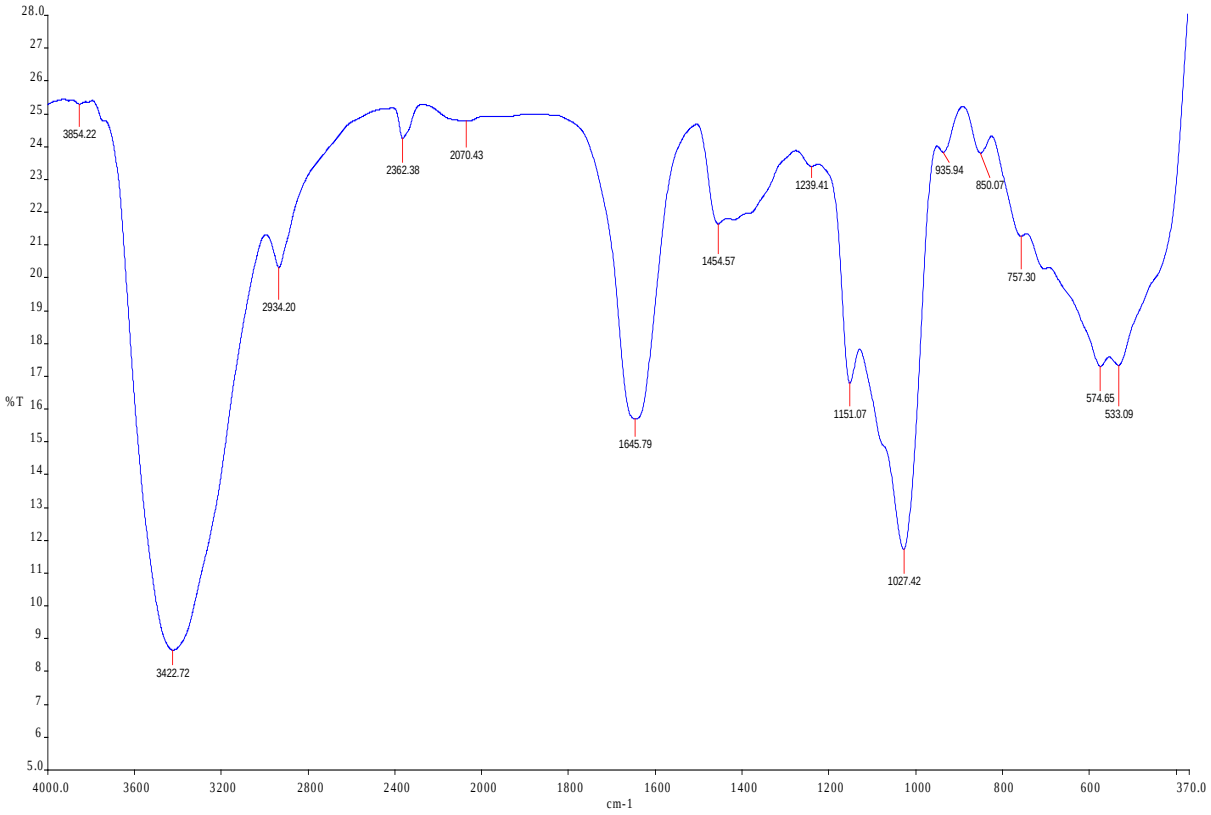

Figure 3.12: Infrared spectrum of the purified bioflocculant produced by *Bacillus* sp. AEMREG4

3.5.3 SEM images of bioflocculant produced by Bacillus sp. AEMREG4

SEM observations were carried out to investigate the mechanism of flocculation of Kaolin clay. Figure 3.13 (A) shows a rod-shaped structure of purified bioflocculant produced by *Bacillus* sp. AEMREG4. Figure 3.13 (B) shows Kaolin clay before the addition of bioflocculant and Figure 3.13 (C) shows flocculated Kaolin clay after the addition of the purified bioflocculant. Comparing Figure B and C, it can be deduced that the addition of the

purified bioflocculant produced by *Bacillus* sp. AEMREG4 to Kaolin clay suspension played a crucial role in connecting the scattered kaolin particles together to form flocs and separate easily from water during the flocculation process.

