PRODUCTION AND CHARACTERIZATION OF A BIOFLOCCULANT FROM A CONSORTIUM OF BACTERIA BELONGING TO THE *HALOMONAS* AND *MICROCOCCUS* GENERA

BY

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DECLARATION BY THE CANDIDATE

I, Okaiyeto Kunle declared that this study was carried out in the Applied and Environment Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa, under the supervision of Mr. L. V. Mabinya and Prof. A. I. Okoh. The research reported in this dissertation, except where otherwise indicated, contains the original research results and has not been submitted to any other university for any degree award or examination purposes. Also, where other written sources have been quoted, their words have been re-written and the general information attributed to them appropriately referenced.

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Okaiyeto K.

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GENERAL ABSTRACT

The physicochemical properties of two bioflocculant producing bacteria; *Halomonas* sp. Okoh and *Micrococcus* sp. Leo were investigated. The optimum culture conditions for the individual species were determined. All the growth conditions examined for the individual bacteria were similar. Glucose and ammonium sulphate as sole carbon and nitrogen sources respectively resulted in optimum production of bioflocculant. The flocculating activity of the bioflocculants was stimulated when Al^{3+} was used as the coagulating aid under acidic medium. The information obtained from individual strains was used to produce a bioflocculant from the consortium of the two bacteria. After purification, the bioflocculant yields from 1L fermentation broths were 1.213 g from *Halomonas* sp. Okoh, 0.738 g from *Micrococcus* sp. Leo and 3.51 g from the consortium. The chemical analyses of the purified bioflocculants showed that they were glycoproteins. The thermostability property of the bioflocculants was investigated between 50-100^{o}C and the results revealed that they are heat-stable. Fourier transform infrared revealed the presence of hydroxyl, carboxyl and amino groups in the bioflocculant molecules. Scaning electron microscope (SEM) images showed the structure of each bioflocculant(s) and kaolin clay before and after flocculation.

From the results obtained, the idea of using the two strains in consortium for bioflocculant production resulted in an improvement in terms of flocculating activity and yield. The bioflocculants appears to have promise as an alternative to chemical flocculants used in various industrial processes such as wastewater treatment and drinking water purification.

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CHAPTER ONE

1.1. Background of the study

Environmental pollution has become one of the major problems in the world (Prasertsan *et al*., 2006). The discharges of wastes from households and industries into the various water bodies have imposed serious problems to the well-being of aquatic animals, humans and the environment (Zaki *et al*., 2001). Flocculants are usually used for the aggregation of colloidal substances and cellular materials in various industrial processes (Salehizadeh and Shojaosadati, 2001).

Flocculants are divided into three groups: inorganic flocculants, such as aluminium sulphate and polyaluminum chloride; organic synthetic flocculants, such as polyacrylamide derivatives and polyethylene amine; naturally occurring flocculants, such as chitosan, sodium alginate and bioflocculants (Salehizadeh and Shojaosadati, 2001; Zhang *et al*., 2007). Among the different kinds of chemical flocculants, polyaluminium salts and polyacrylamide are commonly used in wastewater treatment, drinking water treatment and industrial downstream processing (Salehizadeh and Shojaosadati, (2001).

Acccording to Wu and Ye (2007), the popularity of chemical flocculants was due to their high flocculating capability, low dosage requirement; they do not affect the pH of the media, cost effective and easily available. However, despite these advantages, monomers such as acrylamides have been reported to be both carcinogenic and neurotoxic, thus resulting in their reduced utilisation (Shih *et al*., 2001; Zheng *et al*., 2008).

For the past decades, synthetic organic flocculants have been used because they are cost-effective but they have been reported to be carcinogenic and neurotoxic because their degraded monomers such as acrylamides are carcinogenic and neurotoxic (Shih *et al*., 2001;

Zheng *et al*., 2008). According to Banks *et al*. (2006) aluminium salts are neurotoxic and also traced to be one of the causes of Alzheimer's disease. Ferrite flocculants usually bring out unpleasant metallic taste, colour, odour and corrode easily (Li *et al*., 2008).

As a result of shortcomings that limit the utilisation of these chemical flocculants, the biopolymers secreted during growth of microorganisms have been considered as alternatives (Li *et al*., 2008). Bioflocculants are not only biodegradable but also safe towards humans and the environment (Deng *et al*., 2003). The flocculating activity of the bioflocculant is dependent on its characteristics (Gao *et al*., 2006). Bioflocculants are mainly composed of protein, glycoprotein, polysaccharide, nucleic acid (Labille *et al*., 2005; Salehizadeh and Shojaosadati, 2001). Some of these bioflocculants have been reported more efficient than chemical synthetic flocculants in terms of flocculating activity (Lee *et al*., 1995; Kurane *et al*., 1994). Many bioflocculant-producing microorganisms have been reported in the literature (Fugita *et al*., 2000). For example, Zhang *et al*. (2002a) reported a bioflocculant produced by the marine *myxobacterium Nannocystic* sp. NU-2. Yim *et al*. (2007) reported a bioflocculant p-KG03 produced by a marine dionoflagellate, *Gyrodinium impudicum* KG03. Ugbenyen *et al*. (2012) reported about a thermostable bioflocculant produced by *Cobetia* sp.

According to Zhang *et al*. (2010), bridging mechanism is often used to explain flocculation in biological systems. The bridging mechanism is usually influenced by the molecular weight and charge of the biopolymer, the kind of suspended particles and flocculation conditions such as pH of the solution and the nature of the cation (Zhang *et al*., 2010). The flocculation process of a biopolymer produced by *Aspergillus sojae* and *Pseudoalteromonas* sp. SM9913 was explained in terms of bridging mechanism by Nakamura *et al*. (1976) and Li *et al*. (2008).

Bioflocculants have been widely applied in waste/drinking water treatment downstream processes (Deng *et al*., 2003; Li *et al*., 2009b). Biopolymer from microorganisms can be used to remove soil solids, organic and inorganic suspended particles (Fujita *et al*., 2000). Zouboulis *et al.* (2004) reported that a bioflocculant produced by *Rhizomonas* sp. can be used for the removal of humic acids from destabilized landfill leachates. Deng *et al*. (2005) reported that the polymeric substance secreted by *Aspergillus parasiticus* could be used to solubilize anionic dyes with high decolourization efficiency in aqueous solution. Gong *et al*. (2008) reported the biopolymer produced by *Serratia fiacaria* and discovered that it could be used to flocculate a variety of wastewater and soy brewery wastewater.

The major problems limiting the large-sacle production of bioflocculants are low flocculating efficiency, high production costs and low yield (Kurane *et al*., 1994; Li *et al*., 2003). Consequently, screening of microorganisms from diverse environments with high flocculating potentials which are capable of utilizing low-cost substrates are crucial factors to be considered in bioflocculation. Also, optimization of fermentation conditions to enhance bioflocculant yields has been a subject of interest for researchers in this field (He *et al*., 2002; Wang *et al*., 2007).

1.2. Statement of Research Problem

Conventional synthetic flocculants are frequently used in industrial applications due to their flocculation effectiveness and low cost of production. However, these flocculants have been shown to be non-degradable and detrimental to both humans and the environment. Bioflocculants on the other hand have advantages of being bio-degradable, harmless to humans and the environment. Therefore, it is desirable to produce alternative flocculants from microorganisms to substitute for synthetic flocculants. In order to improve bioflocculant

yield and enhance its flocculating efficiency, it is anticipated that the use of mixed bacteria cultures for bioflocculant production is more likely to deliver better results compared to single-strain culture approach. For large scale industrial application, new microorganisms with potential for increased bioflocculant production need be explored and the production process enhanced for optimum flocculating efficiency.

1.3. Aim and Objectives

- The broad aim is to assess the production and characterization of a bioflocculant produced by a consortium of two bacteria belonging to the *Halomonas* and *Micrococcus* genera.

The specific objectives shall include:

- To validate the bioflocculant production potential of the test bacteria.
- To optimise culture conditions for bioflocculant production by the individual test bacterium.
- To evaluate the kinetics of bioflocculant production by the bacterial consortium.
- To evaluate the effect of various factors on flocculation efficiency of the produced bioflocculant.
- To purify and characterize the bioflocculant produced by the consortium.
- To compare the chemical composition of the bioflocculant produced by the individual strain with the one produced by the consortium.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

Flocculation is a process whereby finely divided or dispersed particles are aggregated together to form larger particles of such a size so as to cause their settling or it is the agglomeration of tiny particles to form flocs which settle and cause clarification of the system (Sharma *et al*., 2006). According to Heitner (1994), flocculants can be defined as any substances which are used in fast solid–liquid separations. They act at a molecular level on the surfaces of the particles to reduce repulsive forces and increase attractive forces.

Due to electrostatic repulsive force that causes like charges to repel each other when they exist in aqueous solution, Hubdard and Arthur (2004) defined flocculating agents as chemicals that promote flocculation by aggregation of colloids and other suspended particles, forming a floc. Particles finer than 0.1 µm (10^{-7}m) in diameter remain continuously in motion in a suspension. The electrostatic repulsive force is overcome by the addition of a flocculant thereby making electrostatic attraction to be effective. This results in the formation of large flocs by the agglomeration of finer particles after collision with each other (Lachhwani, 2005). In other words, flocculating agents (also known as flocking agents), are chemicals that promote flocculation by causing colloids and other suspended particles in liquids to aggregate, forming a floc. These flocculants under defined pH, temperature and salinity conditions react with water to form insoluble hydroxides that precipitate, and form a large floc.

Generally, chemical flocculants have been widely used in industrial processes such as wastewater treatment, drinking water purification and downstream processes in fermentation industries (Shih *et al*., 2001), due to the following advantages:

- They are cheap and easily available (Salehizadeh and Shojaosadati, 2001; Zheng *et al*., 2008).

- They have very strong flocculating capability (Mabinya *et al*., 2011).
- They are effective at very low dosages (Zheng *et al*., 2008).

However, these flocculants have disadvantages which include the following:

- They are carcinogenic and neurotoxic (Dearfield and Abermathy, 1988; Mathys *et al*., 2005; Ruden., 2004; Salehizadeh and Shojaosadati, 2001). Polyaluminium for example is known to be associated with Alzheimer's disease (Christopher *et al*., 2006; Flaten, 2001).
- They are very sensitive to pH (Sharma *et al*., 2006).
- The monomers of polyacrylamide are not easily degraded and as a result thereof result in secondary pollution unfavourable to both humans and the environment (Ho *et al*., 2010; Taniguchi *et al*., 2005).
- They are unable to coagulate very fine particles (Sharma *et al*., 2006).
- They are competent only for a few disperse systems while not working for others (Sharma *et al*., 2006).

Due to the limitations of chemical flocculants, flocculants produced by microorganisms have attracted more attention (Salehizadeh and Shojaosadati, 2001). The major interest of researchers is to produce flocculants from microbes in order to replace chemical flocculants (Jang *et al*., 2001). It was noticed that when microorganisms are cultivated in a rich medium, they are capable of excreting biopolymers either into the medium or as a capsule attached on the surface of the microorganisms (Deng *et al*., 2003; Gao *et al*., 2006; He *et al*., 2002). Different organisms such as algae, bacteria, fungi, yeast have been reported to produce bioflocculants whose structure, composition as well as characteristics are influenced by growth conditions under which the microorganism is cultured (Deng *et al*., 2003).

Bioflocculants are being increasingly utilised as alternatives to chemical flocculants due to observed flocculating properties when applied to colloids or suspensions (Jang *et al*., 2001), as well as to the following advantages over chemical flocculants:

- They are biodegradable (Deng *et al*., 2003; He *et al*., 2004, Zhang *et al*., 2010).
- They are non toxic (Deng *et al*., 2003).
- Their degradation intermediates are not secondary pollutants (Salehizadeh and Shojaosadati, 2001; Joung *et al*., 2007).

Li *et al*. (2010) stated that the molecular weight and chemical composition of the bioflocculant influence its flocculating efficiency and flocculation mechanism. According to Kumar *et al*. (2004), most bioflocculants reported in literature are composed of polysaccharides and proteins as their main constituents. The structure and composition of microbial polysaccharides depend on a number of factors, such as medium-culture compositions which include the nature of carbon and nitrogen sources, and fermentation conditions such as pH, temperature, and oxygen concentration (Chang *et al*. 2005; Margaritis and Pace, 1985).

The flocculation process involving high molecular weight bioflocculants such as polysaccharides with additional hydroxyl and carboxyl functional groups that provide more adsorption points usually leads to strong binding resulting in higher flocculating activity compared to bioflocculants with low molecular weights (Kurane *et al*., 1991). According to Salehizadeh and Shojaosadati (2001), the majority of bioflocculants reported in literature are in the molecular weight range of 10^5 to 2.5×10^6 Da. Kurane *et al*. (1994) reported that bioflocculants which are protein in nature are usually composed of amino and carboxyl functional groups and have low molecular weight compared to polysaccharide bioflocculants. The carboxyl groups present in the molecular chain make the chain to stretch out because of

electrostatic repulsion and the stretched molecular weight chains provide more effective adsorption sites for particle.

Zhang *et al*. (2002a) stated that the interest of researchers in bioflocculation lies in using different microorganisms to synthesize extracellular polymers whose compositions can make them effective flocculating agents for various industrial processes. The other important objective according to Zheng *et al*. (2008) is to produce cation-independent bioflocculants with low dosage requirements in order to contain costs and reduce pollution.

Microorganisms found in the deep sea environment possess special features that made them to adapt to the extreme high pressure, low temperature and low nutrition environment than the terrestrial ones (Mabinya *et al*., 2011). It was also expected that the flocculants extracted from marine microorganisms will exhibit different characteristics from those extracted from terrestrial microorganisms. For example, flocculants from deep sea microorganisms can easily withstand low temperature and high salinity conditions and can be used as effective bioflocculants under these conditions (Li *et al*., 2008, 2009a; Zhang *et al*., 2002a).

In this present study, the flocculating activities and physicochemical properties of two bacteria belonging to *Halomonas* and *Micrococcus* genera were investigated. Results obtained from studies on bioflocculant production by individual strains were used as a basis to investigate and improve bioflocculant production by a consortium of the two bacterial strains. Characterization and biochemical analysis of the properties of the purified consortium-produced bioflocculant were carried out.

2.2. Classes of flocculants

There are different types of flocculants which can either be inorganic, organic or naturally occurring. Flocculants can also either be anionic, cationic, neutral or amphoteric depending on the type of charge they carry.

2.2.1. Inorganic flocculants

Examples of flocculants include; Alum, aluminium chlorohydrate, aluminium sulphate, calcium oxide, calcium hydroxide, iron II sulphate (ferrous sulphate), iron III chloride (ferric chloride), sodium aluminate, and sodium silicate.

2.2.2. Organic flocculants

They are a class of chemical flocculants with high molecular weight; they can flocculate well at a wide range of pH but usually possess low charge density (Lachhwani, 2005). These are subdivided into two groups namely:

- Synthetic organic flocculants: these are based on various monomers such as acrylamide, acrylic acid, diallyldimethyl ammonium chloride (DADMAC), styrene sulphonic acid.
- Natural organic flocculants: These are based on natural polymers such as starch, cellulose, natural gums and mucilage and their derivatives.

2.2.3. Natural organic flocculants

Naturally occurring flocculants include; chitosan, isinglass, *Moringa oleifera* seeds (horseradish tree), gelatine, guar gum, aliginates. These are cheap, less toxic but their flocculating efficiency is very low when compared to inorganic or organic flocculants.

2.3. Mechanism of flocculation

Though the mechanism of flocculation by chemically synthesized flocculants is well described in the literature, the mechanism of flocculation by the biopolymers secreted during growth of microorganisms is still yet to be fully studied and understood (Salehizadeh and Shojaosadati, 2001). Bridging and charge neutralization were both used by Lian *et al*. (2008) and Li *et al*. (2009a) respectively, to explain the mechanism of flocculation in biological systems based on their experimental observations.

2.3.1. Flocculation mechanism by bridging

Bridging in biological systems occurs when a flocculant forms threads or fibers in solution, they usually stretch out like a branch of a tree. They attract suspended particles, making them to come together as aggregates (Li *et al*., 2008). Flocculants with higher molecular weight mean longer molecules and this implies effective bridging because they usually have more attractive (point) sites (Zhang *et al*., 2010). When a polymer molecule comes into contact with a suspended particle, some of the reactive groups on the polymer adsorb at the particle surface, leaving other portions of the polymer molecule extending into the solution (Zhang *et al*., 2010). If excess polymer is added or adsorbed, the particles are restabilized by surface saturation and are statically stabilized (Li *et al*., 2008; Yim *et al*., 2007). The length of the biopolymer always influences effective bridging (Lu *et al*., 2005). The most important thing

is the size of the polymer in solution i.e, hydrodynamic volume rather than merely the molecular weight (high molecular weight means high functional groups). The bridging mechanism was found to play a key role in flocculating efficiency of the bioflocculants EP SM9913 produced by *Pseudoalteromonas* sp. SM9913 and ZS-7 from *Bacillus licheniformis* X14 (Li *et al*., 2008, 2009a). Salehizadeh and Shojaosadati (2001) explained that bridging can be used to describe flocculation by large molecular weight biopolymers and this occurs when the flocculant covers the particle from the particle surface into the solution. The functional groups on the surface of the flocculant can extend and go beyond the distance covered by the particles and thereby making flocculation to be effective. This means that when these flocculants are in solution, they stretch out like linear chains and adsorb particles from the surrounding environment. The number of particles adsorbed by these flocculants depends on a number of factors which include: the charge of the biopolymer, the charge on suspended particles, and most importantly, the molecular weight of the biopolymer (Wang *et al*., 2011).

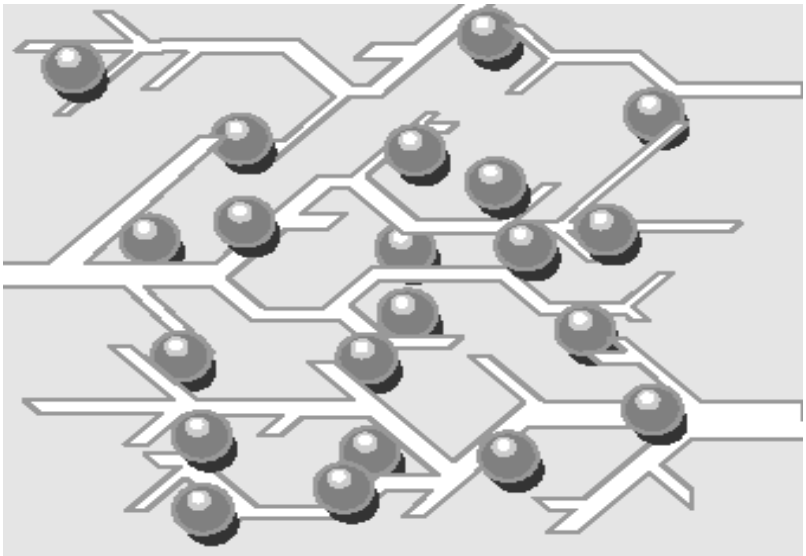

Figure 1: Diagram of polymer chain attaching particle (Lachhwani, 2005)

2.3.2. Flocculation mechanism by charge neutralization

When negatively charged particles are in aqueous solution, they move continuously exhibiting Brownian movement. Since charged particles exhibit electrostatic repulsion forces which are greater than the Vander Waals forces of attraction between them; they inhibit their settling and floc formation (Lachhwani, 2005). For particles in aqueous solution to settle, an opposite charged compound usually a positively charged flocculant is required in order to neutralise and stabilise the negative charge of suspended particles (Salehizadeh and Shojaosadati, 2001). When flocculants are added, flocs are usually formed and this hastens the gravitational settling of particles in solution. Initially the flocculation process involves the formation of small flocs which later aggregate to form a larger floc thus speeding up the sedimentation rate (Lachhwani, 2005). The particle surface charge is reduced when it is adsorbed onto the bioflocculant leading to increased attractive forces compared to repulsive forces (Levy *et al*., 1992).

Like charges repel each other

unlike charges attract each other and form aggregates

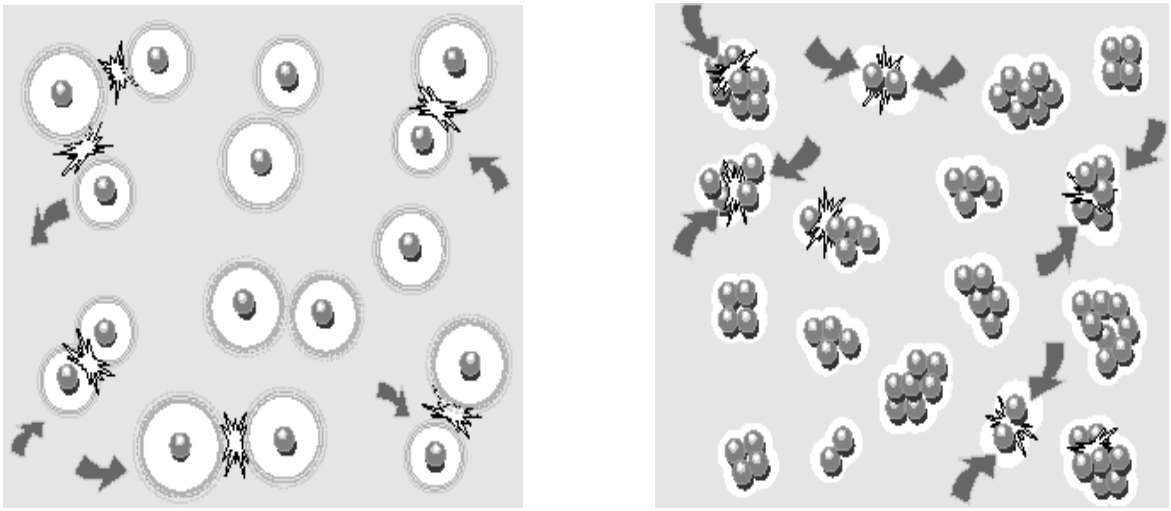

Figure 2: Diagram of electrostatic attraction and repulsion of charges (Lachhwani, 2005).

The above process is the kind of flocculation mechanism exhibited by biopolymers which is the electrostatic interactions or charge neutralization mechanism (Mabire 1984; Gregory 1985). Many researchers have reported this flocculation mechanism in many bioflocculants produced by different microorganisms. Levy *et al*. (1992) stated that when the bioflocculant is oppositely charged compared to the suspended particles, the particle surface charge density is reduced by its adsorption onto the bioflocculant causing the particles to approach sufficiently close to each as attraction forces become more effective than repulsive forces. Adsorption of the particles by the polymers occurs as a result of uneven distribution of charges (Lachhwani, 2005).

2.4. Composition and chemical structure of bioflocculant

The chemical composition of bioflocculants produced by different microorganisms differ (Salehizadeh and Shojaosadati, 2001). Bioflocculants composed of polysaccharides, proteins, nucleic acids, lipids have been reported (Deng *et al*., 2005) and are all known to play a vital role in flocculation mechanism (Li *et al*., 2009b). In the past, several bioflocculant-producing microorganisms have been screened and their chemical compositions documented (Nakamura *et al*., 1976). Takeda *et al*. (1991) reported about a proteinaceous bioflocculant produced by *Rhodococcus erythropolis* which lost its flocculating activity due to bioflocculant-degrading enzyme secreted by the microorganism. Nakata and Kurane (1994) reported that the chemical analysis of extracellular polysaccharide produced by *Klebsiella pneumoniae* revealed that the purified flocculant was composed of polysaccharide with the following monosaccharide unit ratio: galactose (56.04%), glucose (25.92%), galacturonic acid (10.92%), mannose (3.71%) and glucuronic acid (3.37%). Deng *et al*. (2005) reported that the bioflocculant produced by *Aspergillus parasiticus* could be used to remove dye and composed of carbohydrate (76.3%) and protein (21.6%). It had a molecular weight of 3.2×10^5 Da and the FTIR analysis

indicated the presence of amino, amide, and hydroxyl groups. According to Prasertsan *et al*. (2006), the acidic heteropolysaccharide bioflocculant produced by *Enterobactercloacae* WD7 is composed of neutral sugars (29.4%), uronic acids (14.2%), and amino sugars (0.93%) with the FTIR spectroscopy showing the presence of hydroxyl, carboxyl, carbonyl and methoxyl groups. The exopolysaccharide bioflocculant produced by *Sorangium cellulosum* is composed of 38.3% proteins and 58.5% of sugar. The flocculating activity of this bioflocculant was influenced by cations (Zhang *et al*., 2002b). Wu and Ye (2007) reported that *Bacillus subtilis* DYU1 could produce a biopolymer DYU500 composed of sugars, uronic acids, proteins, and polyamides. Also, Yim *et al*. (2007) observed that the exopolysaccharide p-KG03 produced by marine dinoflagellate *Gyrodinium impudicum* contained galactose as the major constituent and exhibited strong flocculating ability. Feng and Xu (2008) reported that MBF3-3 produced by *Bacillus* sp. was mainly composed of acid polysaccharide which was the main effective flocculating component. Zheng *et al*. (2008) reported a bioflocculant produced by *Baccillus* sp. F19 which was composed of neutral sugar (3.6% w/w), uronic acid (37.0% w/w), amino sugars (0.5% w/w) and protein (16.4% w/w). The infrared spectrophotometry analysis revealed the bioflocculant MBFF19 produced by produced by *Bacillus* sp. F19 contained carboxyl, hydroxyl and methoxyl groups. In addition, Li *et al*. (2009a) reported about a novel bioflocculant produced by *Bacillus licheniformis* X14 which was identified as a glycoprotein composed mainly of polysaccharide. He *et al*. (2010) investigated the novel bioflocculant HBF-3 produced by a deep-sea bacterium mutant *Halomonas* sp. V3a' and found it to be composed of mainly a polysaccharide (29.8%) including neutral sugar residues (20.6%), uronic acid (7.6%), amino sugar (1.6%) and a sulphate group (5.3%). Li *et al*. (2010) reported a molecular weight of 8.1×10^4 Da for a bioflocculant produced by *Agrobacterium* sp. M503 which was composed of neutral sugar, uronic acid, amino sugar and protein in weight ratios of 85.0:9.9:2.1:3.0, respectively. Wang

et al. (2011) reported that the bioflocculant obtained from a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus Sphaeicus* F6 composed of sugar (85.82% w/w) and protein (1.86% w/w). In addition, the hydrolysis of purified CBF-F26 mixed culture of *Rhizobium radiobacter* F2 and *Bacillus Sphaeicus* F6 indicated that its constituents were rhamnose, mannose, glucose, and galactose respectively, in a 13:2.1:10.0:1.0 molar ratios (Wang *et al*., 2011). The average molecular weight of CBF-F26 was estimated to be approximately 4.79×10^5 Da and infrared spectrum of CBF-F26 evidenced the presence of carboxyl, hydroxyl and amino groups. The EDS spectrum showed that C and O were present as major elements and N was present as a minor element on the surface of the purified CBF-F26.

2.5. Bioflocculant-producing microorganisms

Due to extreme conditions, the morphological, metabolic and physiological adaptations of deep sea microorganisms are quite different from terrestrial microorganisms (He *et al*., 2010). It is expected that flocculants from deep sea microorganisms can have a special adaptability to low temperature and high salinity conditions and can be used as effective bioflocculants for low temperature water treatments (Li *et al*., 2008; 2009a Zhang *et al*., 2002a). Kurane *et al*. (1986b) reported that *Nocardia restricta*, *Norcardia calcarea* and *Nocardia rhodnii* could produce biopolymer flocculants. Zhang *et al*. (2002a) reported a bioflocculant produced by the marine *Myxobacterium nannocystic* sp. NU-2 with a yield of 14.8 g/l and was composed of protein (40.3% w/w) and polysaccharide (56.3% w/w). The flocculating activity was enhanced by the presence of Fe^{3+} and Al^{3+}. Deng *et al*. (2005) found that *Aspergillus parasitus* could produce a bioflocculant with a flocculating activity for kaolin suspension and waste-soluble dyes, while Lu *et al*. (2005) reported that the bioflocculant produced by *Enterobacter aerogenes* required Zn^{2+} for flocculating activity. Han and co-workers discovered a novel bioflocculant p-KG03 from a marine dionoflagellate, *Gyrodinium*

impudicum KG03 which was characterised as an acidic heteropolysaccharide, with galactose and uronic acid as the main components (Yim *et al*, 2007). The bioflocculant produced by *Klebsiella* sp. S11 was unable to flocculate without the presence of $CaCl_2$ in the solution (Dermalim *et al*., 1999). He *et al.* (2010) reported the production of a novel bioflocculant produced from *Halomonas* sp. V3a by deep-sea bacterium mutant *Halomonas* sp. V3a, while Mabinya *et al*. (2011) reported about a polysaccharide bioflocculant produced by *Halomonas* sp. Okoh whose activity was dependent on Ca^{2+}. *Cobetia* sp. produced a thermostable acidic polysaccharide bioflocculant whose activity was dependent on the presence of cations (Ugbenyen *et al*., 2012).

2.6. Application of bioflocculants

Microbial bioflocculants have long been recognised as having potential applications in a number of different industries. Some of these bioflocculants have been used in the treatment of inorganic solid suspensions such as bentonite, activated carbon, solid clay, $Ca(OH)_2$, and aluminium oxide (Levy *et al*., 1992; Shih *et al*; 2001; Yim *et al*., 2007). Kwon *et al*. (1996) reported a bioflocculant produced by *Rhodococcus erythrpolis* named NOC-1 with an efficient flocculating activity for a wide range of suspended solids. The bioflocculant produced by *Citrobacter* sp. TKF04 could be used to remove soil solids, organic and inorganic suspended particles (Fujita *et al*., 2000). Zheng *et al.* (2002a) reported that the bioflocculant produced by *Myxobacterium nannocystic* sp. NU-2 was able to remove colouring material from an aqueous solution such as dyeing liquors. Deng *et al*. (2003) stated that the bioflocculant produced by *Bacillus mucilaginosus* which showed high flocculating activity for kaolin clay could be used in the treatment of raw water such as river water and wastewater. The bioflocculant purified from the culture broth of *Archuadendron* sp. TS-49 could effectively flocculate various microorganisms and organic/inorganic materials (Li *et*

al., 2003). Zouboulis *et al.* (2004) reported the application of bioflocculant for the removal of humic acids from destabilized landfill leachates. According to the finding of Salehizadeh and Shojaosadati (2003), the bioflocculant produced by *Bacillus firmus* could be used to remove heavy metal residues. He *et al*. (2004) observed that the bioflocculant REA-11 could be used to decolourize molasses wastewater. The bioflocculant secreted by *Aspergillus parasiticus* could be used to solubilize anionic dyes with high decolourization efficiency in aqueous solution (Deng *et al*., 2005). Zhang *et al*. (2007) reported that the bioflocculant produced by microorganism's consortia using brewery wastewater as carbon source showed good flocculating performance in treating indigotin printing and dyeing wastewater and the maximal removal efficiencies of COD and chroma were 79.2% and 86.5%, respectively. Wang *et al*. (2007) reported that a novel polysaccharide produced by a culture of *Klebsiella mobilis* which showed high flocculating activity of 95.4% was effective in flocculating some disperse dyes in aqueous solution. Also, the bioflocculant produced by *Serratia fiacaria* could flocculate a variety of wastewater, and soy brewery wastewater (Gong *et al*., 2008). Ghosh *et al*. (2008) reported that the novel bioflocculant secreted by *Klebsiell taerrigena* was used to remove *Salmonella*, a potent pathogen prevalent in poultry wastewater. Li *et al*. (2009a) reported that a bioflocculant produced by *Bacillus licheniformis* X14 could be applied in low temperature drinking water treatment.

2.7. Factors influencing bioflocculant production and flocculating activity

Various factors in the optimization of culture conditions have to be taken into consideration in order to increase yield and flocculating efficiency of the various bioflocculants produced by different microorganisms (Zufarzaana *et al*., 2012). The structure and composition of microbial polysaccharides depend on a number of factors, such as medium-culture composition, type of carbon and nitrogen sources, type of microbial system employed, and

other fermentation conditions such as pH, temperature, oxygen concentrations (Chang *et al*. 2005; Margaritis and Pace, 1985). Sutherland (1990) and Breedveld *et al*. (1993) reported that through the proper control of fermentation conditions, one may influence not only the amount of exopolysaccharide produced, but also the bioflocculant characteristics and composition. The effect of nutritional conditions on the production of bioflocculants has been extensively studied (Salehizadeh and Shojaosadati, 2001).

2.7.1. Effect of inoculum size on bioflocculant production

Inoculum size is a very important factor that needs to be considered in bioflocculation (Zhang *et al*., 2007). Inoculum sizes used in bioflocculant production by different microorganisms vary thus necessitating determination of an optimum inoculum size (Zhang *et al*., 2007). According to the findings of Salehizadeh and Shojaosadati (2001), small inoculum size prolongs the stagnant phase and large inoculum size also inhibits bioflocculant production. In addition, Zhang *et al*. (2007) reported that inoculum size is a critical factor which influences bioflocculant production. Optimum inoculum size of 1% (v/v) was used in bioflocculant production by *Perilla fructsecens* and *Vagococcus* sp. W31 (Zhong and Yoshida, 1995; Gao *et al*., 2006). In another study, Jang *et al*. (2001) reported an inoculum size of 5% (v/v) for the production of a bioflocculant by *Citrobacter* sp. Similarly, *Klebsiella mobilis* produced maximum yield of bioflocculant when 5% (v/v) inoculum size was utilized (Wang *et al*., 2007). Zhang *et al*. (2007) reported an inoculum size of 1% for bioflocculant production by multiple-microorganism consortia. Inoculum size of 5% (v/v) was also utilised by *Bacillus subtilis* for bioflocculant production (Patil *et al*., 2009). Xiong *et al*. (2010) reported that an optimum inoculum size of 4% (v/v) was required for the production of bioflocculant by *Bacillus licheniformis*. Ugbenyen *et al*. (2012) recorded a 2% inoculum size requirement for optimum bioflocculant production by *Cobetia* sp.

2.7.2. Effect of carbon source on bioflocculant production

The production of bioflocculants by different microorganisms is strongly influenced by the composition of the cultivation medium. Different carbon sources have been shown to support bioflocculant production by different microorganisms to varying degrees (Ntsaluba *et al*., 2011). Kurane and Nakata (1999) observed that ethanol was used for bioflocculant production by *Klebsiella pneumoniae*. Soluble starch was the sole carbon source used by *Sporangium cellulosum* for bioflocculant production (Zhang *et al.,* 2002b). Kumar *et al*. (2004) reported that corn starch was used as the carbon source in the optimized growth conditions in bioflocculant production by *Bacillus* sp. I-450. Sucrose was more favoured by *Corynebacterium glutamicum* compared to other carbon sources (He *et al*., 2004). In the case of *Klebsiella* sp. maltose was found to be the best carbon source (Sheng *et al*., 2006). Wang *et al*. (2007) stated that the flocculating activity of the bioflocculant produced by *Klebsiella mobilis* was optimum when diary wastewater supplemented with 2% ethanol was used as the carbon source. Glucose has been documented in literature to be the carbon source of choice by different microorganisms e.g. bioflocculant production by *Virgibacillus* sp. Rob, *Halomonas* sp. V3a, *Chrysobacterium daeguense* W6, *Halomonas* sp. Okoh, *Methylobacterium* sp. Obi (Cosa *et al*., 2011; Liu *et al*., 2010; He *et al*., 2010; Mabinya *et al*., 2011; Ntsaluba *et al*., 2011).

