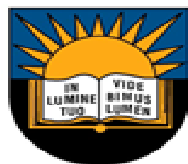

ASSESSMENT OF BIOFLOCCULANT PRODUCTION BY TWO MARINE BACTERIA ISOLATED FROM THE BOTTOM SEDIMENT OF MARINE ALGOA BAY

By

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DECLARATION

I, the undersigned, declared that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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ABSTRACT

Bioflocculants are polymers, mostly, of microbial origin which floc out suspended particles from liquid medium. The ability of these biopolymers to remove suspended particles from solutions is termed bioflocculation, and the efficiency of flocculation activities depends on the characteristics of the flocculants. In comparison with conventionally used flocculants, bioflocculants have the advantage of being safe (no toxic effects known), biodegradable and harmlessness to the environment. The study assessed production of bioflocculant by two marine bacteria from the bottom sediment of marine environment. The 16S rDNA was used for identification, and the two bacteria species were identified as *Enterococcus hirae* and *Bacillus thuringiensis*. Factors affecting the production and activity of the bioflocculants produced by these two organisms were studied. The bacteria optimally produced bioflocculant with fructose (91.7%) and urea (91%) as sole carbon and nitrogen sources respectively. Mg^{2+} (87%) and Ca^{2+} (86%), likewise, served as best cation sources on the production of the bioflocculant at pH 5(93%). Additionally, the flocculating activity of the bioflocculant increased with the addition of Mg^{2+} (81%) and Na^{+} (81%), and the highest flocculating activity was at pH 5 of the kaolin clay. The Fourier transform infrared spectroscopy (FTIR) shows that the bioflocculant is a glycoprotein. The second bacterium (*Bacillus thuringiensis)* produced bioflocculant optimally when the media had mixed nitrogen sources (Urea, ammonium chloride and tryptone (67%)) and glucose (85.65%) as a sole carbon source, also Ca^{2+} (74.6%) was the best cation that induced the production of bioflocculant. After purification, the bioflocculant flocculated optimally in alkaline pH 12 (81%) in the presence of Mn^{2+} (73%) and Ca^{2+} (72.8%). Chemical analysis of the bioflocculant revealed it to be a polysaccharide. Both bioflocculants flocculate efficiently and can be used to replace synthetic flocculants in water treatment, wastewater, in downstream processing, and processing of food and chemicals and other industrial uses of flocculants.

Challenges though (i) are to develop conditions for large scale production of the bioflocculant, (ii) to do further characterization of the both bioflocculants (iii) to assess the bioflocculants for treatments of water/wastewater, and to apply it in various industrial processes.

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CHAPTER ONE

1.1 BACKROUND

Bioflocculants are polymers, mostly, of microbial origin which floc out suspended particles from liquid medium (Xia *et al*., 2008; Ugbenyen *et al*., 2012; Nwodo *et al*., 2012). The ability of these biopolymers to remove suspended particles from solutions is termed *bioflocculation* (Xia *et al*., 2008; Ugbenyen *et al*., 2012; Nwodo *et al*., 2012), and the efficiency of flocculation activities depends on the characteristics of the flocculants. In comparison with conventionally used flocculants, bioflocculants have the advantage of being safe (no toxic effects known), biodegradable and harmless to the environment. Hence, it has been topical as an alternative to conventional flocculants (Salehizadeh and Shojaosadati, 2001; Nwodo *et al*., 2013). Depending on specific environmental conditions, microorganisms produce exopolysaccharides (EPSs) of particular composition and physiochemical properties, and this promotes the survival of microbial populations. Microbes synthesize three types of polysaccharides defined by their cellular location, that is, 1) the intracellular polysaccharides, known as the storage form of carbon, 2) the cell wall polysaccharides such as peptidoglycan and teichoic acids, and 3) the extracellular forms, including the capsule associated with the cell surface and the EPSs secreted in the growth medium (Abdel-Aziz *et al.*, 2012). The capsular structure may protect the cell against unfavourable environmental conditions like oxygen tension, toxic compounds, temperature or high osmotic pressure, and may contribute to the uptake of metal ions (Cerning, 1990). Microbes from the taxa of bacteria, fungi and actinobacteria have been reported to produce extracellular polymeric substances in a form of or combination of saccharides, proteins, nucleic acid and glycoprotein which mediated flocculation effectively. Hence, bioflocculants have been shown to be composed of these biomolecules.

Microbial extracellular polymeric substances have found application in the detergents, adhesives, cosmetics pharmaceutical and dairy industries (Nwodo *et al*., 2013). However, its application as bioflocculants, the textiles and municipal water treatments is highly prospected (Liu *et al*., 2011). Other potential applications of microbial extracellular polymeric substances, worthy of note, include oil recovery, dredging and are found in different downstream processes (Sutherland, 2002; Nwodo *et al*., 2013). The myriad of constituent units of bioflocculants, including uronic acids, have widened the scope of bioflocculant application. Nonetheless, the surface properties of bioflocculants are suggested as being responsible for the numerous applications found in these biomaterials (Lu *et al*., 2005; Wu and Ye, 2007; Dignac *et al*., 1998; Nwodo *et al*., 2012).

Bacteria utilizes available nutrients in the culture media to synthesize high molecular weight polymers under the action of specific enzymes, and these polymers can be excreted into the external environment which may serve varied purpose. Therefore, it is crucial to optimize culture conditions when aiming to manipulate bacterial cells to synthesize these high molecular weight polymers, especially for industrial application and enhanced product-yield (Liu and Cheng, 2010). In this study, marine sediments were screened for bacteria with bioflocculant producing capabilities, and from the numerous positive isolates, two bacteria species with higher bioflocculant production potential were selected for culture conditions optimization and bioflocculant characterization for novelty.

1.2 AIM

This research aimed at assessing bioflocculant production potentials and characterization of the produced bioflocculant by two marine bacterial species isolated from marine sediments belonging to the genera of *Bacillus* and *Enterococcus*.

1.3 OBJECTIVES

The following sets of research objectives were anticipated to achieve the research aim:

1.3.1 To screen the culturable marine bacterial strains isolated from the marine sediments for bioflocculant production and select two of the positive isolates for detailed studies;

1.3.2 To assess the effect of culture conditions on bioflocculant production by the selected bacterial species;

1.3.3 To purify and characterize the bioflocculant produced by the two selected bacterial strains; and

1.3.4 To identify the bacteria by 16S rDNA gene sequence analysis.

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CHAPTER TWO

2 LITERATURE REVIEW

2.1 Bioflocculants Overview

Flocculation, as a process, removes colloidal particles from a solution. However, bioflocculation would imply the removal of suspension by microbial derived flocculants. Flocculation has been widely applied to different industrial processes, including waste-water treatment, drinking water purification, and the downstream process in fermentation processes (Salehizadeh and Shojaosadati, 2001). Flocculants are conventionally classified into three groups or categories based on their origin and or composition. These flocculants are: inorganic flocculants such as aluminium salts (that is, aluminium sulphate); organic synthetic flocculants such as polyacrylamide derivatives; and biologically or naturally occurring flocculants such as chitosan and microbial flocculants. Chemically derived flocculants, (organic and inorganic) besides their high flocculating activity, are easily available and are characterized by low cost; they are, however, associated with toxicity and diseases. Aluminium salts are, likewise, associated with Alzheimer's disease; at the same time, polyacrylamide is not only neurotoxin and carcinogenic, but is also non-degradable in the environment (Salehizadeh and Shojaosadati, 2001; Zhang *et al.,* 2007). Due to these limitations and the continual emphasis on environmental protection, amongst other factors, there is a need for the continual exploration of different microbial habitat for novel microbial flocculants with enhanced flocculation activity, high stability, and innocuous (Qiang *et al.,* 2010). Although bioflocculants may be produced in high quantity, however, the shortcomings to the production of bioflocculants for large-scale applications include low flocculating activity and high production costs (Salehizadeh and Shojaosadati, 2001; Zhang *et al.,* 2007).

The aforementioned shortcomings buttress for the continual search for microbial species with the capacity to produce bioflocculants with enhanced flocculation activity at a cost- effective level. Extremophiles have been regarded to be very beneficial in biotechnology, and various organisms, mostly microbes, have been isolated, characterized and proven to be a source of commercially important biotechnological products such as new pharmaceuticals and enzymes. Due to the extreme nature of marine environment, this has been the source of these commercial value molecules. Following the peculiar nature of the marine environment, it is important to explore it for biotechnologically beneficial microbes such as bioflocculant producers. As mentioned above, the shortcomings of bioflocculants-production (such as cost and yield) to overcome them it is useful to pursue such industrial goals in parallel with fundamental research

Microbes, bacteria, fungi and actinobacteria have been reported to produce extracellular polymeric substances composed of polysaccharides, proteins, nucleic acid and glycoprotein which mediate flocculation effectively. Bioflocculants have, thus, been shown to be composed of these biomolecules (Salehizadeh and Shojaosadati, 2001; Ugbenyen and Okoh, 2013;).

2.2 Prospective application of bioflocculants

In water treatment, aluminium salts, especially polyaluminium chloride, have been widely used due to advantages such as high efficiency of flocculation. However, disadvantages such as highly sensitivity to pH, inefficiency towards very fine particles, neurotoxicity, carcinogenicity and Alzheimer's disease indicate that an alternative should be sought (Wu *et al.,* 2012; Zhang *et al.,* 2013). To overcome or avoid the already-mentioned problems, scientists have looked for alternative flocculants with advantages such as biodegradability,

inherently benign nature, wide availability and safety to human beings, namely bioflocculants. The performance efficiency of bioflocculants produced is evaluated using measurements such as the ability to remove suspended solids, pigments, and the chemical oxygen demand (COD) (Salehizadeh and Shojaosadati, 2001). Due to these advantages and characteristics, which makes them a suitable substitute for chemical flocculants, microbial flocculants (bioflocculants) have been widely used, not only in water treatment but in various industries for downstream processing and processing of food chemicals, mining and milling operations and in petroleum refineries (Salehizadeh and Shojaosadati, 2001).

2.2.1 Prospective Application of bioflocculants

2.2.1.1 Removal of toxic materials

For purposes of public awareness and the endangerment of the environment due to human activities such as industrialisation, more attention has been focused on environmentally friendly measures to combat these problems (Wu *et al.,* 2012). Chemical contamination of water from a wide range of toxic compounds, particularly dyeing pigments from different industries including the textile industry, is problematic. Such contamination includes heavy metal ions and other toxic-suspended particles, and this remains a serious environmental problem which gives potentially serious risk to public health. Therefore, the discharge of these toxic products to the environment, especially bodies of water, should be strictly controlled. Various mechanisms have been proposed for the removal or cleaning of the effluents, and due to the above-mentioned advantages of bioflocculants, it is with certainty that they should be exploited further to remedy

2.2.1.2 Wastewater treatment

Zhu *et al*. (2010) isolated bioflocculant-producing bacterium that produced a bioflocculant named MBF4-13 from *R. philippinarum* conglutination mud, and it was added into dye

solutions, heavy metal solutions, and bulking activated sludge to determine the flocculating abilities in decolourization, removal of heavy mental ions, and improving performance of activated sludge. The bioflocculant removed methylene blue, crystal violet and malachite green with 86.11%, 97.84% and 99.49%, respectively. Zhang *et al.* (2002) and Deng *et al.* (2005) also had similar good removal results of dyes through a bioflocculant named MBF4-13 which also removed or decreased the concentrations of the heavy metals and improved water quality of the sludge. Salehizadeh & Shojaosadati, (2003) and Rawat & Rai, (2012) used bioflocculants for the removal of heavy metals produced from *Bacillus firmus,* and *Paenibacillus validus* respectively. Bioflocculants, mostly exopolysaccharides, have numerous active functional groups such as carboxylate which carry negative charges capable of binding metal ions, thus making them suitable for the removal of these metal ions (Li *et al.,* 2013)

Bioflocculants have been applied in the removal of various materials in the treatment of wastewater or purification of drinking water. Lian *et al.* (2008) tested for the removal or reduction of suspended solids, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) by a bioflocculant produced by *Bacillus mucilaginosus* in different wastewater in domestic, brewing and pharmaceutical wastewaters and found that this particular bioflocculant reduced these in all mentioned wastewaters drastically (Li *et al.,* 2009; Lian *et al.,* 2008). Zouboulis *et al.* (2004) and Li *et al*. (2013) used bioflocculants as well to reduce COD and BOD in wastewater, and results were positive up to 45% of COD at the same percent as aluminium sulphate. Although all these experiments were very successful in the treatment of wastewater by bioflocculants, there is limit because they have not been used in large scales, and have only been used in laboratory work. A need, therefore, exists to extend such use to large-scale research.

2.2.1.3 Removal of inorganic matters in oil from oil–water emulsions

Various proposed techniques have been used for the recovery of oil from dropped crude oil, oil loam and wash-down water. These techniques include gravitationally settling of the inorganic impurities or using surfactants; the mentioned techniques, however, have major disadvantages. Bioflocculants have been used to separate visible oil layers from inorganic matter (Salehizadeh and Shojaosadati, 2001; Jiang *et al.,* 2012). Similar to the previously mentioned experiments on treatment of wastewater by bioflocculants, these have not been done on a large scale.

2.2.2 Coal quality Improvement

The over-utilization of fossil fuel and petrochemicals towards provision of energy for the ever-growing industries of the world has made it inevitable for bi-products and hazardous material to threaten the very existence of man. Coal, being one of the mostly used fossil fuels in the world for generation of electricity, is one of the polluting agents as its mining releases unwanted compounds into the environment. The degree of polluting compounds released from coal mining and processing depends on the quality of coal. Coal quality does not only affect the humans, animals and the environment, but it also affects the efficiency of energy-generation. Therefore, separation of unwanted mineral matter from coal to produce clean coal of the high British thermal unit (BTU) is a major concern, and various techniques have been applied in the removal of contaminant mineral matter such as gravity concentration, flotation, coagulation, and agglomeration (Raichur and Vijayalakshmi, 2003). These techniques, however, have major disadvantages. Bioflocculation appears to be the alternative as reported by various authors such as Misra *et al.,* 1993; Raichur *et al.,* 1996; Attia *et al,*. 1993; Ohmura and Saiki, 1994; Raichur and Vijayalakshmi, 2003). These authors used *Mycobacterium*

phlei, *Thiobacillus ferrooxidans,* and *Paenibacillus polymyxa* to remove some impurities in coal such as sulphur, pyrite and ash respectively.

2.2.3 Reduction of Pathogenicity

Bioflocculants can be also be used to reduce some pathogenicity of some strains as Zhao *et al.* (2013) reported that MBF-5 bioflocculant by *Klebsiella pneumonia* was able to remove cysts amoebae of the genus *Acanthamoeba*; such cysts are responsible for the pathogenicity of the organisms.

2.2 .4 Algal biomass harvesting

The world population is increasing, and this brings about demand biomass for food, and animal feed, at the same time, increase in population has led to the demand of bio-based fuel to replace the environmental polluting fossil-fuel (Vandamme *et al.,* 2013). Due to the unavailability of crops for biofuel production, micro-algae have been used as alternatives due to some advantages such as high growth rate and high lipid content. A setback, though, is the cost of harvesting procedures which requires a low-cost, reliable and high yield product that is free from metallic contaminants. One of the primary concerns is the energy-intensive harvesting process which accounts for up to 20–30% of the total cost of algal biomass production due to the small size of algal cells; these include the cost of electricity, reagents and maintenance of the separation equipment (Zhou *et al.,* 2003). Bioflocculation has been used to harvest microalgae successfully for the production of biodiesel. Zhou *et al.* (2003) used filamentous pellets-forming fungal strain (*A. oryzae*) to harvest algae biomass. Harvesting microalgae at the commercial scale usually involves a flocculant to reduce the time required to separate the medium from the algal cells. Compared to synthetic focculants, flocculants with high molecular weight are preferred because they adsorb several particles at

once, thereby forming a three dimensional matrix, and the cells are aggregated and become easily harvested. Bioflocculants such as chitosan has been reported to allow harvesting up to 98% of the microalgae (Suali and Sarbatly, 2012). Not all the above-mentioned applications have been implemented in large scale experiments; however, most have been very successful in the laboratory.

2.3 Factors affecting bioflocculant production and bioflocculant activity

Bioflocculants production is affected by various factors including the composition or constituents of the culture media and fermentation media, as well as the physical, chemical and biological conditions involved in fermentation. The composition of the media affects the expression of genes and therefore determines which compounds are produced by the cell. All this implies that bioflocculant production is influenced by, broadly, three factors, and these are genotypic, physiologically, and environmental aspects (Salehizadeh and Shojaosadati, 2001). Therefore, it is crucial to vary fermentation conditions so as to achieve conditions that are optimal for the maximization of the production of bioflocculants. A cultivation medium is designed to reflect the elemental composition and the biosynthetic capacity of a given microbial cell.

