

**University of Fort Hare**  
*Together in Excellence*

**EVALUATION OF BIOFLOCCULANT-PRODUCING POTENTIAL OF  
*BACILLUS PUMILUS* STRAIN ISOLATED FROM TYUME RIVER IN  
THE EASTERN CAPE PROVINCE OF SOUTH AFRICA**

By

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## **DECLARATION**

I, Busisiwe Makapela, solemnly declare that this dissertation is my original work, except where otherwise stated and has not been submitted for the award of any other degree or examination in this or any other university.

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**Signature:** .....

**Date:** .....

**Supervisor's signature:** .....

## **DEDICATION**

I dedicate this dissertation to the almighty God for showering me with His heavenly blessings and also to my lovely son, Intando Ivile Makapela.

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## GENERAL ABSTRACT

A bioflocculant is a kind of metabolite produced by microorganisms during their growth. Most bioflocculants are mainly composed of high polymers such as extracellular polysaccharides, glycoprotein, protein and nucleic acids. Bioflocculants promote flocculation by forming bridges between suspended particles in solutions resulting in precipitation of the suspended particles. Generally, when suspended particles are flocculated into large flocs, they settle down thus resulting in a clarified solution and can easily be removed. In this study, a bacterial strain named F45 was investigated for its ability to produce a bioflocculant. Source samples for isolation of bioflocculant-producing microorganisms were collected from the Tyume River, Eastern Cape Province of South Africa. The collected samples included water samples, rock scrapings and sediment samples. In total, 144 isolates were obtained from all the samples in which 13 were found to be bioflocculant producers as evidenced by the ability of the produced metabolite to flocculate kaolin suspension. Notable among these isolates was a bacterial strain F45 which was obtained from rock scrapings and whose bioflocculant exhibited a flocculating activity above 60%. Identification of the F45 strain revealed it to have 95% similarity to *Bacillus pumilus* strain ZAP 028. The optimum culture conditions for bioflocculant production by this strain were inoculum size of 4% (v/v), maltose as carbon source, multiple nitrogen source composed of yeast extract, urea and ammonium sulphate. The highest flocculating activity was recorded at an initial pH of 7. A bioflocculant yield of about 0.289 g/l was recovered from the fermented broth. The purified bioflocculant was a white powder which showed high flocculating activity (96.5%) against kaolin suspension at a dosage of 0.1 mg/ml. All the cations tested stimulated the flocculating activity of the purified bioflocculant except for  $\text{Fe}^{3+}$  which only showed a low flocculating activity of 21%. Thermal stability test of the purified bioflocculant proved it to be stable as it could retain more than 90% of its activity after being heated at 100°C for 1 hour. Fourier-transform infrared (FTIR) spectroscopy analysis revealed that purified bioflocculant contained hydroxyl groups, carboxyl groups and uronic acid. The bioflocculant was

composed of sugar (75.4%), protein (5.3%) and uronic acid (15.4%). Scanning electron microscopy (SEM) revealed a dendritic bioflocculant structure and elemental analysis of the purified bioflocculant showed that the weight fractions of elements C, N, O, S and P were 22.71%, 11.56%, 41.60%, 0.51% and 7.98% respectively. The results obtained suggest that this bioflocculant could be utilized as an alternative for harmful synthetic flocculants in various industrial applications.

**Key words:** *Bacillus pumilus*, Bioflocculant, Flocculating activity

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# CHAPTER

# 1

# 1. INTRODUCTION

## 1.1 General background

Environmental pollution has become one of the world's serious challenges. One of the contributing factors to environmental pollution is the rapid development of industrialization and anthropogenic activities which have led to the increased discharge of waste and wastewater containing organic and inorganic pollutants (Sanayei et al., 2010). Industrial processes are potential sources of water pollution and specific treatment methods are required for the waste produced (Zaki et al., 2011). The wastewater generated by different kinds of industries normally contains a wide range of different particles such as very fine suspended solids, dissolved solids, inorganic and organic particles, metals and other impurities (Lee et al., 2014). Sedimenting these particles out of solution for easy filtration becomes challenging due to the small size of particles as well as the presence of surface charges (Bratby 2006). Hence, removal of these colloidal particles from wastewaters becomes a serious challenge for the industries. Various traditional and advanced technologies have been employed to remove the colloidal particles from wastewater and these include ion exchange, membrane filtration, precipitation, flotation, solvent extraction, adsorption, coagulation, flocculation, as well as biological and electrolytic methods (Radoiu et al., 2004). Among the listed methods, flocculation is one of the most widely used solid-liquid separation process for treating the wastewater in various industries due to the fact that it is a simple and efficient method for wastewater treatment (Renault et al., 2006). Flocculation has been extensively utilized for the treatment of various types of wastewater such as palm oil mill effluent, textile wastewater, pulp mill wastewater, sanitary landfill leachates and oily wastewater (Ahmad et al., 2005; Tatsi et al., 2003; Wong et al., 2006; Yue et al., 2008; Zhong et al., 2003). Flocculation may also be used as an alternative to centrifugation and filtration for separation of microbial cells from broth in food and pharmaceutical and medicine industries (Shih & Van 2001). Flocculating agents or flocculants are chemicals that promote flocculation by causing colloids and other suspended particles in liquids to

aggregate, forming a floc. Flocculants are generally divided into three groups, namely, inorganic flocculants such as aluminum sulfate and polyaluminum chloride, chemically synthetic organic flocculants such as polyacrylamide derivatives and polyethylene amine, and bioflocculants (Zhang et al., 2007). Among these flocculants, chemical flocculants are widely employed in wastewater treatment, tap water production and in the food and fermentation industries for downstream processing due to their low cost and high efficiency (Mabinya et al. 2011; Ugbenyen & Okoh 2014). However, the wide use of chemical flocculants has raised serious environmental and health concerns. It has been reported that aluminium salts are associated with Alzheimer's disease (Pullen et al., 1990; Arezoo 2002) and also that polyacrylamides containing residual acrylamide monomers have been proved to be neurotoxic and carcinogenic to humans (Nie et al. 2011; Piyo et al. 2011). In addition, chemical flocculants are not easily degraded in nature. These inevitable disadvantages of chemical flocculants make microbial flocculants important targets for extensive investigation. Compared with chemical flocculants, bioflocculants are benign in nature because they are environmentally friendly, biodegradable, nontoxic and harmless to human and animal health and are also free of the risk of secondary pollution.

A bioflocculant is a natural type of flocculant that is produced by microorganisms during their growth but is readily degradable (Desouky et al., 2008). Bioflocculants are extracellular polymeric substances (EPS), the majority of which include, glycoproteins, polysaccharides, proteins and nucleic acids produced by microorganisms during their growth (Christensen 1989; Lazarova & Manem 1995). These special macromolecules secreted by microorganisms induce solid particles, bacteria, cells, and colloidal particles in a liquid suspension to flocculate and sediment (Xiong et al., 2010). Bioflocculation promotes aggregation of suspended solid particles from solutions or suspensions and the flocculating activity of bioflocculants depends on the characteristics of the flocculants produced by the microorganism. In microbial systems, flocculation was first reported in 1876 by Louis Pasteur for yeast identified as *Saccharomyces cerevisiae*. Thus, years later many

researchers extensively investigated the process of bioflocculation and a correlation was established between the accumulation of extracellular bioflocculants and cell aggregation (Salehizadeh & Shojaosadati 2001).

Many microorganisms that have been reported to produce bioflocculants include bacteria, fungi, algae, actinomycetes and yeast (Xiong et al., 2010). These microorganisms have been isolated from different environments including soil, activated sludge and wastewater (Desouky et al., 2008). Recently, bioflocculant-producing microorganisms have also been isolated from human saliva (Luo et al., 2014) and mucus (Zhao et al., 2013). Due to their agglutinating properties, bioflocculants may also remove soluble nutrients from the water column, resulting in heterotrophic activity in the sediment region. In addition, bioflocculants with numerous negatively charged binding sites are capable of removing cations from the environment, thereby protecting cells from the toxic effects of heavy metals (Kurek et al., 1991).

Different microorganisms have been reported to produce bioflocculants. These include *Sorangium cellulosum* (Zhang et al., 2002), *Enterobacter aerogenes* (Lu et al., 2005), *Azotobacter indicus* (Patil et al. 2010), *Chlamydomonas reinhardtii* (Zhu et al., 2011) and *Klebsiella pneumonia* (Zhao et al., 2013).

## **1.2 Problem statement**

Chemical synthetic flocculants have been widely used in industrial applications owing to their low cost of production and effectiveness. However, their use has been implicated in serious human health problems in addition to being proven to be harmful to the environment. Consequently, bioflocculants have gained much wider attention because of their benign nature.

Practical application of bioflocculants in industries is however, still limited due to high costs of production as well as low yields. Therefore, it is desirable to screen novel strains for bioflocculant production and optimize culture conditions for enhanced yields.

### **1.3 Aims and objectives**

The aim of this study is to isolate a fresh water bacterial strain, evaluate its potential for biofloculant production and optimize culture conditions for enhanced biofloculant production.

The specific objectives of this study include:

- To isolate microorganisms from freshwater environment.
- To screen the microorganisms for biofloculant production and select the strain showing flocculant-producing potentials.
- To optimize conditions for production of biofloculant by the selected biofloculant-producing microorganism.
- To carry out bulk production of biofloculant following fermentation with the selected bacterial specie.
- To purify and characterize the biofloculant produced by the selected bacterial strain.

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# CHAPTER

# 2

## **2. LITERATURE REVIEW**

### **2.1. Introduction**

Flocculation is a process wherein colloids come out of suspension in the form of a floc; it can either be spontaneous or due to the addition of a clarifying agent. In a broader sense, it is a process whereby finely divided or dispersed particles are aggregated together to form large particles of such a size so as to cause their settling or it is the agglomeration of tiny particles to form flocs which settle resulting in the clarification of the suspension (Sharma et al., 2006).

Flocculants are the materials which facilitate rapid solid–liquid separations. They are said to act on a molecular level on the surfaces of the particles to reduce repulsive forces and increase attractive forces (Sharma et al., 2006). Flocculation has been applied in a wide range of fields, such as dredging, wastewater treatment and fermentation (Lin & Harichund 2012). Flocculants can either be negatively or positively charged and exhibit a wide range of molecular weights (Salehizadeh & Shojaosadati 2001). Louis Pasteur was the first person to report on flocculation in microbial systems (Salehizadeh & Shojaosadati 2001) and since then, the process of flocculation in microbial systems had been explored extensively and a correlation between the accumulation of extracellular bioflocculants and cell aggregation established (Tenney & Verhoff 1973).

Bioflocculation is defined as a dynamic process in which flocculation is mediated by the presence of microorganisms. A bioflocculant is a type of flocculant that is produced by microorganisms but is readily degradable (Desouky et al., 2008).

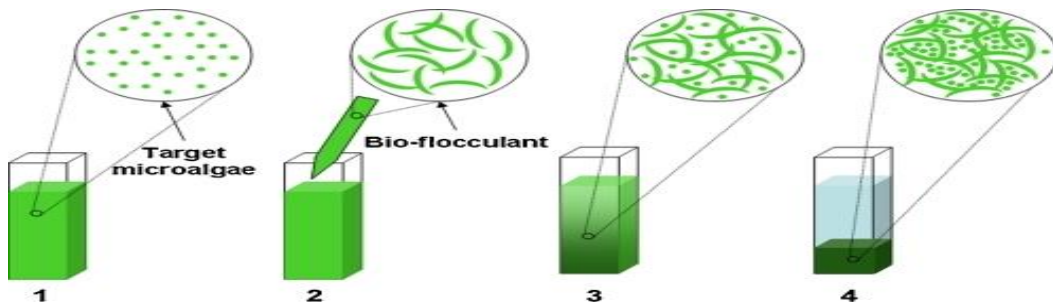


Figure 2. 1 Schematic view of the proposed mechanism involved in bioflocculation (Salim et al., 2011).

## 2.2 Types of flocculants

Flocculants are generally divided into three groups depending upon their composition, namely, inorganic flocculants such as aluminum sulfate and polyaluminum chloride, organic flocculants such as polyacrylamide derivatives and polyethylene amine, and bioflocculants (Salehizadeh & Shojaosadati 2001; Zhang et al., 2007).

### 2.2.1 Inorganic flocculants

Among the three classes of flocculants, these flocculants have been used for a very long time. This class of flocculants includes salts of multivalent metals such as aluminium and iron. Examples of these salts include aluminium sulphate and ferric chloride and high dosages of these salts are usually required for effective flocculation (Sharma et al., 2006; Brostow et al., 2009). Inorganic flocculants have disadvantages that have led to their utilization being minimized. These disadvantages include the following:

- They are required in very large quantities to be effective.
- They result in the production of large amounts of sludge.
- They are sensitive to pH variations (Brostow et al., 2009).

### *2.2.2 Organic flocculants*

These flocculants are polymeric in nature and cause formation of large cohesive aggregates (flocs) and are inert to pH changes. They fall into two categories viz. natural and synthetic flocculants.

#### *2.2.2.1 Natural organic flocculants*

These are exemplified by chitosan, starch, guar gum, alginate and glycogen. Natural polymers are biodegradable, effective at large doses and are shear stable (Sharma et al., 2006).

#### *2.2.2.2 Synthetic flocculants*

Synthetic flocculants are broadly divided into anionic, cationic and non-ionic based on charge. Examples of this category are polyacrylic acid, polydiallylmethyl ammonium chloride and polyacrylamide (PAM) respectively. Synthetic polymers are highly effective at low dosages but have poor shear stability (Brostow et al., 2009).

### *2.2.3 Bioflocculants*

Bioflocculants have an edge over inorganic and organic flocculants because of their benign nature.

Listed below are some of the reported advantages that bioflocculants possess:

- They are convenient to use.
- They need to be added in lesser quantity (1-5 ppm).
- They form strong and larger flocs without affecting the pH of the working medium thus allowing better settling than those of the simple coagulating electrolytes.
- They are also shear stable to a large extent.
- They are biodegradable (Lachhawani 2005).

Due to their advantages over chemical flocculants, bioflocculants have gained much wider scientific and technical attention. Most researchers have focused on microbial screening, production

conditions, flocculating mechanisms, chemical structures and most importantly low-cost production (Salehizadeh & Shojaosadati 2001; Xia et al., 2008).

### **2.3 Composition of bioflocculants**

The predominant components of bioflocculants are extracellular polymeric substances (EPS), such as glycoproteins, polysaccharides, proteins and nucleic acids produced by microorganisms during their growth (Christensen 1989; Lazarova & Manem 1995). The ability of bioflocculants to aggregate particles depends on the characteristics of the components of which they are composed.

Different microorganisms produce bioflocculants that vary in their composition. Bioflocculants produced by *Paenibacillus* sp. CH11, *Bacillus* sp. CH15 and *Halomonas* sp. were composed of protein as the major component while that produced by *Pseudomonas* sp. CH6 contained carbohydrate and protein in almost equal amounts (Lin & Harichund 2012). A bioflocculant produced by *Herbaspirillum* sp. CH7 differed in that it possessed more carbohydrate content (Lin & Harichund 2012). Similar levels of uronic acid and hexosamine composition were observed in all these bacterial flocculants (Lin & Harichund 2012). Zhang et al. (2013) reported that the predominant components of two novel bioflocculants produced by Gram-positive *Bacillus* and *Streptomyces* strains were mainly non-ketose polysaccharides.

Chemical analysis of a bioflocculant produced by *Serratia ficaria* (Gong et al., 2008) revealed the presence of neutral sugar as the major component with no protein detected. Bioflocculants produced by *Alcaligenes cupidus* KT201 (Toeda & Kurane 1991) *Bacillus subtilis* IFO3335 (Yokoi et al., 1996) and *Bacillus* sp. Gilbert (Piyo et al., 2011) were mainly polysaccharides. Generally, flocculants with protein or peptide backbone in their structure are thermally labile, but those made of sugars are thermo-stable (Salehizadeh & Shojaosadati 2001). However, if the major component of a bioflocculant is a glycoprotein, its stability will depend on the relative content ratios of protein and

polysaccharide (Desouky et al., 2008). Gao et al. (2006) reported on a bioflocculant produced by *Vagococcus sp.* which was identified as a polysaccharide. The bioflocculant from this species was named MBFW31 and it contained neutral sugar (71.5%) and uronic acid (15.4%) as its major and minor components respectively, and was proved to be heat-stable. The flocculation efficiency of a bioflocculant produced by *Aspergillus parasiticus* remained at 54% for kaolin suspension when heated in boiling water (100° C) for 30 minutes, indicating that the bioflocculant had moderate heat stability. This heat-stability was associated with the composition of the bioflocculant which was found to be predominantly carbohydrate. The total sugar content was 76.3% as determined by the phenol-sulfuric acid method, and the protein content determined by the Lowry–Folin method was 21.6%, indicating that the bioflocculant was composed mainly of polysaccharide (Deng et al., 2005). It has been noticed that the amino and carboxyl groups of protein bioflocculants are the effective groups mainly responsible for flocculation. Contrary to this, glycoprotein bioflocculants have many functional groups actively involved in the flocculation process (Kurane et al., 1994). Aguilera et al. (2008) proposed that the presence of uronic acid in bioflocculants allows for adhesion of microorganisms to surfaces and is involved in the uptake of metallic ions. Carboxylic and sulfate groups present in acidic exopolysaccharides work as non-specific ion exchange materials which may convey a chelating property (Valls & De Lorenzo, 2002).