2.7.3. Effect of nitrogen source on bioflocculant production

In addition to carbon source, nitrogen source also plays a critical role in influencing cell growth rate and hence bioflocculant production (Ntsaluba *et al*., 2011). The production of a bioflocculant by *B. licheniformis* CGMCC2876 was maximal when urea was used as the sole nitrogen source (Shih *et al*., 2001). In the production of bioflocculant by *Aspergillus parasiticus*, peptone was the nitrogen source of choice (Deng *et al*., 2005). Li *et al*. (2010)

reported that *Chryseobacterium daeguense* W6 produces the bioflocculant by utilizing organic nitrogen sources among which tryptone was the best preferred resulting in flocculating activity of more than 90%, while all inorganic nitrogen sources (sodium nitrate, ammonium sulphate and ammonium nitrate) resulted in poor flocculating activity. Piyo *et al*. (2011) observed that *Bacillus* sp. Gibert effectively utilized an inorganic nitrogen source, ammonium chloride, to produce a bioflocculant with a flocculating activity of 91%. Ugbenyen *et al*. (2012) reported that the bioflocculant produced by *Cobetia* sp. was inhibited by both organic and inorganic nitrogen sources but the production was enhanced when multiple nitrogen sources (urea, yeast extract and ammonium sulphate) were used resulting in 92.25% flocculation activity.

2.7.4. Effect of cations on bioflocculant production and flocculating activity

The role of cations in bioflocculation is for destabilization of the negative charges of both the kaolin particles in solution and the functional groups of the bioflocculant (Salehizadeh *et al*., 2000). Zufarzaana *et al*. (2012) reported that the cation acts as a coagulant, neutralizing the zeta potential in the kaolin suspension thereby giving room for attraction between them before allowing the bioflocculant to flocculate through bridging mechanism. The flocculating rate of the bioflocculant produced by *Nannocystic* sp. NU-2 strongly depended on cations (Zhang *et al*., 2002a). Lu *et al*. (2005) reported that the bioflocculant WF-1 produced by *E. aerogenes* required the presence of Zn^{2+}. The flocculating activity of an *Aeromonas*-produced bioflocculant was increased with the addition of K^{+}, Na^{+} and Ca^{2+} (Li *et al*., 2007). Zheng *et al*. (2008) observed that the flocculating activity of the bioflocculant from a haloalkalophilic *Bacillus* was greatly enhanced by the addition of cations such as Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+} but reduced by the addition of cations such as Al^{3+}, Fe^{3+} and Na^{+}. Cosa *et al*. (2011) reported

that the flocculating activity of the bioflocculant produced by *Virgibacillus* sp. Rob was stimulated by the presence of Fe^{2+}.

Table 1: Cation-dependent bioflocculant-producing microoganisms

Microorganisms	**Cations**	**References**
Bacillus licheniformis	Ca^{2+}, Fe^{3+}, Al^{3+}	Shih *et al*., 2001
Bacillus Substilis	Ca^{2+}, Mg^{2+}	Wu and Ye, 2007
Bacillus circulans	Al^{3+}, Fe^{3+}, Ca^{2+}	Li *et al.*, 2009b
Serratia ficaria	Ca^{2+}, Mg^{2+}	Gong *et al*., 2008
Halomonas sp. V3a'	Ca^{2+}	He *et al*., 2010
Pseudoalteromonas sp. SM9913	Ca^{2+}, Fe^{3+}	Li *et al*., 2008
Enterobacter cloacae WD7	Cu^{2+}	Prasertsan *et al*., 2006
Methylobacterium sp. Obi	Ca^{2+}	Ntsaluba *et al*., 2011
Bacillus subtilis	Fe^{3+},Al^{3+}	Patil *et al*., 2009
Aeromonas sp.	K^{+}, Na^{+}, Ca^{2+}	Li *et al*., 2007

Table 2: Cation independent bioflocculant-producing microoganisms

Microorganisms	Cations	References
Citrobacter sp.	None	Jang *et al*., 2001
Bacillus mucilaginosus	None	Deng *et al*., 2003
Bacillus sp. F19	None	Zheng *et al*., 2008
Chryserbacterium daeguense	None	Liu *et al*., 2010

2.7.5. Effect of initial pH of growth medium on bioflocculant production

The pH of the culture medium may affect or influence the production of the bioflocculant (Salehizadeh and Shojaosadati, 2001). The initial pH requirement may have a different effect with different strains. The pH of the culture medium is said to determine the electric charge of the cells together with the oxidation potential which can affect the nutrient absorption and enzymatic reaction (Xia *et al*., 2008). In addition, Wang *et al*. (2011) stated that pH determines the formation of flocs as well as affect the stability of the suspended particles (i.e. the pH effect as well as affecting the turbidity of the kaolin suspension without the formation of flocs). *Rhodococcus erythropolis* produced a bioflocculant which was best active at alkaline condition (Kurane *et al*., 1994). *Streptomycetes griseus* and *Aspergillus Sojae* produced flocculating substances under acidic conditions (Shimofuruya *et al*., 1995). The production of bioflocculant by *Bacillus licheniformis* CCRC12826 was documented to be best at pH 7 (Salehizadeh *et al*., 2000). Fujita *et al*. (2000) observed that the production of bioflocculant by *Citrobacter* sp. TKF04 was optimal within the pH range of 7.2-10. He *et al*. (2004) reported a bioflocculant produced by *Corynebacterium glutamicum* at pH 7.2. *Vagococcus* sp. W31 produced a bioflocculant at pH 6.5 (Gao *et al*., 2006), while *Bacillus* sp. Gibert and *Gyrodinium impudicum* KG03 required a wide pH range of 3-6 respectively (Piyo

et al., 2011; Yim *et al*., 2007). Zheng *et al*. (2008) reported a bioflocculant produced by *Bacillus* sp. F19 at pH 8.95 while *Bacillus licheniformis* X14 produced the bioflocculant optimally at pH 7.5 (Li *et al*., 2009b). Liu *et al*. (2010) stated that the optimum pH for production of bioflocculant by *Chryseobacterium daeguense* W6 was between pH 4-8. *Proteus mirabilis* produced the bioflocculant at pH 7 (Zhang *et al*., 2010), while pH 7.2 and 7.4 were appropriate for optimum bioflocculant production by marine bacteria *Myxobacterium nanocystic* sp. NU-2 and *Halomonas* sp. V3a, respectively (He *et al*., 2010). Cosa *et al*. (2011) reported that the bioflocculant produced by *Virgibacillus* sp. Rob was best produced at pH 12. In addition, maximum bioflocculant yield from a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 was obtained at pH 8. *Arthrobacter* sp. Raats produced its bioflocculant at pH 7 (Mabinya *et al*., 2012). Zufarzaana *et al*. (2012) investigated the bioflocculant produced by *Bacillus* sp. UPMB13 at pH 5.

2.7.6. Effect of shaking speed on bioflocculant production

Shaking speeds are known to have an influence on aeration levels available to microorganisms' respiration in the culture broth (Salehizadeh and Shojaosadati, 2001). The production of bioflocculant depends on the dissolved oxygen tension (DOT) of the culture broth. Increase or decrease in DOT of the culture broth results in increase or decrease in the respiration of microorganisms, absorption of nutrients and enzymatic reaction (Salehizadeh and Shojaosadati, 2001). The shaking speed required for bioflocculant production by different microorganisms differs. Takagi and Kadowaki (1985) recorded improved production of bioflocculant by *Paecilomyces* sp. at 200 rpm. Toeda and Kurane (1991) reported that *Alcaligenes latus* produced its bioflocculant at 300 rpm. Dermlim *et al*. (1999) reported the bioflocculant produced by *Klebsiella* sp. at 200 rpm. *Proteus mirabilis* TJ1 produced the bioflocculant efficiently at 130 rpm (Zhang *et al*., 2010). *Bacillus firmus* and

Bacillus mucilaginosus produced bioflocculants at 150 rpm (Salehizadeh and Shojaosadati, 2002; Deng *et al*., 2003). Li *et al*. (2007) reported an improved yield of the bioflocculant produced by *Aeromonas* sp. at 170 rpm. *Bacillus licheniformis* X14, *Cobetia* sp., *Halomona* sp. Okoh, *Methylobacterium* sp. Obi produced their bioflocculants optimally at 160 rpm (Xiong *et al*., 2010; Ugbenyen *et al*., 2012; Mabinya *et al*., 2011; Ntsaluba *et al*., 2011).

2.7.7. Effect of temperature on bioflocculant production.

Kurane and Nakata (1999) stated that the metabolism of microorganisms has direct relationship with cultivating temperature. Most of the bioflocculant-producing microorganisms documented in literature produced their bioflocculants within a temperature range of 25-35^{o}C. Optimal temperature used in cultivation experiments activates enzymatic reaction which directly influences bioflocculant production (Kurane and Nakata, 1999). Zhang *et al*. (2007) reported that the bioflocculant production by multiple microoganism consortia was optimal at 30^{o}C. *Aspergillus parasiticus*, *Arthrobacter* sp. Raats, *Cobetia* sp., produced their bioflocculants at 28^{o}C (Deng *et al*., 2005; Mabinya *et al*., 2012; Ugbenyen *et al*., 2012).

2.7.8. Thermal stability of the bioflocculants

According to Kurane *et al.* (1986a) and Salehizadeh and Shojaosadati (2001), bioflocculants produced by *Rhodococcus erythropolis* and *Bacillus firmus*, respectively, could only retain 50% of flocculating activity after being heated in boiling water for 15 min. He *et al*. (2004) reported that there was no significant decrease in flocculating activity of REA-11 when heated at 80^{o}C for 1 h but further increase in temperature up to 100^{o}C drastically reduced the flocculating activity of REA-11. Li *et al*. (2007) reported that the flocculating activity of the

bioflocculant produced by *Aeromonas* sp. decreased by only 9.2% after being heated at 100^{o}C for 60 min. Gong *et al*. (2008) reported that the bioflocculant produced from *Serratia ficaria* decreased its flocculating activity by 15% after heating to 100^{o}C for 15 min and 20% reduction in flocculating activity was noticed when heated at 50^{o}C for 30 min. A bioflocculant which composed mainly polysaccharide as its backbone identified by Wang *et al*. (2011) from a mixed culture of *Rhozobium radiobacter* and *Bacillus sphaeicus* retained its flocculating activity of 90% after being heated at 100^{o}C for 30 min. Ugbenyen *et al*. (2012) reported about the thermostable-bioflocculant produced by *Cobetia* sp. which maintained its flocculating activity of 78% after heating at 100^{o}C for 25 min.

2.7.9. Effect of bioflocculant dosage on flocculating activity

Another important factor which is very important in flocculation by different bioflocculants produced by different microorganisms is the bioflocculant dosage required for achieving maximum flocculating activity (Zufarzaana *et al*., 2012). The interest of researchers in bioflocculation is to produce bioflocculants whose usage will require a low dosage in order to be cost effective. In the past decades, dosages of bioflocculants produced by different microorganisms have been documented. The flocculants produced by *Rhodococcus erythropolis*, *Bacillus subtilis* and *Enterobacter sp*. required 20 mg/l dosage for optimum activity (Takeda *et al*., 1991; Yokoi *et al*., 1995 and Yokoi *et al*., 1997). Lee *et al*. (1995) stated that the bioflocculant dosage that was effective for flocculating activity by a flocculant produced by *Arcualendron* sp. was 2 mg/l. The flocculant produced by *Pestalotiopsis* sp., *Bacillus* sp and *Gyrodinium impudicum* KG03 required a low dosage of 1 mg/l for effective flocculating activity (Kwon *et al*.,1996; Suh *et al*., 1997). According to Shu *et al*. (1997), 3 mg/l of flocculant generated by *Zoogleo ramigera* was the optimum dosage required. The bioflocculant generated by *Enterobacter* sp. BY-29 flocculated best when a dosage of 40

mg/l was used (Yokoi *et al*., 1997) and while 30 mg/l dosage of the bioflocculant produced by *Bacillus coagulants* AS-101 (Salehizadeh *et al*., 2000). Zhang *et al*. (2002b) observed that the optimum dosage needed for exopolysaccharide bioflocculant produced by *Sorangium cellulosum* was 20 mg/l. Deng *et al*. (2003) stated that 0.1 mg/l dosage was needed by bioflocculant produced by *Bacillus mucilaginosus* to perform effectively. According to the work documented by Lu *et al*. (2005) about the bioflocculant generated by *Enterobacter aerogenes*, 90 mg/l of bioflocculant dosage was noticed to give the highest flocculating activity with kaolin clay. With regards to reports documented by Gao *et al*. (2006), 25 mg/l was the required dosage for bioflocculant MBFW31 produced by *Vagococcus* sp. W31. Prasertersan *et al*. (2006) observed that 2 mg/l was the optimum dosage required by the flocculant produced by *Enterobacter cloacae* for effective activity. Liu *et al*. (2009) reported 6 mg/l optimum dosage was required for flocculation by bioflocculants produced from biologically aerated filter backwashed sludge. The bioflocculant MBF3-3 produced by *Bacillus* sp. required 0.675 mg/l dosage for optimum flocculating activity (Feng and Xu, 2008). Wang *et al*. (2011) documented that 12 mg/l was the optimum dosage required by bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6.

2.7.10. Molecular weight of bioflocculants

Bioflocculants of different molecular weights have been reported in literature (Li *et al*., 2009a). According to the report of Salehizadeh and Shojaosadati, (2001), the molecular weight of a bioflocculant also contributes to its flocculating efficiency. Yokoi *et al*. (1997) reported that the molecular weight of a purified bioflocculant from *B. subtilis* was 1.5×10^5 Da. The bioflocculants produced by *Klebsiella sp.* and *Bacillus* sp. DP-152 had molecular weights of $> 2 \times 10^6$ and 2.6×10^6 Da respectively (Dermlin *et al*., 1999 and Suh *et al*., 1997).

Citrobacter sp. TKF04 produced a bioflocculant which had a molecular weight of 3.2×10^5 Da (Fujita *et al*., 2000). Shih *et al*. (2001) reported that the bioflocculant produced by *B. licheniformis* CCRC12826 had a molecular weight of 2.0×10^6 Da. Furthermore, the bioflocculant produced by *Corynebacterium glutamicum* was reported to have a molecular weight of 10^5 Da (He *et al*., 2002). Deng *et al*. (2003) stated that the biopolymer produced by *B. mucilaginosus* had a molecular weight of 2.6×10^6 Da. Kumar *et al*. (2004) reported a molecular weight of 2.2×10^6 Da for a bioflocculant produced by *Bacillus* sp. I-450. In addition, the molecular weights of bioflocculants obtained from *Aspergillus parasiticus* and *Enterobacter aerogenes* were reported to be 3.2×10^5 Da and 2.4×10^6 Da, respectively (Deng *et al*., 2005; Lu *et al*., 2005). Wu and Ye (2007) also recovered a bioflocculant from *Bacillus* sp. DYU1 with a molecular weight of $(3.16\text{-}3.20) \times 10^6$ Da. The bioflocculant p-KG03 produced by a marine dinoflagellate *Gyrodinium impudicum* KG03 had an average mass of 1.87×10^3 kDa (Yim *et al*., 2007). Gong *et al*. (2008) reported that the molecular weight of the bioflocculant generated from *Serratia ficaria* was 3.13×10^5 Da. The average molecular weight of a novel bioflocculant produced by *Bacillus licheniformis* was approximately 1.76×10^6 Da (Xiong *et al*., 2010). A novel bioflocculant TJ-F1 produced from a *Proteus mirabilis* had an average mass of 1.2×10^5 Da (Zhang *et al*., 2010). Li *et al*. (2010) documented that the molecular weight of a thermal and alkaline stable biopolymer produced by *Agrobacterium* sp. M-503 was 8.1×10^4 Da which was a significant factor in aiding the bridging mechanism in flocculation in kaolin suspension. Liu *et al*. (2010) reported that the bioflocculant produced by *Penicillium* sp. had an average weight of 3.0×10^5 Da. According to the findings of He *et al*. (2010), a novel bioflocculant HBF-3 produced by a deep-sea bacterium mutant *Halomonas* sp. V3a had a molecular weight of 590 kDa. Abdel-Aziz *et al*. (2011) reported that the bioflocculant produced by *Bacillus alvei* NR-14 had a molecular weight of 6.9×10^4 Da.

2.7.11. Bioflocculant yields

One of the major problems hindering the large-scale production of bioflocculants is low yield (He *et al*., 2010). The cost of production of a bioflocculant is very high compared to that of chemical synthetic flocculants (Jang *et al*., 2001; He *et al*., 2004). The flocculating activities of the bioflocculants documented in literature are usually low and they require a cation and optimum pH to stimulate their activities. The interest of researchers is to screen microorganisms that are capable of producing bioflocculants with high flocculating activity using low cost substrates which can support high bioflocculant yields (Mabinya *et al*., 2011). Three grams of bioflocculant was produced from 1 L of fermented broth of *Klebsiella pneumonia* (Nakata and Kurane, 1999). Zhang *et al*. (2002b) investigated bioflocculant production by *S. cellulosum* NUST06 and reported a yield of 17.5 g/l when starch and $NaNO_3$ were used as carbon and nitrogen sources, respectively. The yield of a bioflocculant produced by marine *Myxobacterium nannocystics* sp. NU-2 was 14.8 g/l in a medium supplemented with starch (Zhang *et al*., 2002a). Lu *et al*. (2005) reported about 1.30 g of biopolymer generated from 1 L fermented broth of *Enterobacter aerogenes*. The yield of carbohydrate biopolymer secreted by *Enterobacter cloacae* WD7 was 2.27 g/l (Prasertsan *et al*., 2006). The yield of bioflocculant from *Klebsiella mobilis* using diary wastewater was 2.58 g in 1L of fermented broth (Wang *et al*., 2007). In the case of the work done on *Aeromonas* sp. 2.25 g/l of purified biopolymer was recovered (Li *et al*., 2007). The yield of the purified novel bioflocculant MMF1 produced by multiple-microorganism consortia using brewery wastewater as carbon source was 15 g/l (Zhang *et al*., 2007). Li *et al*. (2010) reported that 14.5 g of purified bioflocculant was produced from *Agrobacterium* sp. M-503 from 1 L of fermentation broth while Cosa *et al*. (2011) reported that 0.264 g/l of purified bioflocculant was recovered from a fermented culture of *Virgibacillus* sp. Rob. For a bioflocculant yield from *Bacillus mojavensis* strain 32A, 5.2 g was recovered from 1 L of fermented culture

(Elkady *et al*., 2011). The yield of purified bioflocculant obtained from 1 L of fermented broth of a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 was 2.06 g (Wang *et al*. 2011).

CHAPTER THREE

Production and characterization of bioflocculant from *Halomonas* sp. Okoh isolated from sediment of Algoa Bay in Eastern Cape

Abstract

The flocculating efficiency and physiochemical properties of bioflocculant produced by *Halomonas* sp. Okoh were investigated. The production of the bioflocculant was optimal when inoculum size of 2%, glucose and ammonium sulphate, as the sole carbon and nitrogen sources, were used as culture conditions. The medium was adjusted to pH 4.0 and using Al^{3+} as the coagulating aid, the flocculating activity of about 93.9% was obtained. After purification, a yield of about 1.213 g was recovered from 1 L fermented broth. Jar test experiment revealed the optimum dose of the bioflocculant for the flocculation of 66.1% to be 0.2 mg/ml. The thermostable bioflocculant retained about 74% of its flocculating activity after treatment at 100^{o}C for 30 min. The chemical analysis revealed the bioflocculant to be composed of sugar (26.5%), protein (2.64%) and uronic acid (13.3%). The FTIR spectrum of the purified bioflocculant revealed the presence of hydroxyl and carboxyl as the functional groups in its molecule. Thermo gravimetric analyses showed that about 67.5% of its weight remained after being heated up to 500^{o}C. SEM images revealed the amorphous structure of the bioflocculant and kaolin clay before and after flocculation. These results suggested this bioflocculant could be applied in wastewater treatment and various industrial processes.

Key words: Bioflocculant, *Halomonas* sp. Okoh, flocculating activity

3.1. Introduction

Flocculation was first reported in 1876, by Louis Pasteur (Salehizadeh and Shojaosadati, 2001). The word flocculation is synonymous to agglomeration. Flocculants are kind of materials that are used to separate solid-liquid suspension (Sharma *et al*., 2006). They act on a molecular level on the surface of the particles to reduce repulsive forces and increase attractive forces (Sharma *et al*., 2006). According to Abdel-Aziz *et al*. (2011), the term flocculation is used to describe the aggregation of suspended particles to form flocs with other sediments in the solution.

The usage of commercial flocculating agents such as polyacrylamide, ferric chloride and aluminium salts are common because of their high flocculating performance and time saving merits (Zufarzaana *et al*., 2012). However, their usage had been associated with some humans and environmental problems (Zheng *et al*., 2008). In contrast, bioflocculants are kinds of metabolites which are produced by microorganisms such as bacteria, fungi, algae and actinomycetes during their growth (Deng *et al*., 2003). They are safe, degradable, lack secondary pollution (Salehizadeh and Shojaosadati, 2001).

Presently, many research institutes have considered the development of a flocculant produced from microorganisms as one of their major research work in order to produce bioflocculant in large-scale and to replace chemical synthetic flocculants (Zhi *et al*., 2010). The major limiting factors affecting their industrial application are low flocculating activity and yield (Mabinya *et al*., 2011).

Over the past decades, screening for bioflocculant-producing microorganisms with high flocculating capability, optimization of culture conditions for a high yield and reduced cost have been the topic of interest (Mabinya *et al*., 2011).

According to Salehizadeh and Shojaosadati (2001), the main components of bioflocculants are protein, polysaccharide, nucleic acid and other macromolecular compounds. The characteristics of flocculant are dependent on their composition (Gao *et al*., 2006).

As a result of the advantages attributed to bioflocculants, they have been considered to circumvent the problems associated with chemical synthetic flocculants (Li *et al*., 2009a). Therefore, they have been considered as replacements for chemical flocculants used in wastewater treatment and fermentation processes (Mao *et al*., 2011).

Marine bacteria like *Halomonas* strains are kind of microorganisms that can adapt to high pressure and low temperature conditions. They are halophilic, gram-negative rods whose species are widely distributed in hyersaline habitats (Bouchotroch *et al*., 2001). They are versatile in terms of their ability to grow in a variety of temperature and pH conditions.

Although some studies have been conducted on this strain that looked into culture conditions for bioflocculant production, the present study is aimed at re-evaluating the optimization of culture conditions and conduct further investigation on the physicochemical properties and composition of a bioflocccculant produced by *Halomonas* sp. Okoh.

3.2. Materials and Methods

3.2.1. Source of bacteria

The test bacteria was isolated from the sediment of Algoa Bay in the Eastern Cape Province of South Africa and maintained in 20% glycerol at -80^{o}C as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa.

3.2.2. Growth media and cultivation conditions

The growth medium for bioflocculant production was composed of glucose (20 g), $MgSO_4.7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (0.2 g), K_2HPO_4 (5 g), urea (0.5 g), yeast extract (0.5 g) and KH_2PO_4 (2 g) in a litre of filtered seawater at pH 4 and sterilized by autoclaving (Zhang *et al*. 2007). The culture was incubated at 28^oC in a shaker at 160 rpm for 5 days and centrifuged at 4000 × g for 30 min at 4^oC to sediment the cells. Two millilitres of the cell free culture supernatant was used to determine flocculating activity.

3.2.3. Determination of flocculating activity

Kaolin clay was used as the test material and flocculating activity of the bioflocculants was determined according to the method described by Kurane *et al*. (1994) with minor modifications. A concentration (4 g/l) of kaolin suspension was made. One hundred millilitres of the kaolin suspension was measured into 250 ml flask, 3 ml of 1% $CaCl_2$ and 2 ml of culture supernatant were then added. The mixture was agitated vigorously for 60 sec and then poured into 100 ml measuring cylinder and allowed to sediment for 5 min. The optical density (OD) of the clarified supernatant was measured at 550 nm with a UV spectrophotometer (Thermo Spectronic, USA) and the flocculating activity determined as follows:

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

where A and B are optical densities of control and sample measured at 550 nm, respectively.

3.2.4. Culture conditions for bioflocculant production

3.2.4.1. Effect of inoculum size for bioflocculant production

Optimum inoculum size was evaluated by inoculating different amounts (1, 2, 3, 4, and 5 %v/v) of culture broths after incubating for 18 h into the growth medium and further incubated in a shaker for 5 days, 28^{o}C at 160 rpm (Ugbenyen *et al*., 2012). The flocculating activity was determined in the same manner as described in Section 3.2.3.

3.2.4.2. Effect of carbon and nitrogen sources on bioflocculant production

Using the description of Lachhwani (2005), the effects of different carbon and nitrogen sources on bioflocculant production by the test bacteria were evaluated. Carbon sources such as glucose, fructose, galactose, xylose, sucrose, maltose and starch were used. The nitrogen sources were ammonium nitrate, ammonium chloride, sodium nitrate, (inorganic nitrogen sources) and tryptone, urea, casein, and peptone (organic nitrogen sources).

3.2.4.3. Effect of agitation speed on bioflocculant production

Different shaker speeds for incubation ranging from 120-200 rpm were used to determine the optimal speed for bioflocculant production (Zhang *et al*., 2007). The culture broth was cultivated for 5 days and the flocculating activity was determined in the same manner as described in Section 3.2.3.

3.2.4.4. Effect of incubation temperature on bioflocculant production

Cultures were incubated at different temperatures (25°C, 28°C, 31°C, 34°C, and 37°C) for 5 days (Zhang *et al*., 2007). The flocculating activity of the bioflocculant was determined at room temperature using the same method previously described in Section 3.2.3.

3.2.5. Extraction and purification of the bioflocculant

After 5 days of fermentation, the culture broth was centrifuged at 4 000 × g, 4^{o}C for 30 min in order to remove bacterial cells. To remove the insoluble substances, one volume of distilled water was added to the supernatant phase and then centrifuged at 4, 000 × g for 15 min. Two volumes of ethanol were added to the supernatant, and the solution was agitated and left standing at 4^{o}C for 12 h. To obtain the crude bioflocculant, the precipitate was vacuum dried. The purification of the crude bioflocculant was done according to the method described by Salehizadeh *et al.* (2000). The obtained precipitate was re-dissolved in distilled water (1% w/v) and one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v*)* was added. After agitation, the mixture was then left standing at room temperature for 12 h. The upper phase was centrifuged at 4 000 × g for 15 min at 4^{o}C and dialyzed against distilled water overnight. The bioflocculant solution was then vacuum-dried in order to obtain a purified bioflocculant.

3.2.6. Effect of cations on flocculating activity of both crude and purified bioflocculant

The effects of cations on flocculating activity of the bioflocculant was done in a similar way as described above for flocculating activity except that $CaCl_2$ solution was replaced by various salt solutions. Solutions of 1% (w/v) of different salts NaCl, KCl, $LiCl_2$, $MgCl_2$,

$MnCl_2.4H_2O$, $BaCl_2$, $AlCl_3$ and $FeCl_3 \cdot 6H_2O$ were used as cation sources according to He *et al*. (2010).

3.2.7. Effect of pH on the flocculating activity of crude and purified biofloculant

A solution of bioflocculant concentration 0.1 mg/ml was made. The pH of the kaolin solutions were adjusted ranging from 2 -12 with HCl and NaOH accordingly (Xiong *et al*., 2010). The flocculating activity of the bioflocculant was determined at each of this pH value.

3.2.8. Jar test experiment

Different concentrations of the bioflocculant solution (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were prepared and evaluated to determine the optimum dose of the purified bioflocculant for flocculation of kaolin clay suspension. Four grams of kaolin clay was weighed and dissolved in 1 L of distilled water. Three millilitres of 1% (w/v) $CaCl_2$ and 2 ml of bioflocculant solution were both added to 100 ml kaolin suspension inside 500 ml beakers. The solution was agitated at 200 rpm for 3 min and then reduced to 45 rpm and allowed to agitate further for 10 min (Wang *et al*., 2010). The solution was poured into 100 ml measuring cylinder and allowed to settle for 10 min and 2 ml of the supernatant was carefully withdrawn and the flocculating activity was read at 550 nm.

3.2.9. Composition analysis of the purified bioflocculant

The total protein content of the purified bioflocculant was determined by Bradford method (1976), using bovine serum albumin as the standard solution. The total sugar content was determined by phenol-sulphuric acid method as described by Chaplin and Kennedy (1994)

using glucose as a standard solution. The uronic acid was determined by carbazole method according to Bitter and Muir (1962).

3.2.10. Fourier transfom infrared spectroscopy (FTIR)

The functional groups of the bioflocculant were determined using a Fourier transform infrared spectroscopy (Perkin Elmer System 2000, FT-IR, England). The bioflocculant was ground with KBr salt at 25^{o}C and pressed into a pellet for FTIR analysis over a wave number of 4000-370 cm^{-1} (Wang *et al*., 2011).

3.2.11. Thermo-gravimetric analysis (TGA)

Ten milligrams of the bioflocculant was weighed and analysed with TGA analyzer (STA 449/C Jupiter, Netzsch, Germany Perkin Elmer TGA7 Thermo gravimetric Analyzer, USA) over a temperature range of 40-500^{o}C with a heating rate of 10^{o}C per minute under a constant flow of nitrogen gas (Wang *et al*., 2011).

3.2.12. Scanning electron microscopy (SEM)

The surface morphology structures of the purified bioflocculants were studied with scanning electron microscope (JSM-6390 LV, Japan). The SEM images of bioflocculant powders, kaolin clay before and after flocculation were scanned (He *et al*., 2010).

3.3. Results and Discussion

3.3.1. Effect of inoculum size on bioflocculant production by Halomonas sp. Okoh

The effect of different inoculums size on bioflocculant production by the the *Halomonas* sp was evaluated and results are presented in Figure 3.1. The flocculating activities of the bioflocculant were as follows: 50.3% when 1% (v/v) inoculum size was used; 88.0% with 2% (v/v); 69.0% with both 3 and 4% (v/v) and 75% with 5% (v/v). The highest flocculating activity was observed with 2% (v/v) of inoculum size and it was used as standard for this study. Similarly, Zhong and Yoshida (1995) reported that 2% (v/v) inoculum size was utilized for bioflocculant production by *Perilla sp.* However, on the contrary, Gao *et al*. (2006) reported that 1% (v/v) of inoculum size was used for the production of the bioflocculant by *Vagococcus* sp. W31. Also, Patil *et al*. (2009) reported that inoculum size of 5% (v/v) was utilized by *Bacillus subtilis* for bioflocculant production. Furthermore, Xiong *et al*. (2010) reported that inoculum size of 4% (v/v) was utilized by *Bacillus licheniformis* CGMCC 2876 for bioflocculant production. Small or large inoculum sizes will bring about a decrease in flocculating activity (Zhang *et al*., 2007).

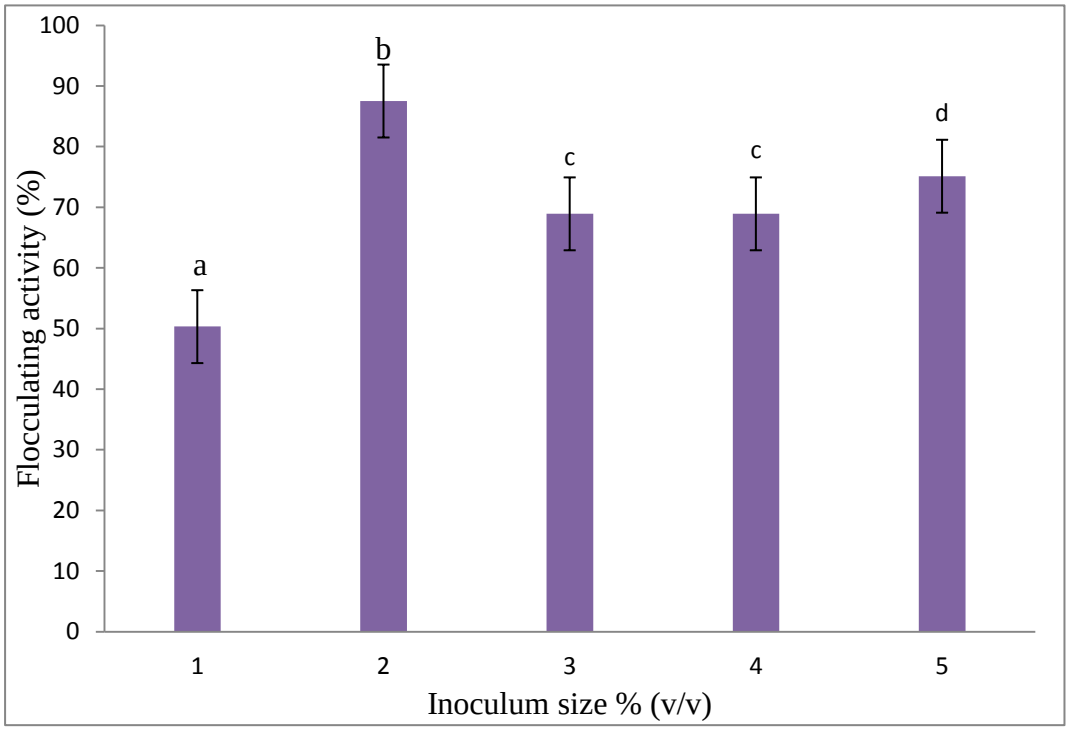

Figure 3.1: Effect of inoculum size on bioflocculant production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.2. Effect of carbon source on bioflocculant production

The effects of different carbon sources on bioflocculant production were investigated. Nakamura *et al*. (1976) stated that carbon source has a significant impact on bioflocculant production. Figure 3.2 shows the flocculating activities of the cell free culture product after incubated for 5 days. Among the different carbon sources tested, the flocculating activity was maximal when glucose was used as the sole carbon source of choice with flocculating activity of 87.5%, followed by starch at 52.4%. It was noticed that bioflocculant production was very poor with the other carbon sources tested and the lowest flocculating activity of 13.5% was obtained with fructose. Similarly, among all the carbon sources tested, maltose, mannose and glucose were preferrable carbon sources for bioflocculant production by *Chryseobacterium daeguense* W6 (Liu *et al*., 2010). Glucose was chosen as the carbon source for bioflocculant

MBF-W6 production *Chryseobacterium daeguense* W6 because it is cheap and exhibited flocculating activity of 88.7% (Liu *et al*., 2010). Deng *et al*. (2005) reported that glycerol, starch, glucose and sucrose favoured bioflocculant production by *Aspergillus parasiticus*. On the contrary, Zhang *et al*. (2002b) reported that glucose completely inhibited the production of bioflocculant by *Sorangium cellulosum* instead; flocculating activity was optimum (about 96%) with starch as the sole carbon source for this organism. In the case of bioflocculant production by *Rhodococcus erythropolis,* the preferable carbon source was ethanol (Kurane *et al*., 1994). Also, Shih *et al*. (2001) reported about *Bacillus licheniformis* where bioflocculant production was highly favoured when multiple carbon sources (glutamic acid, citric acid and glycerol) were used compared to the low flocculating activities observed when glucose, lactose and fructose were utilized. Li *et al*. (2007) reported that sucrose was the preferable carbon source of choice in bioflocculant production by *Aeromonas* sp. N11. In another study conducted by Gong *et al*. (2008) with *Serratia ficaria,* it was reported that lactose was the preferable carbon with a flocculating activity of 97.15% compared to other carbon sources such as lactose, glucose and ethanol with a flocculating activity more than 90%. He *et al*. (2004) reported that gluocose, fructose and sucrose supported both cell growth and bioflocculant REA-11 production from *Corynebacterium glutamicum* CCTCC M201005. The highest flocculating activity was observed with sucrose, thus it was used as a sole carbon source. Xiong *et al*. (2010) reported that sucrose was the carbon source of choice for bioflocculant production by *Bacillus licheniformis* CGMCC 2876.

