PRODUCTION AND BIOCHEMICAL CHARACTERIZATION OF NEW BIOFLOCCULANTS FROM BACTERIA ISOLATED FROM FRESHWATER AND MARINE ENVIRONMENTS OF THE EASTERN CAPE IN SOUTH AFRICA.

By

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DECLARATION

This thesis is the original work produced by Leonard V. Mabinya, carried out under Applied and Environment Microbiology Research Group (AEMREG) and submitted to the Department of Biochemistry and Microbiology, University of Fort Hare in 2013, for a Doctor of Philosophy in Biochemistry. 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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GENERAL ABSTRACT

The production and characterization of bioflocculants produced by three bacteria belonging to *Arthrobacter*, *Halomonas* and *Micrococcus* genera and isolated from freshwater and marine environments were evaluated both as axenic cultures and as consortia. The influences of cultutre conditions such as carbon, nitrogen and metal ions sources, as well as initial pH on bioflocculant production by individual isolates were investigated. Both *Arthrobacter* sp. Raats and *Halomonas* sp. Okoh utilized urea as a nitrogen source of choice for optimal production of the bioflocculants with *Micrococcus* sp. Leo having a preference for peptone. All three strains differed in as far as the carbon source of choice was concerned with lactose, glucose and sucrose the preferred carbon sources respectively. Also, all three bacterial strains produced an extracellular bioflocculant aerobically but an intial pH 7.0 of the culture media was suitable for both *Arthrobacter* sp. Raats and *Halomonas* sp. Okoh with a slightly alkaline pH of 9.0 preferred by *Micrococcus* sp. Leo. The presence of Mg^{2+} cations stimulated bioflocculant production by both *Arthrobacter* sp. Raats and *Micrococcus* sp. Leo while Ca^{2+} resulted in more efficient bioflocculant production by *Halomonas* sp. Okoh. Chemical analyses revealed the bioflocculants produced by both *Halomonas* sp. Okoh and *Micrococcus* sp. Leo to be predominantly polysaccharides whereas *Arthrobacter* sp. Raats produced principally a glycoprotein composed of about 56% protein and 25% total carbohydrate. Response surface methodology (RSM) was used to optimize production medium for bioflocculant production by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. Plackett-Burman experimental design showed that fructose,

ammonium sulphate and $MgCl_2$ were significant in the high yield of the bioflocculant. Furthermore, central composite design showed that optimal concentration of these critical nutritional sources were 16.14 g/L, 1.55 g/L and 1.88 g/L for fructose, ammonium sulphate and $MgCl_2$ respectively. Quantification of the bioflocculant showed a yield of 6.43 g/L which was in close accord with the predicted value of 6.51 g/L. FTIR spectrometry of the bioflocculant indicated the presence of carboxyl, hydroxyl and amino groups, typical for heteropolysaccharide, while SEM imaging revealed a lattice-like structure. The efficiency of the nutrient optimization suggests suitability for industrial applicability.

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LIST OF ABBREVIATIONS

AEMREG : Applied and Environmental Microbiology Research Group

BLAST: Basic Local Alignment Search Tool

CCD: Central Composite Design

C/N: Carbon/Nitrogen

COD: Chemical Oxygen Demand

DOT: Dissolved Oxygen Tension

FTIR: Fourier Transform Infrared Spectroscopy

MAR: Marine Actinomycete (Phylotype)

OD: **Optical Density**

PB: **Plackett-Burman**

γ-PGA: **Poly γ-Glutamic Acid**

PCR: **Polymerase Chain Reaction**

RSD: **Response Surface Design**

RSM: **Response Surface Methodology**

SEM: **Scanning Electron Microscopy**

TGA: **Thermogravimetric Analysis**

WSSV: **White Spot Syndrome Virus**

CHAPTER ONE

INTRODUCTION

The interest in biotechnological methods for the production of bioflocculants lies in the possibility of using different organisms to synthesize extracellular substances with different compositions (Taniguchi *et al*., 2005; Gong *et al*., 2008; Zheng *et al*., 2008). Compared with conventional synthesized flocculants, bioflocculants have special advantages such as safety for ecosystems, strong effect, biodegradability and harmlessness to humans and the environment, thus making them potentially suitable for application in drinking and wastewater treatment, downstream processing, food and fermentation processes (Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001). To utilize bioflocculants widely in industrial fields, it is desirable to find various microorganisms with high bioflocculant-producing ability and improve the flocculating efficiency of the bioflocculant.

In the past, photosynthetic bacteria were used for wastewater treatment because they have a relatively high growth rate and can utilize a wide range of organic compounds (Kobayashi and Kurata, 1978). However, difficulty in cell separation due to their low flocculating ability has been the major disadvantage in this application (Wanatabe *et al*., 1999). Presently, both organic and inorganic flocculating agents are extensively used for sedimentation of colloidal and cellular materials and thus applied in a wide range of industrial fields, such as purification of drinking water, wastewater treatment, food industries, dredging and fermentation processes (Salehizadeh and Shojaosadati, 2001). Notwithstanding their harmfulness to humans and the environment, various chemically

synthesized flocculants such as aluminum salts and polyacrylamide derivatives are commonly employed in these processes due to their effectiveness and cost considerations (Salehizadeh and Shojaosadati, 2001; Zheng *et al*., 2008). However, evidence exists that shows polyacrylamide derivatives such as the acrylamide monomer to be a strong carcinogen, non-degradable and neurotoxic to humans (Yokoi *et al*., 1995, 1997; Kwon *et al*., 1996). Moreover, some reports indicate that aluminium, as the main component of polyaluminium chloride, may induce Alzheimer's disease (Kurane *et al*., 1994b). Although naturally occurring materials including chitosan and gelatin are safe and biodegradable, they show only weak flocculating activities in applications (Takagi and Kadowaki, 1985). In order to circumvent the health and environmental problems attributed to inorganic and synthetic flocculants, flocculants produced by microorganisms have attracted considerable scientific and technological attention in recent years (Li *et al*., 2009a).

The industrial potential of bioflocculants has long been recognized because of their harmlessness, biodegradability and lack of secondary pollution from their degradative intermediates (Salehizadeh and Van Loosdrechtl, 2004; Wang *et al*., 2011). The majority of bioflocculants produced by different microorganisms are usually high molecular weight polymers such as polysaccharides, proteins, glycoproteins and nucleic acids (Wu and Ye, 2007; Feng and Xu, 2008). Although a number of microorganisms have been screened for their bioflocculant-producing capabilities, to-date, very little has been accomplished on a commercial scale (Kurane *et al*., 1994a; Salehizadeh and Shojaosadati, 2001). The high cost of production coupled with low yield seem to be the major deterring factors in the advancement of research in developing bioflocculants for

both scientific and commercial applications (Li *et al*., 2003; He *et al*., 2004; Gao *et al*., 2006; Zheng *et al*., 2008). However, in order to reduce costs and optimize cultivation conditions, strategies such as the use of multi-organisms consortia and fed-batch production processes are being developed (Wu *et al*., 2010).

It is therefore in this context that microbial flocculants, if ascertained by chemical analyses to be harmless toward humans and the environment, are expected to be widely applied not only for wastewater treatment but also for drinking water treatment and downstream processes in food and fermentation industries (Yokoi *et al*., 1995). Screening new microorganisms which could produce flocculants with high flocculating activity (Takagi and Kadowaki, 1985; Toeda and Kurane, 1991; Wanatabe *et al*., 1998) and optimizing the fermentation process to improve productivity (Kurane*, et al*., 1986; 1994b) have, therefore, become another research focus in recent years.

Enhancing flocculating capability of these bioflocculants has advantages which include reduced dosage requirements thus making their application in industry to be cost-effective and economically viable (Gao *et al*., 2006). It therefore becomes imperative to identify new microorganisms (especially from unusual environments such as marine habitat) with high bioflocculant-producing potential and improve upon the flocculating efficiency of the isolated bioflocculants.

The presence of indigenous marine microorganisms in the oceans has been confirmed by studies which also indicate that they are widely distributed in different marine habitats, from ocean shores (Ogunmwonyi *et al*., 2010) to the deep sea floor and coral reefs

(Madrid *et al*., 2001). Since marine microorganisms have evolved the greatest genomic and metabolic diversity, they hold promise as potential sources for the discovery of novel secondary metabolites and consequently, no efforts should be spared in exploring this possibility (Lam, 2006).

As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine microorganisms have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds (Charan *et al*., 2004; Brutner *et al*., 2005; Buchanan *et al*., 2005). The living conditions to which marine microorganisms had to adapt during evolution vary drastically from extremely high pressure and anaerobic conditions at very low temperatures on the deep sea floor, to high acidic conditions at very high temperatures near hot spring vents at the elevated region of the ocean floor. Relative to the deep sea environment, the areas around submarine hydrothermal vents are often biologically more productive due in part to the chemicals dissolved in the vent fluids, and as a consequence, they support and host dirvese and complex communities of organisms (Itoh *et al*., 2003; Kanoh *et al*., 2005., Lam, 2006). It is therefore possible that such extreme conditions would have a significant influence on the genetic and metabolic diversity of these microorganisms which remains largely unknown (Stach *et al*., 2003; Magarvey *et al*., 2004; Bull *et al*., 2005; Jensen *et al*., 2005).

In spite of all the bioflocculants that have been identified, few (or none) of them have been practically applied in industry because of their low flocculating capability and high costs in terms of large dosage requirement (Gao *et al*., 2006). In order to utilize

bioflocculants widely in industry, it is desirable to find and screen various microorganisms with high bioflocculant-producing ability (high flocculating activity) and optimize the fermentation process to improve the flocculating efficiency of the bioflocculant.

AIM AND OBJECTIVES OF THE STUDY

The overall aim of this study is to assess the bioflocculant production potential of selected bacteria belonging to the *Halomonas*, *Arthrobacter* and *Micrococcus* genera isolated from both freshwater and marine environments of the Eastern Cape Province of South Africa. Specific objectives include:

1. Evaluation of bioflocculant production potential of axenic cultures of the three test bacteria: *Halomonas* sp. Okoh, *Arthrobacter* sp. Raats and *Micrococcus* sp. Leo.
2. Optimize culture cultivation conditions for efficient bioflocullant production by individual selected test bacteria.
3. Evaluate bioflocculant production potential of consortia of the bacteria isolates.
4. Purify and characterize the bioflocculants produced by individual strains and the consortium.
5. Assess the potentials of the purified bioflocullants in water/wastewater treatments processes.

CHAPTER TWO

LITERATURE REVIEW

The primary purpose of wastewater treatment is to remove the suspended and soluble organic constituents measured as chemical oxygen demand (COD). The COD of the wastewater is the amount of oxygen required to completely degrade wastewater to carbon dioxide and water. The COD of the wastewater also provides an estimate of the energetics of the wastewater treatment process.

Flocculants are used for the sedimentation of colloidal substances and cellular materials and thus applied in a wide range of industrial fields such as clarification of tap water, wastewater, dredging and industrial processes (Choi *et al*., 1998; Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001). Synthetic chemical polymeric flocculants are commonly used worldwide in water treatment industry prior to the distribution of the final product to consumers (He *et al*., 2002). However, serious health problems associated with the use of these synthetic flocculants have necessitated the need for alternative safe, cost-effective and more environmentally acceptable flocculants (Yokoi *et al*., 1995, 1997; Know *et al*., 1996; Taniguchi *et al*., 2005; Ho *et al*., 2010). In addition to being sensitive to pH changes, these agents also produce sludge whose disposal poses an environmental challenge (Sharma *et al*., 2006). Therefore, the need to develop

microbial flocculants becomes most desirable as they promise to be safe, cost-effective as well as environmentally friendly.

A bacterium, *Serratia ficaria*, isolated from soil by Wen-Xin *et al*. (2008), produced a bioflocculant with the ability to flocculate a variety of wastewaters, including river water, brewery water, meat processing wastewater and soy sauce brewing wastewater. The bioflocculant could further be used to treat pulp effluent. It achieved 99.9% and 72% rates of removal of colour and chemical COD respectively, far better than can be achieved with traditional chemical flocculants. Using the non-living biomass of AK61 actinomyces strain, Kefala *et al*. (1999), demonstrated almost 100% removal of cadmium metal at 5 mg/L from dilute aqueous solutions over a wide range of pH values.

Buthelezi *et al*. (2009) reported removal of more than 90% of both turbidity and bacterial load from river water by bioflocculants produced by indigenous bacteria isolated from wastewater. Furthermore, these bioflocculants were able to remove both Gram-positive and Gram-negative bacteria. It was also observed that there was no significant reduction in the pH of river water after the addition of the bioflocculants, an advantage as no further chemical re-adjustment of pH of the finished water was necessary as would be the case with the use of alum.

Since the advent of research studies into biological waste treatment, bacterial flocculation has received a great deal of attention. Bioflocculants are extracellular biodegradable macromolecules that are known for their ability to clarify turbid water. The production of these biopolymers has been observed in a number of microorganisms

including algae, bacteria, fungi, yeasts and actinomycetes (Kurek *et al*., 1991; He *et al*., 2002; Deng *et al*., 2003; Gao *et al*., 2006). Bioflocculation is basically an aggregation process in which cells, as well as organic and inorganic colloids, are closely bound together, mostly by extracellular biopolymers, thus creating a stable biofloc structure (Takagi and Kadowaki, 1985; Salehizadeh and Shojaosadati, 2001; He *et al*., 2002).

Bioflocculation is responsible for changes in supernatant turbidity and biofloc characteristics that result in variations in settling and dewatering properties with the resulting flocs usually held together by means of exocellular polymers (biopolymers) and divalent cations (Eriksson and Alm, 1991; Bruus *et al.,* 1992). The industrial potential of bioflocculants has long been recognized because of their harmlessness, biodegradability and lack of secondary pollution from their degradative intermediates (Wang *et al*., 2011). Therefore, in order to comprehend the bioflocculation process properly, a clear understanding of the role of biopolymers, biopolymer binding and the role of specific cations is required.

Bridging and charge neutralization are two mechanisms often used to describe or explain the process of flocculation in biological systems (Lian *et al*., 2008; Li *et al*., 2009a) with the biopolymer size, its chemical composition and the nature of the cation playing a critical role in flocculation mechanism (Li *et al*., 2010). Bridging often occurs with larger molecular weight polymers which form linear chains in solution with numerous functional groups acting as adsorption sites for suspended particles in the surrounding environment (Zhang *et al*., 2010). The size of the polymer is one of the key factors infuencing this type of flocculation mechanism (Wang *et al*., 2011). The surface charge of

particles moving randomly in suspension is usually neutralized and reduced when a polymer of opposite charge is added. This increases the attractive force between the particle and the poplymer leading to the formation of flocs which aggregate into larger flocs and subsequently settle out of solution by gravitation. This type of mechanism constitutes flocculation by charge neutralization (Levy *et al*., 1992; Lachhwani, 2005).

Different constituents such as carbon and nitrogen sources, salt ions including the initial pH of the growth medium have an influence on the production of bioflocculants by various microorganisms. Preferences for both carbon and nitrogen source, as well as for metal ions are very diverse amongst a large group of microorganisms showing potential for bioflocculant production (Ntsaluba *et al*., 2011). Carbon source preferences can range from simple sugars such as glucose for both *Halomonas* sp. V3a' and *Virgibacillus* sp. Rob (He *et al*., 2010; Cosa *et al*., 2011), disaccharides such as sucrose and maltose by *Corynebacterium glutamicum* and *Klebsiella* sp. respectively (He *et al*., 2004; Sheng *et al*., 2006), polysaccharides such as corn starch by *Bacillus* sp. I-450 (Kumar *et al*., 2004) to a more complex carbon source such as diary wastewater supplemented with ethanol by *Klebsiella mobilis* (Wang *et al*., 2007).

In addition to the carbon source, changing the nitrogen source has also been observed to influence bacterial growth and hence bioflocculant production (Sheng *et al*., 2006). Improved bacterial growth rates as well as flocculant recovery rates have indeed been reported in the presence of either organic, inorganic or when a combination or multiple nitrogen sources are used (Gong *et al*., 2008; Xia *et al*., 2008; Ugbenyen *et al*., 2012). For *Bacillus* sp. I-450 and *Vagococcus* sp. W31 respectively, urea was the preferred

nitrogen source (Kumar *et al*., 2004; Gao *et al*., 2006) whereas ammonium chloride and peptone were nitrogen sources of choice for both *Halomonas* sp. V3a' and *Halomonas* sp. AAD6 respectively (He *et al*., 2009; Sam *et al*., 2011)

Cations significantly affect bioflocculation by interacting and neutralizing negatively charged functional groups of both biopolymers and suspended particles (Salehizadeh and Shojaosadati, 2001; Li *et al*., 2008), thus weakening the static repulsive force and hence promoting and enhancing flocculation (Kumar *et al*., 2004; Zheng *et al*., 2008; Li *et al*., 2008). The charge bridging between the biopolymers promote an increase in floc size and floc density (Higgins, 1995). Murthy (1998) reported evidence showing that divalent cations such as calcium and magnesium tend to improve effluent quality through improvements in bioflocculation and increased association of biopolymers to the floc compared to monovalent cations such as sodium. This observation is in agreement with the data from selected bacterial bioflocculants shown in Table 1. Moreover, Higgins and Novak (1997a) showed a decrease in settling properties when the monovalent to divalent cation ratio (M/D) on a charge equivalent basis exceeded the value of 2 with sodium as a prevalent cation. Wu and Ye, (2007), Gong *et al*. (2008) and He *et al*. (2010) also reported improved bioflocculant production by *Bacillus subtilis*, *Serratia ficaria* and *Halomonas* sp. V3a' respectively in the presence of either Ca^{2+} or both Ca^{2+} and Mg^{2+}. The role of some cations such as calcium, magnesium and sodium are better defined than other cations such as potassium and ammonium ions. However it would be useful to determine some of the mechanisms involved in order to understand the effect of changes in concentration of these cations on bioflocculation. It is imperative that interaction between different cations and the floc and among themselves be taken into

account when considering their influence on bioflocculation (Murthy, 1998). However, in order to contain costs, screenings of microorganisms with the potential for cation-independent bioflocculant production have been undertaken (Zheng *et al*., 2008; Liu *et al*., 2010).

The pH of the growth medium is also a key factor in flocculation and thus effectively influences the flocculation process to varying degrees depending on initial pH requirements of individual bacterial strains (Salehizadeh and Shojaosadati, 2001; Yokoi *et al*., 1996). pH tolerance is an important factor in determining not only the effectiveness of the bioflocculant but also its suitability and applicability in processes where wide pH variations are prevalent (Wang *et al*., 2011). In addition, the pH of the medium affects the stability of both enzymatic reactions involved in bioflocculant production and suspended particles involved in floc formation (Xia *et al*., 2008; Wang *et al*., 2011). At low pH, the absorption of H^+ ions tends to weaken the bioflocculant-Kaolin complex-formation process with a similar effect also observed at high pH values due to the presence of OH^- ions (He *et al*., 2010). Li *et al*. (2008) also observed that the mediating effect of Ca^{2+} appeared to be strongest at neutral pH values. Bouchtroch *et al*. (2001), Gao *et al*. (2006) as well as He *et al*. (2010) reported optimal flocculating activities at neutral pH values for bioflocculants ERSS-31, MBFW31 and HBF-3 produced by *Halomonas maura* sp. nov, *Vagococcus* sp. W31 and *Halomonas* sp. V3a' respectively. However, bioflocculation by *Bacillus* sp. As-101 (Salehizadeh *et al*., 2000) and *Anabaena* sp. (Choi *et al*., 1998) and was more prevalent in acidic conditions of pH 2 while biopolymer flocculants produced by *Rhodococcus erythropolis* (Kurane *et al*., 1994a) and *Virgibacillus* sp. Rob (Cosa *et al*., 2011) were effective at alkaline pH. Some

bioflocculants have been reported to exhibit flocculating activity over a wide pH range (Fujita *et al*., 2000; Yim *et al*., 2007; Zhang *et al*., 2010; Piyo *et al*., 2011)

Dosage optimization of bioflocculants is another critical aspect when considering the cost effectiveness of treatment processes. An ideal flocculation performance optimization strategy would involve the utilization of lower bioflocculant dosage without compromising the flocculation rate (Zufarzaana *et al*., 2012). Varying dosage requirements for maximum flocculation by a wide range of bioflocculants produced by various microorganisms have been documented. Among the lowest dosage requirements reported for flocculating Kaolin suspension was 1 mg/L for a bioflocculant produced by *Bacillus mucilaginosus* (Deng *et al*., 2003). Li *et al*. (2007) and Salehizadeh and Shojaosadati, (2002) also obtained the highest flocculating percentage for kaolin suspension with a bioflocculant concentration of 1 mg/L, while most bioflocculants showed the highest activity within the concentration range of 10 to 50 mg/L (Zhang *et al*., 2002; Gao *et al*., 2006). Recently, Feng and Xu (2008) reported a bioflocculant, MBF3-3, isolated from a *Bacillus* sp. with flocculating activity of over 98% at a concentration as low as 0.675 mg/L (Table 1), while the highest dosage requirement documented was 90 mg/L attributed to a bioflocculant produced by *Enterobacter aerogenes* (Lu *et al*., 2005). Several other dosage requirements that fall inbetween have been reported for bioflocculants produced by different microorganisms. For example 2 mg/L was reported to be the minimum dosage requirement for a bioflocculant produced by *Enterobacter cloacae* (Prasertersan *et al*., 2006) while 40 mg/L was required for maximum flocculating activity by a bioflocculant generated by *Enterobacter* sp. BY-29 (Yokoi *et al*., 1997).