## **2.4 Factors affecting bioflocculant production and flocculating activity**

There are several factors that affect production and flocculating activity of bioflocculants. Factors such as the components of the production medium and culture conditions have an effect on bioflocculant production (He et al., 2004). Generally, a suitable medium for bioflocculant production comprises glucose or fructose as the carbon source (Kurane et al., 1994) and yeast extract or beef extract as the nitrogen source (Kurane et al., 1986) while moderate amounts of phosphate ions are

also necessary (Fujita et al., 2000). However, different organisms optimally produce biofloculants under different conditions depending on the type of microorganism.

#### **2.4.1 Nutrients**

Carbon and nitrogen sources are among factors that have been reported to have an important impact on the production of biofloculants. A biofloculant-producing bacterium identified as *Arthrobacter* sp. Raats could optimally produce biofloculant when lactose and urea were used as sole sources of carbon and nitrogen respectively with flocculating activities of 75.4% and 83.4% respectively (Mabinya et al., 2012). Xiong et al. (2010) reported that a biofloculant-producing *Bacillus licheniformis* CGMCC 2876 could effectively use urea as the nitrogen source resulting in high yield of biofloculant.

#### **2.4.2 Cations**

Metal cations play an important role in the flocculation process, and their presence often enhances the flocculating activity of the polymer. Cations promote efficient flocculation by neutralizing and destabilizing the residual negative charges of functional groups of suspended particles and the biofloculant and by forming bridges between the particles (Lian et al., 2008; Yim et al., 2007). The enhancing effect of cations on flocculation is highly dependent on both the concentration and valence of added cations (Watabane et al., 1999). Bivalent and trivalent cations increase the adsorption of biofloculants on the suspended particles by decreasing the negative charges on both the polymer and the particle (Levy et al., 1992). This provides an explanation for the stronger synergistic effect of trivalent and bivalent cations on flocculation (Wu & Ye 2007). On the other hand, monovalent cations produce bonds that are loose in structure causing a decrease in floc density, size and floc resistance to shear (Wu & Ye 2007) Most of the reported biofloculants are cation-dependent, and for such biofloculants it is important to determine the optimal cation dosage which will not overcome the positive effect of the biofloculant (Zulkeflee et al., 2012). Feng & Xu (2008) reported that

metal ions such as  $\text{Al}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$  and  $\text{Na}^+$  enhanced the flocculating activity of MBF3-3 and the stimulating effects increased in the order: monovalent < divalent < trivalent. Divalent cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) were more effective in enhancing the flocculating activity of the bioflocculant SF-1 produced by *Serratia ficaria* than monovalent ( $\text{Na}^+$ ) and trivalent cations ( $\text{Al}^{3+}$  and  $\text{Fe}^{3+}$ ). The sedimentation of kaolin particles significantly improved in the presence of  $\text{Ca}^{2+}$  (Gong et al., 2008).

A bioflocculant produced by *Citrobacter* sp. TKF04 was found to be cation-independent when all the cations tested ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Fe}^{3+}$ ) did not enhance its flocculating activity. This bioflocculant could effectively flocculate a variety of organic and inorganic suspended particles without the addition of any cation (Fujita et al., 2000). The flocculating activity of a novel bioflocculant p-KG03 produced by a marine dinoflagellate identified as *Gyrodinium impudicum* was not enhanced by the addition of any cations including  $\text{Ca}^{2+}$ . On the contrary, high concentration of  $\text{Ca}^{2+}$  led to a decrease in the flocculating activity (Yim et al., 2007).

### **2.4.3 Bioflocculant dosage**

Bioflocculant dosage is an important parameter in the process of flocculation because inadequate dosage may decrease the stimulation of the bridging mechanism and result in low flocculating activity (Gong et al., 2008). In the case where the bioflocculant molecules are excessively present in the solution, they generate high viscosity; block the adsorption sites thereby reducing flocculation process and formation of flocs (Zulkeflee et al., 2012; Wang et al., 2011). For instance, a bioflocculant, p-KG03 produced by a marine dinoflagellate showed maximum activity of more than 90% in kaolin suspension at a concentration of 1 mg/l. However, its flocculating activity decreased when used at a concentration of 2.5 mg/l (Yim et al., 2007). This phenomenon was also demonstrated in a study carried out by Wang et al. (2011) where the maximum flocculating activity of  $96.2 \pm 1.19\%$  was observed at 12 mg/l bioflocculant dosage and a decrease in flocculating activity occurred at concentrations higher than 12 mg/l. The aim of bioflocculant dosage experiment is to determine the

lowest amount of bioflocculant to be used while achieving the highest flocculating activity (Zulkeflee et al., 2012). A bioflocculant, MBFA9 had a flocculating activity of 99.6% for kaolin suspension at a dosage of only 0.1 mg/l (Deng et al., 2003).

## **2.5 Bioflocculation mechanism**

Generally, bridging and charge neutralization were proposed as the two main mechanisms of microbial flocculants (Lian et al., 2008; Li et al., 2009b). The flocculating mechanisms of bioflocculants appear to be less well investigated compared to the relatively well developed mechanisms (including bridging and charge neutralization) of conventional chemical and synthetic flocculants (He et al., 2010). However, to further elucidate the mechanisms of bioflocculants, various mechanisms of varying complexity for floc formation have been suggested in literature, which are not necessarily mutually exclusive. Some of these mechanisms include the double layer theory (DLVO type interaction) and the divalent cation bridging theory (Vatansever 2005).

### **2.5.1 Bridging**

More often, this mechanism is used to elucidate the flocculation in biological systems. Bridging mechanism occurs when the bioflocculant extends from the particles surface into the solution for a distance greater than the distance over which the interparticle repulsion acts. In this case, the bioflocculant can adsorb to the surrounding particles to form an aggregate of particles (Salehizadeh & Shojaosadati 2001). Stronger bridging ability depends upon several factors, and these factors include: the molecular weight and charge of the bioflocculant, the charge on the particle, the ionic strength of the suspension and the nature of mixing (Salehizadeh & Shojaosadati 2001). Flocculating with a high molecular weight flocculant involves multiple adsorption points, stronger bridging ability and higher flocculating activity compared with a low molecular weight flocculant (He et al., 2010). Also, linear (non-branched) polymers would be the most effective compared to branched polymers (Sharma et al., 2006).

Bridging mechanism explains flocculation by neutral or like-charged bioflocculants. The bridging was found to play a significant role in flocculating ability of the bioflocculant EPS SM9913 produced by *pseudoalteromonas* sp. SM9913 (Li et al., 2008). Deng et al. (2003) also reported that the bridging mechanism played an important role in flocculating organic particles in starch wastewater using the bioflocculant MBFA9 produced by *Bacillus mucilaginosus*. Bridging is a preferred mechanism for applications of non-ionic polymers with molecular weights of more than 1 million Daltons or for polyelectrolytes of high molecular weight and low charge density (Sharma et al., 2006).

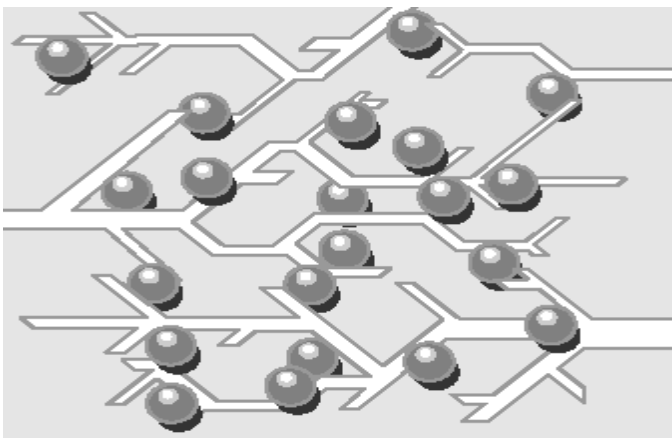


Figure 2. 2 Diagram depicting polymer chain attaching many particles (Lachhwani, 2005)

### 2.5.2 Neutralization

Charge neutralization occurs when suspended particles are oppositely charged against the bioflocculant. It is said that the surface charge of the suspended particles is reduced by the adsorption of the bioflocculant and the particles can approach sufficiently close to each other in order for the forces of attraction to become effective (Li et al., 2009b). A highly cationic polymer is adsorbed on a surface of a negatively charged particle in planar conformation thereby promoting flocculation by reducing the inter-particle repulsion (Sharma et al., 2006). Also, polymer adsorption complex may have a net positive charge as a result of polymer having a high charge density. In this case, the positive regions will be attracted to negative regions of other particles, a process known as

heterocoagulation (Sharma et al., 2006). This description suggests that this mechanism is preferred for polyelectrolytes with low molecular weight and high charge density (Sharma et al., 2006). Most biofloculants and suspended particles are negatively charged by nature thus the charge neutralization mechanism rarely occurs in the flocculating process (He et al., 2010).

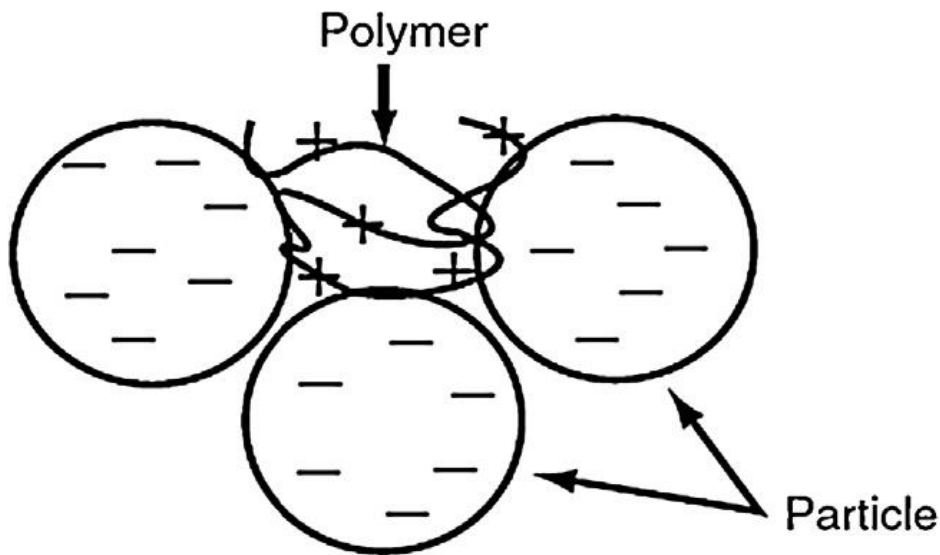


Figure 2.3 Schematic view of a charge neutralization flocculation mechanism (Bohuslav Dobias 2005).

### 2.5.3 DLVO theory

This is also known as the double layer compression and is named after Derjaguin, Landau, Verwey and Overbeek (Lachhwani 2005). According to a classical colloidal theory of DLVO, charged particles are surrounded by a double layer of counter ions. The first layer is known as the Stern layer and is made up of a tightly associated layer of counter ions whereas the second layer is known as the diffuse layer and is made up of less tightly associated counter ions (Adamson 1990). This theory combines the effects of van der Waals attraction and the electrostatic repulsion to explain the tendency of colloids to flocculate due to the double layer of counter ions (Lachhwani 2005). The concentration of ions in the second layer (diffuse layer) decreases with distance from the surface of the particle until the concentration of ions is equivalent to that of the bulk solution and consequently,

the electric potential develops around the particle. This double layer surrounding the particle causes repulsion of adjacent particles and inhibits aggregation. As the ionic strength increases, the size of the double layer decreases thus decreasing the repulsion between particles allowing short-range attractive forces to enhance aggregation. The process of bioflocculation would therefore be improved by the addition of cations to the solution due to a decrease in size of the double layer and the repulsive forces between the particles (Sobeck & Higgins 2002).

This type of mechanism is often associated with inorganic flocculants such as alum and ferric chloride and a good example to elucidate this would be when low ionic strength particles in freshwater of rivers mixes with high ionic strength seawater. As a consequence, the particles are destabilized by double layer compression and accumulation of particles results in the formation of deltas at river mouths (Sharma et al., 2006). Double layer compression effect of  $\text{Ca}^{2+}$  was observed by He et al. (2010) when they reported that the surfaces of kaolin particles were strongly negatively charged and the divalent cation  $\text{Ca}^{2+}$  could compress double layer of kaolin particles thereby weakening the static repulsive force and promoting HBF-3, a bioflocculant produced by *Halomonas sp.* V3a to form flocs with kaolin particles.

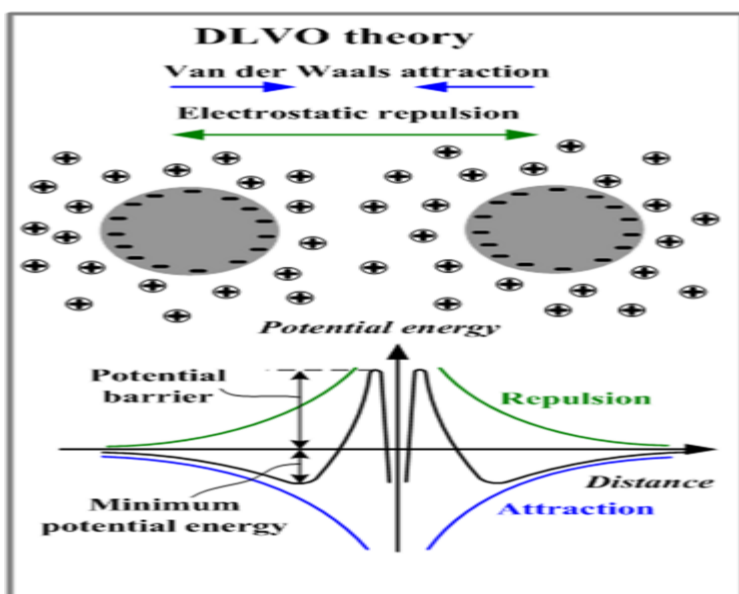


Figure 2. 4 Diagram depicting the DLVO theory (Hwang 2011)

### 2.5.4 Divalent cation bridging

This mechanism describes the role of cations in bioflocculation. Among the polyvalent metal ions, the role of divalent cations, in particular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  has been emphasized by many researchers (Vatansever 2005). According to the divalent cation bridging theory, divalent cations bridge negatively charged functional groups within the flocculant thus promoting the matrix of the biopolymer to aggregate and stabilize thereby promoting bioflocculation (Sobeck & Higgins 2002).

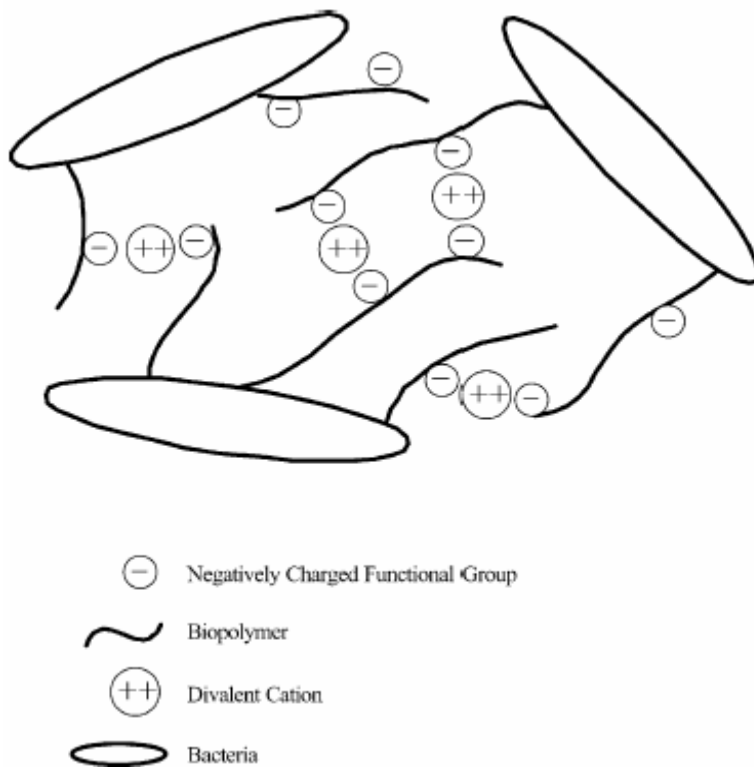


Figure 2.5 Depiction of divalent cation bridging within floc matrix (Sobeck & Higgins, 2002).

### 2.6 Bioflocculant-producing microorganisms

The composition and properties of bioflocculants vary depending on the type of bioflocculant-producing microorganisms (Aljuboori et al., 2013). Bioflocculants are produced by microorganisms during their growth, and their ability to flocculate suspended particles in solutions depends upon the characteristics of the flocculant (Deng et al., 2005). In 2001, Salehizadeh & Shojaosadati (2001)

reported that more than 60 bioflocculant-producing microorganisms have been identified. However, there has been increasing interest in the exploration for microbial flocculants due to their benign nature, an observed increase in the number of reported bioflocculant-producing microorganisms. In recent years, several kinds of microorganisms, which secreted biopolymer flocculants, have been screened and isolated from activated sludge, soil and wastewater. These groups of microorganisms include different species of bacteria, fungi, actinomycetes and algae (Desouky et al., 2008).