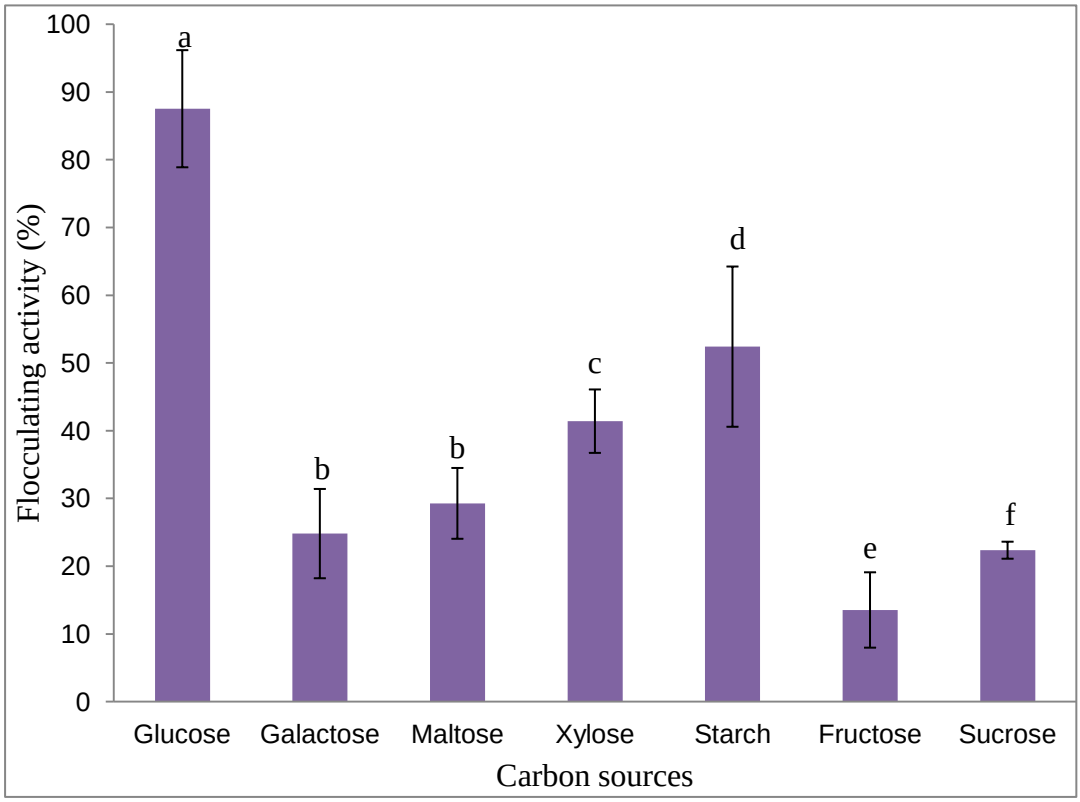

Figure 3.2: Effect of carbon source on bioflocculant production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.3. Effect of nitrogen source on bioflocculant production

Nitrogen source in the medium is an important factor for cell growth and development (Ntsaluba *et al*., 2011). The effect of nitrogen sources on bioflocculant production was also investigated. From Figure 3.3, the highest flocculating activity of 91.2% was observed with ammonium sulphate as a nitrogen source. Other nitrogen sources which supported bioflocculant production includes; ammonium nitrate (52.3%), yeast extract (60.8%), peptone (88.1%), ammonium chloride (79.6%) and mixed nitrogen (ammonium sulphate, yeast extract and urea) (87.5%). The bioflocculant production was completely inhibited when tryptone was used with flocculating activity of 16.7%. Several studies have reported incidences where some microorganisms utilised either organic or inorganic nitrogen source or combination of

both (Deng *et al*., 2005; Ugbenyen *et al*., 2012; Xia *et al*., 2008). The production of bioflocculant by *Aspergillus parasiticus* was supported by both organic nitrogen source (peptone) with a flocculating activity of 98% and inorganic nitrogen source (sodium nitrate) with flocculating activity of 96%. In the same study, bioflocculant production was completely inhibited when ammonium sulphate or beef extract was used (Deng *et al*., 2005). In the case of bioflocculant produced from *Serratia ficaria,* the highest bioflocculant production was observed when two nitrogen sources (urea and beef extract) were used while Ugbenyen *et al*. (2012) reported that the bioflocculant production by *Cobetia* sp. was at peak when multiple nitrogen source (ammonium sulphate, urea, yeast extract) were utilised as nitrogen source. According to the findings of Nakamura *et al*. (1976), bioflocculant production by *A. sojae* was highly favoured when a mixture of casein, glutamic acid and polypeptone was used simultaneously in the medium as a nitrogen sources. Liu *et al*. (2010) reported that all the inorganic nitrogen sources tested inhibited flocculating activity of the bioflocculant MBF-W6 from *Chryseobacterium daeguense* W6, and among the organic nitrogen sources investigated; tryptone gave the highest flocculating activity. He *et al*. (2010) reported that ammonium chloride was the nitrogen source of choice for bioflocculant production by *Halomonas* sp. V3a'. In *Arthrobacter* sp. Raats, the combination of urea and ammonium sulphate resulted into the highest flocculating activity of 83.4% (Mabinya *et al*., 2012). This showed that the bioflocculant production from this strain was supported by a mixture of inorganic and organic nitrogen source. According to Patil *et al*. (2010), yeast extract, urea and ammonium sulphate supported the production of bioflocculant produced by *Azotobacter indicus*. The flocculating activity of bioflocculant REA-11 produced by *Corynebacterium glutamicum* CCTCC M201005 was maximal when a complex nitrogen source of urea and corn steep liquor was used (He *et al*., 2004). Urea was the favourable nitrogen source which favoured bioflocculant production by *Bacillus licheniformis* CGMCC 2876 (Xiong *et al*., 2010).

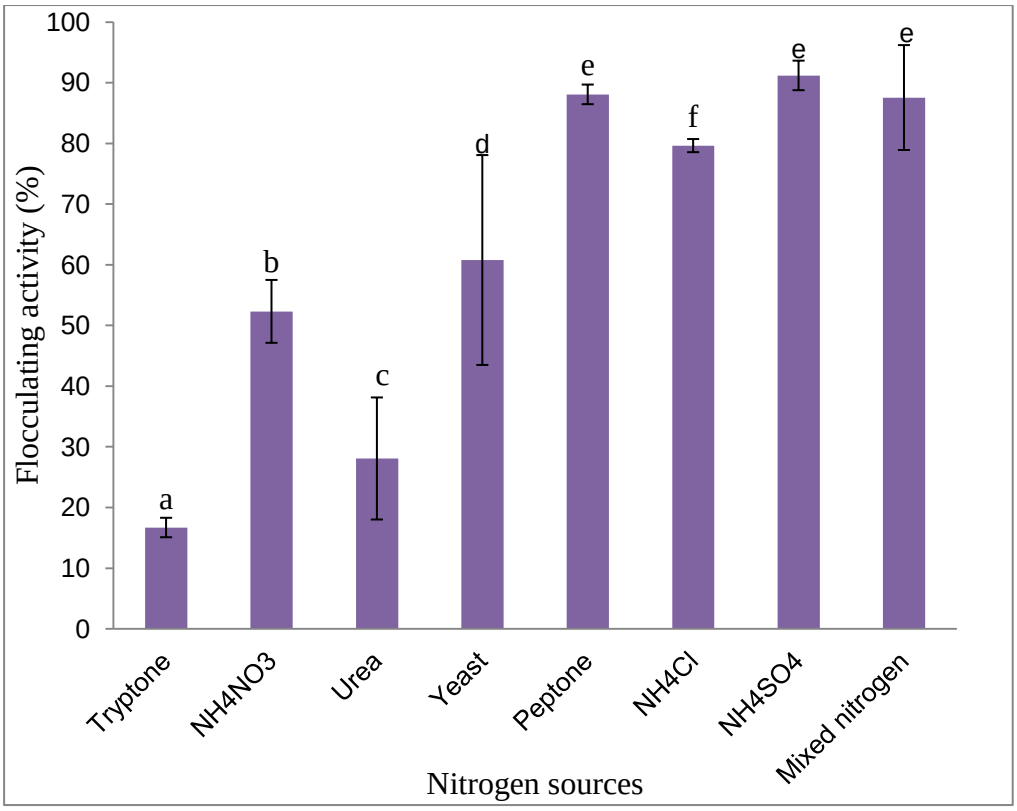

Figure 3.3: Effect of nitrogen source on the flocculating activity of the bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.4. Effect of initial pH on bioflocculant production

The effect of initial pH of production medium on bioflocculant production by *Halomonas* sp. Okoh was examined and is represented in Figure 3.4. The effect of the initial pH ranging from 2-12 was investigated on the bioflocculant production. The initial pH of the medium plays an important role in the flocculation process (Yokoi *et al*., 1997). From the results showed in Figure 3.4, the highest flocculating activity (87.9%) was obtained at pH 4. The lowest flocculating activity (56.3%) was recorded at pH 9. These results showed that bioflocculant production by this bacterium could occur under a wide pH range of 2-12. In the case of *Aspergillus sojae*, alkaline condition was reported to be favourable for its

bioflocculant production (Nakamura *et al*., 1976). Gong *et al*. (2008) reported that *Serratia ficaria* optimally produced the bioflocculant in the pH range of 6-8 and over a wide pH 4.5-10; the flocculating activity was 63-95%. The bioflocculant produced by *Serratia ficaria* attained the highest flocculating activity of 97.0 at pH 7 (Gong *et al*., 2008). According to Zheng *et al*. (2008), a bioflocculant produced by *Bacillus* sp. F19 had the highest flocculating activity at pH 2. Xiong *et al*. (2010) reported that the initial pH of 7.5 gave the highest flocculating activity of the bioflocculant produced by *Bacillus licheniformis* CGMCC 28576. Mabinya *et al*. (2011) reported that the flocculating activity of a bioflocculant produced by *Halomonas* sp. Okoh was maximal at pH 7, while Zheng *et al*. (2008) reported that the production of bioflocculant by *Bacillus* sp. F19 proliferates well at pH 7-12.

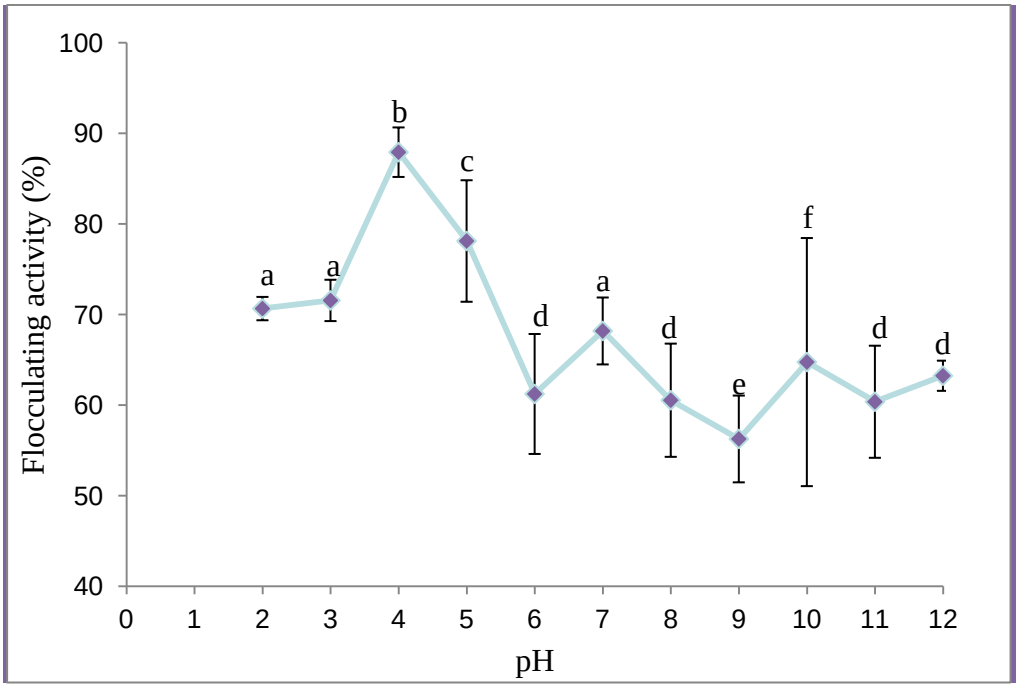

Figure 3.4: Effect of initial pH on bioflocculant production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.5. Effect of cations on the flocculating activity of the crude bioflocculant

The effect of cations on the flocculating activity of crude bioflocculant was investigated and is reported in Figure 3.5. The various cations stimulating flocculating acitivity to varying degrees as follows; Na^{+} (flocculating acitivity 17.9%), Li^{+} (flocculating acitivity 30.65%), Ca^{2+} (flocculating acitivity 87.5%), Mn^{2+} (flocculating acitivity 53.4%), Fe^{3+} (flocculating acitivity 80.8%), Al^{3+} (flocculating acitivity 93.9%), K^{+} (flocculating acitivity 69.7%), Mg^{2+} (flocculating acitivity 69.5%), and Ba^{2+} (flocculating acitivity 77.7%). Among all the cations, it was observed that Na^{+} and Li^{+} inhibited the flocculating activity of the produced bioflocculant while Al^{3+} supported the highest stimulating role of 93.9% flocculating activity. Similarly, Li *et al*. (2009b) reported that in the presence of Al^{3+}, Fe^{3+} and Ca^{2+}, the flocculating activity of the bioflocculant produced by *Bacillus circulans* was greatly enhanced. Also, Patil *et al*. (2009) reported that the flocculating activity of the bioflocculant produced by *Bacillus subtilis* was increased when Fe^{3+} and Al^{3+} were used as coagulating aid. Ugbenyen *et al*. (2012) reported that the flocculating activity of bioflocculant produced by a Cobetia sp. was about 77.4% in the presence of Al^{3+}, whereas Fe^{3+} completely inhibited flocculation. Zufarzaaana *et al*. (2012) reported that the flocculating activity of the bioflocculant produced by *Bacillus* sp. UPMB13 was inhibited by Fe^{2+} and Al^{3+} but was increased when Na^{+}, Ca^{2+} and Mg^{2+} were used as the cations. The flocculating activity of the bioflocculant produced by *Chryseobacterium daeguense* W6 cultured in low nutritional medium was stimulated in the presence of Mg^{2+}, Ca^{2+}, K^{+}, and Mn^{2+} (Liu *et al*., 2010). Among these cations, Mg^{2+} gave the highest flocculating activity (Liu *et al*., 2010). According to Wang *et al*. (2011), the role of cation in bioflocculation is to increase the initial adsorption of the bioflocculant on the kaolin particles by decreasing the distance between them. It plays this role by decreasing the negative charge of both biopolymer and the kaolin particles so that the electrostatic repulsive force between them will be reduced.

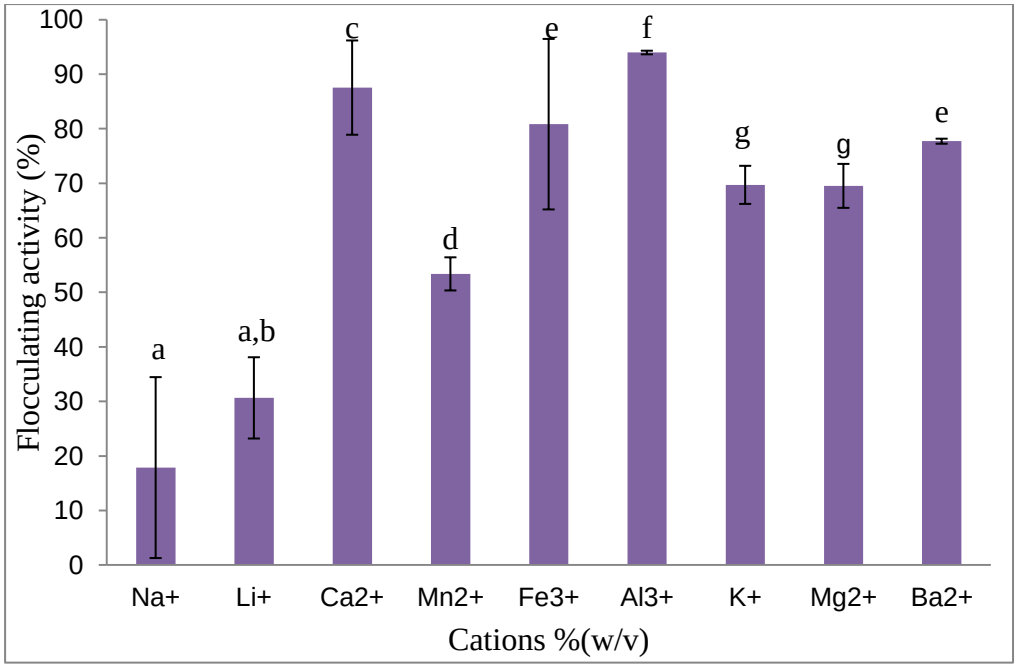

Figure 3.5: Effect of cations on the flocculating activity of crude bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.6. Effect of agitation speed on bioflocculant production

The relationship between shaker speed and bioflocculant production was investigated and is depicted in Figure 3.6. According to Salehizadeh and Shojaosadati (2001), agitation speed determines the aeration levels to the microorganisms and this affects nutrient absorption. Figure 6 shows that all the agitation speed tested resulted into flocculating activity above 60% with highest activity obtained at 160 rpm. A similar work which supported this present study is the bioflocculant production by *Bacillus licheniformis* X14 where 160 rpm gave the highest flocculating activity (Li *et al*., 2009a). On the contrary, Gao *et al*. (2006) reported that agitation speed of 150^{o}C was optimum for bioflocculant production by *Vagococcus* sp. strain W31. In another study carried out by Gong *et al*. (2008) on bioflocculant production by *Bacillus* sp. strain F19, 200 rpm was the shaker speed of choice. Also, Xiong *et al*. (2010)

documented that 200 rpm was used for bioflocculant production by *Bacillus licheniformis* CGMCC 2876.

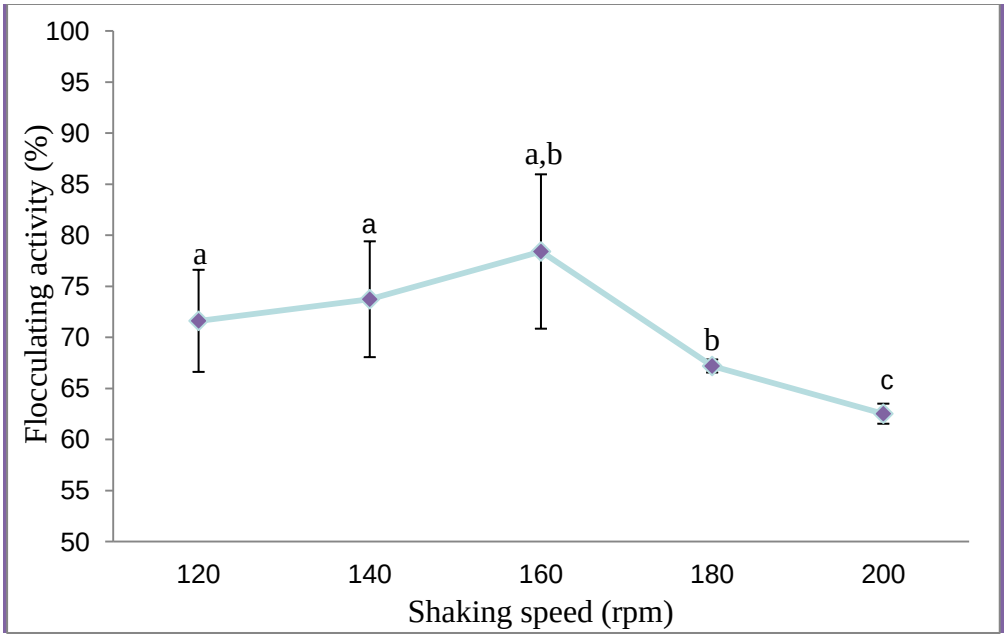

Figure 3.6: Effect of agitation speed on bioflocculant production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.7. Effect of incubation temperature on bioflocculant production

The effect of incubation temperature on bioflocculant production was evaluated and is represented in Figure 3.7. It was observed that the flocculating activity of the bioflocculant was greater than 60% in the fermentation culture temperature range of 25-37^{o}C. These results showed that the bioflocculant production can be achieved at all the temperatures investigated. Since the highest flocculation activity was attained at 28^{o}C, this incubation temperature was used throughout the experiment. Similar observations were reported in the production of bioflocculant from *Virgibaccillus* sp. Rob and *Cobetia* sp. where bioflocculant production was performed at 28^{o}C (Cosa *et al*., 2011; Ugbenyen *et al*., 2012). Xiong *et al*. (2010)

reported that the bioflocculant from *Bacillus licheniformis* CGMCC 2876 attained its maximum production at 37°C. The production of bioflocculant from *Klebsiella* sp., *Aeromonas* sp., *Bacillus firmus*, and was carried out at 30°C (Dermlim *et al*., 1999; Li *et al*., 2007; Salehizadeh and Shojaosadati, 2002) while Gao *et al*. (2006) reported the production of bioflocculant by *Vagococcus* sp. strain W31 at 25°C.

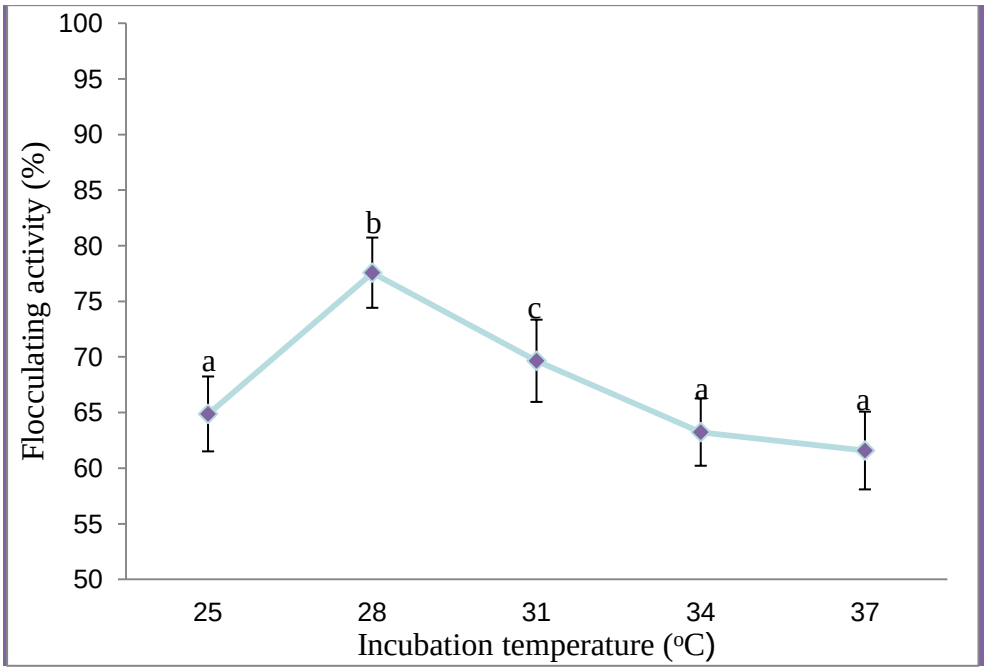

Figure 3.7: Effect of incubation temperature on bioflocculant production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.8. The yield of bioflocculant produced by Halomonas sp. Okoh

About 1.213 g of purified bioflocculant was recovered from 1 L fermented culture broth of *Halomonas* sp. Okoh. In another study, about 14.8 g of purified bioflocculant was produced by marine *myxobacterium Nannocystics* sp. NU-2 from 1 L of fermented broth (Zhang *et al*., 2002a). Lu *et al*. (2005) reported about 1.30 g of biopolymer generated from 1 L of fermented broth of *Enterobacter aerogenes*. The yield of carbohydrate biopolymer produced

by *Enterobacter cloacae* WD7 was 2.27 g/l (Prasertsan *et al*., 2006). For bioflocculant yield from *Bacillus mojavensis* strain 32A, 5.2 g was recovered from 1 L of fermented culture (Elkady *et al*., 2011).

3.3.9. Effect of bioflocculant dosage on the flocculating activity of purified bioflocculant

The relationship between bioflocculant concentration and flocculating activity of purified bioflocculant was evaluated and is depicted in Figure 3.8. The bioflocculant dosage required for optimum flocculation varies with different bioflocculants produced from different microorganisms. It was observed that the flocculating activity of the bioflocculant was optimum at 0.2 mg/ml. Further increase in concentration led to a decrease in flocculating activity. The flocculating activity remained steady between 0.5-0.7 mg/ml, showing that there is no significant different in the flocculanting activity with an increase in bioflocculant dosage. Decrease in flocculating activity observed at high dosage might be due to high viscosity generated, thus increasing the turbidity of the solution (He *et al*., 2010). Deng *et al*. (2003) reported that the optimum bioflocculant dosage required for effective flocculation of the flocculant produced by *Bacillus mucilaginosus* was 0.1 mg/l. Prasertersan *et al*. (2006) observed that 2 mg/l was the optimum dosage required by the biopolymer produced by *Enterobacter cloacae* for effective flocculating activity. Wang *et al*. (2011) documented that 12 mg/l was the optimum dosage required by bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6.

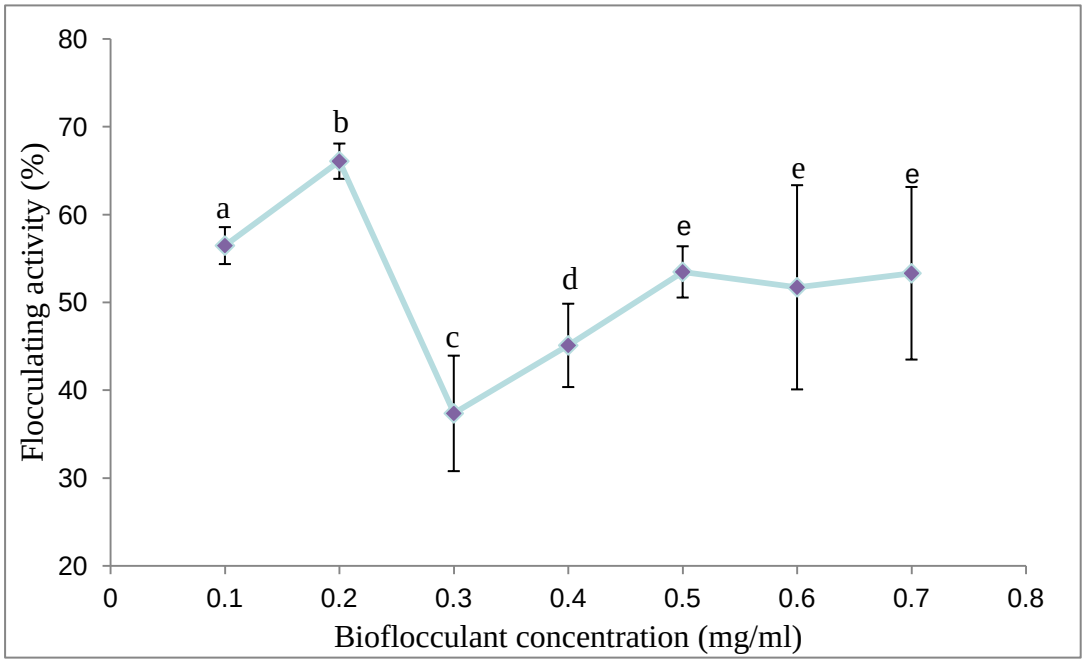

Figure 3.8: Effect of bioflocculant dosage on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.10. Effect of cations on the flocculating activity of purified bioflocculant

The effect of cations on the flocculating activity of purified bioflocculant was evaluated and is represented in Figure 3.9. It was noticed that the presence of K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+} and Al^{3+} stimulated the flocculating activity of the purified bioflocculant. The flocculating activity was maximal with Al^{3+} with flocculating activity of 78.4%. The flocculating activity was inhibited in the presence of Li^+, Na^+ and Fe^{3+}. According to Salehizadeh and Shojaosadati (2001), the role of cations is to stimulate flocculating activity by neutralizing and stabilizing the negative charge of functional group and by forming bridges between particles. Shih *et al*. (2001) reported that the flocculating activity of flocculant by *Bacillus licheniformis* CCRC 12826 was stimulated in the presence of Ca^{2+}, Fe^{3+}, Al^{3+}. The flocculating activity of carbohydrate biopolymer produced by *Enterobacter cloacae* WD7

was stimulated in the presence of Cu^{2+} (Prasertsan *et al*., 2006), while for bioflocculant MBF-W5 produced by *Chryseobacterium daeguense* W6 it was stimulated by all the cations tested except F^{3+} which resulted in a flocculating activity of 50.01%. It was also observed the flocculating activity was highest without cation, showing that the bioflocculant is cation-independent (Liu *et al*., 2010).

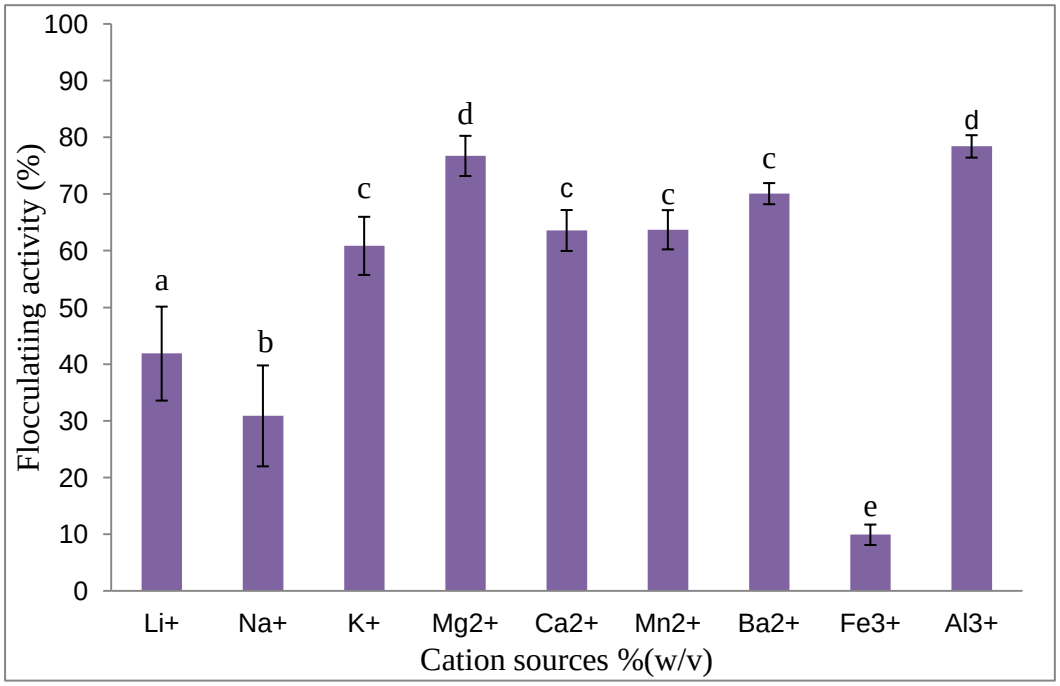

Figure 3.9: Effect of cations on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.11. Effect of pH on the flocculativity activity of purified bioflocculant

The effect of pH on flocculating activity of the purified bioflocculant is summarized in Figure 3.10. It was observed that the bioflocculant had activity at a wide pH range of 2-7 with the flocculating activity more than 65%. Increasing the pH more than 7 resulted in a decreased flocculating activity. The flocculating activity was completely inhibited at pH 11 and 12

suggesting that the OH^- absorbed at alkaline condition destabilized complex formation (Zurfarzaana *et al*., 2012). These results indicated that the bioflocculant is more active at acidic pH conditions with the highest flocculation activity of 78% at pH 3. Similarly, He *et al*. (2004) reported that the bioflocculant REA-11 produced by *Corynebacterium glutamicum* CCTCC M21005 had a pH range of 3-6. Decreasing the pH lower than 3 or increasing it more than 6 resulted into lower flocculating activity. Wang *et al*. (2011) reported that the purified bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 *and Bacillus sphaeicus* F6 maintained high flocculating activity of above 90% over the pH range of 7-9. The decrease in flocculating activity observed at alkaline conditions might be due to restabilization of kaolin particles and thereby increasing the turbidity of the kaolin suspension (Zurfarzaana *et al*., 2012). The pH of the solution determines both floc formation and stability of suspended particles (Wang *et al*., 2011). Li *et al*. (2009b) reported the flocculating activity of the bioflocculant produced by *Bacillus circulans* was optimal within a wide pH range of 4-10. The flocculating activity of a biopolymer produced by *Bacillus* spp. UPMB13 work best at pH 4-8 (Zarfarzaana *et al*., 2012). Fujita *et al*. (2000) reported that the bioflocculant produced by *Citrobacter* sp. TKF04 flocculated best within a pH range of 2-6. According to the findings of Prasertsan *et al*. (2006), the biopolymer flocculated the kaolin suspension over a wide pH range of 2-8. He *et al*. (2010) reported that the bioflocculant produced by *Halomonas* sp. V3a' had a wide pH range of 3-11 with a flocculating activity of more than 80%.

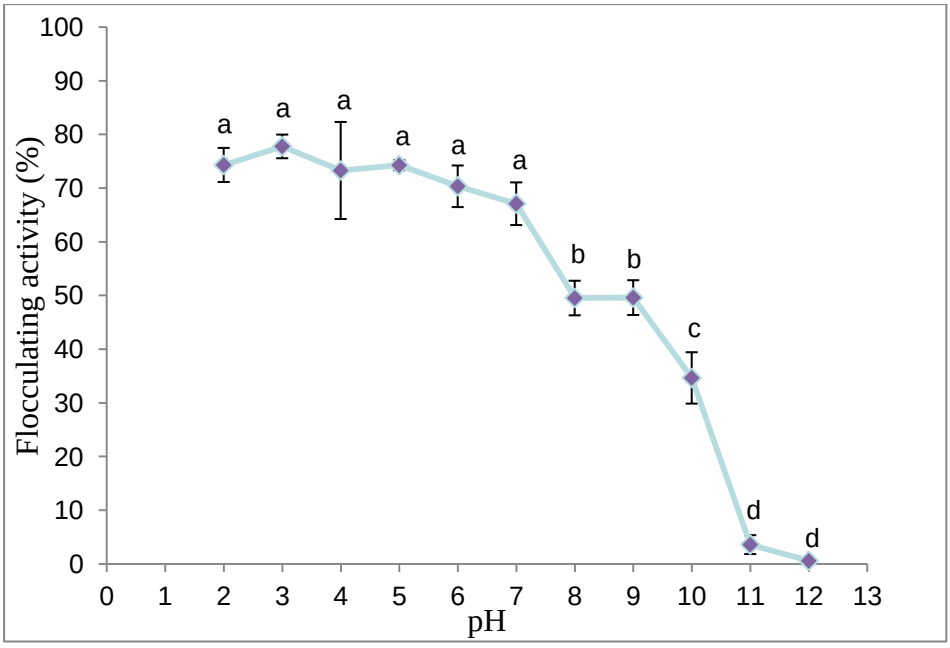

Figure 3.10: Effect of pH on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.12. Effect of temperature on the flocculating activity of purified bioflocculant

The thermal stability property of the purified bioflocculant was investigated and is represented in Figure 3.11. The bioflocculant solution was heated at different temperatures from 50-100^{o}C. It was observed that the flocculating activity was optimum at 50^{o}C with flocculating activity of 89.5%. Increasing the temperature to 100^{o}C resulted into 15.6% decrease in flocculating activity. These results showed the bioflocculant retained its high flocculating activity of 74% at 100^{o}C suggesting that the bioflocculant composed mainly of polysaccharide. Salehizadeh and Shojaosadati (2002) reported that a thermostable bioflocculant produced by *Bacillus firmus* only retained about 50% of flocculating activity after being heated in boiling water for 15 min. Also at high temperature, the rate of reaction increased because the collision frequency between the particles and the molecules (bioflocculant molecules) will be increased and thus, maintaining a high flocculating activity

at 100°C (Gong *et al*., 2008; Lu *et al*., 2005). Gao *et al*. (2006) reported that the bioflocculant produced from *Vagococcus* sp. W31 retained its flocculating activity of 86.5% at 100° C. Li *et al*. (2010) reported that the flocculating activity of the bioflocculant produced by *Agrobacterium* sp. M-503 maintained high flocculating activity at 70-121°C. He *et al*. (2004) reported about a thermostable REA-11 produced by *Corynebaterium glutamicum* CCTCC M201005 which could withstand temperatures of up to 80°C for 1 h without any significant effect on the flocculating activity of REA-11. Further increases in temperature to 100°C for 1 h decreased the flocculating activity of REA-11. Xiong *et al*. (2010) reported that the bioflocculant produced by *Bacillus licheniformis* CGMCC 2876 maintained high flocculating activity of over 90% when heated below 80°C for 30 min. The thermostable-bioflocculant HBF-3 produced by deep-sea bacteria mutant *Halomonas* sp. V3a' retained more than 90% of its flocculating activity within the temperature range of 4-40°C (He *et al*., 2010).