Yokoi *et al*. (1995) isolated a Gram-positive, aerobic *Bacillus* sp. PY-90 strain from soil that grew optimally in a basal medium supplemented with glutamic acid as a nitrogen source. In Kaolin suspension, the highest flocculating activity was attained at a bioflocculant concentration of 20 mg/L. The activity increased in the presence of 2 to 8 mM Ca^{2+} and at an acidic pH range of 3.0 to 5.0 but decreased upon heating at 100^{o}C.

Recently, Wang *et al*. (2011) recorded 12 mg/mL as the minimum dosage required for maximum flocculation by bioflocculant CBF-F26 produced by a consortium of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6. A bioflocculant produced by a consortium of microorganisms (*Staphylococcus* sp. and *Pseudomonas* sp.) was found to have a higher flocculating activity than that produced by either of the strains under the same conditions. Its effectiveness in treating dyed wastewater and the maximal removal efficiencies of COD was recorded above 79% and 86% respectively (Zhang *et al*., 2007).

A study conducted by Kurane and Mutsuyama (1994) on bioflocculant production by a mixed culture of four bacterial strains showed an improvement in the flocculating rate of the bioflocculant produced when an additional 5th strain was included in the consortium. A higher yield with an improved flocculating efficiency was also reported by Zhang *et al*. (2007) for a bioflocculant produced by a combination of strains of microorganisms in consortia. It is therefore envisaged that the optimization of culture conditions together with the use of mixed bacterial cultures will enhance not only bioflocculant production but also its flocculation efficiency.

Temperature plays a critical role in the activation of enzymatic reactions involved in both the production process and flocculating performance of the bioflocculant (Nakata and Kurane, 1999). Although temperature requirements vary among different microorganisms, the majority of bacterial strains documented in the literature produce bioflocculants optimally within a temperature range of 25 to 40^{o}C (Nakamura *et al*., 1976; Deng *et al*., 2005), a finding also supported by the report of Zhang *et al*. (2007) for bioflocculant production by multiple microorganisms in consortia which was optimal at 30^{o}C. Temperature stability, much as pH tolerance, is an important factor when considering the suitability of a biopolymer for water treatment purposes (Patil *et al*., 2011). Bioflocculants containing sugars as the main flocculating component have been shown to be heat-stable and capable of retaining more than 50% of their activity when heated to 100^{o}C (Gao *et al*., 2006; Li *et al*., 2007; Feng and Xu, 2008), whereas those that do not have a sugar component are sensitive to temperature (He *et al*., 2004). A predominantly polysaccharide based bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 retained more than 90% flocculating activity after being heated at 100^{o}C for 30 min. (Wang *et al*., 2011) whereas a drastc reduction in flocculating activity above 80^{o}C was noticeable for bioflocculant REA-11 produced by *Corynebacterium glutamicum* (He *et al*., 2004). A reduction of about 50% in flocculating activity was also noted when bioflocculants produced by *Rhodococcus erythropolis* (Kurane *et al*., 1986) and *Bacillus firmus* (Salehizadeh and Shojaosadati, 2002) respectively were subjected to boiling for 15 min.

The cost-effectiveness of bioflocculation requires that no effort should be spared by scientists to achieve maximum bioflocculant yields at the lowest possible cost (Mabinya *et al*., 2011). Several studies have been reported reflecting bioflocculant yields obtained by altering the composition of the medium used for cultivating different microorganisms as well as by using mixed bacterial cultures for bioflocculant production. These range from as low as 1.30 g/L bioflocculant yield obtained from a 1 L fermented broth of *Enterobacter aerogenes* (Lu *et al*., 2005) to as high as 14.5 g/L generated from 1 L of *Agrobacterium* sp. M-503 fermented broth (Li *et al*., 2010). Of interest is the promising yield of 15 g/L reported by Zhang *et al*. (2007) of a pure novel bioflocculant MMF1 produced by a mixed bacterial culture using brewery wastewater as a carbon source. Also, not to be overlooked when optimizing cultivation conditions for bioflocculant production are aeration level requirements for the microorganism. Dissolved oxygen tension (DOT) of the culture broth has been reported to influence not only the rate of respiration of microorganisms but also nutrient absorption and the rate of enzymatic reactions (Salehizadeh and Shojaosadati, 2001). Varying agitation rates for determining DOT's that are necessary for improved bioflocculant production by different microorganisms have been reported. Zhang *et al*. (2010) reported using an agitation rate of 130 rpm for improved bioflocculant production by *Proteus mirabilis* TJ1 while 170 rpm was suffice for efficient bioflocculant production by *Aeromonas* sp. (Li *et al*., 2007).

Table 1. Comparison of Parameter Requirements by Selected Bacteria for Optimization of Bioflocculant Production and Flocculation Efficiency.

	Parameter Requirements						
Isolate	**Nitrogen Source**	**Carbon Source**	**Cation**	**Dosage (mg/L)**	**pH Stability**	**Temp. Stability ($100^{o}C$)**	**References**
B. sp. PY-90	glutamic acid	glu	Ca^{2+}	20	4	unstable	Yokoi *et al*. (1995)
C. glutamicum	urea	suc			3-6.5		He *et al*. (2002, 2004)
S. ficaria	urea	lac	K^{+}, K^{2+}	4	5-7		Wen-Xin *et al*. (2008)
V. sp.	urea	glu	Ca^{2+}	25-30	4-10	thermostable	Gao *et al*. (2006)
B. sp. I-450	urea	starch	Ca^{2+}		8-12		Kumar *et al*. (2004)
B. firmus	peptone	Ca^{2+} Mg^{2+}	4	7	thermostable		Salehizadeh and Shojaosadati (2002)

K. pneumonia	peptone	glu	Ca^{2+} Mg^{2+} Mn^{2+}		8	unstable	Nakata and (1999) Kurane
A. sp.	YE	suc	Ca^{2+} K^{+}, Na^{+}	1	3-5	thermostable	Li *et al*. (2007)
B. sp.	YE	suc	Ca^{2+}, K^{+}, Mg^{2+}	0.675	4	thermostable	Feng and Xu (2008)
P. purpurogenum			Ca^{2+} Mg^{2+} Fe^{3+}	40	8		Hu *et al*. (2009)
Consortium *(S. sp.+ P. sp.)*	*urea*		Ca^{2+}		6-7		Zhang *et al*. (2007)

Isolate legend: B. *sp.* = ***Bacillus***, ***C***. *glutamicum* = ***Corynebacterium***, ***S***. *ficaria* = ***Serratia, V***. *sp.* = ***Vagococcus***, ***P***. *purpurogenum* = ***Penicillin***, *Consortia* (***S***. *sp.* + ***P***. *sp.)* = ***Staphylococcus*** + ***Pseudomonas***

It is therefore evident that an ideal approach in the production of bioflocculants by microorganisms would involve optimizing cultivation medium components and other significant variables such as intial pH, temperature, agitation speed, aeration rate and dosage requirements. The downside of this approach when using conventional methods is that it is not only laborious and costly but also time-consuming in terms of individual experiments required to determine the content at which each factor would operate optimally (He *et al*., 2009). Response surface methodology (RSM) is a significant statistical procedure that has been successfully utilized for variable screening in order to optimize the production medium during fermentation processes (Wang and Wan, 2008; He *et al*., 2009; Bajaj *et al*., 2009; Liu *et al*., 2010). He *et al*. (2009) reported an increased yield from 2.5 g/L to 5.58 g/L when RSM was used to optimize growth conditions for the production of bioflocculant HBF-3 by *Halomonas* sp. V3a'. Likewise, Liu *et al*. (2010) used RSM technology to increase exopolysaccharide production from *Paenibacillus polymixa* EJS-3 by as much as 1.55-fold.

The majority of bioflocculants produced by different microorganisms are usually high molecular weight polymers such as polysaccharides, proteins, glycoproteins and nucleic acids (Wu and Ye, 2007; Feng and Xu, 2008). The extracellular polymeric substances are the major constituents of the floc structure and can originate from two possible sources: microbial activity such as metabolism and cell lysis (Choi *et al*., 1998; He *et al*., 2002; Gao *et al*., 2006). These biopolymers, which are made up of proteins, polysaccharides, glycoproteins, lipids and nucleic acids (Takeda *et al*., 1991; Koizumi *et al*., 1991; Lee *et al*., 1995; Wang *et al*., 1995; Gao *et al*., 2006; He *et al*., 2009, 2010) provide a matrix for enmeshment of the microbes and cations. In addition, the biopolymers have functional groups such as phosphate and carboxyl contributing

negative charges to the floc structure to enhance bioflocculation by providing additional adsorption sites for suspended particles (Salehizadeh and Shojaosadati, 2002; Gao *et al*., 2006; Sam *et al*., 2011).

Studies carried out on the chemical composition of bioflocculants produced by *Bacillus* sp. I-471 (Kumar *et al*., 2004), *Halomonas* sp. V3a' (He *et al*., 2009, 2010), *Bacillus subtilis* DYU1 (Wu and Ye, 2007), *Vagococcus* sp. W31 (Gao *et al*., 2006), and *Bacillus subtilis* IFO3335 (Yokoi *et al*., 1996) have all been shown to be predominantly polysaccharides. *Rodoccocus erythropolis* (Takeda *et al*., 1991) and *Nocardia amarae* YK-1 (Koizumi *et al*., 1991) produce protein flocculants while *Arcuadendron* sp. TS-4 (Lee *et al*., 1995) and *Arthrobacter* sp. (Wang *et al*., 1995) have been reported to produce glycoprotein bioflocculants.

Extracellular proteins have long been associated with improvements in settling and dewatering properties. Higgins (1995) carried out studies that indicated that at least some of the proteins in activated sludge possess lectin-like activity. Lectins are extracellular proteins that attach to polysaccharides to cause agglutination or bioflocculation and are considered to play a major role in the main mechanism for attachment and agglutination by bacteria in such diverse fields as food microbiology, pathogenic microbiology, industrial microbiology and plant-microbe interactions (Lodeiro *et al.,* 1995; Siero *et al.,* 1995; vanRhizn and Vanderleyden, 1995). The presence of a protein component in a bioflocculant has also been reported by Salehizadeh and Shojaosadati (2001) to indirectly improve the flocculating capacity by increasing its molecular weight.

A novel polygalacturonic acid bioflocculant REA-11 produced by *Corynebacterium glutamicum* utilizing sucrose and urea as carbon and nitrogen sources respectively was reported by He *et al*. (2004). In addition to being thermostable at acidic pH values of 3.0 to 6.5, with a capability to decolourize molasses wastewater, a carbon/nitrogen ratio of 20:1 was also found to enhance the products of REA-11 by 17% compared to the control.

Yokoi *et al*. (1996), demonstrated the flocculating effect of authentic Poly γ-glutamic acid (γ-PGA) produced by *B. subtilis* IFO3335 on various inorganic suspensions such as active carbon, acid clay and solid soil with the highest activity observed in the acid clay suspension. γ-PGA was also shown to flocculate organic suspensions such as yeast and cellulose provided suitable ions were selected and the pH of the reaction mixture was adjusted to the neutral range. As a consequence, γ-PGA has been certified as being harmless toward humans and the environment as well as edible (Yokoi *et al*., 1996).

Wahlberg *et al.* (1994) described an observation in which the rate of floc breakup decreased with an increase in polysaccharides associated with the floc, accompanied by a decrease in supernatant turbidity with an increase in polysaccharides associated with the flocs. Some studies have reported an enhancement in bioflocculation by the presence of uronic acids with suggestions that the negatively charged uronic acids in polysaccharides (specifically alginates) interact with divalent cation through charge bridging to promote bioflocculation (Takeda *et al*., 1991; Bender *et al*., 1994).

The biopolymers appear to affect the physico-chemical properties associated with the flocs such as floc density, floc particle size, specific surface area, charge density, bound water content and hydrophobicity. Research also indicates that an increase in floc density and floc particle size increases settling velocity. Jorand *et al.* (1994) also indicated significant improvements in bioflocculation and settling as a result of an increase in floc hydrophobicity.

Novel Metabolites From Marine Actinomycetes

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics (Sanchez Lopez *et al*., 2003; Strohl, 2004; Berdy, 2005), anti-tumor agents (Maskey *et al*., 2003a, 2004a, 2004b; Cragg *et al*., 2005; Kwon *et al*., 2006), immunosuppressive agents (Mann, 2001; Shin *et al*., 2003; Redlinger et al., 2004) and enzymes (Oldfield *et al*., 1998; Pecznska-Czoch and Mordarski, 1988). The impressive track record of actinomycetes in this regard has led to a concerted effort being made to successfully isolate novel actinomycetes from terrestrial sources for drug screening programs, thus leading to a decrease in the rate of discovery of new compounds (Fenical *et al*., 1999). Thus, it has become crucial that new groups of actinomycetes from unexplored or unexploited habitats be pursued as sources of novel bioactive secondary metabolites (Feling *et al*., 2003; Stritzke *et al*., 2004). Oceans present the greatest diversity for this purpose (Donia and Hamann, 2003), simply because more than 70% of our planet's surface is covered by oceans and life on earth originated from the sea.

As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds. However, the distribution of actinomycetes in the sea is largely unexplored and the presence of indigenous marine actinomycetes in the oceans remains elusive. This is partly caused by the lack of effort spent in exploring marine actinomycetes and skepticism regarding the existence of indigenous populations of marine actinomycetes, the assumption being that they are merely of terrestrial origin (Bull *et al*., 2000).

Indigenous Marine Actinomycetes

Rhodococcus marinonascene was the first marine actinomycete species to be characterized (Helmke and Weyland, 1984). Subsequent data from culture-dependent studies showed that indigenous marine actinomycetes indeed exist in the oceans. These include members of the genera *Dietzia*, *Rhodococcus*, *Streptomycetes*, *Salinispora*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris* and *Verrucosispora* (Bull *et al*., 2005; Jensen *et al*., 2005, Magarvey *et al*., 2004; Stach *et al*., 2004). Among these, the most exciting finding was the discovery of the first obligate new marine actinomycete genus, *Salinispora* and the demonstration of the widespread populations of this genus in ocean sediments by Fenical's research group (Mincer *et al*., 2002, 2005). Furthermore, Mincer *et al*. (2005) demonstrated that *Salinispora* strains were actively growing in some sediment samples, indicating that these bacteria were metabolically active in the natural marine environment.

Therefore, actinomycetes are active components of marine microbial communities. They form a stable, persistent population in various marine ecosystems. It has also been reported that novel compounds with biological activities have been isolated from these marine actinomycetes (Jensen *et al*., 2005; Magarvey *et al*., 2004), indicating that marine actinomycetes are an important source for the discovery of novel secondary metabolites. Cultivation of these novel actinomycetes will facilitate the investigation of their ecological roles and provide an important source for the discovery of novel metabolites.

Distribution of Marine Actinomycetes

The presence of indigenous marine actinomycetes in the oceans was confirmed by studies which also indicated that they are widely distributed in different marine environments and habitats, from deep sea floor to coral reef (Madrid, *et al*., 2001). Regardless of the extreme variation among the different marine environments, the presence of actinomycetes that are distantly related to known taxa has been detected in abundance. Actinomycetes isolated from the samples collected at the marine environments mentioned above, such as deep sea floor, all represent unique ecosystems that cannot be found anywhere else in the world. The isolation of marine actinomycetes that evolved from, and adapted to, these unique ecosystems represents a valuable source for the discovery of novel secondary metabolites. Numerous novel metabolites were isolated between the years 2003 to 2005 as shown in the table below (Lam, 2006). Although this is by no means an exhaustive list of all novel secondary metabolites produced by marine actinomycetes over this period, nevertheless the list covers many different diverse structures which exhibit some biological activities (Table 2).

As marine microorganisms, particularly actinomycetes, have evolved the greatest genomic and metabolic diversity, efforts should be directed towards exploring marine actinomycetes as a source for the discovery of novel secondary metabolites. There is a tremendous potential for the isolation of novel secondary metabolites from marine actinomycetes. Recent investigations using enrichment techniques, new selection methods and media (Magarvey *et al*., 2004; Jensen *et al*., 2005; Mincer *et al*., 2005) have led to the isolation of novel actinomycetes from sediment samples. Further development work in improving isolation strategies in the recovery of marine actinomycetes is of utmost importance for ensuring success in this area.

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, anti-tumor agents, immunosuppressive agents and enzymes (Oldfield *et al*., 1998; Mann, 2001; Berdy, 2005). Actinomycetes have also been reported to account for approximately 70% of the compounds reported in the Dictionary of Natural Products. In the past two decades, however, there has been a decline in the discovery of new lead compounds from common soil-derived actinomycetes as culture extracts yield unacceptably high numbers of previously described metabolites. It is for this very same reason that the cultivation of novel actinomycetes taxa has become a major research focus area (Mincer *et al*., 2002). The MAR 1 group of actinomycetes isolated by Mincer *et al.* (2002) seems to represent a remarkable source of biological active secondary metabolites such as cancer cell cytotoxicities, antifungal and antibiotic activities. The genus *Streptomyces* alone is known to account for a remarkable 80% of the actinomycete natural products reported to

date, a feat that remains without rival in the microbial world. It has therefore become logical to study marine-derived strains in search of new natural products (Jensen *et al*., 2005).

To understand how marine actinomycetes evolved, it is necessary to study the difference between those that evolved due to influence exerted by specific marine environmental conditions and those that are present as dormant spores washed from inland (Bull *et al*., 2000).

Table 2. Novel Metabolites Produced By Marine Actinomycetes During The Period 2003-2005 (Lam, 2006).

Compound	**Source**	**Activity**
Abyssomicins	*Verrucosispora* sp.	Antibacterial
Aureoverticillactam	*S. aureoverticillatus*	Anticancer
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal
Caprolactones	*Streptomyces* sp.	Anticancer
Chandrananimycins	*Actinomadura* sp.	Antialgal; antibacterial; antifungal; anticancer
Chinikomycins	*Streptomyces* sp.	anticancer
Chloro-dihydroquinones	*Novel actinomycete*	Antibacterial; anticancer
Diazepinomicin (ECO-4601)	*Micromonosproa* sp.	Antibacterial; anticancer; anti-inflammatory
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer
Frigocyclinone	*Streptomyces griseus*	Antibacterial

Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutingimycin	*Streptomyces* sp.	Antibacterial
Helquinoline	*Janibacter limosus*	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
IB-00208	*Actinomadura* sp.	Anticancer
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	'*Marinispora*'	Antibacterial; anticancer
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer
MKN-349A	*Nocardiopsis* sp.	Unknown biological activity
Salinosporamide A (NPI-0052)	*Salinispora tropica*	Anticancer
Sporolides	*Salinispora tropica*	Unknown biological activity
Trioxacarcins	*Streptomyces* sp.	Antibacterial; anticancer; Antimalarial

Members of the genus *Salinispora* have yielded a wealth of new secondary metabolites including salinosporamide A, a molecule currently advancing through clinical trials as an anticancer agent (Schwartsmann *et al*., 2003; Prudhomme *et al*., 2008). These sediment-derived actinomycetes include obligate marine taxa (Maldonado *et al*., 2005), and an impressive array of readily cultivable diversity that has not been previously reported from land (Gontang *et al*., 2007). Actinomycetes have also provided bioactive compounds of high prophylactic and therapeutic value whose antiviral properties helped lower White Spot Syndrome Virus (WSSV) infection in penaeid shrimps in Southeast Asia (Kumar *et al*., 2006).

A *Streptomyces* species isolated from the Nahoon beach of South Africa showed a great potential for producing a novel bioflocculant with a diverse mode of action. The results of the study indicated that the extracts possessed compounds with antimicrobial properties which could be used as antimicrobial agents in new drugs for therapy of infectious diseases in humans. (Ogunmwonyi *et al*., 2010)

Rhodococcus marinonascene was the first marine actinomycete species to be characterized (Helmke and Weyland, 1984). Subsequent investigations employing culture-dependent studies supported the existence of indigenous marine actinomycetes in the oceans. These include members of the genera *Dietzia*, *Rhodococcus*, *Streptomycetes*, *Salinispora*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris* and *Verrucosispora* (Stach *et al*., 2003; Magarvey *et al*., 2004; Bull *et al*., 2005; Jensen *et al*., 2005). These strains have an obligatory requirement of sea water for growth and do produce secondary metabolites that possess new carbon skeletons (Jensen *et al*., 2005; Mincer *et al*., 2002). Among these, the most exciting finding was the discovery of the first obligate new marine actinomycete genus, *Salinispora* and the demonstration of the widespread populations of this genus in ocean sediments by Fenical's research group (Mincer *et al*., 2002, 2005). Furthermore, Mincer *et al*. (2005) demonstrated that *Salinispora* strains were actively growing in some sediment samples, indicating that these bacteria are metabolically active in the natural marine environment.

It is however, evident that actinomycetes are active components of marine microbial communities. They form a stable, persistent population in various marine ecosystems. It

has also been reported that novel compounds with biological activities have been isolated from these marine actinomycetes (Jensen *et al*., 2005; Magarvey *et al*., 2004), indicating that marine actinomycetes are an important source for the discovery of novel secondary metabolites. Cultivation of these novel actinomycetes will facilitate the investigation of their ecological roles and provide an important source for the discovery of novel metabolites.