### **2.6.1 Bacteria**

Several bacterial strains have been reported to produce bioflocculants. Among the bioflocculant-producing bacteria is the genus *Bacillus*. Generally, it is known that the genus *Bacillus* includes a variety of industrially important species and has a history of safe use in both the food and industrial processes (Desouky et al., 2008). Xiong et al. (2010) reported on a bioflocculant-producing bacteria isolated from contaminated LB medium and identified as *Bacillus licherniformis*. Chemical analyses of the growth products of this strain showed the presence of a novel extracellular proteoglycan and a protein. The bioflocculant produced from this strain was found to be heat stable and comparable to chemical flocculants in terms of a dosing rate required for optimum flocculation of suspended solids and flocculation efficiency in the sugar refinery process. All these properties suggest its potential in industrial applications (Xiong et al., 2010).

*Bacillus* sp. BF3-3 secreted a bioflocculant MBF3-3 which showed excellent flocculating activity on real and synthetic wastewaters. This bioflocculant required a much lower dosage than that of the widely used polyaluminum chloride (PAC) when flocculating brewery wastewater. MBF3-3 was mainly composed of acidic polysaccharide (66%) and protein (29.3%) in which the acidic polysaccharide was the main effective flocculating component (Feng & Xu 2008). A novel bioflocculant, PX was produced from indigenous flocculant-producing bacterial isolate identified as

*Bacillus circulans* X3. PX was found to be an acidic polysaccharide with uronic (19.8%), pyruvic (6.5%), and acetic acids (0.7%). Studies of its flocculating properties showed that it was thermo-stable at 60-100<sup>0</sup>C and had a pH stability range of 4-10. Optimum flocculating activity was observed at a dosage of 2 mg/l. Bioflocculant PX may find possible application as an alternative for environmental bioremediation and other biotechnological processes (Li et al., 2009a).

Other strains from the *Bacillus* family found to produce bioflocculants include *Bacillus mucilaginosus* (Deng et al., 2003), *Bacillus alvei* (Shadia et al., 2011) and *Bacillus subtilis* DYU 1 (Wu & Ye, 2007).

*Klebsiella pneumonia* isolated from a sputum sample produced a bioflocculant named MBF-5 which was mainly composed of polysaccharide and protein in proportions of 96.8% and 2.1% respectively. MBF-5 showed excellent flocculating activity of kaolin suspension without the addition of any cations. It had a maximum flocculating efficiency of 84% for *Acanthamoeba* cyst removal under optimum conditions which were 129.73 mg/l dosage, temperature of 30.75<sup>0</sup>C and pH 4.36 (Zhao et al., 2013).

A bacterial strain *Proteous mirabilis* TJ-1 produced a bioflocculant TJ-F1 with high flocculating activity. The production of bioflocculant TJ-F1 could be stimulated by the addition of cations Ca<sup>2+</sup>, Mg<sup>2+</sup> and Fe<sup>3+</sup>. The optimum conditions for TJ-F1 production were inoculum size 2% (v/v), initial pH 7.0, culture temperature 25<sup>0</sup>C and shaking speed 130 r/min for 48h, under which the flocculating activity reached 93.13% (Xia et al., 2008).

## **2.6.2 Fungi**

Bioflocculants are produced by a variety of microorganisms including fungi. *Aspergillus parasiticus* was reported to produce a bioflocculant which was effective in the removal of some acid as well as reactive and direct dyes. The bioflocculant mainly consisted of sugar (76.3%) and protein (21.6%).

This bioflocculant had decolorization efficiencies above 92% for reactive blue 4 and acid yellow 25 dyes (Deng et al., 2005).

In the study by Aljuboori et al. (2013), *Aspergillus flavus* was reported as bioflocculant producing fungus which produced a bioflocculant named IH-7 which showed excellent flocculating efficiency of kaolin suspension in the absence of cations. The main component of IH-7 was a polysaccharide hence its observed thermo-stability. The use of waste material such as potato extract in the culture medium reduced the cost of production of this bioflocculant and also improved its feasible utilization in commercial production (Aljuboori et al., 2013). In another study, Zhi et al. (2010) reported on bioflocculation capability of white-rot fungus.

### **2.6.3 Actinomycetes**

A bioflocculant-producing actinomycete, *Norcadia amarae* YK-1, produced a bioflocculant named FIX. Chemical analysis of this bioflocculant revealed the presence of a protein as a predominant component. Flocculating activity of FIX was stimulated by the addition of salts (NaCl, CaCl<sub>2</sub>, Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> and FeCl<sub>3</sub>). However, the flocculation activity was inhibited by excess addition of FeCl<sub>3</sub> (Tadeka et al., 1991). Other strains in the *Norcadia* family found to produce biopolymer flocculants include *Norcadia restricta*, *Norcadia calcarea* and *Norcadia rhodnii* (Kurane et al., 1986; Desouky et al., 2008).

### **2.6.4 Algae**

*Chlamydomonas reinhardtii* (*C. reinhardtii*) was reported by Zhu et al. (2011) as the bioflocculant-producing algae. The bioflocculant of this green algae was examined and isolated under axenic conditions. The highest flocculating activity of 97.06% was observed at a bioflocculant dosage of 4 mg/L. The purified *C. reinhardtii* bioflocculant was composed of 42% (w/w) protein, 48.3% (w/w) carbohydrates 8.7% (w/w) lipids and 0.01% (w/w) nucleic acids. The following parameters were necessary for optimum production of the bioflocculant by *C. reinhardtii*: temperature range of 15 to

25°C, pH range of 6 to 10 and an illumination range of 40 to 60  $\mu\text{mol photons m}^{-2} \text{ S}^{-1}$ , which according to Zhu et al. (2011), indicated that it is potentially more economical production process when compared to the production by heterotrophic bacteria. Flocculating activity of microalgae has also been reported in other strains such as the cyanobacteria *Oscillatoria sp.* (Bender et al., 1994), *Anabaena* PC-1 (Choi et al., 1998) and *Chlorella sp.* (Kaplan et al., 1987).

## **2.7 Applications of bioflocculants**

The application of bioflocculants on treatment of different kinds of wastewater has been widely studied during the past decades, including the treatment of starch wastewater (Deng et al., 2003), indigotin printing and dyeing wastewater (Zhang et al., 2007), inorganic solid suspensions (Shih et al., 2001; Yim et al., 2007), olive mill wastewater (Aguilera et al., 2008) and many other industrial wastewater effluents (Lin & Hirachund 2012). However, an extensive knowledge on the treatment of real wastewaters is still lacking (Deng et al., 2003).

### **2.7.1 Wastewater treatment**

Starch wastewater is one of the most common wastewaters in the food industry. Deng et al. (2003) reported on a bioflocculant MBFA9 produced by *Bacillus Mucilaginosus*, which could flocculate starch wastewater. MBFA9 significantly separated organic particles from starch in the presence of  $\text{Ca}^{2+}$  ions. The removal rate of suspended solids (SS) and chemical oxygen demand (COD) reached 85.5% and 68.5% respectively. The results compared better to those of traditional chemical flocculants. A novel bioflocculant MMF1 produced by a multiple-microorganism consortia MM1 showed good flocculating capability for treating indigotin printing and dyeing wastewater. Maximal removal efficiencies of COD and chroma reached 79.2% and 86.5% respectively, when 1.7 ml of 1% (v/v) MMF1 was added to 100 ml of indigotin printing and dyeing wastewater (Zhang et al., 2007).

### 2.7.2 Defecating trona suspension

According to a study by Lu et al. (2005), *Enterobacter aerogenes* produced a bioflocculant named WF-1 which could be applied in defecating strong alkaline trona suspension. The flocculating ability of WF-1 was indicated by the resultant high sedimentation rate that was reported. A bioflocculant dosage of 90 mg/l was optimum in providing the sedimentation rate of  $2.96 \times 10^{-4}$  m/s and the optical density of the supernatant was determined at 0.05. Based on these results it was deduced that WF-1 was efficient for the treatment of strong alkaline trona suspension compared to conventionally chemically synthesized flocculants such as anionic polyacrylamide or non-ionic polyacrylamide.

### 2.7.3 Flocculation of inorganic and organic solutions

Yim et al. (2007) carried out a study which showed the flocculating efficiency of bioflocculant p-KG03, produced by a marine dinoflagellate *Gyrodinium impudicum* KG03 for kaolin clay suspension, active carbon, aluminium oxide and  $\text{Ca}(\text{OH})_2$  was enhanced in the presence of  $\text{Ca}^{2+}$  with flocculating activities of  $93.6 \pm 4.3\%$ ,  $61.1 \pm 1.4\%$ ,  $83.4 \pm 6.3\%$  and  $91.2 \pm 5.1\%$ , respectively at a flocculant dosage of 1.0 mg/l. From the results they concluded that p-KG03 could potentially be used as a bioflocculant in various industrial processes.

A bacterial strain TKF04 identified as *Citrobacter* was capable of using acetic acid and propionic acid as substrates for the production of a bioflocculant (Fujita et al., 2000). The bioflocculant produced by *Citrobacter* sp. was found to be effective for flocculation of a kaolin suspension at a concentration of 1-10 mg/l. The bioflocculant was tested against a variety of inorganic and organic suspended particles including kaolin, diatomite, bentonite, activated carbon, soil and activated sludge. When added at 10 mg/l, the crude bioflocculant could effectively flocculate all the tested material with the following flocculating activities being observed: kaolin suspension (96.7%), diatomite (79.7%), bentonite (96%) activated carbon (82.1%), soil (95%) and activated sludge (74%). The flocculating activity of this bioflocculant was compared with that of chemical flocculants such as

polyaluminium (PAC) and polyacrylamide (PAA) and was found to exhibit flocculating activity comparable or superior to that of the existing synthetic flocculants.

A psychrophilic bacterium identified as *Pseudoalteromonas* sp. SM9913 secreted an exopolysaccharide (EPS SM9913) which could flocculate various suspended solids such as kaolin clay, activated carbon, soil, magnesium oxide (MgO) and aluminium oxide (Al<sub>2</sub>O<sub>3</sub>) (Li et al., 2008). Addition of divalent cations such as Ca<sup>2+</sup> and Fe<sup>2+</sup> enhanced the flocculating activity of EPS SM9913. It was observed that EPS SM9913 had a better flocculating performance than alum at low temperatures (5-15°C) or in high salinity water.

#### **2.7.4 Flocculation of real wastewaters**

A bioflocculant named SF-1 produced by *Serratia ficaria* could flocculate a variety of real wastewaters including river water, brewery wastewater, meat processing wastewater and soy sauce brewing wastewater. The removal efficiencies of COD, turbidity and color in river water were 87.1%, 84.25 and 90.4% respectively. Comparison of the results with commonly used synthetic flocculants showed that SF-1 achieved a slightly better flocculating efficiency in the clarification of river water (Gong et al., 2008).

In the case of brewery wastewater, meat processing wastewater and soy sauce brewing wastewater the removal efficiency of COD was in the range 60-80% which was similar to that reported for polyaluminium chloride (PAC) and polyacrylamide (PAM). However, the turbidity removal was between 91.8 and 93.7% which was better than that reported for PAC and PAM. From the results it was deduced that the use of SF-1 bioflocculant was feasible in wastewater treatment of some agricultural products-processing industries (Gong et al., 2008).

Luo et al. (2014) reported on a bioflocculant produced by the bacterium *Klebsiella pneumonia* YZ-6 isolated from human saliva. The bioflocculant was named MBF-6 and was used to flocculate different industrial waste wastewaters including wastewaters from brewery, sugar, dairy and textile

industries. MBF-5-treated wastewater samples from each industry exhibited reduction in BOD, COD and suspended solids (SS) in comparison with untreated samples. However, of all the wastewaters treated, sugar wastewater exhibited the highest reduction of COD, BOD and suspended solids with percentages up to 77.8%, 80.7% and 78.6% respectively. These results showed the feasibility of the use of MBF-5 in wastewater treatment for various industries.

*Arthrobacter* sp. B4 produced a bioflocculant which had its active ingredient as a polysaccharide (B4-PS) (Li et al., 2014). B4-PS was utilized to purify a strong alkaline propylene oxide saponified wastewater that contained a large amount of  $\text{Ca}^{2+}$  ions,  $\text{Cl}^-$  ions, organic chloride and other inorganic ions. The results' analyses showed that the pollutant removal efficiencies for COD, TOD,  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$  and turbidity were 51%, 61%, 89%, 20% and 33% respectively. The data obtained suggested that B4-PS may be a promising tool for use in industrial alkaline wastewater pretreatment.

### **2.7.5 Heavy metals removal**

Gomaa, (2012) evaluated a bioflocculant produced by *Pseudomonas aeruginosa* for heavy metals removal. The bioflocculant showed the highest removal of copper (87.39%) and mercury (89.09%) at a concentration of 20ppm and pH 7. The optimum adsorption of lead and cadmium were 79.70% and 79.93% respectively, observed at 40ppm and at pH 7 whereas the highest arsenate and zinc removal of 72.9% and 80.59% respectively was recorded at 660ppm and pH 9. The bioflocculant showed better removal efficiency of heavy metals at lower bioflocculant concentrations and those results were in agreement with those obtained by Das & Santra 2007. Gomaa, (2012) concluded that the bioflocculant had a potential to be applied as an alternative bio-remedial tool for industrial effluents and wastewater treatments which are co-contaminated with heavy metals.

A bioflocculant produced by psychotropic bacterium *Pseudomonas fluorescense* BM07 removed about 45% and 70% cadmium and mercury respectively, while the percentage of cobalt, zinc, nickel and copper cations were between 20 and 30% (Noghabi et al., 2007).

Kaewchai & Prasertsan, (2002) reported on an investigation for adsorption of heavy metals such as nickel and cadmium by bioflocculants produced from three thermo-tolerant isolates (*Enterobacter agglomerans* SM 38, *Bacillus subtilis* WD 90 and *Bacillus subtilis* SM 29). From the results obtained it was observed that the bioflocculants of *B. subtilis* WD 90 and *B. subtilis* SM 29 showed the highest nickel removal of 90.7 and 87% respectively, while the cadmium removal was 90.9 and 91.4% respectively. The adsorption of nickel and cadmium by the bioflocculant of *E. agglomerans* SM 38 was 92.8 and 84.2% respectively.

### **2.7.6 De-ashing of coal**

Vijayalakshmi & Raichur, (2002) studied bioflocculation of high-ash Indian coals using a bacterium, *Paenibacillus polymyxa*. Flocculation tests of the study showed that the bacterium could flocculate coal effectively and efficiently at around pH 2. In the presence of the bacterium, more than 90% of coal flocculated in about a minute compared to 20-30% in the absence of the bacterium. Ash analysis experiments showed that nearly 50-60% of the ash could be removed in a single stage flocculation experiment. The results demonstrated the potential for bioflocculation in de-ashing of high-ash Indian coals.

In another study, *Mycobacterium phlei* (*M. phlei*), a gram-positive, rod-shaped, prokaryotic cell was observed to be useful as a flocculant for phosphate slimes, hematite, and coal fines (Mishra et al., 1993). Selective flocculation of fine coals with respect to the separation of pyritic sulphur could also be achieved in the presence of *M. phlei* with more than 85% of pyritic sulphur being removed from coal by bioflocculation.

Abdel-Khalek & El-Midany (2012) investigated the effect of *Bacillus subtilis* (*B. subtilis*) on the removal of sulphur and ash in Egyptian coal using flotation technique. At optimum conditions the flotation results showed that *B. subtilis* could remove more than 70% of sulphur and ash content. The optimum conditions for the removal of these impurities in coal were as follows: 10 minutes

conditioning time,  $2 \times 10^{-6}$  cells/cm<sup>3</sup> and pH 3. From these results it can be deduced that bioflocculation is much more effective in removing impurities from coal compared with flotation technique.

## **2.8 Limiting factors of bioflocculants applications**

Many researchers attest to the fact that low flocculating activity and high cultivation costs are always the major limiting factors for large scale industrial applications of bioflocculants (Yang et al., 2009; Li et al., 2003; He et al., 2004). To overcome this challenge, several investigators have tried to produce bioflocculants with high flocculating activity using cheap substrates.

### **2.8.1 High production cost**

Peng et al. (2014) reported on a bioflocculant produced by culturing *Rhodococcus erythropolis* in a cheap medium composed of sludge and livestock wastewater. A maximum flocculating activity of 87% could be achieved when the ratio of sludge/livestock wastewater was 7:1 (v/v).

Also, brewery wastewater was used as a carbon source for the production of MM1, a bioflocculant produced by a consortium composed of strains identified as *Staphylococcus* sp. and *Pseudomonas* sp. The flocculating activity of MM1 reached 96.8% under optimal growth conditions which were inoculum size of 2%, initial pH 6.0, cultivating temperature of 30°C, and shaking speed of 160 r/min. This bioflocculant also had a markedly higher yield as 15 grams of purified bioflocculant could be recovered from 1 litre of fermentation broth (Zhang et al., 2007).