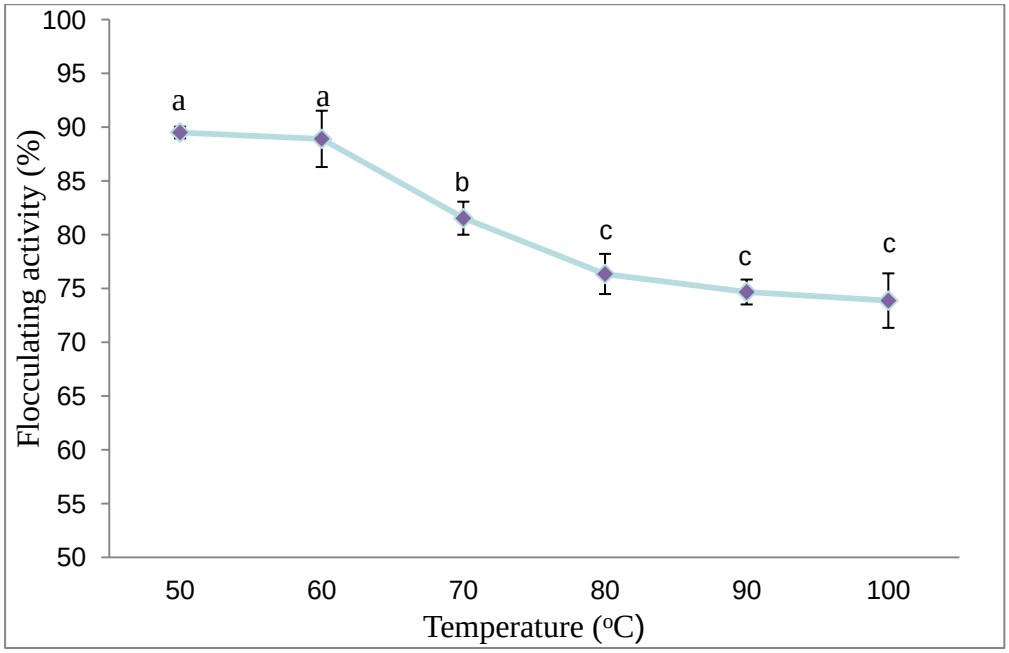

Figure 3.11: Effect of temperature on the flocculating activity of bioflocculant produced by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

3.3.13. Chemical composition of purified bioflocculant

Chemical analyses showed that the purified bioflocculant was composed of 2.64% total protein content, 26.5% total sugar content and 13.3% uronic acid. Similarly, Prasertsan *et al*. (2006) reported that the purified bioflocculant produced *by Enterobacter cloacae* WD7 composed of acid heteropolysaccharide whose units are neutral sugars (29.4% w/w), uronic acids (14.2% w/w) and amino sugars (0.93 %w/w). On the contrary, Deng *et al*. (2005) reported that the bioflocculant produced by *Aspergillus parasiticus* composed of sugar (76.3% w/w), protein (21.6% w/w). Zheng *et al*. (2008) reported that the bioflocculant MBFF19 produced by *Bacillus* sp. F19 composed of neutral sugar (3.6% w/w), uronic acid (37% w/w), amino sugar (0.5% w/w) and protein (16.4% w/w). Li *et al*. (2010) reported that the bioflocculant produced by *Agrobacterium* sp. M-503 consisted of neutral sugar (85% w/w), uronic acid (9.9% w/w), aminosugar (2.1% w/w) and protein (3.0 % w/w).

3.3.14. Determination of functional group by FTIR

In order to correlate the flocculating activity of the bioflocculant and functional groups in the molecule, FTIR analysis of the purified bioflocculant was done. The infrared spectra observed at 3481 cm^{-1} and 3414 cm^{-1} were characteristic of hydroxyl group (Xiong *et al*., 2010). Asymmetrical stretching peak was observed at 1639 cm^{-1} (Xiong *et al*., 2010). The weak vibration peak at 1401 cm^{-1} indicated weak symmetrical stretching which further confirmed the presence of uronic acid in the bioflocculant molecule (Liu *et al*., 2010). The absorption peaks within 1000-1100 cm^{-1} showed the presence of all sugar derivatives (Liu *et al*., 2010). The FTIR analysis results from this purified bioflocculeant was consistent with some results obtained from other bioflocculants (Li *et al*., 2010; Kumar *et al*., 2004; Salehizadeh and Shojaosadati, 2001; Xiong *et al*., 2010).

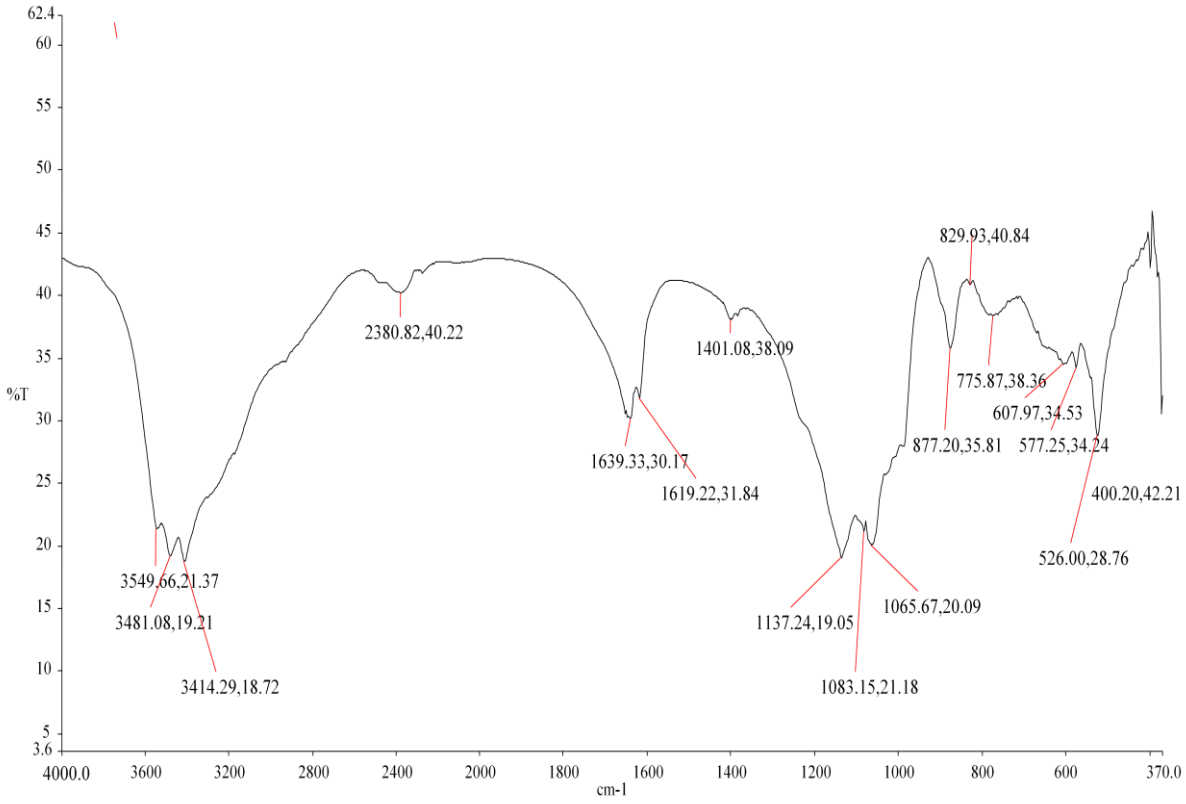

Figure 3.12: Fourier-transform intrared (FTIR) spectroscopy of purified bioflocculant produced by *Halomonas* sp. Okoh.

3.3.15. Thermogravimetric analysis

The purified bioflocculant was subjected to high temperature of about 500^{o}C. The initial temperature was 40^{o}C and increasing the temperature up to 200^{o}C resulted in 18% decrease in weight of the bioflocculant. This initial loss of weight can be due to moisture content in the molecule. This moisture content might be due to the presence of carboxyl group in the molecule. The higher the number of carboxyl groups, the more the moisture content (Kumar and Anand, 1998). Further increase in temperature to 300^{o}C brought about 30% decreases in weight. When the temperature was increase to 500^{o}C, about 32.5% weight loss was observed. Yim *et al*. (2007) reported that the bioflocculant p-KG03 produced by a marine dinoflagellate *Gyrodinium impudicum* KG03 the initial weight loss was observed between 40-230^{o}C. There

was dramatically declined in weight loss of this bioflocculant at about 310^{o}C. Kumar *et al*. (2004) reported about 10% decreased in weight of bioflocculant produced by haloalkalophilic *Bacillus* sp. I-450. With further increase in temperature to 290^{o}C, huge degradation of the bioflocculant was observed which resulted into about 55% decreased in weight. At 600^{o}C, about 70% weight decrease was observed.

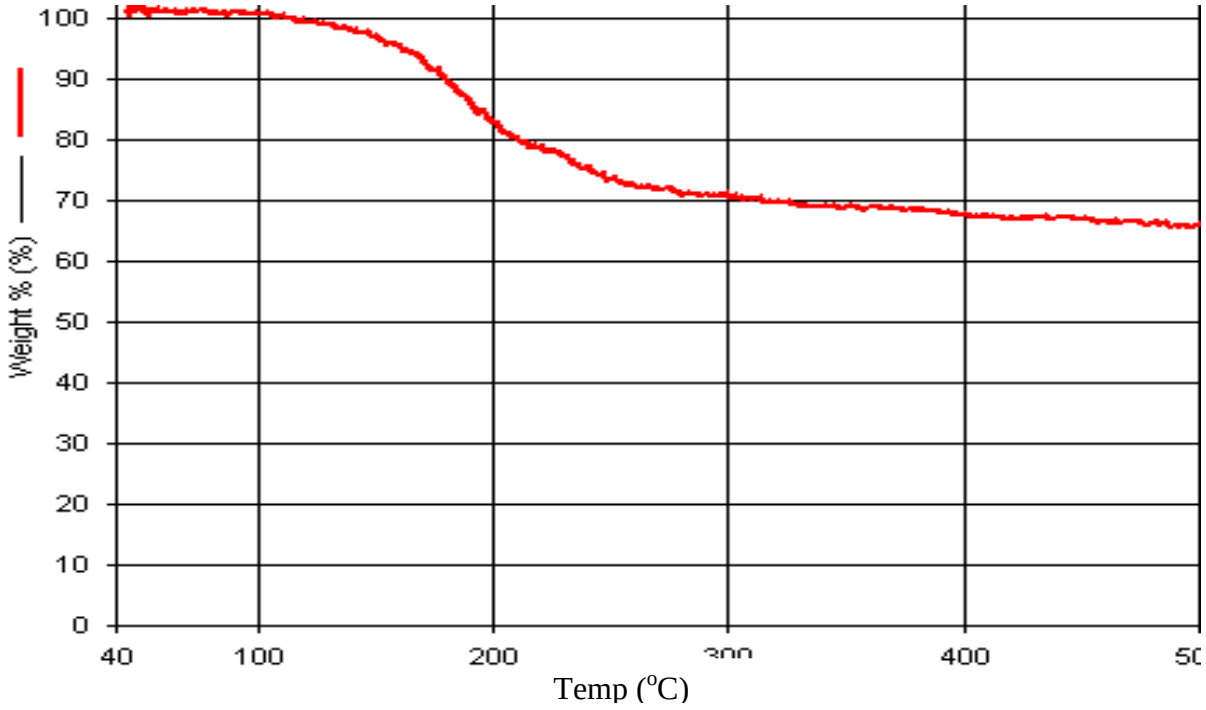

Figure 3.13: Thermo gravimetric analyses of purified bioflocculant produced by *Halonomas* sp. Okoh.

3.3.16. SEM observations of the bioflocculant

SEM images of the bioflocculant and kaolin particles before and after flocculation were observed. Figure 3.14(A) revealed how the structure of bioflocculant is stretched out like a thread and Figure 3.14(B) showed how the kaolin particles were scattered before flocculation. Figure 3.14(C) showed how the bioflocculant efficiently connected the scattered kaolin particles and formed aggregates.

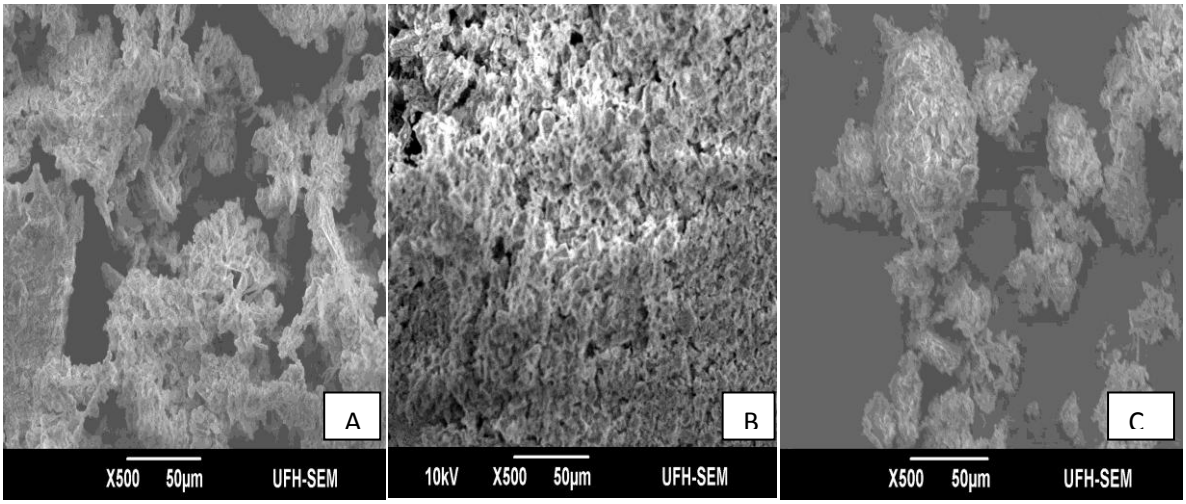

Figure 3.14: Bioflocculant powder A, Kaolin clay B, bioflocculant and kaolin clay after flocculation C.

3.4. Conclusion

The thermostable glycoprotein bioflocculant from *Halomonas* sp. Okoh was optimally produced when glucose and ammonium sulphate were used as sole carbon and nitrogen sources, respectively. The flocculating activity of both the crude and purified bioflocculant was stimulated optimally when Al^{3+} was used as coagulating aid. After purification, the bioflocculant yield was about 1.213 g in 1 L of fermentation broth. The optimum dosage for effective flocculation was 0.2 mg/ml and the bioflocculant had a pH range of 2-7 with flocculating activity of about 78%. FTIR analyses showed the presence of hydroxyl, carboxyl and amino group as the main functional groups which are responsible for its flocculation process. The bacteria appear to be a very useful producer of bioflocculant that could stand as alternative to conventional inorganic and synthetic flocculants.

CHAPTER FOUR

Production and characterization of bioflocculant from *Micrococcus* sp. Leo isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa

Abstract

The culture conditions and physicochemical properties of the bioflocculant produced by *Micrococcus* sp. Leo were investigated. Optimum culture conditions for bioflocculant production included 2% (v/v) inoculums size as well as glucose and $(NH_4)_2SO_4$ as sole carbon and nitrogen sources respectively, Al^{3+} for cation source, initial pH of 4, incubation temperature of 28^oC and agitation speed of 160 rpm. The purified bioflocculant flocculated kaolin suspension optimally at a dosage of 0.2 mg/ml. The flocculating activity was about 88% in the presence of Al^{3+}, under acidic conditions of pH 4. The purified bioflocculant retained more than 70% flocculating activity at 100^oC. Chemical analysis of the purified bioflocculant showed that it was composed of polysaccharide 28.4% (w/w), protein 2.63% (w/w) and uronic acid 9.7% (w/w). Fourier transform infrared revealed the presence of hydroxyl, carboxyl and amino group as the main functional groups. Thermogravimetric analysis showed that the bioflocculant could not decompose completely at 400^oC. These properties showed that the bioflocculant had a good flocculating activity and could be used as alternatives to chemical flocculants commonly used in developing countries.

Key words: *Micrococcus* sp. Leo, Marine environment, Bioflocculant, Flocculating activity.

4.1. Introduction

Discharge of wastes and wastewater containing organic and inorganic pollutants in the environment have increased tremendously as a result of industrialization (Prasertsan *et al*., 2006). These pollutants have adverse effects on human health and the environment, hence the need to develop means of reducing pollution levels through proper waste treatments.

Flocculants are usually used for the aggregation of colloidal substances and cellular materials in various types of effluents (Salehizadeh and Shojaosadati, 2001). They are widely used in a variety of industrial processes such as wastewater treatment, food and fermentation industries, drinking water purification and industrial downstream processes (Shih *et al*., 2001; Wu and Ye, 2007). Despite the effective flocculation performance and low cost of synthetic chemical flocculants, their usages have resulted in some health and environmental problems since they are not biodegradable in nature (Ruden, 2004). Due to the hazardous nature of inorganic and organic flocculants, the flocculants produced by different microorganisms have begun to attract more attention because of their safety to the ecosystem (Salehizadeh and Shojaosadati, 2001).

A bioflocculant is a kind of biodegradable macromolecular flocculant secreted by microorganisms during their growth (Xia *et al*., 2008). In addition to being biodegradable, bioflocculants are also safe and lack secondary pollution (Deng *et al.,* 2003). Despite these advantages, low flocculating activity and yields are still factors affecting their industrial applications of bioflocculants (He *et al*., 2010).

Similar to chemical flocculants, bioflocculants may be applied as treatment in the following industries: starch wastewater, river water, brewery wastewater, soy sauce brewing, meat

processing wastewater, effluent from pulp, paper mills and the removal of dyes (Zhang *et al*. 2007; Zheng *et al*., 2008; Gong *et al*., 2008).

Many studies have shown that bioflocculants can be produced by different microorganisms including fungi, bacteria, yeast and algae (Deng *et al*., 2003). Wu and Ye (2007) reported that sugars, uronic acids, protein, and polyamides were components of a bioflocculant produced by *Bacillus subtilis* DYU1. Yim *et al*. (2007) reported that galactose was the main component of biopolymer p-KG03 produced by marine dinoflagellate *Gyrodinium impudicum*.

Members of the genus *Micrococcus* are aerobic, Gram-positive, oxidase-positive, spherical shape and are always found in tetrad forms ranging from 1 to 1.8 mm in diameter. They are usually nonmotile and do not form spores. Their genomes are very rich in guanine and cytosine (GC), having 65 to 75 GC content (Kocur *et al*, 2006; Bannerman and Peacock, 2007).

This study reports on production and characterization of a bioflocculant by a *Micrococcus* specie isolated from sediment samples from Algoa bay in the Eastern Cape Province, South Africa.

4.2. Materials and Methods

4.2.1. Source of bacteria

The bacteria was isolated from the sediment of Algoa Bay in the Eastern Cape Province of South Africa and maintained in 20% glycerol at -80^{o}C as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa. The bacteria was identified by 16S rDNA sequencing to have a

99% similarity to *Micrococcus* sp. Bg-6 and the nucleotide sequence was deposited in GenBank as *Micrococcus* sp. Leo with the accession number JF799091.

4.2.2. Growth media and cultivation conditions

The growth medium for bioflocculant production was composed of glucose (20 g), $MgSO_4.7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (0.2 g), K_2HPO_4 (5 g), urea (0.5 g), yeast extract (0.5 g) and KH_2PO_4 (2 g) in a litre of filtered seawater at pH 4.0 and was sterilized by autoclaving (Zhang *et al*., 2007). The culture was incubated at 28^{o}C in a shaker incubator at 160 rpm for 5 days and centrifuged at 4000 × g for 30 min at 4^{o}C to sediment the cells. Two millilitres of the cell free culture supernatant was used to determine flocculating activity according to Kurane *et al*. (1994).

4.2.3. Determination of flocculating activity

Flocculating activity was determined using kaolin clay suspension as described by Kurane *et al*. (1994) with minor modifications. A concentration (4 g/l) of kaolin suspension was made in distilled water. One hundred millilitres of the kaolin suspension was measured into 250 ml flask, 3 ml of 1% w/v $CaCl_2$ and 2 ml of culture supernatant were added. The mixture was agitated vigorously for 60 sec and then poured into 100 ml measuring cylinder and allowed to settle for 5 min. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Thermo spectronic, made in USA) and the flocculating activity determined as follow:

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

where A and B are optical densities of control and sample measured at 550 nm respectively.

4.2.4. Effect of culture conditions on bioflocculant production

4.2.4.1. Effect of inoculum size on bioflocculant production

The effect of inoculum size was determined by using varying volumes (0.5, 1, 1.5, 2, and 2.5 ml) representing (1, 2, 3, 4, and 5% v/v) of an 18 h old pre-culture broths to inoculate 50 ml of production medium and incubated at 28^{o}C in a shaker incubator at 160 rpm for 5 days (Ugbenyen *et al*., 2012). Flocculating activity was determined in according with Section 4.2.3.

4.2.4.2. Effect of carbon and nitrogen sources on bioflocculant production

The effects of different carbon and nitrogen sources on bioflocculant production by the test bacteria were investigated according to the method described by Lachhwani (2005). Carbon sources such as glucose, fructose, galactose, xylose, sucrose, maltose and starch were used while the nitrogen sources were ammonium nitrate, ammonium chloride, sodium nitrate, (inorganic nitrogen sources) and tryptone, urea, casein, peptone (organic nitrogen sources).

4.2.4.3. Effect of agitation speed of bioflocculant production

Different shaker speeds for incubation ranging from 120-200 rpm were used to determine the optimal speed for bioflocculant production (Zhang *et al*., 2007).

4.2.4.4. Effect of incubation temperature on bioflocculant production

Cultures were incubated at different temperatures (25°C, 28°C, 31°C, 34°C, and 37°C) following the method of Zhang *et al*. (2007). Flocculating activity of the bioflocculant was determined at room temperature using the same method previously described.

4.2.5. Extraction and purification of the bioflocculant

After 5 days of fermentation, the culture broth was centrifuged at 4 000 × g, 4^{o}C for 30 min in order to remove bacterial cells. To remove the insoluble substances, one volume of distilled water was added to the supernatant phase and the mixture centrifuged at 4 000 × g for 15 min. Two volumes of ethanol were added to the supernatant and the solution was agitated and left standing at 4^{o}C for 12 h. To obtain the crude bioflocculant, the precipitate was vacuum dried. The purification of the crude bioflocculant was done according to the method described by Salehizadeh *et al.* (2000). The obtained precipitate was re-dissolved in distilled water (1% w/v) and one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v*)* was added. After agitating, the mixture was left standing at room temperature for 12 h. The pellet was collected by centrifuging at 4 000 × g, 4^{o}C for 15 min. The upper phase (bioflocculant sample) was centrifuged at 4 000 × g, 4^{o}C for 15 min and dialyzed against distilled water overnight. The bioflocculant solution was then vacuum-dried in order to obtain a purified bioflocculant.

4.2.6. Effect of cations on flocculating activity of crude and purified bioflocculant

The effects of cations on flocculating activity of the produced bioflocculant was done in a similar way as described above for flocculating activity except that $CaCl_2$ solution was

replaced by various salt solutions. Solutions (1% w/v) of NaCl, KCl, $LiCl_2$, $MgCl_2$, $MnCl_2.4H_2O$, $BaCl_2$, $AlCl_3$ and $FeCl_3 \cdot 6H_2O$ were used as cation sources according to He *et al*. (2010).

4.2.7. Effects of pH on the flocculating activity of crude and purified biofloculant

A solution containing 0.1 mg/ml bioflocculant was prepared. Separate kaolin solutions were prepared and the pH of each adjusted with either HCl or NaOH ranging from 2.0-12.0 (Xiong *et al*., 2010). The flocculating activity of the bioflocculant was determined at each of this pH value in according with Section 4.2.3.

4.2.8. Determination of bioflocculant dosage (Jar test)

Different concentrations of bioflocculant solution (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were prepared and evaluated. Three millilitres of 1% (w/v) $CaCl_2$ and 2 ml of bioflocculant solution were both added to 100 ml kaolin suspension (0.4% w/v) contained in 500 ml beakers. The solutions were agitated at 200 rpm, at room temperature for 3 min; the speed reduced to 45 rpm and then allowed to agitate for a further 10 min (Wang *et al*., 2010). The solutions were poured into separate 100 ml measuring cylinders and allowed to settle for 10 min at room temperature. Two millilitre of the clear supernatant was withdrawn and flocculating activity determined as previously described.

4.2.9 Composition analysis of the bioflocculant

The total protein content of the purified bioflocculant was determined by using the Bradford method (1976) with bovine serum albumin as the standard solution. The total sugar content

was determined by the phenol-sulphuric acid method described by Chaplin and Kennedy (1994) using glucose as a standard solution. The uronic acid was determined by carbazole method according to Bitter and Muir (1962).

4.2.10. Fourier transfom infrared spectroscopy (FTIR)

The functional groups of the bioflocculant were determined using a Fourier transform infrared spectroscopy (Perkin Elmer System 2000, FT-IR, England). The bioflocculant was ground with KBr salt at 25^{o}C and pressed into a pellet for FTIR analysis over a wave length of 4000-370 cm^{-1} (Wang *et al*., 2011).

4.2.11. Thermo-gravimetric analysis (TGA)

Ten milligrams of the bioflocculant was weighed and analysed by TGA analyzer (STA 449/C Jupiter, Netzsch, Germany Perkin Elmer TGA7 Thermo gravimetric Analyzer, USA) over a temperature range of 20-400^{o}C with a heating rate of 10^{o}C per minute under a constant flow of nitrogen gas (Yim *et al*., 2007).

4.2.12. Scanning electron microscopy (SEM)

The surface morphology structures of the purified bioflocculants were studied with scanning electron microscope (JSM-6390 LV, Japan). The SEM images of bioflocculant powders, kaolin clay before and after flocculation were scanned (He *et al*., 2010).

4.3. Results and Discussion

4.3.1. Effects of inoculum sizes on bioflocculant production

Different inoculum sizes were used to inoculate the production medium and the results are depicted in the Figure 4.1. It was observed that all the different inoculum sizes used resulted in flocculating activity of more than 80% with the maximum flocculating peak at 4% v/v. Inoculums sizes greater than 4% (v/v) resulted into a decrease in flocculating activity of the produced bioflocculant (Figure 4.1). Salehizadeh and Shojaosadati (2001) stated that small inoculums size will prolong the stagnant phase and large inoculums could result in excessive overlap of the organism's niche and affect the production of the bioflocculant.

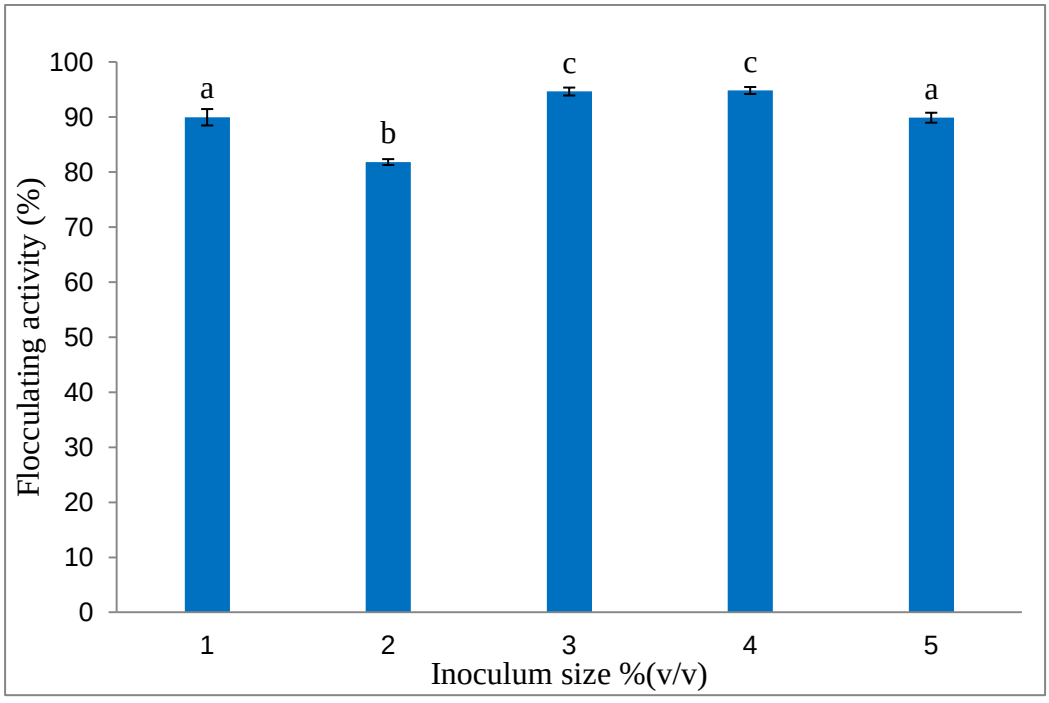

Figure 4.1: Effect of inoculum size on bioflocculant production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.2. Effects of carbon and nitrogen sources on bioflocculant production

Salehizadeh and Shojaosadati (2001) reported that the production of bioflocculant and its characteristics depends on culture conditions. The effect of different carbon and nitrogen sources on bioflocculant production by *Micrococcus* sp. Leo are as represented in Figures 4.2 and 4.3 below. Glucose was more preferred carbon source by the test strain for bioflocculant production with a flocculating activity of 82.1%, followed by starch (64.7%), maltose (61.5%), xylose (58.9%), galactose (45.1%), sucrose (24.4%) and fructose at 23% was the least preferred. Patil *et al.* (2009) observed that glucose was preferably used by *Bacillus subtilis* for bioflocculant production. Ntsaluba *et al*. (2011) also reported that glucose was the carbon source of choice for bioflocculant production by *Methylobacterium* sp. Obi. Furthermore, glucose was the favourable carbon of choice for bioflocculant production from *Rhodococcus erythropolis (*Kurane *et al.*, 1991), *Virgibacillus* sp. Rob (Cosa *et al*., 2011). On the contrary to these findings, Zhang *et al*. (2002b) reported that glucose inhibited cell growth in *Sorangium cellulosum* during bioflocculant production. Nie *et al*. (2011) reported that lactose was the carbon source of choice for bioflocculant (MNXY1) production by a *Klebsiella pneumoniae* strain, while sucrose was a preferred carbon source for bioflocculant production by *Aeromonas* sp (Li *et al*., 2007), *Bacillus* sp. F19 (Zheng *et al*., 2008) and *Bacillus* sp. Gilbert (Piyo *et al*., 2011). Zhang *et al*. (2007) reported using brewery wastewater as a carbon source for bioflocculant production by multiple-microorganism consortia.

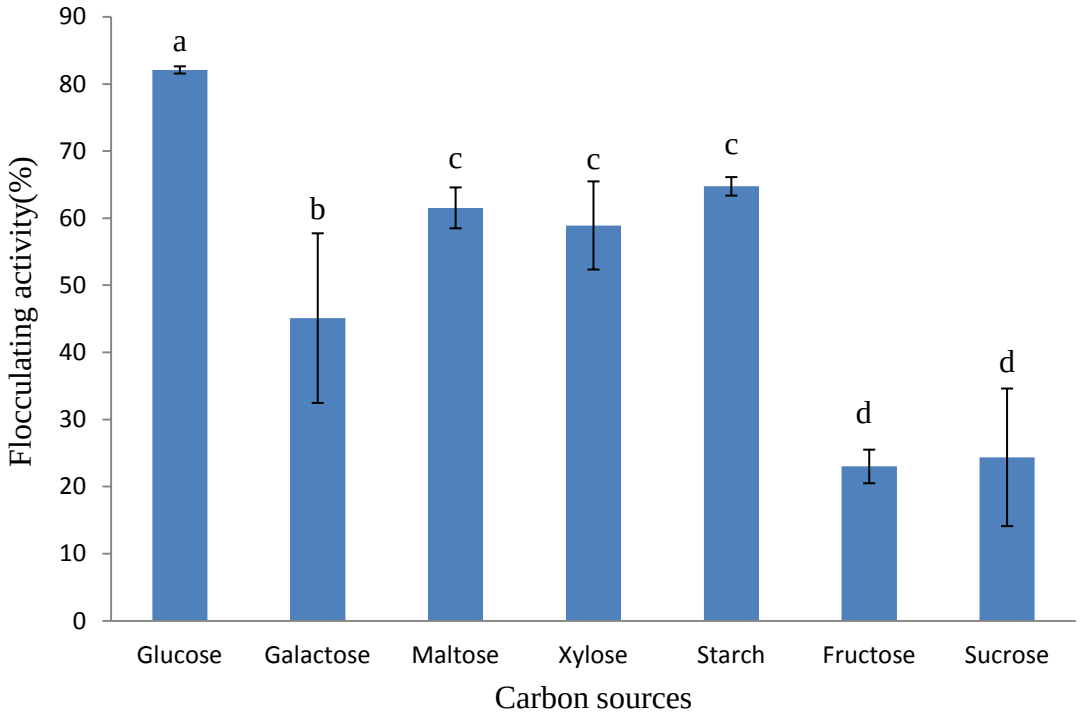

Figure 4.2: Effect of different carbon sources on bioflocculant production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

Different nitrogen sources were investigated for their support of bioflocculant production from different microorganisms (Xia *et al*., 2008). In Figure 4.3, the effect of different nitrogen sources on bioflocculant production is indicated. Inorganic nitrogen source, ammonium sulphate with a flocculating activity of 92.8% was found to be the most preferable nitrogen source by the test strain compared to other inorganic and organic nitrogen sources tested. Different nitrogen sources supported flocculating activity to varying extents as follows: Tryptone (34.4%), NH_4NO_3 (51.9%), urea (59.4%), yeast extract (77.6%), peptone (90.8%), NH_4Cl (84.0%) and mixed nitrogen source; ($(NH_4)_2SO_4$ + urea + yeast extract) (82.1%). Similar results were reported by Piyo *et al*., (2011) where inorganic nitrogen source, ammonium chloride favoured bioflocculant production by *Bacillus* sp. Gilbert.