The assumption that actinomycetes isolated from marine samples are of terrestrial origin has persisted despite evidence that marine-derived actinomycetes can be metabolically active (Moran *et al*., 1995) and physiologically adapted to growth in sea water (Jensen *et al*., 1991). The first major actinomycete taxon exclusively from the sea was reported by Mincer *et al*. (2002) when they isolated and characterized a new actinomycete taxon referred to as MAR 1. These strains were distinguished by morphological characteristics, small-subunit rRNA signature nucleotides and an obligate requirement of sea water for growth. Data obtained from isolates collected from far apart localities such as the Red Sea and the Sea of Cortex indicated that MAR 1 members are widely distributed in tropical and subtropical marine sediments from deep sea floor to carol reefs (Madrid, *et al*., 2001; Mincer *et al*., 2002).

Sodium requirements are not common in Gram-positive bacteria, and the discovery of the MAR 1 group appears to represent the first genus-level Gram-positive taxon to reside exclusively in the ocean. Of interest was the observation that *Micromonospora* isolates (CNB394 and CNB 512) did not require sea water for growth yet were tolerant of a higher concentration of NaCl than MAR 1 strains (Mincer *et al*., 2002). This

observation suggested that certain marine-derived *Micromonospora* isolates may have adapted to osmotically variable intertidal environments, whereas MAR 1 strains were adapted to the relatively constant salinities associated with deeper marine sediments (Mincer *et al*., 2002). In addition, the widespread distribution, phylogenetic divergence, abundance and physiological adaptations of the MAR 1 group is an indication of the presence of a highly evolved member of the marine sediment bacterial community (Mincer *et al*., 2002).

Regardless of the extreme variation among the different marine environments, the presence of actinomycetes that are distantly related to known taxa has been detected in abundance. Actinomycetes isolated from the samples collected at the marine environments mentioned above, such as deep sea floor, all represent unique ecosystems that cannot be found anywhere else in the world.

Substantial progress has been made in understanding marine bacterial diversity through studies carried out with mainly Gram-negative bacterioplankton. Very little is known about bacteria inhabiting marine sediments in spite of existing evidence that Gram-positive bacteria comprise a large proportion of these communities (Gontang *et al*., 2007). Of the more than 1,500 diverse Gram-positive bacteria isolated by Gontang *et al*. (2007), and based on phylogenetic analyses using 16S rRNA gene sequence data, two major classes were identified: *Actinobacteria* comprising about 65% and *Bacilli* comprising about 34%. The high degree of phylogenetic novelty observed during this study highlighted the need for more studies in order to understand the diversity of Gram-positive bacteria in marine sediments (Gontang *et al*., 2007).

Phylogenetic analysis based on 16S rRNA gene sequence on an actinomycete strain isolated from the Northern South China Sea by Tian *et al*. (2009), placed it within the family *Pseudonocardiaceae*. Further studies revealed the strain as representing a new genus, leading Tian *et al*. (2009) to propose a new species name "*Sciscionella marina* sp. nov".

Characterization of more than 3000 actinomycete bacteria isolated by Bredholt *et al*. (2008) from the sediments of the Norwegian fjords, the Trondheim fjord, revealed *Micromonospora* as the dominating actinomycete genus. The deep water sediments contained a higher relative amount of *Micromonospora* compared to the shallow water samples. Of these isolates, 9% required sea water for normal growth, suggesting the presence of obligate marine characteristics. Extracts from these isolates had strong antibacterial properties, an indication of antibiotic-producing potentials (Bredholt *et al*., 2008). Bredholt *et al*. (2008) further discovered that the use of different types of treatments originally designed for selective isolation of soil actinomycetes was detrimental for deep-water sediment samples. Thus, more studies were needed to establish suitable methods specific for marine actinomycetes enrichment. Informed selective isolation procedures in detecting marine-derived actinobacterial species that had previously only been reported from terrestrial habitats were also highlighted by Maldonado *et al*. (2005). The fact that the Trondheim fjord is a sea inlet into the terrestrial environment could explain the presence of a considerable number of actinomycetes of terrestrial origin (Bredholt *et al*., 2008). Moran *et al*. (1995) also noted that the abundance of culturable *Streptomyces* isolates in marine systems located adjacent to

terrestrial environments had been regarded as responsible for the detection of spores of terrestrial origin during isolation. In a study of ecological distribution and frequency of bioactive *Micromonosporae* in aquatic habitats of the Sunshine Coast region in Australia, Eccleston *et al*. (2008) also reported the presence of novelty bioactive compound-producing species.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Source of the test bacteria.

The bacteria used in this study were amongst several isolated from Tyume river in Alice and sediments samples of Algoa Bay in Port Elizabeth in the Eastern Cape Province of South Africa as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa. The bacteria were preserved on agar slants and maintained in 20% glycerol at -80 ^{o}C.

3.2. Identification of the Bioflocculant-Producing Microorganisms.

3.2.1. DNA extraction.

DNA extraction was conducted via the boiling method whereby 2–3 colonies were suspended in 70 µL of sterile double distilled water. The samples were heated in a water bath at 100 °C for 10 min, allowed to cool for 5 min and thereafter centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4 °C. This served as the template in the subsequent the PCR assay.

3.2.2. PCR Amplification.

PCR was carried out in 50 µL reaction volume containing 2 mM $MgCl_2$, 2 U Supertherm Taq polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTAC GACTT-39) and 2 µL template DNA. Primer F1 and R5 binds to base positions 7–26 and 1496–1476 of the *16S rRNA* gene of *Streptomyces ambofaciens* ATCC 23877, respectively (Cook and Meyers, 2003)). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 °C for 2 min), 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s) and extension (72 °C for 2 min), and a final extension (72 °C for 5 min). Gel electrophoresis of PCR products were conducted on 1% agarose gels to confirm that a fragment of the correct size had been amplified. Automated sequencing of the *16S rRNA* genes of the bacterial isolates was performed using the Spectrumedix SCE2410 genetic analysis system with 24 capillaries. The sequencing reactions were performed according to the manufacturer's instructions, using the Big Dye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) and 27F primer. The sequences were edited manually based on the most similar sequences.

3.3. Growth Medium and Confirmation of Bioflocculant Production Potential.

The bacterial isolates were revived by streaking them on sterile growth medium composed of glucose (10 g), $MgSO_4.7H_2O$ (0.3 g), K_2HPO_4 (5 g), peptone (1.0 g) and KH_2PO_4 (0.2 g) in a litre of either filtered seawater or deionized water at pH 7.0 and sterilized by autoclaving (Zhang *et al*., 2007; Cosa *et al*., 2011). A loopful of colonies of the test bacteria was inoculated into the growth medium and incubated in a rotary shaker

at 160 rpm, 28 °C for 5 days. The resultant culture broths were centrifuged at 8000 × g for 15 min at 4 ^{o}C to sediment the cells. The cell-free supernatant (2 ml) was assayed for flocculating activity using the method described below.

3.4. Determination of Flocculating Activity.

Using a suspension of Kaolin clay as test material, flocculating activity was determined according to Kurane *et al*. (1986) as modified by Gao *et al*. (2006). A suspension of Kaolin clay (4 g/L) in deionized water at pH 7.0 was used as a stock solution for all subsequent flocculating activity assays. The following solutions were mixed in a test tube: Kaolin clay suspension (9 mL), culture supernatant (0.1 mL) and 1% $CaCl_2$ (0.25 mL). A control in which the culture supernatant was replaced with deionized water was also included and measured under similar conditions. The final volume of all mixtures was made up to 10 mL with deionized water. The solutions were mixed gently and allowed to settle for 5 min. at room temperature. The optical density (OD) of the clarifying upper phase solution was measured at 550 nm using a ThermoSpectronic spectrophotometer (Helios Epsilon, USA) and the flocculating activity determined as follows:

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

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

3.5. Effect of Culture Conditions on Bioflocculant Production.

3.5.1. Effect of Inoculum Size.

Inoculum size for optimal bioflocculant production was assessed by using varying amounts ranging between 1% and 5% (v/v). Culture conditions and the determination of flocculating activity were as described previously.

3.5.2. Effect of Carbon and Nitrogen Sources.

The effects of different carbon and nitrogen sources on bioflocculant production by the test bacteria were assessed according to the method described by Lachhwani (2005). Carbon source candidates included glucose, sucrose, fructose, starch and lactose, while the nitrogen source candidates included ammonium sulphate, ammonium chloride (inorganic nitrogen sources) urea and peptone (organic nitrogen sources). Maltose and ammonium nitrate as carbon and nitrogen sources respectively, were also assessed for their effects on bioflocculant production by the consortium.

3.5.3. Effect of Agitation Speed.

Different agitation rates ranging between 120 and 200 rpm were used to determine the most suitable aeration rate for optimal bioflocculant production (Zhang *et al*., 2007). Culture cultivation of each strain using the growth medium previously described was carried out for a period of 5 days prior to measuring flocculating activity.

3.5.4. Effect of Cations and pH on Bioflocculant Production.

The effects of initial pH and cations on bioflocculant production were assessed in accordance with the description of Liu *et al*. (2010). The initial pH of the production medium varied in the range of 3-12 using 0.1M HCl and NaOH, while the cation candidates included Na^+, K^+, Li^+, Mg^+, Mn^+, Al^+ and Fe^{3+} as their chloride salts. With

regards to the effects of cation assays, flocculant tests were conducted as described above, but $CaCl_2$ solution was replaced by a solution of the above cation candidates, and the flocculating activity was measured.

3.6. Time Course Assay of Bioflocculant Production.

The media for time course assays was composed based on the previously determined optimal growth conditions. Samples (2 mL) were drawn every 12 h over 5 days and centrifuged at (8,000 g, 30 min). The cell-free supernatant was then used to determine the flocculating activity. The optimal density (OD) of the broth was also recorded at a wavelength of 660nm.

3.7. Extraction and Purification of the Bioflocculant.

After fermentation, the culture broths were centrifuged at 8000 × g, 4^{o}C for 15 min to remove bacterial cells. One volume of distilled water was added to the supernatant phase and centrifuged at 8000 × g, 4^{o}C for 15 min to remove insoluble materials. The supernatants were then mixed with 2 volumes of ethanol, stirred and left standing at 4°C for 12 h. The supernatants were decanted and the precipitates vacuum-dried to obtain crude biopolymers. The crude products were then dissolved in distilled water (1% w/v) and mixed with 1 volume of chloroform/*n*-butyl alcohol (5:2, v/v). After stirring, the mixtures were left standing at room temperature for 12 h. The upper phases were separated by centrifugation at 3000 × g, 4°C for 15 min and the supernatants concentrated at 40°C. Two volumes of ethanol were added, the precipitates recovered by centrifugation at 3000 × g, 4°C for 15 min, vacuum-dried and re-dissolved in an appropriate volume of distilled water.

3.8. Bioflocculant Production by Bacterial Consortium and Nutrient Optimization by Response Surface Methodology

3.8.1. Media Optimization

3.8.1.1. Plackett-Burman (PB) *Design*

The carbon, nitrogen and cation sources yielding optimal flocculation activity were evaluated with other media components to ascertain the role of every media component in bioflocculant production. PB design; used for n variable screening in n + 1 experiments was employed for obvious reasons of less laborious and time effectiveness (He *et al*., 2009). Five independent variables (media components); fructose, $MgCl_2$, $(NH_4)_2SO_4$, K_2HPO_4 and KH_2PO_4 were investigated using PB design to ascertain individual role in bioflocculant production. Two levels (concentrations) of each variable, ‘‘high” and ‘‘low”, were used and were designated as + 1 and – 1 respectively (He *et al*., 2009; Liu *et al*., 2010).

NCSS 2007 program (Kaysville, Utah) was used to develop the PB experimental design; which was based on the following first-order model:

$$Y = bo + \sum_{i=1}^{k} bixi$$

Y = the response (flocculation activity), bo = model intercept, bi = linear coefficient, xi

= level of the independent variable (concentrations), and k = number of involved variables (media components). Every run (assay) was carried out in triplicates while regression analysis was used to determine components with significant effect ($P < 0.05$) on bioflocculant production.

3.8.1.2. Central Composite Design (CCD)

Media components with significant input in bioflocculant production, as identified by PB design, were optimized through the response surface design (RSD). Thus, a central composite design (CCD) model was generated and this model was applied to the independent variables; fructose, $MgCl_2$ and $(NH_4)_2SO_4$ using 3-factor-5-level CCD (Liu *et al*., 2010). The correlation of response variable (flocculation activity) to the independent variables, showed flocculation activity to fit into second order polynomial model as shown below:

$$Y = bo\sum_{i=1}^{k} bixi + \sum_{i=1}^{k} biix^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} bijxixj, \quad i \neq j$$

Y = response variable (flocculation activity), bo = coefficient of interception, bi = coefficient of linear effect, bii, = coefficient of the quadratic effect, bij = coefficient of interaction effect when i < j and k which are the involved variables (media components).

3.9. Characterization of Purified Bioflocculant.

3.9.1. Composition Analyses of the Bioflocculants.

Total protein content of the bioflocculants was determined by using the Folin-Lowry method with bovine serum albumin as standard (Lowry *et al*., 1951). Total sugar content was determined by employing the Phenol-Sulphuric acid method with glucose as standard (Dubois *et al*., 1956).

3.9.2. Thermal Stability of the Purified Bioflocculant.

Thermal stability was examined by incubating 10 mL of bioflocculant solution made up in distilled water (0.1%, w/v) at temperatures ranging between 50°C and 80°C for 30 min. The stability was then determined by measuring the residual flocculating activity for Kaolin suspension (pH 7) at room temperature using the method previously described.

3.9.3. Fourier Transform Infrared Spectroscopy (FTIR).

The functional groups of the bioflocculants were analyzed using FTIR (Perkin Elmer System 2000, FT-IR, England). Bioflocculants were mixed with KBr salt, ground at 25 ^{o}C and pressed into pellets for FTIR analyses over a wave number of 4000-370 cm^{1}.

3.9.4. Thermo-Gravimetric Analysis (TGA)

TGA analyses on 10 mg of each bioflocculant were performed using 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.

3.9.5. Scanning Electron Microscopy (SEM).

Images of surface morphology structures of the purified bioflocculants were obtained by using a scanning electron microscope, JEOL JSM-6390LV FEI XL30 (JEOL; USA). SEM images of Kaolin clay, bioflocculant powders and the mixture of bioflocculant and Kaolin after flocculation were obtained.

3.9.6. Effect of Bioflocculant Dosage on Flocculating Activity (Jar test).

Various 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, w/v) were prepared in distilled water and their flocculating rates of Kaolin solution evaluated. The following reaction mixtures: 100 mL Kaolin suspension, 3 mL $CaCl_2$ (1%, w/v) and 2 mL of bioflocculant solution were prepared in 500 mL beakers for each bioflocculant concentration. The reaction mixtures were stirred at 200 rpm for 3 min before reducing the speed to 45 rpm for a further 10 min of agitation (Wang *et al*., 2010). The solutions were each transferred into 100 mL measuring cylinders, allowed to settle for 10 min at room temperature before withdrawing 2 mL of clear supernatant for determining flocculating activity as described previously.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Bioflocculant Production by *Halomonas* sp. Okoh.

This bactetria was identified by 16S RNA gene nucleotide sequencing and BLAST analyses revealed the nucleotide sequence to have 99% similarity to that of *Halomonas* sp. Au160H and the nucleotide sequence was deposited in GenBank as *Halomonas* sp. OKOH with accession number HQ875722. This bacteria was confirmed to be a bioflocculant producer.

The genus *Halomonas* is comprised of Gram-negative rods which are extremophiles and whose species are widely distributed in hypersaline habitats (Bouchotroch *et al*., 2001; Reddy *et al*., 2003), varying from moderate halophiles such as *Halomonas elongate*, to extreme halophiles such as *Halobacteria salinarum*. In order to survive and adapt in high ionic strength environments such as the sea, halophiles produce proteins which are designed to function under these conditions and in addition, are also capable of growing anaerobically in the presence of glucose (Prescott *et al*., 2002).

4.1.1. Effect of Culture Conditions on Bioflocculant Production.

4.1.1.1. Effect of Inoculum Size.

The effect of varying inoculum size on bioflocculant production was evaluated (Figure 4.1). The flocculating activities fluctuated within the volume range used with optimal activity of 88% achieved at 2% (v/v) and the lowest activity of 50.3% obtained with 1% (v/v) inoculum sizes respectively. Increasing the inoculums size above 2% did not improve the flocculation rate (Figure 4.1)**.** Inoculum size of 2% (v/v) was therefore used as standard for all subsequent experiments for the test bacteria. Inoculum size requirements for optimal bioflocculant production vary substantially among different microorganisms ranging from 1% (v/v) for *Vagococcus* sp. W31 (Gao *et al*., 2006) to 5% (v/v) for *Klebsiella mobilis* (Wang *et al*., 2007). Similar to the test bacteria, *Perilla fructescens* required a 2% (v/v) inoculum size for maximum bioflocculant production (Zhong and Yoshida, 1995). Xiong *et al*. (2010) reported a 4% inoculum size requirement for the enhancement of bioflocculant production by *Bacillus licheniformis* CGMCC 2876.

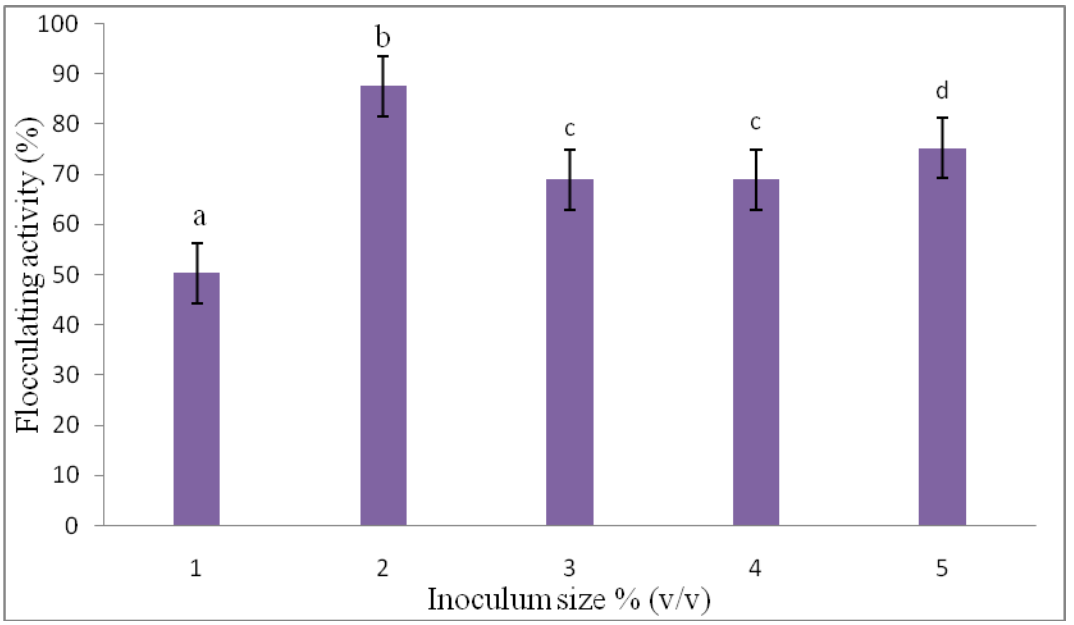

Figure 4.1. Effect of Inoculum Size on Bioflocculant Production by *Halomonas* sp. Okoh. Flocculating activities with different letters are significantly different (P < 0.05) from each other.

4.1.1.2. Effect of Carbon and Nitrogen Sources.

It has been well documented that changing the carbon and nitrogen sources highly influences bacterial growth and bioflocculant production (Sheng *et al*., 2006). Different carbon and nitrogen sources were also evaluated for their effects on flocculant production by this bacteria. From Figure 4.2, glucose seems to be the preferred carbon source for bioflocculant production by the bacteria, as it yielded about 87% flocculating activity compared to sucrose, fructose and starch, which yielded about 75%, 66% and 0% flocculating activities, respectively.

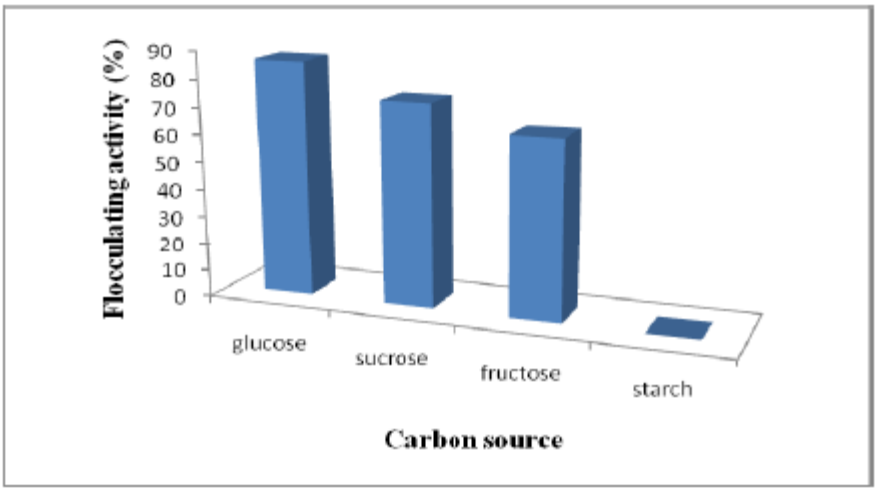

Figure 4.2. Effect of Carbon Source on Bioflocculant Production *Halomonas* sp. Okoh.

