ASSESSMENT OF BIOFLOCCULANT PRODUCTION BY SOME MARINE BACTERIA ISOLATED FROM THE BOTTOM SEDIMENT OF 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

Several problems concerning the use of conventional synthetic flocculants has necessitated the need for alternative cost effective, safe and efficient bioflocculants from microorganisms inhabiting many environments, particularly those from unusual environments. Hence, this study assessed bioflocculant production by three marine bacteria isolated from the bottom sediment of Algoa Bay in the Eastern Cape Province of South Africa. Analysis of the 16S rDNA sequences led to their identification as *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky. Several factors affecting the production and activity of the bioflocculant(s) were studied. *Halobacillus* sp. Mvuyo produced bioflocculant optimally with glucose (76%) and ammonium chloride (93%) as sole carbon and nitrogen sources, respectively and at neutral pH and in the presence of Ca^{2+}. On the other hand, *Virgibacillus* sp. Rob preferred glucose (70.4 %) and iron sulphate (74%) as carbon and nitrogen source respectively; an alkaline pH (12.0) and $Fe^{2+.}$ *Oceanobacillus* sp. Pinky produced bioflocculant optimally when sucrose (80%) and peptone (72.4 %) were used as carbon and nitrogen source respectively, at neutral pH, and in the presence of Ca^{2+} cation. The chemical analyses of the partially purified bioflocculants revealed that the bioflocculants produced by *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky were glycoproteins, while that produced by *Virgibacillus* sp. Rob was a polysaccharide. We thus conclude that *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky hold promise as producers of new and efficient bioflocculant(s). We recommended development of process conditions for large scale production of the bioflocculants followed by their detailed characterization, as well as pilot scale assessment of the

applicability of the purified bioflocculant in water/wastewater treatments and other industrial uses.

CHAPTER ONE

INTRODUCTION

1.1 General background

Bioflocculation is defined as a process whereby flocculation is mediated by the presence of microorganisms or bioflocculants (which are biodegradable macromolecular flocculant secreted by microorganisms) (Gao *et al*., 2005). In a broader sense, it is the removal of colloidal particles from solution by flocculating substances of biological origins. In microbial systems, the process of flocculation was first depicted by Loius Pasteur in 1876 for yeast (*Saccharomyces cerevisiae*). A similar observation was reported in bacterial cultures. Thus, years later bioflocculation became vastly examined and a correlation was established between the accumulation of extracellular bioflocculants and cell aggregation (Salehizadeh and Shojaosadati, 2001). Since then, many bioflocculant-producing microorganisms i.e. yeast, bacteria, fungi, algae were screened or isolated from soil and wastewater (Salehizadeh *et al*., 2000).

A number of microorganisms (i.e. bacteria, algae, fungi and actinomycetes) are described as producers of bioflocculants (Desouky *et al*., 2008). Microbial bioflocculants are polymers produced by microorganisms during their growth, with their flocculating activity being dependent on the characteristics of the flocculants (Deng *et al*., 2005). These macromolecules have been described to be readily biodegradable (Yasaman *et al.,* 2010), and it is this characteristic along with their harmlessness and lack of secondary pollution that have gained bioflocculants much wider attention and research to date (Li *et al*., 1999).

Flocculants have been defined as agents, which are used, in fast solid–liquid separations. Their addition enables dispersed particles to aggregate together and form flocs of a size to make them settle speedily and to clear the system (Sharma *et al*., 2006). They are said to act on a molecular level on the surfaces of the particles to reduce repulsive forces and increase attractive forces (Sharma *et al*., 2006). For the past years, the chemical synthetic flocculants have been widely used due to their effectiveness and low costs (Yim *et al*., 2006). These (chemical or synthetic flocculants) included the three different classes of flocculants: i.e. organic flocculants which include polyacrylamide, polyethyleneimine, inorganic flocculants (polyaluminium chloride and aluminium sulphate) and natural flocculants (gelatin, chitosan) (Yim *et al*., 2006). They have been vastly utilized in chemical and mineral industries treatment of water/wastewater, dredging, food and in fermentation industries. However, synthetic flocculants have been reported to be hazardous and have been shown to be carcinogenic, neurotoxic and non-degradable, and also associated with induction of Alzheimer's disease (He *et al*., 2002; Zhang *et al*., 2006). These negative properties of synthetic flocculants have encouraged exploration for new safer alternatives such as bioflocculants.

Thus far, according to recent reports, over sixty bioflocculants have been screened and identified (Deng *et al*., 2005). Some of these have been noted for their dependency on added cations for flocculating capabilities. As an example, according to Dermlim *et al*. (1999), the bioflocculant produced by *Klebsiella* sp. S11 cannot flocculate without the aid of calcium chloride solution. Also, *Enterobacter aerogenes* could not show any bioflocculant activity without the addition of zinc (Dermlim *et al*., 1999). These cations

thus consequently increase costs and secondary pollution. Hence, it is crucial and desirable to find novel bioflocculants that are independent of cations to reduce costs (Zheng *et al*., 2008).

Much focus have been based on isolating and screening terrestrial microorganisms for bioflocculant production, with very little effort based on marine microorganisms. The marine environment embodies a largely untapped source for microorganisms with ability to produce biologically active secondary metabolites (Bredholt *et al*., 2008). The marine realm covers up to 70% of the earth's surface and over 90% the volume of its crust, thus providing the largest inhabitable space for microorganisms (Lam, 2006; Fenical, 1993). It is regarded as massively complex and contains diverse assemblage of life forms, which occur in environments with extreme variations in pressure, salinity, and temperature. The conditions of marine environment are found entirely different from that of terrestrial environment suggesting that marine microorganisms have unique characteristics, thus may produce different novel bioactive compounds (Stach *et al*., 2003; Lam, 2006). For example, those conditions under which marine microorganisms survive and or adapt to include, extremely high pressure and anaerobic conditions at temperature below 0 ^{o}C on the deep sea floor to high acidic conditions (pH ~2.8) at temperatures of over 100 ^{o}C near the hydrothermal vents at the mid-ocean ridges (Lam, 2006). These organisms have developed exclusive metabolic and physiological capabilities that ensure survival in such extreme habitats and ability to produce metabolites not found with terrestrial isolates (Fenical, 1993).

According to Kumar *et al*. (2004), most of the bioflocculants produced from marine environment are extracted from seaweeds (agar, alginates and carrageenans) (Kumar

et al., 2004), with extremely scanty information on bioflocculants produced by marine bacteria. Hence, there is great interest in exploring marine bacteria diversities of underexploited environment such as the marine environment of the Algoa bay in the Eastern Cape Province of South Africa, for bioflocculant producing capabilities. To the best of our knowledge, there is no evidence in literature to show that such work has been done before in this area.

1.2 The aims and objectives

The aim of this study is to assess the bioflocculant production in three marine bacteria isolated from the bottom sediment of Algoa Bay in the Eastern Cape Province of South Africa. The specific objectives of the study included:

- To screen the culturable marine bacteria strains isolated from the Algoa bay for bioflocculant production and select three of the positive isolates for detailed studies.
- To assess the effect of culture conditions on bioflocculant production by the selected bacteria.
- To isolate, purify and characterize the bioflocculant produced by the selected bacteria.
- To identify the bacteria by 16S rRNA gene sequence analysis.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Brief history of bio-flocculation

According to Vatansever (2005), bioflocculation of newly formed biomass is believed to be primarily as a result of the adhesion of particles by gelatinous material excreted by bacteria which forms a polymeric network that holds everything together causing the particles to coalesce. In the early findings, a theory of flocculation whereby capsulated bacteria are flocculated by direct chemical interactions between adjacent cells was proposed by McKinney in 1952, but in 1965 Tenney and Stunn then proposed that the flocculation of microorganisms occurred due to polymers excreted by microorganisms. That material was extracted from cell free supernatant and examined and showed that such bacterial polymers were able to destabilize dispersions of bacteria. Quite a number of studies were carried out but it is now generally accepted that bio-flocculation takes place as a result of polymeric substances derived from microorganisms (Vatansever 2005).

By definition flocculation is a process whereby colloids come out of suspension in the form of flocs. This process is widely used and most commonly in wastewater treatment. It is mainly achieved via the aid of flocculants which quicken the process (Kurane *et al*., 1986; Lachhwani, 2005). Flocculating agents are substances having a synthetic or natural origin utilized as the sedimentation aids thus bringing about the solid-liquid separations by process of flocculation (Lachhwani, 2005). Based on literature in Lachhwani (2005) and Sharma *et al*. (2006), these flocculants have a wide range of applications viz. in surface treatment industry (petroleum refinery effluents),

sewage and municipal waste (in physicochemical reactions, prior sludge dewatering), chemical industry (water reuse), and in drainage improvement, raw water treatment and potable water treatment, to mention a few. These are further discussed below.

2.2 Categories of flocculants

Flocculants are materials used in fast solid-liquid separations, which act on the molecular level on surface of the particles thus reducing repulsive forces and mounting attractive forces (Sharma *et al*., 2006). Their addition enables dispersed particles to aggregate together to form flocs of a size thus allowing them to settle speedily and clear the system (Heitner, 1994 in Sharma *et al*., 2006). These flocculants can be classified into three different categories as denoted:

2.2.1 Inorganic flocculants

These are said to be commonly used and according to Sharma et al. (2006), have been used since time immemorial. This category incorporates salts of multivalent metals viz. polyaluminium chloride (PAC) usually used for wastewater treatment (Kurane *et al*., 1986); ferric chloride; and according to Lachhwani (2005), polyaluminium silicate sulphate, which is one of the commercially available inorganic flocculants used in drinking water production (Lachhwani, 2005).

Some of the reported disadvantages of inorganic flocculants include the following:

- Very large amount needed to cause solid-liquid separation of the dispersion, thus also results in large amounts of sludge produced, posing another disposal problem;
- Very sensitive to pH;
- Competent only to few disperse systems while not working for others;
- Cannot coagulate very fine particles (Sharma *et al*., 2006).

2.2.2 Organic flocculants

According to Kurane *et al*. (1986) these are frequently used as economical and powerful flocculating agents for dredging fields, plant wastewater treatment and so forth. These organic synthetic high-polymer flocculants have been most commonly utilized due to their flocculating effectiveness and low cost (Salehizadeh and Shojaosadati, 2001). According to Kurane *et al*. (1994) organic flocculants enhance the flocculation of suspended solids in treatment of process water, wastewater and effluents. They are available commercially in the form of dry powder and are said to be more suitable for flocculating suspended solids, also in clarification of non-portable raw water (Lachhwani, 2005). These are exemplified by polyacrylamide and polyacrilic acid (Kurane *et al*., 1994). Analyses of such flocculants indicate that they have high molecular weight; are effective at different pH; and have low charge density (Lachhwani, 2005). However, the following disadvantages have been noted:

- None -biodegradable
- Toxic to the environment

2.2.3 Natural organic flocculants

These are exemplified by chitosan, gelatine and sodium alginate (Kurane *et al*., 1986; He *et al*., 2001). According to He *et al*. (2001) and Sharma *et al*. (2006), these naturally occurring flocculants are based on renewable biomass are safe and biodegradable, cheap and non-toxic, but show weak activity in applications.

2.3 Mechanisms of bioflocculation

Extensive research has been done to better understand the mechanisms of bioflocculation and solid/ liquid separation processes (Lachhwani, 2005 and Vatansever, 2005). According to Salehizadeh and Shojaosadati (2001), flocculants (bioflocculants) cause aggregation of particles and cells by bridging or charge neutralization, colloid entrapment and double layer compression.

2.3.1 Bridging

Bridging occurs when a flocculant forms threads or fibers that adsorb to more than one particle, capturing and binding them together. The polymer molecule comes into contact with the colloidal particles, the reactive groups of the polymer attach to the surface of the particle thus leaving other portions of the molecule extending into the solution. The polymer/ flocculant adsorbs on the surface in loops (i.e. the segments extending on the solution), and tails (i.e. segments adsorbed on the surface). Thus, when another particle (with free adsorption site) comes into contact with these extended

loops and tails it attaches thus forming a particle-polymer-particle aggregate, thereby acting as a bridge (Figure 2.1) (Lachhwani, 2005). This attachment of segments leading to formation of strong bonds is made more effective due to the short distances involved, which likely overcome some local electrostatic repulsion between particles having the same net charge (Ganczarczyk, 1983 and Vatansever, 2005). This mechanism is dependent on the molecular weight of the bioflocculant; the charge on the polymer and particle; ionic strength of the suspension; and the nature of mixing (Salehizadeh and Shojaosadati, 2001; Ebeling *et al*. (2005). The mechanism is much preferred for high molecular weight polyelectrolytes of low large density (Sharma *et al*., 2006). Hence, high molecular weight flocculants produce more, large, loosely packed flocs and more fragile flocs (Ebeling *et al*., 2005). For more effective bridging, the mechanism can be used together (i.e. function simultaneously) with the charge neutralization thus enhancing settling and/ or shear resistant flocs (Lachhwani, 2005).

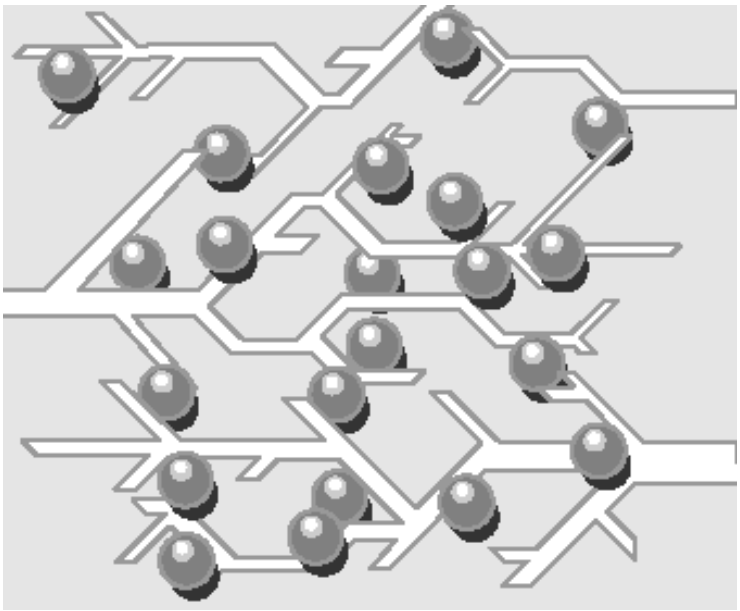

Figure 2.1: Scheme showing polymer chain attaching many particles (Lachhwani, 2005)

2.3.2 Charge neutralization

Charge neutralization is said to occur when the flocculant is oppositely charged to the particles. The particle surface is said to be reduced by adsorption of the bioflocculant and the particles therefore can effectively approach close to each other, thus attractive forces become effective (Salehizadeh and Shojaosadati, 2001). In other words, a highly cationic polymer/flocculant may be adsorbed on the surface of a negatively charges particle in a flat conformation thus the flocculation is promoted thereby reducing the overall negative charge on the particle and reducing inter particle repulsion. This kind of mechanism is linked with reduced electrophoretic mobility (Sharma *et al*., 2006).

Also, the polymer/ flocculant adsorption may have a net positive charge due to the polymer having a charge density that is high. In such a case the positive regions will be adsorbed to the negative regions on the particle and this is referred to as heterocoagulation (Sharma *et al*., 2006).