On the contrary to these findings, several other studies have indicated that different nitrogen sources were utilized by different microorganisms for bioflocculant production. For example, Zhang *et al*. (2002b) showed that sodium nitrate ($NaNO_3$) was the favourable nitrogen source for bioflocculant production by *Sorangium cellulosum*. For production of bioflocculant by *Bacillus* sp. Gilbert, ammonium chloride was the nitrogen source of choice and ammonium sulphate completely inhibited the growth (Piyo *et al*., 2011). Mabinya *et al*. (2011) reported that urea was the choicest nitrogen source for *Halomonas* sp. Okoh. Also, Mabinya *et al*. (2012) reported that peptone was utilized as a nitrogen source for bioflocculant production by *Arthrobacter* sp. Raats. In addition, several other studies showed that multiple nitrogen (ammonium sulphate, urea and yeast extract) were preferred by different microorganisms. For example, the production of bioflocculant by *Vagococcus* sp. W31, *Aeromonas* sp. *Methylobacterium* sp. Obi utilised multiple nitrogen sources (Gao *et al*., 2006; Li *et al*., 2007; Ntsaluba *et al*., 2011).

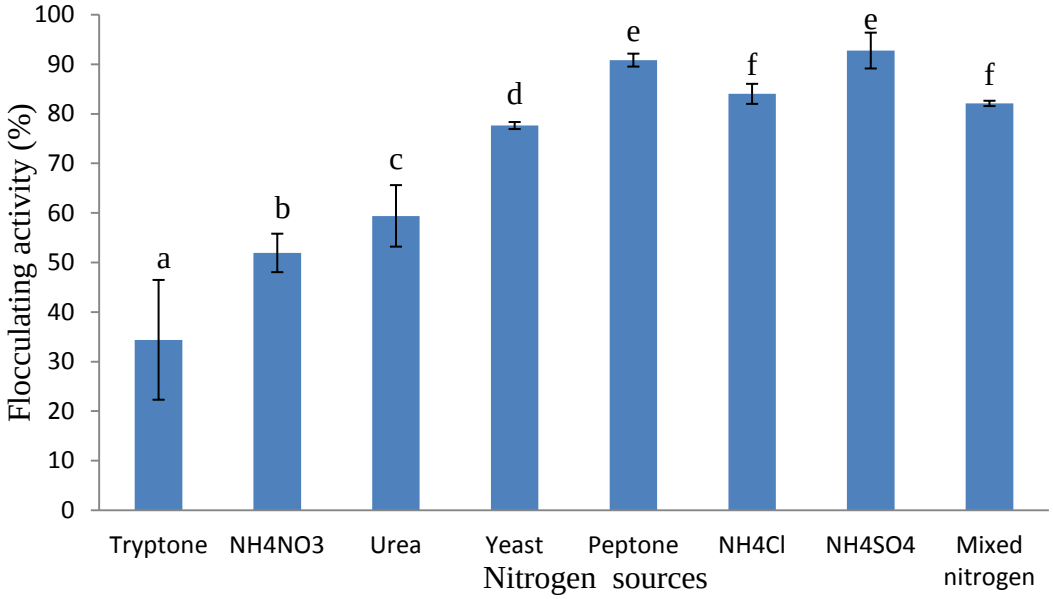

Figure 4.3: Effect of different nitrogen sources on bioflocculant production by *Micrococcus* sp. Leo. (Mixed nitrogen source = $(NH_4)_2SO_4$ + urea + yeast extract). Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.3. Effect of cations on flocculating activity of crude bioflocculant

Wu and Ye (2007) stated that cations can neutralize negative charges of both bioflocculant produced and kaolin particles in suspension thereby increasing the adsorption of the bioflocculant onto the supended particles. The effects of cations on the flocculating activity of bioflocculants differ from each other (Salehizadeh and Shojaosadati, 2001). The mechanism of bioflocculation can be deduced from the functional groups and molecular weight of the produced bioflocculant (Kumar *et al*., 2004). Also, in accordance with Deng *et al*. (2005) observations, the flocculation mechanism could be due to the interaction of the bioflocculant with its targets molecules or particles via absorption, charge neutralization, bridging and aggregation. The results represented in Figure 4.4 shows that divalent cations generally stimulated the flocculating activity of the bioflocculant produced by *Micrococcus* sp. Leo compared to monovalent cations. The cations which stimulated flocculation activity includes K^{+}, Ca^{2+} Mn^{2+}, Ba^{2+}, Fe^{3+}, Al^{3+}. The maximum flocculating activity was observed with Al^{3+} at 85.2% and the inhibitory effects of the cations were observed with Na^{+}, Li^{+} and Fe^{3+} resulting in a flocculating activity of less than 50%. On the other hand, Zhang *et al*. (2002a) observed that the flocculating activity of the bioflocculant produced by *myxobacterium Nannocystics* sp. NU-2 was stimulated by Fe^{3+}. Also, Li *et al*. (2008) reported that the bioflocculant produced by deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913 was stimulated in the presence of Ca^{2+} and Fe^{2+}. Similar observations were also noticed by Mabinya *et al*. (2011) and Ntsaluba *et al*. (2011) where flocculating activity of the bioflocculants produced by *Halomona* sp. Okoh and *Methylobacterium* sp. Obi respectively were stimulated by the presence of Ca^{2+}. Cosa *et al*. (2011) reported that the flocculating activity of the bioflocculant produced by *Virgibacillus* sp. Rob was stimulated by Fe^{2+}. The bioflocculant produced by *Cobetia* sp. flocculated best when Mn^{2+} was used as the cation (Ugbenyen *et al*., 2012). On the contrary, addition of

cations had no positive results on the flocculating activity of the bioflocculant produced by *Bacillus* sp. F19 (Zheng *et al*., 2008).

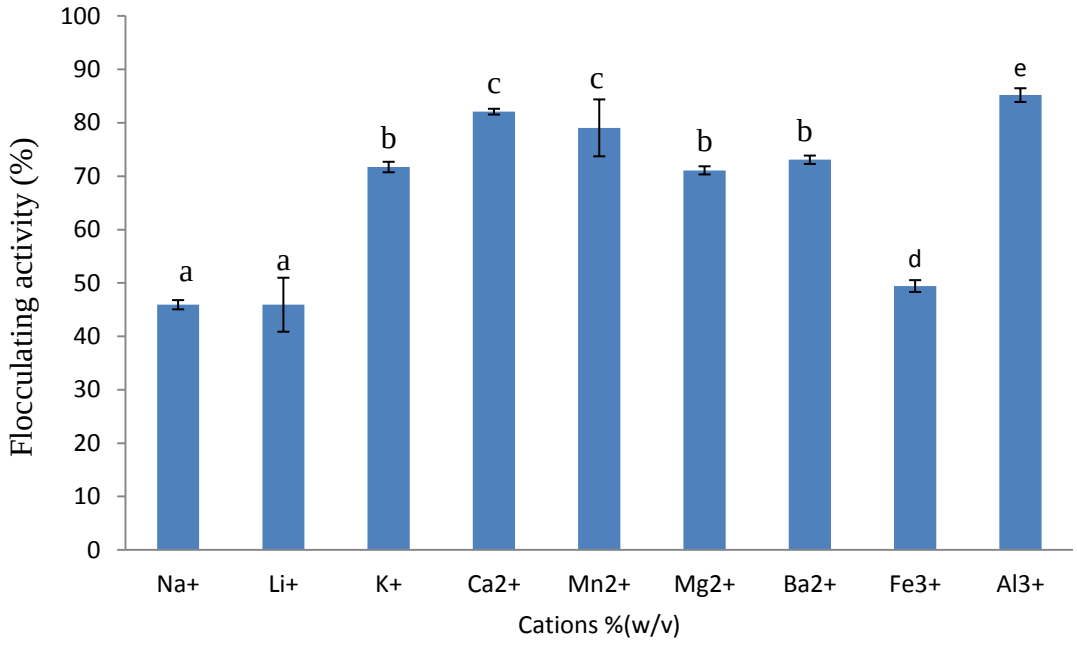

Figure 4.4: Effect of cations (1% w/v) on the flocculating activity of a bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.4. Effect of initial pH of the production medium on bioflocculant production

The initial pH of production medium was adjusted to pH values ranging from 2-12 with either HCl or NaOH and the flocculating activity at each pH value was measured. The highest flocculating activity was obtained at pH 4 (Figure 4.5). The initial pH of the fermentation medium determines the electric charge of the cells or the nutrient absorption potential that affect the enzymatic reaction (Nakata and Kurane, 1999; Xia *et al*., 2008).

The results represented in Figure 4.5 shows the effect of initial pH of the production medium on bioflocculant production. It was observed that the bioflocculant can be produced at a wide

range of pH ranging from 2-9 with flocculating activity more than 60% peaking (87.4%) at pH 4.

On the other hand, Nie *et al*. (2011) reported that a slightly acidic pH was favourable for MNXY1 produced by *Klebsiella pneumonia* strain. The growth of the strain was completely inhibited at low pH or at basic medium. Also, Li *et al*. (2007) reported bioflocculant production by *Aeromonas* sp. at pH 8. *Halomonas* sp. Okoh and *Methylobacterium* sp. Obi produced their bioflocculants at pH 7 (Mabinya *et al*., 2011; Ntsaluba *et al*., 2011). Furthermore, *Baccillus* sp. Gilbert and *Chryseobacterium daeguense* W6 produced their bioflocculants at pH 6.2 and 5.6 respectively (Liu *et al* 2010; Piyo *et al*., 2011). While Li *et al*. (2008) reported that the production of bioflocculant by *Pseudoalteromonas* sp. SM9913 was achieved at a pH range 5-8, *Bacillus licheniformis* produced its bioflocculant best at pH 7.2 (Xiong *et al*., 2010).

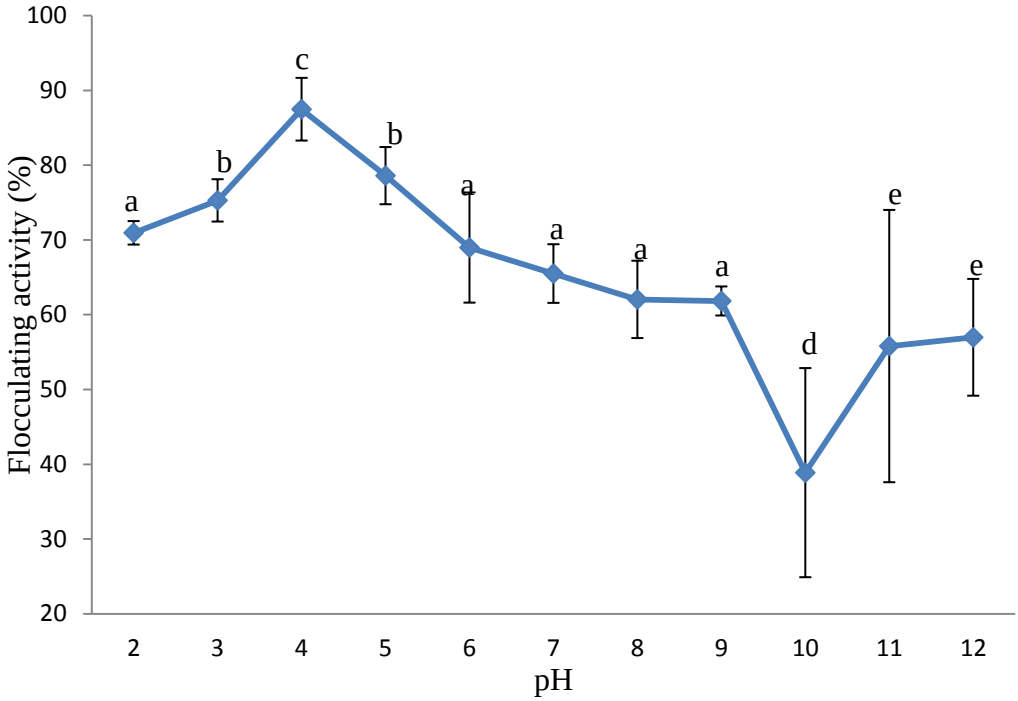

Figure 4.5: Effect of initial pH on bioflocculant production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.5. Effect of agitation speed on bioflocculant production

The effect of agitation speed on bioflocculant production is as shown in the Figure 4.6. Salehizadeh and Shojaosadati (2001) reported that agitation speed determines the concentration of the dissolved oxygen, which can also affect the absorption of nutrients and the rate of enzymatic reaction. It can be seen from Figure 4.6 that the bioflocculant production could be achieved at a wide range of agitation speeds ranging from 120-200 rpm. The flocculating activities were over 60% in all the speeds with the maximum flocculation rate of 79% at 160 rpm and this was used to follow the experiment throughout. Increase or decrease in the speed decreased the production of bioflocculant by the bacterium. Different agitation speeds for bioflocculant production by different microorganisms have been documented by many researchers. For example, *Citrobacter* sp. TKF04, *Agrobacterium* sp. M503, *Methylobacterium* sp. Obi, *Baccillus subtilis*, produced their bioflocculants at 120 rpm (Fujita *et al*., 2000; Li *et al*., 2010; Ntsaluba *et al*., 2011; Patil *et al*., 2009;). Also, *Bacillus alvei* NRC-14 produced a bioflocculant at 130 rpm (Abdel-Aziz *et al*., 2011). Deng *et al*. (2005) reported an agitation speed of 140 rpm that was used for the production of bioflocculant by *Aspergillus parasiticus*. Deng *et al*. (2003) documented the production of bioflocculant by *Bacillus mucilaginosus* at 150 rpm. Furthermore, a novel bioflocculant HBF-3 was produced at 160 rpm by the deep-sea mutant bacterium *Halomonas* sp. V3a (He *et al*., 2010). The bioflocculant produced by multiple-microorganism consortia using brewery wastewater as a carbon source was achieved at 160 rpm. At this speed (160 rpm), *Halomona* sp. Okoh, *Bacillus* sp. Gilbert, *Arthrobacter* sp. Raats, and *Cobetia* spp. (Mabinya *et al*., 2011, Piyo *et al*., 2011; Ugbenyen *et al*., 2012) produced their bioflocculants. At 170 rpm, *Aeromonas* sp. (Li *et al*., 2007) and *Bacillus* sp. (Feng and Xu, 2008) produced bioflocculants. *Enterobacter cloacae* WD7, *Pseudoalteromonas* sp. SM9913 and *Bacillus* sp. F19 produced their bioflocculants at 200 rpm (Li *et al*., 2008; Prasertsan *et al*., 2006; Zheng

et al., 2008). Although extracellular polysaccharide bioflocculant was produced at 220 rpm (Nakata and Kurane, 1999), Zhang *et al*. (2002b) reported an exopolysaccharide biofloculant that was produced at 220 rpm by *Sorangium cellulosum*.

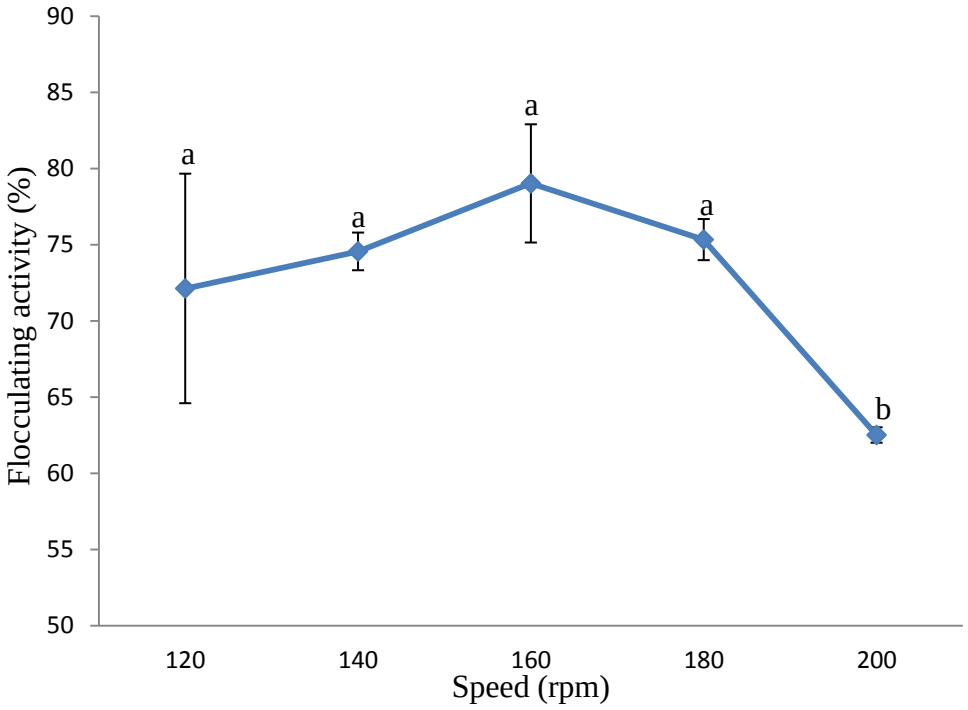

Figure 4.6: Effect of agitation speed on bioflocculant production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.6. Effect of incubation temperature for bioflocculant production

The effect of different incubation temperatures on bioflocculant production was investigated. Different temperatures have been used by various researchers in the production of bioflocculants from different microorganisms. Kurane and Nakata (1999) stated that the optimal activity of the enzyme could only be achieved at optimal temperature. Since enzymes are proteins and they are very sensitive to heat and they get easily denatured with high temperature. Figure 4.7 shows the results of using different temperatures for the production

of bioflocculant and it was observed that the maximum flocculating activity of the bioflocculant was achieved at 28^{o}C. Similarly, Ugbenyen *et al*. (2012) and Mabinya *et al*. (2012) documented that the bioflocculants produced by *Cobetia* sp. and *Arthrobacter* sp. Raats respectively flocculated optimally at 28^{o}C. Different observations were recorded by Zhang *et al*. (2002a, 2002b), and Patil *et al*. (2010) in the production of bioflocculants by marine *Sorangium cellulosum*, *myxobacterium Nannocystis* sp. Nu-2, and *Azobacter indicus* at 30^{o}C.

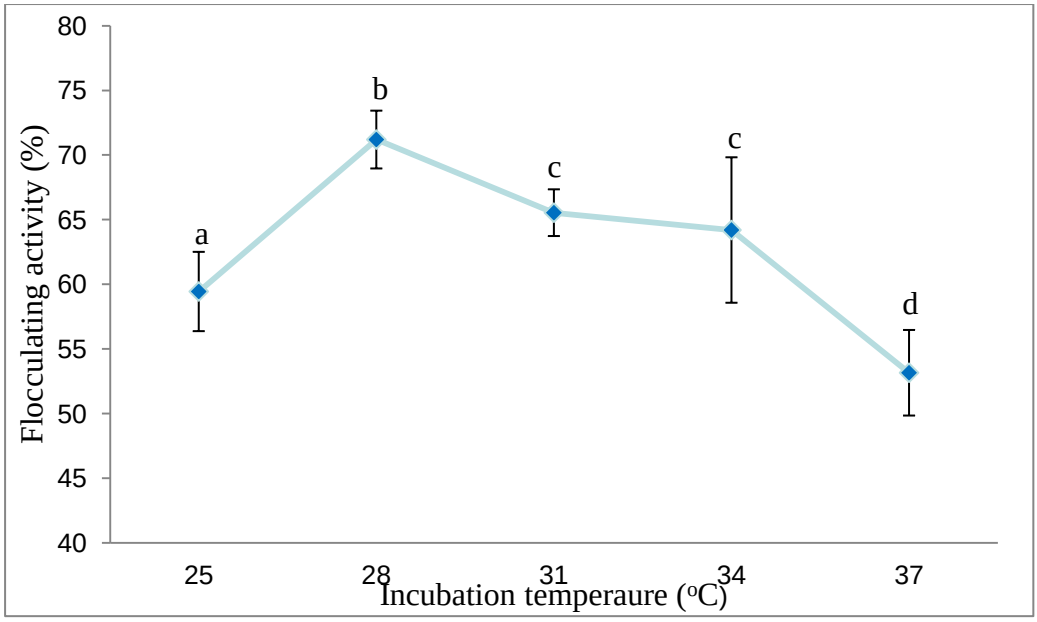

Figure 4.7: Effect of incubation temperature on bioflocculant production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.7. The yield of purified bioflocculant produced by Micrococcus sp. Leo

From 1 L fermented broth of *Micrococcus* sp. Leo, 0.738 g of purified biolocculant was obtained. Different amounts of purified flocculants have been extracted from different microorganisms and documented. For example, 3 g of bioflocculant was produced from 1 L of fermented broth of *Klebsiella pneumonia* (Nakata and Kurane, 1999). Also, the yield of a

bioflocculant produced by marine *Myxobacterium nannocystics* sp. NU-2 was 14.8 g/l in a medium supplemented with starch (Zhang *et al*., 2002a). Cosa *et al*. (2011) reported that 0.264 g/l of purified bioflocculant was recovered from a fermented culture of *Virgibacillus* sp. Rob.

4.3.8. Effect of cations on flocculating activity of purified bioflocculant

Almost all the cations tested except Fe^{3+} which gave a flocculating activity of 36.9% supported a flocculating activity more than 50% with Al^{3+} and Mn^{2+} supporting the highest flocculating activity of 77.4% and 77.3%, respectively in Figure 4.8. These results showed that Fe^{3+} inhibited the flocculating activity of the bioflocculant. In other studies on different bacterial strains, it was reported that the flocculating activities of the biopolymers produced by *Bacillus licheniformis* and *Bacillus circulans* were stimulated in the presence of Ca^{2+}, Fe^{3+} and Al^{3+} (Shih *et al*., 2001; Li *et al*., 2009b). Ntsaluba *et al*. (2011) and Mabinya *et al*. (2011) reported that Ca^{2+} gave the highest flocculating activity for the flocculants produced by *Methylobacterium* sp. Obi and *Halomonas* sp. Okoh, respectively. Li *et al*. (2008) stated that cations have the ability to neutralize negatively charged functional groups of both bioflocculant molecues and suspended particles, thereby increasing the adsorption of the bioflocculant onto the suspended particles. The surfaces of kaolin particles are strongly negatively charged, Al^{3+} could compress the double layer of kaolin particles, thereby weakening the electrostatic repulsive force, and stimulate effective flocculation (Li *et al*., 2008, 2009a; Kumar *et al*., 2004; Suh *et al*., 1997; Zheng *et al*. 2008).

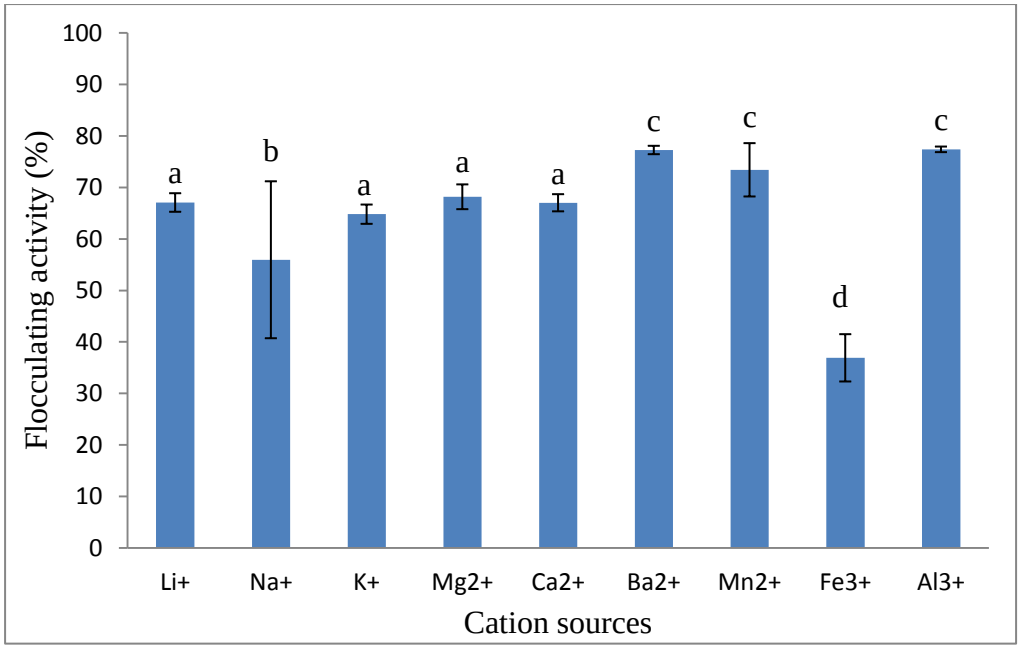

Figure 4.8: Effect of cations on the flocculating activity of the purified bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.9. Effect of pH on the flocculating activity of purified bioflocculant

The effect of pH on the flocculating activity of the purified bioflocculant was determined at pH values ranging from 2-12 as shown in Figure 4.9. The flocculating activity of the bioflocculant was more than 50% in the pH range 2-9 and the flocculating activity was optimal (81%) at pH 4. The flocculating activity decreases with an increase in pH and very low flocculating activity was observed at pH 12 (1.2%). This showed that at high basic conditions, the hydroxyl ion (OH^-) absorbed destabilized the complexes between the bioflocculant and kaolin particles resulted in the low flocculating activity observed at pH 12 (Li *et al*., 2008). Yim *et al*. (2007) reported that the bioflocculant p-KG03 produced by a deep sea microorganism *Gyrodinium Impudicum* KG03 gave optimum flocculating activity at pH 4 and was active in a pH range of 3-6. Many studies have been done on different bioflocculant- producing microorganisms which flocculant best at pH 7. For example, Li *et*

al. (2008) showed that the flocculating activity of EPS SM9913 was optimal at pH 7 and maintained a high flocculating activity in a pH range 6-8. The flocculating activity of the bioflocculant produced by *Agrobacterium* sp. M-503 was greatly enhanced at pH range of 8-12.

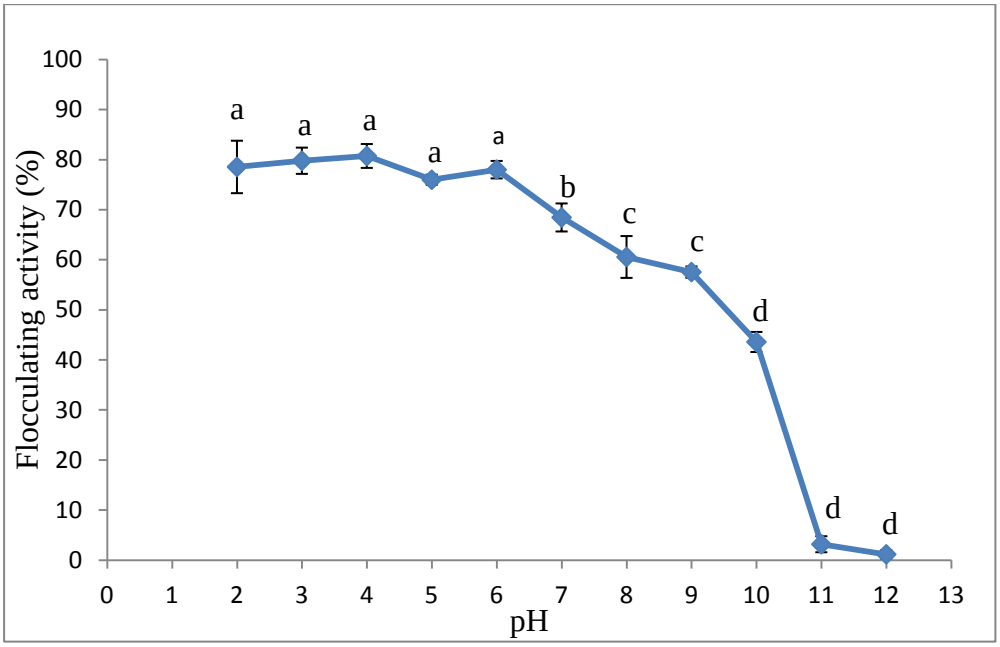

Figure 4.9: Effect of pH on the flocculating activity of the purified bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.10. Effect of bioflocculant dosage on flocculating activity (Jar test)

Bioflocculant dosage is the amount of the purified bioflocculant powder (concentration) that is required for optimal flocculation. It was found that the purified bioflocculant produced by *Micrococcus* sp. Leo was very effective for flocculation at low dosage requirements. Using kaolin suspension as a test material, the relationship between bioflocculant dosage and flocculating activity is illustrated in Figure 4.10. The highest flocculating activity (63.1%) was attained under optimized dosage concentration of 0.2 mg/ml with higher or lower

dosages inducing lower flocculating rates. The flocculating activity decreased as the dosage concentration increased to 0.6 mg/ml but the observed increase in flocculating activity at 0.7 mg/ml did not follow the same pattern and could not be explained. The bridging phenomena could not effectively form when the bioflocculant dosage was insufficient (Gong *et al*., 2008). Excessive addition of negatively charged bioflocculant will restabilize the kaolin particles in suspension thereby leading to repulsion of negatively charged kaolin particles (Wang *et al*., 2011). Yim *et al*. (2007) and Salehizadeh *et al*. (2002) obtained the highest flocculating activity with a bioflocculant concentration of 1 mg/l while most bioflocculants show best flocculating activity within the concentration range of 10-50 mg/l (Gao *et al*. 2006; Wu and Ye, 2007; Zhang *et al*., 2002b). He *et al*. (2010) reported that 4 mg/l of HBF-3 produced by mutant *Halomonas* sp. V3a' was the optimum bioflocculant concentration required for maximum flocculation and further increases in the concentration resulted in a decrease in flocculating activity. From the observations of Feng and Xu (2008), the decrease in flocculating activity as the bioflocculant concentration increases may be due to the blocking of the binding sites of one dispersive kaolin particle by one or more bioflocculants in high concentration. Instead of the bioflocculant molecules forming stronger bridging among them, they increase the viscosity of the solution thereby inhibiting flocculating activity (Wang *et al*., 2011).

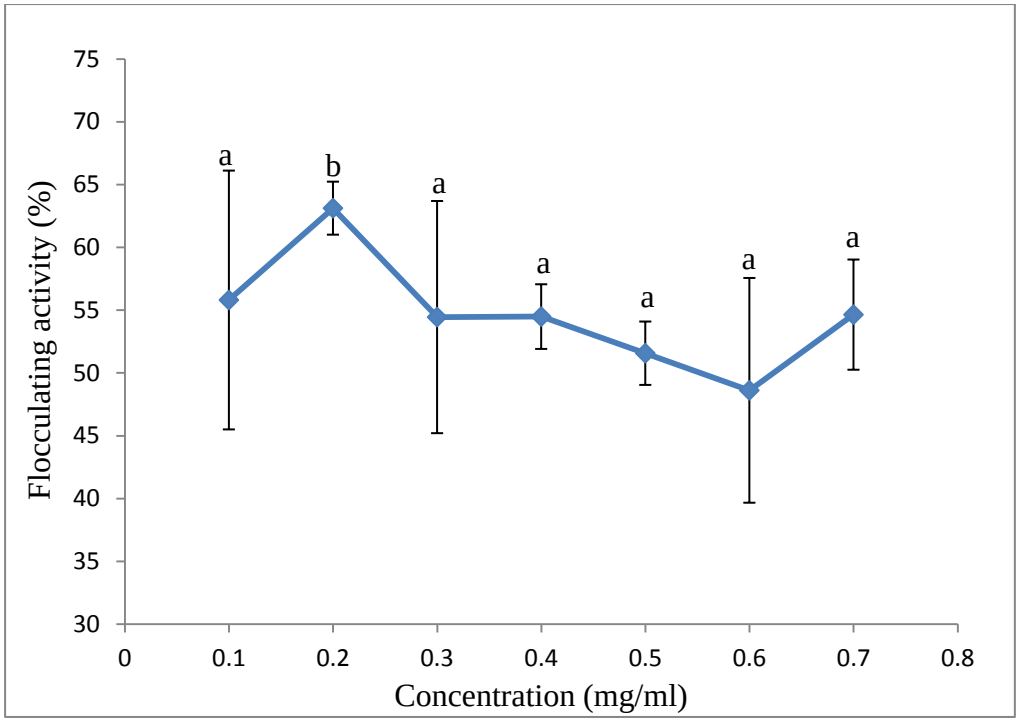

Figure 4.10: Effect of bioflocculant concentration on the flocculating activity of purified bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.11. Thermal stability of the purified bioflocculant

The flocculating activity of the bioflocculant solution was stable after being heated to 100^{o}C. The bioflocculant could retain about 70% of flocculating activity at 100^{o}C (Figure 4.11). Bioflocculants containing polysaccharides as their main components are usually thermo-stable and retained more than 70% of their flocculating activity at high temperatures up to 100^{o}C (Deng *et al*., 2003; Lu *et al*., 2005; Suh *et al*., 1997). On the contrary, bioflocculants that are made up protein as the main components are sensitive to temperature because they easily get denatured by heat, hence affecting their flocculating activities (He *et al*., 2004; Shimofuruya *et al*., 1995; Yokoi *et al*., 1995). Wang *et al*. (2011) reported the thermally-stable bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 could retain its flocculating activity above 90% after being heated at 100^{o}C.

Salehizadeh *et al*. (2000) reported a 50% decrease in flocculating activity of bioflocculant As-101 after heated for 15 min at 100^{o}C. He *et al*. (2004) reported the collapse of bioflocculant REA-11 after heating up to 100^{o}C. Ugbenyen *et al*. (2012) documented that the bioflocculant produced by *Cobetia* sp. retained its flocculating activity of more than 70% after heating to 100^{o}C. On the contrary, Yokoi *et al.* (1995) observed no flocculating activity of PY-90 after being heated for 40 min at 100^{o}C.

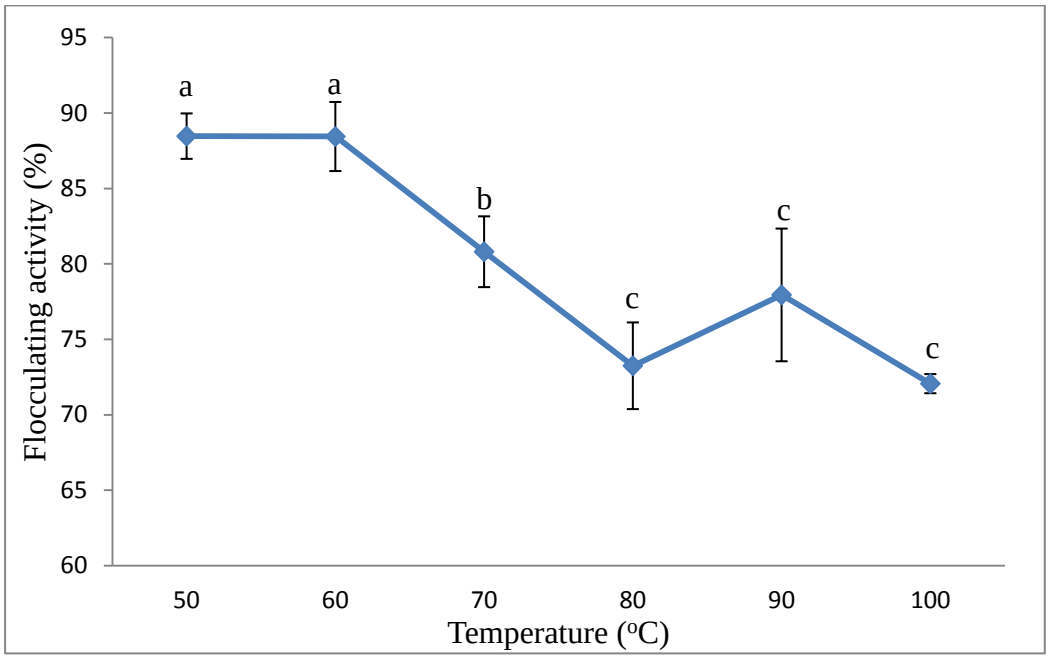

Figure 4.11: Effect of temperature on the flocculating activity of the purified bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.12. Effect of increase in concentration of cation on flocculation

He *et al*. (2010) reported that an increase in the concentration of Ca^{2+} increases the flocculating activity of the novel bioflocculant HBF-3 produced by the deep-sea mutant bacterium *Halomonas* sp. V3a. The findings of He *et al*. (2010) were in contrary to the results obtained in this study as shown in the Figure 4.12 where an increase in the concentration of

the cation decreased the flocculating activity of the bioflocculant. From Figure 4.12, the flocculating activity was optimal at 1% (w/v) of $AlCl_3$. Further increased in the concentration of $AlCl_3$ resulted into decreased in the flocculating activity of the bioflocculant.