Glucose was also reported as the carbon source of choice for enhancing the production of bioflocculants by *Vagococcus* sp. W31 (Gao *et al*., 2006) and *Halomonas* sp. V3a' (He *et al*., 2009). However, in their study of the bioflocculant production by *Klebsiella pneuminiae*, Nakata and Kurane (1999) found that the use of ethanol as a carbon source supported the highest production of the polysaccharide flocculant, while Kumar *et al*. (2004) used an optimized cultivation medium containing corn starch in their investigation of bioflocculant production by *Bacillus* sp. I-450.

With respect to nitrogen source, urea gave the optimal production of bioflocculant with the highest flocculating activity of about 88.4%, compared to peptone, ammonium sulphate and ammonium chloride (Figure 4.3). Urea was also the preferred nitrogen source for the cultivation of haloalkalophilic *Bacillus* sp. I-450 (Kumar *et al*., 2004) and *Vagococcus* sp. W31 (Gao *et al*., 2006). This observation is contrary to the findings of He *et al*. (2009) and Sam *et al*. (2011) in which NH_4Cl and peptone were found to be

significant factors affecting bioflocculant production by *Halomonas* sp. V3a' and *Halomonas* sp. AAD6, respectively. In addition, Bouchotroch *et al*. (2001) reported a requirement for an L-amino acid such as alanine, arginine, histidine or leucine as the sole nitrogen source for the growth of *Halomonas maura* sp. nov.

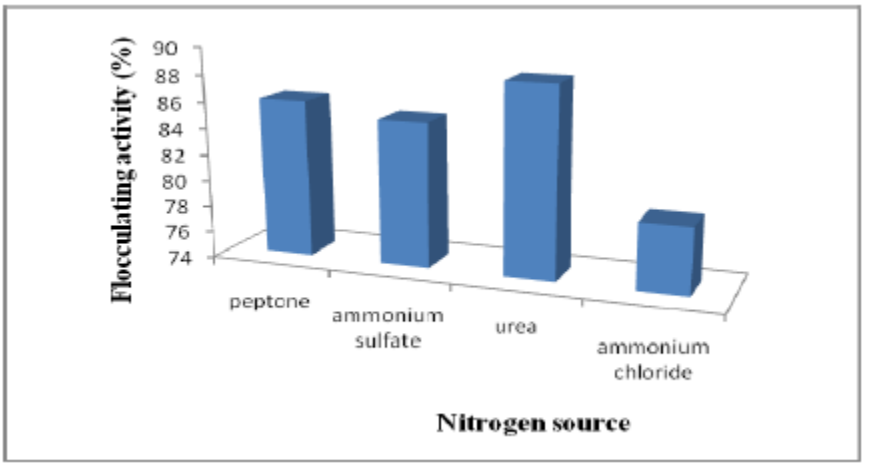

Figure 4.3. Effect of Nitrogen Source on Bioflocculant Production by *Halomonas* sp. Okoh.

4.1.1.3. Effect of Agitation Rate on Bioflocculant Production.

Varying agitation rates have been reported to influence not only the rate of respiration of different microorganisms but also nutrient absorption, the rate of enzymatic reactions and hence bioflocculant production (Salehizadeh and Shojaosadati, 2001). Figure 4.4 is an illustration of the relationship between shaker speed and bioflocculant production by *Halomonas* sp. Okoh. On average the rates investigated resulted in flocculating activity of abot 70% with the maximum activity obtained at 160 rpm (Figure 4.4). Zhang *et al*. (2010) reported using an agitation rate of 130 rpm for improved bioflocculant production by *Proteus mirabilis* TJ1 while 170 rpm was suffice for efficient bioflocculant production

by *Aeromonas* sp. (Li *et al*., 2007). Xiong *et al*. (2010) documented 200 rpm as being the required shaking rate for improved bioflocculant production by *Bacillus licheniformis* CGMCC 2876. Several other agitation rates that are required for enhanced bioflocculant production by different microorganisms have been reported (Gao *et al*. 2006; Gong *et al*. 2008).

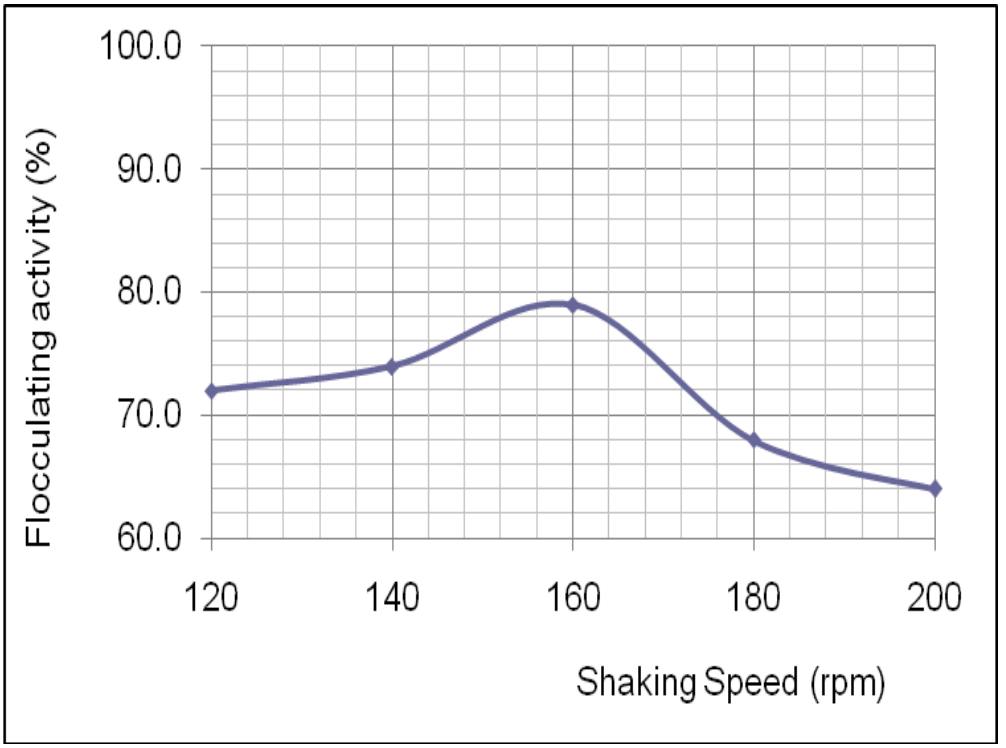

Figure 4.4. Effect of Agitation Rate on Bioflocculant Production by *Halomonas* sp. Okoh.

4.1.1.4. Effect of Cations and pH.

A dosage of the bioflocculant solution was mixed with Kaolin solution supplemented with commercial cationic electrolytes and the rate of flocculation measured spectrophotometrically at 550 nm. Solutions of $CaCl_2$, $MgCl_2$, $FeSO_4 \cdot 7H_2O$ and KCl were used as sources of cations. As shown in Figure 4.5, flocculating activity was

markedly increased by the addition of divalent cations with Ca^{2+} being marginally better than Mg^{2+} and Fe^{2+} in this respect. $CaCl_2$ was also reported to significantly increase the flocculating activity of the bioflocculants MBFW31 produced by *Vagococcus* sp. W31 (Gao *et al*., 2006) and HBF-3 produced by a mutant *Halomonas* sp. V3a' (He *et al*., 2010). For *Serratia ficaria*-produced bioflocculant, both divalent cations, Ca^{2+} and Mg^{2+} enhanced the flocculating activity whereas the co-presence of trivalent cations Al^{3+} and Fe^{3+} negatively affected the flocculating activity (Gong *et al*., 2008). On the other hand, Li *et al*. (2007) noted an increase in flocculating activity in the presence of monovalent cations K^{+} and Na^{+} and a divalent cation Ca^{2+} for a bioflocculant produced by *Aeromonas* sp. Cations have been reported to be capable of neutralizing negatively charged functional groups of both the bioflocculant molecules and the suspended particles (Salehizadeh and Shojaosadati, 2001; Li *et al*., 2008) and consequently weaken the static repulsive force thus enhancing the flocculation effect (Kumar *et al*., 2004; Zheng *et al*., 2008; Li *et al*., 2008).

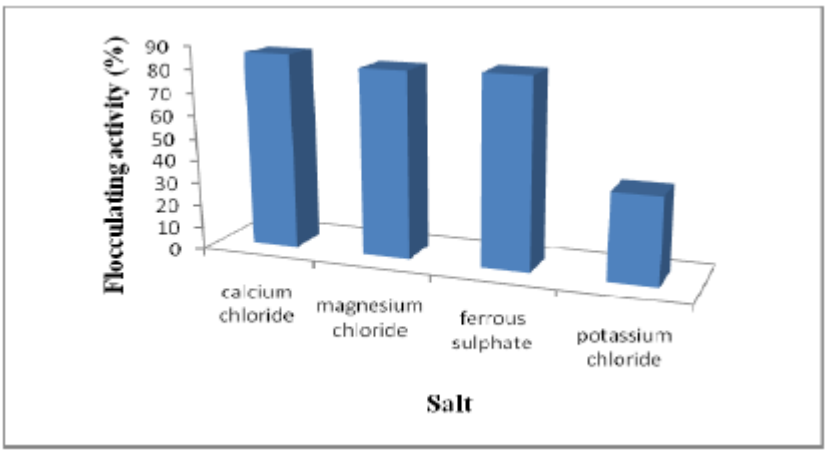

Figure 4.5. Effect of Cations on Bioflocculant Production by *Halomonas* sp. Okoh.

The effect of initial pH of the culture medium on flocculating activity was examined at pH values ranging from 3-12 (Figure 4.6). The activity was found to be highest (87%) at pH 7. Bouchtroch *et al*. (2001) and Gao *et al*. (2006) reported similar optimal pH values (pHs 7.2 and 7.0) for the activity of the bioflocculants ERSS-31 produced by *Halomonas maura* sp. nov. and MBFW31 produced by *Vagococcus* sp. W31, respectively. Bioflocculant HBF-3, produced by a mutant *Halomonas* sp. V3a' also attained the highest flocculating activity at pH 7 (He *et al*., 2010). At low pH, the absorption of H^+ ions tends to weaken the bioflocculant-kaolin complex formation process and a similar effect is also observed at high pH values (He *et al*., 2010). According to Li *et al*. (2008), the mediating effect of Ca^{2+} appears to be strongest at neutral pH values. However, these observations differ from the results of studies carried out by Choi *et al*. (1998) and Zheng *et al*. (2008) in which the maximum flocculating activities of bioflocculants produced by *Anabaena* sp. and *Bacillus* sp. F19, respectively, were observed at pH 2. The bioflocculant produced by *Bacillus* sp. PY-90 was found to be actively high in an acidic pH range of 3.0 to 5.0 (Yokoi *et al*., 1995), whilst *Serratia ficaria* produced a bioflocculant effective over a weakly acidic pH range of 5.0 to 7.0 (Gong *et al*., 2008).

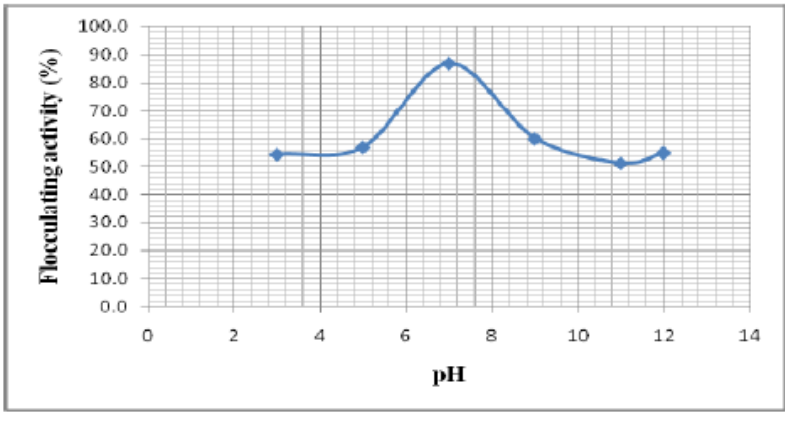

Figure 4.6. Effect of pH on Bioflocculant Production.

4.1.1.5. Time Course Assay of Bioflocculant Production.

In this study, the bioflocculant produced by *Halomonas* sp. Okoh growing on filtered seawater medium supplemented with different carbon and nitrogen sources was evaluated for its bioflocculant properties. According to Sam *et al*. (2011), the flocculating capability of flocculants produced by halophilic bacterial strains is enhanced when the evaluation is carried out in seawater suspensions. *Halomonas maura* sp. nov. was also reported to have a strict growth requirement for sea salts with no growth observed in their absence (Bouchotroch *et al*., 2001). In addition, the high-salinity condition of seawater tends to eliminate any risk of contamination during microbial cultivation (Sam *et al*. 2011).

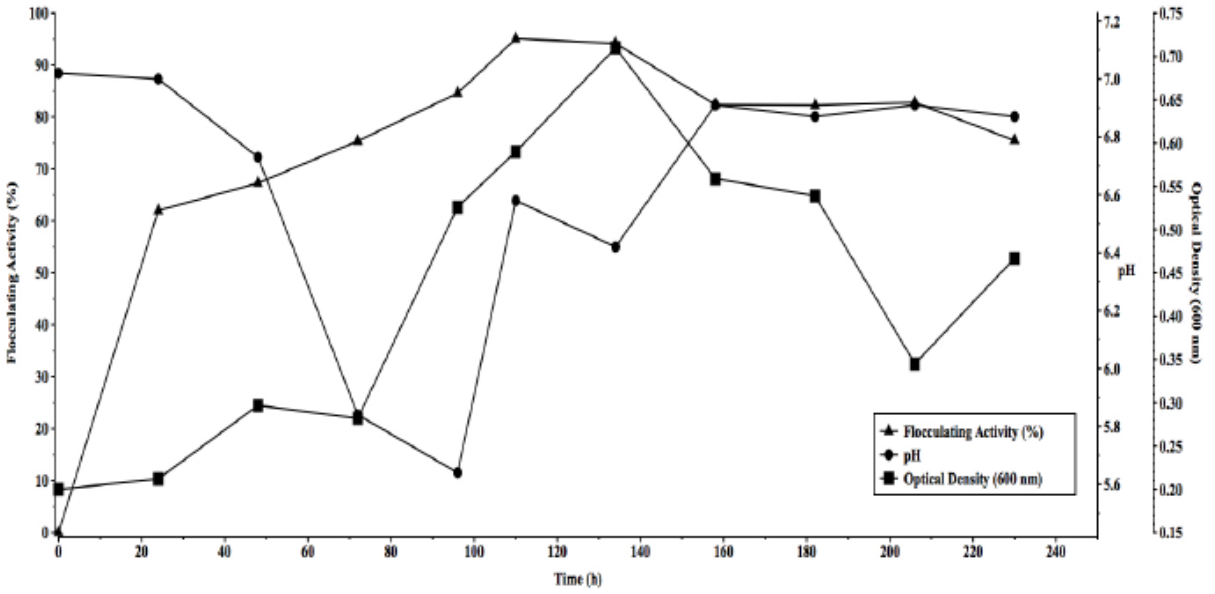

Figure 4.7. Time Course Assay of Bioflocculant Production by *Halomonas* sp. Okoh. ***[Legend are as follows: ■ (pH); ▲ ([flocculating activity (%)]; ● [optical density (600 nm)].***

The growth pattern, flocculating activity and pH variation of the culture broth were as shown in Figure 4.7. During growth, the flocculating activity increased as the cultivation period increased, attaining a peak flocculating activity of about 95% after 135 h of cultivation, beyond which flocculating activity began to decline (Figure 4.7). The observed decrease in flocculating activity might be due to partial enzymatic degradation of the polymer flocculant in the late phases of cell growth (Choi *et al* 1998). He *et al.* (2002) reported a decrease in flocculating activity of a bioflocculant produced by *Corynebacterium glutamicum* which was attributed to a decrease in the molecular weight of the polymer when it was subjected to protease hydrolysis. A corresponding increase in optical density with cultivation time was also observed and ran almost parallel to the flocculating activity curve (Figure 4.7). The observed direct relationship between growth and activity is an indication that the bioflocculant was produced by biosynthesis during growth of the bacteria and not by cell autolysis (He *et al*., 2002; Gao *et al*., 2006). Figure 4.7 also shows a decrease in the pH of the culture broth from about 7.0 to about 5.6 during the first 96 h of incubation. This observed decrease in pH may, according to Dermlin *et al*. (1999), be caused by the production of organic acids from either the metabolism of glucose, or from the produced bioflocculant, although a contrary observation was reported for *Halomonas maura* sp. nov. (Bouchotroch *et al*., 2001) where no such acid production was observed. The observed increase in pH in the later stages of cultivation could not be accounted for, but may be attributed to excretion of extracellular materials resulting from the partial degradation of the bioflocculant.

4.1.1.5.1. Bioflocculant Yield.

In order to be cost-effective maximum bioflocculant production at the minimum cost input possible should be targeted for (Mabinya *et al*., 2011). A purified bioflocculant yield of 1.2 g was recovered from 1 L fermented culture broth of *Halomonas* sp. Okoh. Several studies have been reported reflecting bioflocculant yields obtained by altering the composition of the medium used for cultivating different microorganisms for bioflocculant production. These range from as low as 1.30 g/L bioflocculant yield obtained from fermented broth of *Enterobacter aerogenes* (Lu *et al*., 2005) to as high as 14.5 g/L generated from *Agrobacterium* sp. M-503 fermented broth (Li *et al*., 2010), as well as 14.8 g/L produced by marine *myxobacteria Nannocystics* sp. NU-2 (Zhang *et al*. 2002).. About 1.2 g of purified bioflocculant was recovered from 1 L fermented culture broth of *Halomonas* sp. Okoh. Different bioflocculant yield recovery rates have been reported for different microorganisms (Prasertsan *et al*. 2006; Elkady *et al*., 2011). On a comparative basis, the bioflocculant yied by *Halomonas* sp. Okoh was on the low side.

4.1.1.5.2. Characterization of Purified Bioflocculant.

(a) *Composition Analyses of the Bioflocculant.*

Total carbohydrate was determined by the phenol-sulphuric acid method, which gave a total carbohydrate content of 23 mg/mL, thus confirming the presence of carbohydrates as a major component of the bioflocculant produced by the bacteria. No obvious protein absorption peak was detected at 280 nm and further quantification of protein content using the Folin Lowry method proved negative. These findings corroborated the results

obtained from chemical analyses of bioflocculants produced by other halophilic species belonging to the same genus, such as *Halomonas maura* sp. nov (Bouchotroch *et al*., 2001), *Halomonas* sp. V3a' (He *et al*., 2009, 2010), and *Halomonas* sp. AAD6 (Sam *et al*., 2011) which indicated the presence of polysaccharide as the main component. In their investigation of the chemical nature of the polymer flocculant produced by a haloalkalophilic *Bacillus* sp. I-450, Kumar *et al*. (2004) also showed it to be composed of a polysaccharide. According to Sam *et al*. (2011), the low charge density of polymers such as polysaccharides usually leads to enhanced performance in suspensions with high ionic strengths. Other microorganisms producing different kinds of polysaccharide flocculants have been investigated. These include *Anabaena* sp. (Choi *et al*., 1998), *Klebsiella pneuminiae* (Nakata and Kurane, 1999), *Corynebacterium glutamicum* (He *et al*., 2002) and *Vagococcus* sp. W31 (Gao *et al*., 2006).

(b) *Thermal Stability of Purified Bioflocculant.*

Temperature stability, much as pH tolerance, is an important factor when considering the suitability of a biopolymer for a wide range of different treatment purposes (Patil *et al*., 2011). The temperature stability of the bioflocculant produced by *Halomonas* sp. Okoh was verified and the results thereof are represented by Figure 4.8. After subjecting the bioflocculant solution to different temperatures ranging from 50-100^{o}C, optimum flocculating activity of 89.5% was achieved at 50^{o}C with further increases in temperature of up to 100^{o}C only resulting in a decrease in flocculating activity (Figure 4.8). Of note is the observation that the bioflocculant still retained its high flocculating activity of 74% at 100^{o}C, an indication that, most probably, it was of a polysaccharide nature. Contrary to this observation, Salehizadeh and Shojaosadati, (2002) reported a thermostable

bioflocculant generated by *Bacillus firmus* which retained only about 50% of flocculating activity after being heated in boiling water for 15 min. Gao *et al*. (2006) working on bioflocculant production by *Vagococcus* sp. W31, reported a retention rate of 86.5% in flocculating activity at 100^{o} C. The bioflocculant produced by *Agrobacterium* sp. M-503 maintained significantly high flocculating activity within a broad temperature stability of 70-121^{o}C (Li *et al*. 2010). A thermostable REA-11 bioflocculant produced by *Corynebaterium glutamicum* CCTCC M201005 could withstand temperatures of up to 80^{o}C for 1 h without any significant effect on the flocculating activity while a further increase in temperature to 100^{o}C for the same duration resulted in a decrease in flocculating activity (He *et al*. 2004). The thermostable-bioflocculant HBF-3 produced by deep-sea bacteria mutant *Halomonas* sp. V3a' retained more than 90% of its flocculating activity only within the temperature range of 4-40^{o}C (He *et al*., 2010).