Pei et al. (2013) recycled piggery wastewater and used it as a cheap alternative medium for a bioflocculant-producing bacterial flora B-737. The cost of the production medium was reduced by 90% when this medium was used for bioflocculant production and the bioflocculant yield reached 1.5g/l.

Carbon sources generally used in culture media for bioflocculant production have a direct impact on production costs of bioflocculants which limits the market potential of these biopolymers. Therefore, strains which are able to produce bioflocculants in nutrient poor conditions would be an advantage from the industrial point of view. Attempts are being made to search novel strains capable of producing bioflocculants in low cost substrate containing medium. A facultative oligotrophy *Klebsiella* sp. produced an exopolysaccharide in nutrient-poor medium. This EPS produced in nutrient-poor medium showed flocculating rate above 80% for activated carbon suspensions (Mandal et al., 2013).

Aljboori et al. (2013) proposed that addition of cations to the flocculation process does not only produce secondary pollution but also increases costs. Consequently, finding cation-independent bioflocculants is desirable. *Aspergillus flavus* is a bioflocculant-producing strain which showed excellent flocculating rate of kaolin suspension without cation addition, thereby avoiding secondary pollution and decreasing the production cost.

In another study, *Klebsiella pneumonia* produced a bioflocculant named MBF-5 which showed excellent flocculating activity without addition of any cations. Maximum flocculation efficiencies of 98% and 84% were observed under optimal flocculation parameters for kaolin suspension and *Acanthamoeba* cysts removal, respectively (Zhao et al., 2013).

Cation-independent bioflocculants have also been reported in various studies (Zheng et al., 2008; Yim et al., 2007; Fujita et al., 2000).

### **2.8.2 Low yield**

Low yield has also been reported to be one of the factors limiting the application of bioflocculants in industrial processes such as wastewater treatment (He et al., 2009). Using two or more microbes in consortium was first reported by Ma et al. (2003), the idea behind this approach was to improve yield and flocculating efficiency of the bioflocculant produced by individual strains. Okaiyeto et al. (2013)

reported on a bioflocculant produced by a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. They reported that about 3.51g of the purified bioflocculant was recovered after fermenting 1L of a mixed culture for 5 days. This yield was higher than that produced by individual strains which were 1.213 g/l and 0.738g/l for *Halomonas* sp. Okoh and *Micrococcus* sp. Leo respectively.

There are also other techniques which have been put in place to overcome the challenge of poor yield. These include optimization of fermentation conditions, microbial mutation analyses to obtain more efficient strains, utilization of low cost substrates for bioflocculant production and using statistical models with high efficiency towards yield optimization such as factorial experiment and surface response design (SRD) (Nwodo et al., 2014; Wang et al., 2007; Zhang et al., 2007).

Also in a bid to enhance bioflocculant yield, Wang et al. (2007) used dairy wastewater as a cheap substrate for the production of a novel bioflocculant by *Klebsiella mobilis*. A yield of 2.58 g/l of crude bioflocculant was recovered with a flocculating activity of 95.4%. This bioflocculant was reported to be effective in flocculating dyes in solutions with a decolourization efficiency of 91%. Also, *Paenibacillus elgii* B69 was reported as a bioflocculant-producing microorganism. The exopolysaccharide (EPS) it produced was composed of glucose, gluco-uronic acid, mannose and xylose. A maximum production of about 25.63 g/l EPS could be obtained in the optimized medium with sucrose at 51.53 g/l, peptone at 6.78 g/l and yeast extract at 0.47 g/l optimized by response surface methodology (RSM) (Li et al., 2013).

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# CHAPTER

# 3

## Bioflocculant production by *Bacillus pumilus* strain

### Abstract

A bioflocculant produced by bacterial specie identified as *Bacillus pumilus* strain was investigated with regards to its physiochemical properties and flocculating activity. The optimum culture conditions for the production of the bioflocculant were inoculum size of 4% (v/v), maltose as carbon source, multiple nitrogen source composed of yeast extract, urea and ammonium sulphate. The highest flocculating activity was recorded at an initial of pH 7. A bioflocculant yield of about 0.289 g was recovered from 1L of fermented broth. The purified bioflocculant was a white powder which showed a high flocculating activity (96.5%) against kaolin suspension (0.4% w/v) at a dosage of 0.1 mg/ml. All the cations tested stimulated the flocculating activity of the purified bioflocculant except for Fe<sup>3+</sup> which only supported a flocculating activity of 21%. Thermal stability test of the purified bioflocculant showed that the bioflocculant was thermo-stable as it could retain more than 90% of its flocculating activity with only 4.8% decrease after being heated at 100°C for 1 hour. Fourier-transform infrared (FTIR) spectroscopy analysis revealed that purified bioflocculant contained hydroxyl groups, carboxyl groups and uronic acid. The bioflocculant was composed of sugar (75.4%), protein (5.3%) and uronic acid (15.4%). Scanning electron microscopy (SEM) revealed a dendritic bioflocculant structure and elemental analysis of the purified bioflocculant showed that the weight fractions of elements C, N, O, S and P were 22.71%, 11.56%, 41.60%, 0.51% and 7.98% respectively. The bioflocculant produced by *Bacillus pumilus* strain had strong flocculating activity and high stability which affords high possibility of its possible utilization in industrial processes.

**Key words:** *Bacillus pumilus*, bioflocculant, flocculating activity

### 3.1 INTRODUCTION

Bioflocculants are said to be natural product metabolites of microorganisms (Nie et al., 2011) that can flocculate various suspended solids, cells, colloidal solids etc. (Zaki et al., 2011). Chemical flocculants have been widely used in various industries owing to their low cost and high efficiency (Li et al., 2007), however many researchers have now opted for the use of bioflocculants as a result of health concerns implicated in the use of chemical flocculants. Polyaluminum chloride (PAC) and polyacrylamide are among chemical flocculants that have been used in wastewater treatment, downstream processing, food and fermentation industries (Li et al., 2007). However, polyaluminum salts are associated with Alzheimer's disease (Arezoo 2002) and also there is evidence that polyacrylamide monomers are neurotoxic and carcinogenic to humans (Dearfield et al., 1988). Consequently, the use of these chemical flocculants is now restricted. These inevitable disadvantages of chemical flocculants have shifted the focus of many researchers to investigating a variety of microorganisms for their flocculant-producing potentials (Nie et al., 2011). At present, many universities and research institutes consider the development of new bioflocculants as their research priority and are engaged in the production of new bioflocculants with improved prospects for application (Zhi et al., 2010). This can be attributed to the unrivalled benefits of bioflocculants such as being environmentally friendly, biodegradable, free of the risk of secondary pollution, nontoxic and harmless to humans, animals and the environment (Zhang et al., 2002a; Nie et al., 2011; Yang et al., 2012).

Bioflocculant-producing microorganisms have been isolated from almost all kinds of different environments such as soil, wastewater, activated sludge and rivers (Deng et al., 2005; Yim et al., 2007; Gong et al., 2008; You et al., 2008, Wan et al., 2013). Recently, bioflocculants have also been

produced by microorganisms isolated from unusual environments such as sputum (Zhao et al., 2013) and human saliva (Luo et al., 2014).

Biofloculants are not only useful in wastewater treatment but have a wide range of applications. For an example, biofloculants produced by *Sobacillus Silvestries* WO1 and *Paenibacillus* sp. AM49 were used to harvest marine microalgae *Nannochloropsis oceanica* and *Chlorella vulgaris* respectively (Wan et al., 2013; Oh et al 2001). Also, in another study by Zaki et al. (2014), biofloculant producing *Bacillus Mojavensis* strain 32A and its biofloculant were used to synthesize silver nanoparticles.

The practical application of biofloculants in industries however, is somewhat limited due to their high cost of production. To overcome this challenge, a number of cost-effective substrates for biofloculant production have been utilized. As an example, dairy wastewater was used as a cheap substrate for biofloculant production by *Klebsiella mobilis* (Wang et al., 2007); also a facultative oligotrophic *Klebsiella* sp. PB12 produced an exopolysaccharide in nutrient-poor medium thus reducing production costs (Mandal et al., 2013). It has therefore become apparent that novel microbial strains need to be identified and screened for biofloculant production and the fermentation conditions optimized to enhance biofloculant activity and yield. In this study, the effect of culture conditions on a biofloculant produced by fresh water bacteria identified as *Bacillus Pumilus* strain was evaluated. Furthermore, the produced biofloculant was characterized and purified. To the best of my knowledge, there are no reports on *Bacillus pumilus* for biofloculant production.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Sample collection and processing

Samples were collected randomly from different points of the Tyume River in Alice, South Africa and stored in sterile containers. The samples included water samples, rock scrapings and sediment samples. After collection, the sample containers were transported in ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) lab for processing within four hours of collection.

Isolation of colonies was done following the methods of Jensen et al. (1991; 2005) with some modifications.

**Water samples:** Water sample (1 ml) was diluted with 9 ml of sterilized saline solution in a test tube. The diluted solution was agitated for 30 seconds and serially diluted up to a dilution factor of  $10^{-3}$ . Diluted samples (100  $\mu$ l) were inoculated onto the surface of M1 agar with the following composition (18 g/l of agar, 10 g/l of starch, 4 g/l of yeast extract, 2 g/l of peptone) and spread with an alcohol-sterilized glass spreader.

**Sediment samples:** Wet sediment samples were vigorously shaken by hand to ensure uniformity and serial dilutions were made by aseptically removing 2 ml of wet sediment and transferring it into 8 ml of sterile saline solution in a test tube and vortexed for 30 seconds. Serial dilutions were made up to a dilution factor of  $10^{-3}$ . The diluted sediments (100  $\mu$ l) were inoculated onto the surface of M1 agar plates using a sterilized glass spreader.

**Rock scrapings:** Rock scraping (0.5 g) was dissolved in 5 ml of sterile saline water. The serial dilutions of the solution were made by transferring 1 ml of the diluted solution to 9 ml of sterilized saline solution and dilutions were made up to a dilution factor of  $10^{-3}$ . Similarly, 100  $\mu$ l of the diluted solutions were inoculated on M1 agar plates using a sterile glass rod.

The medium (M1 agar) was prepared with sterile distilled water and contained the anti-fungal agents; cyclohexane (100 µg/ml) and nystatin (50 µg/ml) added after the medium had been autoclaved and cooled down to room temperature. All plates were incubated at 28°C for 1 week. After the incubation period, the isolates were purified by streak plate method and incubated at 28 °C for 5 days.

### **3.2.2 Screening of isolates for bioflocculant production**

#### **3.2.2.1 Medium preparation**

Activation medium was prepared by dissolving the following components in 1L of sterile distilled water: 3 g of beef extract 10 g of tryptone, 5 g of NaCl. The medium was dispensed (5 ml) into separate test tubes and autoclaved at 121°C for 15 minutes.

The growth medium reported by Zhang et al. (2007) was used for screening of isolates for bioflocculant production. The medium was made up in 1 L of distilled water and contained the following: glucose (20 g), K<sub>2</sub>HPO<sub>4</sub> (5 g), KH<sub>2</sub>PO<sub>4</sub> (2 g), urea (0.5 g), yeast extract (0.5 g), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.2 g), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.2 g). After sterilizing by autoclaving at 121°C for 15 minutes, the initial pH of the medium was adjusted to 7.0 with either NaOH (0.1M) or HCl (0.1M).

#### **3.2.2.2 Evaluation of bioflocculant production**

A loop full of colonies was inoculated into each of the test tubes containing activation medium and incubated with shaking (160 rpm) for 24 hours at 28°C (Zhang et al., 2007). The activation medium was used as a seed culture and 1 ml was inoculated into 50 ml of growth medium contained in 250 ml flasks and incubated in a rotary shaker (160 rpm) at 28°C for 5 days. At the end of the incubation period, 2 ml of the fermentation broth was centrifuged at 4000 x g, for 30 minutes to separate the cells. The cell-free culture supernatant was analyzed for flocculating activity.

### **3.2.2.3 Determination of flocculating activity**

The determination of flocculating activity was done according to the method described by Kurane et al. (1994) using kaolin suspension (0.4% w/v) as a test material. Two milliliter of the culture supernatant and 3 ml of 1% CaCl<sub>2</sub> (w/v) were added into 100 ml of kaolin clay suspension (0.4% w/v) in 250 ml flask, gently shaken and left to stand still for 5 min at room temperature. The control was prepared following the same procedure except that culture supernatant was replaced with fresh broth. The turbidity in the upper phase was measured with a spectrophotometer at 550 nm and the flocculating activity was estimated as follows: Flocculating rate =  $\{(A-B)/A\} \times 100\%$ , where A and B are the optical densities of the control and the sample at 550 nm respectively. All experiments were performed in triplicates and the mean value determined. A total of 144 isolates were screened for bioflocculant production potential and all the positive isolates were maintained in 20% glycerol stocks at -80°C for further experiments.

### **3.2.3 Effect of culture conditions on bioflocculant production by *Bacillus pumilus* strain**

#### **3.2.3.1 Effect of carbon sources on bioflocculant production**

Carbon and nitrogen sources are critical factors which greatly affect the production of bioflocculants by microorganisms (Xia et al., 2008). The assessments for the effect of carbon and nitrogen sources on bioflocculant production were done following the procedure of Lachhwani (2005). Isolate F30 was inoculated in separate growth media containing different carbon sources such as glucose, sucrose, starch, lactose, maltose, fructose, phthalate and incubated in a rotary shaker (120 rpm) at 28°C for a period of 5 days. The flocculating activity was determined as previously described and the results were studied after 120 hours.

### **3.2.3.2 Effect of nitrogen sources on bioflocculant production**

For optimization of the effect of nitrogen sources on bioflocculant production, individual nitrogen sources (1.2 g/l) were incorporated into separate flasks containing the production medium. The nitrogen source candidates included the organic nitrogen sources such as yeast extract, urea, peptone, tryptone, inorganic nitrogen sources such as ammonium sulphate, and also the mixed nitrogen source (yeast extract, ammonium sulphate, and urea) was tested. Flocculating activity was determined as previously described.

### **3.2.3.3 Effect of the initial pH of the medium**

Using pre-determined optimum culture conditions of nitrogen and carbon sources, the pH of the medium contained in separate flasks was adjusted from pH 3 to pH 11 with either NaOH (0.1M) or HCl (0.1M) prior to inoculating the medium with the seed culture. The medium was incubated in a rotary shaker at 28°C for a period of 5 days. The flocculating activity assay was done as previously described (Yim et al., 2007).

### **3.2.3.4 Inoculum size effect**

The effect of inoculum size on bioflocculant production was also investigated. This experiment was done by using different inoculum sizes ranging from 0.5 to 2% (v/v) to inoculate into the production medium. The assessments were done in accordance with the description of Zhang et al. (2007).

### **3.2.3.5 Effect of various cations on the flocculating activity**

The effect of cations on flocculating activity was done following the same procedure elaborated above for determining flocculating activity but CaCl<sub>2</sub> solution was substituted with different cation solutions (1%, w/v). These included solutions of KCl, NaCl, MnCl<sub>2</sub>, MgCl<sub>2</sub>, FeSO<sub>4</sub> and AlCl<sub>3</sub> (Yim et al., 2007).

### **3.2.4 Time course assay of bioflocculant activity**

The time course experiment was done following the method of Gao et al. (2006) with some minor modifications. The isolate was cultured under optimal culture conditions determined from previous experiments. The growth medium was prepared by mixing the following components in 1 litre of distilled water: maltose (20.0 g),  $\text{KH}_2\text{PO}_4$  (2.0 g),  $\text{KH}_2\text{PO}_4$  (5.0 g),  $(\text{NH}_4)_2\text{SO}_4$  (0.2g),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2 g), urea (0.5 g) and yeast extract (0.5 g) (Zhang et al., 2007). For inoculum preparation, the isolate was cultured in 50 ml activation medium (3 g/l of beef extract 10 g/l of tryptone, 5 g/l of NaCl) in a 250 ml flask on a rotary shaker (160 rpm) at 28°C for 24 hours. After 24 hours of incubation, the optical density ( $\text{OD}_{600}$ ) of the culture broth was adjusted to give 0.1 using 0.85% sterile saline solution. The diluted culture broth (4 % v/v) was used as a seed culture to inoculate 200 ml of growth medium in 250 ml flasks. About 15 ml of medium samples was withdrawn at 12 hour intervals and monitored for pH, cell growth, cell count and flocculating activity. At each withdrawal, 2 ml of culture broth was centrifuged at  $4,000 \times g$ , 4 °C for 30 minutes and the cell free supernatant was used as the test bioflocculant to determine the flocculating activity. The bacterial growth was monitored by measuring the optical density ( $\text{OD}_{600}$ ) and bacterial counts were determined by standard spread plate technique using nutrient agar and all plates were incubated at 37°C for 24 hours.