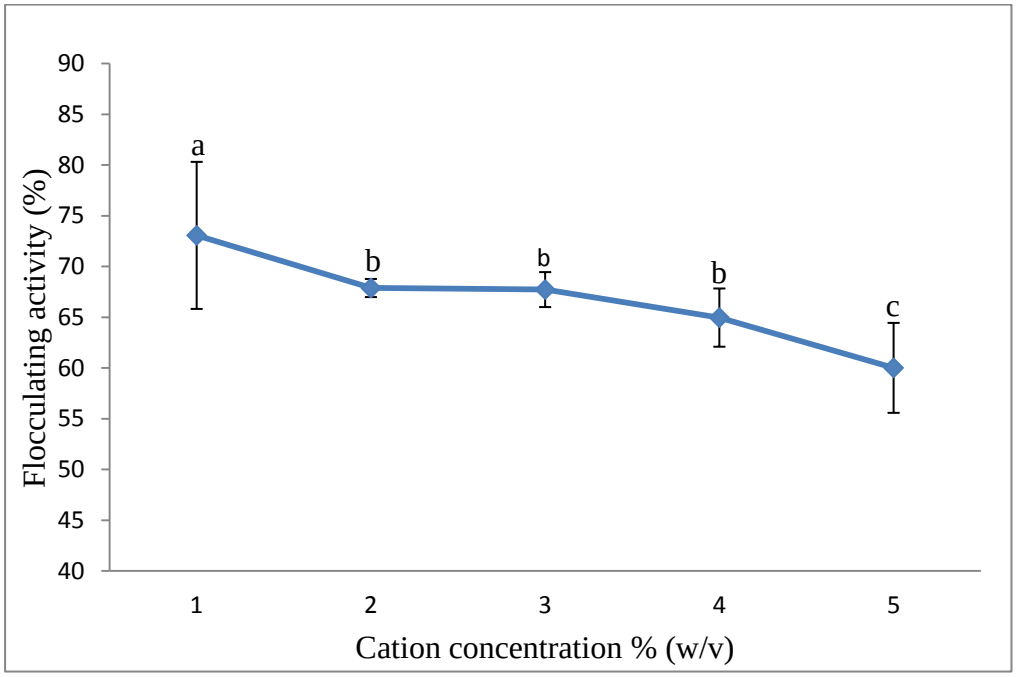

Figure 4.12: Effect of increase in the concentration of $AlCl_3$ on flocculating activity of the purified bioflocculant produced by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

4.3.13. Chemical composition of purified bioflocculant

Chemical analyses showed that the purified bioflocculant was composed of 2.64% total protein content, 28.4% total sugar content and 9.7% uronic acid. Similarly, a glycoprotein bioflocculant produced by *Aspergillus parasiticus* composed of sugar (76.3% w/w) and protein (21.6% w/w). Xiong *et al*. (2010) reported a glycoprotein bioflocculant produced by *Bacillus licheniformis* composed of carbohydrate (89% w/w) and protein (11% w/w).

On the contrary, the polysaccharide bioflocculant produced by *Klebsiella pneumonia* composed galactose (56.04 % w/w), glucose (25.92% w/w), galacturonic acid (10.92% w/w), mannose (3.71% w/w) and glucuronic acid (3.37% w/w) (Nakata and Kurane, 1999). The bioflocculant MBFA9 produced by *Bacillus mucilaginosus* composed of uronic (19.1% w/w), neutral sugar (47.4% w/w), and amino sugar (2.7% w/w). Also, the bioflocculant HBF-3 produced by deep-sea bacterium mutant *Halomonas* sp. V3a composed of neutral sugar (20.6% w/w), uronic acid (7.6% w/w), amino sugar (1.6% w/w) and sulphate groups (5.3% w/w) (He *et al*., 2010).

4.3.14. Thermogravimetric property of purified bioflocculant

The thermogravimetric property of the purified bioflocculant was used to elucidate its behaviours when subjected to heat. This enables us to understand its pyrolysis property when exposed to a very high temperature. Figure 4.13 shows that there was about 12.5% decreased in weight at 200^{o}C and increase in temperature resulted into a further decreased of about 20% in weight. These weights losses could be due to the loss of moisture content in the bioflocculant. Kumar and Anand (1998) reported that the moisture content in the bioflocculant sample may be due to the presence of carboxyl and hydroxyl groups. The higher the carboxyl group presence, the higher will be its affinity to absorb water molecule. These results showed that the bioflocculant produced by *Micrococcus* sp. Leo retained more than 80% of its weight after being subjected to a high temperature of about 400^{o}C. Prasertsan *et al*. (2006) reported that the bioflocculant produced by *Enterobacter cloacae* WD7 decomposed at 300^{o}C. Also, with the findings Yim *et al*. (2007) with the bioflocculant p-KG03 produced by a marine dinoflagellate *Gyrodinium impudicum* KG03, a decrease in weight was observed between 40-230^{o}C. Further decrease in weight loss was observed at about 310^{o}C.

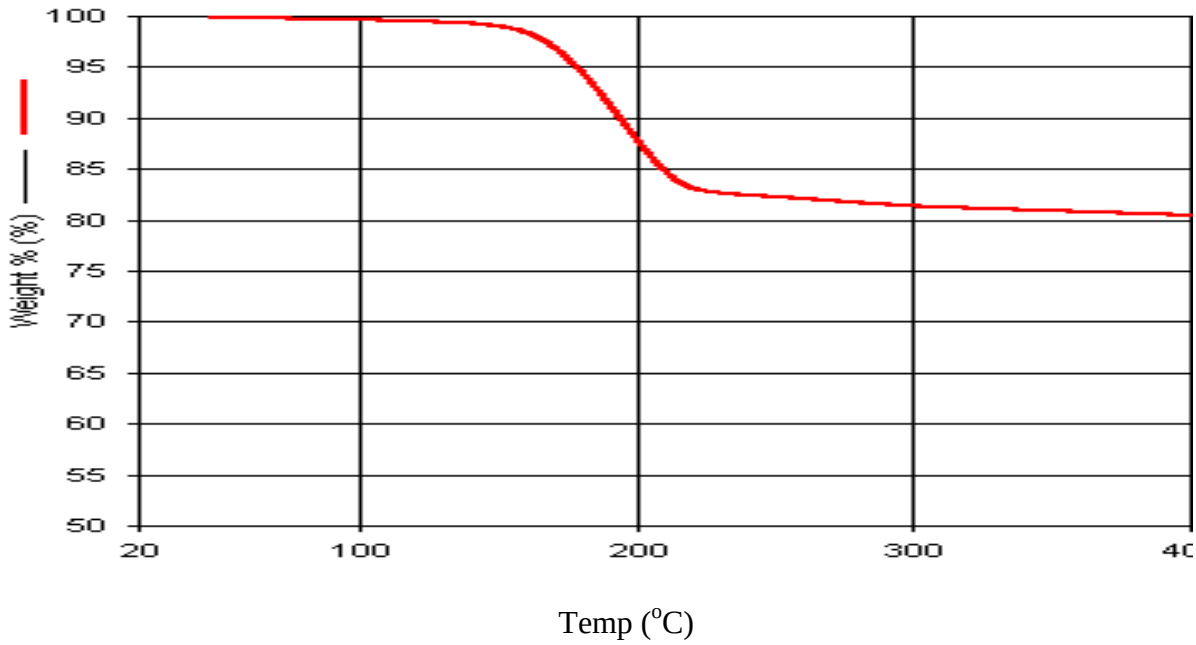

Figure 4.13: Thermogravimetric analyses of bioflocculant produced by *Micrococcus* sp. Leo.

4.3.15. Functional groups determination by FTIR

The chemical compositions of the bioflocculant produced from different microorganisms differ (Salehizadeh and Shojaodasati 2001). The flocculating activity of the purified bioflocculant solely depends on the chemical composition which can be shown to be related to the functional groups in the molecule. The Fourier-transform infrared (FTIR) spectrum analysis revealed different functional groups in the molecule. In Figure 4.14, the spectrum peak at 3412 cm^{-1} showed the presence of OH and NH_2 groups in the molecule (Desouky *et al*., 2008). A weak band noticed at 2248 cm^{-1} indicated the presence of aliphatic bonds. The spectrum at 1652 cm^{-1} suggested the presence of C=O group stretching peak (Li *et al*., 2008) and the sharp peak at 1622 cm^{-1} is an indication of the presence of amide group (Fujita *et al*., 2000). The vibration peak at 1014 cm^{-1} corresponding to the C-O stretching in alcohols further suggests the presence of OH group in the bioflocculant molecule (Deng *et al*., 2005).

The spectrum peaks in between 1000-1100 cm^{-1} suggested the presence of saccharide derivatives. The vibration peaks in between 757-839 cm^{-1} suggested the presence of furan saccharides (Wang *et al*., 2011) and the spectra peaks from 472-670 cm^{-1} represent the presence of benzene ring-structured compound in the bioflocculant molecule.

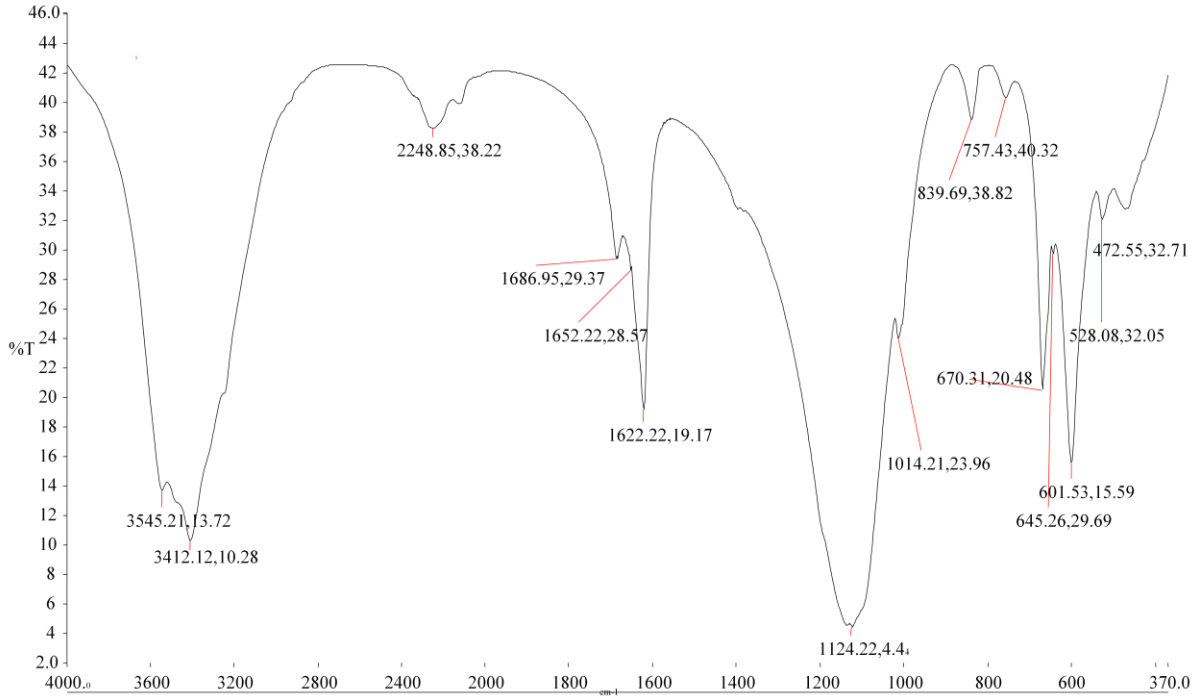

Figure 4.14: Fourier transform infrared spectroscopy analyses of purified bioflocculant produced by *Micrococcus* sp. Leo.

4.3.16. SEM images

The surface morphology of the purified bioflocculant and flocculation with kaolin clay was elucidated by scanning electron microscope observations (Wang *et al*., 2011). Figure 4.15 shows SEM images of bioflocculant and kaolin clay. Figure 4.15(A) reveals the amorphous structure of the purified bioflocculant, Figure 4.15(B) shows the scattered kaolin particles and Figure 4.15(C) depicts the structure of the kaolin particles and how they are connected

together by the bioflocculant to form aggregates and large flocs which can sediment quickly. Similar observations were recorded for bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F26. On the contrary, the bioflocculant produced by *Proteus mirabilis* TJ-1 had a crystal-linear structure (Xia *et al*. 2008).

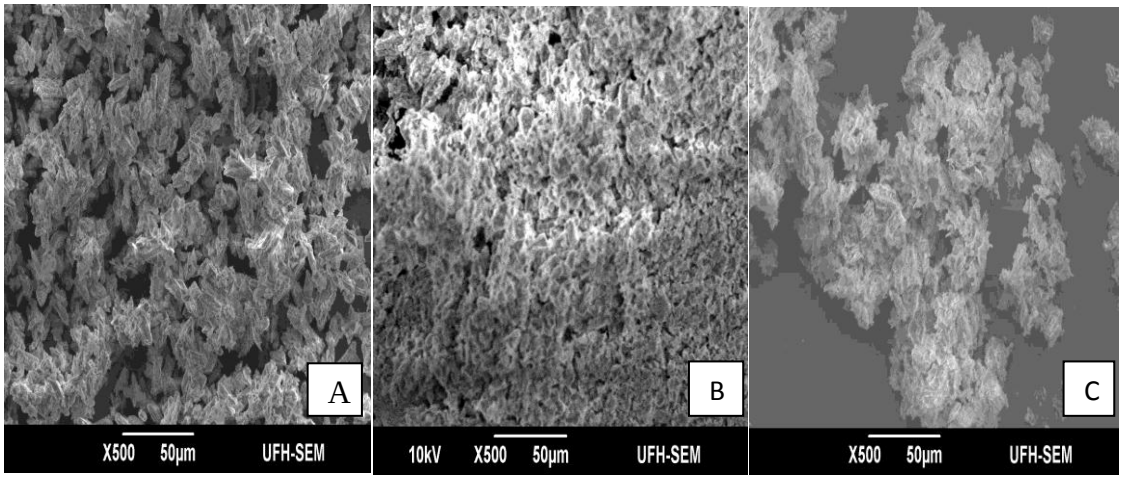

Figure 4.15. Bioflocculant powder A, kaolin clay B, bioflocculant and kaolin after flocculation C.

4.3.17. Composition analysis of the bioflocculant

Several bioflocculants have been produced from different microoraganisms and their chemical compositions differs (ABD-EL-Haleen *et al*., 2008). The characteristics of the bioflocculants are determined by the chemical nature and the molecular weight of the bioflocculant. The total carbohydrates content of the bioflocculant produced by *Micrococcus* sp. Leo was determined by phenol sulphuric acid which showed that it is composed of polysaccharide 28.4% (w/w) and 2.64% (w/w) of protein. Similar observations have been confirmed with *Klebsiella pneumonia* (Nakata and Kurane, 1999), *Baccillus mucilaginosus* (Deng *et al*., 2003), *Vagococcus* sp. W31 (Gao *et al*., 2006), *Bacillus subtilis* (Patil *et al*., 2009), *Halomona* sp. V3a (He *et al*., 2010) and *Cobetia* sp. (Ugbenyen *et al*., 2012). On the contrary, glycoprotein bioflocculants have been documented to be produced by

Myxobacterium nannocystics sp. Nu-2 (Zhang *et al*., 2002a), *Sorangium cellulosum* (Zhang *et al*., 2002b), *Chryseobacterium daeguense* W6 (Liu *et al*., 2010) and *Arthrobacter* sp. Raats (Mabinya *et al*., 2012).

4.4. Conclusion

To the best of our knowledge, this is the first report on the bioflocculant produced by *Micrococcus* sp. Leo. The bioflocculant produced by *Micrococcus* sp. Leo is predominantly composed of a polysaccharide. It was optimally produced under acidic medium conditions at pH 4 when glucose and nitrogen were used as carbon and nitrogen sources, respectively with Al^{3+} stimulating its flocculating activity up to 87.5%. *Micrococcus* sp. Leo is a potential source for the production of a bioflocculant with a high flocculating activity which could be used to replace the harmful chemically synthesised chemicals widely used in water purification and wastewater treatment plants. Further studies on the characterization of the purified bioflocculant are needed in order to enhance its potential for large-scale industrial usage.

CHAPTER FIVE

Characterization of bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo

Abstract

The physicochemical and flocculating properties of a bioflocculant produced by a bacterial consortium composed of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo were investigated. The purified bioflocculant was cations and pH dependent, optimum flocculating activity (58.5%) was reached at a low dosage of 0.1 mg/ml. The flocculating activity of the bioflocculant was stimulated by the presence of Ca^{2+}, Mn^{2+}, Al^{3+} and had a wide pH range of 2-10 with the highest flocculating activity of 86% at pH 8. The bioflocculant was thermostable and retained more than 70% of its flocculating activity after being heated at 80^{o}C for 30 min. The TGA analyses revealed a partial thermal decomposition of the biofloculant at 400^{o}C. The infrared spectrum showed the presence of hydroxyl, carboxyl and amino groups as functional groups. SEM images showed the structure of the bioflocculant molecule and kaolin particles before and after flocculation. The bioflocculant produced by a mixed cultured of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo has a strong flocculating efficiency and high thermal stability which positioned it as a promising alternative to inorganic and synthetic organic flocculants**.**

Key words: Bioflocculant, Flocculating activity, Consortium, *Halomonas* sp. Okoh, *Micrococcus* sp. Leo.

5.1. Introduction

Flocculants are widely used in various industrial processes such as drinking water treatment, downstream processes, wastewater treatment plants, and in different fermentation processes (Shih *et al*., 2001). Although chemical flocculants have their numerous advantages of being effective in term of flocculating efficiency, cheap and easily available, their usages have been reported to be harmful to humans (Vanhoric and Mones, 1983). They are neurotoxic, carcinogenic and recalcitrant to degradation, thus constituting environmental nuisance (Dearfield and Abermathy, 1988). Due to the adverse nature of chemical synthetic flocculants, more attention has been diverted to the use of flocculants produced by microorganisms (Li *et al*., 2009a).

Xia *et al*. (2008) reported that the biopolymers produced by microorganisms during their growth are harmless, degradable and do not lead to secondary pollution. As results of merits associated with bioflocculants, they are being considered as a good replacement for chemical flocculants used in wastewater treatment and other industrial processes (Salehizadeh and Shojaosadati, 2001).

In recent years, many studies have been undertaken where different microorganisms such as algae, fungi, bacteria and actinomycetes have been used in bioflocculant production (Deng *et al*., 2003; Xia *et al*., 2008). For example, Takeda *et al*. (1991) reported that a proteinaceous bioflocculant produced by *Rhodococcus erythropolis* lost its flocculating activity due to enzymatic digestion. Zhang *et al*. (2002a) reported on a bioflocculant produced by the marine *Myxobacterium nannocystic* sp. NU-2 composed of 40.3% protein and 56.3% polysaccharide. Recently, it was reported that *Cobetia* sp. produced a thermostable acidic polysaccharide bioflocculant whose activity was dependent on cations (Ugbenyen *et al*., 2012).

Although, many bioflocculants have been produced by different microbes, low flocculating activity and high production costs have been limiting factors hindering large-scale production (Li *et al*., 2003). According to the findings of Xia *et al*. (2008), screening for microorganisms with high bioflocculant-producing capability and high flocculating efficiency is vital for success in this field. Furthermore, Fujita *et al*. (2000) investigated the use of low-cost substrates in growth media for bioflocculant production as a possible cost-cutting measure. Some low-cost substrates such as soybean juice, fishmeal wastewater have been documented as alternative nitrogen source components in production media (Huang *et al*., 2001; Zhou *et al*., 2003). Brewery wastewater was used as a carbon source in bioflocculant production by multiple microorganisms (Zhang *et al*., 2007).

The idea of using two or more microbes in consortium for bioflocculant production was first reported by Ma *et al*. (2003). The concept behind this approach was to improve the yield and flocculating efficiency of the bioflocculant produced by individual strains. Furthermore, Zhu *et al*. (2004) reported that a compound bioflocculant produced by a mixed culture of strains F2 and F6 had higher flocculating activity than compared to individual strains. In addition, Wang *et al*. (2011) reported that the compound bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus shaeicus* F6 possessed higher flocculating efficiency than those from individual strains.

To the best of my knowledge, no previous work has been reported in literature about bioflocculant production by a mixed culture of *Halomonas* and *Micrococcus* species. This paper reports on the characterization of a bioflocculant produced by a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo isolated from sediment samples of Algoa Bay, Eastern Cape.

5.2. Materials and Methods

5.2.1. Source of bacteria

The bacteria was isolated from the sediment of Algoa Bay in the Eastern Cape Province of South Africa and maintained in 20% glycerol at -80^{o}C as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa.

5.2.2. Growth media

The growth medium for bioflocculant production was composed of glucose (20 g), $MgSO_4.7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (0.2 g), K_2HPO_4 (5 g), urea (0.5 g), yeast extract (0.5 g) and KH_2PO_4 (2 g) in a litre of filtered seawater at pH 6.5 and sterilized by autoclaving at 121-124^{o}C for 15 min (Zhang *et al*., 2007).

5.2.3. Evaluation of bioflocculant production

The bacteria were inoculated into a 250 ml flask containing 50 ml of production medium prepared according to description of Zhang *et al.* (2007) and incubated at 28^{o}C in a shaker at 160 rpm for 5 days and centrifuged at 4000 × g for 30 min at 4^{o}C. The cell free culture supernatants were used to determine flocculating activities of the bioflocculants produced by the consortium.

5.2.4. Determination of flocculating activity

Using the description of Kurane *et al*. (1994) with minor modifications, kaolin clay was used as the test material for determining the flocculating activity of the produced bioflocculant.

Four grams of kaolin clay was suspended in 1 L of distilled water to make a concentration (4 g/l). One hundred millilitres of kaolin suspension was measured into 250 ml flask, 3 ml of 1% w/v $CaCl_2$ and added 2 ml of culture supernatant were added. The mixture was agitated vigorously for 60 sec and then poured into 100 ml measuring cylinder and allowed to sediment for 5 min at room temprature. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Thermo spectronic, made in USA) and the flocculating activity determined as follows:

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

where A and B are optical densities of control and sample measured at 550 nm respectively.

5.2.5. Time course assay for bioflocculant production

The compositions of the medium for bioflocculant production were prepared according to the method described by Zhang *et al*. (2007) with minor modifications. The optimum culture conditions and cultivation conditions previously described for the individual strains were used. The strains were pre-cultured in 50 ml growth medium contained in 250 ml flask on the rotary shaker (160 rpm) at 28^{o}C for inoculation preparation. After 18 hours of cultivation, 2% (v/v) culture broth of *Halomonas* sp. Okoh and 4% (v/v) culture broth of *Micrococcus* sp. Leo were inoculated into 200 ml of the production medium in 500 ml flask (Wang *et al*., 2011). Batch fermentation was carried out under the same cultivation conditions as those for pre-cultivation. Medium samples (15 ml) were withdrawn at 12 h intervals and monitored for pH, cell growth, cell count and flocculating activity. Two millilitres of culture broth was centrifuged at 4 000 × g, 4^{o}C for 30 min, 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 (OD_{600}) and bacterial counts was determined by standard spread plate technique using nutrient agar and all plates were incubated at 35^{o}C for 36 h.

5.2.6. Extraction and purification of the bioflocculant

After 5 days of fermentation, the culture broth was centrifuged at 4 000 × g at 4^{o}C for 30 min in order to remove bacterial cells. In order to remove the insoluble substances, one volume of distilled water was added to the supernatant phase and then centrifuged at 4, 000 × g for 15 min, 4^{o}C. Two volumes of ethanol were added to the supernatant, and the solution was agitated and left standing at 4^{o}C for 12 h. To obtain the crude bioflocculant, the precipitate was vacuum dried. The purification of the crude bioflocculant was done according to the method described by Salehizadeh *et al.* (2000). The recovered precipitate was re-dissolved in distilled water (1% w/v) and one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v*)* was added. After agitating, the mixture was left standing at room temperature for 12 h. The supernatant was then be centrifuged at 4 000 × g for 15 min at 4^{o}C and dialyzed overnight against distilled water overnight. The dialysate was then vacuum-dried in order to obtain a purified bioflocculant.

5.2.7. Determination of bioflocculant dosage (Jar test)

Different concentrations of the bioflocculant solution (0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were prepared and their flocculating activities evaluated. Four grams of kaolin clay was weighed and dissolved in 1 L of distilled water. Three millilitres of 1% (w/v) $CaCl_2$ and 2 ml of bioflocculant solution were both added to 100 ml kaolin suspension inside 500 ml beakers. The solution was agitated at 200 rpm for 3 min and the speed reduced to 45 rpm for further 10 min of agitation (Wang *et al*., 2010). The solution was poured into 100 ml

measuring cylinder, allowed to sediment for 10 min and 2 ml of clear supernatant withdrawn and the flocculating activity was read at 550 nm.

5.2.8. Effect of cations on flocculating activity of purified bioflocculant

A solution of bioflocculant concentration 0.1 mg/ml was prepared. The effects of the following salt solutions at 1% w/v NaCl, KCl, $LiCl_2$, $MgCl_2$, $MnCl_2.4H_2O$, $BaCl_2$, $AlCl_3$ and $FeCl_3 \cdot 6H_2O$ on flocculating activity of the purified bioflocculant were determined according to Kurane *et al*. (1994).

5.2.9. Effect of pH on flocculating activity of purified biofloculant

Concentration of 0.1 mg/ml solution of bioflocculant solution was prepared. The adjusted pH of individual kaolin solutions in separate flasks ranged from 2.0-12.0 prior to determining flocculating activity at each of these pH values (Xiong *et al*., 2010).

5.2.10. Effect of temperature on flocculating activity of purified bioflocculant

The purified bioflocculant was dissolved in distilled water to give a concentration 0.1 mg/ml. Ten millilitre of the bioflocculant solution was heated at 50^oC, 60^oC, 70^oC and 80^oC for 30 min, and then the temperature dependence was determined by measuring the residual flocculating activity for kaolin suspension (4g/l) at room temperature (Wang *et al*., 2011).

5.2.11. Fourier transfom infrared spectroscopy (FTIR)

The functional groups of the bioflocculant were determined using a Fourier transform infrared spectroscopy (Perkin Elmer System 2000, FT-IR, England). The bioflocculant was ground with KBr salt at 25^{o}C and pressed into a pellet for FTIR analysis over a wave number of 4000-370 cm^{-1} (He *et al*., 2010).

5.2.12. Thermo-gravimetric analysis (TGA)

Ten milligrams of the bioflocculant was analysed by TGA analyzer (STA 449/C Jupiter, Netzsch, Germany Perkin Elmer TGA7 Thermo gravimetric Analyzer, USA) over a temperature range of 20-400^{o}C with a heating rate of 10^{o}C per minute under a constant flow of nitrogen gas (Wang *et al*., 2011).

5.2.13. Chemical composition of the bioflocculant

The total protein content of the purified bioflocculant was determined as described by Bradford (1976) using bovine serum albumin (BSA) as the standard solution. The total sugar content was determined by phenol-sulphuric acid method described by Chaplin and Kennedy (1994) using glucose as a standard solution. The uronic acid content of the bioflocculant was determined by carbazole method described by Bitter and Muir (1962).

5.2.14. Scanning electron microscopy (SEM)

The surface morphology structure of the purified bioflocculant and kaolin clay was investigated with scanning electron microscope (JSM-6390 LV, made in Japan). The SEM

images of the bioflocculant powder, and kaolin clay before and after being flocculated were scanned (He *et al*., 2010).

5.3. Results and Discussion

5.3.1. The yield of purified bioflocculant produced by consortium

After fermenting 1 L of a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo for 5 days, 3.51 g of purified bioflocculant was recovered. Increase in amount of bioflocculant recovered from consortium compared to the individual strains (*Halomonas* sp. Okoh: 1.213 g/l; *Micrococcus* sp. Leo: 0.738 g/l) might be due to the synergistic effect observed resulting in their combination. This implies that both of them contributed to the effective yield obtained. Zhang *et al*. (2002b) reported that bioflocculant production by *S. Cellulosum* NUST06 gave a yield of 17.5 g/l when starch and $NaNO_3$ were used as carbon and nitrogen sources respectively. Zhang *et al*. (2007) reported that about 15 g of purified bioflocculant MMF1 was produced by multiple-microorganism consortia using brewery wastewater as carbon was obtained from 1 L of culture broth. Li *et al*. (2010) also reported that 14.9 g of purified bioflocculant was obtained from 1 L fermented broth of *Agrobacterium* sp. M-503. While Wang *et al*. (2011) reported that 2.06 g of purified bioflocculant was obtained from 1 L of fermented broth of a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6.

5.3.2. Time course assay for bioflocculant production by consortium

Optimum culture conditions that were used for bioflocculant production by individual strains (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo) were adopted for culturing the consortium

of the two strains. Figure 5.1 shows the time course assay for bioflocculant production. As expected, no cell growth was observed within the first 10 h of cultivation (lag phase). However, a steady increase in cell growth accompanied by a corresponding increase in flocculating activity was observed after this period. The stationary phase of the bacteria growth was attained after 120 h of cultivation. It was also observed that the flocculating activity of the bioflocculant produced was in parallel with cell growth. This indicated that increase in cultivation period resulted in an increase in cell growth with concomitant increase in bioflocculant production. The flocculating activity of the bioflocculant reached its maximum flocculating peak of 63.2% at late stationary phase of 120 h and further increase in cultivation period resulted into a decrease in both flocculating activity and cell growth. This observation indicated that the production of bioflocculant was as a result of biosynthesis during the bacteria growth and not by cell autolysis (Gao *et al*., 2006). The decrease in flocculating activity observed after 120 h could be attributed to the presence of bioflocculant-degrading enzyme produced by the microorganisms (Li *et al*., 2009a). A similar observation was reported by Mabinya *et al*. (2011) for the bioflocculant produced by *Halomonas* sp. Okoh which attained its maximum peak at 132 h.

The initial pH of the production medium that supported optimal growth was 4, so the pH of the production medium was adjusted to 4. According to Salehizadeh and Shojaosadati (2001), the pH of the production medium determines the electric charge of the cells and oxidation-reduction potential thus affecting nutrient absorption and enzymatic reactions. It was observed that there was a decrease in pH of the medium as cultivation time progresses. The decrease in pH of the medium may be due to the production of organic acids as a result of glucose metabolism since glucose was a component of the cultivation medium or the decrease in pH might be due to the presence of organic acids produced during metabolism by bacteria (Deng *et al*., 2003; Lu *et al*., 2005).

Most researchers in this field had reported bioflocculant production by different microorganisms either in the late logarithmic growth phase or early stationary phase (Lu *et al*., 2005; Shih *et al*., 2001). Flattom, (1984) reported that the flocculating activity of the bioflocculant produced by *Phormidium starin* J-1 attained its highest flocculating peak at 96 h. The maximum bioflocculant production by *Alcaligenes latus* was achieved at the middle and late stage of logarithmic growth phase between 2-3 days and flocculating activity dropped due to the action of bioflocculant-degrading enzymes (Kurane and Nohata, 1991). Shih *et al*. (2001) observed that the bioflocculant produced by *B. licheniformis* reached maxima production during the stationary phase at 96 h. Fujita *et al*. (2000) reported that the flocculating activity peak of the flocculant produced by *Citrobacter* sp. TKF04 was obtained at 24 h of culture time and beyond in which a decrease in flocculating activity was observed. The bioflocculant produced by *Bacillus firmus* attained the highest flocculating activity after 33 h (Salehizadeh and Shojaosadati, 2002). According to the findings of Lu *et al*. (2005), bioflocculant production by *Enterobacter aerogenes* was observed to be parallel to cell growth and reached its flocculating activity peak at 60 h indicating that the bioflocculant was produced by biosynthesis during growth. The bioflocculant production pattern of *Aspergillus parasiticus* and *Bacillus licheniformis* showed good correspondence to the cell growth curve, indicating that the bioflocculant was formed during cell growth but not by cell-lysis (Deng *et al*., 2005; Xiong *et al*., 2010). With the observations of Gao *et al*. (2006), the flocculating activity of the biopolymer produced by *Vagococcus* sp. reached its maximal flocculating activity at 60 h. Li *et al*. (2007) observed that the production of biofloculant by *Aeromonas* sp. reached its maximal flocculating activity at 72 h and the production of this bioflocculant was as a result of cell autolysis because the cell growth decreased after 36 h of cultivation. The growth curve of *P. Mirabilis* TJ1 showed that bioflocculant production was almost parallel with cell growth, and pH of the production medium dropped from 6.5 to 5.5 (Xia *et*

al., 2008). The flocculating activity of the bioflocculant produced by *Serratia fiacaria* and *Bacillus* sp. F19 reached its maximal at early stationary phase of 72 h (Gong *et al*., 2008; Zheng *et al*., 2008). Li *et al*. (2009a) reported that the biopolymer produced by *B. licheniformis* X14 attained its optimal flocculating activity at 48 h. Liu *et al*. (2010) reported that the flocculating activity of the bioflocculant produced by *Chryseobacterium daeguense* W6 cultured in low nutrition medium and observed that it reached its maximal flocculating activity after 54 h. The cell growth decreased after 10 h indicating that the production of bioflocculant was as result of cell-lysis. In *Agrobacterium* sp. M503, the biopolymer production was as at its flocculating activity peak at 48 h (Li *et al*., 2010). Cosa *et al*. (2011) reported that the flocculating activity peak of the bioflocculant produced by *Virgibacillus* sp. Rob was attained after 4 days of cultivation time. The bioflocculant produced by *Bacillus* sp. Gilbert had the highest peak of flocculation at 240 h of cultivation time (Piyo *et al*., 2011). Ugbenyen *et al*. (2012) reported about the flocculant produced by *Cobetia* sp. which reached its highest flocculating activity at 72 h.

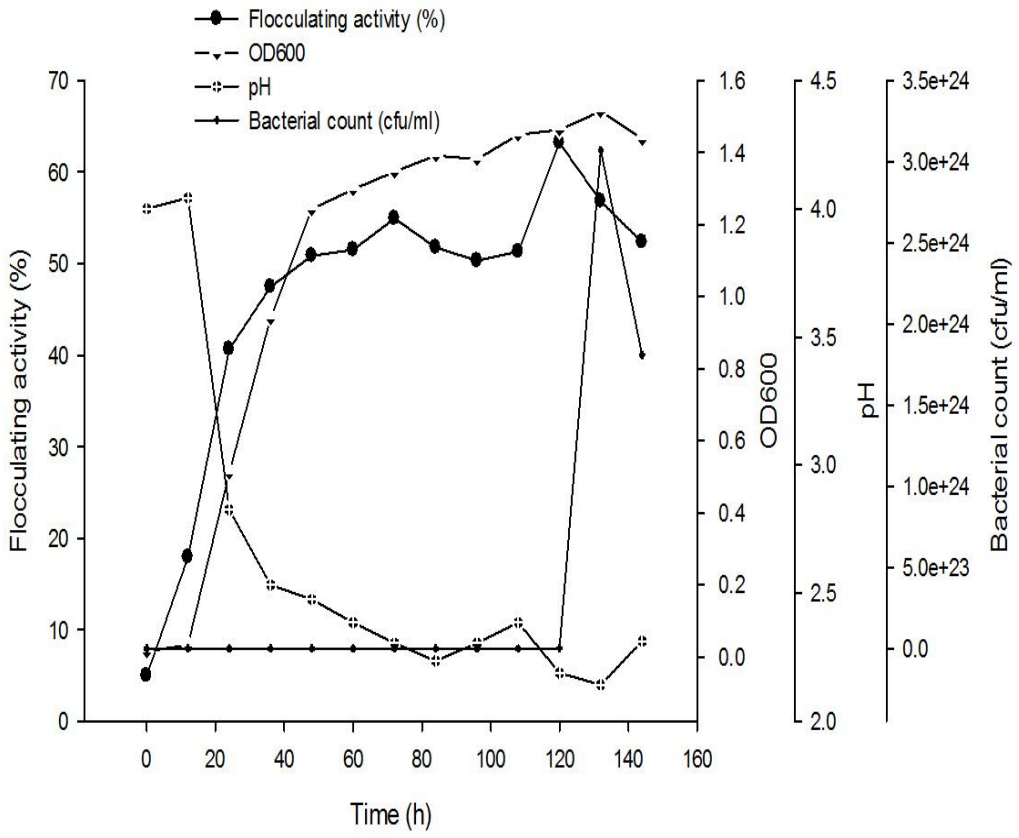

Figure 5.1: Time course for bioflocculant production by a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo.