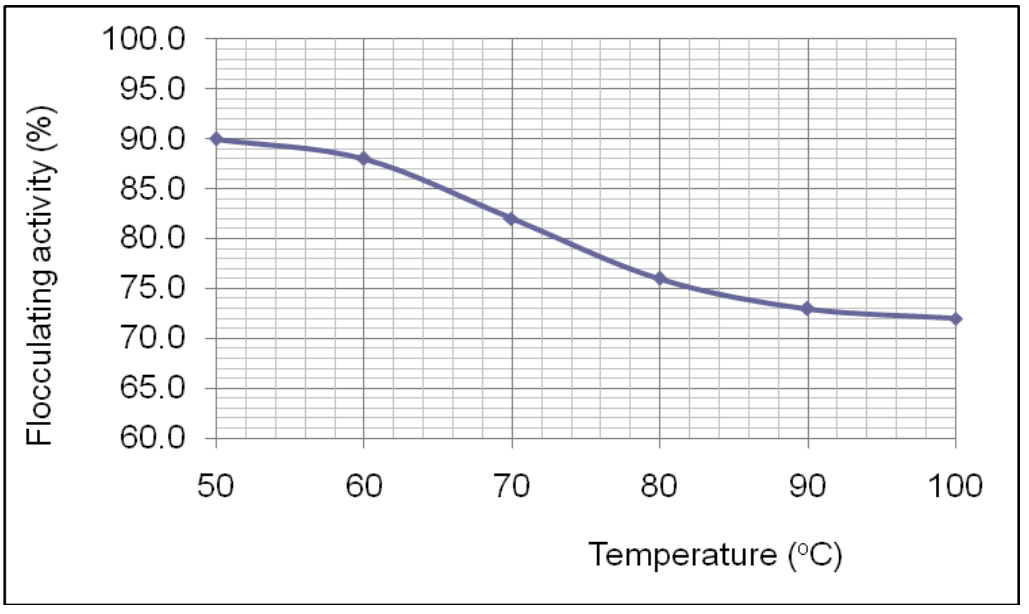

Figure 4.8: Effect of Temperature on the Flocculating Activity of the Bioflocculant.

(c)*. Determination of Functional Groups by FTIR.*

FTIR analysis of the purified bioflocculant was done in order to establish the presence within the molecule of functional groups known to be essential for flocculating activity of the bioflocculant (Figure 4.9). 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 of this *Halomonas*-produced bioflocculant was consistent with results obtained from other bioflocculants (Salehizadeh and Shojaosadati, 2001; Kumar *et al*., 2004; Li *et al*., 2010; Xiong *et al*., 2010).

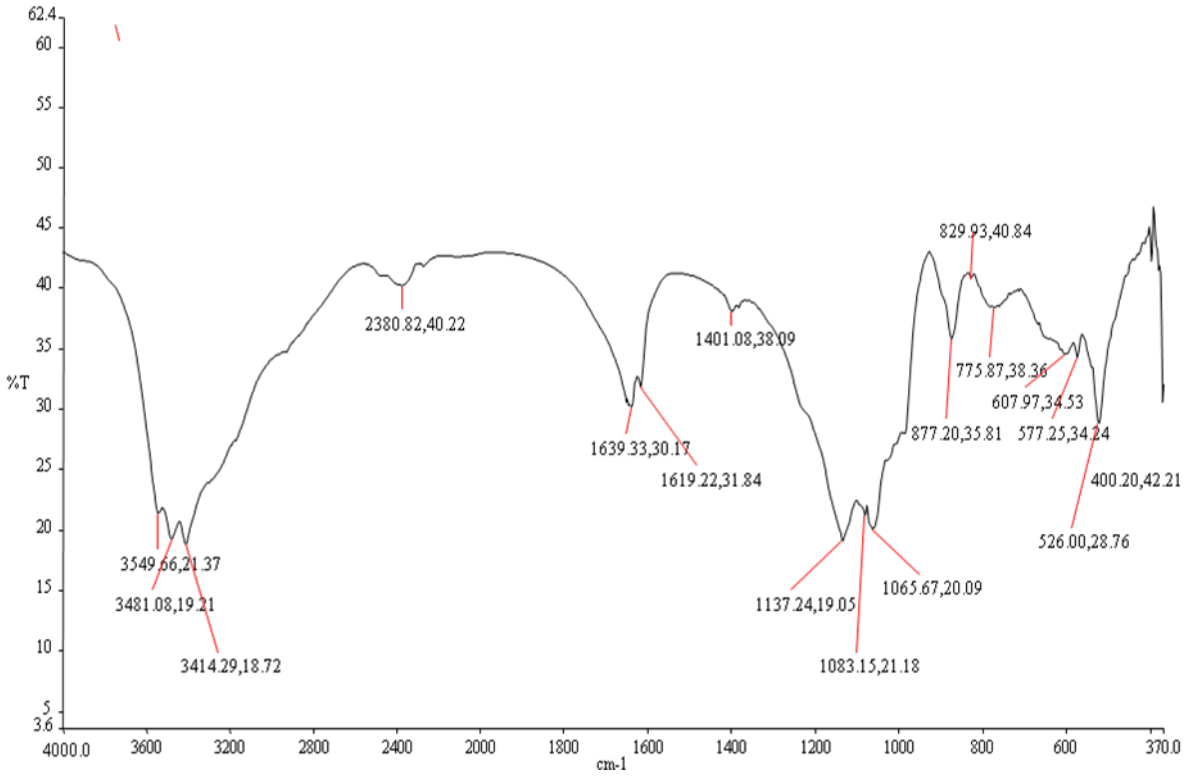

Figure 4.9. Fourier-Transform Infrared (FTIR) Spectroscopy of Purified Bioflocculant.

(d) Thermogravimetric Analysis.

The result that show the decomposition of the bioflocculant when subjected to high temperatures of about 500^{o}C is depicted in Figure 4.10. From an initial temperature of 40^{o}C a steady decrease in weight of about 18% was observed when the temperature was to 200^{o}C (Figure 4.10). This initial weight loss can be attributed to moisture content in the molecule as a result of the presence of carboxyl groups. The higher the number of carboxyl groups, the higher the moisture content (Kumar and Anand, 1998). Further increases in temperature to 300^{o}C resulted in a 30% decrease in weight which further decreased by approximately 32.5% when the temperature was increased to 500^{o}C (Figure 4.10).

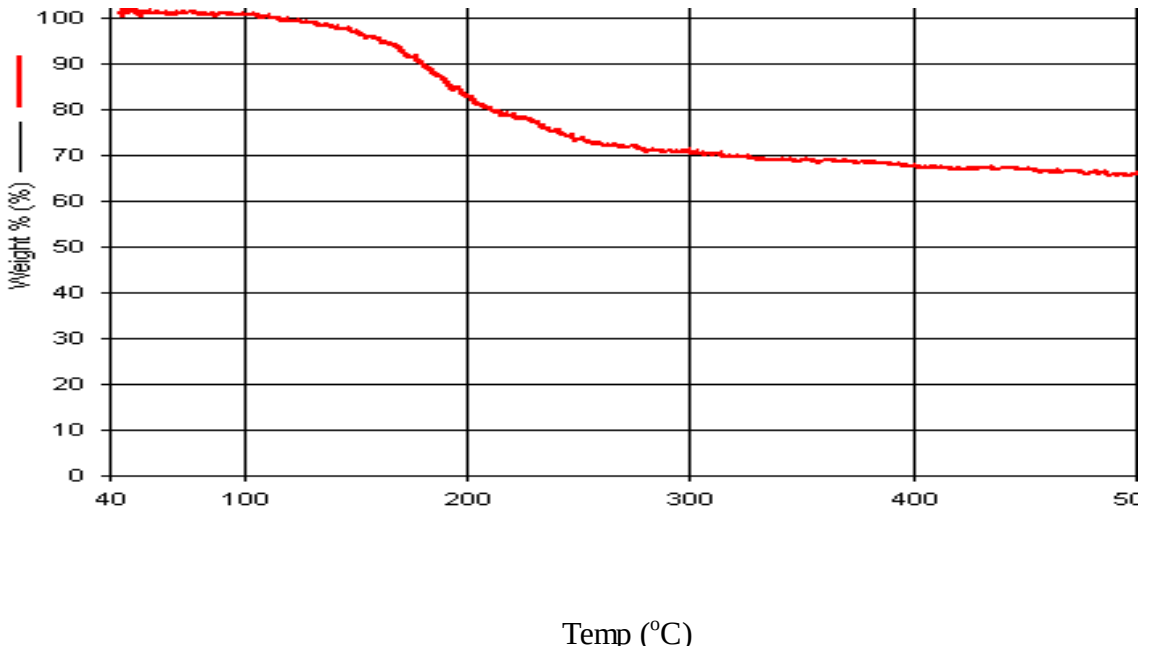

Figure 4.10. Thermo Gravimetric Analyses of Purified Bioflocculant produced.

(e) SEM Morphology Images.

SEM images of the bioflocculant powder (A) Kaolin clay (B) before flocculation and a mixture of Bioflocculant and Kaolin (C) after flocculation were scanned (Figure 4.11). The images revealed how the structure of bioflocculant was stretched out like a thread (Figure 4.11 A), how the Kaolin particles were scattered before flocculation (Figure 4.11 B), and how the bioflocculant efficiently connected the scattered Kaolin particles and formed aggregates (Figure 4.11 C).

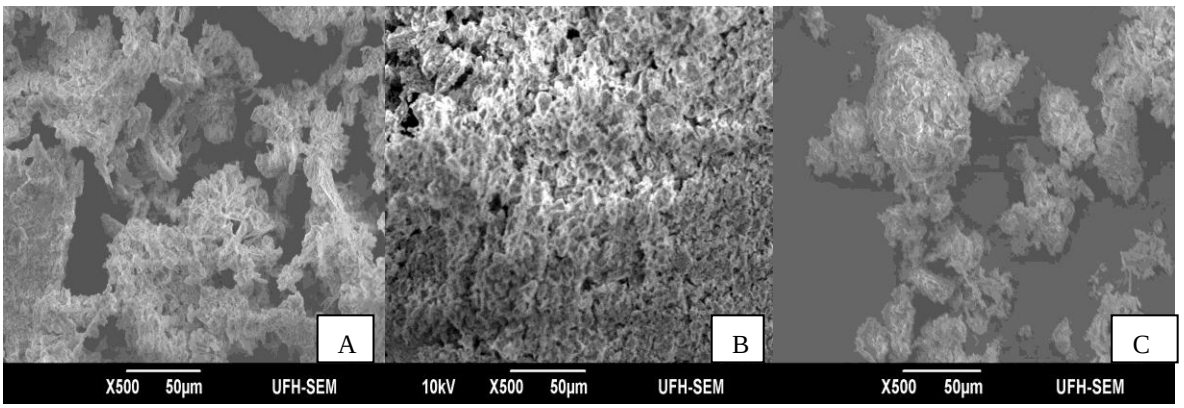

Figure 4.11. Bioflocculant powder (A), Kaolin clay (B), Bioflocculant and Kaolin clay after Flocculation (C).

4.1.1.5.3. Effect of Bioflocculant Dosage on the Flocculating Efficiency.

The relationship between bioflocculant dosage and its flocculating activity is depicted in Figure 4.12. The bioflocculant dosage required for optimum flocculation varies with bioflocculants produced by different microorganisms. For *Halomonas* sp. Okoh, an optimum flocculating activity was achieved at a dosage of 0.2 mg/mL with further increases in dosage resulting in a decrease in flocculating activity which remained relatively steady between 0.5-0.7 mg/mL (Figure 4.12). A decrease in flocculating activity observed at high bioflocculant dosage might be due to high viscosity being generated, thus leading to an increase in the turbidity of the solution (He *et al*., 2010). Deng *et al*.

(2003) reported an optimum bioflocculant dosage requirement of 0.1 mg/mL for a bioflocculant produced by *Bacillus mucilaginosus* while Prasertersan *et al*. (2006) recorded 2 mg/mL as optimal for flocculation by the biopolymer produced by *Enterobacter cloacae*.

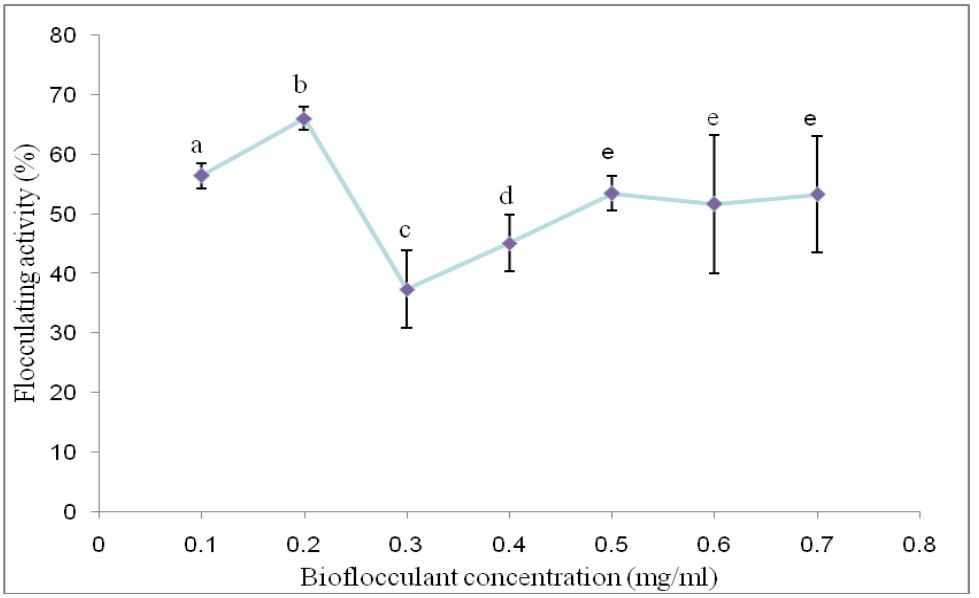

Figure 4.12. Effect of Bioflocculant Dosage on the Flocculating Activity. Flocculating activities with different letters are significantly different (P < 0.05) from each other.

4.2. Bioflocculant Production by *Micrococcus* sp. Leo.

This bacteria was amongst the several bacteria isolated from the bottom sediments of Algoa Bay in the Eastern Cape Province of South Africa and confirmed to be a bioflocculant producing bacteria. The identity of the bioflocculant producing-bacteria was confirmed by 16S rRNA gene nucleotide sequence analysis following PCR amplification of the gene. Analyses of the nucleotide sequence of the amplified 16S rDNA gene revealed a 99% similarity to that of *Micrococcus* sp. Bg-6. The nucleotide sequence was

therefore deposited in the Genbank as *Micrococcus* sp. Leo with accession number JF799091.

Gram-positive bacteria have a stronger cell envelope than Gram-negative bacteria and this allows them to thrive in the highly variable intertidal sediment environment where sediment temperatures are high in the day and osmotic pressures and nutrient supply may change periodically over a daily cycle (Zhuang *et al*., 2003). *Micrococcus* falls into the Gram-positive cocci group which is made up of spherical or slightly oval cells and are relatively resistant to reduced water potential and tolerate drying and high salt concentrations fairly well. *Micrococcus* are obligate aerobes with a typical respiratory metabolism and often aerobically produce acid from utilizable carbohydrates with the exception of *Micrococcus* antarcticus s. nov. in which acid production from carbohydrates is negative (Liu *et al*., 2000). They are catalase-positive, which helps distinguish them from other Gram-positive *cocci*, known to be non-motile, and can also be found in freshwater environments or in soil. Some strains of *Micrococcus* can be used for hydrocarbon and wax degradation (Auletta and Kennedy, 1966; Liu *et al*., 2000; Zhuang *et al*., 2003). *Micrococcus luteus* has the ability to tolerate and utilize very toxic organic molecules and metals and thus has a potential role in bioremediation (Sandrin and Maier, 2003; Greenblat *et al*., 2004), in addition to its ability to use biphenyl as a carbon source and degrade phthalates (Eaton, 1982).

4.2.1. Effect of Culture Conditions on Bioflocculant Production.

The composition of the production medium and culture conditions are known to influence bacterial growth and bioflocculant production (Nakata and Kurane, 1999; He *et al*., 2004;

Sheng *et al*., 2006). Inoculum size, carbon and nitrogen sources, agitation speed, salt ions and the pH were some of the factors investigated for their effects on the production and flocculating activity of the bioflocculant produced by the test bacteria.

4.2.1.1. Effect of Inoculum Size.

Of the varying inoculum volumes used to initiate bacterial growth, both 3% (v/v) and 4% (v/v) supported optimal bioflocculant production with the resultant flocculating activities of more than 80% and a noticeable decrease at 5% (v/v) (Figure 4.13). Various factors, including the size of the inoculum used, affect bacterial growth differently (Salehizadeh and Shojaosadati, 2001) necessitating the need to establish suitable inoculum size for optimal growth by different microorganisms.

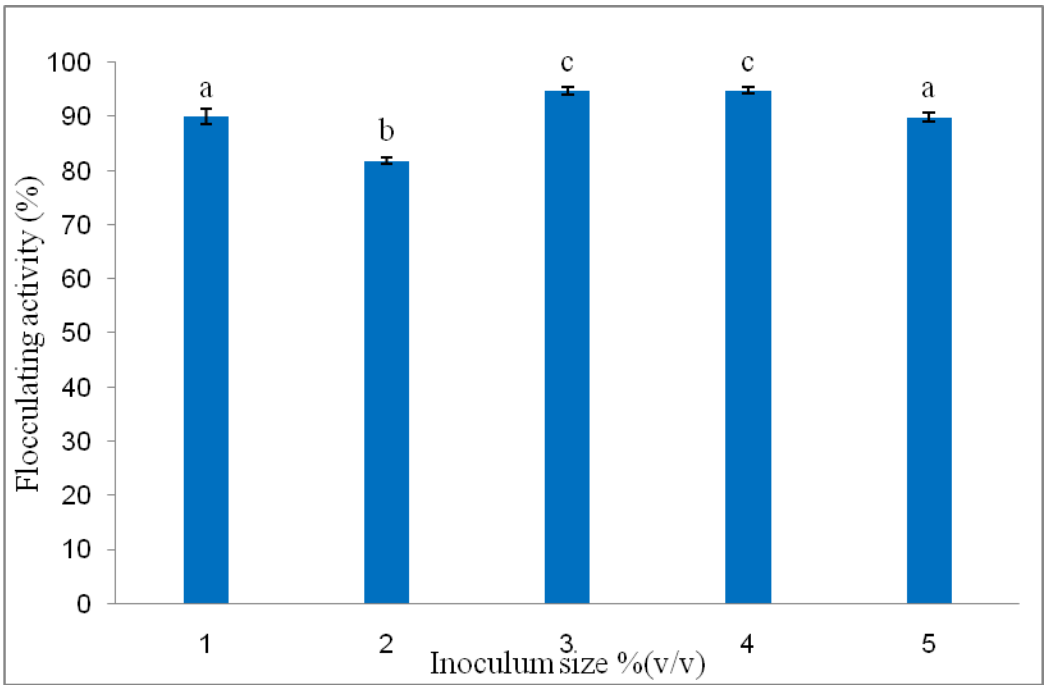

Figure 4.13. Effect of Inoculum Size on Bioflocculant Production by *Micrococcus* sp. Leo. Flocculating activities with different letters are significantly different (P < 0.05) from each other.

4.2.1.2. Effect of Carbon Source.

The effects of glucose, sucrose, fructose and starch on bioflocculant production were assessed and the results are as shown in Table 4.1. Sucrose and glucose seemed to be preferred carbon sources with resultant flocculating activities of 90% and 76.7% respectively. No flocculating activity was detected with either fructose or starch as carbon sources (Table 4.1). These results are in agreement with those reported by Dong *et al*. (2009) and Huang *et al*. (2011) for *Micrococcus* sp. S-11 in as far as glucose as a preferred carbon source, is concerned. On the contrary, Liu *et al*., (2000) reported a positive fructose but negative glucose utilization by the cold-adapted marine *Micrococcus antarcticus* sp. nov. Although starch has also been reported as a poor carbon source for bioflocculant production by *Bacillus* sp. Gilbert (Piyo *et al*., 2011), however, in their assessment of bioflocculant production by *Bacillus licheniformis* X14, Li *et al*. (2009a) found both starch and sucrose to be effective carbon sources in enhancing its production. Sucrose was also shown as a carbon source of choice for enhancing the production of bioflocculants by *Vagococcus*. W31 (Gao *et al*., 2006). However, some reports seem to indicate a significant improvement in bioflocculant production by constructing consortia of different microorganisms. Wang *et al*., (2011) reported high flocculating activities maintained over a wide concentration range for a compound bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 grown on glucose as carbon source. Similar results of an improved bioflocculant production by a consortium of *Staphylococcus* sp. and *Pseudomonas* sp. were also reported by Zhang *et al*. (2007).

4.2.1.3. Effect of Nitrogen Source.

The effect of both organic and inorganic nitrogen sources on biofloccdulant production and activity was evaluated and both peptone and ammonium chloride enhanced flocculating activity with peptone being more effective (77%) compared to ammonium chloride (68%). No flocculating activity was detected with urea and ammonium sulphate as carbon sources (Table 4.1). Contrary to this finding, Dong *et al*., (2009) and Huang *et al*., (2011) reported urea as a nitrogen source of choice for *Micrococcus* sp. S-11. A number of strains have been reported to optimally produce bioflocculants in the presence of organic nitrogen sources (or sometimes combined organic and inorganic sources) (Gong *et al*., 2008; Xia *et al*., 2008; Mabinya *et al*., 2011). He *et al*. (2009) and Sam *et al*. (2011) reported NH_4Cl and peptone to be significant factors affecting bioflocculant production by *Halomonas* sp. V3a' and *Halomonas* sp. AAD6 respectively. For *Arthrobacter albidus* LC13^T both yeast extract and peptone were key parameters influencing optimal production of the bioflocculant (Su *et al*., 2011).