### **3.2.5 Extraction and purification of bioflocculant**

The bioflocculant was purified according to the method described by Okaiyeto et al. (2013) using media formulation based on the pre-determined optimum culture conditions. Briefly, after 24 hours of fermentation, the fermentation broth was transferred to centrifuge bottles and centrifuged at 4000 x g at 4°C for 30 minutes to remove bacterial cells. The supernatant was mixed with one volume of distilled water and centrifuged again at 4000 x g, 4°C for 15 minutes to remove insoluble substances. Two volumes of ethanol were added to the supernatant, stirred and left to stand at 4°C overnight. The supernatant was decanted and the precipitate was vacuum-dried to obtain the crude

biopolymer. The crude product was then dissolved in distilled water and mixed with 1 volume of chloroform/n-butyl alcohol (5:2 v/v). After stirring, the mixture was left standing at room temperature for 12 hours. The upper phase was separated, centrifuged at 4000 x g for 15 minutes at 4°C and the supernatant was dialyzed overnight against distilled water. The dialysate was then vacuum-dried to obtain a purified bioflocculant.

### **3.2.6 Effects of various factors on flocculating activity of the purified bioflocculant**

#### **3.2.6.1 Determination of bioflocculant dosage (Jar Test)**

For bioflocculant dosage determination, the method of Wang et al. (2010) was used with minor modifications. Different concentrations of bioflocculant solutions were prepared to determine the optimum bioflocculant dosage. Concentrations of 0.05, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml were used. Two milliliters of the bioflocculant solution was added to 100 ml of kaolin suspension (0.4% w/v) containing 1% (w/v) CaCl<sub>2</sub> in 500 ml flasks. The solution was stirred rapidly at 120 rpm for 3 minutes during the addition of the bioflocculant, followed by slow stirring at 45 rpm for 5 minutes. The solution was then transferred into 100 ml measuring cylinder and allowed to sediment for 10 minutes at room temperature before removing the clear upper phase of the supernatant for determination of flocculating activity. The flocculating activity was measured at 550 nm as previously described (section 3.2.2.3).

#### **3.2.6.2 Thermo-stability test**

The effect of temperature on flocculating activity of the purified bioflocculant was determined following the method of Wang et al. (2011). The desired concentration of the bioflocculant was prepared in 10 ml of distilled water. Two milliliters aliquots of the solutions were transferred into different Eppendorf tubes and heated for 30 minutes using heating blocks set at different temperatures (50°C, 80°C, 100°C). The residual flocculating activity was measured at room temperature.

### **3.2.6.3 Effect of pH on flocculating activity**

To investigate the effect of pH on flocculating activity, the pH of the kaolin suspension was adjusted using HCl and NaOH in the pH range of 3–11 in 250 ml flasks. The desired bioflocculant concentration was added to each flask and the flocculating activity was measured as previously described (He et al., 2010).

### **3.2.6.4 Effect of cations on flocculating activity**

The effect of various cations on the flocculating activity of the purified bioflocculant was also investigated. This was done by replacing the 1% (w/v) CaCl<sub>2</sub> solution with various salt solutions including KCl, NaCl, MnCl<sub>2</sub>, MgCl<sub>2</sub>, FeCl<sub>3</sub>, and AlCl<sub>3</sub> (Lachhwani 2005). The flocculating activity was measured as previously described using the optimum bioflocculant concentration.

## **3.2.7 Chemical analysis of the purified bioflocculant composition**

### **3.2.7.1 Sugar content**

The total sugar content of the bioflocculant was determined by the Phenol-Sulphuric method as described by Chaplin & Kennedy (1994) using glucose as a standard.

### **3.2.7.2 Protein content**

The protein content of the bioflocculant was determined using the Folin-Lowry method as described by Lachhwani (2005) with bovine serum albumin as a standard.

### **3.2.7.3 Uronic acid content**

The uronic acid content of the bioflocculant was determined using the carbazole–sulfuric acid method (Chaplin & Kennedy 1994).

#### **3.2.7.4 Fourier Transform Infrared Spectroscopy (FTIR)**

Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, FT-IR, England) was used to determine the functional groups of the bioflocculant. The bioflocculant was ground with KBr salt at 25°C and pressed into a pellet for FTIR spectroscopy over a wave number range of 4000-370 cm<sup>-1</sup> (He et al., 2010).

#### **3.2.7.5 Scanning Electron Microscope (SEM)**

The surface morphology structure of the purified bioflocculant was attained with scanning electron microscope (Japanese Electron Optical Lab, JSM-6390 LV SEM). Images of bioflocculant powder, kaolin clay before and after flocculation were scanned.

#### **3.2.7.6 Elemental analysis of the bioflocculant**

For elemental analysis of the bioflocculant, energy dispersive X-ray (EDX) analysis was conducted using a JOEL (JSM-6390 LV SEM, Japan) and the elements were analyzed using Noran system six software package.

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1 Screening and identification of bioflocculant-producing bacteria

Out of the 144 bacterial strains isolated from fresh water samples, rock scrapings and sediment samples of the Tyume River in Alice, 13 were found positive for bioflocculant production as they gave a flocculating activity above 50% when tested against kaolin suspension. These included a bacterial strain named F45 which exhibited high flocculating activity against kaolin suspension. The 16S rDNA analysis of the bacterium revealed it to have 95% similarity to *Bacillus Pumilus* strain ZAP 028.

The genus *Bacillus* is ubiquitous in nature and includes a variety of species which are commercially useful (Harwood 1989). *Bacillus* is a genus of gram-positive, rod shaped bacteria and is a member of the phylum *firmicutes*. *Bacillus* species can be oxygen reliant, aerobic or anaerobic. The microorganisms of *Bacillus* genus are one of the most important sources of enzymes and other biomolecules of industrial interest, being responsible for the supply of about 50% of the market for enzymes (Schallmey et al., 2004). However, studies on *Bacillus* species have been reported for a number of applications. For example, *Bacillus subtilis* has been used as a food source in Japan (Djien & Hesseltine, 1979). Some *Bacillus* species are commonly utilized in biological control of plant diseases (Korsten & Cook 1996).

Compared with other industrially relevant *Bacilli* such as *B. subtilis* or *B. licherniformis*, *Bacillus pumilus* is characterized by a higher oxidative stress resistance (Handtke et al., 2014). This natural potential and resistances of *B. pumilus* could be a major benefit for the improvement of industrial production processes, since oxidative stress can occur in all phases of fermentation processes (Schweder & Hecker 2004; Stadtman & Levine 2003; Farr & Kogoma 1991). In another study, a novel *Bacillus pumilis* strain was used for the production of xylanase at alkaline pH and temperatures above 40°C (Handtke et al., 2014).

In bioflocculation, a number of *Bacillus* species have been reported for their potential to produce bioflocculants. These include *B. licherniformis* (Xiong et al., 2010), *B. mucilaginosus* (Lian et al., 2008; Deng et al., 2003), *B. circulans* (Li et al., 2009b), *B. subtilis* (Kaewchai & Prasertsan 2002) and *B. alvei* (Shadia et al., 2011).

### **3.3.2 Effect of carbon sources on bioflocculant production**

The effect of carbon sources on the production of bioflocculant by the bacterium was investigated. The choice of a carbon source has a profound influence on the production of bioflocculants. This may be attributed to the fact that different microorganisms prefer different carbon sources for optimum production of bioflocculants. The results of the effect of different carbon sources on bioflocculant production by *B. pumilus* strain are shown in figure 3.1. Among the various sources of carbon tested, maltose was the preferred carbon source for the production of the bioflocculant by the test bacterium with the flocculating activity of 72%, followed by glucose with a flocculating activity of 68%. Sucrose, lactose and starch supported the production of the bioflocculant with flocculating activities of 69.9%, 61.3% and 55.3% respectively. Fructose showed the least flocculating activity at 35%. Maltose was then used as a carbon source of choice for all subsequent experiments. Similar findings were reported by Wan et al. (2013) for *Solibacillus silvestris* in which maltose was the preferred carbon source for bioflocculant production exhibiting a flocculating activity of 88.7%. Similarly, Sheng et al. (2006) reported that maltose was the best carbon source for the production of bioflocculant by *Klebsiella* sp. In another study, fructose, together with lactose and glucose, were not favored for the production of bioflocculant by *Bacillus licherniformis* (Shih et al., 2001). Contrary to these findings, He et al. (2004) reported that fructose, glucose and sucrose were favorable for REA-11 production, a bioflocculant produced by *Corynebacterium glutamicum*. It is well documented in literature that many bioflocculant-producing microorganisms prefer organic carbon sources for optimum bioflocculant production whilst on the other hand, the utilization of inorganic carbon sources for bioflocculant production is still scanty in literature.

Zhang et al. (2002a) reported that glucose completely inhibited the production of bioflocculant by *Sorangium cellulosum* instead, the flocculating activity was optimum (about 96%) when starch was used as the sole carbon source.

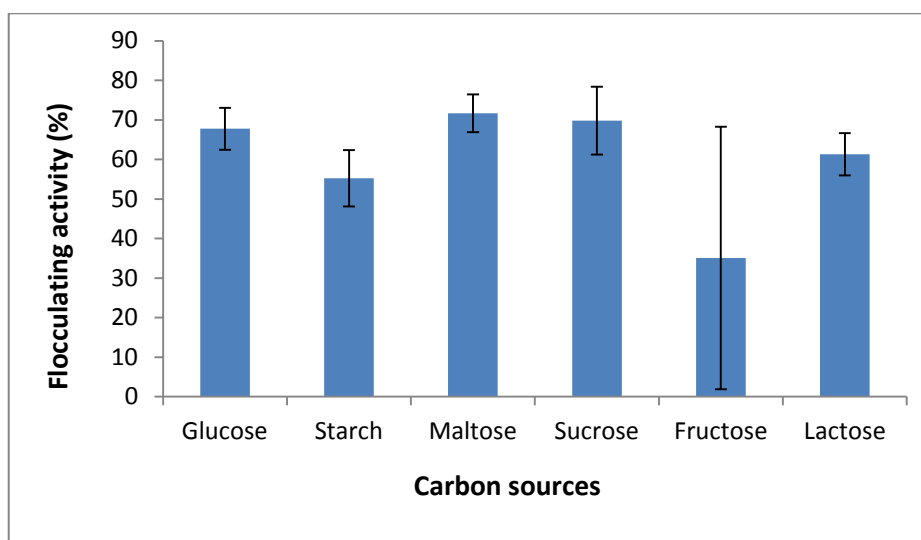


Figure 3.1 Effect of carbon sources on bioflocculant production by *Bacillus pumilus*

### 3.3.3 Effect of nitrogen sources on bioflocculant production

Nitrogen sources have been reported to have a crucial role in bioflocculant production (Cosa et al., 2011). Different microorganisms utilize either organic or inorganic nitrogen sources, or both, to produce bioflocculants (Ugbenyen et al., 2012). Consequently, the effect of organic (yeast extract, urea, peptone, tryptone), inorganic (ammonium sulphate) and multiple nitrogen sources on bioflocculant production by the test bacterium was assessed. As shown in figure 3.2, it is evident that the complex nitrogen source composed of yeast extract; urea and ammonium sulphate was the most favorable for bioflocculant production by *Bacillus pumilus* strain as opposed to the individual nitrogen sources. Urea as a sole nitrogen source also supported bioflocculant production (54%) when compared with other single nitrogen sources such as peptone, yeast extract, tryptone and ammonium sulphate. Similarly, Ugbenyen et al. (2012) also observed that *Cobetia* spp poorly utilized all the tested organic and inorganic nitrogen sources when used individually as sole nitrogen

sources, but the combination of urea, yeast extract and ammonium sulphate resulted in high flocculating activity of 92.2%. In another study by Lixi et al. (2006), the optimum medium for bioflocculant production by *Klebsiella* sp. contained three nitrogen sources (urea, ammonium sulphate, yeast extract) in which the flocculating activity reached 94.7%. Gong et al. (2008) investigated the effect of various nitrogen sources on bioflocculant production by *Serratia ficaria* and observed that a multiple nitrogen source (beef extract and urea) supported a higher flocculating activity of 94.1% when compared to sole organic or inorganic nitrogen sources. These results suggest that it is necessary to test not only for the effect of individual nitrogen sources on bioflocculant production but for the effect of combined nitrogen sources as well since some microorganisms seem to prefer a combination of nitrogen sources to optimally produce bioflocculants. As it is the case with carbon sources, nitrogen sources required for optimum bioflocculant production differ for different microorganisms. All inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, sodium nitrate) tested for bioflocculant production by *Chryseobacterium daeguense* W6 led to poor production of both the bioflocculant and cell growth, while the organic nitrogen sources were better sources for bioflocculant production for which tryptone was the most preferred exhibiting a flocculating activity of more than 90% (Liu et al., 2010). Generally, it has been reported in literature that organic nitrogen sources are more suitable for bioflocculant production and are more easily absorbed by the cells when compared to inorganic nitrogen sources (Cosa et al., 2013a).

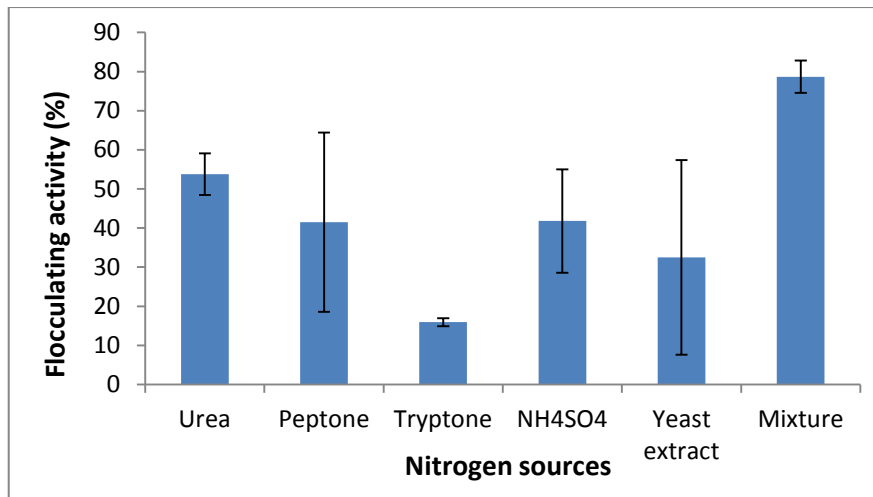


Figure 3. 2 Effect of various nitrogen sources on bioflocculant production by *Bacillus pumilus*

### 3.3.4 Effect of inoculum size on bioflocculant production

Inoculum size is one of the factors that have been reported to affect the production of bioflocculant by microorganisms (Zhang et al., 2007). The effect of inoculum size on bioflocculant production was investigated using different inoculum sizes ranging from 0.5 ml to 2.5 ml. Specifically, inoculum sizes of 0.5 ml, 1 ml, 1.5 ml, 2 ml and 2.5 ml were used. As the results are depicted in figure 3.3, flocculating activity steadily increased with increasing inoculum size with an optimum activity attained at 4% (v/v) inoculum size beyond which a steady decline in activity was observed. Consequently, an inoculum size of 4% (v/v) was used for all subsequent experiments. A similar phenomenon was reported by Xiong et al. (2010) where it was observed that an inoculum size of 4% (v/v) was optimum for bioflocculant production by *Bacillus licherniformis*. These results are in agreement with the phenomenon observed by Salehizadeh & Shojaosadati (2001) that a small inoculum prolonged the lag phase of the strain, whereas a large inoculum made the niche of *Bacillus pumilus* strain overlap excessively and restrained bioflocculant production. Previous studies have reported optimum bioflocculant production by different microorganisms at varying inoculum sizes ranging from 1-5% (v/v) (Wang et al., 2009; Luo et al., 2014, Cosa et al., 2013b). The following

inoculum sizes were reported to be optimum for bioflocculant production by various microorganisms: an inoculum size of 1% (v/v) for bioflocculant production by *Klebsiella pneumoniae* YZ-6 (Luo et al., 2014), 2% (v/v) for bioflocculant production by *Oceanobacillus* sp. Pinky (Cosa et al., 2013b), 3% (v/v) for production of bioflocculant by *Bacillus* sp. Gilbert (Ugbenyen et al., 2014), and 5% (v/v) for bioflocculant production by *Pseudomonas Alcaligenes* (Wang et al., 2009).