2.3.1 Effect of pH

The pH influences flocculating activity in various ways, mainly by affecting the stability of suspended particles and the formation of floccules (Ugbenyen and Okoh, 2013). The pH of the fermentation medium determines the electric charge of the cells and the oxidative-reduction potential which also contributes to the absorption of nutrients and enzymatic reaction. Bioflocculant production has been observed under various pH values. Ntsaluba *et*

al. (2013) reported to have used alkaline pH of 9 to reach a maximum flocculating activity of 89% using a consortium. While a neutral, weak alkaline and weak acidic pH were observed to produce maximum flocculating activity for *Rhizobium radiobacter* F2, *Bacillus sphaeicus* F6 and *Streptomyces* sp. Gansen was reported by Wang *et al*,. (2010) and Nwodo *et al*. (2012) respectively.

2.3.2 Effect of carbon and nitrogen sources

Carbon and nitrogen sources are the backbone of any media composition, as the availability of these nutrients affect the consumption and growth of the organism being cultured. These nutrients have been emphasized in the production of bioflocculants (Ugbenyen and Okoh, 2013, Nwodo *et al.,* 2012). According to Bura *et al*. (1998), nitrogen and carbon sources do not only affect the growth of the organisms, but also affect the composition of the flocs and even the structure of the floc. Due to the fact that different organisms have different nutrient requirements, it is imperative to optimize bioflocculant production to elucidate the optimum carbon and nitrogen sources. Gong *et al.* (2008) reported that *Serratia ficaria* (S-14) produced a bioflocculant SF-1 using three different carbon sources that had flocculating activities of a similar range and observed maximum flocculating activities of 91.11%, 96.69%, and 97.15% using ethanol, glucose and lactose respectively. Moreover, Cosa *et al.* (2013) reported glucose as the best carbon source with a 70.4% flocculating activity for a bioflocculant produced by *Virgibaccilus* sp. Rob. The same results were reported by Xia *et al.* (2008) where glucose was the preferred carbon, including *Proteus mirabilis* TJ-1. Due to the low flocculation efficiency and production cost for most of the bioflocculants produced, it is necessary that production alternatives (production optimization) be devised in the exploration of microbes for high bioflocculant yield and high flocculation efficiency at

minimal production cost via the utilization of cost-effective materials (Zhang *et al.,* 2007; Qiang *et al.,* 2010).

In nature, microorganisms grow mostly through a combination of substrates, and growth may not be controlled by only a single nutrient, but by two or more nutrients simultaneously; additionally, kinetic properties of a cell might change due to adaptation. A noted problem with introducing an organism that has been isolated from a natural environment and exposing it to single substrate, especially in a closed fermentation system, is that the cell's environment and the cell's composition and physiological state changes during the experiment. It is for this reason that organic available substrates such as biomass and wastewater, which are complex substrates, have been used to reduce production cost of bioflocculant and increase bioflocculant efficiency (Zhang *et al,* 2007).

Nitrogen sources are as imperative as carbon sources in medium composition as they provide the appropriate biochemical and biophysical environment, and therefore, its optimization is crucial in bioflocculant production. Cosa *et al.* (2013) optimized for a nitrogen source and deduced that peptone was the preferred nitrogen source for *Virgibacillus* sp. Rob. In most instances, organic nitrogen sources (urea, yeast extract, peptone and so on.) are more favourable than inorganic nitrogen sources $(NH_4)_2SO_4$, KNO_3, NH_4NO_3), and this could be because nitrogen is easily available from the organic compounds. Furthermore, these organic compounds are complex media that have other compounds in them that might add nutritional value on the growth or and the production of bioflocculants. Pathak *et al.* (2013) experienced this when optimizing the activated sludge-borne bacteria *Pseudomonas aeruginosa* strain, whereby yeast extract and peptone showed the highest flocculating activity compared to other nitrogen sources. Nie *et al.* (2011) also discovered that organic nitrogen sources give higher

flocculating activity. The preference of inorganic nitrogen by some bioflocculant producers, as sources of nitrogen, is only seen in some, maybe, most of the bioflocculant producers, however, some such as those reported by (Ling 2009; Nwodo and Okoh, 2012; Mabinya *et al.,* 2012) preferred inorganic nitrogen sources to produce the bioflocculant maximally.

2.3.3 Inoculums size, Temperature and Agitation speed

Inoculum size usually is the first parameter in the optimization of bioflocculant production, as consequent (optimization) experiments depends on it. Inoculum size is the amount or cell percent used to inoculate into the bioflocculant production media. It has an effect on the growth patterns of the cultured organism and also on the final product produced by the organism. Inoculum size gives optimum bioflocculant activity and also differs with regards to organisms. Ugbenyen and Okoh, (2013) reported that 2% gave the best flocculating activity, while Zhang *et al.* (2007) reported that they had high flocculating activity at 1%; the same was observed with *Bacillus* strain, *Bacillus licheniformis* X14 (Lie *et al.,* 2009) even though Wang *et al.* (2010) and Jang *et al.* (2001) observed an optimal flocculation at inoculums size 5%. Optimization of inoculums size is crucial because small inoculum sizes delay the lag phase in the bacterial growth, whereas large inoculum sizes cause the niche of the strain to overlap excessively, resulting in inhibition of bioflocculant production (Zhang *et al.,* 2007)

2.3.4 Temperature and shaking or agitation speed

The cultivation temperature is crucial in any microbial growth as metabolism of microorganisms is directly influenced by the cultivation temperature. At an optimal temperature maximum growth, activity and enzymatic activity is observed (Zhang *et al.,* 2007; Li *et al.,* 2008). Shaking or agitation is applied to aerobic fermentation so as to allow dissolved oxygen which is needed for nutrient absorption and enzymatic reactions. Different

bioflocculant strains have different optimal shaking speed which is used as a determinant for the concentration of dissolved oxygen (Lie *et al.,* 2009).

2.3.5 Metal ions

The use of metal ions, more specifically, cations in bioflocculation processes has been explored; this has three theories to support it. These theories are: Double Layer Theory or DLVO Theory, Alginate Theory, and Divalent Cation Bridging Theory, and such theories are proposed to explain the aid of cations on bioflocculation process (Sobeck and Higgins, 2002). The above-mentioned theories explain the fact that the charges of these metals makes them to neutralise and stabilise opposite charges on the functional group of the bioflocculant, and also tend to reduce the particle surface charge density of the suspended particle so that the bioflocculant molecule and the suspended particle can come together. Cations stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups and by forming bridges between particles. The role of bivalent and trivalent cations is to increase the initial adsorption of biopolymers on suspended particles by decreasing the negative charge on both the polymer and the suspended material to be flocculated.

2.4 Composition of flocculants

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 (Mabinya *et al.,* 2011). Bacteria, fungi and actinobacteria have been reported to produce extracellular polymeric substances such as saccharides, proteins, nucleic acid and glycoprotein during their growth, with functional groups which mediate flocculation effectively. Hence, bioflocculants have been shown to be composed of these biomolecules (Nwodo and Okoh, 2012; Mabinya *et al,* 2012; Salehizadeh and Shojaosadati, 2001). Various

methods or techniques have been employed to determine the composition of bioflocculants. For example, Phenol sulphuric acid determination of total carbohydrates, Lowry-Folin assay for protein content or Bradford method, Carbazole-sulfuric acid method for uronic acid, Friedman method for pyruvic acid and acetic acid, Emission Scanning Electron Microscope (SEM) to obtain images of the bioflocculant, and IR spectrophotometer and an X-ray photoelectron spectroscopy (XPS) have be used to determine the functional groups of the bioflocculant (Lie *et al,* 2009, Mabinya *et al.,* 2011). Extracellular porimeric bioflocculants, which are biosynthetic polymers of microbial origin, are usually composed of majorly polysaccharides. Studies on bioflocculants produced by *Halomonas maura* sp. Nov *Halomonas* sp. V3a, *Halomonas* sp. OKOH, *Bacillus firmus*, *Serratia ficaria*, and *Virgibacillus* sp. Rob and many others have shown these to be composed of polysaccharide, with little or no protein or other contents at all (Bouchotroch *et al.,* 2001; Salehizadeh and Shojaosadati, 2003; Sheng *et al.,* 2006; He *et al.,* 2009; Mabinya *et al.,* 2011). Although most bioflocculants reported are mainly composed of polysaccharides, there are those that are a combination of components ranging from proteins, nucleic acid and glycoprotein. Xiong *et al.* (2010) reported a bioflocculant from *B. Licheniformis* which was comprised of 89% carbohydrate and 11% protein, and also *Cobetia* spp produced a bioflocculant that was composed mainly of uronic acid (93%), protein (5%) and neutral sugar (1.8%). Similar results were observed by Okaiyeto *et al.* (2013) with a bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo (polysaccharide 62.3%, protein 4.73% and uronic 25.7%). Extracellular substances that are typically made of polysaccharides and proteins are thought to mediate cell-cell interactions or adherence of cells to surfaces.

2.5 Classification of flocculants

Flocculants are divided into three categories based on the origin of the flocculants, and these are: Inorganic flocculants, such as aluminium salts (that is, aluminium sulphate); organic synthetic flocculants, such as polyacrylamide derivatives; and biologically or naturally occurring flocculants, such as chitosan and microbial flocculants (Zhang *et al.,* 2007).

2.5.1 Inorganic flocculants

The inorganic coagulants include: ferric chloride, aluminium sulphate and calcium chloride. Ferric chloride is cheaper compared to all the inorganic flocculants, and a small amount of it is used or required as compared to other inorganic flocculants. Inorganic flocculants are divided into two categories based on their differences in molecular weight; these are inorganic small molecular flocculants and inorganic high molecular flocculants. Inorganic small molecular flocculants, although less costly, are the least utilized because of poor functionality (Salehizadeh and Shojaosadati, 2001). Inorganic high molecular flocculants, on the other hand, are frequently used because of their high molecular weight and complex structure, which contributes to their effectively high flocculation efficiency; these include polymers of aluminium salt and iron salt. Although polymers of aluminium salts, as mentioned previously, are used more because of less dosage, higher efficiency, bigger flocculating constituent, quicker subsiding and better purifying effect, they are poisonous. Treatment of water using aluminium compounds can substantially increase the soluble aluminium concentration in drinking water. Moreover, it has been reported that even at low levels, exposure to aluminium can lead to several neuro-degenerative diseases mainly, Alzheimer's disease, dementia, and Parkinson's disease (Tsunoda and Sharma, 1999).

2.5.2 Organic flocculants

Organic flocculants such as polyethylene imine and the derivatives of polyacrylamide which are mostly used in the group of organic flocculants are sometimes preferable as flocculants as compared to inorganic flocculants due to their inertness to pH changes, easy handling and high efficiency with low dosage (Lee *et al.,* 2014). Nonetheless, acrylamide is a rodent carcinogen and human carcinogen. Acrylamide, or any of its derivatives, also has a strong affinity for mammalian sperm cells and inducing genetic damage with great efficiency in developmental stages of spermal cells (Ghanayem *et al.,* 2005). Besides its carcinogenicity and neurotoxicity, organic flocculants are also non-biodegradability and unfriendly to the environment (Salehizadeh and Shojaosadati, 2001; Tyl and Friedman, 2003).

2.5.3 Extracellular Polymeric Substances (EPS)

EPS are a mixture that is composed of macromolecular polyelectrolytes including polysaccharides, proteins, and nucleic acids. Each comprises variable molecular mass and structural properties (Sun *et al.,* 2009). They are excreted extra-cellular by bacteria of different taxa and serve various functions in the bacteria strain producing it. EPS has different functions which include the following: adherence of cells to surfaces, migration of prokaryotes in groundwater, protection from engulfment by other predatory organisms such as protozoa, protection from adverse environmental conditions and white blood cells (phagocytes), (Nwodo *et al.,* 2012) protection from environmental effects such as drying or desiccation and also protection from attack by antimicrobial agents of plant or animal origin (Nwodo *et al.,* 2012). All these depend on the habitat of the bacteria. EPS are metabolic products that accumulate on the microbial cell surface, and the accumulation of these metabolic products varies. EPS can either be soluble (S-EPS) and bound (B-EPS). S-EPS are

dissolved in solution; B-EPS are bound to cells and are generally subdivided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The composition of the EPS varies and is organism-reliable (Hou *et al.,* 2013). Micro-organism flocculants (EPS) are extracted and purified from micro-organisms or their secretion by using biological technology. It is safe, high efficiency and degrades naturally. Various micro-organisms have been used for the production of flocculants, and these include bacteria, yeast, actinomycetos and others. Most bioflocculant producing microorganisms were isolated from moderate environmental conditions, like soil, and fresh water. Recently, scientists are trying to isolate and screen floculants from microorganisms that are found in extreme environments so as to improve flocculants activity.

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CHAPTER THREE

Characterization of bioflocculant produced by *Enterococcus* sp. isolated from a marine sediment in Algoa Bay

ABSTRACT

The bacterium was isolated from the marine sediment of Algoa Bay, and its potentials for bioflocculant production were assessed. A comparative analysis of the 16S rDNA sequence of the isolate in the GenBank datebase showed 97% similarity to *Enterococcus hirae* ARBS1. Bioflocculant production and flocculation activity optimization were assessed. The bacteria optimally produced bioflocculant with fructose (91.7%) and urea (91%) as sole carbon and nitrogen sources respectively. Mg^{2+} (87%) and Ca^{2+} (86%), likewise, served as best cation sources on the production of the bioflocculant at pH 5 (93%). Additionally, the flocculating activity of the bioflocculant increased with the addition of Mg^{2+} (81%) and Na^{+} (81%), and the highest flocculating activity was achieved at pH 5. The FTIR analysis suggests the presence of carboxyl, hydroxyl and amine groups, hence it is proposed that the bioflocculant is a glycoprotein.

1.0 INTRODUCTION

Water constitutes about 70% of the surface of the planet earth, and 97% of this water is present as sea water, and 2% of the remaining 3% is locked up as frozen water. Therefore, only 1% is available for usage, so it is crucial conserve water. One of the stages of water-treatment process, namely, the flocculation stages, involves debris removal consequent to the addition of inorganic flocculants like alum (poly-aluminium chloride). The major problem currently is that the use of these inorganic flocculants has adverse effects on both the environment and health. Several problems concerning the use of conventional synthetic flocculants has necessitated the need for alternative cost-effective, safe and efficient bioflocculants from micro-organisms inhabiting many environments, particularly those from unusual environments.

Besides being used in water-treatment, flocculants have been widely used in industrial processes including downstream processing in food and fermentation processes (Salehizadeh, and Shojaosadati, 2001). During the flocculation stage by flocculants, the surface property of such colloidal particles can be changed, or dissolved material can be precipitated so as to facilitate the separation of solids by gravity or filtration, and this is called flocculation (Tripathy *et al*., 2000). The suspended particles vary considerably in source, composition charge, particle size, shape and density. Flocculants are categorised into three groups, namely, inorganic flocculants (such as aluminum sulfate and polyaluminium chloride); organic flocculants (such as polyacrylamide derivativates and polyethylene imine); and naturally occurring flocculants (such as chitosan and microbial flocculants) (Yim *et al*., 2007; Salehizadeh and Shojaosadati, 2001). Use of all of these, despite the effective flocculating performance and low-cost of the synthetic chemical flocculants, has resulted in some health and environmental problems. Inorganic and organic flocculants are termed ‘synthetic

chemical flocculants' and are widely used. However, synthetic flocculants have adverse effect on human beings and environment and can lead to unwanted effects such as carcinogenic, neurotoxic effects and non-degradable conditions and also lead to human diseases such as Alzheimer's disease and cancer (He *et al*., 2004; Zhang *et al*., 2007). These adversities of these chemical flocculants have led to scientist exploring other means such as bioflocculants. At the beginning of the bioflocculants discovery, a problem of then identified bioflocculants were cation dependent for flocculating capabilities.

2.0 METHODS AND MATERIALS

2.1 Screening for bioflocculant- producing bacteria

Several hundred marine bacteria were isolated from the bottom sediments of Algoa Bay in South Africa as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, South Africa.
In the laboratory, the samples were serially diluted into sterile saline water. About 100 micro-litres of the diluted solution were spread on Marine Agar (MI) and Reasoner´s 2A agar (R2A). About 2 ml of the sediment samples was transferred into 8ml of sterile saline water and agitated for 30 seconds. From these, serial dilutions were made. Similarly 100 micro-litres of the serially diluted sample were spread on the surface of M1 and R2A plates, following the method of Jensen *et al*. (1990)

The R2A Agar plates were incubated for 4 days at 28 ºC (M1), and these agar plates were incubated for 8 days on similar conditions. Colonies from both media were randomly picked and isolated on fresh plates and incubated for 2 and 5 days for R2A and M1 respectively.