Table 4.1. Effect of constituents of culture medium on the production and flocculating activity of the bioflocculant produced by *Micrococcus* sp. Leo.

Carbon Source	**Glucose**	**Sucrose**	**Fructose**	**Starch**
Flocculating Activity %	76.7	90.0	-	-
Nitrogen Source	**Ammonium Chloride**	**Ammonium Sulphate**	**Urea**	**Peptone**
Flocculating Activity %	68.0	-	-	77.0

Cations	Calcium Chloride	Magnesium Chloride	Ferrous Sulphate	Potassium Chloride
Flocculating Activity %	77.0	85.0	70.0	72.0

Note: (-) denotes no flocculating activity detected

4.2.1.4. Effect of Agitation Speed

Aeration level requirements which are essential for adequate respiration rate differ among microorganisms. Dissolved oxygen tension (DOT) of the culture broth has been reported to influence the rate of growth by microorganisms and varying agitation rates for determining DOT's that are necessary for improved bioflocculant production by different microorganisms have been reported (Salehizadeh and Shojaosadati, 2001). *Micrococcus* sp. Leo required an agitation rate of 160 rpm for maximum bioflocculant production with an optimal flocculating activity of 79% (Figure 4.14). A similar agitation speed was reported by both He *et al*. (2010) for optimal production of a novel bioflocculant HBF-3 from a deep-sea bacteria mutant *Halomonas* sp. V3a' and Ugbenyen *et al*. (2012) for the bioflocculant produced by *Cobetia* spp. As is well documented in the literature, different microorganisms require specific aeration levels for adequate growth hence different shaking speeds. Ntsaluba *et al*. (2011) and Abdel-Aziz *et al*. (2011) documented 120 rpm and 130 rpm agitation rates for maximum bioflocculant production by *Methylobacterium* sp. Obi and *Bacillus alvei* NRC-14 respectively. For *Bacillus mucilaginosus* and *Aspergillus parasiticus* agitation rates of 150 rpm and 140 rpm respectively improved bioflocculant production (Deng *et al*., 2003, 2005). At 170 rpm, *Aeromonas* sp. (Li *et al*., 2007) and *Bacillus* sp. (Feng and Xu, 2008) produced bioflocculants maximally while *Bacillus* sp. F19, *Pseudoalteromonas*

sp. SM9913, *Enterobacter cloacae* WD7 required shaking at 200 rpm to enhance bioflocculant production (Prasertsan *et al*., 2006; Zheng *et al*., 2008; Li *et al*., 2008). A higher shaking speed of 220 rpm has also been reported for enhanced bioflocculant production by both Nakata and Kurane, (1999) and Zhang *et al*. (2002).

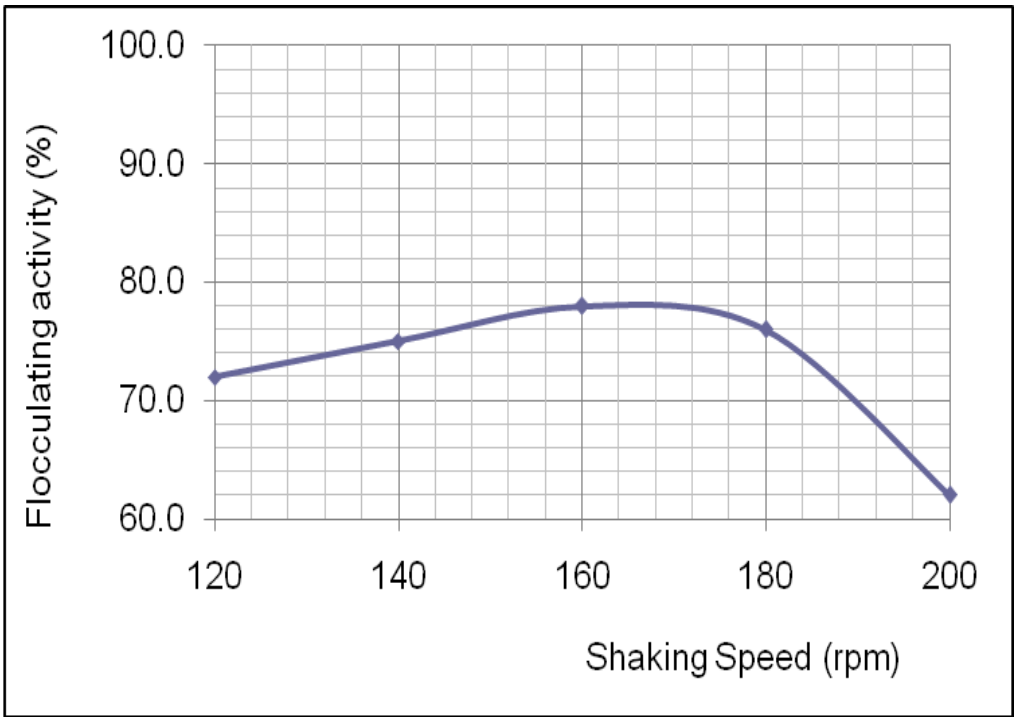

Figure 4.14. Effect of Agitation Speed on Bioflocculant Production by *Micrococcus* sp.

4.2.1.5. *Effect of Cations.*

The production of bioflocculant is influenced by salt ions present in the culture medium. The effects of cations on flocculating activity of the bioflocculant produced by *Micrococcus* sp. Leo were evaluated using solutions of $CaCl_2$, $MgCl_2$, $FeSO_4.7H_2O$ and KCl as sources of cations. As can be seen in Table 4.1, all cations used stimulated flocculating activity albeit to varying degrees but the strongest stimulation (85%) was achieved with Mg^{2+}. Several studies have reported on the stimulating effect of both Mg^{2+}

and Ca^{2+} divalent cations on bioflocculant activity. For *Serratia ficaria*-produced bioflocculant, both Ca^{2+} and Mg^{2+} enhanced the flocculating activity although a negative effect was recorded for Fe^{3+} (Gong *et al*., 2008). Mabinya *et al*. (2011, 2012) also reported an increase in flocculation activity of bioflocculants produced by *Halomonas* sp. Okoh and *Arthrobacter* sp. Raats respectively in the presence of Mg^{2+}, an effect similar to the one obtained for flocculating activity of MBF3-3 produced by a *Bacillus* sp. (Feng and Xu, 2008). For bioflocculant LC13-SF produced by *Arthrobacter albidus* $LC13^{T}$, maximum flocculating efficiency required the presence of Ca^{2+} (Su *et al*., 2011). In contrast, Li *et al*. (2007) noted an increase in flocculating activity in the presence of both monovalent cations K^{+} and Na^{+} and a divalent cation Ca^{2+} for a bioflocculant produced by *Aeromonas* sp. Salehizadeh and Shojaosadati, (2002) noted that both Mg^{2+} and Ca^{2+} seem to accelerate the initial adsorption of the biopolymer on Kaolin particles thus decreasing the negative electrical charge of Kaolin particles and the biopolymer flocculant which consequently enhanced the flocculating effect.

4.2.1.6. Effect of pH

The pH of the solution is also a key factor in flocculation and thus effectively influences the flocculation process. The pH can affect both the dispersion stability of suspended particles and the formation of floccules. The flocculating efficiency of different bioflocculants varies greatly with pH (Salehizadeh and Shojaosadati, 2001).

The effect of pH on flocculating activity of the bioflocullant produced by *Micrococcus* sp. Leo was investigated at pH values ranging from 3-12 (Figure 4.15). Amaximum flocculating activity of 94% was recorded at weak alkaline conditions (pH 9) with the activity distinctly much lower at strong acidic conditions (pH 3) than in neutral and

alkaline conditions (Figure 4.15). Optimum flocculating efficiencies have also been recorded at weakly alkaline pH (7.0-9.0) for bioflocculants produced by *Arthrobacter* sp. (Su *et al*., 2011, Mabinya *et al*., 2012), *Halomonas* sp. (Bouchtroch *et al*., 2001; Mabinya *et al*., 2011; He *et al*., 2010). At low pH, the absorption of H^+ ions tends to weaken the bioflocculant-kaolin complex formation process and a similar effect is also observed at high pH values (He *et al*., 2010).

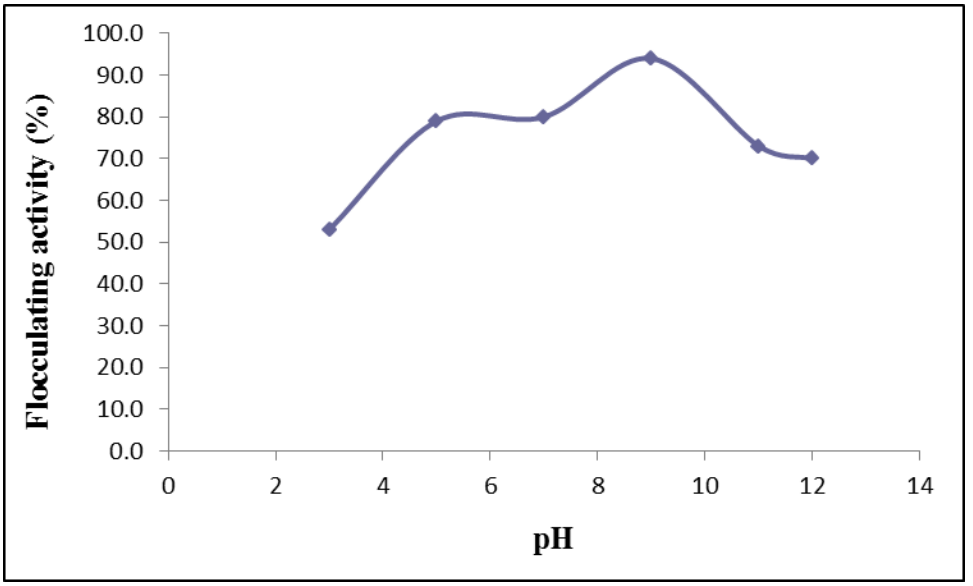

Figure 4.15. Effect of initial pH on Bioflocculant Production.

According to Li *et al*. (2008), the mediating effect of cations appears to be strongest at neutral pH values. However, these observations are different from studies carried out by Choi *et al*. (1998) and Zheng *et al*. (2008) in which the maximum flocculating activities of bioflocculants produced by *Anabaena* sp. and *Bacillus* sp. F19 respectively were observed at pH 2. The bioflocculant produced by *Bacillus* sp. PY 90 was found to be actively high in an acidic pH range of 3.0 to 5.0 (Yokoi *et al*., 1995), while *Serratia ficaria* produced a bioflocculant effective over a weakly acidic

pH range of 5.0 to 7.0 (Gong *et al*., 2008).

4.2.2. Time Course Assay of Bioflocculant Production.

Figure 4.16 shows the time course of bioflocculant production by *Micrococcus* sp Leo with an illustration of the relationship between optical density, flocculating rate and pH over a cultivation period of over 9 days. As shown in the figure, the rate of flocculation was observed to increase correspondingly with optical density, reaching a peak of 59.4% in 48 h of cultivation followed by a steady decline in both flocculating activity and optical density up to 4 days of cultivation (Figure 4.16). Sudden and rapid declines in flocculating activity and optical activity followed and in 6 days of cultivation approximately 80% and 60% declines in flocculation rate and optical activity were respectively observed. A significant recovery in flocculation rate with both the optical density and pH remaining relatively steady was then observed in the following 3 days of growth (Figure 4.16).

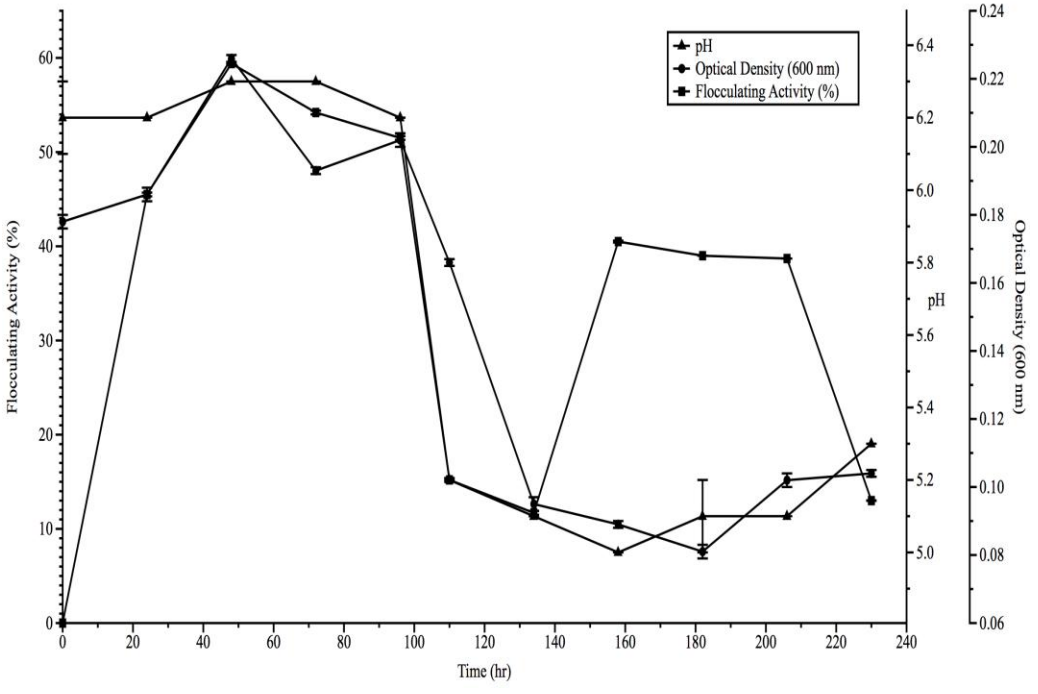

Figure 4.16. Time Course Assay of Blocculant Production by *Micrococcus* sp. Leo.

According to a study carried out by Jang *et al*. (2001) on bioflocculant production by fed-batch culture of *Citrobacter* sp., specific flocculating activity and bioflocculant production were influenced by fermentation conditions which included among other things, carbon source, inoculum size, dissolved oxygen tension (DOT) and carbon/nitrogen (C/N) source ratio. Jang *et al*. (2001) also noted that the initial growth rate increased with an increase in carbon source concentration. However, carbon source consumption was severely limited at higher carbon source concentrations. Inoculum size has also been reported to affect not only cell growth but also product formation with the dynamic behavior of cells of *Citrobacter* sp. cell cultures at different inoculum sizes affecting bioflocculant activity (Jang *et al*., 2001; Kallos and Behie, 1999). The synthesis of bioflocculant and the flocculating activity were also shown to be significantly affected by the C/N ratio of the growth medium with a higher C/N ratio not necessarily translating to a higher yield of flocculant synthesis (Jang *et al*., 2001). The influence of the initial pH of fermentation medium can determine both the electric charge of the cells and the oxidation-reduction potential which, in turn, can affect nutrient absorption and enzymatic reaction (Su *et al*., 2011). Thus the corresponding steady increase in cell growth (OD_{600}) in direct relation to flocculating activity observed over the first 2-day growth period is possibly an indication that the bioflocculant was produced by biosynthesis during growth of the bacteria and not by cell autolysis (Gao *et al*., 2006). The observed decrease in flocculating activity might be due to the activity of deflocculation enzymes (Gong *et al*., 2008; Choi *et al*., 1998). He *et al.* (2002) reported a decrease in flocculating activity of a

bioflocculant produced by *Corynebacterium glutamicum* which was attributed to a decrease in the molecular weight of the polymer when it was subjected to protease hydrolysis.

Figure 4.16 also shows that optimum flocculating activity was reached at pH 6.3 in 48 h of cultivation and in 6 days the pH had dropped by about 1.2 pH units to 5.12 which also corresponded to a decline of about 80% in flocculating activity. The observed fluctuations in the pH of the medium during bacterial growth may be attributed to two opposite phenomena: a decrease of pH due to the bacterial activity resulting in the production of organic acids from either the metabolism of glucose, or from the produced bioflocculant (Dermlin *et al*., 1999; Lors *et al*., 2009), and an increase of pH due to the release of hydroxide ions associated to the cations that are leached out (Lors *et al*., 2009).

The correlation between bioflocculant production and culturing times may differ among different organisms. Maximum production levels and a flocculating peak of 87.5% for a bioflocculant produced by *Arthrobacter* sp. Raats were obtained in 5 days of fermentation time under neutral conditions (pH 7.0) (Mabinya *et al*., 2012). For *Bacillus licheniforms*, maximum values were obtained for both flocculant production and cell growth during the stationary phase (Shih *et al*., 2001), while Li *et al*. (2009) and Xia *et al*. (2008) showed that for *Bacillus licheniforms* X14, and *Proteus mirabilis* TJ-1, maximum production of the bioflocculant was achieved in 24 h with flocculating activity peaking at 48 h. The relationship between growth kinetics and the concentration of a substrate is fundamental in studying the enormous inconsistencies that exist especially for pure cultures growing with single substrates. In addition, the fact that microorganisms tend to

change their kinetic properties in order to adapt to changing environment further complicates the understanding of this relationship (Kovárová-Kovar and Egli, 1998). According to Mabinya *et al*. (2012), these observations seem to underscore a fed-batch culture approach in order to fully understand the dynamics involved in a growing cell culture.

4.2.2.1. Bioflocculant Yield

From 1 L fermented broth of *Micrococcus* sp. Leo, close to 0.75 g of purified biolocculant was recovered. This recovery rate is still far less than what has been reported in the literature for some microorganisms such as *Klebsiella pneumonia* at 3 g/L (Nakata and Kurane, 1999) and *Myxobacteria nannocystics* sp. NU-2 at 14.8 g/L (Zhang *et al*. 2002) although much better than the yied of 0.26 g/L reported by Cosa *et al*. (2011) for *Virgibacillus* sp. Rob. Further studies still need to be conducted in order to establish appropriate growth conditions for the improvement of bioflocculant production by *Micrococcus* sp. Leo.

4.2.2.2. Characterization of Purified Bioflocculant

4.2.2.2.1. Composition Analysis.

Chemical analysis of the bioflocculant showed it to be predominantly carbohydrate in content with the presence of protein and uronic acid also detected. Several growth factors are known to influence the chemical nature of the bioflocculant and different microorganisms have also been reported to produce bioflocculants showing the presence of components reported for *Micrococcus* sp. Leo (Wu and Ye, 2007; Zheng *et al*., 2008).

4.2.2.2.2. Thermal Stability of the Bioflocculant.

The bioflocculant showed better stability at 50^{o}C to 60^{o}C maintaining a flocculating activity of approximately 90% which decreased to about 70% at 100^{o}C (Figure 4.17). A similar observation was noted by Ugbenyen *et al*. (2012) for the bioflocculant produced from *Cobetia* spp. which retained more than 70% flocculating activity after being heated at 100^{o}C. Bioflocculants containing sugars as the main flocculating component have been shown to be heat-stable and capable of retaining more than 50% of their activity when heated to 100^{o}C (Gao *et al*., 2006; Li *et al*., 2007; Feng and Xu, 2008), whereas those that do not have a sugar component are sensitive to temperature (He *et al*., 2004). A predominantly polysaccharide based bioflocculant produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 retained more than 90% flocculating activity after being heated at 100^{o}C for 30 min. (Wang *et al*., 2011) whereas a drastic reduction in flocculating activity above 80^{o}C was noticeable for bioflocculant REA-11 produced by *Corynebacteria glutamicum* (He *et al*., 2004). A reduction of about 50% in flocculating activity was also noted when bioflocculants produced by *Rhodococcus erythropolis* (Kurane *et al*., 1986) and *Bacillus firmus* (Salehizadeh and

Shojaosadati, 2002) respectively were subjected to boiling for 15 min. Bioflocculant PY-90 was completely inactivated after heating for 40 min at 100°C (Yokoi *et al.,* 1995). The noticeable decrease in flocculating activity of the bioflocculant produced by *Micrococcus* sp. Leo at 100°C (Fig. 4.17) can presumably be ascribed to the presence of the protein component.

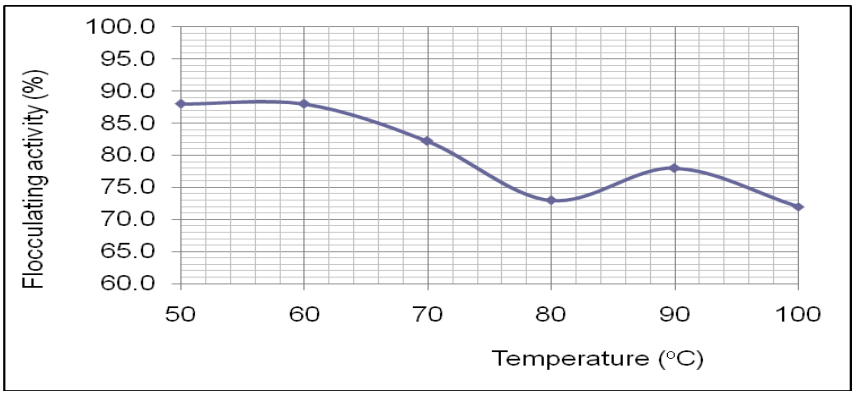

Figure 4.17: Effect of Temperature on the Flocculating Activity of the Purified Bioflocculant.