2.3.3 Colloid entrapment/Sweep mechanism

Colloid entrapment entails the addition of relatively large amount of flocculants, for example, inorganic salts (viz. aluminium or iron) that precipitate as hydrous metal oxides entrain. The dose of the flocculant added is far in excess of the amount needed for the neutralization of the charge on the colloidal particle (Lachhwani, 2005). According to Sharma *et al*. (2006), this flocculation entails the combination of destabilization and transport. It is vastly used in treatments of water of variable turbidity

and dissolved organic carbon in conjunction with rapid mixing, flocculation, sedimentation and filtration facilities (Sharma *et al*., 2006).

2.3.4 Double layer compression

This is also known as DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, which is a classical colloidal theory that describes charged particles as having a double layer of counter ions surrounding the particle. According to Adamson (1990) the first layer is referred to as the Stern layer which is comprised of a tightly associated layer of counterions, while the second layer is the diffuse layer which is made up of less tightly associated counterions (Figure 2.2). The concentration of ions in the diffuse layer decreases with the distance from surface of the particle, hence the equivalence of ion concentration to that of the solution, and consequently, the electric potential that develops around the particle. This double layer surrounding the particle therefore result in repulsion of adjacent particles and inhibits aggregation. The ionic strength thus increases and the size of the double layer decreases, thus decreasing the repulsion between particles, allowing short-range attractive forces to promote aggregation. Consequently, adding the cations to a solution would therefore result in an improvement in bioflocculation due to a decrease in the size of the double layer and the repulsive forces between particles (Sobeck and Higgins, 2002; Vatansever, 2005).

This mechanism is also linked for inorganic flocculants i.e. alum and ferric salts, and a good example to explain this would be when the particles in freshwater (like rivers) with low ionic strength mix with that of high ionic strength (such as seawater). The

particles are destabilized by double-layer compression and therefore resulting in the formation of deltas at river mouths (Sharma *et al*., 2006).

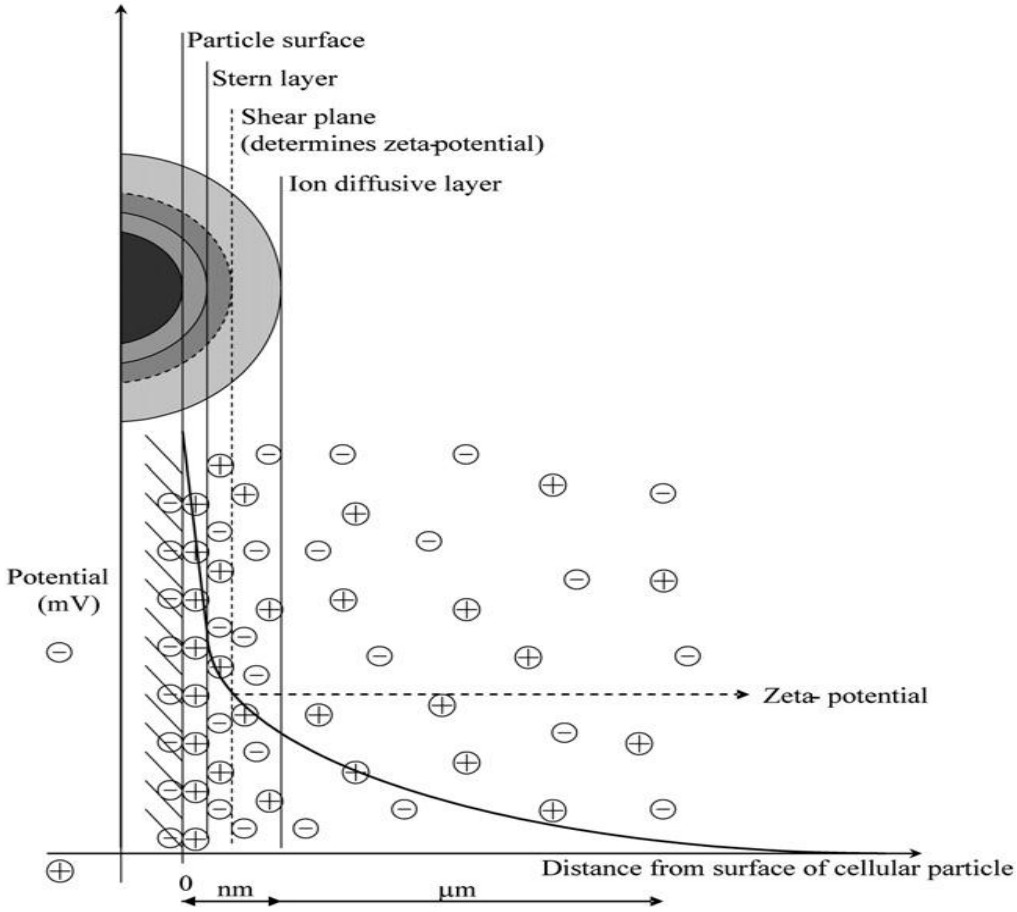

Figure 2.2: Schematic diagram showing the charged cellular particle with its counter charges and the potential in the area of a particle surface (Schryver *et al*., 2008).

2.4 Composition of bioflocculants

According to reported studies of extraction and analysis of bioflocculants, most of their composition includes mainly polysaccharides/carbohydrates, proteins and nucleic acids (Deng *et al*., 2005); lipids (Vatansever, 2005); and heteropolymers such as

glycoproteins (Vatanser, 2005). Also, some flocculants produced by microorganisms isolated from activated sludge under maintained conditions (intensive growth, intensive starvation or decomposition) had sugars (viz. rhamnose, fucose, mannose, galactose, glucose), amino sugars (viz. galactosaminuronic acid, glucosamine, galactosamine), and amino acids (viz. lysine, histidine, argigine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine) (Vatansever, 2005). .

A bioflocculant produced by *Rhodococcus erythropolis* which causes flocculation of different suspended solids it was reported to consists of assemblies of polypeptide and lipids (i.e. mycolate-containing glycolipids) (Bell *et al*., 1998). Also, Zhang *et al*. (2002) reported on a bioflocculant produced by *Nannocystis* sp. Nu-2 deduced to be a glycoprotein. Other organisms including *Bacillus subtillis, Bacillus licheniformis, Pacilomyces sp., Nocardia amarae YK* were reported to produce proteinaceous bioflocculants (Deng *et al*., 2005).

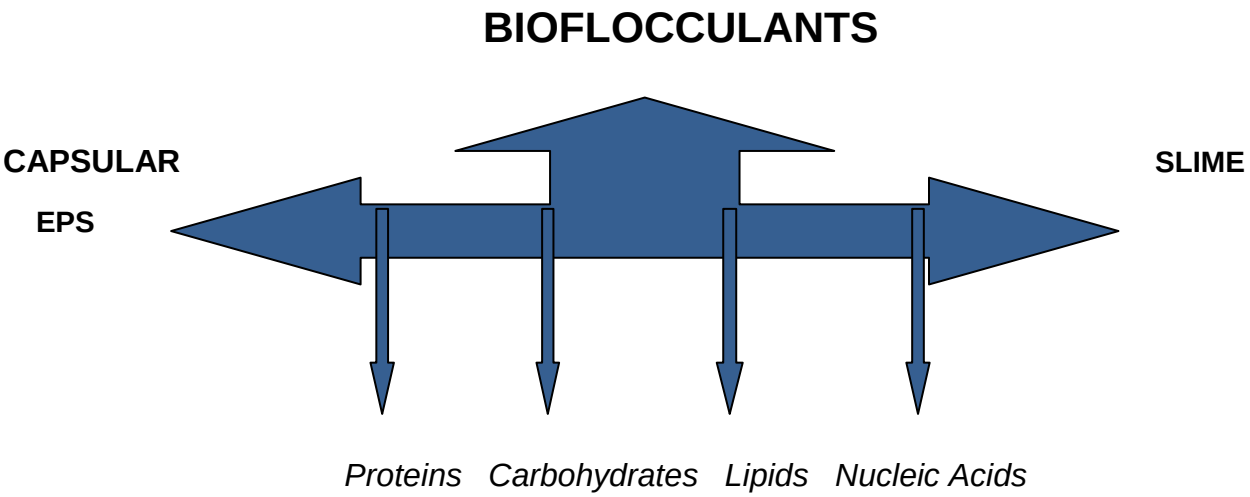

Figure 2.3: Schematic diagram of the components of bioflocculants

2.4.1 Factors affecting bioflocculant production

Type of microorganism: The differences in the composition of bioflocculants have been reported to be dependent on organism type. As example, Bejar *et al*. (1998) investigated the chemical composition of the bioflocculant produced by the 19 strains of *Halomonas eurihalina* and found out that the carbohydrate contents were different among the strains.

Growth phase of microorganism: According to early findings Busch and Stumn, 1968; Pavoni *et al*., 1972 in Vatansever (2005), the flocculant production increased dramatically at the early stages of death phase and thus after no further production of flocculant. In recent study by Lu *et al*. (2005) the production of bioflocculant was found to reach its maximum flocculating activity in early stationary phase, thus indicating that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis. Thereafter, the production drops at death phase, probably due to cell autolysis and decreased enzymatic activity depending nevertheless on the microbial cell. For example, in *Pseudomonas aeroginosa* bioflocculant production occurs mainly during the exponential phase of growth, while in other *Pseudomonas* species production starts late in the exponential phase and continued to reach a maximum in the stationary phase (Vatansever, 2005).

Nutrients: Carbon and nitrogen sources have been emphasized for the production of bioflocculant (Kurane *et al*., 1994). These nutrients can also influence the composition of the flocs and even the structure of the floc (Bura *et al*., 1998). As example, according to Allison and Sutherland (1987), bacterium grown under glucose limiting conditions

only a small amount of carbohydrate was detected in connection with the attached cells. Also in another study under carbon limited growth, the production of polymer/ flocculant was unlikely (Harris and Mitchell, 1973).

Nutrient requirement for the production of the bioflocculant may differ with each different microorganism. For example in the case of bioflocculant production by *Rhodococcus erythropolis*, sorbitol, mannitol and ethanol were the favored carbon sources and also these were favorable for its growth (Kurane *et al*., 1991). Also, the production of bioflocculant by *Aspergillus sojae* was greatly improved when casein, yeast extract, polypeptone and other amino acids were added to the medium (Salehizadeh and Shojaosadati, 2001).

Metal ions: the production of bioflocculant is greatly influenced by metal ions present in the culture medium (Li *et al*., 2009; Salehizadeh and Shojaosadati, 2001). These may also differ with studied microorganism. For example the flocculant production of *Paecilomyces* sp. was greatly enhanced by the presence or addition of Ca^{2+} ion but its cell growth was inhibited by Cu^{2+} (Takagi and Kadowaki, 1985). In another study cations like Na^{+}, Fe^{2+}, Ca^{2+} and Mg^{2+} did not have much effect on the bioflocculant production by *Bacillus licheniformis* X14 but Cu^{2+} inhibited its cell growth (Li *et al*., 2009). According to Sousa *et al*. (1992) metal ions like Ca^{2+}, Co^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+} and Al^{3+} improved the flocculation of *K. marxianus* cells but Fe^{2+} and Sn^{2+} were not effective.

2.5 Bioflocculant- producing microorganisms

Over the past years, there has been increasing interest in the exploration for biodegradable flocculants as a consequence of the hazardous attributes of synthetic flocculants. According to recent studies, quite a number of bioflocculants have been identified (He *et al*., 2001; You *et al*., 2008) with potential to replacing chemical synthetic flocculants, as they are biodegradable; not harmful to humans; and do not pollute the environmental (Fugita *et al*., 2001; Kurane *et al*., 1986). Among the bioflocculant producing organisms includes the genera *Rhodococcus*, *Norcadia, Corynebacterium* and *Mycobacterium* (Kurane *et al*., 1986).

Rhodococcus erythropolis S-1 have been reported to produce a protein bioflocculant that loses its flocculating capabilities by enzymatic digestion (Tadeka *et al*., 1991; Zheng *et al*., 2008). This bioflocculant has an efficient activity resulting in flocculation of a number of suspended solids (Kurane and Tomizuka 1992; Bell *et al*., 1998). The bioflocculant from this species was named NOC- (Kwon *et al*., 1996; Kurane *et al*., 1994; Tadeka *et al*., 1991). According to Bell *et al*. (1998) the bioflocculant consist of assemblies of polypeptide and lipids (mycolate-containing glycolipids) and has been suggested for utilization wastewater treatment to remove the suspended solids (Bell *et al.*, 1998).

Norcadia amarae YK-1 was reported as the bioflocculant producing actinomycetes (Tadeka *et al*., 1992). The bioflocculant it produces is known as FIX. It is also found to be a protein flocculant. According to He *et al*. (2002) the ingredients of this bioflocculant include high contents of glycine (25.6%), alanine (13.8%) and serine (12.3%) (He *et al*.,

2002). The other strains from the *Norcadia* family found to produce flocculants include *Norcadia restricta*, *Norcadia calcarea* and *Norcadia rhodnii*. These are said to produce biopolymer flocculants (Kurane *et al*., 1986; Desouky *et al*., 2008).

In the study by Shimmofuruya *et al*. (1995), *Streptomyces griseus* was deduced to produce certain kinds of flocculating substances that may aggregate suspended solids (i.e. kaolin clay) (Shimmofuruya *et al*., 1995). According to Cheng *et al*. (2004) a strain *Saccharomycete* STSM-1 with flocculating activity was isolated from activated sludge (Cheng *et al*., 2004).

Vagococcus sp. W31 was reported as bioflocculant producing bacteria. The exhibited bioflocculant had very strong flocculating activity on kaolin clay and was deduced to be heat stable. According to Gao *et al*. (2005), the bioflocculant (named MBFW31) was anticipated for utilization in wastewater treatment, clarification of water, fermentation industries (Gao *et al*., 2005). The *Enterobacter cloacae* WD7 was also found amongst the organisms producing or exhibiting flocculant activity (Desouky *et al*., 2008). Based on study reported by Zheng *et al*. (2008), *Bacillus* sp. F19, isolated from soil produced bioflocculant named MBFF19. Its bioflocculant was a biopolymer with components: neutral sugar (3.6%), uronic acid (37%), amino acids (0.5%) and protein (16.4%). This bioflocculant was also deduced to be cation-independent (Zheng *et al*., 2008)

Marine bacteria: The marine myxobacterium strain NU-2, is a bacteria found to be able to grow on high concentrations of salts (i.e. up to 7% NaCl). According to Zhang *et al*. (2002), this strain *Nannocystis sp* NU2 was able to produce a new kind of flocculant.

The flocculant was deduced to be composed of 40.3% proteins and 56.5% polysaccharides and it was confirmed that it had high flocculating activity of 90%. Further study revealed that the flocculant was capable of bleaching basic pink (i.e. bleaching dyeing liquors) (Zhang *et al*., 2002).