2.1.2 Activation of the isolates for fermentation

One litre of the activation medium containing 3 g of beef extract, 10 g of tryptone and 5 g of sodium chloride was prepared. About 5 ml of the activation media was measured into different test tubes and autoclaved at 121 ºC. After inoculation of the isolates into the tubes, they were incubated in a rotary shaker for 24 hours at 28 degrees Celsius, 160 rpm.

2.1.3 Bioflocculant producing medium and cultivation

Screening of the isolates for bioflocculants production was done following the method of Zhang *et al*, 2007. The medium composition includes: 20 g glucose, 5 g K_2HPO_4, 2g KH_2PO_4, 0.3 g $MgSO_4$, 0.5 g Yeast extract, 0.5g Urea, and 0.3 g NH_4SO_4 in 1L of distilled water. The components were autoclaved and mixed aseptically. The seed culture (1 ml), after 24 hours of incubation was inoculated into 50 ml of the growth medium in 250 ml flask. The culture was incubated in a rotary shaker at 28 ºC, 160 rpm for 5 days. After 72 hours of incubation, samples were withdrawn and flocculating activity of the produced bioflocculants were checked according to the method of Kurane *et al*. (1994)

2.1.4 Assay of flocculating activity

The flocculating activity was determined using the method previously described by Kurane *et al*. (1994) in which Kaolin clay was chosen as the suspended solid. 2 milli-litres of the culture supernatant and 3 ml of 1% $CaCI_2$ were added into 100 ml of Kaolin clay suspension (4 g/l) in a 100 ml flask, gently shaken and left to stand still for 5 min. The control was prepared following the same procedure, but the bioflocculant was replaced by fresh broth. The turbidity in the upper phase was measured with a spectrophotometer at 550 nm, and thus the flocculating activity was estimated as follows:

Flocculating rate = {(A-B)/A} × 100%

Where A is the optical density of the control at 550 nm and B is the optical density of the sample at 550nm. All experiments were performed in triplicates for the mean calculation.

2.2 The effect of culture conditions on bioflocculant activity

2.2.1 The effect of carbon and nitrogen sources

In various studies, it has been proven that alteration or changing of nitrogen and carbon sources influence both bacterial growth and flocculating activity (Cosa *et al*, 2013). Therefore, in this study, the effect of different carbon and nitrogen sources on flocculating activity were assessed. These carbon sources included glucose, sucrose, fructose, starch, lactose and maltose, while the nitrogen source candidates included, ammonium chloride, urea, tryptone, peptone and yeast extract. The assessments were done in accordance with the description of Lachhwani (2005).

2.2.2 Effects of various cations and pH on flocculating activity

The effect of various salts on both the production and flocculation of the bioflocculant by the strain was evaluated. Flocculant tests were conducted utilizing the procedure elaborated above, however, the $CaCl_2$ solution was replaced by various metal salt solutions, and the flocculating activity was measured. These salts are: Al^{3+}, Fe^{3+}, Mg^{2+}, K^{+}, Na^{+}, Li^{+}, Mn^{2+}. To assess the effect of pH on flocculating activity and production, the pH of the kaolin suspension and culture medium was adjusted using HCl and NaOH in the pH range of 3-12 respectively (Yim *et al*., 2007).

2.3 Time course of bioflocculant activity

2.3.1 Composition of culture medium:

For the time course experiment, Optimum media was used, and the composition of the medium was as follows: 20 g of glucose; 5 g of KH_2PO_4; 1 g of urea; 0.3 g of $MgSO_4$, $7H_2O$ per litre of filtered natural sea water (Zhang *et al*., 2007). Pertaining the s*tandardization of the inocula*, **s**aline solution was prepared by adding 0.45 g NaCl in 50ml of distilled water. Fifty milli-litres of saline solution were inoculated with a loop full of colonies for each of the strains. The optical density (660nm) of each was measured by taking 100 µl (culture) into 1 ml of distilled water in 3 ml cuvettes and re-adjusted when the need arose to give 0.1 (Cosa *et al*., 2013).

Time course assays, according to the method of Gao *et al*. (2006) and Cosa *et al*., 2013, were that the inoculated saline solution was used as seed culture for inoculum preparation. Seed culture (4% v/v) was inoculated into 200 ml of medium in 500 ml flasks on a rotatory shaker (160rpm) at 28 degrees Celsius. A sample was drawn at appropriate time intervals (every 24 hrs) for a period of 10 days. 2 milli-litres of culture broth were centrifuged at 4000 g for 30 minutes, and the cell-free supernatant was used to determine the flocculating activity. The pH of the broth sample was also measured, and cell growth was determined by plating the broth after every 24 hours. All experiments were performed in triplicates.

2.4 Purification and characterization of the bioflocculant compound

2.4.1 Extraction and purification of the bioflocculant

Purification and characterization of the bioflocculant was done following the methods described by Chen *et al*., 2002, Mabinya *et al.,* 2012 and Cosa *et al*., 2013) using media formulation based on the pre-determined optimum culture conditions.

2.4.2 Analysis/ characterization of purified bioflocculant

The effect of pH, thermal stability, and cation salts on the flocculating activity of the purified bioflocculant was evaluated. The protein content of the purified bioflocculant was measured using the Folin-Lowry method. Total sugar content was measured using phenol-sulphuric acid protocol, as described by Lachhwani (2005).

2.5 Identification of the bacteria

The bioflocculant-producing bacterial was identified by molecular technique as described by Cook and Mayers (2003) and Nwodo *et al.*, (2012). The bacterial 16S rDNA gene was amplified by a polymerase chain reaction (PCR) followed by sequence analysis of the amplified gene. The bacterial DNA was extracted through the boiling method, and the PCR amplification was carried out in a 50 µL reaction volume containing 2 mM MgCl2, 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-ACGGITACCTTGTTACGAC TT-39) and 2 mL of the template DNA. Primer F1 and R5 binds were used to base positions 7–26 and 1496–1476 of the 16S rRNA gene of Streptomyces ambofaciens ATCC23877 respectively (Cook and Meyers, 2003). The PCR condition included the following: initial denaturation (96 °C for 2 min), 30 cycles of

denaturation (96 °C for 45 s), annealing (56 °C for 30 s), 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.

3.0 RESULTS AND DISCUSSION

3.1 Screening and identification of bioflocculant- producing bacteria

Close to 300 marine bacteria were isolated from the marine environment and were screened for bioflocculant production. Amongst these was the test bacterium which exhibited significant bioflocculant activity of over 80% against Kaolin suspension. PCR revealed the bacterium to be *Enterococcus hirae.* The genus *Enterococcus* consists of Gram-positive, facultative anaerobic bacteria, catalase negative, non-spore-forming that can occur both as single cocci and in chains. This genus also belongs to a group known as lactic acid bacteria (LAB) (Devriese, *et al*, 2002). Enterococcus *hirae* is among human and animal gastrointestinal bacteria and play a role in physiology, clinical relevance and a large application in technology. This bacterium grows under anaerobic conditions fermenting glucose which is accompanied by an acidification of the medium, changes in the proton-motive force and environment oxidation-reduction potential (Vardanyan and Trchounian, 2012)

3.2 Optimization of conditions for bioflocculants production

3.2.1 Carbon, nitrogen and cation source

Table 1 shows the effects of various carbon (20 g/L) sources on bioflocculant production. Since carbon source plays a very significant role in growth, particularly in extracellular polysaccharide production (Cosa *et al*, 2013), several sugars were investigated. The bacteria effectively utilized all organic carbon sources for bioflocculant-production with flocculating activities of more than 80% except for lactose and starch which showed flocculating activity of 21% and 20% respectively. Sucrose and fructose were favourable carbon sources for the test bacteria, with flocculating activities of 87% and 92% respectively. The source of carbon is dependent on the organism; different organisms prefer different carbon sources. Optimization of factors like carbon sources is essential since productivity and distribution of bioflocculant is dependent on the culture conditions, and therefore, optimum culture conditions give optimum flocculation activity. There are various factors involved that affect culture conditions during bioflocculants production; these include nitrogen source and cations. Table 3.1 below shows the effect of these factors (carbon, nitrogen and cation sources) on the test bacterium (*Enterococcus hirae*). The nitrogen source that produced optimum flocculating activity was Peptone and Urea, both having 91% flocculating activity, yeast, tryptone, NH_2SO_4, and they produced flocculating activities of 85%, 83.5% and 81.4% respectively. In addition, calcium chloride and magnesium chloride gave the highest flocculating activity for the test bacterium.

Optimum conditions are organism-dependant. For instance, *Bacillus* sp. Gilbert favoured glucose as the best carbon source for the production of the bioflocculant (Piyo, *et al*, 2011). *Virgibacillus* sp. Rob was observed to produce bioflocculant optimally in the presence of

glucose and peptone as sole sources of carbon and nitrogen respectively (Cosa *et al*., 2011). Furthermore, *Aspergillus flavus* produced a bioflocculant optimal when sucrose was used as a carbon source (Aljuboori, *et al*, 2013). Most micro-organisms utilise either organic or inorganic nitrogen sources, or both, to produce bioflocculant. Piyo *et al.* (2011) reported that *Bacillus* sp. Gilbert utilised an inorganic nitrogen source ammonium chloride effectively to produce bioflocculant with a flocculating activity of 91%, while organic nitrogen sources like urea and peptone were poorly utilised. On the other hand, *Proteus mirabilis* T-1 preferred organic nitrogen sources such as beef extract, yeast extract, and peptone, while peptone was the optimal nitrogen source (Xia *et al*, 2008). The same was observed by Noghabi, *et al*, 2007 where *Pseudomonas fluorescens* BM07 utilized glycerol as carbon source and organic nitrogen peptone. The test organism preferred organic nitrogen sources (urea and peptone) as well. Effects of cations (monovalent and divalent) were also evaluated, and it was observed that flocculation was stimulated by these cations, with Mg^{2+} enhancing the strain to reach a flocculating activity of 81.4%, followed by Na^{+}, Mn^{2+}, Li^{+}, Fe^{2} and K^{+} flocculating activity of 81%, 75,6%, 75.5%, 75.2% and 65.5% respectively. The role of cation is to neutralize and stabilize the negative charge of both functional groups of kaolin particle in solution and the bioflocculants (Okaiyeto *et al*, 2013). The surface properties, specifically the distribution of charges on the surface of the bioflocculants lead to variety of ion valences being preferred by different bioflocculants-producing strains (Ntsaluba *et al*, 2013). *Achromobacter* sp. TERI-IASST N produced a bioflocculant that preferred Ca^{2+} as cation source, and urea as nitrogen source, which is a similar case to ourteest bacterium (*Enterococcus hirae)* (Subudhi *et al.* 2014)

Table 3.1. Effects of composition medium on the bioflocculant production and activity

Carbon source	**Fructose**	**Sucrose**	**Maltose**	**Glucose**	**Lactose**	**Starch**
Flocculating Activity (%)	91.7	86.9	83.6		21	20.5

Nitrogen source	**Peptone**	**Urea**	**Yeast**	**Tryptone**	**NH_2SO_4**	**combined**
Flocculating Activity (%)	91.8	91	85	83.5	81.4	

Cations	**Mg^{2+}**	**Na^+**	**Mn^{2+}**	**Li^+**	**Fe^{2+}**	**K^+**	**Ca^{2+}**
Flocculating Activity (%)	81.4	81	75.6	75.5	75.2	65.5	

3.2.2 Effect of pH on production of the bioflocculant

The initial pH of the production medium is one of the factors affecting the production and flocculating activity of the bioflocculants (Xia, *et al*, 2008, Piyo *et al*, 2011). Similarly, in this case, the bioflocculant production and activity of the studied bacteria strain was affected by initial pH of the production medium, as a consequence affecting flocculating activity. The figure 3.1 below shows that the strain produced the bioflocculant optimally at acidic pH of 5 (93.4%), and the flocculating activity gradually decreased as the pH was increased towards alkalinity to a point where no flocculating activity was observed at 9 to 11. Moreover, Zhang *et al.*, 2012 reported that at pH 5 a bioflocculant produced for bacteria decolorization, Cr removal and swine wastewater application, produced its bioflocculant optimally. In addition, with pH (on production media), its effect varies with the organism, and this is because change of pH might vary the charge status of the bioflocculant and surface characteristics of suspended materials, thereby resulting in variation of flocculating activity. Therefore pH

variation is dependent on the composition of the bioflocculant which is strain-dependant (Piyo *et al*, 2011)

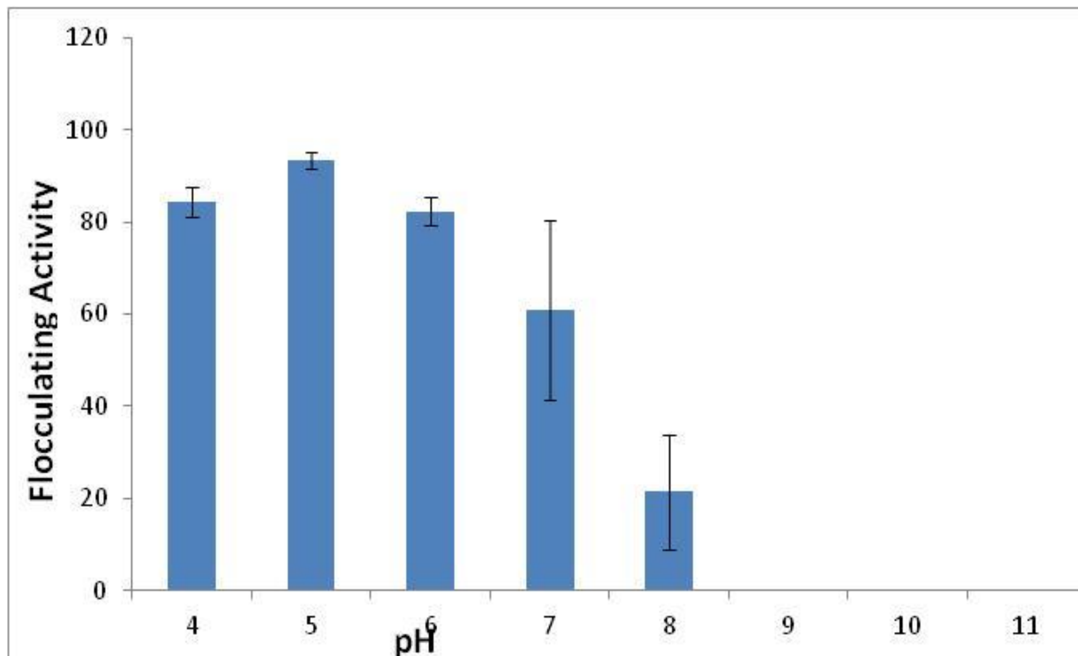

Figure 3.1 Effect of pH on bioflocculant production

3.2.3 Time course of bioflocculant production

The pH of the production media decreased from 6.21 to 4.96 during the 9 days of the experiment. The decrease in pH is thought to be due to the production of organic acids either from fructose metabolism (since fructose is a component of the cultivation medium) or the presence of organic acid components in the bioflocculant polymer being produced (Ntsaluba, *et al*., 2011). The initial pH of the culture medium determines the electric charge of the cells and the oxidation-reduction potential which can affect nutrient absorption and enzymatic reaction. This observed sharp decrease in activity and may be attributed to the action of a bioflocculant-degrading enzyme being produced by the micro-organism. Time (culture time) that is taken to produce a bioflocculant differs per organism. Our test bacteria produced bioflocculant maximally on the fifth day of fermentation, and on this day, cells were growing

exponentially, suggesting that the bioflocculant was produced by biosynthesis. Similarly, Piyo *et al.*, 2011 observed the same phenomenon whereby *Bacillus* sp. Gilbert produced a bioflocculant with flocculating activity increasing with the increase of cultivation time. This important phenomenon implies that the production of this bioflocculant can be manipulated by manipulating the growth pattern of the producing organisms as it is produced through biosynthesis. This could be done to minimise cost. The same was observed by Cosa *et al*, (2011) where *Virgibacillus* sp. Rob showed highest flocculating activity at day 4. Additionally, Cobetia Spp produced a bioflocculant during the exponential phase (Ugbenyan, *et al*, 2012), including *Halomonas* sp. OKOH where the bioflocculant was produced at about 135 hours of cultivation.