4.2.2.2.3. Thermogravimetric Analysis of Purified Bioflocculant.

The thermal properties of the purified bioflocculant were studied by TGA in the temperature range of 200°C to 400°C in the presence of nitrogen. The loss in bioflocculant weight with concomitant increase in temperature is shown in Figure 4.18 and is an indication of its rate of decomposition when exposed to high temperatures. At 200°C the weight dropped to about 87%, equivalent to a 13% loss which was followed by a further decrease to just above 80% of its original weight at 400°C (Figure 4.18). These bioflocculant weight losses could be attributed to changes in the moisture content with decomposition and were indicative of the probable presence of both the carboxyl and hydroxyl groups in the bioflocculant produced by *Micrococcus* sp. Leo (Kumar and Anand 1998).

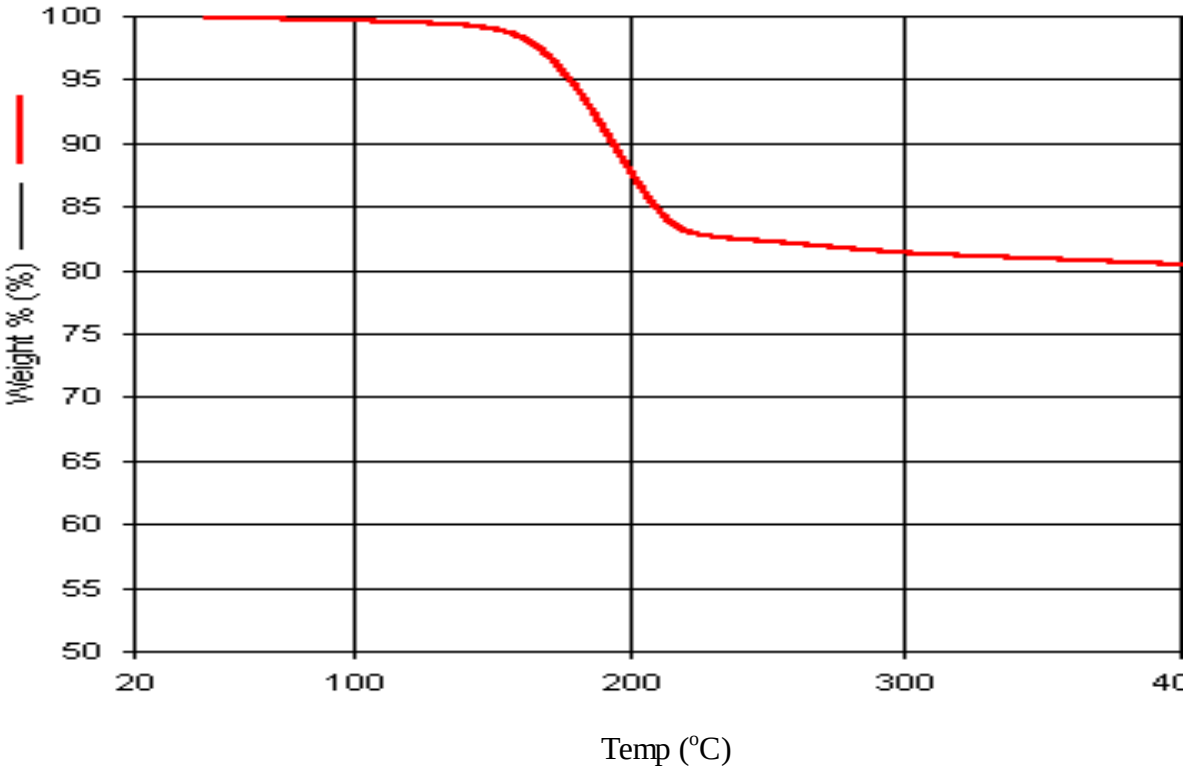

Figure 4.18. Thermogravimetric Analysis of the Bioflocculant.

4.2.2.2.4. FTIR Analysis.

Fourier Transform Infrared spectrum of the bioflocculant indicated the presence of different functional groups (Figure 4.19). The stretching peak at around 3545.12 to 3412.12 is characteristic of OH and NH_2 groups in the polymer (Desouky *et al*., 2008) while the presence of both aliphatic bonds and the C=O stretch were indicated by a weak band at 2248 cm^{-1} and the spectrum showing at 1652 cm^{-1} respectively (Li *et al*., 2008). Functional groups, as indicative of the bioflocculant chemical composition, have been reported to play an influential role in the flocculation process by different microorganisms (Salehizadeh and Shojaodasati, 2001). The sharp peak at 1622 cm^{-1} is an indication of the presence of an amide group (Fujita *et al*., 2000), and the streching band at 1014 cm^{-1} indicates the presence of carboxylic groups with the spectrum peaks

in between 1000-1100 cm^{-1} suggestive of the presence of saccharide derivatives (Deng *et al*., 2005). The vibration peaks in between 757-839 cm^{-1} suggest the presence of furan saccharides (Wang *et al*., 2011) and in addition, the spectra peaks from 472-670 cm^{-1} represent the presence of benzene ring-structured compound in the bioflocculant molecule.

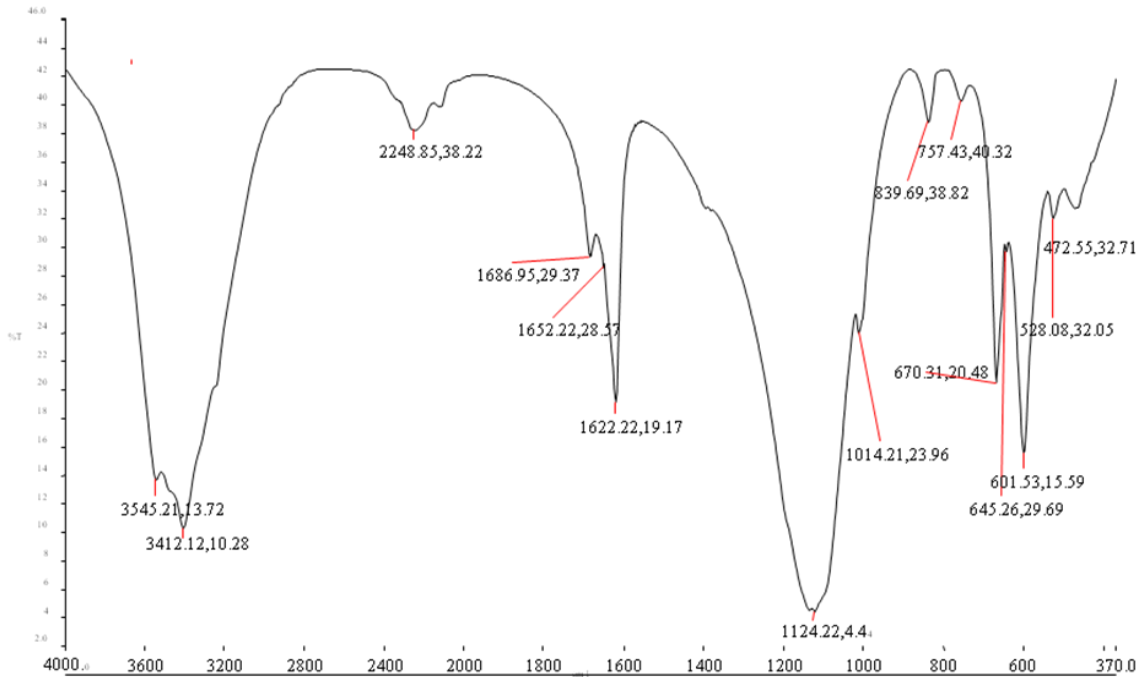

Figure 4.19. Fourier Transform Infrared Spectroscopy Analyses of Purified Bioflocculant.

4.2.2.2.5. *SEM.*

Morphological elucidation of the purified bioflocculant and Kaolin clay before and after flocculation is represented in Figure 4.20. A marked difference in the morphology of the three samples was noticeable with (A) representative of the amorphous meshy clump like structure of the purified bioflocculant. The scattered Kaolin particles are shown in (B) with (C) showing how the fluffy like Kaolin-bioflocculant complex is connected together

into aggregates to form large flocs for efficient and rapid sedimentation. Nwodo *et al*. (2012) reported a similar clump like structure of a partially purified bioflocculant produced by *Streptomyces* sp. Gansen while the bioflocculant produced by *Proteus mirabilis* TJ-1 displayed a crystal-linear structure (Xia *et al*. 2008).

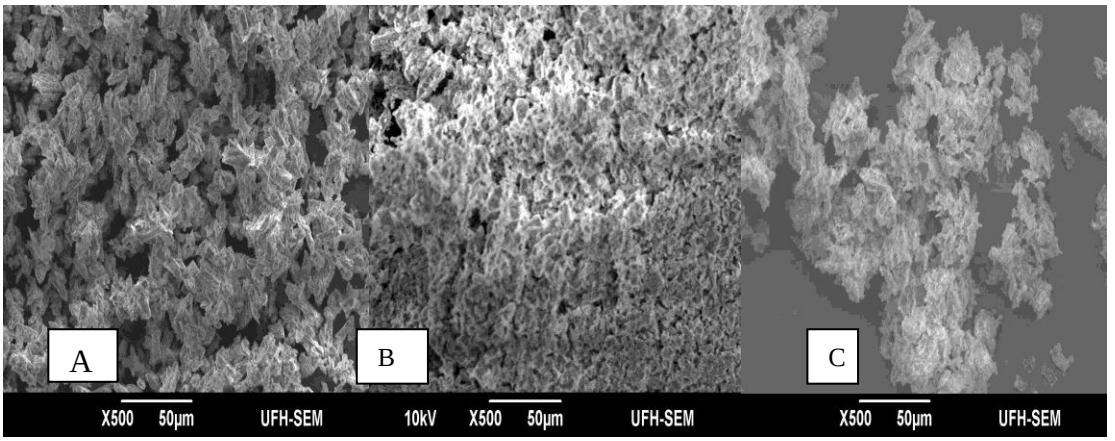

Figure 4.20. Bioflocculant powder A, Kaolin clay B, bioflocculant and Kaolin after flocculation C.

4.2.3. Effect of Bioflocculant Dosage

The bioflocculant produced by *Micrococcus* sp. Leo had a comparatively low dosage requirement of 0.2 mg/mL for maximum flocculation (63%) of Kaolin suspension (Figure 4.21). Beyond 0.2 mg/mL, a steady decrease in flocculating activity with increasing bioflocculant dosage was observed but a sudden spike in flocculating activity at 0.7 mg/mL could not be accounted for (Figure 4.21). Bioflocculants produced by different microorganisms require varying dosages for effective flocculating rate with the majority of bioflocculants reported in the literature having dosage requirements that are

comparatively higher than the bioflocculant produced by *Micrococcus* sp. Leo. Yim *et al*. (2007) and Salehizadeh and Shojaosadati, (2002) obtained the highest flocculating activity with a bioflocculant concentration 1 mg/ml while most bioflocculants show best flocculating activity within concentration range of 10-50 mg/ml (Zhang *et al*. 2002; Gao *et al*. 2006; Wu and Ye, 2007). Feng and Xu, (2008), suggested that the observed decrease in flocculating activity with increasing bioflocculant concentration may be the result of saturation by concentrated bioflocculant solution thus blocking the binding sites of Kaolin particles leading to weakened bridge formation.

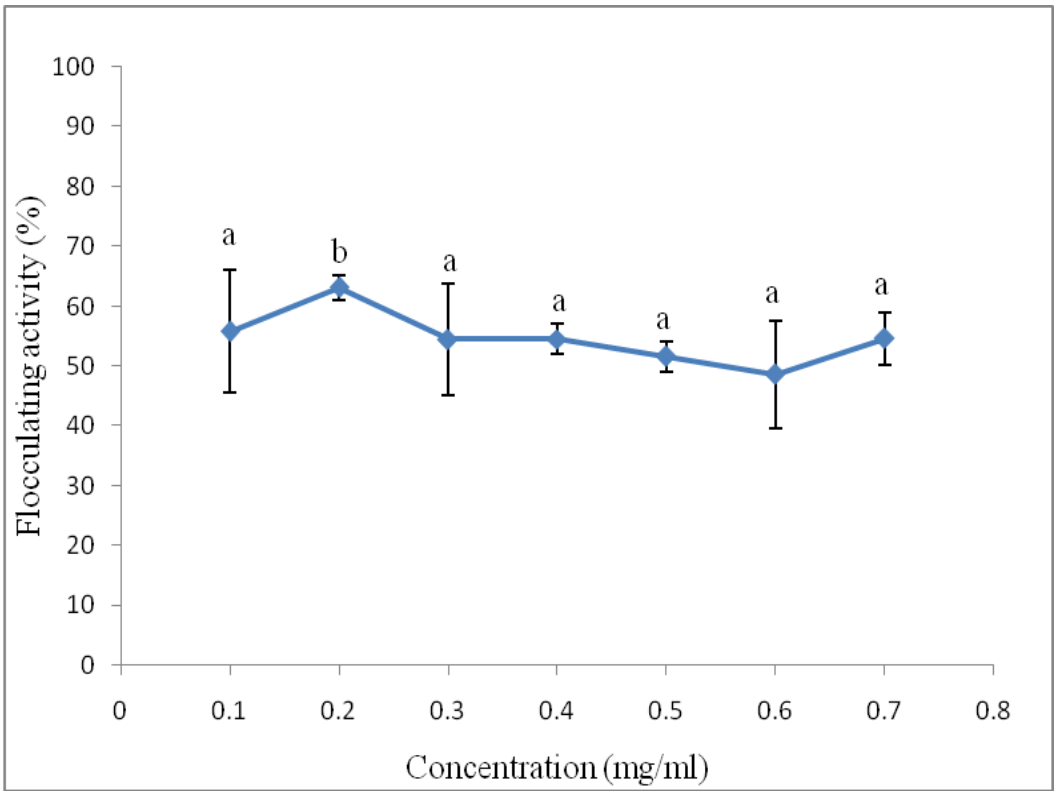

Figure 4.21. Effect of Bioflocculant Concentration on the Flocculating activity of Purified Bioflocculant. Flocculating activities with different letters are significantly different (P < 0.05) from each other.

4.3. Bioflocculant Production by *Arthrobacter* sp. Raats

The test bacteria was one of several isolated from Tyume River in the Eastern Cape Province of South Africa as party of the bioflocculant producing bacteria culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa. The nucleotide sequence of the *16S rRNA* gene of the bacteria revealed a 91% similarity to that of *Arthrobacter* sp. 5J12A, and the nucleotide sequences was deposited in GenBank as *Arthrobacter* sp. Raats with accession number HQ875723.

Arthrobacter is a common genus of soil bacteria with all species being generally non-sporulating, Gram-positive and obligate aerobes that exhibit pure respiratory metabolism with the exception of at least two species, *Arthrobacter globiforms* and *Arthrobacter nicotianae* which exhibit anaerobic metabolism (Eschbach *et al*., 2003). Due to their metabolic diversity, *Arthrobacter* species have been used in industrial applications in the bioremediation of contaminated water (Eschbach *et al*., 2003). The usefulness of *Arthrobacter globiformis* for bioremediation of industrial wastes rich in nitrates was demonstrated by its ability to remove all nitrate from solutions containing from 100 to 150 mM NO_3^- **(**Piñar and Ramos, 1998), while Shen *et al*. (2009) reported on an *Arthrobacter nitroguajacolicus* strain with the capability to transform acrylonitrile into acrylic acid. Su *et al*. (2011) also studied the flocculability of the *Arthrobacter* sp. and demonstrated *Arthrobacter albidus* LC13T to be an effective flocculant-producing strain with high flocculating efficiency obtained for the produced bioflocculant LC13-SF.

4.3.1. Effect of Culture Conditions on Bioflocculant Production.

Factors such as the constituents of the production medium and culture conditions have an influence on bioflocculant production (He *et al*., 2004). Different key factors such as inoculum size, carbon source, nitrogen source, agitation rate, metal ions and the pH were evaluated for their effects on the flocculating activity of the bioflocculant produced by the test bacteria.

4.3.1.1. Effect of Carbon and Nitrogen Sources.

The effects of lactose, sucrose, fructose and starch on flocculation were assessed and the results are as shown in Table 4.2. Lactose and sucrose were favorable carbon

sources with flocculating activities of 75.4% and 73.4% being attained respectively, while fructose and starch did not support any flocculating activities (Table 4.2). With respect to sucrose, these results seem to support the findings reported by Li *et al*. (2009a) for bioflocculant production by *Bacillus licheniformis* X14. However, contrary to the present study, starch was found to enhance the production of the bioflocculant by *Bacillus licheniformis* X14 while lactose was not favored at all (Li *et al*., 2009a). Studies carried out by Patil *et al*. (2010) and He *et al*. (2009) on bioflocculant production by *Azotobacter indicus* and *Halomonas* sp. V3a' respectively, also showed a better bioflocculant recovery rate with sucrose as a carbon source. Starch has also been reported as a poor carbon source for bioflocculant production by *Bacillus* sp. Gilbert (Piyo *et al*., 2011) and *Virgibacillus* sp. Rob (Cosa *et al*., 2011) respectively with fructose also shown not to be a favorable carbon source among sugars tested for bioflocculant production by *Enterobacter* sp. BY-29 (Yokoi *et al*., 2001).

The effect of nitrogen sources on bioflocculant production was investigated by cultivating the strain in the same medium, except that the nitrogen source was varied (Table 4.2). *Arthrobacter* sp. Raats was able to utilize both organic (urea) and inorganic (ammonium sulphate) nitrogen sources with urea being more effectively used and resulting in the highest flocculating activity of 83.4%, followed by ammonium sulphate at 79% and ammonium chloride at 61% (Table 4.2). Patil *et al*. (2010) assessed the influence of nitrogen sources on bioflocculant production by *Azotobacter indicus* and showed that in addition to yeast extract, both urea and ammonium sulphate were also effective in promoting flocculation. On the other hand, ammonium chloride has been reported to be an effective nitrogen source for bioflocculant production by *Halomonas*

sp. V3a' and AAD6 respectively (He *et al*., 2009; Sam *et al*., 2011). Nevertheless, contrary to the present studies with *Arthrobacter* sp. Raats, both yeast extract and peptone were found to be key parameters influencing optimal production of the bioflocculant produced by *Arthrobacter albidus* LC13^{T} (Su *et al*., 2011). A number of strains have also been reported to optimally produce bioflocculants in the presence of organic nitrogen sources (or sometimes combined organic and inorganic sources) (Gong *et al*., 2008; Xia *et al*., 2008).

Table 4.2. Effect of Constituents of Culture Medium on the Production and Flocculating Activity of the Bioflocculant Produced by *Arthrobacter* sp. Raats.

Carbon Source	**Lactose**	**Sucrose**	**Fructose**	**Starch**
Flocculating Activity %	75.4	73.4	2.8	2.1
Nitrogen Source	**Ammonium Chloride**	**Ammonium Sulphate**	**Urea**	
Flocculating Activity %	60.9	78.5	83.4	
Cations	**Ferric Chloride**	**Magnesium Chloride**	**Ferrous Sulphate**	**Potassium Chloride**
Flocculating Activity %	-	76.9	-	2.8

Note: - denotes no flocculating activity detected.

4.3.1.2. Effect of Cations on Bioflocculant Production.

The production of bioflocculant is influenced by the presence of cations in the culture medium (Salehizadeh and Shojaosadati, 2001). The effects of various cations on flocculating activity were evaluated using solutions of $FeCl_3$, $MgCl_2$, $FeSO_4 \cdot 7H_2O$ and KCl as sources of cations. As can be seen in Table 4.2, the addition of a divalent cation, Mg^{2+} enhanced flocculation when compared to the monovalent cation, K^+. Both Fe_3^+ and Fe_2^+ had no effect on flocculation. Increased flocculation in the presence of Mg^{2+} was also reported for *Virgibacillus* sp. Rob (Cosa *et al*., 2011). In contrast, for bioflocculant LC13-SF produced by *Arthrobacter albidus* $LC13^T$, maximum flocculating efficiency required the presence of Ca^{2+} (Su *et al*., 2011). A similar requirement was also reported for optimal flocculation by bioflocculant-producing *Vagococcus* sp. W31 and *Halomonas* sp. V3a' (Gao *et al*., 2006; He *et al*., 2010). For *Serratia ficaria*-produced bioflocculant, addition of both divalent cations, Ca^{2+} and Mg^{2+} enhanced the flocculating activity whereas the co-presence of trivalent cations Al^{3+} and Fe^{3+} negatively affected the flocculating activity (Gong *et al*., 2008). Li *et al*. (2007) reported a requirement for the co-presence of both monovalent and divalent cations for enhancing flocculating efficiency by *Aeromonas* sp. Cations were often added to achieve high flocculating activity by neutralizing negatively charged functional groups of both the bioflocculant molecules and the suspended particles (Salehizadeh and Shojaosadati, 2001; Li *et al*., 2008) and consequently weaken the static repulsive force thus enhancing the flocculating effect (Zheng *et al*., 2008). The influence of these cations on flocculating rates varies among bioflocculant-producing microorganisms.