A marine dinoflagellate *Gyrodium impudicum* KG03 was investigated and was found to produce an exopolysaccharide p-KG03, with high flocculating activity (viz. more than 90% flocculating activity in kaolin suspension). The flocculating activity of p-KG03 was high in the range of 0.5-2.5 mg/l and was found to be most effective at low concentration in the flocculating reaction. Thus it was concluded that this (p-KG03) can be utilized as a bio-flocculant in different industrial processes (Yim *et al*., 2006)

2.6 Biotechnological importance and application of bioflocculants

2.6.1 Wastewater treatment

According to Salehizadeh and Shojaosadati (2001), the phenomena of bioflocculation and secretion of flocculants are mostly eminent in the process of activated sludge of wastewater. As example, the flocculant derived from *Aspergillus sojae* AJ 7002 was found to be able to remove microorganisms of the activated sludge produce during wastewater treatment. The effectiveness of the bio-flocculant was compared to that of the synthetic flocculant i.e. ferric chloride and polyacrylamide, it was deduced that this bioflocculant had useful potential in wastewater treatment and other downstream processing (Nakamura *et al*., 1976). Another study carried out by Ghosh *et al*. (2008), evaluated the potential of novel biopolymeric flocculant produced by a strain of

Klebsiella terrigena for the removal of *Salmonella* (a potent pathogen prevalent in poultry wastewater). It was then revealed that an optimized dosage of 2 mg/l of the purified bioflocculant was enough for the removal of 80.3% *Salmonella* sp. within 30 minutes at ambient temperature. The poultry wastewater contained 3log CFU cells *Salmonella*. This bioflocculant demonstrated effectiveness far better than that of synthetic flocculants and could flocculate kaolin particles up to 90% flocculating activity (Ghosh *et al*., 2008). Zhang *et al*. (2006) reported a novel bio-flocculant MMF1 (protein and polysaccharide bio-flocculant) produced by multiple-microorganism consortia (*Staphylococcus* sp. BAFRT4 and *Pseudomonas* sp. CYGS1) which remarkably showed good flocculating performance in treating indigotin printing and dyeing wastewater, and the maximal removal efficiencies of COD and chroma were 79.2% and 86.5% . Based on Buthelezi *et al*. (2009) from their study, the application of bacterial bioflocculants in the treatment of river water is a promising alternative to using alum. The bioflocculants were also capable of removing bacteria (*Staphylococcus aureus* and *Streptococcus faecalis*, *Escherichia coli* and *Klebsiella oxytoca*) used to individually spike the autoclaved river water samples, thus resulting in complete removal of *S. aureus*, *K. oxytoca* and *E. coli* and up to 98.35% removal of *S. faecalis* in some cases (Buthelezi *et al*., 2009).

2.6.2 Removal of metal ions

As previously noted microorganisms have the ability to produce extracellular polymeric substances, a mixture of polysaccharides, mucopolysaccarides and proteins and these

bind potentially toxic metals (Gadd, 2004, White and Gadd, 1998). These are said to also adsorb or entrap particulate matter such as precipitated metal sulfides and oxides (Gadd, 2004). According to Howe *et al*. (1997) low molecular weight (6000–10,000 Da) metal-binding proteins, are produced by microorganisms in response to the presence of toxic metals. According to Bender *et al*. (1994) one process uses floating cyanobacterial mats to remove metals from waters, the metal-binding process being due to large polysaccharides. In such study, the potential of cyanobacterium Oscilatoria sp to remove metal ion (viz. Mn^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, CO^{2+}, Cr^{3+} and Fe^{3+}) was reported (Bender *et al*., 1994 and Salehizadeh, Shojaosadati, 2001). Based on Salehizadeh and Shojaosadati (2003) reported adsoption of Pb, Cu and Zn on the polysaccharide produced by *Bacillus firmus*.

2.6.3 Cells and cell debris removal in culture broths

This is said to be one of the most significant applications in brewing and fermentation industries. In view of Salehizadeh and Shojaosadati (2001) the best beer is as a result of flocculating yeasts rather than non-flocculating yeasts. Also it is said that the use of bioflocculation in classical brewing is utilized in the production of bio-ethanol and baker's yeast. It is said that id the suspended solids are flocculated the removal by filtration and centrifugation, that removal of cells and cell debris achieved during downstream processing could be greatly improved. The introduction of flocculating strains in such processes is said to greatly increase productivity thereby reducing the size of fermenters required (Salehizadeh and Shojaosadati, 2001).

2.6.4 Removal of humic acids

According to a study by Zouboulis *et al*. (2004) they evaluated bio-flocculant in comparison with traditional inorganic coagulants, for the removal of humic acids from landfill leachates. Based on their study this bioflocculant was effective applying lower initial concentrations and the maximum humic acids removal was observed for concentration of 20 mg/l. From the study they can deduce that only a small concentration of bio-flocculant was needed for efficient removal of humic acids, thus giving an advantage over the conventional (chemicals) flocculants (Zouboulis *et al*., 2004).

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CHAPTER THREE

Production and characterization of bioflocculant produced by *Halobacillus* sp. Mvuyo isolated from bottom sediment of Algoa Bay

Abstract

A bioflocculant producing bacterium isolated from marine sediment of Algoa Bay was assessed for its bioflocculant producing potentials. Based on 16S rDNA sequence analysis the isolate was identified as *Halobacillus* sp. and deposited in the Genbank as *Halobacillus* sp. Mvuyo with accession number HQ537125. The bacterium produced bioflocculant optimally in the presence of glucose (76%) and ammonium chloride (93%) as sole sources of carbon and nitrogen respectively. The flocculating capabilities of the flocculant were increased by the addition of Ca^{2+} (76%) and the highest flocculating activity was observed at neutral pH (7.0). The chemical analysis of the bioflocculant revealed that it was composed of polysaccharide and protein and we propose the compound to be a glycoprotein bioflocculant.

Keywords: *Halobacillus* sp.; Bioflocculant; Characterization.

1. Introduction

Flocculants such as organic, inorganic and naturally occurring flocculants have been commonly utilized in different industrial processes such as wastewater, drinking water purification, food and fermentation, dredging and downstream processing due to their effectiveness and low cost (Yokoi *et al*., 1994; He *et al*., 2009). Although these flocculants have been frequently used, recent studies reveal that some are hazardous and pose a threat to human health and increased environmental risk (Yokoi *et al*., 1994; Zheng *et al*., 2008). As example, the use of alum which is said to result in the problem of residual aluminum. Recent epidemiological, neuropathological and biochemical studies show an association between neurotoxicity of aluminum and pathogenesis of Alzheimer's disease (Banks *et al*., 2006). Also, polyacrilamine which includes monomers of acrylamide have been shown to be neurotoxic and carcinogenic to humans (Zheng *et al*., 2008). According to Li *et al*. (2008), ferrite flocculants may be expensive and also lead to excess iron causing unpleasant metallic taste, odor, color corrosion, foaming or staining. Although these synthetic flocculants are most frequently used but because of their limitations, flocculants produced by microorganisms have been anticipated as better alternatives. This is due to their biodegradability, harmlessness, environmentally friendly and no secondary pollution characteristics (Yokoi *et al*., 1994; He *et al*., 2004; Zheng *et al*., 2008).

Bioflocculants are essential polymers produced by microorganisms during their growth with their flocculating activity being dependent on the characteristics of flocculants (Gao *et al*., 2006). They are readily biodegradable, safe with strong flocculating effect and thus may be potentially applied in industrial processes (Gao *et*

al., 2006). With regard to chemical nature, bioflocculants are regarded as extracellular polymers containing polysaccharides, protein, glycoprotein, cellulose, lipids, glycolipids and nucleic acids (He *et al*., 2009). As example, flocculant AI-201 produced by *Alcaligenes cupidus* KT201 was found to be composed of polysaccharides (glucose, lactose, glucironic acid and acetic acid) (Toeda and Kurane, 1991), so also is the bioflocculant produced by *Alcaligenes latus* B-16 (Kurane and Nohata, 1994).

Several kinds of microorganisms including algae, bacteria, actinomycetes and fungi have been screened and isolated for bioflocculant production mostly from soil and activated sludge (Zhang *et al*., 2002; Sheng *et al*., 2006). According to Sheng *et al*. (2006) soil and activated sludge samples have been regarded as best sources for isolating flocculant-producing microorganisms. As example, bioflocculant producing *Bacillus* sp., *Enterobacter* sp. and *Alcaligenes latus* B-16 were isolated from soil samples (Kurane and Nohata, 1994; Yokoi *et al*., 1996; Suh *et al*., 1997) while a *Saccharomycete* STSM1 strain and a *Klebsiella* sp. with high flocculating capability were isolated from activated sludge (Cheng *et al*., 2004; Sheng *et al*., 2006).

However, novel efficient bioflocculants from microorganisms inhabiting other unusual environments such as the marine environments are beginning to be of great interest. The marine environment is rarely studied. It is anticipated that such environment may be a reservoir of novel bioflocculant producing organisms due to the uniqueness of the marine environmental conditions which is very different from terrestrial environment. In this paper, we report the bioflocculant producing potentials of marine bacteria identified as *Halobacillus* sp. Mvuyo isolated from bottom sediment of Algoa Bay in South Africa.

2. Materials and methods

Screening for bioflocculant- producing bacteria

2.1 Microorganisms

Several hundred marine bacteria previously isolated and characterized 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 and maintained in 20% glycerol at -80^0 C were screened for bioflocculant production.

2.2 Bioflocculant producing medium and cultivation

The medium used for cultivation of marine bacteria for the bioflocculant screening was composed of glucose (10 g), peptone (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.3 g), K_2HPO_4 (5 g) and KH_2PO_4 (0.2 g) in 1 liter of filtered marine water. The initial pH was adjusted to 7.0 with NaOH (0.1M) and HCI (0.1M) (Zhang *et al*., 2007). Two loopfuls of the bacterial colonies were inoculated in 5 ml of the cultivation medium and incubated with shaking (120 rpm) at 28 ^{o}C for 5 days (Zhang *et al*., 2007). At the end of the incubation period 2 ml of the fermentation broth was centrifuged (4000 g, 30 min) to separate the cells. The cell-free culture supernatant was analyzed for flocculating activity.

2.3 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 milliliter of the culture supernatant and 3 ml of 1% $CaCl_2$ were added into 100 ml of Kaolin clay suspension (4 g/l) in 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 550nm; and B is the optical density of the sample at 550nm. All experiments were performed in triplicates for the mean calculation.

The effect of culture conditions on bioflocculant activity

2.4 The effect of carbon and nitrogen sources

It has been well documented that changing the carbon and nitrogen sources highly influenced bacterial growth and production of bioflocculant (Sheng *et al*., 2006). Hence, we assessed the effects of different carbon and nitrogen sources on bioflocculant activity in the test bacterium. Carbon source candidates included glucose, sucrose, fructose and starch, while the nitrogen source candidates included ammonium sulphate,

ammonium chloride (inorganic nitrogen sources) and urea (organic nitrogen sources) replacing peptone. The assessments were done in accordance with the description of Lachhwani (2005).

2.5 Effects of various cations and pH on flocculating activity

Studying the effects of salts, flocculant tests was conducted utilizing the procedure elaborated above, but the $CaCl_2$ solution was replaced by various metal salt solutions, and the flocculating activity was measured. Solutions of KCl, $MgCl_2$ and $FeSO_4$ were used as salt sources. To assess the effect of pH on flocculating activity, the pH of the culture medium was adjusted using HCl and NaOH in the pH range of 3-12 (Yim *et al*., 2006).

Time course of bioflocculant activity

*2.6 Composition of culture medium***:** For the time course experiment, the composition of the medium for the bioflocculant production was as follows: 10g of glucose; 5g of KH_2PO_4; 1g of ammonium chloride; 0.3g of $MgSO_4$, $7H_2O$ per liter of filtered natural sea water (Zhang *et al*., 2007). The isolate was cultured under optimal growth conditions.

*Standardization of the inocula***:** Saline solution was prepared by adding 0.45g NaCl in 50ml of distilled water for each of the selected isolates. Fifty milliliters of saline solution was 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 cuvets and readjusted if need be to give 0.1.

Time course assays (modified method of Gao *et al*. (2006): The inoculated saline solution was used as seed culture for inoculum preparation. Seed culture (1% v/v) was inoculated into 150 ml of medium in 500ml flasks on a rotatory shaker (160rpm) at 28 ^{o}C. Sample was drawn at appropriate time intervals (every 24 hrs) for a period of 10 days.Two milliliter of culture broth was centrifuged at 4000 g for 30 min and the cell free supernatant was used to determine the flocculating activity. The pH of the broth sample was also measured. All experiments were performed in triplicates.

Purification and characterization of the bioflocculant compound

2.7 Extraction and purification of the bioflocculant

Purification and characterization of the bioflocculant was done following the methods described elsewhere (Chang *et al*., 1998; Chen *et al*., 2002) using media formulation based on the pre-determined optimum culture conditions. Briefly, after five days of fermentation, the culture solution was centrifuged at 4 600 rpm for 30 min to remove bacterial cells. One volume of distilled water was added to the upper phase and centrifuged at 4 600rpm for 15 min to remove insoluble substances. To the supernatant, two volumes of ethanol were added, and the mixed solution was stirred and left to stand at 4 °C for 12 h. The precipitate was vacuum dried to obtain the crude biopolymer. The crude product was directly dissolved in distilled water to yield a solution, to which one volume of the mixed solution of chloroform and *n*-butyl alcohol (5:2 v/v) was added.

After stirring, the mixture was set aside for 12 h at room temperature (about 20 °C). The upper phase was centrifuged at 3000×g for 15 min and the supernatant was concentrated at 40 °C. Thereafter two volumes of ethanol were added to recover the precipitate.

2.8 Analysis/ characterization of purified bioflocculant

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).

Characterization of the bioflocculant-producing microorganism

2.9 DNA Extraction

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

2.10 PCR Amplification

PCR was carried out in 50 µl reaction volume containing 2 mM $MgCl_2$, 2 U Supertherm *Taq* polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-

AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 µl template DNA. Primer F1 and R5 binds to base positions 7 – 26 and 1496 – 1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877, respectively (Cook and Meyers, 2003). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 ºC for 2 min), 30 cycles of denaturation (96 ºC for 45 s), annealing (56 ºC for 30 s) and extension (72 ºC for 2 min), and a final extension (72 ºC 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.

2.11 DNA sequence analysis

Automated sequencing of the 16S rRNA genes of the bacterial isolate 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 sequence was edited manually based on the chromatogram readings and used in the BLAST (Basic Local Alignment Search Tool) search to determine the most similar sequences.

Statistical Analyses

Time course of bioflocculant production, in relation to bioflocculant activity and pH of the medium, and effects of composition of the medium on the activity biofloccant with carbon, nitogen and cation sources in perspective were analysed for levels of significance using analysis of varience (ANOVA). The initial pH of the medium with respect to flocculating activity of the bioflocculant was represented with mean values.

3. Results and discussion

3.1 Screening and identification of bioflocculant- producing bacteria

Over three hundred marine bacteria previously isolated and characterized from the Algoa Bay were screened for bioflocculant production. Amongst these included our test bacterium which exhibited significant bioflocculant activity (about 76%) against Kaolin suspension. The morphological characteristics of the bacterium revealed the colony to be creamy, smooth surfaced, round with radiating margin, branching, umbonate and colony size of approximately 2 mm diameter. The 16S rDNA PCR yielded product of expected size (approx. 1.5 kb) (Figure 3.1). BLAST (Basic Local Alignment Search Tool) analysis of the nucleotide sequence of the 16S rDNA revealed the bacterium to have 99% similarity to *Halobacillus* sp. S25-1 and the sequence was deposited in the Genbank as *Halobacillus* sp. Mvuyo with accession number HQ537125.