5.3.3. Effect of bioflocculant dosage on flocculating activity of the purified bioflocculant

The appropriate bioflocculant concentration to be used for subsequent experiments was determined by investigating different concentrations ranging from 0.02-0.5 mg/ml as depicted in Figure 5.2. It was observed that the flocculating activity of the bioflocculant increased as the concentration increases. The optimum flocculating activity was obtained at 0.1 mg/ml and further increases in bioflocculant concentration resulted in a decline in the flocculating activity. According to the observation of Zufarzaana *et al*. (2012), low dosage will not make bridging flocculation mechanism of the bioflocculant to be effective and high dosage will generate high viscosity which will inhibit the settling of suspended particles by restabilization

of kaolin particles. When the bioflocculant molecules are excessively presence in the solution, they usually generate high viscosity, blocked the adsorption sites thereby reducing flocculating processing and flocs formation (Gong *et al*., 2008; Wang *et al*., 2011, Zufarzaana *et al.,* 2012). A similar observation was observed by Deng *et al*. (2003) of the bioflocculant produced by *Bacillus mucilaginosus* that required a dosage of 0.1 mg/ml bioflocculant for optimum flocculating activity. On the contrary, Lee *et al*. (1995) reported that the dosage required for optimal flocculating activity of a bioflocculat produced by *Arcualendron* sp. was 2 mg/l. Also, the dosage required by the bioflocculant produced by *Pestalotiopsis* sp., *Bacillus* sp and *Gyrodinium impudicum* KG03 was 1.0 mg/l (Kwon *et al*., 1996; Suh *et al*., 1997). According to the reports documented by Gao *et al*. (2006), 25 mg/l dosage of bioflocculant MBFW31 produced by *Vagococcus* sp. was needed for effective flocculation. Lu *et al*. (2005) reported that 90 mg/l was the appropriate dosage for effective kaolin flocculation by the bioflocculant produced by *Enterobacter aerogenes.* Yim *et al*. (2007) investigated dosage requirement for optimum flocculating activity for bioflocculant p-KG03 produced by a marine dinoflagellate, *Gyrodinium impudicum* KG03 and found it to be 1.0 mg/l. In a similar way, Li *et al*. (2007) reported that the flocculating activity of the bioflocculant produced by *Aeromonas* sp. was optimal at a dosage of 1.0 mg/l. The compound bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 required a dosage of 12 mg/l for effective flocculating activity (Wang *et al*., 2011).

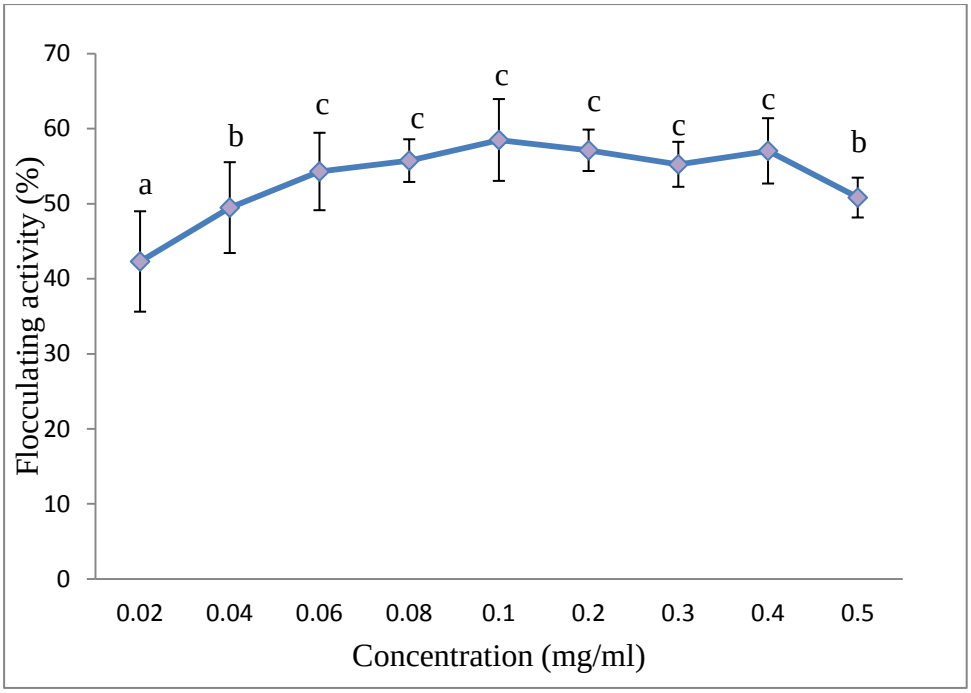

Figure 5.2: Effect of bioflocculant concentration on the flocculating activity of the bioflocculant produced by a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo). Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

5.3.4. Thermostability of the purified bioflocculant

The results for thermostability of the bioflocculant are illustrated in Figure 5.3. After heating 0.1 mg/ml solution of the purified bioflocculant at different temperatures ranging from 50-80^{o}C for 30 min, flocculating activity of the residual bioflocculant was measured at room temperature. Figure 5.3 shows a decreased in flocculating activity from 77.7% at 50^{o}C to approximately 70% at 80^{o}C. The bioflocculant maintained and retained about 70% of its flocculating activity at 80^{o}C due to its structure which is mainly composed of polysaccharide. Li *et al*. (2007) reported that the bioflocculant produced by *Aeromonas* sp. could retain its flocculating activity with only 9.2% decreased in flocculating activity after being heated at 100^{o}C for 60 min. Gong *et al*. (2008) observed that the bioflocculant produced by *Serratia ficaria* could retain its flocculating activity after being heated at 100^{o}C for 15 min, mainly

due to the backbone being a polysaccharide. Li *et al.* (2010) reported that the bioflocculant produced by *Agrobacterium* sp. M503 retained its flocculating activity up to 70^{o}C and further increase in temperature up to 121^{o}C had no effect on flocculating activity. High thermostability property of a compound bioflocculant CBF-F26 was observed when the purified bioflocculant was heated over 100^{o}C for 30 min. The residual flocculating activity of this bioflocculant was more than 90% (Wang *et al*., 2011). Ugbenyen *et al*. (2012) reported about the crude bioflocculant produced by *Cobetia* sp. which maintained its flocculating activity of 87% at 50^{o}C, 82% at 80^{o}C and 78% at 100^{o}C after heated for 25 min. On the contrary, some bioflocculants produced by different microorganisms have been reported to have low thermal stability. For example, according to the findings of Kurane *et al.* (1986a) and Salehizadeh *et al*. (2001), the bioflocculants produced by *Rhodococcus erythropolis* and *Bacillus firmus* respectively could only retained 50% of their flocculating activity after being heated in boiling water for 15 min. The thermostability test that was done on the bioflocculant produced by *Bacillus* PY-90 showed that it lost its flocculating activity when heated at 100^{o}C for 40 min. He *et al*. (2004) showed that an increase in temperature up to 100^{o}C, resulted in a complete loss of flocculating activity for bioflocculant REA-11 produced by *Corynebacterium glutamicum*.

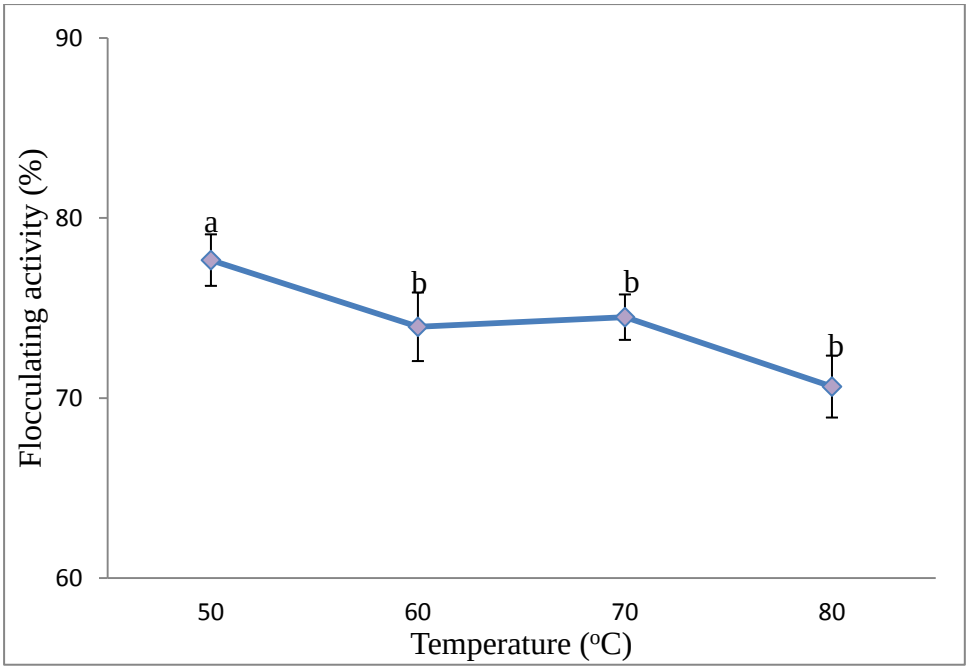

Figure 5.3: Effect of temperature on the flocculating activity of the bioflocculant produced by a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo). Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

5.3.5. Effect of cations on the flocculating activity of bioflocculant produced by the consortium

The role of cations on the flocculating activity of the bioflocculant produced by the consortium was investigated and the results are depicted in Figure 5.4. Of all the cations tested, the flocculating activity of the bioflocculant was enhanced albeit to varying degrees by Ca^{2+} (72%), Mn^{2+} (59.8%) and Al^{3+} (80%) and inhibited to different extents by Li^{+} (12.2%), Na^{+} (18.8%), K^{+} (7.4%), Mg^{2+} (31.5%) , Ba^{2+} (43.6%) and Fe^{3+} (36%). The role of cation is to neutralize and stabilize the negative charge of both functional groups of kaolin particle in solution and the bioflocculant (Salehizadeh and Shojaosadati, 2002; Wu and Ye, 2007). Levy *et al*. (1992) stated that the role of bivalent and trivalent cations is to increase the adsorption of bioflocculants on the suspended particles by decreasing the negative charge on both the

polymer and the particle. Li *et al*. (2009a and 2009b) also reported enhancement of flocculating activity of a bioflocculant produced by *Bacillus licheniformis* and *Bacillus circulans* in the presence of Al^{3+}, Fe^{3+}, and Ca^{2+}. According to the investigation of Patil *et al*. (2009) about the bioflocculant produced by a *Bacillus substilis,* the flocculating activity was stimulated in the presence of Al^{3+} and Fe^{3+}. Salehizadeh *et al*. (2000) observed that the flocculating activity of the bioflocculant produced by *B. coagulants* As 101 was enhanced by the addition of Al^{3+}, Fe^{3+} and Ca^{2+}. The compound bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6, displayed flocculating activity of 97% when Al^{3+} was used as a coagulating aid (Wang *et al*., 2011). According to the findings of Salehizadeh and Shojaosadati, (2002), the bioflocculant MS-102 produced by *Bacillus firmus* was a good example of cation-dependent bioflocculant whose flocculating activity was greatly enhanced by Ca^{2+} and Mg^{2+}. Zhang *et al*. (2002a) reported that the flocculation efficiency of a bioflocculant produced by marine *mycobacterium Nannocystis* sp. NU-2 was enhanced strongly on addition of Fe^{3+} and Al^{3+}. Deng *et al*. (2005) reported that the flocculating activity of the bioflocculant produced by *Aspergillus parasiticus* was stimulated by Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{2+}. The flocculating activity of the bioflocculant MBF-3 produced by *Bacillus* sp. was stimulated by Al^{3+}, Mg^{2+}, Ca^{2+}, K^{+} and Na^{+} with Al^{3+} being the most effective (Feng and Xu, 2008).

On the contrary, Kumar *et al*. (2004) reported that the flocculating activity of the bioflocculant produced by a haloalkalophilic *Bacillus* was stimulated when divalent cations Ca^{2+}, Cu^{2+}, Zn^{2+} were present but a decrease in the flocculating activity was observed when trivalent cations Fe^{3+} and Al^{3+} were present. In the presence of Zn^{2+}, the bioflocculant WF-1 greatly improved the separation solids from Trona suspension (Lu *et al*., 2005). Wu and Ye, (2007) observed that the flocculating activity of the extracellular biopolymer produced by a *Bacillus subtilis* DYU1 isolate greatly increased in the presence of Ca^{2+} and Mg^{2+}. A similar

effect was observed by Gong *et al*. (2008) where divalent cations Ca^{2+} and Mg^{2+} stimulated the flocculating activity of the bioflocculant produced by *Bacillus* sp. F19 greatly. Numerous studies have been conducted with bioflocculants from different microorganisms where the flocculating activities of the bioflocculants were enhanced by Ca^{2+}. These include bioflocculants produced by *Halomonas* sp. Okoh and *Methylobacterium* sp. Obi whose flocculating activities were enhanced by the addition of Ca^{2+} (Mabinya *et al*., 2011; Ntsaluba *et al*., 2011). Cosa *et al*. (2011) reported that the flocculating activity of the bioflocculant produced by *Virgibacillus* sp. Rob was greatly stimulated when Fe^{2+} was used as the cation. Fujita *et al.* (2000) reported that the flocculating activity of the biofloculant produced by *Citrobacter* sp. TKF04 was not stimulated by the addition of any cations. In a similar way, addition of cations had no significant effect on the flocculating activity of the bioflocculant MBFF19 produced by *Bacillus mucilaginosus (*Deng *et al*., 2003). Liu *et al*. (2010) reported that the bioflocculant produced by *Chryserbacterium daeguense* required no cation for its flocculation efficiency. Zheng *et al*. (2008) reported that the addition of cations had no effect on the flocculating activity of the bioflocculant produced by *Bacillus* sp. F19 and addition of Fe^{3+} inhibited the flocculating activity.

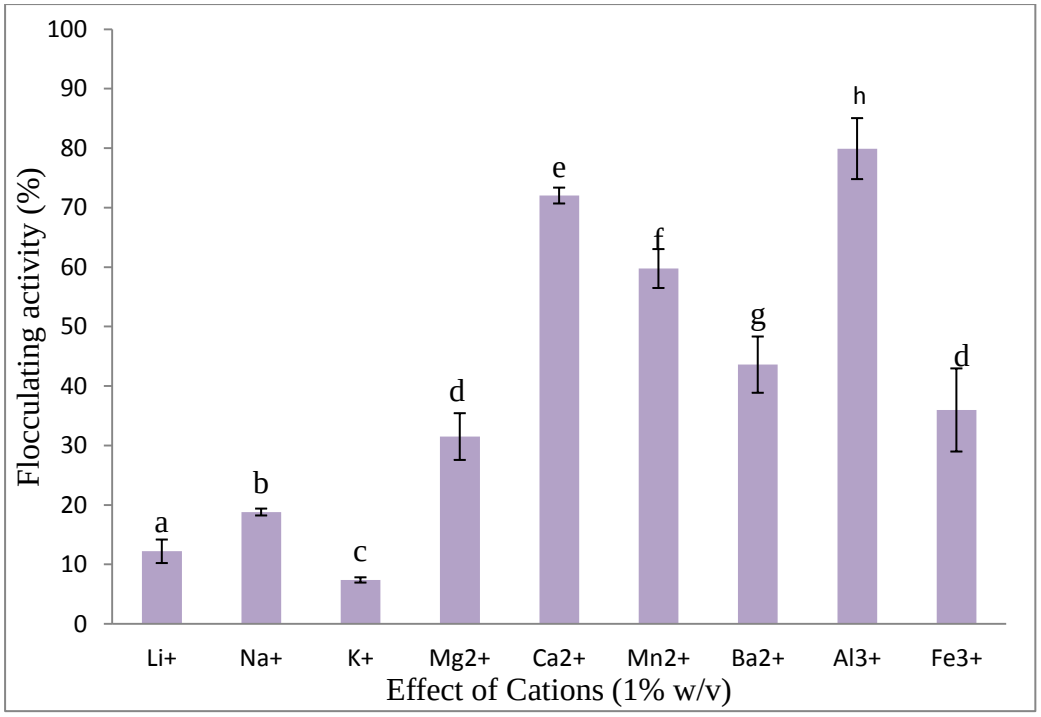

Figure 5.4: Effect of cations on the flocculating activity of the bioflocculant produced by a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo). Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

5.3.6. Effect of increasing concentration of cation on flocculating activity of the bioflocculant

The effect of increasing the concentration of $AlCl_3$ on the flocculating activity of bioflocculant produced by the consortium was investigated and the results are depicted in Figure 5.5. The flocculating activity of the bioflocculant decreased with an increase in concentration of $AlCl_3$. The highest flocculating activity peak of 65.5 % was attained at 1% (w/v). Therefore, increasing the concentration of this cation beyond 1% resulted into a decreased in flocculating activity (Figure 5.5). The role of cations is to increase the initial adsorption of bioflocculant on the suspended particles by decreasing the negative charges of both bioflocculant molecules and kaolin particles (Li *et al*., 2008). Feng and Xu (2008)

reported a similar observation with the bioflocculant MBF3-3 produced by *Bacillus* sp. where an increase in the concentration of $AlCl_3$ resulted into a decrease in the flocculating activity of the produced bioflocculant and no flocculation was observed when the concentration was increased to 50 mg/l.

On the contrary, He *et al*. (2010) reported that an increase in the concentration of Ca^{2+} stimulated the flocculating activity of the bioflocculant produced by *Halomonas* sp. V3a' and this could be attributed to double layer compression effect of Ca^{2+}. Since the kaolin particles exhibits a negative charge in aqueous solution, the divalent cation Ca^{2+} could compress the double layer of kaolin particles thereby weakening the electrostatic repulsive force.

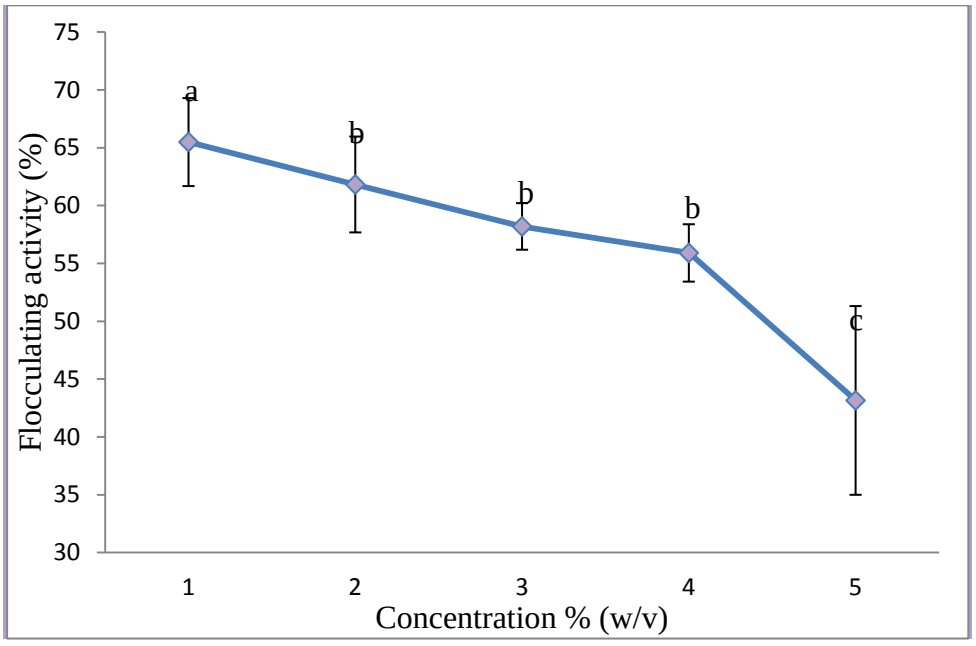

Figure 5.5: Effect of increasing cation ($AlCl_3$) concentration on the flocculating activity of the bioflocculant produced by a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

5.3.7. Effect of pH on the flocculating activity of bioflocculant produced by the consortium

The effect of pH on the flocculating activity of the compound bioflocculant was investigated using bioflocculant dosage of 0.1 mg/ml at different pH values ranging from 2-11. Figure 5.6 shows the results of the effect of pH on flocculating activity of the purified bioflocculant. It was observed that an increase in pH resulted into an increase in the flocculating activity of the produced bioflocculant. A sharp decreased in flocculating activity was recorded at pH 7 with an immediate rise up to pH 8 followed by a slight decrease and relative stability in pH. It was observed that the bioflocculant produced flocculated well at a wide pH range of 2-10 with the maximum flocculating activity peak of 86% at pH 8. Yokoi *et al*. (1995) stated that the pH of the solution plays an important role in flocculating efficiency of the bioflocculant. Wang *et al*. (2011) stated that the pH affected stability of suspended particles and the formation of flocs. Prasertsan *et al*. (2006) reported that the bioflocculant p-KG03 produced by a marine dinoflagellate, *Gyrodinium impudicum* KG03 flocculated best under acidic conditions of pH 4. The optimum pH for flocculating activity of the biopolymer produced by *Enterobacter cloacae* WD7 was 6 (Prasertsan *et al*., 2006). The compound biopolymer CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 had flocculating activity between pH 7-9. The flocculating activity of extracellular bioflocculant produced by a *Bacillus subtilis* DYU1 isolate was highest or enhanced at a weak acid or neutral pH (Wu and Ye, 2007). The flocculating activity of the exopolysaccahride bioflocculant produced by the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913 was optimum between pH 5-8. The bioflocculant produced by *Bacillus alvei* NRC-14 displayed effective flocculating activity at pH 4.5-5.5 (Abdel-Aziz *et al*., 2011). He *et al*. (2010) reported that the flocculating activity of the bioflocculant HBF-3 produced by *Halomonas* sp. V3a' had a wide pH range with average flocculating activity above 80% and a

flocculating activity peak of 97% at pH 7. The bioflocculant produced by *Bacillus* sp. F19 flocculated best at pH 2 and had a wide pH range of 2-9 (Zheng *et al*., 2008). Some biofloccualnts flocculated well at neutral pH conditions, indicating that the complexes formed between bioflocculant molecules and kaolin particles are destabilized by the hydrogen ion (H^+) or (OH^-) (He *et al*., 2010). Also, Prasertsan *et al*. (2006) stated that at high pH, the hydroxyl ion (OH^-) absorbed at alkaline condition destabilized the formation of complexes between bioflocculant molecules and kaolin particles resulting in low flocculating activity. The flocculating activity of thermal and alkaline stable bioflocculant produced by *Agrobacterium* sp. M-503 increased at the pH range of 8-12 (Li *et al*., 2010). Wang *et al*. (2011) reported that the flocculating activity of a compound bioflocculant CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 was recorded at more than 90% at a pH range of 7-9. The flocculating activity of CBF-F26 was maximal under neutral and weak alkaline condition (Wang *et al*., 2011). Li *et al*. (2009a) reported that the flocculating activity of EPS SM9913 was maximally maintained at a pH range of approximately 6-8, had the flocculating activity peak at pH 7.0 but decreased when the pH was lower than 6 or greater than 8. Yim *et al*. (2007) observed that the flocculating activity of the bioflocculant produced by *Gyrodinium impudicum* KG03 was maintained at a high level under acid conditions of pH range 3-6, but the flocculating activity dropped when the pH was greater than 6. The flocculating activity of the bioflocculant was highest at pH 4. According to the findings of Shimofuruya *et al*. (1996), the biopolymer produced by *Streptomyces griseus* was active in acidic conditions ranging from pH 2-6, with the maximum flocculating activity peak at pH 4. For *Enterobacter* sp. BY-29, the highest flocculating activity was observed at pH 3, and the flocculating activity decreased with an increase in pH (Yokoi *et al*., 1997). Deng *et al*. (2005) reported that the flocculating activity of the bioflocculant produced by *Aspergillus parasiticus* was optimal at acidic conditions. The flocculating activity of the

bioflocculant produced by *Chryseobacterium daeguense* W6 was over 90% in the pH range of 4-8. The flocculating activity of a polysaccharide bioflocculant produced by a marine *Bacillus subtilis* MSBN17 was highly stable at alkaline pH and showed 75.62% of flocculating activity at pH 12.

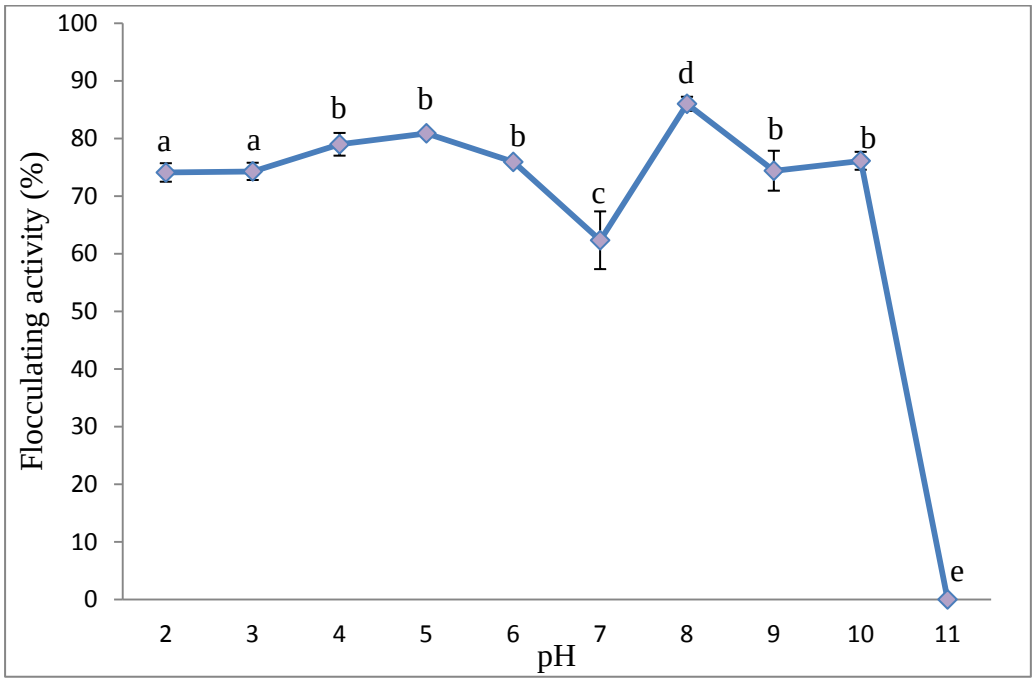

Figure 5.6: Effect of pH on the flocculating activity of the bioflocculant produced by a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo). Flocculating activities with different letters are significantly different ($P < 0.05$) from each other.

5.3.8. Chemical composition of the purified bioflocculant

Chemical analysis showed that the purified bioflocculant was composed of 4.73% total protein content, 62.3% total sugar content and 25.7% uronic acid. Wang *et al*. (2011) reported the purified bioflocculant CBF-F26 mainly composed of polysaccharide with

monosaccharide units of rhamnose, mannose, glucose and galactose respectively in a 1.3: 2.1: 10.0: 1.0 molar ratios.

5.3.9. Thermogravimetric property of the purified bioflocculant

The thermogravimetric property analysis of the purified bioflocculant was used to elucidate its behaviours when subjected to heat. This enables us to understand its pyrolysis property when exposed to a very high temperature. From Figure 5.7, there was about 20% decrease in weight at 200^{o}C and about 29% loss of weight at 500^{o}C. The first weight loss could be due to loss of moisture content in the bioflocculant (Kumar and Anand, 1998). Similarly, in the case of bioflocculant p-KG03 produced by a marine dinoflagellate *Gyrodinium impudicum* KG03 (Yim *et al*., 2007), the initial weight loss was observed between 40-230^{o}C. Further decrease in weight loss of this bioflocculant was observed at about 310^{o}C. Wang *et al*. (2011) reported on a study conducted on a compound bioflocculant by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 where initial loss of about 10% was observed between 20 and 150^{o}C. Further decreased in weight of 40% was observed at 400^{o}C and there was a total loss of weight at 1000^{o}C.

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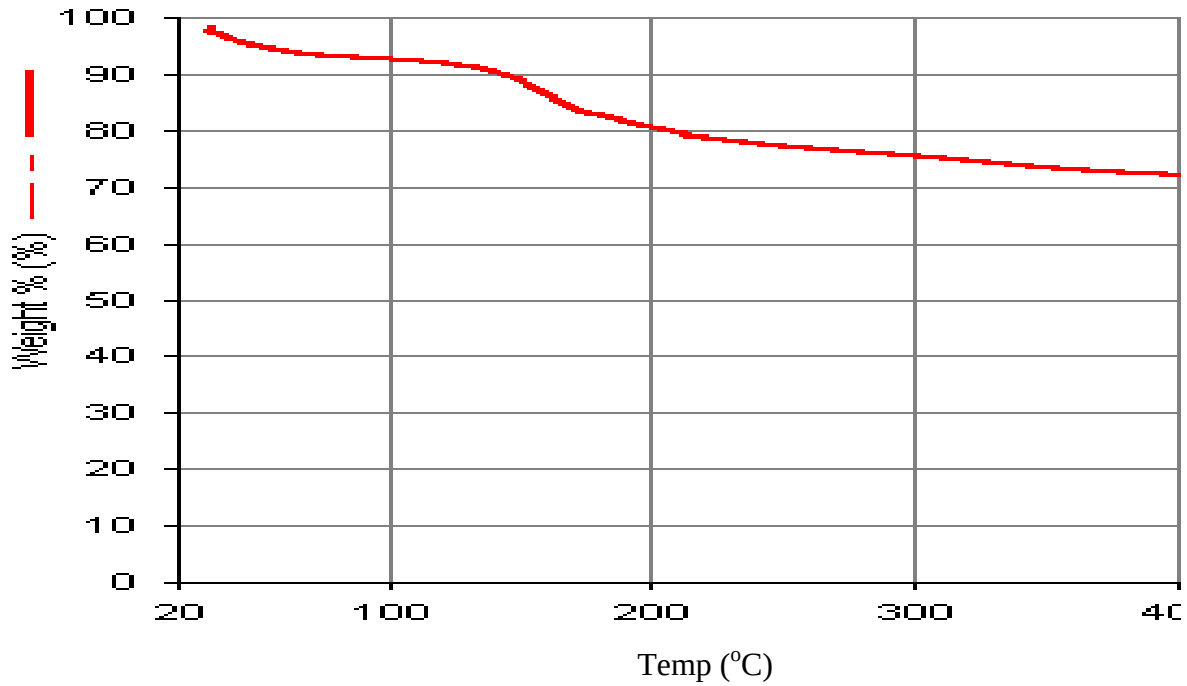

Figure 5.7: Thermogravimetric analyses of the purified bioflocculant

5.3.10. Functional groups determination by FTIR

The composition of the bioflocculants produced by different microorganisms differ (Salehizadeh and Shojaodasati, 2001). The flocculating activity of the purified bioflocculant solely depends on the chemical structure which is related to the functional groups in the molecule. The Fourier-transform infrared (FTIR) spectrum analysis revealed the presence of different functional groups in the molecule. In Figure 5.8, the spectrum peak at 3412 cm^{-1} showed the presence of OH group and NH_2 group in the molecule (Desouky *et al*., 2008). The weak band noticed at 2113 cm^{-1} indicated the presence of aliphatic bonds. The spectrum peak at 1622 cm^{-1} is an indication of the presence of an amide group (Fujita *et al*., 2000). The vibration peak at 1139 cm-^{1} corresponding to the C-O stretching in alcohols and this further suggests the presence of OH group in the bioflocculant molecule (Deng *et al*., 2005). The spectrum peaks in between 1000-1100 cm^{-1} suggested the presence of saccharide derivatives.

The spectra peaks from 604-670 cm^{-1} represent the presence of benzene ring-structured compound in the bioflocculant molecule.

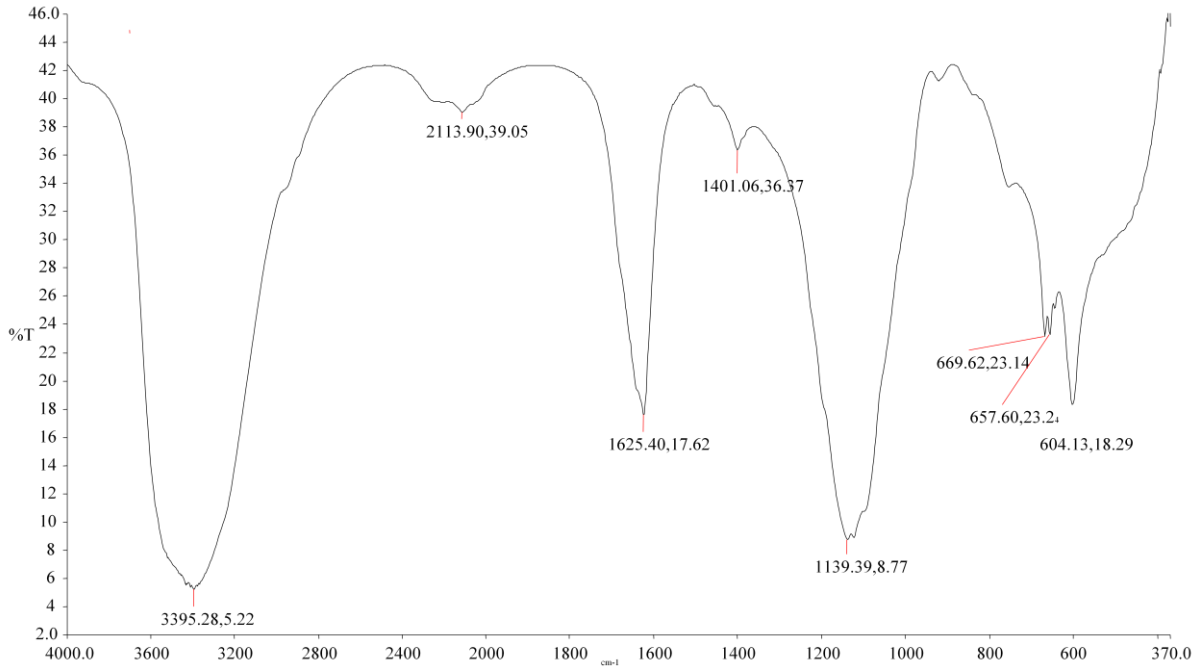

Figure 5.8: Fourier transform intrared (FTIR) spectroscopy of the purified bioflocculant

5.3.11. SEM observations

The surface structures of the bioflocculant, kaolin particles before and after flocculation were observed under a scanning electron microscope and the images are depicted in Figure 5.9. The amorphous structure of the purified bioflocculant is represented in Figure 5.9(A), showing a thread-like structure. Figure 5.9(B) shows how the kaolin particles are scattered before flocculation with Figure 5.9(C) depicting how the scattered kaolin particles are linked together with the help of bioflocculant after flocculation. Similar observations were made about the structure of the purified compound bioflocculant.