A number of factors influence bioflocculant production and thereby influence the bioflocculation process. These factors include: culture time, cell growth and pH. Culture time amongst these factors, may influence the production, distribution and flocculating capabilities of the bioflocculant (Cosa *at el*, 2011). Although culture time for flocculant release into the medium and its activity may differ with different organisms, most are produced biosynthetically. After 144 hours of incubation, flocculating activity began to decrease, while cell growth decreased gradually after 168 hours. The decrease in flocculating activity is due or could be due to cell autolysis and/or presence of a bioflocculant-degrading enzyme (Ugbenyen, *et al*, 2012).

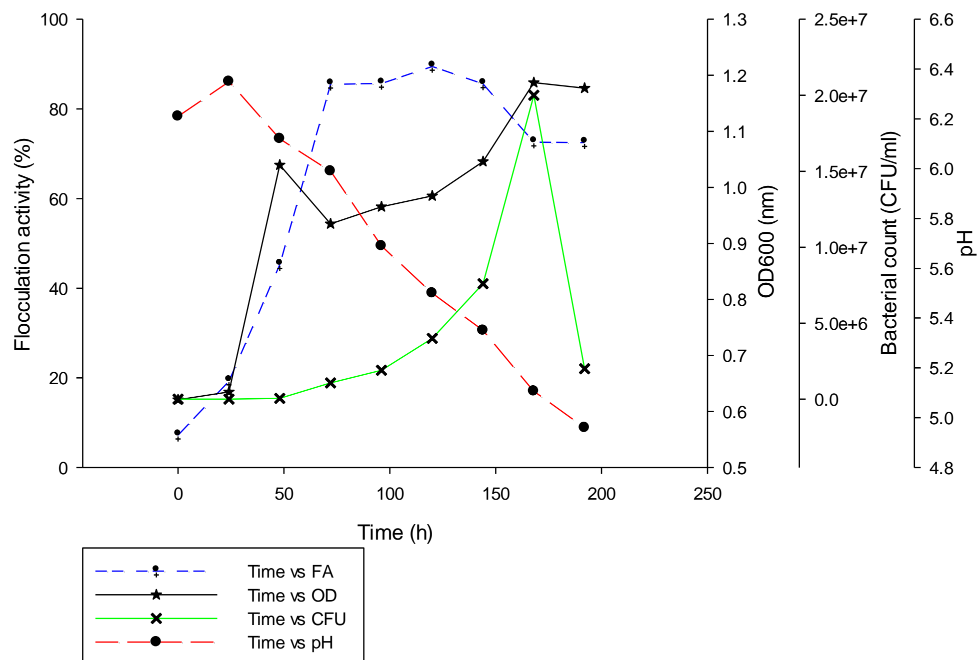

Figure 3.2 Time course of bioflocculant production *Enterococcus* sp.

3.2.4 Effect of heat on bioflocculant production

This strain had strong thermal stability within the range of 50-100 °C, and the flocculating efficiency was over 60% on 60 ℃. Some researches indicate that heat resistance of bioflocculant is consistent with the general understanding that flocculants rich in polysaccharides have a better thermal resistance than those of proteins and nucleic acids (Zhang *et al*, 2012). Although the bioflocculants have a strong thermal stability, increase in heat had an effect of the flocculating activity. As the temperature was increased, flocculating activity decreased with 60% from 60°C to 34.8% from 100°C.

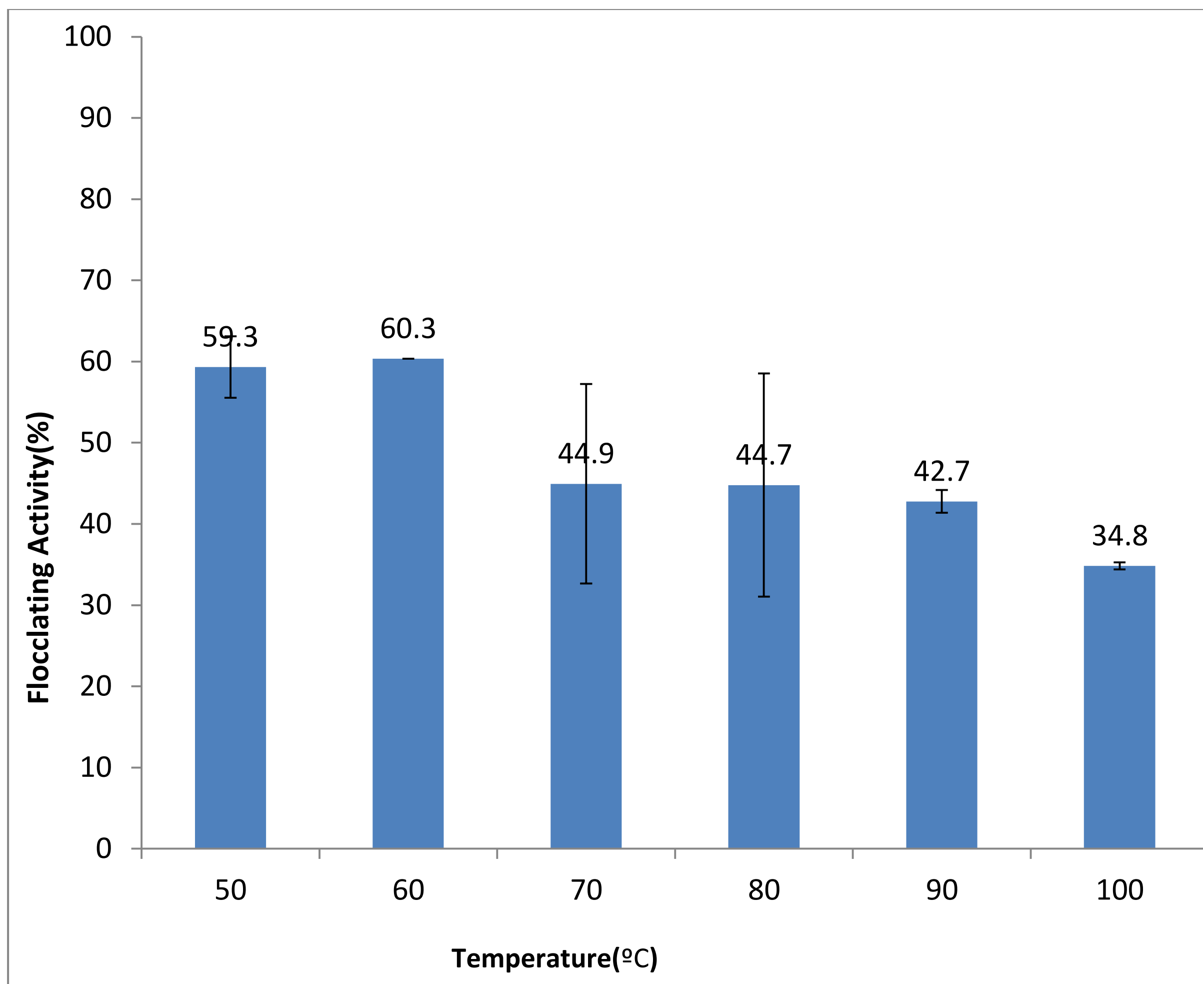

Figure 3.3 Effect of heat on bioflocculant activity

3.2.5 Effect of cation on flocculating Activity

Cations stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups of bioflocculants and also by forming bridges between particles. The role of bivalent and trivalent cations is to increase the initial adsorption of biopolymers on suspended particles by decreasing the negative charge on both the polymer and the particle (Levy *et al*. 1992, Ugbenyen, *et al*, 2014). Figure 3.4 below shows the effect of cation on the crude bioflocculant before purification and extraction. The highest flocculating activity was observed when Mg^{2+} and Na^{+} were used. The assistance of flocculation by addition of

divalent and trivalent cation is of two possible scenarios, and these are (i) that the addition of the cation to Kaolin suspension decreases the negative charge of the particles and (ii) cation bridging whereby the bioflocculant absorbs onto the Kaolin clay particles, thus flocculating them (Sheng *et al.*, 2006; Li *et al.*, 2008). Because divalent and trivalent cations have more surface area for adsorption, they are usually the ones that aid in flocculation, however, in this instance, Na^{+} is a monovalent, but had the second best flocculation activity of 81% following Mg^{2+}. Ugbenyen, *et al*, 2012 observed the same result whereby monovalent cations had high flocculating activity compared to some divalent and trivalent cations, and Na^{+} and K^{+} showed flocculating activity of 93.6% and 94.3% respectively

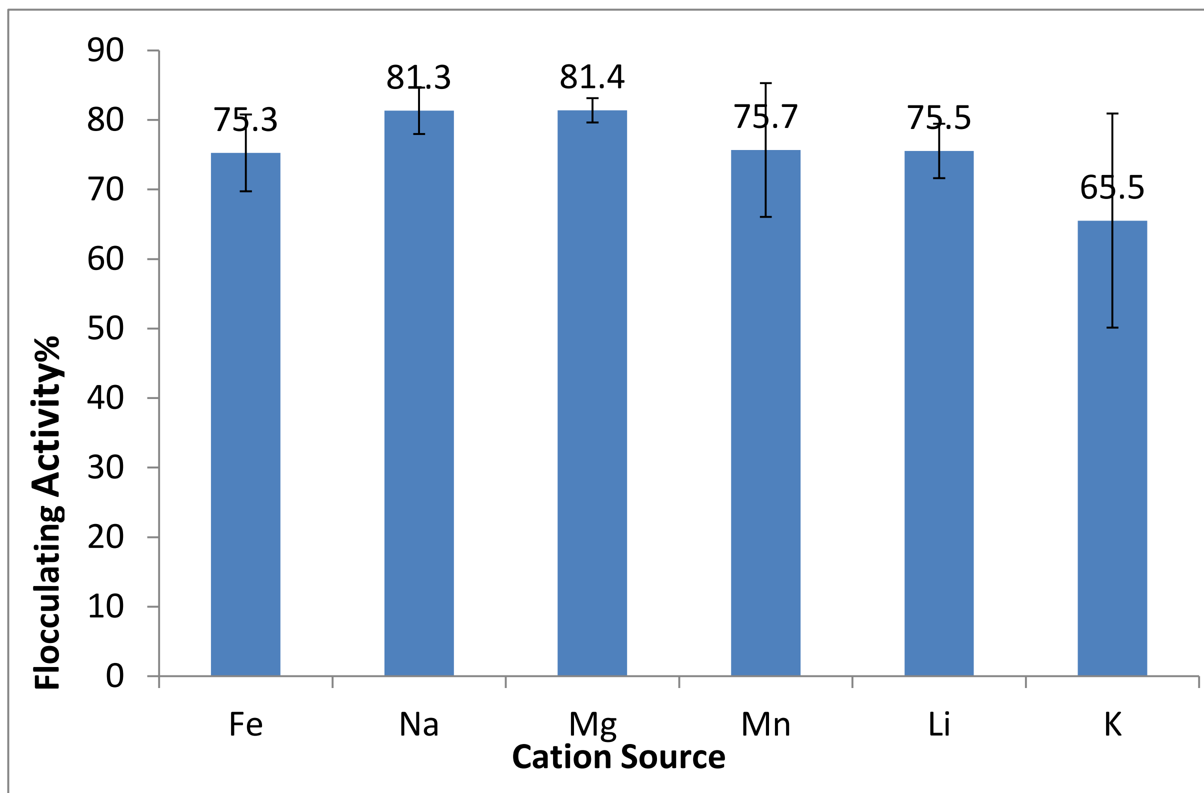

Figure 3.4 Effect of cation on flocculating Activity

3.2.6 Effect of pH on flocculating activity on the supernatant bioflocculant

A pH of the kaolin clay was adjusted so as to test the effect the change in pH will have on the flocculating activity. The clay was adjusted from pH 5 to 10, although this did not have much

effect on the flocculating activity of the supernatant, but pH 10 showed the highest flocculating activity of 72.1%.

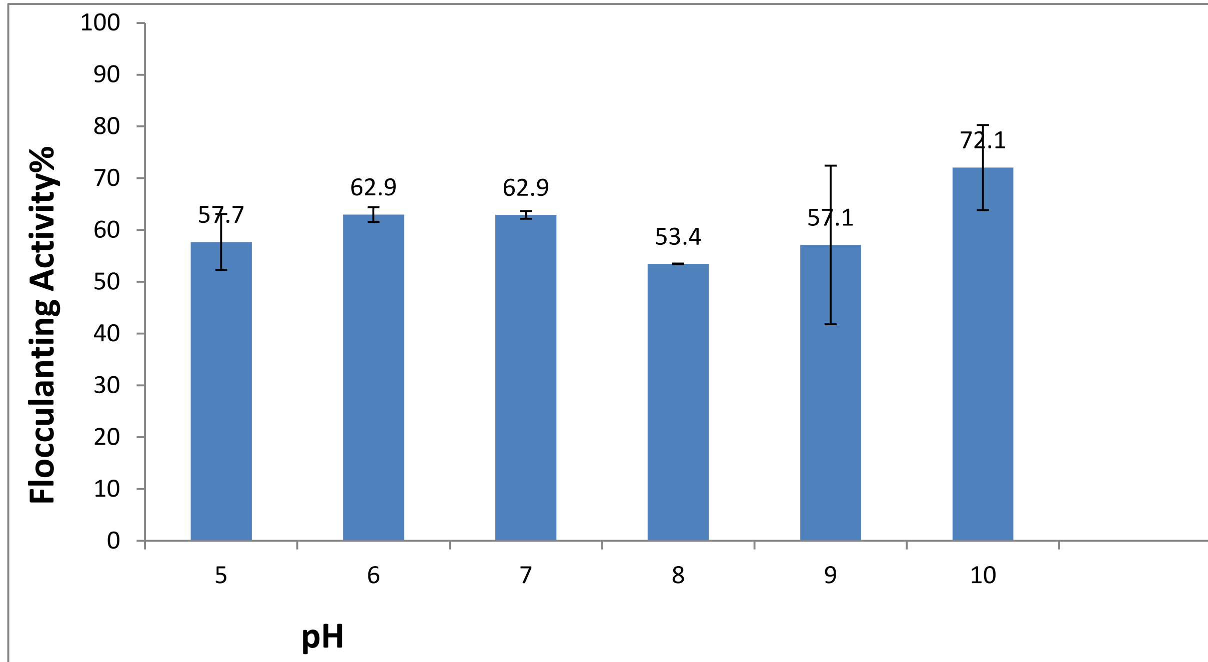

Figure 3.5 Effect of pH on flocculating activity on the Supernatant bioflocculant

3.2.7 Inoculum size on production of the bioflocculants

Amongst a number of the bacteria's physiological properties, inoculum size may play a significant role in biological development (Cosa *et al.*, 2013). Inoculum size affects not only the cell growth of microorganisms but also their product formation (Ugbenyen *et al*, 2012). Some studies have reported correlations between inoculum size and bioflocculant production by some organisms. In our test, bacterium inoculum size had a big role to play in flocculation rate, and a significant increase was observed from 1% to 5%. Furthermore, an optimal flocculation was observed at 4% inoculum size.

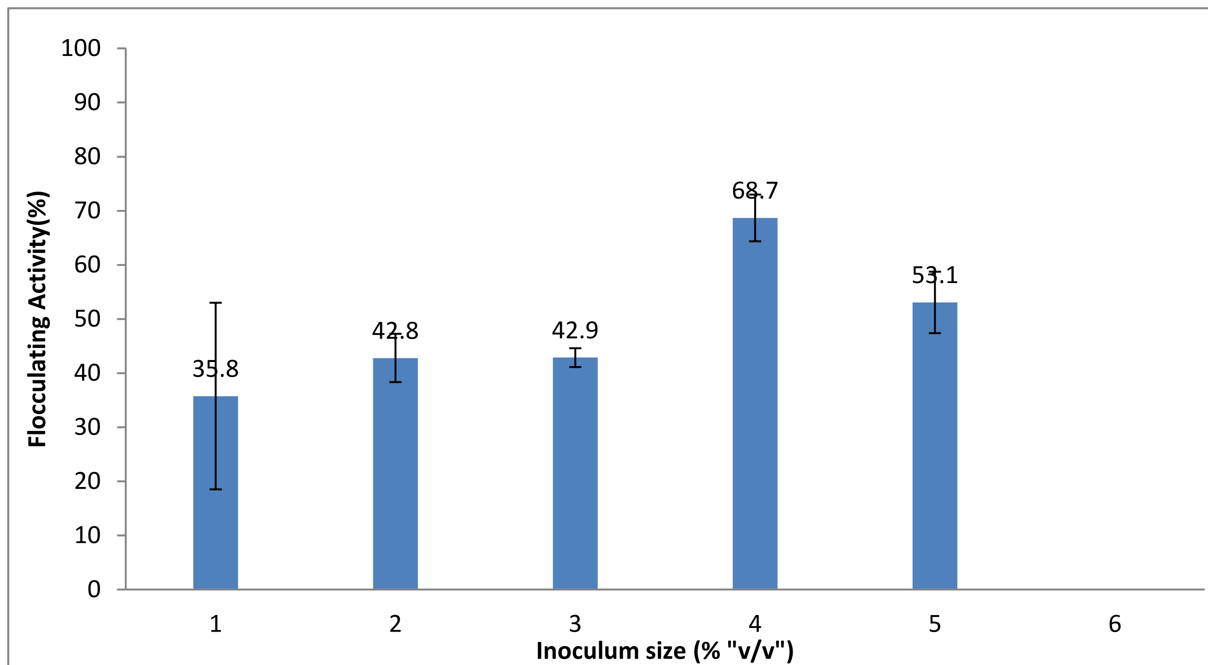

Figure 3.6 Effect of Inoculum size

3.2.8 Distribution of bioflocculants on the culture broth

To determine the distribution of the bioflocculants, after 5 days of fermentation, the culture broth was centrifuged at 4000 g for 30 minutes. After centrifugation, the precipitate (the composition included mycelium cells, cellulose and so on and was collected and dissolved in distilled water. The flocculating efficiency of the supernatant (89.7%) was high. Comparatively speaking, the residual precipitate (42%) has a very weak flocculating activity. This indicates that most of the flocculating activities are mainly found in the extracellular materials that were produced by mycelium cells. Zhang and Wang *et al.*, 2012 also their experience regarding a similar phenomenon whereby bioflocculant produced from bacteria for decolorization Cr removal and swine wastewater was mainly distributed on the supernatant, entailing that the bioflocculant is also produced by extracellular materials.