The genus *Halobacillus* are gram- positive, motile, spore forming, rod shaped, moderate or halophilic bacteria. They are mainly differentiated or noted for their

peptidoglycan cell wall based on Orn-D-Asp type, DNA G+C content ranging from 40-44 mol%. The genus was initially proposed by Spring *et al*. (1996) to accommodate two recently identified species i.e. *H. litoralis* and *H. trueperi* and now *H. halophilus* (Spring *et al*., 1996, Chen *et al*., 2009).

A number of applications have been stated for such halophiles. As example, they have found application in antimicrobial production and production of bioactive compounds and in the food industry as fish and meat fermenting microorganisms and for the degradation of toxic compounds. Typical examples for bioactive compounds from these halophilic bacteria include enzymes, ectoines and pralines (Ventosa *et al*., 1998). Three extracellular proteases were identified from *Halobacillus thailandensis* in Thailand which include two serine proteases and metaprotease. Thus the strain has been successfully utilized in the improvement of fermentation process in industrial production of fish sauce in Thailand (Chaiyanan *et al*., 1999). In another study, an enzyme amylase was produced by newly isolated moderate *Halobacillus* sp. strain MA-2 under stress conditions of high temperature and high salinity with promising commercial value. This enzyme plays an important role in the biogeochemical cycle of carbon and also has a wider application in the biotechnological-based food, detergent, and pharmaceutical industries (Amoozegar *et al*., 2003). Also, ectoines (ectoine and hydroxylectoine) which are synthesized by *Halobacillus halophilus* are considered of great biotechnological importance, for they are used as stabilizers of enzymes and whole cells. Proline, N-acetylornithine and N-acetyllysine are found in moderate halophiles including *Halobacillus halophilus* (Ventosa *et al*., 1998).

In a recent study *Halobacillus salinus* was found to be a producer of biosurfactants, which aid with the transportation of hydrophobic low water soluble substrates by increasing their bioavailability. Other functions include for example heavy metal binding, quorum sensing and biofilm formation. This gram-positive bacterium produces two phenetrylamide metabolites that inhibit quenching quorum sensing-controlled processes of gram-negative strains i.e. bioluminescence production by *Vibrio harveyi*, inhibit quorum sensing regulated violacein biosynthesis by *Chromobacterium violaceum* CV026 and green fluorescent protein production by *Escherichia coli* JB525 (Teasdale *et al*., 2008; Carvalho and Fernandes, 2010).

In another study, halotolerant or moderately halophilic strains from DSM culture collection were also reported to demonstrate antifungal activity against *Bacillus cinerea* on tomato fruits. The most effective strains included *Halobacillus litoralis, Halobacillus halophilus, Halomonas subglaciescola, Marinococcus halophilus, Salinococcus roseus, Halovibrio variabilis* (Sadfi-Zouaoui *et al*., 2008).

Although a number of active compounds have been characterized from such marine bacteria, not much information has been reported from this particular genus especially on the production of the bioflocculant.

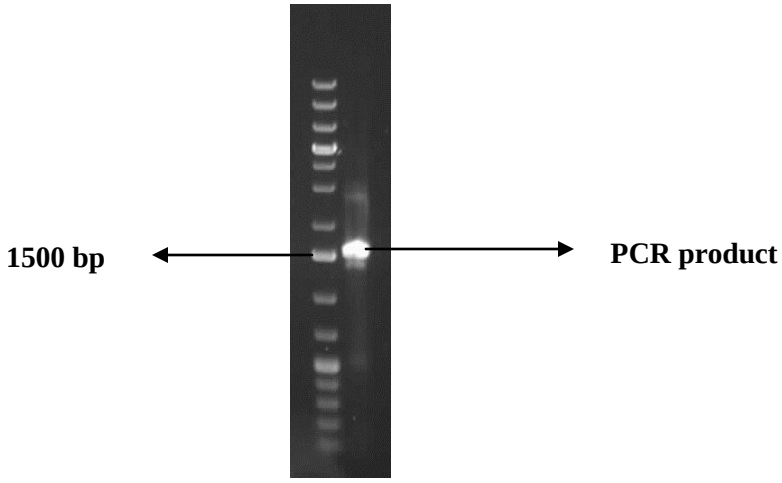

Figure 3.1: PCR product of the 16S rDNA of the test bacterium. The left lane is DNA ladder; while the right lane is the PCR product.

3.2 Factors affecting the bioflocculant production and flocculating activity

The production of bioflocculant may be affected by various factors i.e. constituents of the culture medium and cultivation conditions (Zhang *et al*., 2007). Key factors such as carbon and nitrogen sources, cations, and pH of the medium that could impact on bioflocculant production by the bacterium, were examined to determine the optimum requirements for these conditions. Table 3.1 summarizes the effect of the different culture conditions on bioflocculant activities in the test bacterium. Glucose appears to be the best carbon source for bioflocculant production with a flocculating activity of 75.7 % ($\alpha \geq 0.05$), while ammonium chloride was the best nitrogen source with a flocculating activity of 93%. With regards to cations, calcium chloride was the cation of choice with a flocculating activity of 75.7%. All these were significant at α level of 0.05.

The importance of carbon and nitrogen source for bioflocculant production has been reported before (Xia *et al*., 2008). As example, according to a study by Kurane and Nohata (1991), flocculant production of *Rhodococcus erythropolis* was highly increased in medium using 0.5% glucose and 0.5% sucrose as carbon source when compared to that of 1% sucrose. The production of a bioflocculant (MBFF19) by *Bacillus* sp F19 was enhanced by addition of glucose, lactose and fructose in the medium but the sucrose medium resulted in the highest generation of MBFF19. Also, the bioflocculant was optimally produced when ammonium nitrate was used as the nitrogen source (Zheng *et al*., 2008). As shown in Table 3.1 glucose when compared to other carbon sources i.e. sucrose, fructose and starch was more suitable for the production of the bioflocculant produced by *Halobacillus* sp. Mvuyo. Similar findings by Xia *et al*. (2008) for *Proteus mirabilis* TJ-1 were observed, wherein glucose was the preferred source of carbon for optimal production of TJ-1 bioflocculant. Also a study by Lacchwani (2005), glucose was the most effective carbon source for strain RDL-1.

As nitrogen sources ammonium chloride and peptone were effectively used by the bacterium for the production of the bioflocculant. Hence, the bioflocculant was optimally produced in the presence of ammonium chloride as the sole nitrogen source.

Flocculation by induction of cations is generally accepted to occur by bridging and charge neutralization (Kurane and Nohata, 1994). From Table 1, the flocculating rate was increased by the addition of cations particularly Ca^{2+}. Possible reasons for this maybe (i) the addition of the cation to Kaolin suspension decreased the negative charge of the particles and (ii) cation bridging, in which the bioflocculant absorbs onto the Kaolin clay particles thus flocculating them i.e. bioflocculant and kaolin clay could form

solid complexes mediated by Ca^{2+}. (Sheng *et al*., 2006; Li *et al*., 2008). Similar results were observed with TJF1 bioflocculant produced by *Proteus mirabilis* where Ca^{2+} increased flocculating activity (Zhang *et al*., 2010). Addition of Mg^{2+}, Fe^{2+} and K^{+} resulted in little and no flocculating activity. The addition of cations to suspended particles is known to induce more effective flocculating capabilities; however, the effect of cations on flocculating activity may vary depending on the bioflocculant (Li *et al*., 2007).

Table 3.1: Effects of composition of medium on the bioflocculant activity

Carbon Source	**Glucose**	**Sucrose**	**Fructose**	**Starch**
Flocculating Activity %	75.7	-	6	-
Nitrogen Source	**Peptone**	**Ammonium Sulphate**	**Urea**	**Ammonium Chloride**
Flocculating Activity %	75.7	-	26	93
Cations	**Calcium Chloride**	**Magnesium Chloride**	**Iron Sulphate**	**Potassium Chloride**
Flocculating Activity %	75.7	10.6	44	-

Note: (-) denotes no flocculating activity; ($\alpha \geq 0.05$).

The effect of initial pH of the production medium on bioflocculant production is shown in Figure 3.2. Flocculating activity of the bioflocculant increased as the pH increased from pH 3 and attained optimum activity at pH 7 beyond which activity declined steadily to zero from pH 9 and above.

Bioflocculant production by *Halobacillus* sp. Mvuyo was affected by initial pH of the medium. The pH of the production medium has been documented to affect and or influence the bioflocculant production (Salehizadeh and Shojaosadati, 2001). It determines the electric charge of cells and oxidation-reduction potential which may affect the absorption of nutrients and the enzymatic reaction (Salehizadeh and Shojaosadati, 2001; Xia *et al*., 2008). However, the initial pH requirement may differ with different strains. As example, *Aspergillus parasiticus* preferred acidic conditions for synthesis, secretion and production of bioflocculant in the culture medium (Deng *et al*., 2005). While *Bacillus licheniforms* X14 appeared to be more productive for the bioflocculant at alkaline conditions (pH above 7) (Li *et al*., 2009). Also, flocculant production by *Rhodococcus erythropolis* was higher at alkaline pH values of 8.0-9.5 (Kurane *et al*., 1994; Salehizadeh and Shojaosadati, 2001). In the case of *Halobacillus* sp. Mvuyo, neutral pH (7.0) was required for optimal production of bioflocculant. A similar result was obtained for TJ-1 bioflocculant production by *Proteus morabilis* TJ-1 (7.0) (Xia *et al*., 2008). The production of bioflocculant at neutral pH allows saving of large amounts acid and alkali needed for the adjustment of pH (Xia *et al*., 2008).

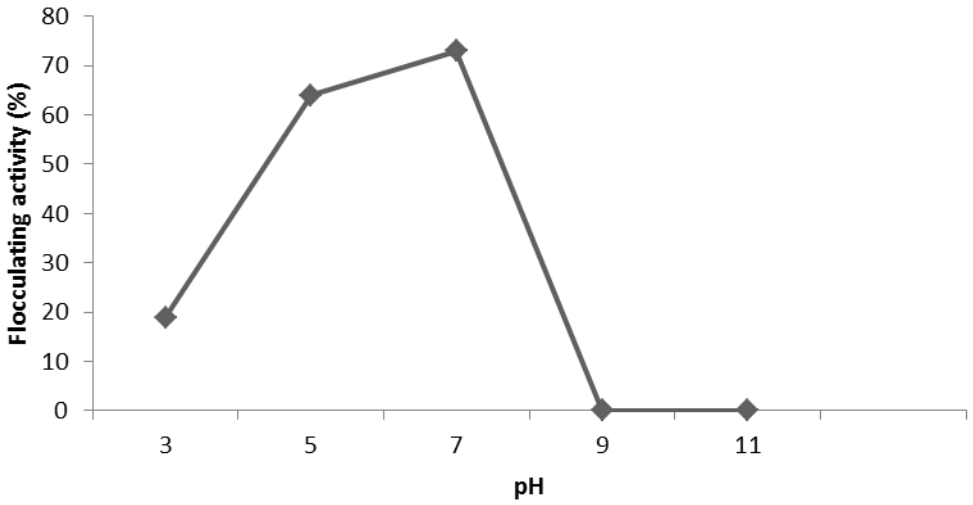

Figure 3.2: Effect of pH on bioflocculant activity.

3.3 Time course of bioflocculant production

Figure 3.3 shows the time course of the bioflocculant production by *Halobacillus* sp. Mvuyo in relation to pH. There was a rapid exponential increase in bioflocculant production within the first four days of cultivation, after which it slowed but reached peak flocculating activity of about 84% on the eight day of fermentation before declining. This decline may be due to cell autolysis and enzymatic activity (Gong *et al*., 2008). The pH of the medium was relatively constant during the first three days of incubation and thereafter increased slightly to peak level of pH on the fifth day before declining to the lowest level of pH 5.8 on the tenth day. The pH change may be due to the utilization of nutrients in the medium by test bacterium *Halobacillus* sp. Mvuyo.

Microorganisms differ in the culture times required for the production of bioflocculant. Our test bacterium released increasing amount of bioflocculant as culture time

progressed and maximum flocculating activity was reached after eight days of fermentation, thus suggesting that the bioflocculant was produced by biosynthesis (Fugita *et al*., 2001; Gong *et al*., 2008). In contrast to our finding, *Aspergillus parasiticus* produced a bioflocculant optimally after 72 h of cultivation (Deng *et al*., 2005). Also, flocculating activity induced by *Flavobacterium* was detected at the end of logarithmic growth phase (Hantula and Bramford, 1991), while a *Streptomycetes griseus* was reported to release large amount of flocculating substance into the medium after four days of cultivation (Shimforuya *et al*., 1995).

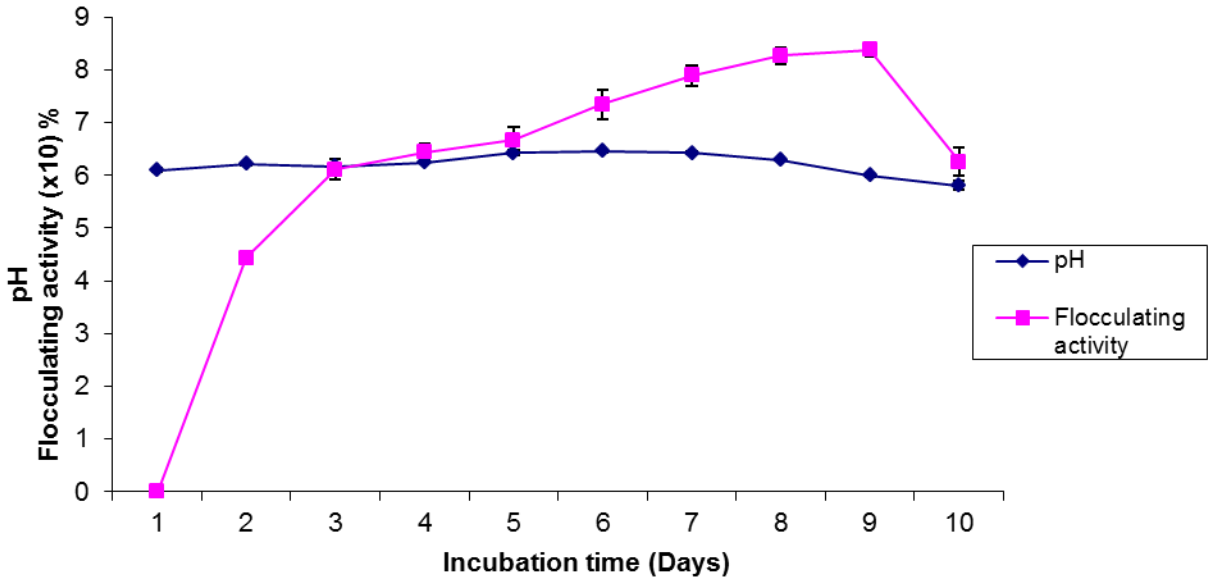

Figure 3.3: Time course of bioflocculant activity by *Halobacillus* sp. Mvuyo.