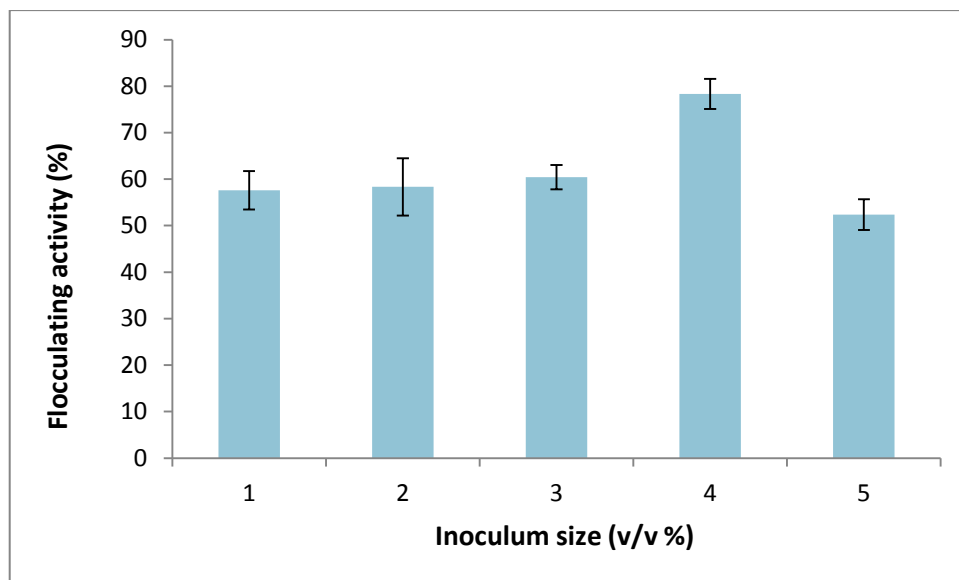


Figure 3. 3 Effect of inoculum size on bioflocculant production by the bacteria

### 3.3.5 Effect of the initial pH of the medium

The initial pH of the culture medium does not only affect the electric charge of the cells and the oxidation–reduction potential but also affects nutrient absorption and enzymatic reaction of bioflocculant producers (Salehizadeh & Shojaosadati 2001; Xia et al., 2008). Hence the effect of initial pH of the culture medium on bioflocculant production was examined at pH values ranging from 3-11 and the results are presented in figure 3.4. As can be seen from the figure, the bioflocculant was significantly produced at pH 7.0-9.0 with the optimum flocculating activity

(80.6%) being observed at pH 7. At both acidic pH (3-6) and high alkaline pH (10-11) ranges, flocculating activity was significantly reduced with no flocculating activity being observed at pH 11. Consequently, the initial pH of the culture medium for all subsequent experiments was adjusted to 7. He et al. (2010) stated that at low pH (acidic) both the bioflocculant and the kaolin particles are likely to absorb the hydrogen ions ( $H^+$ ) thus consequently weakening the complex formation between the bioflocculant and kaolin particles mediated by  $Ca^{2+}$ . Similarly, at high alkaline pH the hydrogen ions ( $OH^-$ ) may interfere with the combination of flocculant molecules and kaolin particles mediated by  $Ca^{2+}$  resulting in lower flocculating activity. According to Li et al. (2008), the mediating effect of  $Ca^{2+}$  appeared to be the strongest at neutral pH values. Mabinya et al. (2011) and Aljuboori et al. (2013) observed similar optimal pH values (pH 7) for bioflocculants produced by *Halomonas* sp. OKOH and *Aspergillus flavus*, respectively. A bioflocculant named MBF-6 produced by *Klebsiella pneumonia* also reached its highest flocculating activity at pH 7 (Luo et al., 2014). However, the initial pH of the medium required for optimum bioflocculant production differs with different organisms. The optimum initial pH for the production of bioflocculants by *Bacillus* xn12 and *Streptomyces* xn17 strains was in the range 3-10 and 3-9 respectively, with the highest flocculating activity (97%) being observed at pH 5 for both strains (Zhang et al., 2013). In the case of *Solibacillus silvestris* W01, the bioflocculant was produced at pH between 7 and 9, and the optimum flocculation efficiency was attained at pH 8 (Wan et al., 2013). For bioflocculant production by *Arthrobacter* sp. B4, Li et al. (2014) observed that the highest flocculation was when the pH was more than 12.0, accompanied by the formation of white particles and a decrease at pH 6.0.

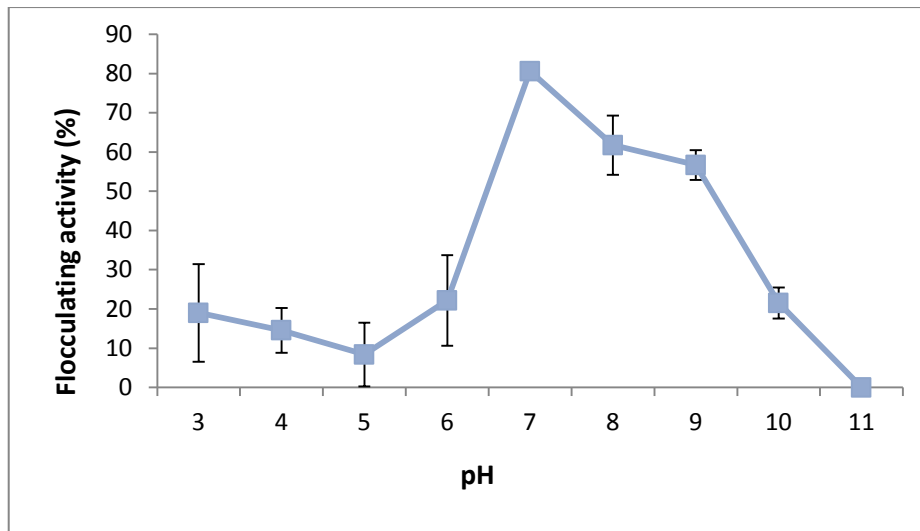


Figure 3. 4 Effect of initial pH on bioflocculant production

### 3.3.6 Effect of cations on the flocculating activity of the crude bioflocculant

The effect of various cations on the activity of the crude bioflocculant was investigated and the results are depicted in figure 3.5. The stimulating effect of the added cations on flocculation is not only dependent on the concentration of the cations added but is also highly dependent on the valence ions (Zulkeflee et al., 2012), hence the effect of cations on the flocculating activity was investigated using cations with different valence ions (i.e. monovalent, divalent and trivalent). All the divalent cations tested stimulated the flocculating activity of the bioflocculant to varying degrees as follows:  $\text{Ca}^{2+}$  (67.9%),  $\text{Mg}^{2+}$  (74.5%) and  $\text{Mn}^{2+}$  (82.9%) (Fig. 3.5). Among these cations,  $\text{Mn}^{2+}$  gave the highest flocculating activity and was therefore used as a replacement for  $\text{Ca}^{2+}$  in all subsequent experiments. It was noticed that  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Fe}^{3+}$  completely inhibited the flocculating activity of the bioflocculant while  $\text{Al}^{3+}$  had a stimulating effect with a flocculating activity of 77.2%. Ugbenyen et al. (2012) also reported that  $\text{Mn}^{2+}$  was the best metal ion for the activity of the bioflocculant produced by *Cobetia* spp. The flocculating activity of the bioflocculant produced by the consortium of *Cobetia* sp. and *Bacillus* sp. was enhanced by all of the divalent metal ions tested ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ) but it was completely inhibited by  $\text{Li}^+$  and  $\text{K}^+$  (Ugbenyen & Okoh 2014). This observation is

supported by the phenomenon proposed by Wu & Ye (2007) which stated that monovalent cations produce bonds that are loose in structure and therefore result in decrease in floc density and size which in turn result in a decrease in flocculating activity compared to divalent cations. The flocculating activity of BFB4 bioflocculant produced by *Arthrobacter* sp. B4 was  $\text{Ca}^{2+}$ -dependent and unaffected by other metal ions tested, no flocculation occurred in the absence of  $\text{Ca}^{2+}$  ions (Li et al., 2014). On the other hand, Liu et al. (2010) reported a cation-independent bioflocculant MBF-W6 produced by *Chryseobacterium daeguense* since it was observed that the addition of any cations could not evidently enhance its flocculating rate. The function of cations is to stimulate the flocculating activity by neutralizing and destabilizing the residual negative charge of functional groups and to form bridges between particles (Yim et al., 2007). However, metal ions can also inhibit flocculating activity or have no effect at all on the flocculating activity of bioflocculants (Li et al., 2014; Ugbenyen & Okoh 2014;)

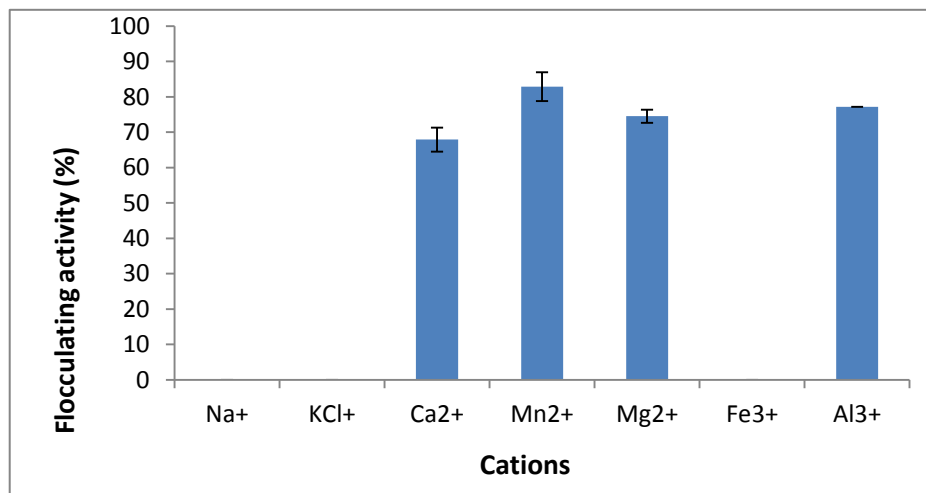


Figure 3. 5 Effect of cations on the flocculating activity of the bioflocculant

### 3.3.7 Time course for bioflocculant production

The time course profile of bioflocculant production by *Bacillus pumilus* strain was investigated over a growth period of 120 hours and the results are depicted in figure 3.6. There was a sharp increase in flocculating activity between 0 and 24 hours of fermentation after which a steady but low decline was noticeable up to 96 hours, beyond which a very sharp drop in flocculating activity was observed. Maximum flocculating activity of 89.7% was attained at early stationary phase (24 h) (Fig. 3.6). The decline in flocculating activity observed at 120 hours could be associated with the presence of bioflocculant-degrading enzymes produced by the microorganism (Li et al., 2009a). It was also observed that the bacterial growth, as represented by CFU/ml paralleled the flocculating activity and also reached its peak ( $1.80 \times 10^{13}$ ) at early stationary phase of 24 hours. There was a decrease in bacterial cell count at 72 hours which coincided with a gradual decrease in flocculating activity (Fig 3.6). These results suggest that the bioflocculant was produced by biosynthesis during its growth and not by cell autolysis (Gong et al., 2008). In order to allow for test organism's potential flocculant yield capability with a possible resultant high flocculating activity, culturing period was prolonged for 24 hours. The short time recorded for optimum bioflocculant production by *Bacillus Pumilus* strain suggest that bulk production of the bioflocculant may be achieved in a short period of time which may substantially reduce production costs in an industrial scale process. Similarly, *Bacillus mojavensis* reached its maximum flocculating efficiency in the early stationary phase which was also recorded at 24 hours, while the cell production reached its maximum in the stationary phase (at 72 hours) indicating that the bioflocculant was produced as a result of biosynthesis (Elkady et al., 2011). Similarly, 24 hour culturing time was chosen in order to produce bioflocculant with high flocculating efficiency (Elkady et al., 2011). The optical density curve which may also be representative of the cell growth also showed a similar trend to that of flocculating activity curve until 96 hours of fermentation where it started to increase. The increase in optical density after this

period, while flocculating activity decreased, may be related to the turbidity of the bioflocculant produced by *Bacillus pumilus* strain mainly as a result of dead bacterial cells (Elkady et al., 2011).

The initial pH of the medium is reported to be a very important factor in a bioflocculation process. The pre-determined optimum initial pH of the medium was pH 7 (refer to section 3.3.5). The pH of the production medium determines the electric charge of the cells and oxidation-reduction potential thus affecting nutrient absorption and enzymatic reactions (Salehizadeh & Shojaosadati 2001). In contrast to the profile of flocculating activity, the pH profile showed that the pH decreased from 7.0 to 5.39 within 24 hours, followed by a slow decline until it reached a pH of 4.57 at 120 hours of incubation (Fig.3.6). The decline in pH may be indicative of the production of organic acids during metabolism by the microorganism (Deng et al., 2003; Lu et al., 2005) or may be attributed to the acidic nature of the bioflocculant produced by *Bacillus pumilus* strain (Batta et al., 2013).

Time course profiles for production of bioflocculants differ with microorganism. Bioflocculant produced by *Bacillus licherniformis* X14 reached its maximum flocculating activity at 48 hours while the cell production reached a maximum at 20 hours (Li et al., 2009a). For example, Gao et al. (2006) reported that flocculating activity of the bioflocculant produced by *Vagococcus* sp. W31 reached its maximum (90%) at 60 hours. For *Bacillus* sp. F19, the produced bioflocculant reached its maximum flocculating activity at early stationary phase of 72 hours (Zheng et al., 2008). In the case of a bioflocculant produced by *Bacillus* sp. Gilbert, the flocculating activity increased rapidly with incubation time and reached its peak activity of 72.4% after 96 hours of fermentation, suggesting that the bioflocculant was produced by biosynthesis (Piyo et al., 2011). Growth profile of *Achromobacter* sp. TL-3 with respect to bioflocculant activity revealed that maximal activity (95%) was observed in the late stationary phase (120 hours) (Batta et al., 2013). In the majority of studies reported in literature, it has been well documented that the production of bioflocculants was mainly parallel to cell growth and reached its maximum flocculating activity in early stationary phase,

indicating that most bioflocculants are produced by biosynthesis via nutrient assimilation during their growth, not by cell autolysis (Lu et al., 2005; Vatansever 2005; Aljuboori et al., 2008; Cosa 2010). However, Li et al. (2007) reported that the flocculating activity of a bioflocculant from *Aeromonas* sp. increased rapidly after 36 hours of cultivation while the growth ceased and reached its maximum after 72 hours of cultivation indicating that the production of the bioflocculant was not associated with cell growth, which was produced only during the stationary phase. Also, in the case of *Chryseobacterium daeguense* W6, the cell growth increased sharply in the first 8 hours of fermentation and the flocculating rate during this period was very low. When the cell growth entered the death phase, the flocculating rate increased sharply and reached over 90% indicating that the bioflocculant produced was an intracellular bioflocculant (Liu et al., 2010).

- Bacterial count (cfu/ml)
- pH
- .. - - - OD at 660nm
- ..... Flocculating activity (%)

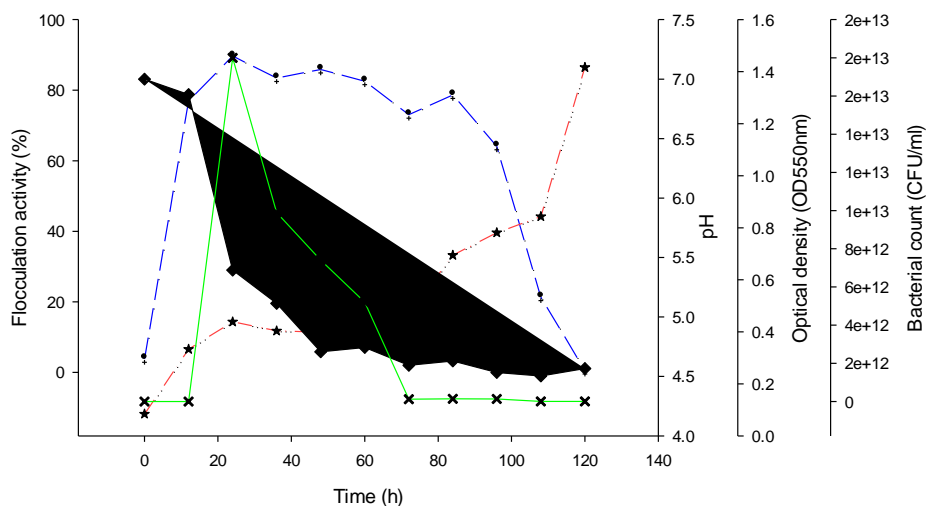


Figure 3. 6 Time course profile for bioflocculant production by *Bacillus pumilus*

### 3.3.8 Bioflocculant yield

The bioflocculant was extracted and purified as outlined in section 3.2.5. A bioflocculant yield of about 0.289 g was recovered from 1L of fermented broth. The freeze-dried purified bioflocculant was a white powder which was completely soluble in water. The bioflocculant yield obtained in this study was much lower than the reported bioflocculant yields in literature (Zhang et al., 2002b; Lu et al., 200; Aljuboori et al., 2013) However, Cosa et al. (2011) reported that 0.264 g/l of purified bioflocculant was recovered from a fermented culture of *Virgibacillus* sp. Rob. The product yield of crude bioflocculant produced by *Aeromonas* sp. was 2.25 g/l (Li et al., 2007). In another study, a bioflocculant yield of about 14.8 g/l was reported. This bioflocculant was produced by a on a *Bacillus mojavensis* strain 32A capable of producing 5.2 g/l of purified biopolymer.