Strains	FA supernatant (%)	FA Pellet (%)
M86	89.70399	42.11068

Figure 3.7 Distribution of bioflocculants

3.3 Factors that affect the flocculation of purified bioflocculant

3.3.1 Effect of concentration dose on the production of the bioflocculant

The effect of different concentration doses on the flocculating activity of purified bioflocculant from the M86 strain was investigated. Appropriate bioflocculant concentration to be used in subsequent experiments was determined by testing different bioflocculants concentrations ranging from 0.1-1 mg/mL. It was observed that as bioflocculants' concentration increases, so does flocculation to the rate of 0.4mg/ml after which flocculation began to decrease steadily. According to Akaiyeto *et al*. (2013) and Zufarzaana *et al.*(2012), low dosage will not make bridging flocculation mechanism of the bioflocculant to be effective, and high dosage will generate high viscosity which will inhibit the settling of suspended particles by re-stabilization of kaolin particles. Furthermore, Wang *et al*. (2011) emphasize that when the bioflocculant molecules are excessively present in the solution, they usually generate high viscosity and block the absorption sites, thereby reducing flocculating processing and flocs formation. From this study, we observed the optimum flocculating activity (77%) when the bioflocculant concentration was 0.4 mg/ml. Decrease in flocculating activity occurred at concentrations of higher than 0.4mg/ml, which was probably because sedimentation of floccules was inhibited by the viscosity generated at high bioflocculants concentrations.

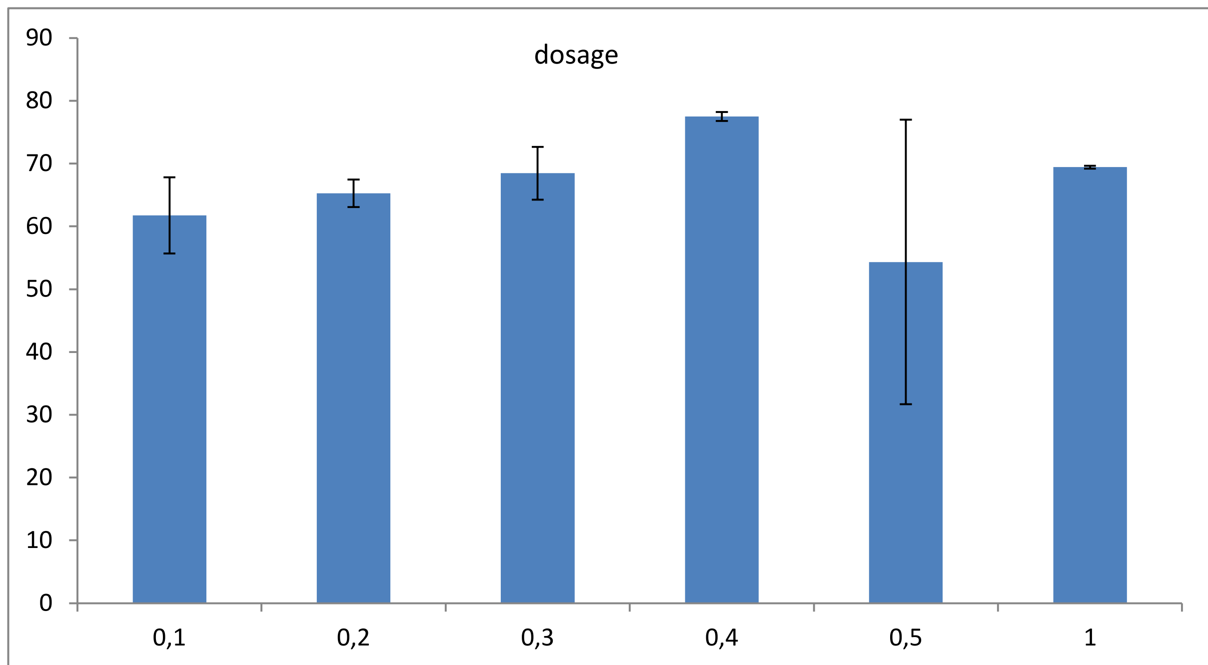

Figure 3.8 Effect of concentration dose

3.3.2 Effect of cations on flocculating activity

Various cations were used to evaluate the effect of cation on the purified bioflocculant. Due to large capacities, divalent and trivalent cations are expected to be more favourable compared to monovalent cations (Wang *et al*, 2011). Cations are added during flocculating activity tests to neutralize and stabilize negative charge of both functional groups of kaolin particles in the solutions and the bioflocculants (Akaiyeto *et a.l*, 2013). As expected, divalent cations were Mg^{2+} Ca^{2+}, Mn^{2+} (with flocculating activity of 65%, 62% and 61.5% respectively) and trivalent were Al^{+++} and Fe^{+++} (with 68.9% and 5% respectively), while all monovalent cations had a flocculating activity of less than 30%. Wang *et al.* (2011) observed the same results whereby Ca^{2+} Zn^{2+}, $Fe^{2+}$$Al^{3+}$ and Fe^{3+} enhanced flocculation greatly. Moreover, Cosa *et al*, 2013 observed the same phenomenon where monovalent cations (Na^{+}, Li^{+} and K^{+}) and trivalent Fe^{3+} had very little or no effect on flocculation activity although divalent ($Ca^{2+,}$ Mn^{2+} and $Mg^{2+)}$ and trivalent ($Al^{3+)}$ promoted flocculation, with Ca^{2+} and Al^{3+} showing the highest flocculation activities.

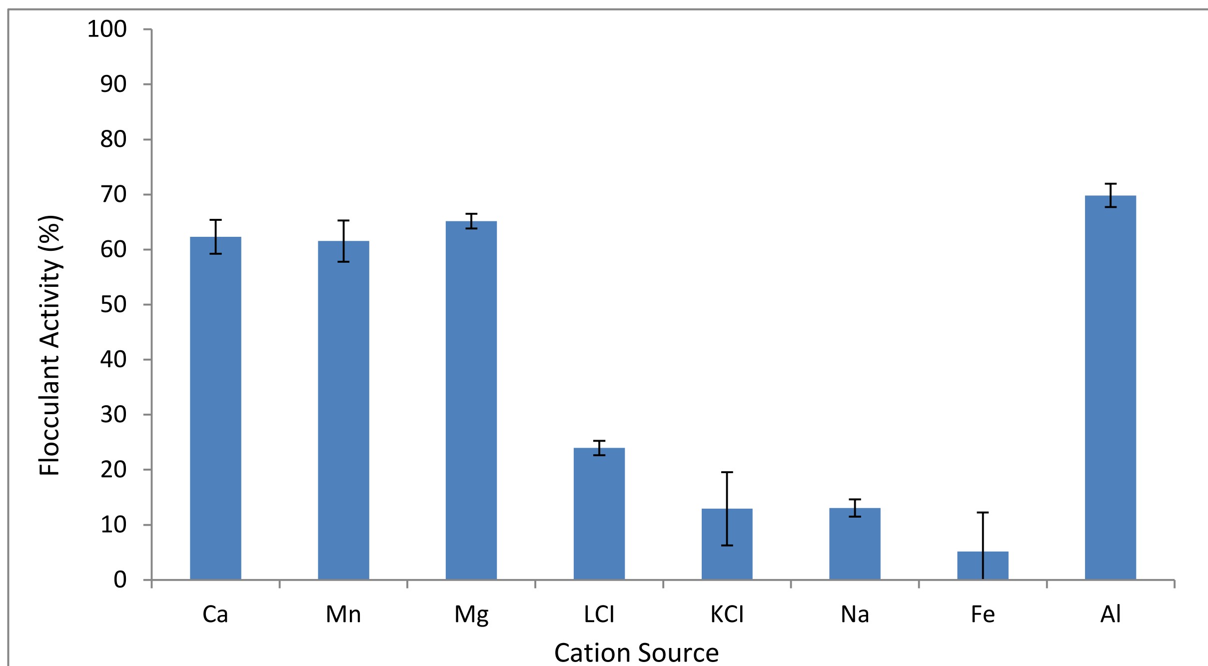

Figure 3.9 Effect of cations on flocculating Activity

3.3.3 Effect of heat on flocculation of the purified bioflocculants

On investigating the effect that heat had on the flocculation of the bioflocculants, five different temperatures (50 °C -100°C) were applied. It was observed that heat had little or no effect on the physical and chemical properties of the bioflocculant up to 100°C. From 50 °C to 90 °C, flocculation was averagely steady, and slight decline was seen at 100°C.

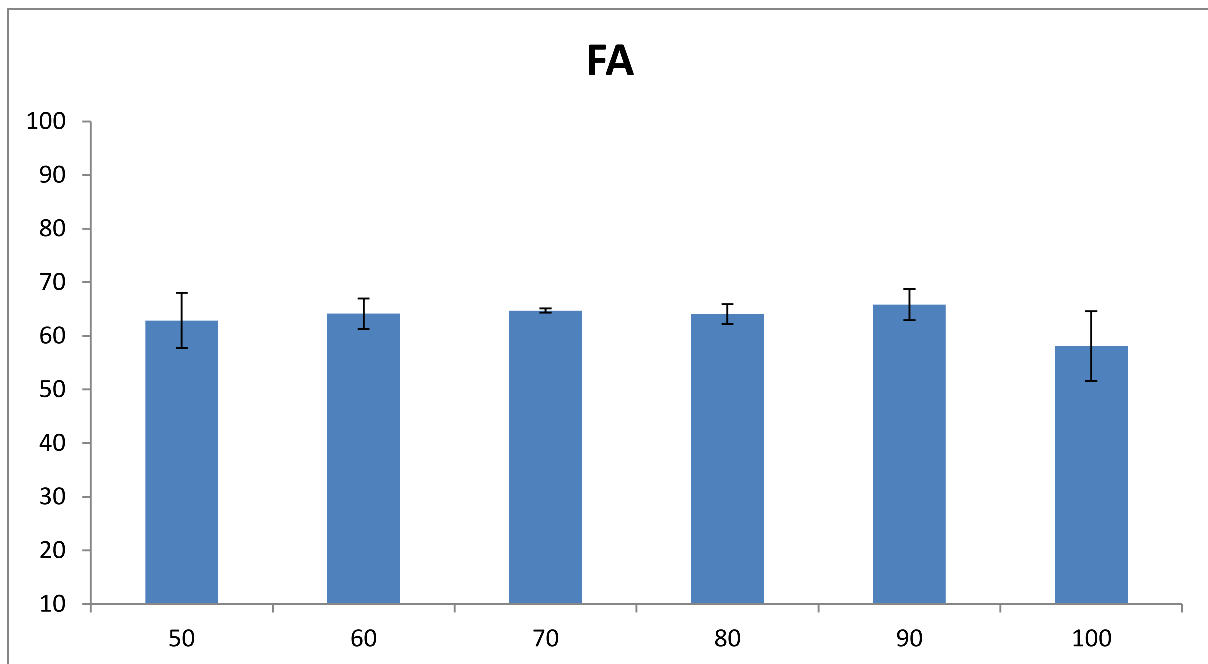

Figure 3.10 Effect of heat on flocculation of the purified bioflocculants

3.3.4 The effect of pH on the flocculating activity of the bioflocculants

A pH of the solution significantly influences the flocculation process of a bioflocculants (Wang *et al.*, 2011). Dispersion stability of suspended particles and the formation of flocs are highly affected or dependent on the surround pH. Furthermore, different microorganisms produce different bioflocculants of different compositions which react different on pH ranges (Wang *et al.*, 2011). This organism produced a bioflocculant that had highest flocculating activity at the extreme pHs ranges (acidic and alkaline pH); pH 12 showed highest flocculation of 83.3%, while, from the acidic range, pH 3 had a flocculation of 76.2%. Ntsaluba *et al.* (2013) observed the same phenomenon whereby the extreme pH ranges showed high flocculation, with the highest flocculating activity of 79% obtained at pH 11. Similarly, high flocculation activity (76%) was observed at the acidic pH of 3. The reason behind the dual flocculation optimum, at an acidic and alkaline pH, is unclear. Various pHs have been documented to maximally support flocculation activity of bioflocculants (Ntsaluba

et al, 2013). Okaiyeto *et al.* (2014) reported that a consortium (*Halomonas* sp. Okoh and *Micrococcus* sp. Leo) produced a bioflocculant that flocculated optimally at pH 8. Yim *et al.,* 2007 reported that the bioflocculant p-KG03 produced by a marine dinoflagellate, *Gyrodinium impudicum* KG03, flocculated best under acidic conditions of pH 4.

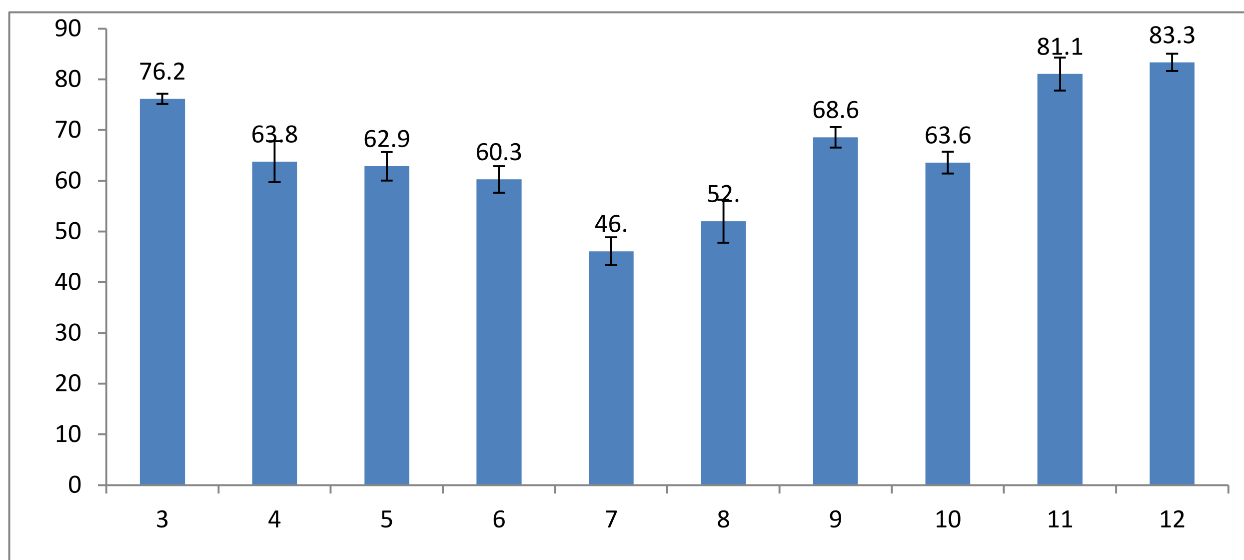

Figure 3.11 Effect of pH on the flocculating activity of the bioflocculants

3.3.5 Compositional analysis of the purified bioflocculant

A broad stretching peak was observed at 3474.13 and 3417.63 cm^{-1} which was indicative of hydroxyl groups and amine groups. This may also be as a result of vibration of -OH or -NH present in the sugar ring (Xiong *et al*., 2010; Cosa *et al*., 2013 and Akaiyeto *et al*., 2013). A weak peak at 2,927 cm^{-1}, known to be typical of carbohydrates indicated C-H asymmetrical stretching vibration (Xiong *et al*., 2010; Wang *et al*, 2011). Two peaks at 2476.95 cm^{-1} and 2364.85 cm^{-1} is indicative of C-H alphatic bonds (He *et al*., 2010). A weak peak at 2964.84 cm^{-1} and 2924.08 cm^{-1} known to be typical of carbohydrates indicated COH asymmetrical stretching vibration. An asymmetrical stretching peak observed at 1647.58 cm^{-1} was characteristic of C=O stretching vibration in $-NHCOCH_3$. The weak peak at 1,384.15 cm^{-1}

may be assigned to be C=O symmetrical and asymmetrical stretching of a carboxylate group in the bioflocculant. The peak at 1544.51 cm^{-1} was attributed to the N–H bending vibration (Wang *et al*, 2011). In addition, the strong absorption band at 1069.52 cm^{-1} indicated asymmetrical stretching vibration of a C-O-C ester linkage, and the weak peaks (873.58 cm^{-1}) identified are indicative of the presence of sugar derivatives. Furthermore, according to Xiong *et al*. (2010), the small absorption peaks may be suggestive of β-glycosidic linkages amid the sugar monomers. Hence, the FTIR spectrophotometric analysis suggests the presence of carboxyl, hydroxyl, and amine groups which are major adsorptive forces of the bioflocculant and crucial for the process of flocculation (Wang et al., 2011). The observation also suggests that the carboxyl groups, as the binding sites for cation, allow improved flocculation. For example, the OH, COOH, and COO- groups may link with the H+ and OH- present on the surface of the particles, thus forming hydrogen bonds when the bioflocculant chains approach the particles' surface (Zheng *et al*., 2008).