3.4 Characterization of bioflocculant

Various types of bioflocculants have been examined and reported as polysaccharides, proteins, glycoproteins, lipids and glycolipids (Xia *et al*., 2008). In this study, analysis of the partially purified bioflocculant was performed. As shown in Table

2, the yield of the purified bioflocculant stood at 0.34 g/L. The product yield was lower than those documented in literature. As example, the amount of crude bioflocculant produced by *Aeromonas* sp. was 2.25 g/L (Li *et al*., 2007). Also, the yield of MMF1 bioflocculant produced by multiple – microorganism consortia was up to 15 g/L (Zhang *et al*., 2007). The chemical analysis showed that the flocculant was composed of protein and carbohydrate. Carbohydrate content was found to be more than that of protein indicating that this bioflocculant is glycoprotein. These results are similar to that of bioflocculant ZS-7 produced by *Bacillus licheniformis* X14 where the flocculant was also found to be a glycoprotein made up of polysaccharide (91.5%) and protein (8.4%) (Li *et al*., 2009). Also, the composition of a bioflocculant obtained from *K. terrigena* was mainly polysaccharide and protein (Ghosh *et al*., 2008) and based on literature reported few bioflocculants are glycoproteins.

Table 3.2: Chemical properties of the partially purified bioflocculant produced by *Halobacillus* sp. Mvuyo.

Composition		
Dry weight (g/l)	**Total protein (mg/ml)**	**Total carbohydrate (mg/ml**)
0.34	3.5	4.5

4. Conclusion

This study has shown *Halobacillus* sp. as a potential source of new glycoprotein bioflocculant(s), the production of which could be optimal using glucose and ammonium chloride as sole carbon and nitrogen sources, as well as pH of 7. A detailed characterization of the purified bioflocculant as well as development of process condition for large scale production of the bioflocculant for pilot field trials are required and is a subject of further investigation in our group.

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CHAPTER FOUR

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

Abstract

A bioflocculant-producing marine bacterium previously isolated from marine sediment of Algoa Bay was screened for flocculant production. Comparative analysis of 16S rDNA sequence identified the isolate to have 99% similarity to *Virgibacillus* sp. XQ-1 and was deposited in the GenBank as *Virgibacillus* sp. Rob with accession number HQ537127. The bacterium produced biflocculants optimally in glucose (70.4%) and peptone (70.4%) as sole sources of carbon and nitrogen, alkaline pH (12) (74%); and the presence of Fe^{2+} (74%). Chemical analysis of the bioflocculant revealed it to be a polysaccharide.

Keywords: *Virgibacillus* sp.; Bioflocculant; Flocculating activity; Polysaccharide.

1. Introduction

Bioflocculants have recently attracted and received a considerable scientific and biotechnological attention, especially due to their biodegradability, non-toxicity, benign nature and freeness of secondary pollution (Li *et al*., 2009; Salehizadeh and Shojaosadati, 2001; Zhang *et al*., 2002). As example, bioflocculants have been practically applied in various processes i.e. in treatment of dye solutions, removal of humic acids, and removal of metal ions from polluted effluents (Deng *et al*., 2005; Zouboulis *et al*., 2004; Salehizadeh and Shojaosadati, 2003; Li *et al*., 2009). According to Oh *et al*. (2001) bioflocculants also have been used to successfully harvest *Chlorella vulgaris* from culture broth. Although few bioflocculants have been successfully identified but low flocculating capability, large dosage requirement and high costs are still a major concern in development for such practical applications (He *et al*., 2010; Li *et al*., 2009).

Bioflocculants mostly come from the natural secretions of bacteria and cell lysis (Liu *et al*., 2009). Furthermore, they are kinds of extracellular biopolymers of macromolecular substances such as protein, glycoprotein, polysaccharide and nucleic acids (Lian *et al*., 2007; Zheng *et al*., 2008; He *et al*., 2010). As example *Rhodoccocus erythropolis* S-1*, Bacillus subtillis*, *Bacillus licheniforms*, are found to produce proteinaceous bioflocculant while *Alcaligenes cupidus* KT201 was found to contain polysaccharides (Kurane *et al*., 1986; Yokoi *et al*., 1996; Toeda and Kurane, 1991; Shih *et al*., 2001) and according to Lee *et al*. (1995), *Arcuadendron* sp. TS-4 exhibited glycoprotein bioflocculant. Deng *et al*. (2005) reported that the effective groups for flocculation in proteinaceous bioflocculants include the amino and carboxyl groups while

for polysaccharide bioflocculants are mostly based on high molecular weights. These bio-flocculants produced by bacteria exhibit different flocculating activity or properties for various target compounds. Thus far, many bioflocculant-producing microorganisms (i.e. bacteria, fungi, algae and yeast) have been identified (Deng *et al*., 2005). However, only a few of these bacterial strains have been successfully used industrial processes due to their low flocculating capabilities, low productivity and high cost (Gao *et al*., 2006; He *et al*., 2002). Thus a need to overcome such problems is imperative and discovering novel efficient bioflocculants from varied environments becomes active areas of research (He *et al*., 2010). In this paper, we report on the bioflocculant producing potential of *Virgibacillus* sp. Rob isolated from the marine environment of Algoa Bay, Eastern Cape of South Africa as part of our exploration of the South African marine environment as a source of new bioflocculant-producing organisms.

2. Materials and methods

2.1 Screening for bioflocculant- producing bacteria

Many types of marine bacteria previously isolated and characterized from bottom sediment samples of Algoa Bay in South Africa as part of the culture collections of Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, South Africa and maintained in 20% glycerol at -80^0 C were screened for bioflocculant production.

The medium and cultivation were as follows: 10 g of glucose, 1.0 g of peptone, 0.3 g of $MgSO_4 \cdot 7\ H_2O$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 in 1 liter of filtered natural sea

water. The initial pH was adjusted to 7.0 by NaOH (0.1M) and HCl (0.1M). Two loopfuls of bacterial colonies were inoculated into five milliliters of the cultivation medium and incubated with shaking at 160rpm for five days at 28 ^{o}C (Zhang *et al*., 2007). At the end of inculation period, two milliliters of the fermentation broth was centrifuged (4000 g, 30min) to separate the cells. The cell free culture supernatant was analyzed for flocculating activity.

2.2 Determination 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 milliliter of the culture supernatant and 3 ml of 1% $CaCl_2$ were added into 100 ml of Kaolin clay suspension (4 g/l) in 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 absorbances of the upper phase were measured at 550nm using spectrophotometer. The flocculating activity was then calculated as follows:

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

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

2.4 The effect of carbon and nitrogen sources on bioflocculant activity

It has been well documented that changing the carbon and nitrogen sources highly influenced bacterial growth and production of bioflocculant (Sheng *et al*., 2006). Hence, we assessed the effects of different carbon and nitrogen sources on bioflocculant production in the test bacterium. Carbon source candidates included glucose, sucrose, fructose and starch, while the nitrogen source candidates included ammonium sulphate, ammonium chloride (inorganic nitrogen sources) and urea (organic nitrogen sources) replacing peptone. The assessments were done in accordance with the description of Lachhwani (2005).

2.5 Effects of various cations and pH on bioflocculant activity

Studying the effects of metal ions, flocculant tests was conducted utilizing the procedure elaborated above, but the $CaCl_2$ solution was replaced by various metal salt solutions, and the flocculating activity was measured. Solutions of KCl, $MgCl_2$ and $FeSO_4$ were used as salt sources. To assess the effect of pH on flocculating activity, the pH of the culture medium was adjusted using NaOH (0.1M) and HCI (0.1M) in the pH range of 3-12 (Yim *et al*., 2006).

2.6 Time course of bioflocculant activity

*Composition of culture medium***:** For the time course experiment, the composition of the medium for the bioflocculant production was as follows: 10 g of glucose, 1.0 g of

peptone, 0.3 g of $MgSO_4 \cdot 7\ H_2O$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 in 1 liter of filtered natural sea water (Zhang *et al*., 2007). The isolate was cultured under optimal growth conditions.

*Standardization of the inocula***:** Saline solution was prepared by adding 0.45 g NaCl in 50 ml of distilled water for each of the selected isolates. Fifty milliliters of saline solution was 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 cuvets and readjusted if need be to give OD_{660} of 0.1.

Time course assays (modified method of Gao *et al*. (2006): The inoculated saline solution was used as seed culture for inoculum preparation. Seed culture (1% v/v) was inoculated into 150 ml of medium in 500 ml flasks (prepared in duplicates) on a rotatory shaker (160rpm) at 28 ^{o}C. Sample was drawn at appropriate time intervals (every 24 hrs) for a period of 10 days.Two milliliter of culture broth was centrifuged at 4000 g for 30 min and the cell free supernatant was used to determine the flocculating activity. The pH of the broth samples was also measured.

2.7 Extraction and purification of bioflocculant

Purification and characterization of the bioflocculant was done following the methods described elsewhere (Chang *et al*., 1998; Chen *et al*., 2002) using media formulation based on the pre-determined optimum culture conditions. Briefly, after five days of fermentation, the culture solution was centrifuged at 4 600 rpm for 30 minutes to remove bacterial cells. One volume of distilled water was added to the upper phase and

centrifuged at 4 600 rpm for 15 minutes to remove insoluble substances. To the supernatant, two volumes of ethanol were added, and the mixed solution was stirred and left to stand at 4 °C for 12 hours. The precipitate was vacuum dried to obtain the crude biopolymer. The crude product was directly dissolved in distilled water to yield a solution, to which one volume of the mixed solution of chloroform and *n*-butyl alcohol (5:2 v/v) was added. After stirring, the mixture was set aside for 12 hours at room temperature (about 20 °C). The upper phase was centrifuged at 3000×g for 15 minutes and the supernatant was concentrated at 40 °C. Thereafter two volumes of ethanol were added to recover the precipitate.

2.8 Analysis/ characterization of purified bioflocculant

The protein content was measured using the Folin-Lowry method. Total sugar content was measured using phenol-sulphuric acid protocol as described by Lachhwani (2005).

2.9 Characterization of the bioflocculant-producing microorganism

DNA Extraction

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

PCR Amplification

PCR was carried out in 50 µl reaction volume containing 2 mM $MgCl_2$, 2 U Supertherm *Taq* polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 µl template DNA. Primer F1 and R5 binds to base positions 7 – 26 and 1496 – 1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877, respectively (Cook and Meyers, 2003). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 ºC for 2 min), 30 cycles of denaturation (96 ºC for 45 s), annealing (56 ºC for 30 s) and extension (72 ºC for 2 min), and a final extension (72 ºC 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.

DNA sequence analysis

Automated sequencing of the 16S rRNA genes of the bacterial isolate 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 sequence was edited manually based on the chromatogram readings and used in the BLAST search to determine the most similar sequences.

2.10 Statistical Analyses

The analyses of varience (ANOVA) were performed for levels of significance for the effect of carbon and nitrogen source, various cations respectively, as well as for the time course assays, all in relation to bioflocculant activity. The mean values were used to represent the initial pH of the medium with respect to flocculating activity of the bioflocculant.

3. Results and discussion

3.1 Screening for flocculant producing microorganism

Over 300 bacterial isolates previously obtained from marine sediments of Algoa Bay in the Eastern Cape Province, South Africa were screened for bioflocculant production. One of these marine bacterial isolates exhibited appreciable ability to flocculate kaolin suspension with a 70.4 % activity observed. Morphologically, the bacterium colonies were round, smooth and glistening with entire margin, cream in color, mostly flat and about 1 mm in diameter. The 16S rDNA of the analysis yielded PCR product of expected size (approx. 1.5 kb) (Figure 4.1). BLAST (Basic Local Alignment Search Tool) analysis of the nucleotide sequence of the 16S rDNA revealed the bacterium to have 99% similary to *Virgibacillus* sp. XQ-1 and the sequence was deposited in the Genbank as *Virgibacillus* sp. Rob with accession number HQ537127.

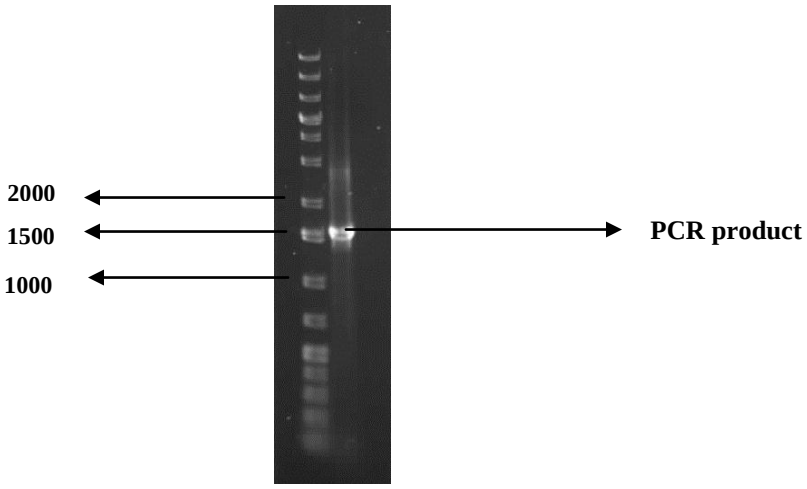

Figure 4.1: PCR product of the 16S rDNA of the test bacterium in 1% agarose gel electrophoresis. The left lane is DNA marker; while the right lane is the PCR product.

The genus *Virgibacillus* are gram-positive, motile, endospore-forming, irregular rod-shaped and halophilic bacteria (Peng *et al*., 2009), and was first proposed by Heyndrickx *et al*. (1998) and later amended by Heyrman *et al*. (2003) and now the genus is comprised of sixteen recognized species (Wang *et al*., 2008; Peng et al., 2009). The members of the genus are noted for meso-diaminopimelic acid presence in their cell-wall peptidoglycan, MK-7 menaquinone, diphosphatidylglycerol and phosphatidylglycerol polar lipids and the G+C content is in the range 36–43 mol% (Wang *et al*., 2008; Peng *et al*., 2009).

Like many halophiles, the genus *Virgibacillus* has been implicated in the production of a number of biotechnologically relevant biomolecules. A typical example of enzymes would include proteases. The enzyme proteases are found to be produced or secreted most by the genus *Virgibacillus* and are of particular interest due to their wide applications in laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, meat tenderization, detergents, food industry, photography, and

pharmaceuticals (Gupta *et al*., 2008). As example, protease was produced by a moderately halophilic bacterium isolated from *Pla-ra,* a fermented fish product in Thailand, and identified as *Virgibacillus marismortui* NB2-1 (Chamroensaksri et al., 2008). In another study, an extracellular proteinase produced by *Virgibacillus* sp. SK37 was found very useful in fish sauce fermentation (Sinsuwan *et al*., 2007). Also, *Virgibacillus pantothenticus* (MTCC 6729) isolated from fresh chicken meat samples produced a newly identified serine alkaline protease that was found useful as an additive in detergents for stain remover and other bio-formulations (Gupta *et al*., 2008). Other studies reported amylases (Namwong, 2006) and antifungal compound (Essghaier *et al*., 2009) production by some *Virgibacillus* species.

Although a number of bioactive compounds have been documented to be produced by *Virgibaccillus* species, to the best of our knowledge, this is the first report implicating the *Virgibaccillus* genus in bioflocculant production.

3.2 Factors affecting the bioflocculant production and flocculating activity

Constituents of the culture medium and culture conditions have been well documented to have effect on the production of bioflocculants (He *et al*., 2004; Xia *et al*., 2008). Carbon and nitrogen sources have also been emphasized (Salehizadeh and Shojaosadati 2001). Therefore, the effect of culture conditions on bioflocculant production by the test bacterium was assessed and the results are as shown in Table 4.1. It was found that using glucose as carbon source in the medium yielded bioflocculant with the highest flocculating activity (70.4%) ($\alpha \geq 0.05$) compared to other

carbon sources. Peptone was deduced to be the preferred nitrogen source as it resulted in production of bioflocculant with the highest flocculating activity (70.4%) ($\alpha \geq 0.05$) compared to other nitrogen sources, while Iron sulphate as cation considerably induced high flocculating activity (74%) ($\alpha \geq 0.05$) in comparison to other salts. Hence, all were significant at α level of 0.05.