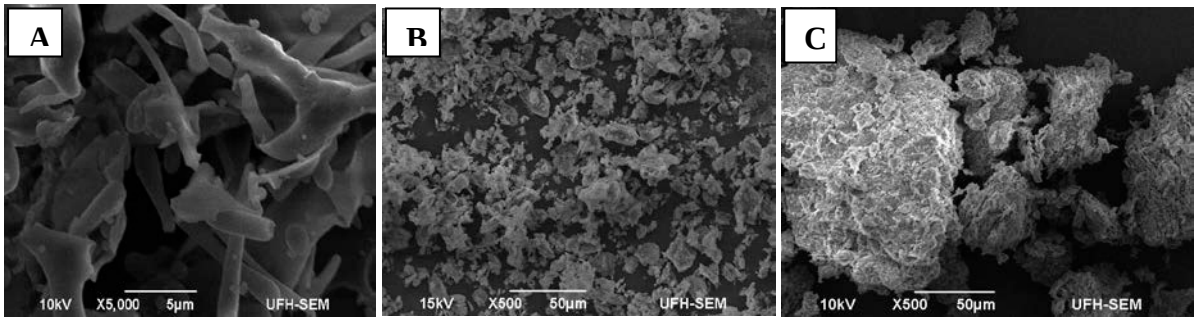

Figure 3.13: SEM images of (A) the purified bioflocculant, (B) Kaolin clay, and (C) Kaolin clay floculated by purified bioflocculant produced *Bacillus* sp. AEMREG4

4. CONCLUSION

This study demonstrated that the bioflocculant produced by *Bacillus* sp. AEMREG4 is mainly composed of a polysaccharide, a property that is also evidenced by its observed thermo-stability. The bioflocculant optimally produced high flocculating rate in the presence of starch and yeast extract as sole carbon and nitrogen sources respectively, at a wide range of pH 4-10. From this study it can be concluded that divalent cations are more effective at enhancing flocculating activity of the bioflocculant produced by *Bacillus* sp. AEMREG4 compared to monovalent or trivalent cations. Bioflocculant production was observed to be associated with cell growth. Bioflocculant produced by *Bacillus* sp. AEMREG4 demonstrated a high flocculating activity at a low dosage and from these findings, it can be inferred that dosage requirement for a bioflocculant produced by *Bacillus* sp. AEMREG4 compares

favourably with published data on microbial flocculants and augurs well for its future industrial applications. The bioflocculant showed excellent flocculating activity in both strong acidic and basic conditions and these findings suggest that *Bacillus* sp. AEMREG4 is a promising alternative source for application in water treatment. The FTIR spectrum illustrated the presence of carboxyl, hydroxyl and methoxyl groups in its molecular chain as the functional groups. Further assessment of the application of the purified bioflocculant to tap water processing or heavy metal removal is required.

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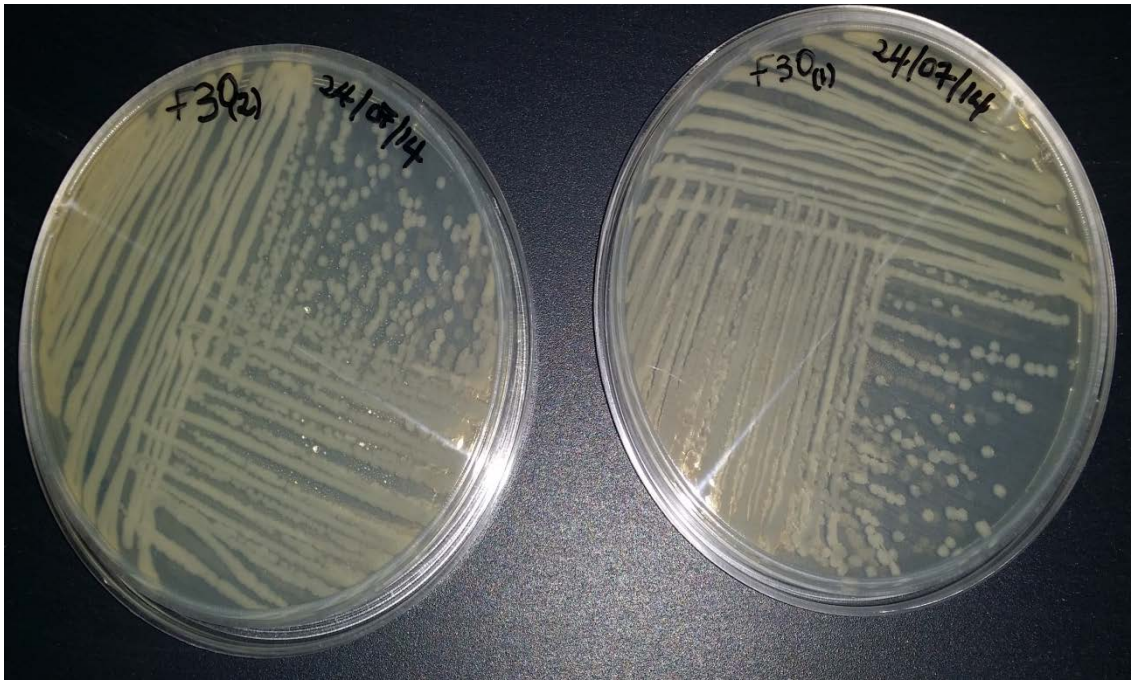
APPENDIX A