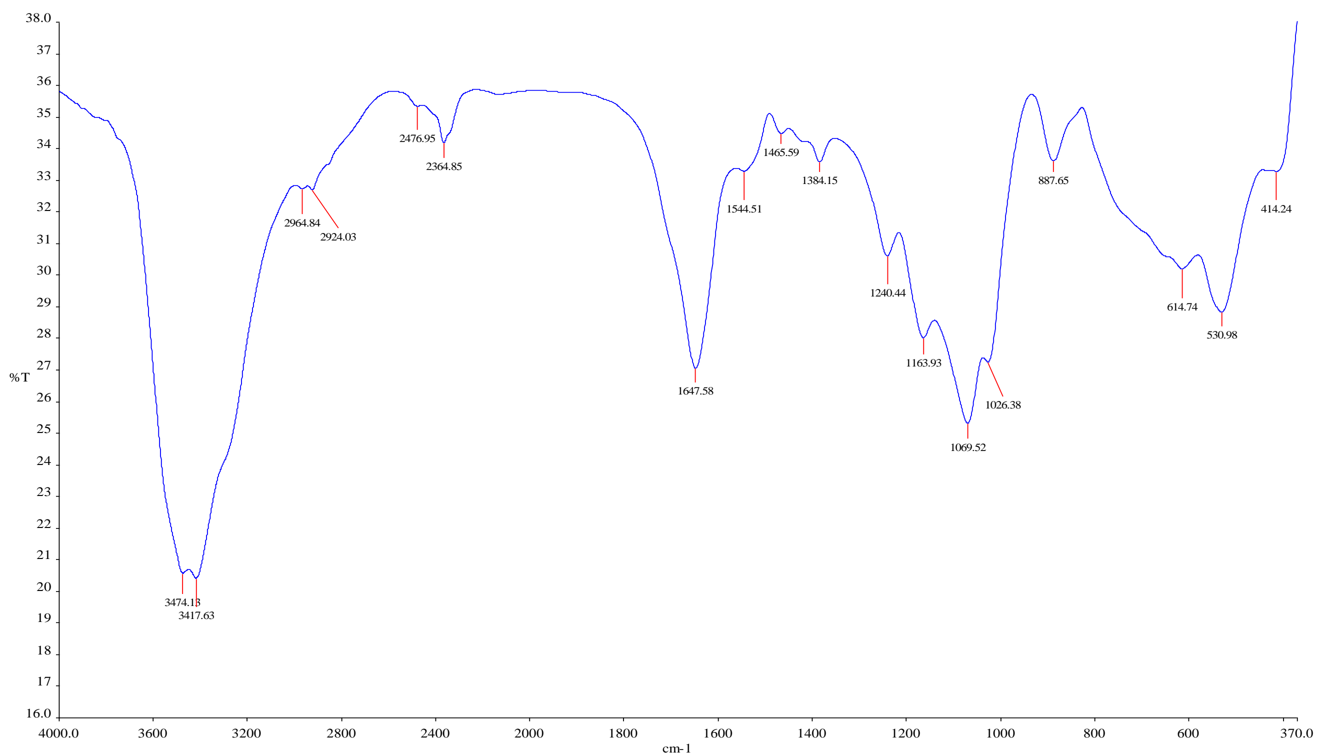

Figure 3.12 FTIR spectroscopy of purified bioflocculant obtained from *Enterococcus* sp.

3.3.6 SEM imaging analysis

SEM observations were carried out to elucidate the surface morphology of the biopolymer and its flocculation to Kaolin clay. As we can see from figure 3.13 below, the bioflocculant shows a crystal-linear structure which reveals an excellent flocculating performance structure that the bioflocculant would have when it interact with any molecules, examples are kaolin clay molecules

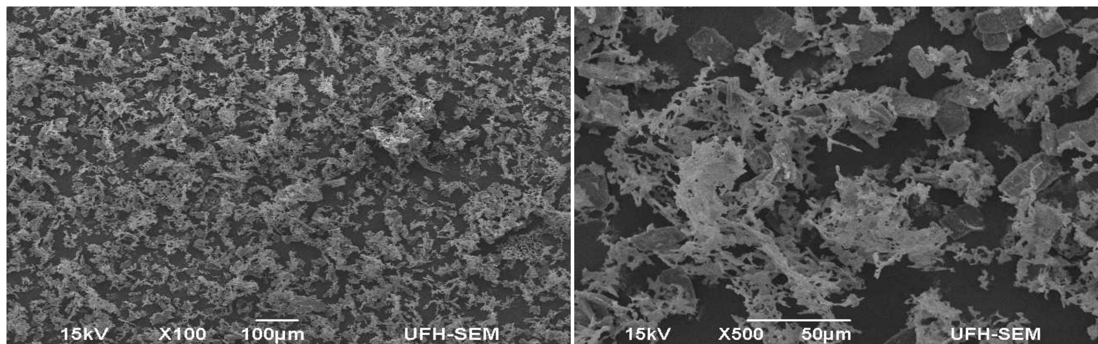

Figure 3.13 SEM spectroscopy of purified bioflocculant obtained from Enterococcus sp

4.0 CONCLUSION

Although there are challenges that need to be looked at, such as to developing conditions for large-scale production of the bioflocculant, and doing further characterization of the bioflocculants, as a way of concluding, the study showed that the bacterial species isolated from marine sediments efficiently produced bioflocculants, and a need, therefore, exists to further expand the scope of the application of this bioflocculant.

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CHAPTER FOUR

Bioflocculant production by *Bacillus* sp. isolated from the bottom of marine sediment of Algoa Bay in the Eastern Cape, South Africa

ABSTRACT

A bioflocculant-producing marine bacterium previously isolated from marine sediment of marine environments was screened for bioflocculant production. A comparative analysis of the 16S rDNA sequence of the isolate in the GenBank database showed 98% similarity to *Bacillus thuringiensis* MR-R1. The bacteria produced bioflocculants optimally when the media had mixed nitrogen sources (Urea, ammonium chloride and tryptone) (67%) and glucose (85.65%) as a sole carbon source. Moreover, Ca^{2+} (74.6%) was the best cation that induced the production of bioflocculant. After purification, the bioflocculant flocculated optimally in alkaline pH (12) (81%) in the presence of Mn^{2+} (73%) and Ca^{2+} (72.8%). Chemical analysis of the bioflocculant revealed it to be a polysaccharide.

4.0 INTRODUCTION

Over the years, researchers have been trying to displace the use of inorganic flocculants such as aluminum sulfate and polyaluminum chloride and organic synthetic flocculants such as polyacrylamide derivatives and polyethylene imine (Zhang *et al*., 2007; and Salehizadeh, and Shojaosadati, 2001). Although these flocculants have advantages such as low cost and effectiveness in flocculation, they have been associated with health problems, and their monomers are non-biodegradable (Zhang *et al*., 2007). These adversities of these chemical flocculants have led to scientists exploring other means such as bioflocculants. This is because it has been discovered that bioflocculants produced by microorganisms during their growth are safe and biodegradable polymers. At the beginning of the bioflocculants discovery, a problem of the identified bioflocculants was their dependency on cations for high flocculation. Since then, various bioflocculant-producing organisms have been discovered such as bacteria, fungi, yeast, actinomycetes and algae (Salehizadeh, and Shojaosadati, 2001).

Although microbial flocculants have discovered to have health and environmental advantages, one of the disadvantages or concerns is the low yield and high rates. Therefore, the challenge is to discover a bioflocculant that can be economically produced, has high yield, and can be produced economically in large-scale culture (Zhang *et al*., 2007; and Salehizadeh, and Shojaosadati, 2001). To achieve this, unusual environments such as the marine environments are beginning to be of great interest to isolate organisms with unique metabolic features to produce such bioflocculatants, and in this study, the organism of study was isolated from the marine environment. It is anticipated that such an environment may be a reservoir of novel bioflocculant that produces organisms due to the uniqueness of the marine environmental conditions which are very different from terrestrial environment (Cosa

2010). According to Cosa (2010) and Zhang *et al*. (2002), soil and activated sludge are the typical environments for isolation of bioflocculant producing organism, therefore, screening unfamiliar environments like marine could lead to unusual novel bioflocculant.

METHODS AND MATERIALS

4.1 Screening for bioflocculant- producing bacteria

Several hundred marine bacteria were isolated from the bottom sediments of Algoa Bay in South Africa as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, South Africa.
In the laboratory, the samples were serially diluted into sterile saline water. About 100 micro-litres of the diluted solution was spread on Marine Agar (MI) and Reasoner´s 2A agar (R2A). About 2ml of the sediment samples was transferred into 8ml of sterile saline water and agitated for 30 seconds. From there, serial dilutions were made. Similarly 100 micro-litres of the serially diluted sample were spread on the surface of M1 and R2A plates, following the method of Jensen *et al*. (1990)

The R2A Agar plates were incubated for 4 days at 28 ℃, and M1 agar plates were incubated for 8 days in similar conditions. Colonies from both media were randomly picked and isolated on fresh plates and incubated for 2 and 5 days for R2A and M1 respectively.

4.2 Activation of the isolates for fermentation

1L of the activation medium containing 3 g of beef extract, 10 g of tryptone and 5 g of sodium chloride was prepared. About 5 ml of the activation media was measured into

different test tubes and autoclaved at 121 ºC. After inoculation of the isolates into the tubes, they were incubated in a rotary shaker for 24 hours at 28 ºC, 160 rpm.

4.3 Bioflocculant producing medium and cultivation

Screening of the isolates for bioflocculants production was done following the method of Zhang *et al*, 2007. The medium composition includes: 20 g glucose, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.3 g $MgSO_4$, 0.5 g Yeast extract,0.5 g Urea, and 0.3 g NH_4S0_4 in 1L of filtered marine water. The components were autoclaved and mixed aseptically. The seed culture (1ml), after 24 hours of incubation, was inoculated into 50 ml of the growth medium in 250 ml flask. The culture was incubated in a rotary shaker at 28 ºC, 160 rpm for 5 days. After 72 hours of incubation, samples were withdrawn and flocculating activity of the produced bioflocculants were checked according to the method of Kurane *et al.* (1994)

2.4 Assay of flocculating activity

The flocculating activity was determined using the method previously described by Kurane *et al*. (1994) in which Kaolin clay was chosen as the suspended solid. Two milli-litres of the culture supernatant and 3 ml of 1% $CaCI_2$ were added into 100 ml of Kaolin clay suspension (4 g/l) in a 100 ml flask, gently shaken and left to stand still for 5 min. The control was prepared following the same procedure, but the bioflocculant was replaced by fresh broth. The turbidity in the upper phase was measured with a spectrophotometer at 550 nm and thus, the flocculating activity was estimated as follows:

Flocculating rate = $\{(A-B)/A\} \times 100\%$

Where A is the optical density of the control at 550 nm and B is the optical density of the sample at 550nm. All experiments were performed in triplicates for the mean calculation.

4.5 The effect of culture conditions on bioflocculant activity

4.5.1 The effect of carbon and nitrogen sources

In various studies, it has been proven that alteration or changing of nitrogen and carbon sources influence both bacterial growth and flocculating activity (Cosa *et al*., 2013). Hence, in the study, the effect of different carbon and nitrogen sources on flocculating activities was assessed. These carbon sources included: glucose, sucrose, fructose, starch, lactose and maltose, while the nitrogen source candidates included ammonium chloride, urea, tryptone, peptone and yeast extract. The assessments were done in accordance with the description of Lachhwani (2005).

4.5.2 Effects of various cations and pH on flocculating activity

The effect of various salts on both the production and flocculation of the bioflocculant by the strain was evaluated. Flocculation tests were conducted utilizing the procedure elaborated above, but the $CaCl_2$ solution was replaced by various metal salt solutions, and the flocculating activity was measured. These salts are: Al^{3+}, Fe^{3+}, Mg^{2+}, K^{+}, Na^{+}, Li^{+}, Mg^{2+} and Mn^{2+}. To assess the effect of pH on flocculating activity and production, the pH of the kaolin suspension was adjusted using HCl and NaOH in the pH range of 3-12 respectively (Yim *et al*., 2007).

4.5.3 The effect of heat on the flocculation and production of the bioflocculants

To evaluate the effect heat has on both the production and flocculation of the bioflocculant, kaolin clay and supernatant were heated at different temperatures (50, 60, 70, 80, 90, and100 ºC), and the same was done to the purified powder.

4.6 Time course of bioflocculant activity

Composition of culture medium: for the time-course experiment, optimum media was used, and the composition of the medium was as follows: 20 g of glucose; 5 g of KH_2PO_4; 1 g of urea; 0.3 g of $MgSO_4$, $7H_2O$ per litre of filtered natural sea water (Zhang *et al*., 2007). *Standardization of the inocula*: a saline solution was prepared by adding 0.45 g NaCl in 50ml of distilled water. Fifty milli-litres of saline solution were inoculated with a loop full of colonies for each of the strains. The Optical 40 density (660 nm) of each was measured by taking 100 µl (culture) into 1 ml of distilled water in 3 ml cuvettes and readjusted when need arose to give 0.1 (Cosa *et al*., 2013).

Time course assays, according to the method of Gao *et al*. (2006) and Cosa *et al*., 2013, were that the inoculated saline solution was used as seed culture for inoculum preparation. Seed culture (4% v/v) was inoculated into 200 ml of medium in 500 ml flasks on a rotatory shaker (160rpm) at 28 degrees Celsius. A sample was drawn at appropriate time intervals (every 24 hrs) for a period of 10 days. 2 milli-litres of culture broth were centrifuged at 4000 g for 30 minutes, and the cell-free supernatant was used to determine the flocculating activity. The pH of the broth sample was also measured, and cell growth was determined by plating the broth after every 24 hours. All experiments were performed in triplicates.

4.7 Purification and characterization of the bioflocculant compound

4.7.1 Extraction and purification of the bioflocculant

Purification and characterization of the bioflocculant was done following the methods described by Chen *et al*., 2002, Mabinya *et al.,* 2012 and Cosa *et al*., 2013) using media formulation based on the pre-determined optimum culture conditions.

4.7.2 Analysis/ characterization of purified bioflocculant

Effect of pH, thermal stability, and cation salts on the flocculating activity of the purified bioflocculant was evaluated. The protein content of the purified bioflocculant was measured using the Folin-Lowry method. Total sugar content was measured using phenol-sulphuric acid protocol, as described by Lachhwani (2005).

4.8 Identification of the Bacteria

The bioflocculant producing bacteria was identified by molecular technique as described by Cook and Mayers, (2003) and Nwodo *et al*. (2012). The bacterial 16S rDNA gene was amplified by polymerase chain reaction (PCR) followed by sequence analysis of the amplified gene. The bacterial DNA was extracted through the boiling method, and the PCR amplification was carried out in 50 µL reaction volume containing 2 mM MgCl2, 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-ACGGITACCTTGTTACGAC TT-39) and 2 mL of the template DNA. Primers F1 and R5 bound to base positions 7–26 and 1496–1476 of the 16S rRNA gene of Streptomyces ambofaciens ATCC23877, respectively (Cook and Meyers, 2003). The PCR condition included the following: 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.