As stated earlier, carbon and nitrogen sources have been well documented to have crucial effect on the bioflocculant production (Deng *et al*., 2005). However, these may vary with different strains. As example, for *Sorangium cellulosum*, soluble starch was optimal carbon source (Zhang *et al*., 2002). In the case of *Klebsiella* sp., maltose and urea were found to be best carbon and nitrogen source (Sheng *et al*., 2006).

Table 4.1: Effects of composition of medium on the bioflocculant production by *Virgibaccilus* sp. Rob

Carbon Source	**Glucose**	**Sucrose**	**Fructose**	**Starch**
Flocculating Activity %	70.4	-	18	5
Nitrogen Source	**Peptone**	**Ammonium Sulphate**	**Urea**	**Ammonium Chloride**
Flocculating Activity %	70.4	22	38	37
Salts	**Calcium Chloride**	**Magnesium Chloride**	**Iron Sulphate**	**Potassium Chloride**
Flocculating Activity %	70.4	-	74	-

Note: (-) denotes no flocculating activity; ($\alpha \geq 0.05$).

In another study, glucose, lactose and fructose were unfavorable for the bioflocculant production by *Bacillus licheniformis*, while glutamic acid, citric acid and

glycerol were favorable for its growth and bioflocculant production (Shih *et al*., 2001). Also, for *Aspergillus parasiticus*, corn starch and peptone were found to produce a bioflocculant with high flocculating activity (Deng *et al*., 2005). In the present study, *Virgibacillus* sp. preferred glucose and peptone as carbon and nitrogen sources, respectively. Similar results were obtained with *Proteus mirabilis* TJ-1 wherein both glucose and peptone were the best favorable carbon and nitrogen sources for TJ-1 bioflocculant production (Xia *et al*., 2008). Also, with *Serratia ficaria*, glucose amongst other carbon sources was suitable for the production of bioflocculant, although peptone and other nitrogen sources were not favorable (Gong *et al*., 2008). In a study by Lachhwani (2005), strain RDL-1 also effectively utilized glucose as the best carbon source.

It is generally accepted that the flocculation induced by bioflocculant can occur by bridging and charge neutralization (Sheng *et al*., 2006; Kurane and Nohata, 1994). It may also be noted that these cations may vary depending on the organism. As example, a bioflocculant from a haloalkalophilic *Bacillus* was greatly enhanced by the addition of cations like Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+} but the addition of cations like Al^{3+}, Fe^{3+} and Na^{+} led to drop in flocculating activity (Zheng *et al*., 2008). Also in another study, the flocculating activity of an *Aeromonas* produced bioflocculant were increased with the addition of K^{+}, Na^{+} and Ca^{2+} (Li *et al*., 2007). In our current study, the bioflocculant production by *Virgibacillus* sp. Rob was most enhanced in the presence of Iron sulphate in support of the findings of Sheng *et al*. (2006) and Li *et al*. (2008).

The effect of initial pH on bioflocculant production by *Virgibaccilus* sp. Rob was investigated (Figure 4.2). A steady decrease in flocculation activities were observed

from pH 3 to the lowest level of about 8% at pH 9 beyond which activity increased to optimal level (74%) pH 12.

The pH of the culture medium may affect or influence the production of the bioflocculant (Salehizadeh and Shojaosadati, 2001). This initial pH of the culture medium is said to determine the electric charge of the cells together with the oxidation potential which can affect the nutrient absoption and enzymatic reaction (Xia *et al*., 2008). This initial pH requirement may have different effect with various strains. For example, strain *Streptomycetes griseus* and *Aspergillus sojae* produced flocculating substances at acidic conditions (Shimforuya *et al*., 1995).

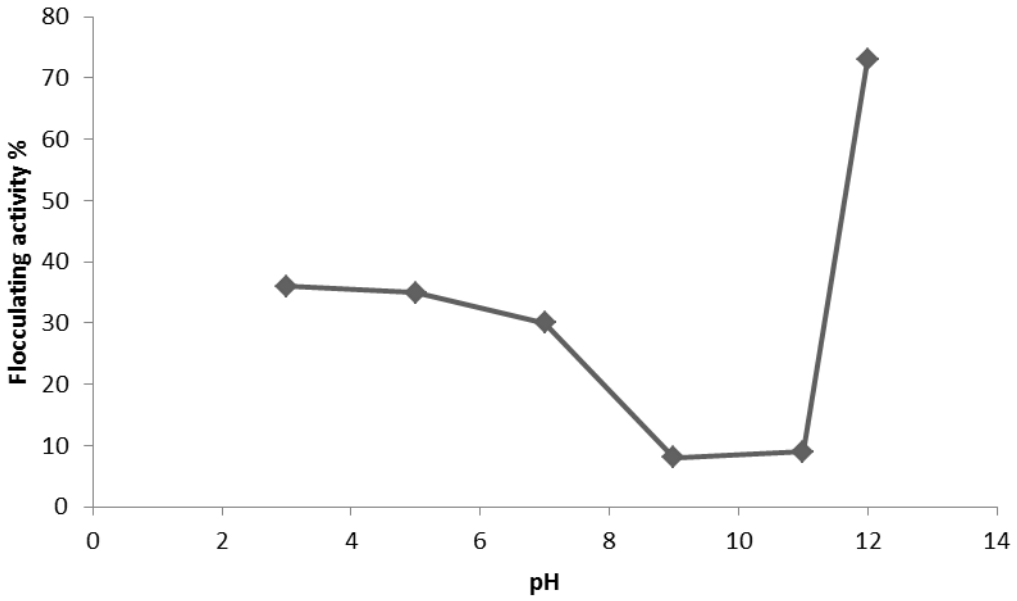

Figure 4.2: Effect of initial pH of cultivation medium on bioflocculant activity by *Virgibaccilus* sp. Rob.

The bioflocculant production by *Rhodococcus erythropolis* was higher at alkaline pH values (8.0-9.5) (Kurane *et al*., 1994), while in another study it showed activity at neutral

pH (Takagi and Kadowaki, 1985). In a study by Lachhwani (2005), the flocculant production by isolates RDL-1 and RDL-2 was greatly stimulated at slightly alkaline pH 7.5 while at acidic pH 6.0 and highly alkaline pH 10.5, it was low. In the current study, *Virgibacillus* sp. Rob preferred high alkaline pH (12.0) for optimal bioflocculant production. Li *et al*. (2009) had also reported *Bacillus licheniformis* X14 to produce a bioflocculant ZS-7 optimally under alkaline conditions.

3.3 Time course of bioflocculant activity

Figure 4.3 shows the time course of flocculating activity of strain *Virgibacillus* sp. Rob in relation to pH. The flocculating activity was observed to increase steadily to peak value of 81.5% in 4 days, and thereafter a steady decline in flocculating activity and from the 8^{th} day onwards flocculating activity was completely lost. Similar findings were obtained with *Streptomycetes griseus* where the flocculating activity increased with time and reached maximum activity on the fourth day (Shimforuya *et al*., 1995). Culture time for flocculant release into the medium and its activity may differ with different organisms. As example, the peak flocculating activity of a bioflocculant produced by *Citrobacter* sp. TKF04 was obtained in 24 h of cultivation and thereafter the activity dropped (Fugita *et al*., 2001). In another study (Gao *et al*., 2006), the maximum flocculating activity of a bioflocculant produced by *Vagococcus* sp. W31 was reached after 60 h. In the case of *Proteus morabilis* TJ-1 and *Bacillus licheniformis* X14, maximum cell production was reached in 24 hr while maximum flocculating activity was achieved in 48 h, thereafter the activity decreased (Xia *et al*., 2008; Li *et al*., 2009). A number of factors influence

bioflocculant production and thereby influence the bioflocculation process (Salehizadeh and Shojaosadati, 2001). Culture time amongst other factors may influence the production, distribution and flocculating capabilities of the bioflocculant. The pH on the other hand remained similar (6.2-6.4) throughout the incubation period.

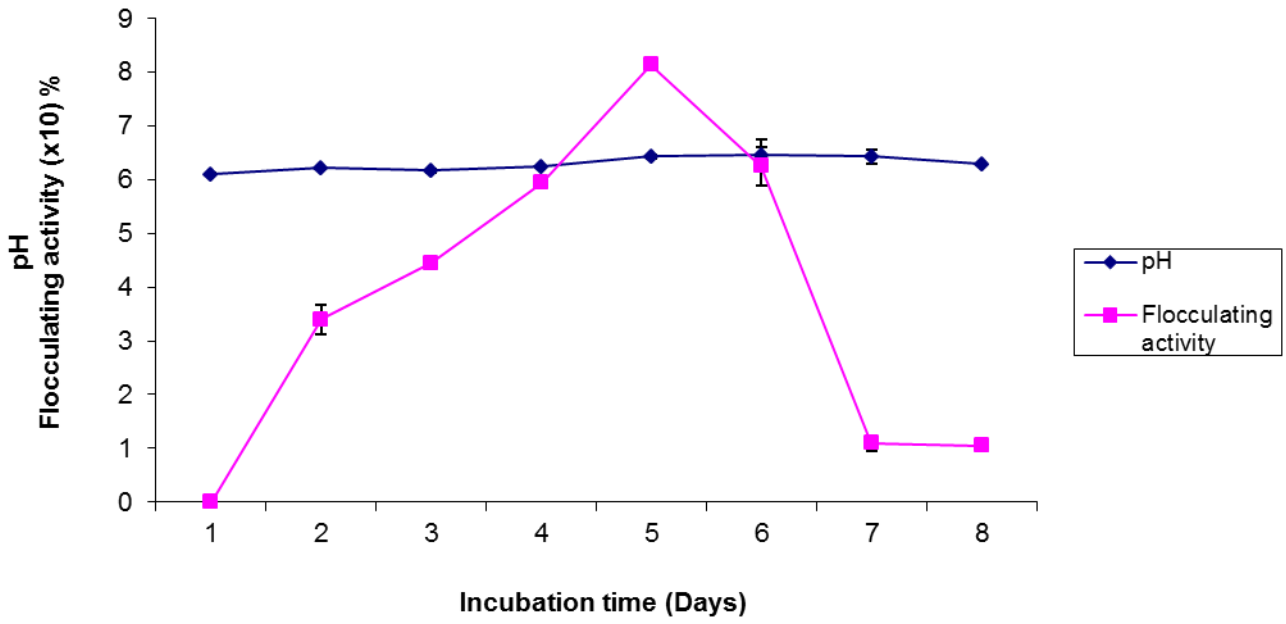

Figure 4.3: Time course of bioflocculant activity *Virgibaccilus* sp. Rob.

3.4 Analysis of bioflocculant composition

An amount of 0.264 g purified bioflocculant was recovered from a culture broth (1L) and the freeze dried bioflocculant was white in colour. The chemical analysis of the bioflocculant showed that it contained carbohydrate (9 mg/ml) (Table 4.2), and no protein was detected in the compound. Hence, the bioflocculant is composed mainly of polysaccharide. Quite a number of flocculants are reported to be polysaccharide. As

example bioflocculant produced by *Bacillus firmus* was found to be polysaccharide (Salehizadeh and Shojaosadati, 2003). Bioflocculant HBF-3 produced by a deep sea bacterium mutant (*Halomonas* sp. V3a) was reported to be mainly polysaccharide (He *et al*., 2010) and a novel *Serratia ficaria* bioflocculant was found to be a polysaccharide (Gong *et al*., 2008).

Table 4.2: Chemical properties of purified bioflocculant

Dry weight (g/l)	**Total protein (mg/ml)**	**Total carbohydrate (mg/ml)**
0.264	0	9

Conclusion

This study has shown *Virgibacillus* sp. Rob as a potential source of new polysaccharide bioflocculant(s), the production of which could be optimal using glucose and peptone as sole carbon and nitrogen sources, 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

Screening and characterization of a bioflocculant produced by *Oceanobacillus* sp. Pinky

Abstract

We report on the bioflocculant production potential of *Oceanobacillus* sp. Pinky, a marine bacteria isolated from the bottom sediments of Algoa Bay. Assessment of the effect of culture conditions on bioflocculant production by the bacterium revealed that the bacteria produce bioflocculants optimally when sucrose was used as the sole carbon source with flocculating activity of 80%, while peptone was the preferred nitrogen source with flocculating activity of 72.4%. Ca^{2+} was found to stimulate bioflocculant production, and neutral pH appeared to be preferred for bioflocculant production. The chemical analysis of the purified bioflocculant revealed it to be a glycoprotein. We conclude that *Oceanobacillus* sp. Pinky hold promise as potential producer of new bioflocullant(s).

Keywords: *Oceanobacillus* sp; Bioflocculant; Glycoprotein.

1. Introduction

In recent years, marine microorganisms have been investigated as potential sources of bioflocculants. Typical examples include exopolysaccharides produced by marine microorganisms such as *Zooglea* sp., *Pseudomonas* sp., and *Altermonas maleolii* (Yim *et al*., 2007). According to Zhang *et al*. (2002) the marine myxobacterium *Nannocystis* sp. NU-2 produced a new kind of flocculating substance (NU-2 flocculant) composed of proteins and polysaccharides, and in another report (He *et al*., 2010), a novel bioflocculant known as HBF-3 was produced by a deep sea bacterium mutant *Halomonas* sp. V3a.

The marine environments have been purported as good sources of interesting bacteria (Zhang *et al*., 2002), especially as a result of the unique environmental conditions of marine habitats, such as high pressure, low temperature and low nutrition. These characteristics could influence the diversity of microorganisms whose morphological, physiological and metabolic adaptations are aligned towards survival in such environments as opposed to those of terrestrial microorganisms. It is thus anticipated that the marine environments could be a good reservoir of unique organisms producing unique bioflocculants that could be of relevance in various industrial processes (He *et al*., 2010).

Bioflocculants secreted by microorganisms (i.e. bacteria, algae, fungi and yeast) are kinds of extracellular biopolymer including proteins, glycoproteins, polysaccharides, lipids and glycolipids (Zheng *et al*., 2008). These have greatly received considerable attention mainly in biotechnology due to the various limitations of synthetic flocculants

some of which have been reported as being carcinogenic, neurotoxic and dangerous source of pollution. Thus, flocculating substances of biological origin have been anticipated and proved as alternatives to such chemical synthetic flocculants. These bioflocculants are found to be important in environmental protection with no detrimental effects on fauna and flora as a result of their biodegradability and safeness for the ecosystem at large (Yokoi *et al*., 1995; He *et al*., 2001; Zhang *et al*., 2002). They are classified as naturally occurring flocculants, but mostly show weak flocculating activities in application (He *et al*., 2001). Hence the need for new biodegradable bioflocculants with strong flocculating activity. In this paper, we report on the potential of a marine bacterium identified as *Oceanobacillus* sp. Pinky for the production of bioflocculant.

2. Materials and Methods

2.1 Screening for bioflocculant- producing bacteria

A number of marine bacteria previously isolated and characterized from bottom sediment samples of Algoa Bay in South Africa as part of the culture collections of Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, South Africa and maintained in 20% glycerol at -80 ^{0}C were screened for bioflocculant production.