4.3.1.3. Effect of pH on Bioflocculant Production.

The pH of the solution is also a key factor in flocculation and thus effectively influences the flocculation process (Yokoi *et al*., 1996). The effect of initial pH of the medium on flocculating activity by *Arthrobacter* sp. Raats was investigated at pH values ranging from 3 to 11, and the flocculating activity was found to be distinctly higher (84%) in neutral pH conditions (pH 7.0) than in acidic and alkaline conditions (Figure 4.22). Similar pH values (pH 7.2 and 7.0) were reported for optimum activity of bioflocculants ERSS-31 produced by *Halomonas maura* sp. nov. (Bouchotroch *et al*., 2001) and MBFW31 produced by *Vagococcus* sp. W31 (Gao *et al*., 2006) respectively. Bioflocculant HBF-3, produced by a mutant *Halomonas* sp. V3a' also attained the highest flocculating activity at pH 7 (He *et al*., 2010). At low pH, the absorption of H^+ ions tends to weaken the bioflocculant-Kaolin complex formation process and a similar effect is also observed at high pH values due to OH^- ions (He *et al*., 2010). According to Li *et al*. (2008), the mediating effect of cations appears to be strongest at neutral pH values. However, these observations are different from studies carried out by Choi *et al*. (1998) and Zheng *et al*. (2008) in which the maximum flocculating activities of bioflocculants produced by *Anabaena* sp. and *Bacillus* sp. F19 respectively were observed at pH 2. The bioflocculant produced by *Bacillus* sp. PY-90 was found to be actively high in an acidic pH range of 3.0 to 5.0 (Yokoi *et al*., 1995), while *Serratia ficaria* produced a bioflocculant effective over a weakly acidic pH range of 5.0 to 7.0 (Gong *et al*., 2008).

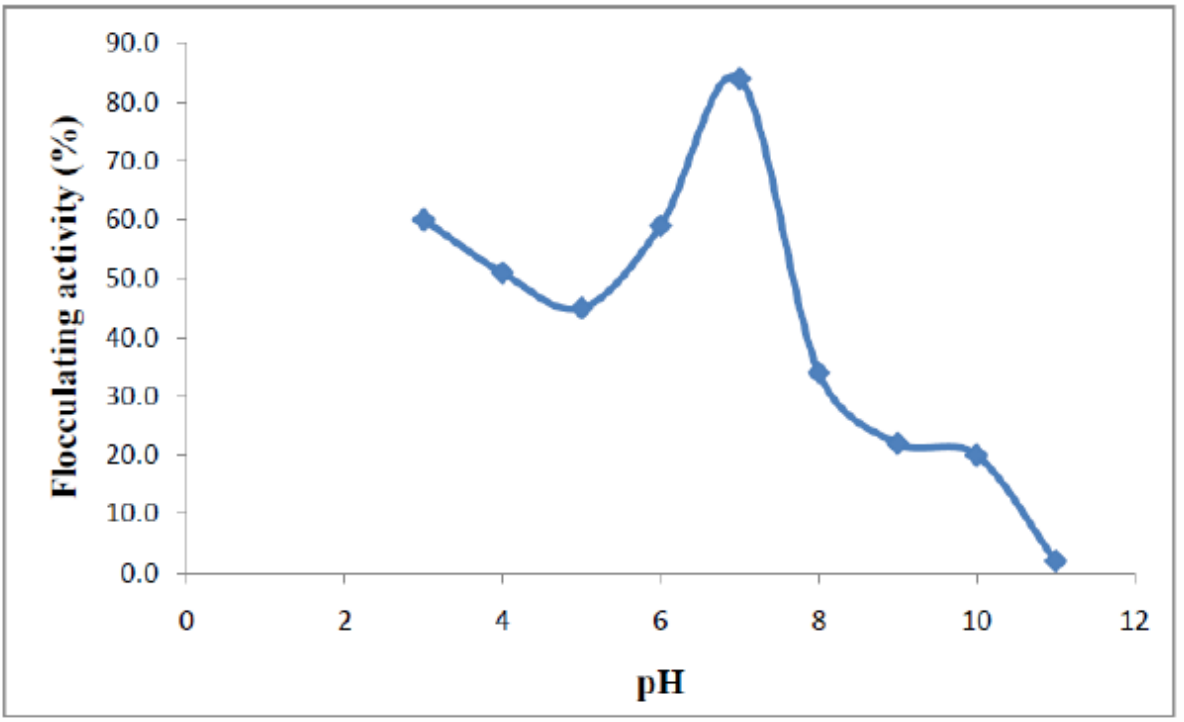

Figure 4.22. Effect of Initial pH on Bioflocculant Production.

4.3.2. Time Course Assay of Bioflocculant Production.

Figure 4.23 shows the time course of bioflocculant production by *Arthrobacter* sp. Raats in relation to optical density, pH and flocculating activity over a 10 days cultivation period. During growth, the flocculating activity was observed to increase reaching a maximum of 87.5% in 5 days beyond which a rapid decline was noted with a complete loss of activity over the last 4 days of growth (Figure 4.23). The observed decrease in flocculating activity might be due to the activity of deflocculation enzymes in the late phase of cell growth (Gong *et al*., 2008; Choi *et al*., 1998). According to a study by Jang *et al*. (2001) on bioflocculant production by fed-batch culture of *Citrobacter* sp., specific flocculating activity and bioflocculant production was influenced by fermentation conditions which included among other things, carbon source, inoculum size, dissolved oxygen tension (DOT) and carbon/nitrogen (C/N) source ratio. Jang *et al*. (2001) also noted that the

initial growth rate increased with an increase in carbon source concentration. However, carbon source consumption was severely limited at higher carbon source concentrations. Inoculum size has also been reported to affect not only cell growth but also product formation with the dynamic behavior of cells of *Citrobacter* sp. cell cultures at different inoculum sizes affecting bioflocculant activity (Jang *et al*. 2001; Kallos and Behie, 1999). The synthesis of bioflocculant and the flocculating activity were also shown to be significantly affected by the C/N ratio of the growth medium with a higher C/N ratio not necessarily translating to a higher yield of flocculant synthesis (Jang *et al*. 2001). The influence of the initial pH of fermentation medium can determine both the electric charge of the cells and the oxidation-reduction potential which, in turn, can affect nutrient absorption and enzymatic reaction (Su *et al*., 2011). Thus the corresponding steady increase in cell growth (OD_{600}) in relation to flocculating activity observed over the first 5 day growth period is possibly an indication that the bioflocculant was produced by biosynthesis during growth of the bacteria and not by cell autolysis (Gao *et al*., 2006). From 72 to 120 h, the pH increased steadily until optimal flocculating activity was reached at pH 7.0. After 6 days of cultivation, a complete loss of flocculating activity was observed while the pH increased sharply and remained around pH 8.2 (Figure 4.23). The correlation between bioflocculant production and culturing times may differ among different organisms. Maximum production levels and a flocculating peak of 90.6% for a bioflocculant produced by *Arthrobacter albidus* $LC13^T$ were obtained at 72 h of fermentation time under neutral conditions (pH 7.0) (Su *et al*., 2011). For *Bacillus licheniforms*, maximum values were obtained for both the flocculant production and cell growth during the stationary phase (Shih *et al*., 2001). Li *et al*. (2009a) and Xia *et al*. (2008) showed that for *Bacillus licheniforms* X14, and *Proteus mirabilis* TJ-1, maximum

production of the bioflocculant was achieved in less time than required for other strains (24 h) while the flocculating activity peaked during the early stationary phase (48 h). The observed fluctuations in the pH of the medium during bacterial growth may also be attributed, according to Lors *et al*. (2009), to two opposite phenomena: a decrease of pH due to the bacterial activity and an increase of pH due to the release of hydroxide ions associated to the cations that are leached out. Taken together, these observations seem to strongly support a fed-batch culture approach in order to fully understand the dynamics involved in a growing cell culture. The relationship between growth kinetics and the concentration of a substrate is fundamental in studying the enormous inconsistencies that exist especially for pure cultures growing with single substrates. Confounding the problem further is the fact that microorganisms tend to change their kinetic properties in order to adapt to changing environment (Kovárová-Kovar and Egli, 1998).

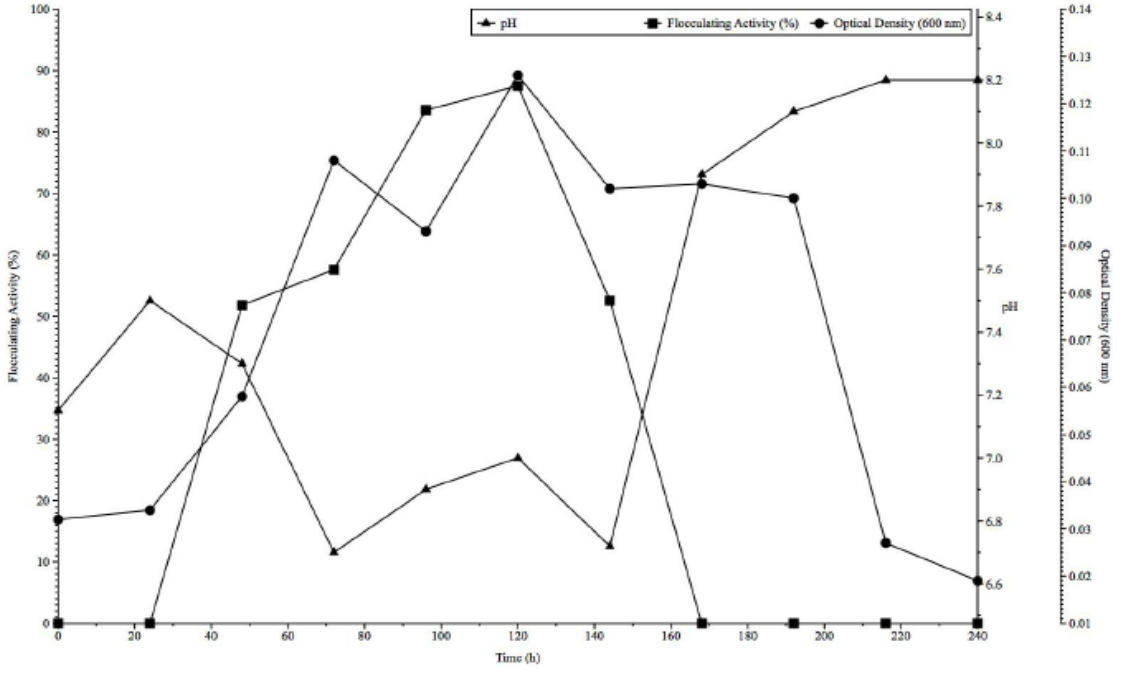

Figure 4.23. Time Course Assay of Bioflocculant Production.

4.3.3. Characterization of Purified Bioflocculant

4.3.3.1. Composition Analyses of the Bioflocculant

An aliquoted suspension (1 mg/mL) of the purified bioflocculant was found to contain 0.25 mg/mL total carbohydrate and 0.56 mg/mL protein respectively, thus confirming that the biofluccculant consisted primarily of a glycoprotein. Inoue *et al*. (1982) investigated a biopolymer produced by *Arthrobacter* sp. and revealed it to be a heterogenous polysaccharide which was principally a galactan sulfate. A number of other organisms have been reported to produce different kinds of glycoprotein bioflocculants (Feng and Xu, 2008; Zhang *et al*., 2007).

4.4. Bioflocculant Production by a Consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo

Microbial products termed bioflocculants have been found to mediate flocculation and no known harmful effects has been ascribed to these natural flocculants; as they are environmentally friendly thus, biodegradable (Nwodo *et al*., 2012; Mabinya *et al*., 2012). The growing interest in bacterial products from marine environments, particularly extreme marine environments, has led to the isolation of products with antiviral, pharmacological and immunomodulatory applications (Maugeri *et al*., 2002; Poli *et al*., 2010). The exploration for bioflocculant producing bacteria has included search in extreme habitats like the marine environment (Piyo *et al*., 2011). Nonetheless, high production cost has been a limitation to industrial scale production and application of bioflocculants. Consequently, we investigated the consortia of two marine bacteria for bioflocculant

production and media optimization through response surface methodology (RSM). RSM is a well known statistical modelling technique utilized to optimize fermentation processes through multiple regression analysis using quantitative data obtained from properly designed methods to solve multivariables that are critical for the optimum production of biomolecules (Bajaj *et al*., 2009; He *et al*., 2009; Liu *et al*., 2010). Plackett-Burman design was developed in order to screen growth medium components with respect to their main effects and its main advantage is that it requires fewer experimental trials compared to time-consuming and tedious conventional single factor methods (He *et al*., 2009; Liu *et al*., 2010). Optimization of physicochemical parameters and measurement of flocculation activity (Table 4.3) was as seen in Nwodo *et al*. (2012).

4.4.1. Media Optimization.

4.4.1.1. Plackett-Burman (PB) Design.

Five independent variables (media components); fructose, $MgCl_2$, $(NH_4)_2SO_4$, K_2HPO_4 and KH_2PO_4 were investigated at two levels of “high” and “low” (concentrations) each which were designated as + 1 and – 1 respectively (Table 4.4).

4.4.1.2. Central Composite Design (CCD)

Media components with significant input in bioflocculant production, as identified by PB design, were optimized through the response surface design (RSD). Thus, a central composite design (CCD) model was generated and this model was applied to the independent variables; fructose, $MgCl_2$ and $(NH_4)_2SO_4$ using 3-factor-5-level CCD

(Table 4.5). The correlation of response variable (flocculation activity) to the independent variables, showed flocculation activity to fit into second order polynomial model as shown under Materials and Methods.

Table 4.3. The Effects of Carbon, Nitrogen and Cation Sources on Bioflocculant Production.

Carbon source	**Glucose**	**Lactose**	**Fructose**	**Sucrose**	**Maltose**	**Starch**
Max. Flocculation activity (%)	81	63	87	54	32	41
Bioflocculant yield (g/L)	4.03 ± 0.64	3.62 ± 0.77	4.6 ± 0.41	3.2 ± 0.9	2.4 ± 0.62	2.6 ± 0.49
Nitrogen source	Urea	$(NH4)_2SO_4$	$(NH4)_2NO_3$	$(NH_4)_2Cl_4$	Peptone	
Max. Flocculation activity (%)	71	88	62	47	79	
Bioflocculant yield (g/L)	2.94 ± 0.09	4.07 ± 0.33	3.37 ± 0.74	2.26 ± 0.55	3.88 ± 0.42	
Cation source	KCl	NaCl	$MgCl_2$	$CaSO_4.H_2O$	$MnCl.4H_2O$	$FeSO_4$
Max. Flocculation activity (%)	48	31	74	55	58	21
Bioflocculant yield (g/L)	2.0 ± 0.05	1.74 ± .041	2.98 ± 0.72	1.88 ± 0.81	2.94 ± 0.07	1.44 ± 0.06

Table 4.4. Plackett Burman Design Showing Coded Levels (concentrations) and Runs for Five Media Components.

Runs	Coded levels/concentrations (g/L)					Flocculation activity (%)	
	Fructose	$(NH4)_2SO_4$	$MgCl_2$	K_2HPO_4	KH_2PO_4	Observed	Predicted
1	-1 (10)	+1(1.5)	+1(0.5)	+1(6.5)	-1(2)	74.0	74.0
2	+1(12.5)	-1(1)	-1(0.3)	-1(5)	1(2.5)	71.0	77.0
3	-1 (10)	+1(1.5)	+1(0.5)	-1(5)	1(2.5)	58.0	79.0
4	+1(12.5)	+1(1.5)	-1(0.3)	+1(6.5)	1(2.5)	82.0	79.0
5	+1(12.5)	+1(1.5)	+1(0.5)	-1(5)	-1(2)	89.0	74.0
6	-1(10)	+1(1.5)	-1(0.3)	+1(6.5)	1(2.5)	91.0	81.0
7	+1(12.5)	-1(1)	+1(0.5)	+1(6.5)	1(2.5)	72.0	69.0
8	-1(10)	-1(1)	+1(0.5)	-1(5)	1(2.5)	88.0	75.0
9	+1(12.5)	+1(1.5)	-1(0.3)	-1(5)	-1(2)	74.0	79.0
10	-1(10)	-1(1)	-1(0.3)	-1(5)	-1(2)	83.0	77.0
11	-1(10)	-1(1)	-1(0.3)	+1(6.5)	-1(2)	67.0	74.0
12	+1(12.5)	-1(1)	+1(0.5)	+1(6.5)	-1(2)	59.0	67.0

Table 4.5. The Matrix of Central Composite Design Showing the Five Levels of Three Critical Media Components with Observed and Predicted Values.

Runs	Critical media components (g/L)			Flocculation activity (%)	
	Fructose	$(NH_4)_2SO_4$	$MgCl_2$	Observed	Predicted
1	0(14)	0(2.0)	0(0.7)	79	83.85
2	-1(12)	-1(1.5)	-1(0.5)	84	77.56
3	0(14)	0(2.0)	0(0.7)	77	83.85
4	0(14)	0(2.0)	0(0.7)	79	83.85
5	0(14)	0(2.0)	0(0.7)	81	83.85
6	-1.73(10.64)	0(2.0)	0(0.7)	87	80.19
7	0(14)	0(2.0)	+1.73(1.03)	94	90.53
8	0(14)	-1.73(1.16)	0(0.7)	87	83.32
9	+1(16)	-1(1.5)	+1(0.9)	96	89.52
10	0(14)	0(2.0)	-1.73(0.37)	72	77.16
11	0(14)	+1.73(2.84)	0(0.7)	86	84.37
12	0(14)	0(2.0)	0(0.7)	75	83.85
13	+1(16)	-1(1.5)	-1(0.5)	79	81.79
14	-1(12)	+(2.5)	+1(0.9)	83	85.90
15	-1(12)	-1(1.5)	+1(0.9)	81	85.29
16	+1(16)	+1(2.5)	+1(0.9)	91	90.13
17	+1.73(17.36)	0(2.0)	0(0.7)	96	87.50
18	-1(12)	+1(2.5)	-1(0.5)	88	78.17
19	0(14)	0(2.0)	0(0.7)	78	83.85
20	+1(16)	+1(2.5)	-1(0.5)	84	82.40

He *et al*. (2009) and Liu *et al*. (2010) utilized RSM to optimize the media components for HBF-3 production by *Halomonas* sp. V3a' and EPS from *Paenibacillus polymyxa* EJS-3 and reported increases in production of more than 2-fold and 1.55-fold respectively.

In a study of the production of poly (γ-glutamic acid) from *Bacillus* licheniformis NCIM 2324, Bajaj *et al*. (2009) demonstrated through PB design, the significant effect of glycerol, $(NH_4)_2SO_4$, $CaCl_2$, glutamic acid, citric acid, K_2HPO_4 and $MnSO_4$ in the culture medium. Further optimization of these most significant factors by RSM resulted in almost a 5-fold increase in PGA production thus demonstrating the effectiveness of PB design followed by RSM (Bajaj et al., 2009). Likewise Wang and Wan, (2008) proved RSM to be a more efficient method for the optimization of fermentative hydrogen production by mixed sludge cultures.

CONCLUSIONS

This study assessed bioflocculant production by axenic and consortia cultures of three bacterial isolates belonging to the *Arthrobacter*, *Halomonas* and *Micrococcus* genera. Detailed characterizations of the purified bioflocculants were also carried out.

The polysaccharide bioflocculant produced by *Halomonas* sp. Okoh showed good flocculating activity for Kaolin suspension with an optimal flocculating activity above 80%. Glucose, urea and $CaCl_2$ were carbon, nitrogen and cation sources yielding optimal flocculation at a dosage of 0.2 mg/mL at pH 7. A yield of 1.2 g/L of the thermostable bioflocculant was recovered after purification. FTIR spectrometry of the bioflocculant indicated the presence of carboxyl, hydroxyl and amino groups, typical for heteropolysaccharides, while SEM imaging revealed a lattice like structure. The bioflocculant from *Halomonas* sp. Okoh showed promising suitability for future industrial applicability. *Micrococcus* sp. Leo produced a bioflocculant with a predominant polysaccharide backbone containing traces of protein and uronic acids. Sucrose, in the presence of peptone as an organic nitrogen source and the divalent cation (Mg^{2+})

supported optimal bioflocculant production at a cost-effective dosage of 0.2 mg/mL at pH 6.3. The pure bioflocculant exhibited strong thermal stability at 60^{o}C with 70% flocculating activity retained at 100^{o}C. FTIR showed the presence of carboxyl, amino and hydroxyl groups known to be critical in the flocculation process. Optimization of culture conditions may enhance flocculation performance of the bioflocculant from *Micrococcus* sp. Leo and thus increasing its prospects as a viable candidate for future industrial applications. The bioflocculant produced by *Arthrobacter* sp. Raats showed good flocculating activity for Kaolin suspension. Lactose and urea were preferred as sole carbon and nitrogen sources for bioflocculant production by the bacteria. The divalent cation (Mg^{2+}) as well as initial medium pH 7.0 resulted in optimal production of bioflocculant, and chemical analyses indicated the bioflocculant to be a glycoprotein made up of about 56% protein and 25% total carbohydrate.

The application of Plackett Burman design for the consortia showed carbon, nitrogen and cation sources to be the only significant ($\alpha \leq 0.05$) variables responsible for bioflocculant production and further enhancement of media concentration by central composite design showed an improved bioflocculant yield. Optimal concentrations of the critical nutritional source were 16.14 g/l, 1.55 g/l and 1.88 g/l for fructose, ammonium sulphate and $MgCl_2$ respectively with an optimal bioflocculation activity of 94% and bioflocculant yield of 6.43g/L. The efficiency of the nutrient optimization procedure suggests suitability for industrial application of the bioflocculant which is a subject of future investigation in our group. The genera of *Halomonas*, *Micrococcus* and *Arthrobacter* might play a future role in mixed culture production of bioflocculants.

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