### 3.3.9 Bioflocculant dosage

The relationship between bioflocculant dosage and flocculating activity of the purified bioflocculant was investigated (Figure 3.7). The main objective of the bioflocculant dosage experiment was to determine the lowest amount of bioflocculant to be used while achieving the highest flocculating activity. As depicted in figure 3.7, the flocculating activity was above 90% within a dosage range of 0.1-0.5 mg/ml, and the maximum flocculating activity (96.5%) was achieved at an optimum bioflocculant dosage of 0.1 mg/ml. The flocculating activity was linear between the range 0.2-0.5 mg/ml, showing that there was no significant difference in the flocculating activity as the bioflocculant dosage increased. The results showed that the bioflocculant produced by *Bacillus pumilus* strain exhibited high flocculating activity at very low dosage which could be cost containing in industrial applications. A lower dosage of bioflocculants with a resultant high flocculating activity will contribute towards cost effectiveness (Zulkeflee et al., 2012). Bioflocculant dosage required for optimum flocculation differs with various bioflocculants produced from different microorganisms. Inadequate

biofloculant dosage may decrease the stimulation of the bridging mechanism and result in low flocculating activity (Gong et al., 2008). In the case where the biofloculant molecules are excessively present in the solution, they generate high viscosity; block the adsorption sites thereby reducing flocculation process and the formation of flocs (Zulkeflee et al., 2012; Wang et al., 2011). Liu et al. (2009) reported a maximum flocculating activity at an optimum biofloculant dosage of 6.0 mg/l for a biofloculant extracted from sludge with higher or lower dosage inducing lower flocculating activity. The biofloculant produced by *Paenibacillus mucilaginosus* achieved the highest flocculating activity at a dosage of 2.0 mg/l, similarly, higher or lower dosages reduced flocculating activity (Tang et al., 2014). Wang et al. (2011) observed that 12 mg/l was the optimum dosage required for maximum flocculation by biofloculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6.

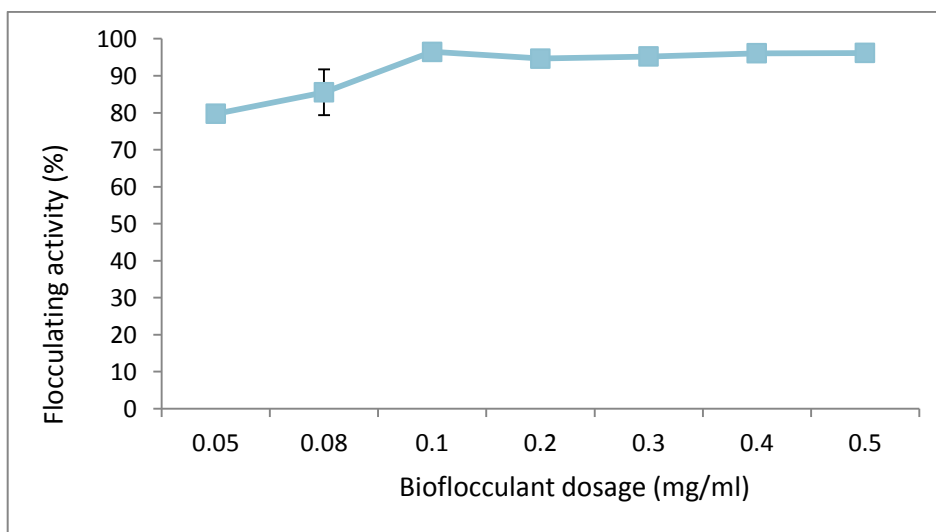


Figure 3. 7 Effect of biofloculant dosage on the flocculating activity of purified biofloculant

### 3.3.10 Thermal stability test of the purified biofloculant

Figure 3.8 shows the effect of temperature on the flocculating activity of the purified biofloculant. From the results, it is clear that there was no significant decrease in flocculating

activity of the heated bioflocculant and the unheated sample, suggesting that the bioflocculant was not affected by heat. The purified bioflocculant could maintain over 90% of its activity with only 4.8% decrease in flocculating activity after being heated at 100°C for 1 hour thus exhibiting its thermostability characteristic (Fig. 3.8). Salehizadeh & Shojaosadati (2001) reported that flocculants with protein or peptide backbone in their structure are generally sensitive to heat but those made of sugars are thermo-stable, thus it can be deduced that the thermal stability exhibited by the bioflocculant produced by *Bacillus pumilus* strain may be due to the presence of a polysaccharide backbone in its structure. A bioflocculant produced by *Aeromonas* sp. maintained its flocculating activity with only 9.2% decrease in activity after being heated at 100°C for 60 min suggesting that it was thermo-stable (Li et al., 2007). Wang et al. (2011) reported that the residual flocculating activity of the compound bioflocculant from *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6 was more than 90% after being subjected to heating at 100°C for 30 minutes. The results show that the bioflocculant produced by *Bacillus pumilus* strain has high thermal stability compared to the other bioflocculants reported in the literature (Kurane et al., 1986; Salehizadeh & Shojaosadati 2002)

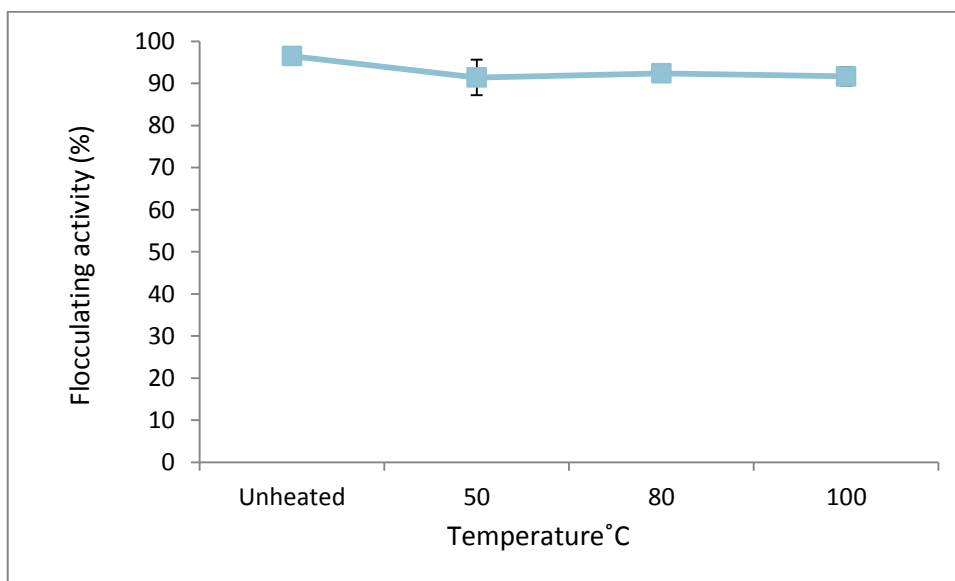


Figure 3. 8 Thermal stability of the purified bioflocculant

### 3.3.11 Effect of pH on the flocculating activity of the purified bioflocculant

The effect of heat on the flocculating activity of the purified bioflocculant was examined. Figure 3.9 depicts the results of the pH stability test of the purified bioflocculant. The results show that the bioflocculant was active over a wide range of pH from 3-11 with a flocculating activity of more than 80% being observed at all pH ranges. The maximum flocculating activity of 95.2% was observed at pH 6. These results suggest that the produced bioflocculant could be applied in various industries to treat various wastewaters without having to adjust the pH of the water. The results obtained in this study were consistent with the results obtained by He et al. (2010), where they observed that the bioflocculant produced by *Halomonas* sp. V3'a had a wide pH range from 3-11 with a flocculating activity of more than 80%. The effect of pH on flocculating activity of the produced bioflocculant was similar to a bioflocculant NU-2 produced by marine *myxobacterium* which had a wide pH range of 2-13 (Zhang et al., 2002b).

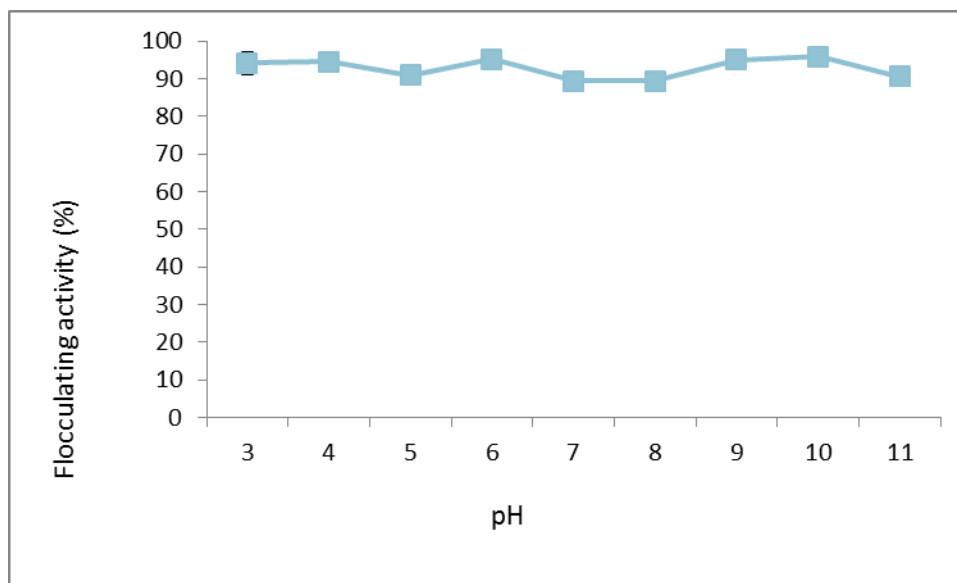


Figure 3. 9 Effect of pH on the flocculating activity of the purified bioflocculant produced by *Bacillus pumilus* strain

### 3.3.12 Effect of cations on the flocculating activity of the purified bioflocculant

The effect of cations on the flocculating activity of the purified bioflocculant was investigated and the results are depicted in figure 3.10. It was found that all the cations tested stimulated the flocculating activity of the bioflocculant, albeit to varying degrees except for  $\text{Fe}^{3+}$  which only showed a flocculating activity of 21%. The flocculating activities observed with all the other cations were above 70% (Fig. 3.10). Specifically, the following flocculating activities in the presence of different cations were observed:  $\text{K}^+$  (91.8%),  $\text{Na}^+$  (90.9%),  $\text{Mg}^{2+}$  (85.9%),  $\text{Ca}^{2+}$  (94.1%),  $\text{Mn}^{2+}$  (96.5%),  $\text{Fe}^{3+}$  (73.5%). Compared with the results reported for crude bioflocculant previously,  $\text{Mn}^{2+}$  gave the highest flocculating activity for the purified bioflocculant when compared with all the other cations tested (Fig. 3.10).  $\text{Mn}^{2+}$  could compress the double layer of the negatively charged kaolin particles thereby weakening the electrostatic repulsive force, and stimulating flocculation (Zheng et al., 2008; Li et al., 2008, Li et al., 2009a). The flocculating activity of the bioflocculant MBF-3 produced by *Bacillus* sp. was stimulated by  $\text{Al}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ , and  $\text{Na}^+$  with  $\text{Al}^{3+}$  being the most effective cation (Feng & Xu 2008).

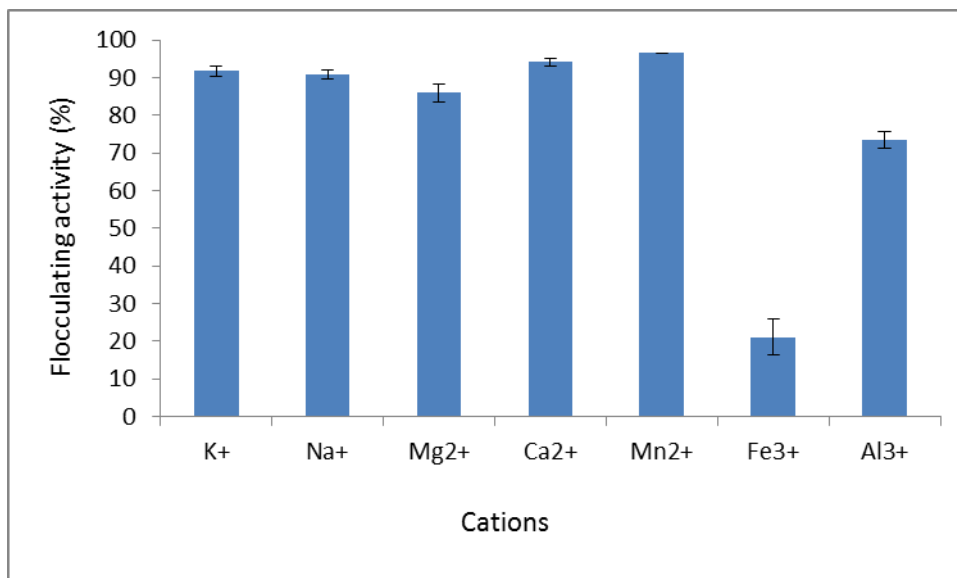


Figure 3. 10 Effect of cations on the flocculating activity of the purified bioflocculant

### 3.3.13. Composition of the bioflocculant

Biochemical analysis of the purified bioflocculant confirmed the presence of carbohydrates at 75.4% as a predominant component of the bioflocculant produced by the bacterium, with uronic acid (15.2%) and protein (5.3%) constituting the other significant proportion (Table 3.1). This finding collaborated the results obtained from chemical analyses of bioflocculants produced by other microorganisms which were found to be mainly polysaccharide in nature. In their investigation of the chemical nature of the polymer flocculant produced by *Rhodococcus erythropolis*, Peng et al. (2014) reported it to be predominantly of a carbohydrate nature. Bioflocculants produced by a halophilic *Halomonas* sp. V3a (He et al., 2010) and *Serratia ficaria* (Gong et al., 2008) were also found to be polysaccharides. The low charge density as well as the size of polymers such as polysaccharides, have been reported to enhance their flocculating capabilities particularly in suspensions with high ionic strengths hence, flocculation is often regarded as a function of molecular weight (He et al., 2010). On the contrary, a bioflocculant produced by *Solibacillus silvestris* was found to be a proteoglycan (Wan et al., 2013).

**Table 3.1: Composition of purified bioflocculant**

Component	Percentage (%)
Total sugar	75.4
Protein	5.3
Uronic acid	15.6

### 3.3.14 Functional group analysis of the purified bioflocculant

FT-IR spectroscopy of the purified bioflocculant was performed to investigate the relationship between functional groups and the flocculating activity of the purified bioflocculant, and the results are depicted in figure 3.11. The infrared spectrum of the bioflocculant displayed a broad stretching peak at around  $3429\text{ cm}^{-1}$  which is characteristic of hydroxyl group and a weak C-H stretching band at  $2928\text{ cm}^{-1}$  (Liu et al., 2009). Two characteristic absorptions of carboxyl groups were indicated by peaks at  $1647\text{ cm}^{-1}$  (C=O asymmetric stretching vibrations) and  $1416\text{ cm}^{-1}$  (C=O symmetric stretching vibrations) (Tang et al., 2014). The symmetric stretching vibration is also indicative of the presence of uronic acid in the bioflocculant molecule (Liu et al., 2010). The absorption peaks displayed in the range from  $1000\text{-}12000\text{ cm}^{-1}$  were known to be characteristics of all sugar derivatives (Gong et al., 2008). The FTIR spectrum of the produced bioflocculant was consistent with the results of other bioflocculants produced by different microorganisms (Xiong et al., 2010; Liu et al., 2010; Zhao et al., 2013; Tang et al, 2014). Based on the above results that the bioflocculant contains hydroxyl and carboxyl groups, it was deduced that the main component of the bioflocculant is a polysaccharide (Zhao et al., 2013).

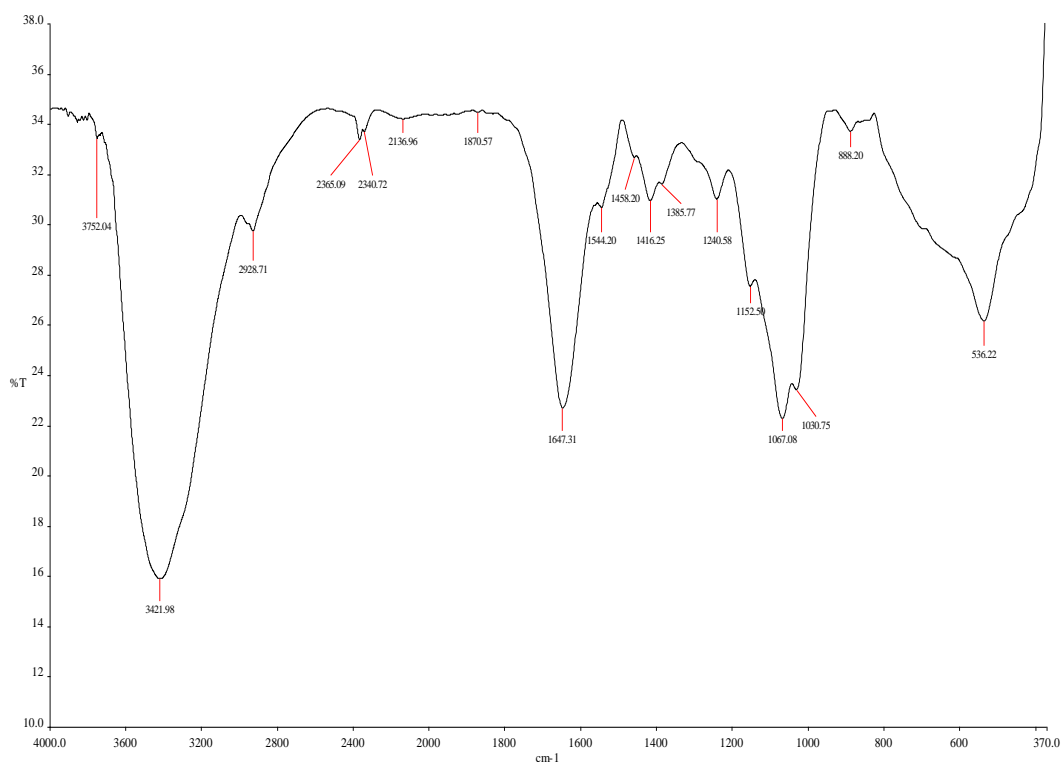


Figure 3. 11 FTIR spectra of the purified bioflocculant produced by *Bacillus pumilus* strain

### 3.3.15 SEM images

SEM observations of the bioflocculant and kaolin clay before and after forming a complex (flocculation) with the produced bioflocculant were performed to elucidate the surface morphology of the bioflocculant and its flocculation of kaolin clay. Figure 3.12 (a) shows the SEM image of the bioflocculant, Figure 3.12 (b) shows the kaolin clay before flocculation and figure 3.12 (c) shows the flocs from precipitated kaolin clay after the addition of the bioflocculant. The SEM image of the bioflocculant molecule revealed it to be dendritic in structure. Comparison of figure 3.12 (b) and (c) illustrates that the bioflocculant molecule successfully connected the kaolin particles to form flocs, which settled down and separated from the suspension. These results reveal that the bioflocculant showed excellent flocculating properties of kaolin clay. It can also be deduced that bridging of the bioflocculant molecule played a significant role in the flocculation process (He et al., 2010).