2.2 Media and culture conditions

The medium used in this study was as follows: 10 g of sucrose, 1.0 g of peptone, 0.3 g of $MgSO_4 \cdot 7\ H_2O$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 in 1 liter of filtered natural sea water. The initial pH was adjusted to 7.0 by NaOH (0.1M) and HCl (0.1M). Two loopfuls of bacterial colonies were inoculated in five milliliters of the cultivation medium and incubated with shaking at 160rpm for five days at 28 ^{o}C (Zhang *et al*., 2007). At the end of incubation period, two milliliters of the fermentation broth was centrifuged (4000 g, 30min) to separate the cells. The cell free culture supernatant was analyzed for flocculating activity.

2.2 Measurement 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 milliliter of the culture supernatant and 3 ml of 1% $CaCl_2$ were added into 100 ml of Kaolin clay suspension (4 g/l) in 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 absorbances of the upper phase were measured at 550nm using spectrophotometer. The flocculating activity was then calculated as follows:

Flocculating rate = {(A-B)/A} × 100%

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

2.3 The effect of carbon and nitrogen sources on bioflocculant production

It has been well documented that changing the carbon and nitrogen sources highly influenced bacterial growth and production of bioflocculant (Sheng *et al*., 2006). Hence, we assessed the effects of different carbon and nitrogen sources on bioflocculant production in the test bacterium. Carbon source candidates included glucose, sucrose, fructose and starch, while the nitrogen source candidates included ammonium sulphate, ammonium chloride (inorganic nitrogen sources) and urea (organic nitrogen sources) replacing peptone.

2.4 Effects of various cations and pH on bioflocculant production

Studying the effects of metal ions, flocculant tests was conducted utilizing the procedure elaborated above, but the $CaCl_2$ solution was replaced by various metal salt solutions, and the flocculating activity was measured. Solutions of KCl, $MgCl_2$ and $FeSO_4$ were used as salt sources. To assess the effect of pH on flocculating activity, the pH of the culture medium was adjusted using HCl and NaOH in the pH range of 3-12 (Yim *et al*., 2006).

2.5 Time course of flocculating activity

*Composition of culture medium***:** For the time course experiment, the composition of the medium for the bioflocculant production was as follows: 10 g of sucrose, 1.0 g of peptone, 0.3 g of $MgSO_4 \cdot 7\ H_2O$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 in 1 liter of filtered

natural sea water. (Zhang *et al*., 2007). The isolate was cultured under optimal growth conditions.

*Standardization of the inocula***:** Saline solution was prepared by adding 0.45 g NaCl in 50 ml of distilled water for the selected isolate. Fifty milliliters of saline solution was inoculated with a loop full of colonies for the strain. The Optical density (660nm) of each will be measured by taking 100 µl (culture) into 1 ml of distilled water in 3 ml cuvets and readjusted if need be to give OD_{660} 0.1.

Time course assays (modified method of Gao *et al*. (2006): The inoculated saline solution was used as seed culture for inoculum preparation. Seed culture (1% v/v) was inoculated into 150 ml of medium in 500 ml flasks (prepared in duplicates) on a rotatory shaker (160rpm) at 28 ^{o}C. Sample was drawn at appropriate time intervals (every 24 hrs) for a period of 10 days .Two milliliter of culture broth was centrifuged at 4000 g for 30 min and the cell free supernatant was used to determine the flocculating activity. The pH of the broth samples was also measured.

2.6 Extraction and purification of bioflocculant

Purification and characterization of the bioflocculant was done following the methods described elsewhere (Chang *et al*., 1998; Chen *et al*., 2002) using media formulation based on the pre-determined optimum culture conditions. Briefly, after five days of fermentation, the culture solution was centrifuged at 4 600 rpm for 30 minutes to remove bacterial cells. One volume of distilled water was added to the upper phase and centrifuged at 4 600 rpm for 15 minutes to remove insoluble substances. To the supernatant, two volumes of ethanol were added, and the mixed solution was stirred

and left to stand at 4 °C for 12 hours. The precipitate was vacuum dried to obtain the crude biopolymer. The crude product was directly dissolved in distilled water to yield a solution, to which one volume of the mixed solution of chloroform and *n*-butyl alcohol (5:2 v/v) was added. After stirring, the mixture was set aside for 12 hours at room temperature (about 20 °C). The upper phase was centrifuged at 3000×g for 15 minutes and the supernatant was concentrated at 40 °C. Thereafter two volumes of ethanol were added to recover the precipitate.

2.7 Analysis/ characterization of purified bioflocculant

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).

2.8 Characterization of the bioflocculant-producing microorganism

DNA Extraction

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

PCR Amplification

PCR was carried out in 50 µl reaction volume containing 2 mM $MgCl_2$, 2 U Supertherm *Taq* polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 µl template DNA. Primer F1 and R5 binds to base positions 7 – 26 and 1496 – 1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877, respectively (Cook and Meyers, 2003). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96 ºC for 2 min), 30 cycles of denaturation (96 ºC for 45 s), annealing (56 ºC for 30 s) and extension (72 ºC for 2 min), and a final extension (72 ºC 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.

DNA sequence analysis

Automated sequencing of the 16S rRNA genes of the bacterial isolate 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 sequence was edited manually based on the chromatogram readings and used in the BLAST search to determine the most similar sequences.

2.9 Statistical Analyses

The analyses of varience (ANOVA) were performed for levels of significance for the effect of carbon and nitrogen source, various cations respectively, as well as for the time course assays, all in relation to bioflocculant activity. The mean values were used to represent the initial pH of the medium with respect to flocculating activity of the bioflocculant.

3. Results and discussion

3.1 Screening and identification

Ten bacteria isolates with ability to flocculate Kaolin clay were chosen amongst over three hundred bacteria previously obtained from marine sediments of Algoa Bay, South Africa. Among these included our test bacterium which had the highest flocculating activity (80% activity). Amplification of the 16S rRNA gene of the bacterium yielded the expected amplicon size of approximately 1.5 kb (Figure 5.1). Nucleotide sequence analysis of the amplified product revealed the bacterium to have 98% similarity to *Oceanobacillus iheyensis* HTE831 and the sequences was deposited in GenBank as *Oceanobacillus* sp. Pinky with accession number HQ537126.

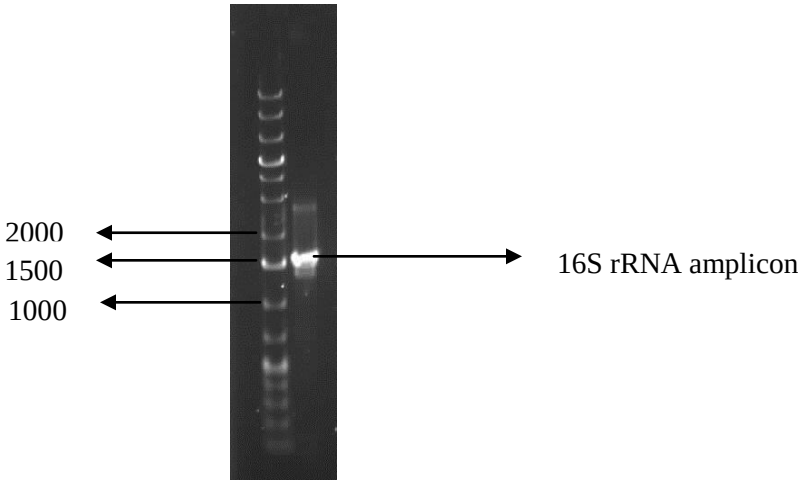

Figure 5.1: PCR product of the 16S rDNA of the test bacterium in 1% agarose gel electrophoresis. The left lane is DNA marker; while the right lane is the PCR product

Bacteria belonging to *Oceanobacillus* genus are aerobic, gram-positive, rod-shaped, motile, spore-forming and moderately halophilic and alkaliphilic, and strains have been isolated from various environments i.e. soils and related environments, marine settings and freshwater (Nam *et al*., 2008; Yumoto *et al*., 2005). The genus was originally assigned to the genus *Bacillus*, but due to 16S rRNA gene sequence comparisons and chemotaxonomic characteristics were later reclassified within the genus *Oceanobacillus* (Nam *et al*., 2008; Lu *et al*., 2001). The genus *Oceanobacillus* was first proposed with the single species *Oceanobacillus iheyensis* however the genus is currently comprised of six species (Kim *et al*., 2007; Nam *et al*., 2008).

Alkaliphilic or halophilic microorganisms have been documented as very important in basic research as well as in industrial applications (Yumoto *et al*., 2005). They offer a multitude of actual or potential applications in various fields of biotechnology (Margesin and Schinner, 2001). As example, they play a crucial role in food biotechnology for the production of fermented food and food supplements (Margesin and Schinner, 2001).

They have also been implicated in the production of bacteriorhodopsin (viz. holography, spatial light modulators and optical memories), biopolymers or use in microbially enhanced oil recovery, and also compatible solutes and enzymes (Ventosa *et al*., 1995; Margesin and Schinner, 2001; Choudhry *et al*., 2003) in several industrial processes.

A member of the genus, *Oceanobacillus iheyensis* has been reported to produce laccase-like enzymes (Claus, 2003). In another study (Sato *et al*., 2005), *Oceanobacillus iheyensis* JCM 11309 together with other microorganisms (i.e. *Escherichia coli* K1, *Synechocystis* sp. PCC 6803 and *Thermotoga maritima* ATCC 43589) were found to contain the genes encoding the enzyme D-Alanine-D-alanine ligase which is an important enzyme in the synthesis of bacterial peptidoglycan. Also, in a recent study, a novel thermo-alkali-stable catalase-peroxidase was purified and characterized from *Oceanobacillus oncorhynchi* subsp. *incaldaniensis* subsp. nov., strain 20AG (Carlandrelli *et al*., 2008). Although a number of enzymes and active compounds have been reported for the *Oceanobacillus* genus, to the best of our knowledge ours is the first report implicating the *Oceanobacillus* genus in bioflocculant production.

3.2 Factors affecting the bioflocculant production and flocculating activity

Carbon, nitrogen and other factors have been reported to have an important effect on the production and flocculating activity of bioflocculants (Deng *et al*., 2005). The effect of carbon source, nitrogen source, cations and initial pH of the production medium

were examined on flocculating activity of the test bacterium, and the results are as shown in Table 5.1.

Amongst the various carbon sources examined, glucose and sucrose were effective for the bioflocculant production. However, sucrose yielded higher flocculating activity (80%) ($\alpha \geq 0.05$). On the other hand, peptone, among the tested nitrogen sources appeared more favorable for bioflocculant production with the highest flocculation activity of 72.4% ($\alpha \geq 0.05$).

A number of studies have been documented emphasizing on the importance of carbon and nitrogen sources for the production of bioflocculant (Salehizadeh and Shojaosadati, 2001). However, carbon and nitrogen source requirement may differ with different strains. For example, *Bacillus licheniformis* X14 preferred sucrose, starch and ethanol as favorable carbon sources for the production of ZS-7 bioflocculant, while ammonium chloride was effectively utilized as nitrogen source (Li *et al*., 2009). In another study (Sheng *et al*., 2006), *Klebsiella* sp., preferred maltose and urea as best carbon and nitrogen source. In the case of our test bacterium, sucrose and peptone were most favorable for the bioflocculant prodcution. Similar results were obtained with *Aeromonas* sp. N11, wherein sucrose was found as best favorable carbon source (Li *et al*., 2007). Also, Zheng *et al*. (2008) reported sucrose as the preferred carbon source in their study. In the case of *Aspergillus parasiticus*, peptone was the suitable nitrogen source while corn starch was the preferred carbon source for the production of bioflocculant (Deng *et al*., 2005).

Cations stimulate flocculating activity by neutralization and stabilizing the residual negative charge of functional groups and thereby forming bridges between particles (Salehizadeh and Shojaosadati, 2001). Their role is therefore to enhance initial adsorption of flocculants on suspended particles by decreasing the negative charge on both the polymer and the particle (Salehizadeh and Shojaosadati, 2001). In the case of our test bacterium, calcium chloride was the most stimulating cation on flocculating activity. Similarly, the flocculating ability of MBFF19 bioflocculant produced by Bacillus sp F19 was enhanced by calcium (Zheng *et al*., 2008). Also, a biopolymer flocculant produced by *Bacillus* sp. PY-90 increased flocculating activity with the addition of Ca^{2+} (Yokoi *et al*., 1995).

Table 5.1: Effects of composition of medium on the flocculating activity produced by *Oceanobacillus* sp. Pinky.

Carbon Source	**Glucose**	**Sucrose**	**Fructose**	**Starch**
Flocculating Activity %	72.4	80	-	12
Nitrogen Source	**Peptone**	**Ammonium Sulphate**	**Urea**	**Ammonium Chloride**
Flocculating Activity %	72.4	-	15	36
Salts	**Calcium Chloride**	**Magnesium Chloride**	**Iron Sulphate**	**Potassium Chloride**
Flocculating Activity %	72.4	-	61	25

Note: (-) denotes no flocculating activity; ($\alpha \geq 0.05$).

The results of the effect of initial pH on bioflocculant production by *Oceanobacillus* sp. Pinky are represented in Figure 5.2. The flocculating activity increased with the

increase in pH and reached optimum (72.4%) at neutral pH and thereafter flocculating activities decreased as pH increased to pH 11, and at pH 12 flocculating activity increased again to the second highest level of about 70%. Similar results were obtained with the bioflocculant PG.a21 Ca where the flocculation efficiency is lower at pH lesser than seven but exhibit maximum activity at exactly neutral pH but this flocculants's activity then decreases with the increase of pH after pH 7 (Pan *et al*., 2009). Also, in the case of strain TJ-1, pH 7.0 was the optimum for the bioflocculant production (Xia *et al*., 2008).

One of the factors documented to influence the production and flocculating activity of the bioflocculant is the initial pH of the medium (Salehizadeh and Shojaosadati, 2001). The initial pH determines the electric charge of cells and oxidation-reduction potential which may affect the absorption of nutrients and the enzymatic reaction (Salehizadeh and Shojaosadati, 2001; Xia *et al*., 2008). However, this may differ with different strains. As example, higher pH (above 7) appears more favorable for ZS-7 bioflocculant production by *Bacillus licheniformis* X14 (Li *et al*., 2009). *Rhodococcus erythropolis* preferred alkaline pH values (8.0-9.5) for the flocculant production (Kurane *et al*., 1994). In another study (Deng *et al*., 2005) *Aspergillus parasiticus* preferred acidic conditions for synthesis, secretion and production of bioflocculant in the culture medium (Deng *et al*., 2005).