Picture 1: *Bacillus* sp. AEMREG4 strain on nutrient agar

APPENDIX B

Table 1: Effect of carbon sources on bioflocculant production

Carbon source	Flask 1	Flask 2	Flask 3
Glucose	0.668	0.663	1.048
Maltose	1.587	1.015	0.989
Fructose	1.137	0.368	1.233
Starch	0.314	0.320	0.305
Sucrose	1.190	1.032	1.045
Lactose	1.085	1.056	1.235

Control= 1.681

Absorbance @ 550nm

Table 2: Effect of nitrogen sources on bioflocculant production

Nitrogen Sources	Flask 1	Flask 2	Flask 3
Urea	0.613	0.917	1.261
Peptone	0.530	0.706	0.323
Tryptone	0.644	0.444	0.733
Ammonium Sulphate	0.843	0.803	0.674
Yeast extract	0.380	0.321	0.567

Control= 1.894

Absorbance @ 550nm

Table 3: Effect of cations on the flocculating activity

Cations	Flask 1	Flask 2	Flask 3
Na^{+}	2.083	2.099	2.122
K^{+}	2.066	2.042	2.070
Ca^{2+}	0.503	0.424	0.805
Mn^{2+}	0.496	0.540	0.854
Mg^{2+}	0.795	0.838	0.883
Fe^{3+}	1.455	1.681	2.063
Al^{3+}	0.552	0.555	1.709

Control=1.894

Absorbance @ 550nm

Table 4: Effect of inoculum size on bioflocculant production

Inoculum size (%)	Flask 1	Flask 2	Flask 3
1	0.398	0.346	0.387
2	0.406	0.454	0.398
3	0.410	0.396	0.354
4	0.341	0.343	0.355
5	0.390	0.339	0.572

Control=1.680

Absorbance @ 550nm

Table 5: Effect of initial pH of the medium on bioflocculant production

pH	Flask 1	Flask 2	Flask 3
4	0.360	0.387	0.506
5	0.409	0365	0.572
6	0.356	0319	0.493
7	0.332	0.318	0.397
8	0.328	0.30	0.398
9	0.374	0.429	0.500
10	0.355	0.411	0.455

Control= 1.545

Absorbance @ 550nm

Table 6: Effect of temperature on the flocculating activity of the liquid bioflocculant

Temperature (˚C)	Flask 1	Flask 2	Flask 3
50	0.282	0.434	0.368
60	0.379	0.370	0.417
70	0426	0.352	0.339
80	0.455	0.385	0.409
90	0.394	0.388	0.339
100	0.376	0.370	0.370

Control= 1.545

Absorbance @ 550nm

Table 7: Time course assay of bioflocculant production

Time (h)	Absorbance @ 550nm			OD @ 600 nm			pH			CFU/ml		
0	0.577	0.640	0.778	0.061	0.043	0.043	7.0	7.0	7.0	168	163	165
24	0.636	0.507	0.506	0.013	0.006	0.008	8.9	8.9	8.9	210	281	271
48	0.690	0861	0.729	0.104	0.051	0.056	9.0	9.0	9.0	144	182	170
72	0.390	0.322	0.280	0.088	0.097	0.098	9.3	9.3	9.3	120	145	155
96	0.236	0.389	0.398	0.083	0.089	0.087	9.5	9.4	9.4	99	102	86

Control= 0.630

Table 8: Effect of bioflocculant dosage

Concentration (mg/ml)	Flask 1	Flask 2	Flask 3
0.1	0.377	0.366	0.322
0.2	0.335	0.419	0.569
0.3	0.527	0.486	0.493
0.4	0.760	0.553	0.549
0.5	0.687	0.698	0.616

Control=1.576

Absorbance @ 550nm

Table 9: Effect of cations on the flocculating activity of the purified bioflocculant

Cations	Flask 1	Flask 2	Flask 3
K^{+}	0.704	0.504	0.708
Na^{+}	0.778	0.926	0.793
Mg^{2+}	0.426	0.484	0.489
Ca^{2+}	0.370	0.286	0.393
Mn^{2+}	0.252	0.447	0.390
Fe^{3+}	0.817	0.846	0.797
Al^{3+}	0.361	0.252	0.333