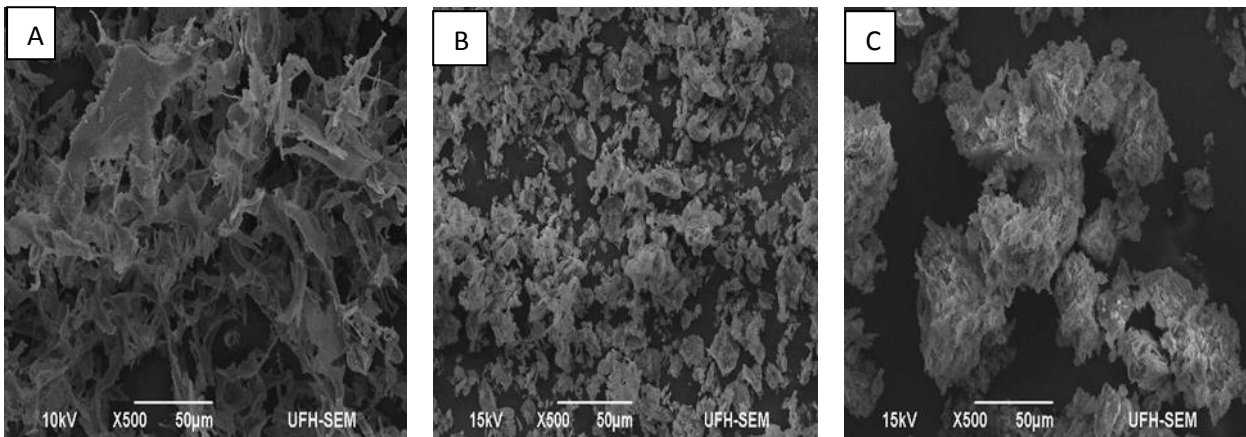


Figure 3. 12 SEM images of biofloculant (A), kaolin clay before flocculation (B), kaolin clay flocculated by the biofloculant.

### 3.3.16 Elemental analysis of the purified biofloculant

Elemental analysis of the purified biofloculant was performed and the results are shown in table 3.2 below. The results show that oxygen was present in high percentage (41.60%) compared to other elements followed by carbon (22.71%). Silicon was present in small proportion (0.50%) compared to other elements present in the biofloculant molecule. The results obtained were consistent with the results of the biofloculant produced by *Chryseobacterium daeguense* W6 (Liu et al., 2010).

**Table 3.2: Elemental analysis of the purified biofloculant**

Element	Percentage (%)
C	22.71
N	11.56
O	41.60
Mg	4.56
Al	0.60
Si	0.50

P	7.98
S	0.51
K	9.98

### 3.4 CONCLUSION

This study has shown *Bacillus pumilus* strain to hold promise as an important source of bioflocculant(s). To the best of my knowledge, there are no reports on bioflocculant production by *Bacillus Pumilus* strain. The bacteria produced a thermostable polysaccharide optimally when maltose and multiple nitrogen sources were used as sources of carbon and nitrogen respectively. After purification, 0.289 g of the bioflocculant was recovered from 1L of fermented broth. The optimum dosage for effective flocculation by the bioflocculant was 0.1 mg/ml and the produced bioflocculant had a wide pH range from 3.0-11 with flocculating activity of more than 80%. The results obtained in this study suggest that the bioflocculant produced could be utilized as an alternative for harmful chemical flocculants.

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# CHAPTER

# 4

## 4.1 GENERAL DISCUSSION AND CONCLUSION

In recent years, biofloculants have attracted much wider attention as biodegradable and nontoxic substituents for conventional chemical flocculants (Salehizadeh & Yan 2014). This is attributed to the research findings that chemical flocculants have shown detrimental effects to humans and the environment. For an example, aluminium salts have been reported to be associated with Alzheimer's disease (Arezoo 2002) and also that polyacrylamides containing residual acrylamide monomers have been proved to be neurotoxic and carcinogenic to humans (Shih et al., 2001).

Biofloculant-producing microorganisms have been isolated from different environments, mostly from soil, activated sludge and marine environments (Cosa et al., 2013b; Jiang et al., 2013; Xia et al., 2008). Feng & Xu (2008) reported on a biofloculant MBF3 produced by *Bacillus* sp. BF3-3 isolated from activated sludge which showed excellent flocculating activity on real and synthetic wastewaters. Also, *Pseudomonas aeruginosa* was reported to produce a biofloculant which was effective for heavy metals removal (Gomma 2012). Although a number of biofloculant-producing microorganisms have been reported, high production cost and low yields have remained the major limiting factors for large scale industrial applications of biofloculants (Yang et al., 2009; Li et al., 2003; He et al., 2004).

To overcome these challenges, researchers have opted for the use of cost-effective substrates such as sludge and livestock wastewater as cheap culture medium (Pei et al., 2013; Peng et al., 2014). To overcome the challenge of low yields, many researchers are investigating the effect of utilizing two or more microbes in combination for biofloculant production (Zhang et al., 2007; Okaiyeto et al., 2013). Other techniques which have been employed by researchers to try and improve yield include optimization of fermentation conditions, using statistical models with

high efficiency towards yield optimization such as factorial experiment and surface response design (SRD) (Nwodo et al., 2014; Wang et al., 2007).

In this study, we attained a total of 144 isolates from water samples, rock scrapings and sediment samples in which 13 isolates were found to be bioflocculant producers. Notable among these isolates was a bacterial strain named F45 which was obtained from rock scrapings and exhibited a flocculating activity above 60% for kaolin suspension during initial screening. Optimal bioflocculant production conditions for F45 included maltose as a carbon source, multiple nitrogen source, initial pH 7 and inoculum size of 4% (v/v). The produced bioflocculant showed excellent flocculating activity (above 80%) for kaolin suspension at an optimum dosage of 0.1 mg/ml. The bioflocculant produced by *Bacillus pumilus* strain also proved to be thermostable and had a wide pH range from 3-11.

In conclusion, the results obtained in this study suggest that the produced bioflocculant has the potential to be utilized in various industries as an alternative to harmful synthetic flocculants.

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# APPENDIX

## APPENDIX A



Image 1: *Bacillus pumilus* strain on M1 agar

## APPENDIX B



Image 2: *Bacillus pumilus* strain on nutrient agar

## APPENDIX C

Table 1: Effect of carbon sources on biofloculant production

Absorbance @ 550nm

<b>Carbon source</b>	<b>Flask 1</b>	<b>Flask 2</b>	<b>Flask 3</b>
Glucose	0.648	0.573	0.464
Maltose	0.426	0.467	0.587
Fructose	0.870	1.794	0.730
Starch	0.637	0.837	0.864
Sucrose	0.511	0.384	0.682
Lactose	0.567	0.730	0.725

Control= 1.742

Table 2: Effect of nitrogen sources on biofloculant production

Absorbance @ 550nm

<b>Nitrogen Sources</b>	<b>Flask 1</b>	<b>Flask 2</b>	<b>Flask 3</b>
Urea	0.699	0.853	0.865
Peptone	0.560	1.284	1.214
Tryptone	1.447	1.463	1.483
Ammonium Sulphate	1.274	0.930	0.837
Yeast extract	0.689	1.320	1.520
Mixture	0.452	0.312	0.350

Control= 1.742

Table 3: Effect of initial pH of the medium on biofloculant production

Absorbance @ 550nm

<b>pH</b>	<b>Flask 1</b>	<b>Flask 2</b>	<b>Flask 3</b>
3	1.352	1.652	1.230
4	1.466	1.403	1.597
5	1.433	1.687	1.667
6	1.496	1.446	1.125
7	0.337	0.313	0.363
8	0.642	0.548	0.808
9	0.808	0.681	0.774
10	1.428	1.292	1.381
11	1.787	1.892	1.677

Control= 1.742

Table 4: Effect of inoculum size on bioflocculant production

Absorbance @ 550nm

Inoculum size (%)	Flask 1	Flask 2	Flask 3
1	0.657	0.768	0.791
2	0.612	0.740	0.825
3	0.722	0.657	0.655
4	0.467	0.352	0.313
5	0.789	0.870	0.832

Control=1.742

Table 5: Effect of cations on the flocculating activity

Absorbance @ 550nm

Cations	Flask 1	Flask 2	Flask 3
Na <sup>+</sup>	2.012	2.150	2.176
K <sup>+</sup>	2.186	2.166	2.224
Ca <sup>2+</sup>	0.594	0.735	0.679
Mn <sup>2+</sup>	0.288	0.451	0.332
Mg <sup>2+</sup>	0.539	0.489	0.567
Fe <sup>3+</sup>	1.214	1.077	1.093
Al <sup>3+</sup>	0.573	0.515	0.339

Control=2.086

Table 7: Time course assay of bioflocculant production

Time (h)	Absorbance @ 550nm			OD @ 600 nm			pH			CFU/ml		
	0	1.349	1.499	1.426	0.071	0.076	0.105	7.0	7.0	7.0	262	273
12	0.327	0.394	0.303	0.322	0.342	0.335	6.9	6.9	6.9	95	68	72
24	0.182	0.174	0.103	0.445	0.443	0.427	5.4	5.4	5.4	205	71	265
36	0.356	0.297	0.245	0.418	0.399	0.393	5.0	5.2	5.2	75	196	27
48	0.200	0.231	0.196	0.424	0.382	0.388	4.6	4.8	4.7	54	67	102
60	0.324	0.227	0.224	0.425	0.399	0.409	4.7	4.8	4.8	68	42	48

72	0.350	0.390	0.457	0.557	0.423	0.428	4.6	4.6	4.6	123	136	105
84	0.386	0.312	0.250	1.261	0.407	0.415	4.7	4.6	4.6	163	155	115
96	0.508	0.285	0.802	10518	0.416	0.411	4.6	4.5	4.5	143	141	116
108	1.510	0.901	1.086	1.662	0.459	0.409	4.6	4.5	4.5	41	53	43
120	1.621	1.510	1.291	1.809	1.295	1.145	4.6	4.6	4.6	31	37	35

Control= 1.482

Table 8: Effect of bioflocculant dosage

Absorbance @ 550nm

Concentration (mg/ml)	Flask 1	Flask 2	Flask 3
0.05	0.259	0.274	0.298
0.08	0.101	0.233	0.258
0.1	0.033	0.056	0.055
0.2	0.070	0.074	0.075
0.3	0.061	0.052	0.082
0.4	0.066	0.052	0.038
0.5	0.072	0.035	0.050

Control=1.365

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

Absorbance @ 550nm

Cations	Flask 1	Flask 2	Flask 3
K <sup>+</sup>	0.151	0.114	0.159
Na <sup>+</sup>	0.143	0.172	0.613
Mg <sup>2+</sup>	0.284	0.246	0.200
Ca <sup>2+</sup>	0.122	0.101	0.083
Mn <sup>2+</sup>	0.060	0.061	0.063
Fe <sup>3+</sup>	1.365	1.285	1.449
Al <sup>3+</sup>	0.423	0.496	0.458

Control=1.365

Table 10: Thermal stability of the purified bioflocculant

Absorbance @ 550nm

Temperature (°C)	Flask 1	Flask 2	Flask 3
50	0.182	0.070	0.100
80	0.090	0.122	0.100
100	0.111	0.085	0.014

Control=1.365

Table 11: Effect of pH of the flocculating activity of the purified bioflocculant

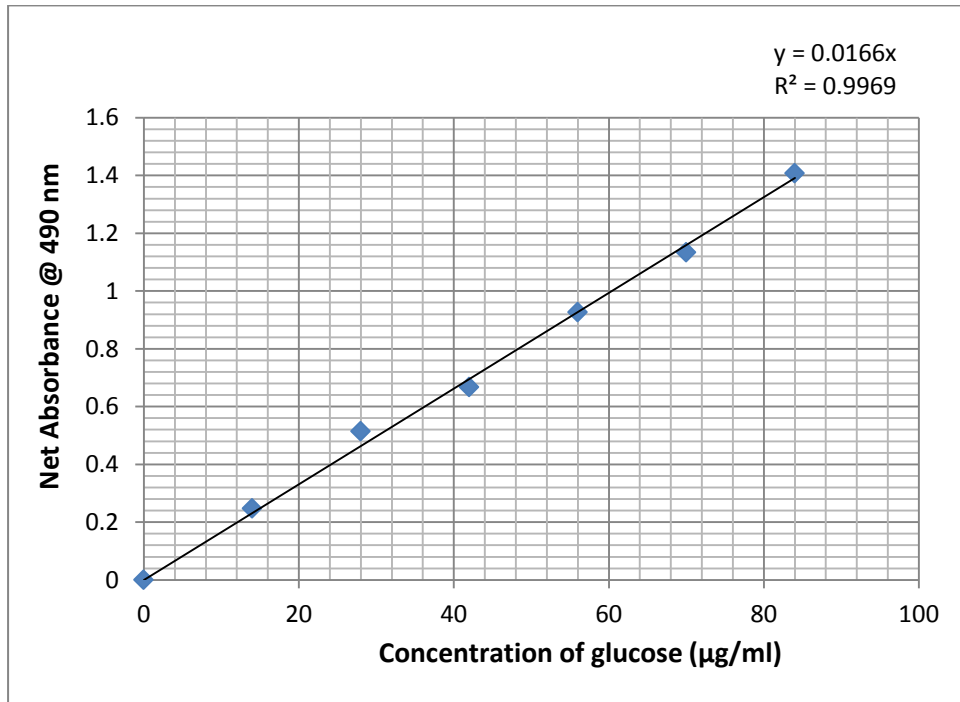
Absorbance @ 550nm

pH	Flask 1	Flask 2	Flask 3
3	0.036	0.044	0.092
4	0.057	0.054	0.050
5	0.071	0.103	0.095
6	0.042	0.044	0.057
7	0.105	0.104	0.108
8	0.080	0.126	0.106
9	0.042	0.041	0.062
10	0.040	0.044	0.037
11	0.091	0.077	0.108

Control= 0.986

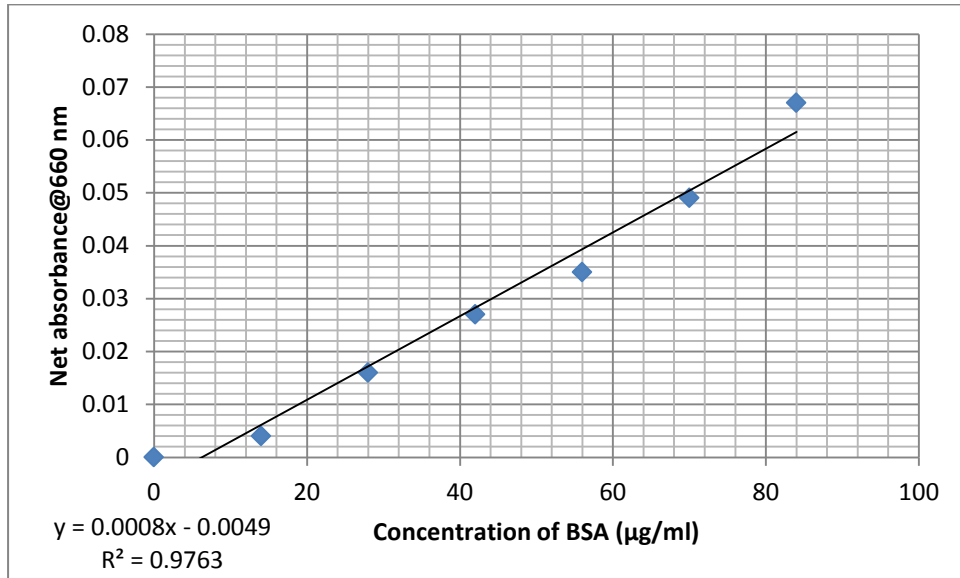
## APPENDIX D

### Standard curve for glucose



## APPENDIX E

### Standard curve for protein estimation



## APPENDIX F

### Standard curve for uronic acid determination