3.0 RESULTS AND DISCUSSION

The initial screening media contains glucose (20 g/L) as the sole carbon source and cocktail of three nitrogen sources (urea, yeast extract, and ammonium sulphate) that mount to 1.3 g/L. To find out a suitable carbon source that would improve flocculation, various carbon sources were evaluated so as to improve the flocculation of the bioflocculant. The table below shows the effects of various carbon sources on bioflocculant production. Glucose with flocculating activity of 85.65% was the best carbon source for the production of bioflocculant by the strain, followed by sucrose, lactose, maltose, and starch with flocculating activity of 60.6%, 22.45%, 18.5% and 10.2% respectively. Glucose was then used is subsequent reactions. Glucose has been well-documented in other studies as the best organic, sole carbon source (Cosa *et al.*, 2013). Furthermore, a literature search shows that numerous bioflocculant producing bacteria prefer organic carbon sources for bioflocculant production (Gong *et al.*, 2007, Chen *et al.*, 2008, Ntsaluba *et al.*, 2011, Cosa *et al.*, 2013).

In addition, the influence of nitrogen on bioflocculants production was investigated with glucose as sole carbon source; screening media used was composed of 3 different nitrogen sources, amounting to 1.3 g/L. on optimizing for the pre-eminent nitrogen source, In place of the multiple-nitrogen sources (yeast, urea and ammonium sulphate) used, individual nitrogen source were incorporated. However, the concentration used amounted to the sum of that multiple-nitrogen source used in the screening media. Out of the 5 sole nitrogen sources used, peptone was the best sole nitrogen source with 63.6%, but the combined nitrogen

sources (urea, yeast extract, and ammonium sulphate) had the highest activity of 75.9%. Cosa *et al.* (2013) observed that complex nitrogen substrate consisting of urea, yeast extract and $(NH_4)_2SO_4$ enhanced cell activity more significantly and was better suitable for the bioflocculant production than the other nitrogen sources tested. Furthermore, in correlation with our results, Gong *et al*. (2008) also reported that higher flocculating activity (94.1%) was obtained when complex nitrogen source used consisted of beef extract and urea. Therefore, complex nitrogen source was better than the sole inorganic or organic nitrogen sources.

The effect of cation on bioflocculants production was investigated, of 8 cation sources, Ca^{2+} and Al^{3+} and Mn^{2+} showed the highest flocculating activity of 62.3%, 69.8% and 65.1% respectively. The initial screening media, with complex nitrogen source (urea, yeast extract and $(NH_4)_2SO_4$) and glucose as sole carbon source, was used in subsequent reactions and also in production of the purified powder because it was observed that any change in the composition of the media would decrease flocculation. A study by Ugbenyen *et al*. (2012) reported that *Cobetia* Spp produced a bioflocculants that were stimulated by Na^+, K^+, Li^+, $Ca^{2+,}$ Mg^{2+} and Mn^{2+} resulting in flocculating activities of over 90%, Feng and Xu 2008. They also reported that bioflocculant production by *Bacillus* sp. BF3-3 was stimulated by $Al^{3+,}$ Mg^{2+} ,$Ca^{2+,}$ K^+ and Na^+.

Table 3.1. Effects of composition medium on the bioflocculant production and activity

Carbon source	**Fructose**	**Sucrose**	**Maltose**	**Glucose**	**Lactose**	**Starch**		
Flocculating Activity (%)	0	60.6	18.5	85.65	22.45	10.2		
Nitrogen source	**Peptone**	**Urea**	**Yeast**	**Tryptone**	**NH_2SO_4**	**combined**		
Flocculating Activity (%)	63.6	27.7	33	22.1	35.25	75.9		
Cation	**Ca^{2+}**	**Mg^{2+}**	**Mn^{2+}**	**L^+**	**Na^+**	**Fe^{3+}**	**Al**	**K^+**
Flocculating Activity (%)								
	62.3	61.5	65.1	23.9	13	5	69.8	12.9

The table above below shows two parameters and these are thermal stability and cation source. The parameters were tested during the supernatant and on the purified bioflocculant. Results shows that the bioflocculant was not adversely affect by increase in heat, and only a slight decrease was observed from 50 to 100 degrees Celsius. Comparing the stability of the bioflocculant in a form of supernatant and purified powder shows that the powder has much resistance to heat. He *et al*. 2004 reported that increasing temperature up to 100℃ caused the tability of REA-11 to collapse. The purified powder showed an increase in flocculation at 100 degrees Celsius. This may be explained by the release of extra and inner-cell polysubstances after heat at higher temperature, and the same phenomenon was observed by Gong *et al*., (2007). Additionally, the effect of cations on flocculating activity on both the supernatant and the purified powder were investigated. Mostly, the cation is used as coagulant aid in achieving high flocculation activity by neutralizing the negatively charged functional groups on the bioflocculant and suspended particles, thereby increasing the adsorption of bioflocculant to the suspended particles (He *et al*., 2010, and Cosa *et al*., 2013. Of all the 8

cation sources investigated, all showed good flocculating activities with only Fe^{3+} that showed flocculating activity of less than 50%. In comparing the effect that each cation source had on the supernatant and on purified bioflocculants powder, not much change occurred in terms of the preference of cation after purification stages. The bioflocculants preferred Al^{3+} on both supernatant and powder 75.2%, and 73.9% respectively. Ca^{2+} was the second preferred, with 74.9% and 72.8% for supernatant and powder respectively; a correlation was observed between the supernatant and powder, and there was not much change in terms of cation source preference. Similar results were also reported by Cosa *et al*. (2013) where Al^{3+} and Ca^{2+} aided to flocculation by improving flocculation. Gong *et al*. (2010) and He *et al*. (2010) observed similar results of Ca^{2+} enhancing flocculation greatly.

Table 3.2. Effects heat and cation on the flocculating activity of both supernatant and the purified bioflocculants

TS (℃)		50	60	70	80	90	100		
	SRB (%)	56.7±4.3	58.0±0.7	58.8±1.7	56.5±2.4	48.8±3.8	43.2±3.9		
	PB (%)	66.6±0.64	70.0±1.2	70.8±0.0	72.2±1.5	69.4±2.3	73.8±1.6		
cation		**Mg^{2+}**	**Na^{+}**	**Al^{3+}**	**Li^{+}**	**Fe^{2+}**	**K^{+}**	**Ca^{2+}**	**Mn**
	SRB	53.8±1.5	53.2±2.0	75.2±2.8	49.2±8.9	9.1±48.8	52.8±7.1	74.9±9.4	73.2±3.2
	PB (%)	70.9±2.1	57.2±3.9	73.9±3.4	57.3±2.6	8.7±6.8	65±0.8	72.8±4.3	-

TS = Thermostability; PB = Purified bioflocculant; Supernatant rich bioflocculant;

3.1 Effect of inoculums size on production of the bioflocculant

Different inoculums were used to determine the optimum inoculum size for the strain. Inoculum sizes of 0.5 mL, 1.0 mL, 1.5 mL and 2.0 mL in 50 mL production medium were

investigated, representing 1%, 2%, 3% and 4% (v/v) respectively. Inoculum size of 4 % (v/v) was the best, with 85.1% flocculating activity. Increase in inoculums size resulted in decrease of flocculating activity, and the inoculums size of 4%, thereafter, was used in subsequent experiments. The finding proves that inoculums size plays an essential role in flocculation, as small volumes prolong lag phase during growth, while too large volume make the niche of strains overlap and restrain the bioflocculant production due to the limit of nutrient allocation (Zhang *et al*, 2012). A bioflocculant by *Virgibacillus* species was produced optimally at inoculum size of 2% Cosa *et al*, 2013. The inoculum size of 5% though was observed to be optimum by Kallos & Behie (2000) and Wang *et al*. (2007).

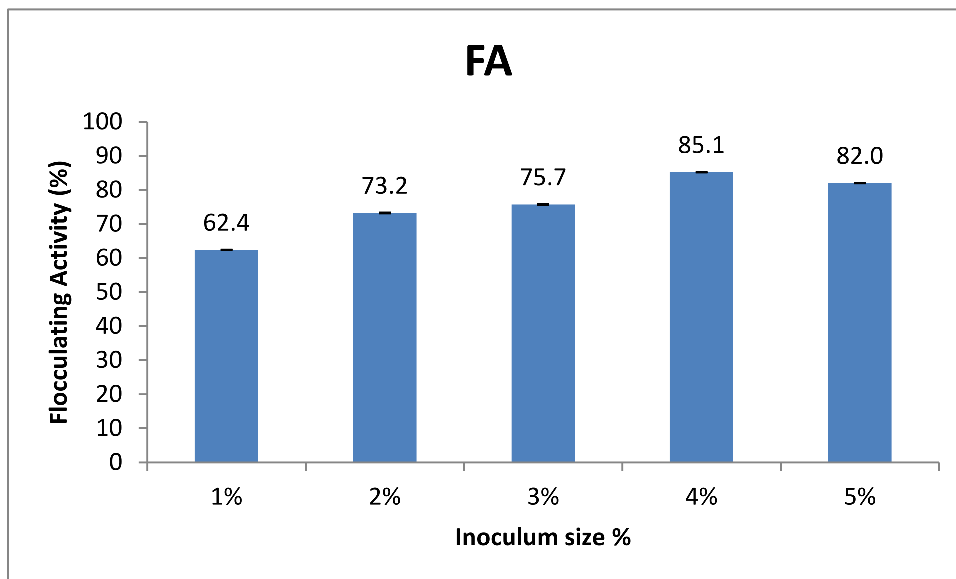

Figure 3.1 Effect of inoculum size on bioflocculant production

3.3 Bioflocculant distribution on culture broth

To determine the distribution of the bioflocculant, after 5 days of fermentation, the culture broth was centrifuged at 4000g for 30 minutes. After centrifugation, the precipitate (the composition included mycelium cells, cellulose and so on) was collected and dissolved in distilled water. The distribution of flocculating efficiency of the culture broth was examined.

The highest flocculating activity (75.95 %) was present in the culture broth. The flocculating efficiency of the cell was only 62%. Bacillus licheniformis also produced a bioflocculant that was distributed in the supernatant (Xiong *et al*., 2010). It was evident that the bioflocculant was the material excreted in the form of culture broth, or supernatant. Liu and Cheng (2010) also observed the same phenomenon in a bioflocculant produced by *Penicillium sp.*

Table 3.3: Bioflocculant distribution

Strains	FA supernatant (%)	FA Pellet (%)
M90	75.95	62.772

3.4 Time course of bioflocculant production

The time course of the bioflocculant production by *Bacillus thuringiensis* MR-R1, in relation to pH, OD, and cell growth was evaluated. There was a rapid exponential increase in bioflocculant production starting from the first 24 hours, and then it continued until it reached a peak after 60 hours. Thereafter, a decline or levelling of growth was observed, and this decline may be due to cell autolysis and enzymatic activity (Cosa 2010). From the flocculating activity curve, it was observed that the cell growth is parallel to the flocculating activity, indicating that the bioflocculant is produced during the growth of the bacterium. In this study, OD and cell count were simultaneously used to detect cell growth, biosynthetically not by cell autolysis. A gradual decrease in pH was observed from 5.89 on the first day to 2.96 after 72 hours, and the decrease in pH, with increase in cultivation time may be due to the production of organic acids from the metabolism of glucose or to the production of organic acid components of the polymer produced (Gao *et al*., 2006). In most studies, OD is

usually used as a tool to measure cell growth, and this is not as accurate as OD measures both viable and dead cells, while doing cell count gives only viable cells.

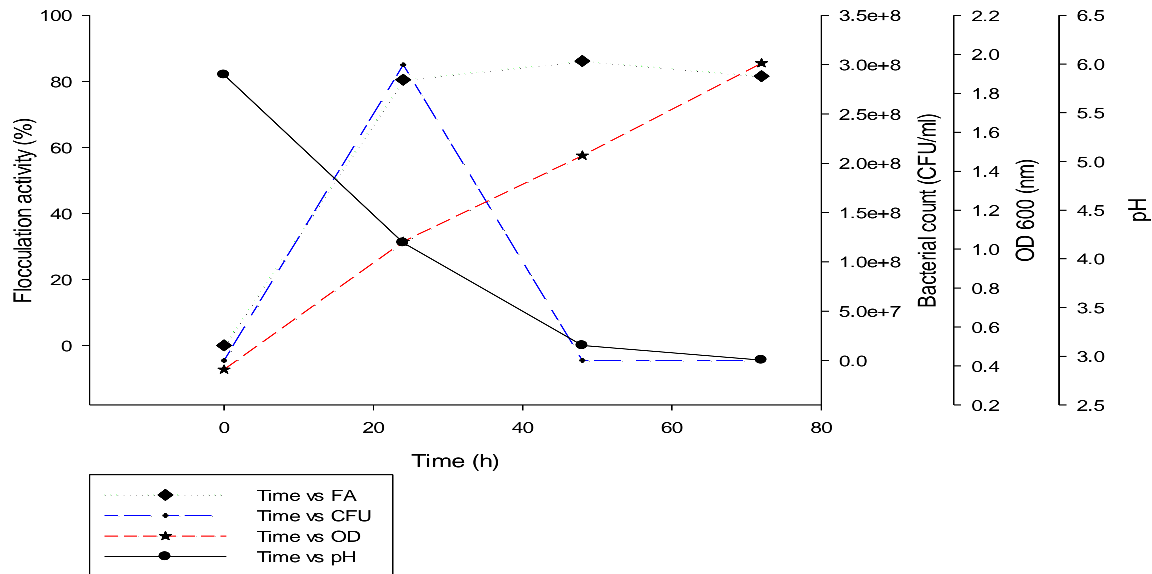

Figure 3.3 Time course of bioflocculant production by *Baccilus* sp

3.5 Factors that affect the flocculation of purified bioflocculant

3.5.1 Effect of Bioflocculant Dosage on Flocculating Activity of Purified Bioflocculant

The appropriate bioflocculant concentration to be used for subsequent experiments was determined by investigating different bioflocculant concentrations ranging from 0.1–0.5 mg/ml. Optimum activity was obtained at 0.5 mg/ml, and further increase to 1 mg/ml did not yield any increase. Bioflocculant dosage is strain-dependant, and Okaiyeto *et al*. (2013) observed that optimum dosage for bioflocculant is produced by Consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. Similar results were observed in a study by Deng *et al.* (2003) of the bioflocculant produced by *Bacillus mucilaginous* that required a dosage of 0.1 mg/ml bioflocculant for optimum flocculating activity. Ugbenyen *et al*. (2014) observed a concentration of 2 mg/ml, while Wang *et al*. (2011) reported mixed culture of *Rhizobium*

radiobacter F2 and *Bacillus sphaeicus* F6 to produce a bioflocculant with dosage of 12 mg/ml where a maximum flocculating activity of 96.21% was observed.

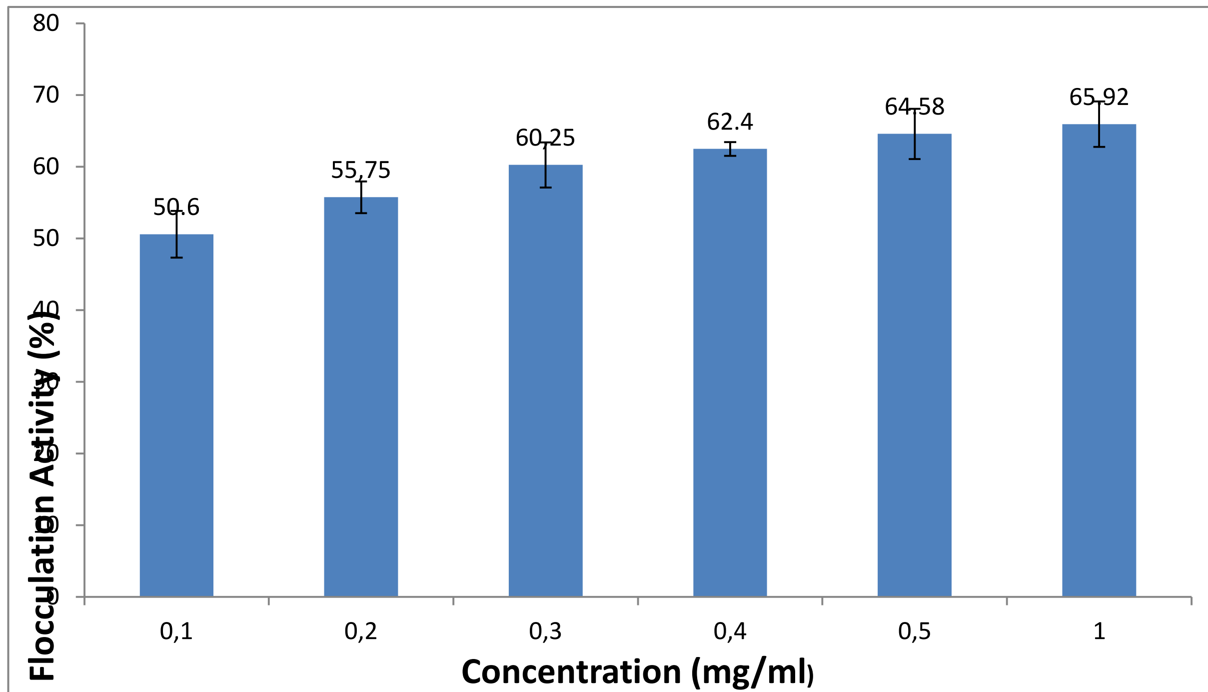

Figure 3.4 Effect of bioflocculant dosage on bioflocculant activity.