Control=1.576

Absorbance @ 550nm

Table 10: Thermal stability of the purified bioflocculant

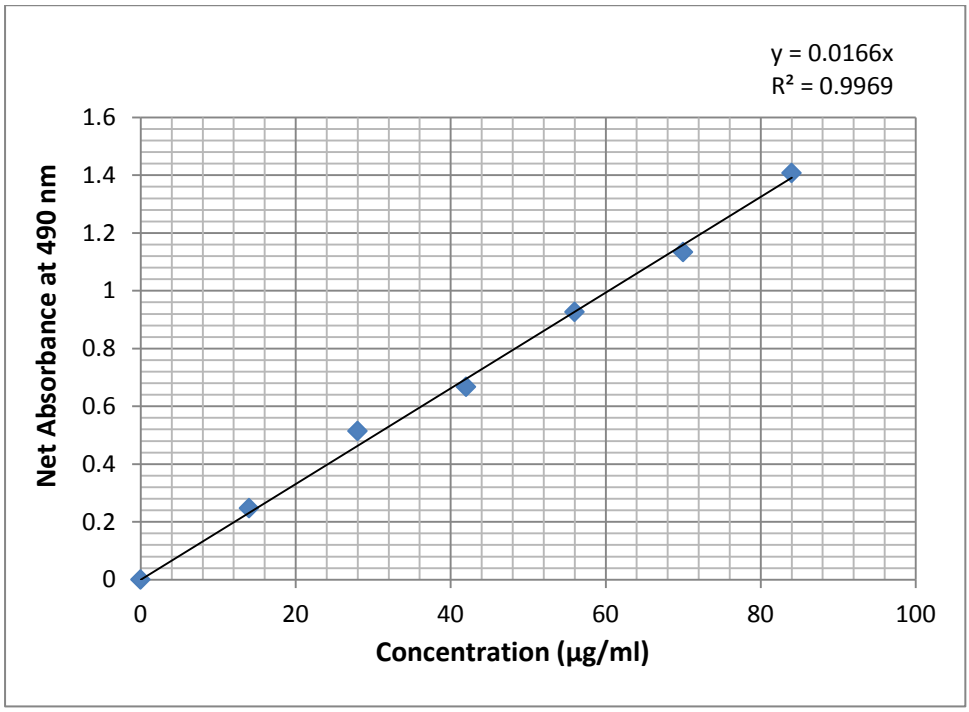
Temperature (˚C)	**Flask 1**	**Flask 2**	**Flask 3**
50	0.570	0.342	0.280
80	0.379	0.316	0.404
100	0.344	0.396	0.407

Control=1.576

Absorbance @ 550nm

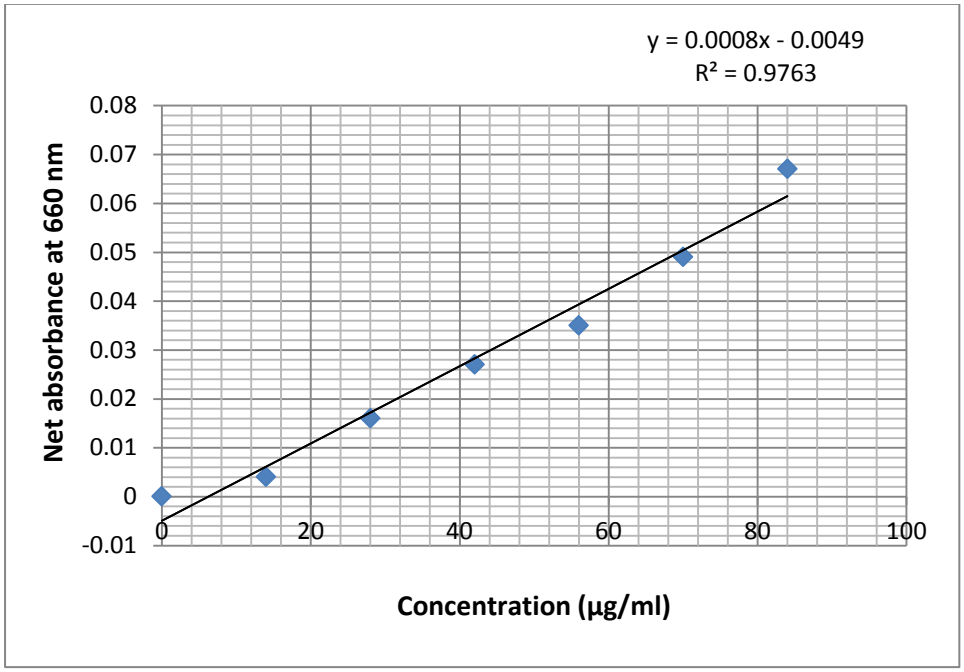
APPENDIX C

GLUCOSE STANDARD CURVE



APPENDIX D

STANDARD CURVE OF PROTEIN ESTIMATION



APPENDIX E

URONIC ACID STANDARD CURVE