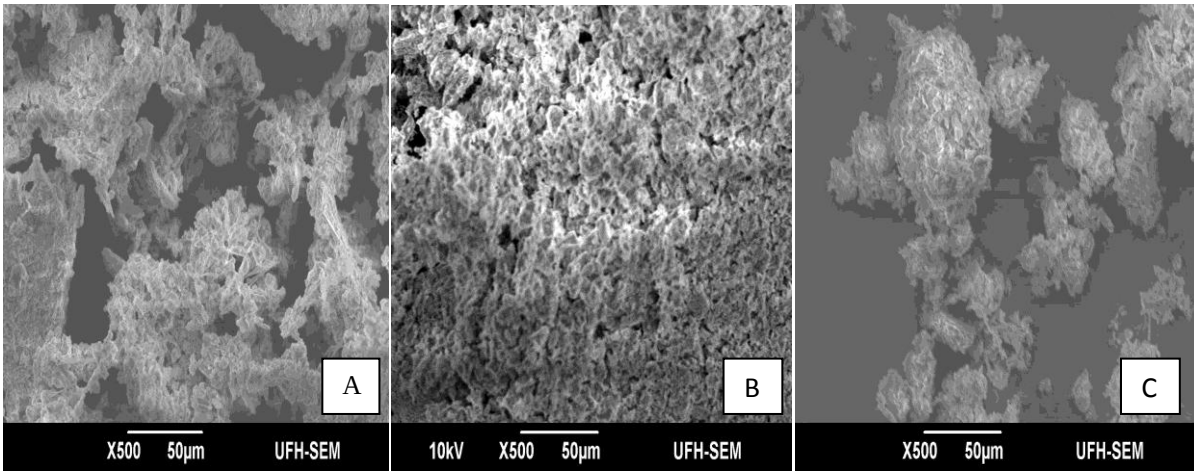

Figure 5.9: SEM images of purified bioflocculant A, Kaolin particles B and Kaolin particles flocculated with bioflocculant.

5.4. Conclusions

The flocculating efficiency and physicochemical properties of the compound bioflocculant produced by a mixed culture of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo were investigated. The bioflocculant maintained wide pH range flocculating activity with a maximum peak of 86% at pH 8. The glycoprotein bioflocculant possessed hydroxyl, carboxyl and amino groups in its molecule as the main functional groups which were responsible for the flocculation mechanism. Further studies on the characterization of the bioflocculant are needed in order to optimize its large-scale production.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

Chemical flocculants are widely used in various industrial processes due to low cost and flocculating efficiency, some of their degraded monomers such as acrylamide are neurotoxic and carcinogenic (Shih *et al*., 2001). As a result of these demerits, biopolymers secreted during growth of microorganisms have been given more attention as potential flocculating agents (Salehizadeh and Shojaosadati, 2001).

Bioflocculants have been reported to be produced from different sources including green algae (Kaplan and Christiaen, 1987), bacteria (Takagi and Kadowaki, 1985), fungi (Kurane *et al*, 1986a). Deng *et al*. (2005) found that *Aspergillus parasitus* could produce a bioflocculant with a flocculating activity for kaolin suspension and waste-soluble dyes. He *et al.* (2010) reported the production of a novel bioflocculant produced by deep-sea bacterium mutant *Halomonas* sp. V3a. *Cobetia* sp. produced a thermostable acidic polysaccharide bioflocculant whose activity was dependent on the presence of cations (Ugbenyen *et al*., 2012).

Despite these bioflocculant-producing microbes, low flocculating efficiency, high production costs and poor yields are limiting factors hindering their large production (Li *et al*., 2003; Li *et al*., 2009a). In addition, the chemical composition of the bioflocculants produced by different microorganisms differs considerably (Salehizadeh and Shojaosadati, 2001).

In this present study, culture conditions for the cultivation of individual strains (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo) were optimised in order to improve the production of a compound bioflocculant from a consortium of these strains.

Marine bacteria such as *Halomonas* strains are halophilic, gram-negative rods whose species are widely distributed in hyersaline habitats. They are versatile in terms of their ability to

grow in a variety of temperature and pH conditions (Bouchotroch *et al*., 2001). He *et al*. (2010) reported about the bioflocculant HBF-3 produced by deep-sea bacterium mutant *Halomonas* sp. V3a.

Also, the genus *Micrococcus* is aerobic, Gram-positive, oxidase-positive bacteria, spherical in shape and always found in tetrad forms ranging from 1 to 1.8 mm in diameter. They are usually non-motile and do not form spores. Their genome is very rich in guanine and cytosine (GC), having 65 to 75 GC content (Kocur *et al.,* 2006; Bannerman and Peacock, 2007). From literature search, no species of *Micrococcus* have been reported for bioflocculant production.

When bacteria are cultivated in a favourable environment, they have the ability to excrete biopolymers into the medium or attached as a capsule on the cell which have so many biological functions (Deng *et al*., 2003). In order to optimise the production of flocculants from any bioflocculant-producing microorganisms, there are certain factors to be considered (Salehizadeh and Shojaosadati, 2001). These factors include: inoculum size, carbon and nitrogen sources, cation, pH effects, incubation temperature, and agitation speed (Salehizadeh and Shojaosadati, 2001; Zufarzaana *et al*., 2012). In the optimization of culture conditions, aforementioned factors were investigated for bioflocculant production by *Halomonas* sp. Okoh and *Micrococcus* sp. Leo.

The first crucial factor considered was inoculums size (Salehizadeh and Shojaosadati, 2001). From the experimental observations, it was observed that there was a variation in inoculums size requirement of the two strains. Two percent (2%) inoculum size was optimum for bioflocculant production by *Halomonas* sp. Okoh (Figure 3.1) while for *Micrococcus* sp. Leo, 4% supported the highest bioflocculant production (Figure 4.1). According to Salehizadeh

and Shojaosadati (2001), small inoculum size prolongs the stagnant phase and large inoculums size in turn inhibits bioflocculant production. Xiong *et al*. (2010) reported maximum bioflocculant production by *Bacillus licheniformis* when 4% (v/v) inoculum size was utilized whilst Ugbenyen *et al*. (2012) reported a 2% inoculum size requirement for optimum bioflocculant production by *Cobetia* sp.

Other factors which play an important role in bioflocculant production by enhancing cell growth are carbon and nitrogen sources (Liu *et al*., 2010; Ntsaluba *et al*., 2011; Coas *et al*., 2011). Carbon and nitrogen sources requirement differs from one organism to the other. Some microorganisms require organic or inorganic carbon and nitrogen sources while others require a combination of organic and inorganic nitrogen (Liu *et al*., 2010; Piyo *et al*., 2011; Ugbenyen *et al*., 2012). In this study, glucose was the preferable sole carbon source of choice for bioflocculant production in both bacterial strains used (Figures 3.2 and 4.2). Similar preferences for carbon source were reported for bioflocculant production by *Chryseobacterium daeguense* W6 (Liu *et al*., 2010), *Methylobacterium* sp. Obi (Ntsaluba *et al*., 2011). On the contrary, He *et al*. (2004) reported that sucrose was the favourable carbon source for bioflocculant production by *Corynebacterium glutamicum*. Different nitrogen sources were tested and it was observed that for both cell growth and bioflocculant production, the two bacterial strains used in this study utilized peptone, ammonium sulphate, ammonium chloride and multiple nitrogen (urea, yeast extract and ammonium sulphate) effectively (Figures 3.3 and 4.3).

The flocculating activity of the bioflocculant produced was measured according to the description of Kurane *et al*. (1994). Throughout the whole study, kaolin clay was used as the test material. Kaolin particles in solution exhibit a negative charge and the repulsive forces in

between the particles are greater than the Van der Waal force of attration (Lachhwani, 2005). Chemical composition analyses of the bioflocculants revealed that polysaccaharide and uronic acid constituted the major components. The predorminant functional groups of the bioflocculants are carboxyl and hydroxyl group. Therefore, there is a need to reduce the negative charge on the kaolin particles (surface charge density) in order to shorten the distances in between them (Levy *et al*., 1992). According to the observations by Wu and Ye, (2007), cations stimulate flocculation by neutralizing negative charges of both functional groups of suspended particles and polysaccharide by increasing the initial adsorption of the polysaccharide onto suspended particles. The flocculating activities of both crude and purified bioflocculants produced by *Halomonas* sp. Okoh, *Micrococcus* sp. Leo and their consortium were highly stimulated in the presence of Al^{3+} (Figures 3.5, 3.9, 4.4 and 4.8). On the contrary, Ca^{2+} stimulated the compound bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 due to double layer compression mechanism by the cation. In addition, Cosa *et al*. (2011) reported that the flocculating activity of the bioflocculant produced by *Virgibacillus* sp. Rob was stimulated by the presence of Fe^{2+}.

The pH of the culture medium may affect or influence the production of the bioflocculant (Salehizadeh and Shojaosadati, 2001). The initial pH requirement may have a different effect with different strains. Initial pH 4 was observed to be effective for optimal bioflocculant production by both *Halomonas* sp. Okoh and *Micrococcus* sp. Leo (Figures 3.4 and 4.5); while the flocculating activity of the purified bioflocculant by *Halomonas* sp. Okoh was best in acidic to neutral conditions (Figure 3.9). On the other hand, the flocculating activity of the purified bioflocculant produced by *Micrococcus* sp. Leo had a pH range of 2-9. The bioflocculant produced by the consortium required a pH range of 2-10 for effective

flocculation (Figure 4.9). According to Xia *et al*. (2008), the initial pH of production medium determines the electric charge of the cells, influence oxidation-reduction potential and also affect nutrient absorption and enzymatic reactions. Zhang *et al*. (2007) observed that initial pH 6 was optimal for bioflocculant production by multiple-microorganism consortia. Zheng *et al*. (2008) reported that bioflocculant production by *Bacillus* sp. F19 was optimal at pH 8.95. Cosa *et al*. (2012) reported that the production of bioflocculant by *Virgibacillus* sp. Rob was highly favourable at pH 12. According to Zufarzaana *et al*. (2012), the pH of the solution plays an important role in the flocculation process. In addition to this, Wang *et al*. (2011) stated that the pH of a solution determines both floc formation and stability of suspended particles. The flocculating activity of purified bioflocculant obtained from a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo was optimised at pH 8.

Different agitation speeds have been reported in literature which favours the cultivation of microorganisms for cell growth and bioflocculant production (Salehizadeh and Shojaosadati, 2001). In this study, it was observed that 160 rpm favours the growth of both *Halomonas* sp. Okoh (Figure 3.6) and *Micrococcus* sp. Leo for bioflocculant production (Figure 4.6). Similarly, Ugbenyen *et al*. (2012) reported the production of thermostable polysaccharide bioflocculant at 160 rpm. On the contrary, Li *et al*. (2007) reported the production of bioflocculant by *Aeromonas* sp. at 170 rpm. According to the observations of Zhang *et al*. (2007), the agitation speed determines the level of dissolved oxygen which will be available for microorganisms during cultivation, nutrient absorption and optimum enzymatic reaction.

For optimal enzymatic reaction, an optimum temperature is required (Nakata and Kurane, 1999). Incubation temperature of 28^{o}C was optimal for bioflocculant production by both *Halomonas* sp. Okoh (Figure 3.7) and *Micrococcus* sp. Leo (Figure 4.7). In another reported

study, 37^{o}C was the optimal temperature for bioflocculant production by *Bacillus licheniformis* (Xiong *et al*., 2010).

Optimum culture conditions that were determined for cultivating individual strains were utilized for the production of a compound bioflocculant from the consortium of the two microorganisms. The time course profile for bioflocculant production was monitored for 144 h. It was observed that the flocculating activity of the produced bioflocculant was parallel with cell growth. Increase in cultivation time led to increase in cell growth and flocculating activity of the produced bioflocculant Figure 5.1. This implies that bioflocculant production was as a result of biosynthesis and not by cell autolysis (Fugita *et al*., 2000). The highest flocculating activity of 63.2% was attained after 120 h. Thereafter, both the flocculating activity and cell growth decreased (Figure 5.1) due probably to the presence of bioflocculant-degrading enzymes (Gong *et al*., 2008). The pH of the production medium was adjusted to 4.0, but a decrease in pH with respect to time was observed. This decrease in pH might be due to the production of organic acids from glucose metabolism or due to the presence of organic acids as part of the components of the bioflocculant produced (Lu *et al*., 2005). Shih *et al*. (2001) noticed that the bioflocculant produced by *B. licheniformis* reached maxima activity during the stationary phase at 96 h. According to the findings of Lu *et al*. (2005), bioflocculant produced by *Enterobacter aerogenes* was observed to be parallel to cell growth and reached its maximum flocculating activity at 60 h indicating that the bioflocculant was produced by biosynthesis during growth. Liu *et al*. (2010) investigated the flocculating activity of the bioflocculant produced by *Chryseobacterium daeguense* W6 cultured in low nutrition medium and reported that it reached its maximum flocculating activity after 54 h. On the contrary, Li *et al*. (2007) observed that the production of biofloculant by *Aeromonas*

sp. reached its maximum activity at 72 h and the production of this bioflocculant was as a result of cell autolysis because the cell growth decreased after 36 h of cultivation.

The major problems affecting large-scale production of bioflocculants is the low flocculating efficiency, high cost of production and low yields (Li *et al*., 2003). Several approaches have been tried by different researchers in order to increase the output. The problem of low flocculating activity of the produced bioflocculant had been solved by optimising the conditions under which the bioflocculant works best (Wu *et al*., 2010). According to Fujita *et al*. (2000), looking for a low-substrate to support growth might help in reducing the production costs. For example, brewery waste water was used as a carbon source in bioflocculant production by multiple-microorganism consortia. The idea of using microorganisms in consortia was introduced by Ma *et al*. (2003) in order to increase yield. In this study, the purified compound bioflocculant (3.51 g/l) yield by a mixed cultured of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo was observed to be much higher than those produced from pure cultures (*Halomonas* sp. Okoh 1.213 g/l; *Micrococcus* sp. Leo 0.738 g/l). This increase in yield might be due to synergistic effects of their combination.

The chemical analysis revealed that the bioflocculant produced by *Halomonas* sp. Okoh composed of sugar (26.5%), protein (2.64%) and uronic acid (13.3%). The bioflocculant produced by *Micrococcus* sp. Leo composed of polysaccharide 28.4% (w/w), protein (2.63%) and uronic 9.7% acid and the consortium composed of protein 4.73%, sugar 62.3%, and uronic acid 25.7%. From the results of chemical compositions analyses, it was observed that both the sugar and uronic acid of the bioflocculant obtained from the consortium of the two bacterial strains were higher than those obtained from individual strains. The thermal stability

property displaced by the purified bioflocculants from *Halomonas* sp. Okoh, *Micrococcus* sp. Leo and the consortium at a temperature range of 50-100^{o}C revealed that the major constituent in their structure is polysaccharide (Lu *et al*., 2005) and the protein content in these bioflocculant does not influence flocculation process. According to Ugbenyen *et al*. (2012), the high uronic acid of these bioflocculants implies high carboxyl and functional groups in their backbones; they interact within the molecule and generating strong hydrogen bonds responsible for their high thermal stability. A similar observation was reported by Li *et al*. (2007), only about 9.2% decreased in flocculating activity of the bioflocculant produced by *Aeromonas* sp. was recorded after heated at 100^{o}C for 60 min. Also, Gong *et al*. (2008) reported about 15% decreased in flocculating activity of the bioflocculant produced by *Serratia ficaria* after being heated at 100^{o}C for 15 min. Further characterization was carried out to determine the functional groups in the molecules. The Fourier transform infrared spectrophotoscopy (FTIR) analyses of the bioflocculants indicated that they all composed of hydroxyl groups, carboxyl groups and amino groups (Figure 3.12, 4.14 and 5.8). It can easily conclude that the presence of these functional groups in their molecules is responsible for their flocculation mechanism. Thermogravimetric (TGA) analyses of the purified bioflocculant either from *Halomonas* sp. Okoh or *Micrococcus* sp. Leo or consortium showed the pyrolysis property that the bioflocculants did not burn completely at 500^{o}C for *Halomonas* sp. Okoh (Figure 5.13), at 400^{o}C for both *Micrococcus* sp. Leo (Figure 4.13) and Consortium (Figure 5.7). The correlation between their thermal stability properties and TGA results implies that the bioflocculants are of high molecular weight and are composed mainly of polysaccharide and uronic acids (Prasertsan *et al*., 2006). SEM images of the bioflocculant and kaolin clay before and after flocculation revealed their surface structure and provided little information about their flocculation mechanism (Figures 3.14, 4.15 and 5.9).

In conclusion, the flocculating efficiency and yield of the bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo is an improvement when compared to individual strains. For these bioflocculants to be of practical use industrially, the following points should be considered:

- ✓ Establish fementations (fed-batch versus continous fermentation) conditions for scale-up process for the production of the bioflocculant.
- ✓ Explore the use of low-cost substrates in the production process in order to reduce production cost.
- ✓ Further characterization of the purified bioflocculant can be carried-out. For instance, determination of molecular weight, zeta potential, flocculation mechanisms (enzymatic pathway for bioflocculant production), and purification of enzymes that are involved in bioflocculant production.
- ✓ Carry out pilot plant studies of the application of the purified bioflocculants in various industrial processes such as drinking water purification and other downstream processing.
- ✓ Carry out detailed comparative analyses of flocculation efficiency of the bioflocculant with other commercially available flocculants.
- ✓ Determine the shelve-live of the bioflocculant as well as establish appropriate packaging regimes.
- ✓ Carry out feasibility study on the marketability of the final bioflocculant product.

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APPENDIX

Table 1: Effect of inoculum size on bioflocculant production by *Halomonas* sp. Okoh.

Abs@550nm

Inoculums size (%)	Tube 1	Tube 2	Tube 3
1	0.761	0.789	0.821
2	0.114	0.357	0.124
3	0.601	0.418	0.521
4	0.967	0.866	0.851
5	0.951	0.717	0.763

Table 2: Effect of carbon source on bioflocculant production by *Halomonas* sp. Okoh.

Abs@550nm

Carbon source	Tube 1	Tube 2	Tube 3
Glucose	0.114	0.357	0.124
Galactose	1.108	1.312	1.168
Maltose	1.215	1.051	1.109
Xylose	1.017	0.878	0.901
Starch	0.904	0.545	0.822
Fructose	1.349	1.303	1.474
Sucrose	1.258	1.222	1.225

Table 3: Effect of nitrogen source on bioflocculant production by *Halomonas* sp. Okoh.

Abs@550nm

Nitrogen source	Tube 1	Tube 2	Tube 3
Tryptone	1.326	1.351	1.3
NH_4NO_3	0.695	0.852	0.73
Urea	1.241	1.233	0.96
Yeast	0.307	0.769	0.797
Peptone	0.219	0.181	0.17
NH_4Cl	0.344	0.312	0.317
$(NH_4)_2SO_4$	0.12	0.116	0.185
Mixed nitrogen	0.114	0.357	0.124

Table 4: Effect of cation on the flocculating acivity of bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
Li^{+}	1.24	1.029	1.041
Na^{+}	1.578	1.291	1.051
K^{+}	0.51	0.418	0.518
Mg^{2+}	0.564	0.441	0.45
Ca^{2+}	0.114	0.357	0.124
Ba^{2+}	0.349	0.363	0.352
Mn^{2+}	0.752	0.784	0.689
Fe^{3+}	0.712	0.756	0.305
Al^{3+}	0.106	0.104	0.096

Table 5: Effect of pH on the flocculating acivity of bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
2	0.426	0.497	0.467
3	0.491	0.419	0.447
4	0.163	0.171	0.242
5	0.465	0.323	0.256
6	0.636	0.711	0.503
7	0.573	0.463	0.482
8	0.545	0.738	0.6
9	0.608	0.746	0.733
10	0.81	0.465	0.407
11	0.593	0.556	0.742
12	0.558	0.611	0.585

Table 6: Effect of shaker speed on the bioflocculant production by *Halomonas* sp. Okoh.

Abs@550nm

Speed (rpm)	Tube 1	Tube 2	Tube 3
120	0.53	0.454	0.371
140	0.456	0.483	0.315
160	0.386	0.437	0.208
180	0.524	0.511	0.531
200	0.582	0.613	0.594

Table 7: Effect of incubation temperature on the bioflocculant production by *Halomonas* sp. Okoh.

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
25	0.542	0.516	0.619
28	0.415	0.327	0.329
31	0.545	0.476	0.428
34	0.576	0.542	0.637
37	0.568	0.592	0.674

Table 8: Effect of concentration on the flocculating activity of the purified bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

Concentration (mg/ml)	Tube 1	Tube 2	Tube 3
0.1	0.368	0.393	0.358
0.2	0.298	0.271	0.303
0.3	0.58	0.557	0.473
0.4	0.5	0.487	0.424
0.5	0.374	0.424	0.398
0.6	0.401	0.321	0.519
0.7	0.303	0.454	0.443

Table 9: Effect of cation on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
L^{i+}	0.654	0.733	0.866
Na^{+}	0.911	0.77	0.998
K^{+}	0.581	0.458	0.477
Mg^{2+}	0.32	0.333	0.248
Ca^{2+}	0.426	0.466	0.519
Mn^{2+}	0.52	0.448	0.438
Ba^{2+}	0.409	0.361	0.389
Fe^{3+}	1.189	1.143	1.16
Al^{3+}	0.261	0.267	0.308

Table 10: Effect of pH on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
2	0.324	0.376	0.295
3	0.318	0.281	0.262
4	0.478	0.298	0.259
5	0.331	0.321	0.345
6	0.326	0.42	0.403
7	0.39	0.484	0.401
8	0.663	0.606	0.687
9	0.699	0.632	0.622
10	0.877	0.773	0.883
11	1.227	1.239	1.271
12	1.285	1.279	1.29

Table 11: Effect of temperature on the flocculating activity of purified bioflocculant produced by *Halomonas* sp. Okoh.

Abs@550nm

Temperature (oC)	Tube 1	Tube 2	Tube 3
50	0.143	0.135	0.129
60	0.105	0.169	0.156
70	0.253	0.247	0.216
80	0.326	0.312	0.279
90	0.323	0.315	0.344
100	0.348	0.301	0.364

Table 12: Effect of inoculum size on bioflocculant production by *Micrococcus* sp. Leo.

Abs@550nm

Inoculums size (%)	Tube 1	Tube 2	Tube 3
1	0.153	0.186	0.14
2	0.284	0.294	0.277
3	0.085	0.097	0.074
4	0.077	0.094	0.076
5	0.177	0.157	0.149

Table 13: Effect of carbon source on bioflocculant production by *Micrococcus* sp. Leo.

Abs@550nm

Carbon source	Tube 1	Tube 2	Tube 3
Glucose	0.284	0.294	0.277
Galactose	1.072	0.878	0.67
Maltose	0.585	0.583	0.668
Xylose	0.712	0.716	0.533
Starch	0.537	0.566	0.58
Fructose	1.248	1.248	1.179
Sucrose	1.28	1.314	1.016

Table 14: Effect of nitrogen source on bioflocculant production by *Micrococcus* sp. Leo.

Abs@550nm

Nitrogen source	Tube 1	Tube 2	Tube 3
Tryptone	1.242	1.032	0.858
NH_4NO_3	0.715	0.834	0.746
Urea	0.532	0.704	0.702
Yeast	0.349	0.369	0.35
Peptone	0.17	0.134	0.134
NH_4Cl	0.291	0.241	0.231
$(NH_4)_2SO_4$	0.174	0.113	0.059
Mixed nitrogen	0.284	0.294	0.277

Table 15: Effect of cation on the flocculating acivity of bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
Li^{+}	0.84	0.792	0.949
Na^{+}	0.877	0.85	0.868
K^{+}	0.458	0.432	0.46
Mg^{2+}	0.453	0.474	0.453
Ca^{2+}	0.284	0.294	0.277
Ba^{2+}	0.414	0.436	0.435
Mn^{2+}	0.414	0.341	0.245
Fe^{3+}	0.807	0.821	0.786
Al^{3+}	0.018	0.031	0.038

Table 16: Effect of pH on the flocculating activity of bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
2	0.445	0.451	0.491
3	0.396	0.347	0.437
4	0.275	0.174	0.149
5	0.296	0.41	0.316
6	0.629	0.426	0.527
7	0.587	0.477	0.583
8	0.545	0.698	0.569
9	0.643	0.588	0.591
10	1.228	0.82	0.87
11	0.494	0.582	1.034
12	0.556	0.694	0.804

Table 17: Effect of shaker speed on the bioflocculant production by *Micrococcus* sp. Leo.

Abs@550nm

Speed (rpm)	Tube 1	Tube 2	Tube 3
120	0.411	0.343	0.576
140	0.427	0.397	0.39
160	0.402	0.282	0.317
180	0.417	0.382	0.378
200	0.604	0.597	0.588

Table 18: Effect of incubation temperature on the bioflocculant production by *Micrococcus* sp. Leo.

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
25	0.625	0.701	0.61
28	0.451	0.427	0.497
31	0.544	0.522	0.579
34	0.479	0.572	0.658
37	0.685	0.782	0.769

Table 19: Effect of concentration on the flocculating activity of purified bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

Concentration (mg/ml)	Tube 1	Tube 2	Tube 3
0.1	0.349	0.478	0.309
0.2	0.318	0.333	0.297
0.3	0.318	0.378	0.475
0.4	0.392	0.367	0.411
0.5	0.433	0.391	0.421
0.6	0.352	0.49	0.479
0.7	0.346	0.417	0.403

Table 20: Effect of cations on the flocculating activity of purified bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
Li^{+}	0.415	0.409	0.452
Na^{+}	0.795	0.435	0.477
K^{+}	0.482	0.446	0.436
Mg^{2+}	0.445	0.384	0.404
Ca^{2+}	0.425	0.448	0.405
Mn^{2+}	0.356	0.271	0.403
Ba^{2+}	0.294	0.283	0.304
Fe^{3+}	0.882	0.794	0.769
Al^{3+}	0.295	0.284	0.297

Table 21: Effect of pH on the flocculating activity of purified bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

pH	Tube 1	Tube 2	Tube 3
2	0.307	0.342	0.283
3	0.3	0.247	0.237
4	0.272	0.214	0.261
5	0.298	0.309	0.324
6	0.268	0.31	0.275
7	0.397	0.378	0.448
8	0.559	0.518	0.452
9	0.564	0.535	0.547
10	0.752	0.734	0.701
11	1.228	1.268	1.257
12	1.266	1.288	1.277

Table 22: Effect of temperature on the flocculating activity of purified bioflocculant produced by *Micrococcus* sp. Leo.

Abs@550nm

Temperature (^{o}C)	Tube 1	Tube 2	Tube 3
50	0.156	0.164	0.127
60	0.117	0.156	0.175
70	0.213	0.265	0.266
80	0.381	0.349	0.307
90	0.345	0.278	0.232
100	0.359	0.37	0.354

Table 23: Time course of bioflocculant production by consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo.

Time (h)	Abs@550nm			OD@600nm			pH			CFU/ml		
0	0.827	0.835	0.826	0.013	0.017	0.014	4.00	4.00	4.00	98	80	64
12	0.716	0.744	0.689	0.035	0.028	0.037	4.16	4.12	3.85	114	120	101
24	0.585	0.521	0.448	0.525	0.46	0.548	2.85	2.89	2.74	395	324	385
36	0.523	0.456	0.397	0.96	0.884	0.978	2.53	2.59	2.48	235	175	184
48	0.443	0.443	0.401	1.241	1.20	1.288	2.51	2.49	2.43	233	323	452
60	0.437	0.451	0.327	1.298	1.234	1.361	2.5	2.41	2.25	285	250	321
72	0.424	0.429	0.327	1.327	1.305	1.408	2.36	2.38	2.18	418	340	484
84	0.406	0.436	0.421	1.4	1.362	1.414	2.28	2.31	2.12	136	167	154
96	0.426	0.456	0.419	1.397	1.352	1.394	2.34	2.29	2.29	342	248	289
108	0.385	0.452	0.438	1.38	1.419	1.55	2.28	2.41	2.27	244	268	338
120	0.362	0.297	0.305	1.485	1.43	1.474	2.21	2.29	2.07	235	189	227
132	0.388	0.327	0.416	1.486	1.415	1.643	2.25	2.22	1.96	210	342	368
144	0.442	0.449	0.357	1.426	1.391	1.497	2.32	2.31	2.31	228	116	139

Table 24: Effect of bioflocculant concentration on the flocculating activity of purified bioflocculant produced by consortium.

Abs@550nm

Concentration (mg/ml)	Tube 1	Tube 2	Tube 3
0.02	0.46	0.481	0.57
0.04	0.41	0.411	0.502
0.06	0.425	0.347	0.425
0.08	0.414	0.379	0.366
0.1	0.371	0.405	0.311
0.2	0.363	0.402	0.358
0.3	0.363	0.415	0.394
0.4	0.365	0.417	0.343
0.5	0.423	0.455	0.41

Table 25: Effect of cations on the flocculating activity of purified bioflocculant produced by Consortium.

Abs@550nm

Cation	Tube 1	Tube 2	Tube 3
Li^{+}	1.592	1.651	1.585
Na^{+}	1.498	1.477	1.49
K^{+}	1.699	1.689	1.705
Mg^{2+}	1.305	1.289	1.173
Ca^{2+}	0.534	0.518	0.486
Mn^{2+}	0.725	0.685	0.803
Ba^{2+}	0.979	1.134	0.989
Fe^{3+}	1.215	1.276	1.03
Al^{3+}	0.467	0.28	0.357

Table 26: Effect of pH on the flocculating activity of purified bioflocculant produced by Consortium.

Abs@550nm

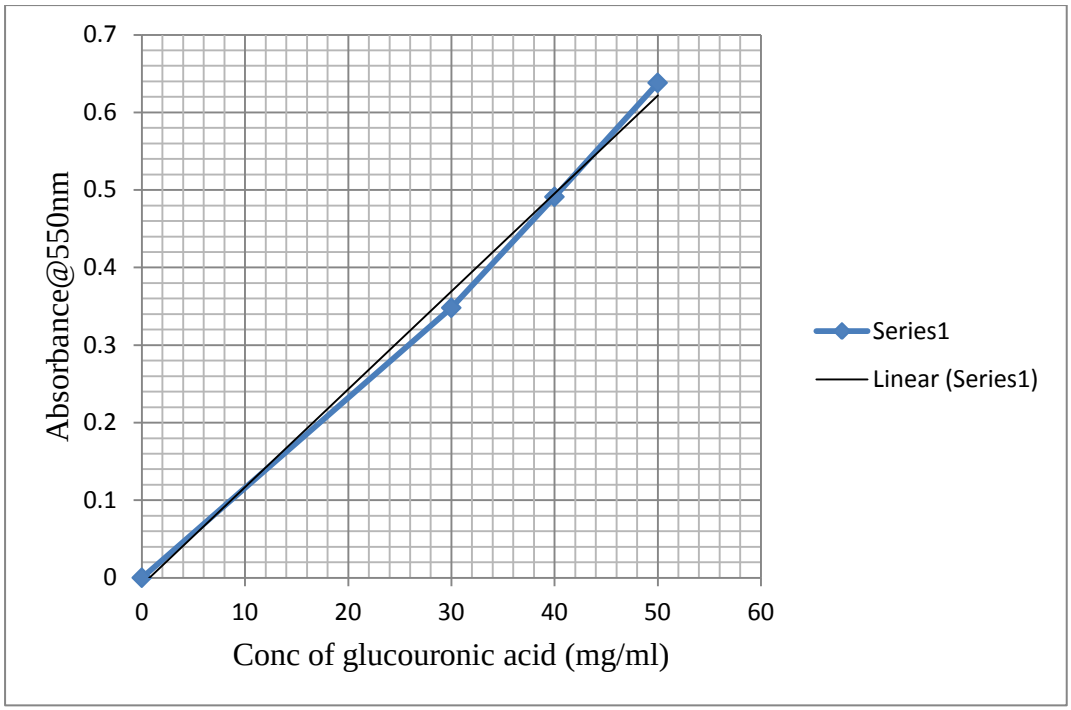
pH	Tube 1	Tube 2	Tube 3
2	0.215	0.242	0.222
3	0.226	0.211	0.237
4	0.196	0.164	0.191
5	0.165	0.164	0.172
6	0.209	0.214	0.208
7	0.379	0.31	0.298
8	0.174	0.12	0.143
9	0.216	0.198	0.257
10	0.217	0.216	0.198
11	1.72	1.798	1.799

Table 27: Effect of temperature on the flocculating activity of purified bioflocculant produced by Consortium.

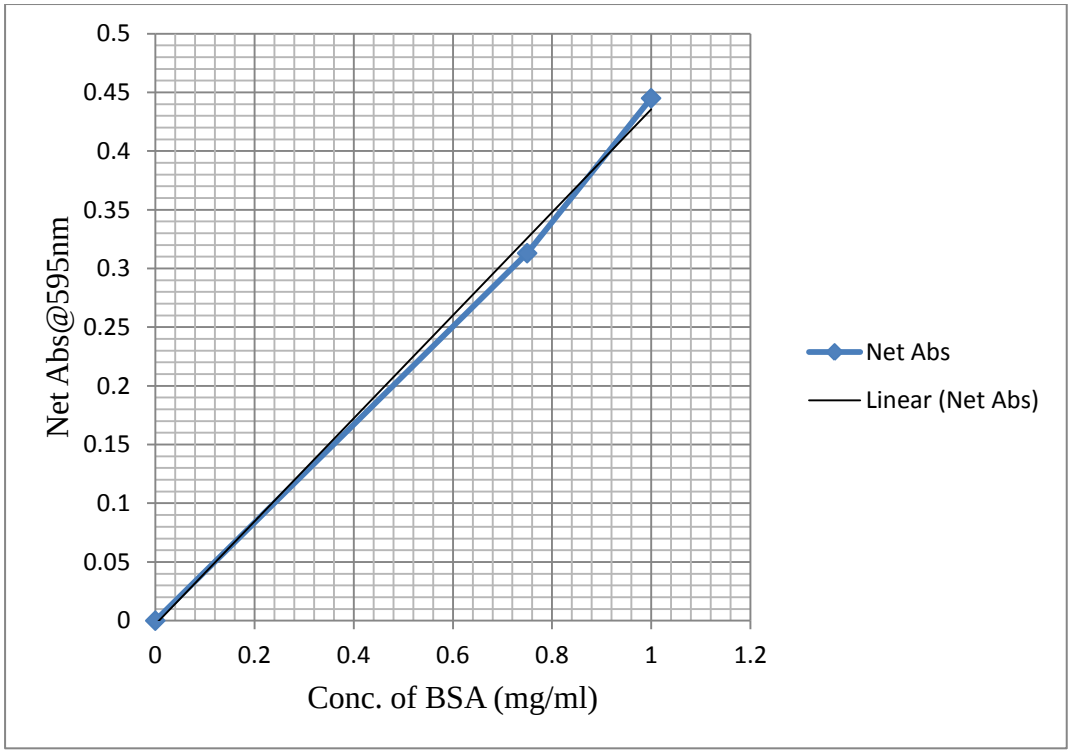
Abs@550nm

Temperature (oC)	Tube 1	Tube 2	Tube 3
50	0.185	0.191	0.209
60	0.245	0.212	0.225
70	0.222	0.212	0.234
80	0.241	0.271	0.257

Graph 1: Standard curve for glucose



Graph 2: Standard curve for protein estimation



Graph 3: Standard curve of glucouronic acid