3.5.2 Effect of pH of the purified bioflocculants

Flocculation by the purified bioflocculant was greatly affected by change in pH. To assess the effect of pH on flocculating activity of the purified bioflocculant, the optimum dosage of 5mg/ml was used, and different pH values were adjusted from 3 to 12. In the present study, the flocculating activity was retained all through the pH range (3-12). However, it was observed that highest flocculation activities were observed at the extremes, with maximum activity at pH 12 (81.25%). This implies that the bioflocculant can be used to treat or used at various pH ranges especially at acidic and alkaline conditions. Literature stipulates that the alteration of pH may ultimately alter the bioflocculant charge status and surface characteristics of suspended materials, consequently changing the flocculating ability (Cosa *et al*., 2013 and Zhang *et al*., 1999)

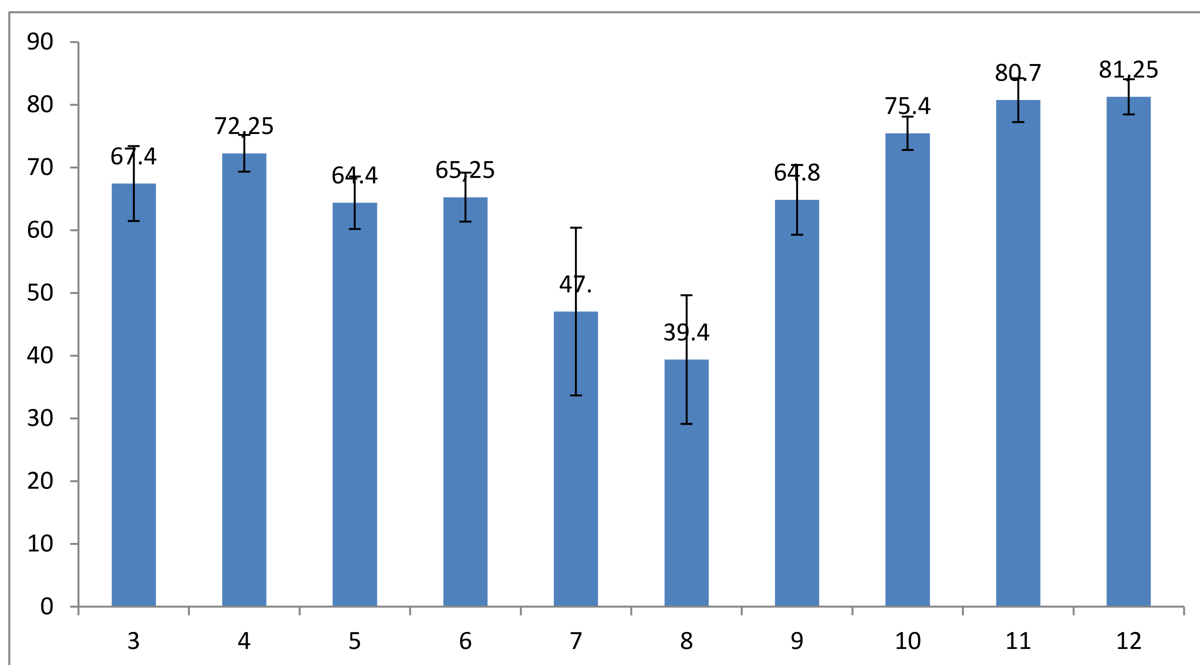

Figure 3.5 Effect of pH on bioflocculant Activity

3.6 FTIR analysis of the purified bioflocculant

A broad stretching peak was observed at 3418.54 cm^{-1} which was indicative of hydroxyl groups and amine groups. This may also be as a result of vibration of -OH or -NH present in the sugar ring (Xiong *et al*., 2010, Cosa *et al*., 2013 and Akaiyeto *et al*., 2013), and the weak peaks (844.90 cm^{-1}) identified are indicative of the presence of sugar derivatives, 1460.62 cm^{-1} which indicated the presence of uronate in the polysaccharide. The vibration peak at 1135.81 cm^{-1} corresponded to the C-O stretching in alcohols and this further suggests the presence of OH group in the bioflocculant molecule (Akaiyeto *et al*., 2013). A peak at 1637.65 and 1460.62 (cm^{-1}) represents the presence of carboxyl and hydroxyl groups from polymeric and dimeric OH stretches of phenol or tertiary alcohol bends. The strong absorption peak observed at 530.03 cm^{-1} was known to be typical characteristics of sugar derivatives. The infrared spectrum of the bioflocculant thus indicated the presence of phospho-, carboxyl, hydroxyl, and amino- groups (Liu and Cheng, 2010).

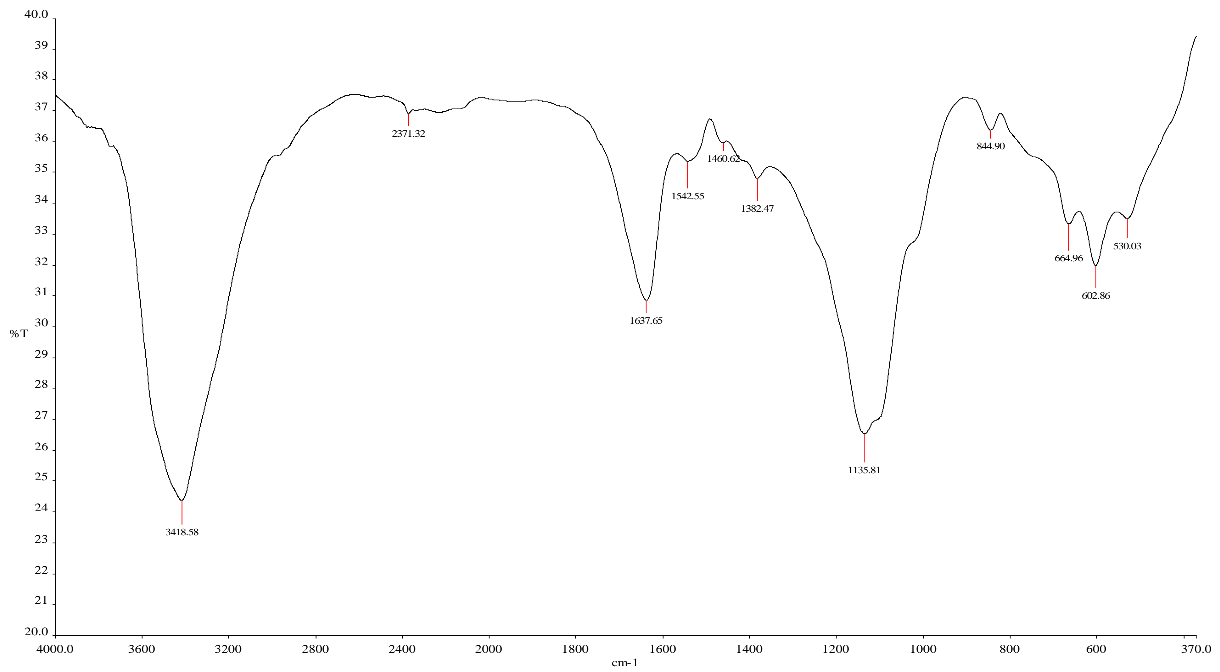

Figure 3.6 FTIR spectroscopy of purified bioflocculant obtained from *Bacillus* sp.

3.7 SEM analysis of the purified bioflocculant

Scanning Electron Microscope (SEM) observations were carried out to elucidate the surface morphology of the biopolymer, and it showed that the bioflocculant was whitish in colour and porous. The morphology of the purified bioflocculant shown in the figure above is crystal, linear, and spongy-like structure.

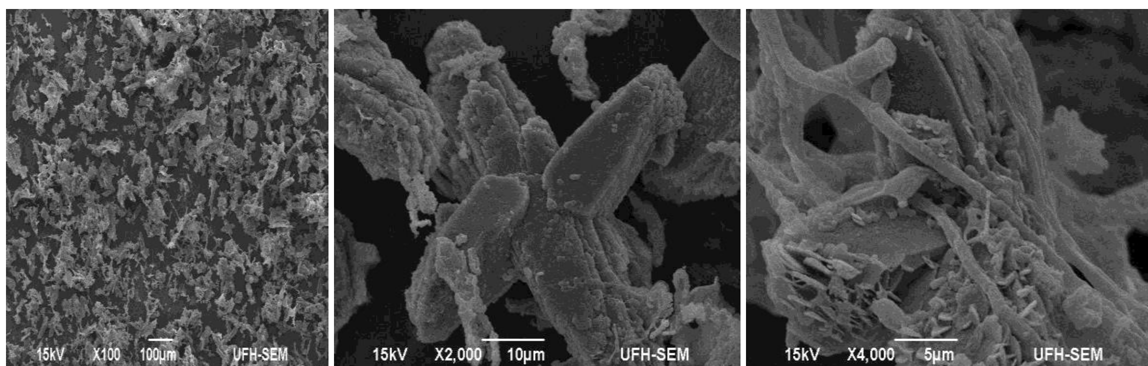

Figure 3.7 SEM spectroscopy of purified bioflocculant obtained from *Bacillus* sp

4.0 CONCLUSION

This study has shown that *Bacillus thuringiensis* is a potential source of new polysaccharide bioflocculant(s), the production of which could be optimal using glucose as a carbon source and a combination of nitrogen sources (Urea, ammonium chloride and tryptone), as well as pH of 12. Further characterization of the purified bioflocculant, as well as development of process condition and practical application for large scale production of the bioflocculant, would be developed in further progress in our group.

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CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

The environment at which the flocculant producing organism is isolated from (whether its bacteria, fungi, actinomycetes or yeast) contributes on the composition, stability and flocculating efficiency of the produced bioflocculants. Soil, activated sludge and very few from marine environments have been documented (Cosa 2010; Deng *et al*., 2005). Therefore exploring rarely explored environments could possible discover a novel efficient bioflocculants with characteristics that could overcome the problem hindering large scaling of production of bioflocculants i.e. high production costs and low flocculating efficiency (Li *et al*., 2009). In this study, we screened over 200 bacteria isolated from marine sediments for bioflocculants production and *Bacillus thuringiensis* sp and *Enterococcus hirae*, showed to produce bioflocculant efficiently. Enterococcus is a Gram positive, catalase negative, facultative anaerobe, bacteria, a normal human flora of the GI and urogenital tract of human and animals. Virulence factors such as Fimbriae and adhesins for attachment and has small white colonies, while bacillus is gram positive, forms endospores, and catalase positive Graeter *et al*., 2015.

Due to the ability that bacteria utilize nutrients in the environment (culture medium) to synthesize polymers present within the cell under the action of specific enzymes, it is therefore possible manipulate the extent at which the polymers are produced by varying or optimizing the nutrients at which the bacteria is expose to. In this manner bacteria converts the simple substances in their environment into polymers that can be utilized as flocculants (EPS) (Desouky *et al*., 2008; Cosa 2010). Various aspects affect the production of

bioflocculants, and these include physiological and environmental aspects (Salehizadeh, and Shojaosadati, 2001). Such aspects include carbon and nitrogen concentration (C/N ratio), the culture pH, temperature, cations to mention just the few, needs to be optimized for efficiency of production. This optimization is essential because productivity and distribution of bioflocculants depend on the culture conditions (Salehizadeh, and Shojaosadati, 2001). Of the two bacteria (*Bacillus thuringiensis* and *Enterococcus hirae*) studied in this study, the effect of the above mentioned factors (and even more) were optimized for the production of the bioflocculants and flocculation efficiency. Observed is that these factors are strain dependant, for example, *Enterococcus hirae* preferred fructose as the carbon source and urea while glucose was the best carbon source for *Bacillus thuringiensis*, and a cocktail of three nitrogen sources. Still on the factors that affect the production of bioflocculants, the effect of cation and pH on the medium was also evaluated and differed with organism. Time course of bioflocculant formation and activity *Bacillus thuringiensis* and *Enterococcus hirae* to pH and cell growth using both spread plate and OD were examined. Cell growth and the secretion of bioflocculants are correlated, for bioflocculants to be secreted, the microorganism have to reach a certain level of growth phase (endogenous) (Zhang *et al*, 2007; Salehizadeh and Shojaosadati, 2001). As noticed in the study, production and activity of the bioflocculant depends on culture time of the specific strain. For both strains, bioflocculants production was steadily increased with culture time, and was observed that the bioflocculants are produced during the active growth of each strain (biosynthetically), although each strain reached its peak activity at different culture times. Cell autolysis occurred after reaching of maximum peak. It was noticed that for both strains pH decreased gradually, this could be due to the production of organic acids either from metabolism of sole carbon source sugars (fructose and glucose) or the presence of organic acid components in the bioflocculant polymer being produced (Ntsaluba *et al*., 2011).

Now on the factors affecting flocculation, Kaolin clay was used as a suspension to test for the flocculation of the produced bioflocculants (Lachhwani, 2005). As a solution kaolin clay is negatively charged (Cosa, 2010; Li *et al*., 2008), therefore for the bioflocculant to affectively flocculate. During the process of kaolin flocculation the bioflocculant approaches the particles in the solution and the attractive force exceeds the electrostatic repulsion force (Cosa, 2010). Therefore to achieve that, the addition of cations may stimulate the flocculating activity of the bioflocculant by neutralizing and stabilizing the charge of the particles on solution and also enabling bridging whereby the bioflocculant easily absorbs onto the particles leading to effective flocculation. During the screening stages Ca^{2+} was used, due to the fact that the required cation differs with different strains, various cations were tested, and *Enterococcus hirae*, *Bacillus thuringiensis* both preferred $Ca^{2+.}$ On Optimization of conditions for factors affecting flocculation, both were done on the final purified product and supernatant, these factors includes temperature of the kaolin clay, metal ions and pH. They all play a crucial role in the flocculation, the flocculants with protein or peptide backbone in the structure are generally thermally labile, but those made of sugars are heat-stable, it was observed that both bioflocculants produced were heat stable. The flocculating activity of bioflocculants varies with pH (Salehizadeh and Shojaosadati, 2001), on both of the bioflocculants, they efficiently flocculated at the extreme pHs. Cations aid in flocculation by neutralizing the residual negative charge of the function groups between particles, forming bridges and stable flocs (Salehizadeh, and Shojaosadati, 2001). *Enterococcus hirae* flocculated efficiently as a powder and a supernantant when Mg^{2+} was used, while Ca^{2+} was preferred by *Bacillus thuringiensis*.Chemical analysis of the purified bioflocculants were done, this was done to elucidate structure, chemical nature and functional groups of these bioflocculants, as these determine mechanism of flocculation

In conclusion, the study showed that two bacterial species isolated from marine sediments efficiently produced bioflocculants. Upon optimization it was observed that (i) Enterococcus hirae: fructose and urea as carbon and nitrogen source respectively, with initial pH of 5, and Mg^{2+} as a preferential metal ion, and (ii) *Bacillus thuringiensis*: glucose and mixed nitrogen sources (Urea, ammonium chloride and tryptone) as carbon and nitrogen source respectively as well as pH 12.0. Both bioflocculants flocculates efficiently and can be used to replace synthetic flocculants in water treatment, wastewater, in downstream processing, and processing of food and chemicals and other industrial uses of flocculants.

5.1 RECOMMENDATION

Challenges though (i) are to develop conditions for large scale production of the bioflocculant, (ii) to do further characterization of the both bioflocculants (iii) Further to assess the bioflocculants for treatments of water/wastewater, and to apply it in various industrial processes

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