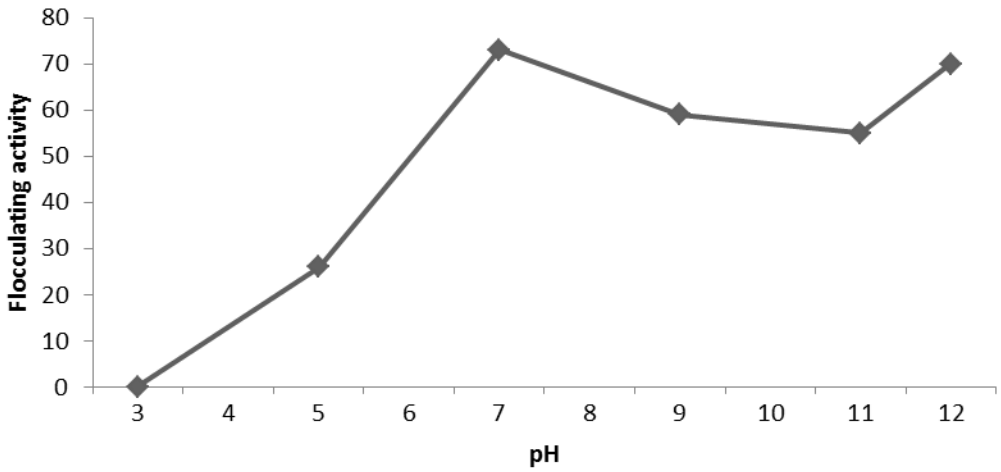

Figure 5.2: Effect of initial pH on bioflocculant activity by *Oceanobacillus* sp. Pinky.

3.3 Time course of the bioflocculant activity

A time course of the bioflocculant production by *Oceanobacillus* sp. Pinky in relation to pH was studied as shown in Figure 5.3. The flocculating activity was initially observed to increase steadily with culture age. Peak activity (72.4%) was reached after 6 days of cultivation and thereafter a dramatic decrease in flocculating activity was observed. The decrease of flocculating activity could be due to cell autolysis and enzymatic activity (Gong *et al*., 2008). The consequent pH was observed to be at constant rate within the first four days of cultivation time however, after the flocculating activity reached maximum peak, the pH starts to decline. The change in pH confirms to the utilization of the nutrients in the medium by the strain (Gong *et al*., 2008).

In case of other microorganisms, cultivation time for the release of flocculant substance into culture broth may differ. For example, in a study by Shimforuya *et al*. (1995), *Streptomycetes griseus*, the flocculating activity increased with the increase of cultivation time however, the maximum flocculating activity was reached after four days

and then decreased linearly with cultivation time. In another study (Desouky et al., 2008), the flocculating activities of three isolates (QUST2, QUST6 and QUST9) belonging to *Bacillus* genus were observed to increase with increasing cultivation time. In the case of *Proteus mirabilis* TJ-1 and *Bacillus licheniformis* X14, maximum flocculating activity were achieved in 48 h, thereafter the activity decreased (Xia *et al*., 2008; Li *et al*., 2009).

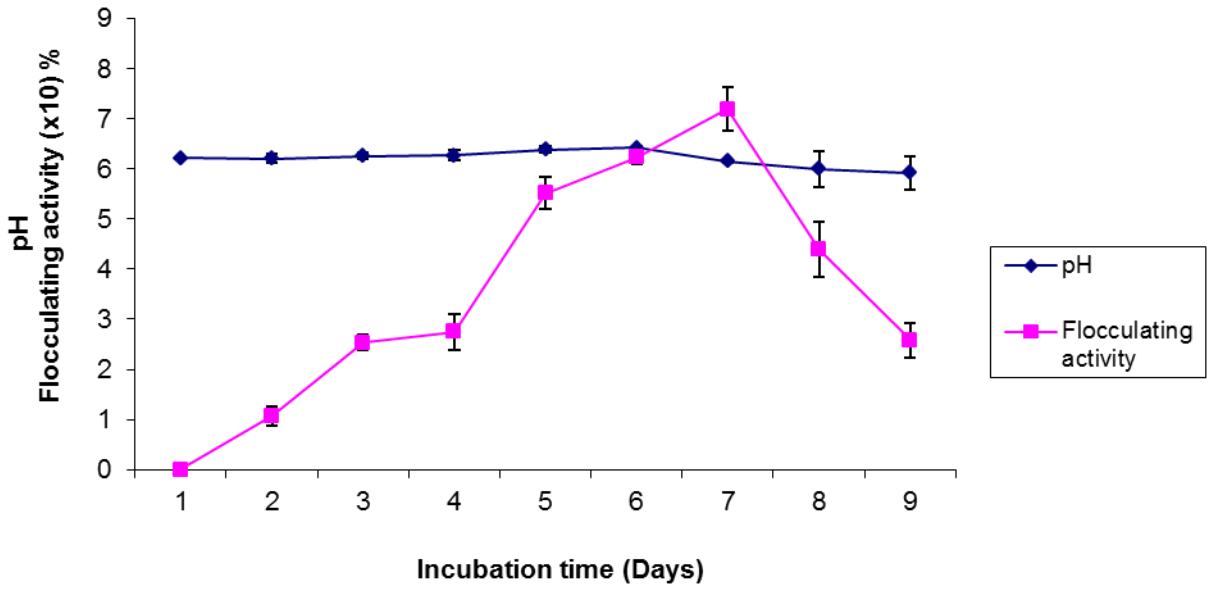

Figure 5.3: Time course of bioflocculant activity of *Oceanobacillus* sp. Pinky.

3.4 Composition analysis of bioflocculant

The yield of the purified bioflocculant stood at 0.34 g as shown in Table 2. Chemical analysis of the purified bioflocculant revealed that it contained both protein (4.9 mg/ml) and carbohydrate (3.3 mg/ml), thus suggesting the compound to be a glycoprotein. Similar bioflocculants have been reported before (Madla *et al*., 2005; Lungmann *et al*., 2007), and glycoprotein flocculants can be applied in various fields including

biotechnology and nanotechnology (i.e. silicon wafers, lipid films and liposomes) (Lungmann *et al*., 2007).

Table 5.2: chemical properties of purified bioflocculant

Dry wt. bioflocculant (g/l)	**Total protein (mg/ml)**	**Total carbohydrate (mg/ml)**
0.34	4.9	3.3

Conclusion

This study has shown the bioflocculant-producing bacterium *Oceanobacillus* sp. Pinky as potential source of glycoprotein bioflocculant(s). The optimal bioflocculant production conditions included sucrose and peptone as carbon and nitrogen sources respectively, as well as pH of 7. It is suggested that further characterization and development of process condition for large scale production of the purified bioflocculant be pursued further and this is a subject of further investigation in our group.

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CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

To date, a number of studies on flocculating substances from microorganisms (viz. bacteria, fungi, actinomycetes and yeast) mostly isolated from soil, activated sludge and very few from marine environments have been documented (Deng *et al*., 2005; Oh *et al*., 2001). As example, Xia *et al*. (2008), reported studies on novel bioflocculant TJ-F1 produced by strain *Proteus mirabilis* TJ-1 from mixed activated sludge. Pan (2009), reported on flocculation property of bioflocculant PG.a21 Ca. Also, in another study by Li *et al*. (2007), *Aeromonas* sp. N11 bioflocculant was reported with flocculating capabilities. Although a number of bioflocculants have been reported, high production costs and practical application of such bioflocculant(s) have remained a major problem, leading to exploration for novel efficient bioflocculant from microorganisms in varied environments (Li *et al*., 2009) especially from unusual environments.

In this study, we screened several hundred bacteria isolated from marine sediments of Algoa Bay in South Africa for bioflocculant production and amongst these were three positive bacteria identified as *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky based on 16S rDNA nucleotide sequence analysis. All three bacteria are documented as halophilic and alkaliphilic *Bacillus* related species and are gram-positive, aerobic, rod shaped, motile and spore-forming (Spring *et al*., 1996; Peng *et al*., 2009, Nam *et al*., 2008; Yumoto *et al*., 2005). Such halophilic and alkaliphilic bacteria have been reported to live in saline environments, and offer multitude of actual or potential applications in various fields of biotechnology (Margesin and Schinner, 2001). Although these organisms have been associated with production of valuable

compounds, to the best of our knowledge they have not been implicated in bioflocculant production.

Bacteria have the ability to utilize nutrients in culture medium to synthesize polymers present within the cell under the action of specific enzymes, and thus excrete such polymers into the medium or surface of the bacteria as capsule (Desouky *et al*., 2008). The action of bacteria converts the simple substances in their environment into polymers that can be utilized as flocculants (Desouky *et al*., 2008). Certain factors may affect or influence the production of flocculant thereby affecting the process of bioflocculation (Salehizadeh and Shojaosadati, 2001). These factors include carbon and nitrogen sources, cations and initial pH of the production medium (Salehizadeh and Shojaosadati, 2001). In this study the effect of the mentioned factors on the production and flocculating capabilities of bioflocculant produced by three bacteria - *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky were examined. As observed, different strains may vary on the requirement of these factors. In the case of this study, amongst the studied carbon and nitrogen sources, both *Halobacillus* sp. Mvuyo and *Virgibacillus* sp. Rob preferred glucose as carbon source while *Oceanobacillus* sp. Pinky favored sucrose. On the other hand, for nitrogen source, both *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky effectively utilized peptone; however, *Halobacillus* sp. Mvuyo preferred ammonium chloride.

One other major condition for the process of flocculation is that the molecules of the flocculant adsorb onto the surface of the particle (Li *et al*., 2008). In this study, Kaolin clay suspension was used as the test material in determining the flocculating activity of the bioflocculant(s) as previously utilized by other researchers (Lachhwani, 2005; Deng

et al., 2005; Gong *et al*., 2008). The charge on Kaolin particles in solution are said to be negative (Li *et al*., 2008; Desouky *et al*., 2008). Thus, when the bioflocculant is added on kaolin suspension, it approaches the particles in the solution and the attractive force exceeds the electrostatic repulsion force (Li *et al*., 2008; Desouky *et al*., 2008). 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. However, the required cation may differ with different strains. In the case of this study, the flocculating activity of bioflocculant produced by *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky was stimulated by the addition of Ca^{2+} while *Virgibacillus* sp Rob required the use of Fe^{2+}.

The initial pH of the production medium is one of the factors affecting the production and flocculating activity of the bioflocculant (Salehizadeh and Shojaosadati, 2001). In this case, the bioflocculant production of the studied bacterial strains was affected by the initial pH of the production medium. As observed from the results, flocculating activity of bioflocculant produced by *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky increased steadily with the increase of pH and reached maximum peak at neutral pH (7.0). The flocculating activity of bioflocculant by *Virgibacillus* sp. Rob obtained peak activity at alkaline pH (12.0). As noticed, effective pH values differ with strains in support of previous reports. As example, Yim *et al*. (2007) reported bioflocculant produced by *Gyrodium impudicum* KG03 to have maximum activity at acidic pH (4.0). In another study (Lachhwani, 2005), bioflocculant production and activity were greatly stimulated at alkaline pH 7.5.

Time course of bioflocculant formation and activity of *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky in relation to pH were examined. As observed, the production and activity of bioflocculant of different strains may vary with culture time. In this study, the flocculating activities of bioflocculants produced by *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky were observed to increase steadily with culture times. However, the peak activities were reached at different culture times. For example, for *Halobacillus* sp. Mvuyo, the peak activity was obtained after eight days; *Virgibacillus* sp. Rob after four days; and *Oceanobacillus* sp. Pinky was observed after six days. The increase of flocculant and activity with culture time may significantly indicate that the bioflocculant(s) are produced by biosynthesis (Fugita *et al*., 2001). In all three cases, after the maximum peak was reached, a dramatic decrease in flocculating activity was observed. Cell autolysis and enzymatic activity may have been the cause of this decrease (Fugita *et al*., 2001). The corresponding pH of the culture media varied. In the case of *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky the pH initially was constant within the first 3-4 days of cultivation, followed by a slight increase before declining to the lowest level of pH 5 on the tenth day. In contrast, the culture medium of *Virgibacillus* sp. Rob remained at similar pH throughout the cultivation period.

Microorganisms have the ability to convert the simple substances in their environment into complex polymers i.e. polysaccharides and glycoproteins (Desouky *et al*., 2008). Also, different bioflocculants may be produced by different microorganisms. In this study, chemical analysis of the purified bioflocculant(s) produced by *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky revealed them to be glycoproteins containing

both carbohydrate and protein. However, *Oceanobacillus* sp. Pinky had higher protein content than carbohydrate while with *Halobacillus* sp. Mvuyo the reverse was the case. *Virgibacillus* sp. Rob was however found to produce a polysaccharide bioflocculant. Lee *et al*. (1995) and Wang *et al*. (1995) had both reported on glycoprotein bioflocculant(s) by some organism, while Yokoi *et al*. (1996) reported on a *Bacillus subtilis* IFO 3335 producing polysaccharide bioflocculant.

In conclusion, this research has revealed three marine bioflocculant-producing bacteria: *Halobacillus* sp. Mvuyo, *Virgibacillus* sp. Rob and *Oceanobacillus* sp. Pinky as potential sources of glycoprotein and polysaccharide bioflocculant(s). Their different optimal bioflocculant production conditions included: (i) *Halobacillus* sp. Mvuyo: glucose and ammonium chloride as carbon and nitrogen source respectively, with initial pH of 7. (ii) *Virgibacillus* sp. Rob: glucose and peptone as carbon and nitrogen source respectively as well as pH 12.0; and (iii) *Oceanobacillus* sp. Pinky: Sucrose and peptone as carbon and nitrogen source respectively with initial pH of 7.

Considering such findings, the following recommendations are suggested:

- Development of process condition for large scale production of the bioflocculant
- Further detailed characterization of the bioflocculants such as elemental analysis and their mechanisms of action.
- Pilot scale assessment of the applicability of the purified bioflocculants for water/wastewater treatments.

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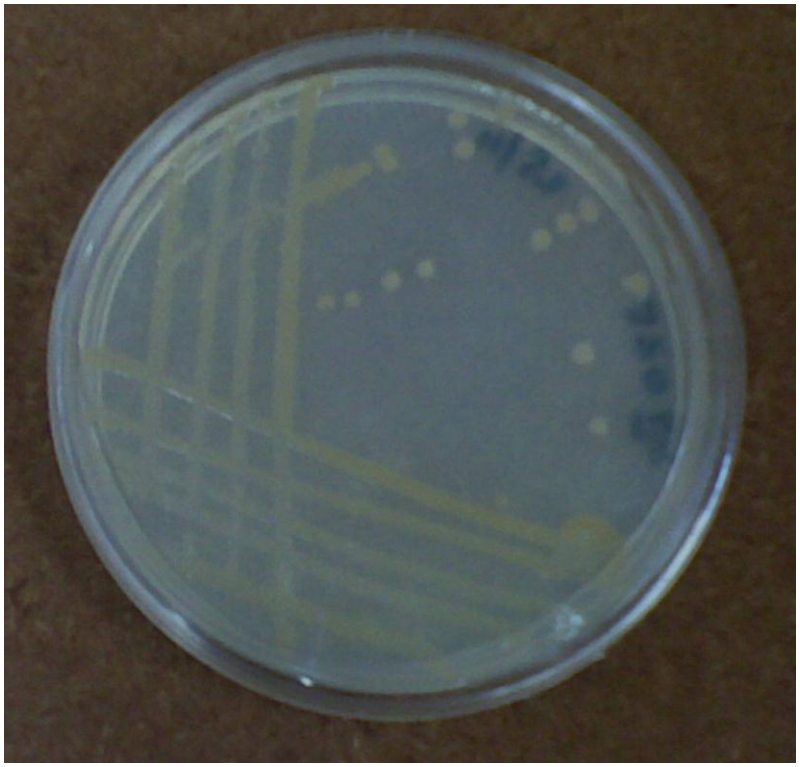
APPENDIX A

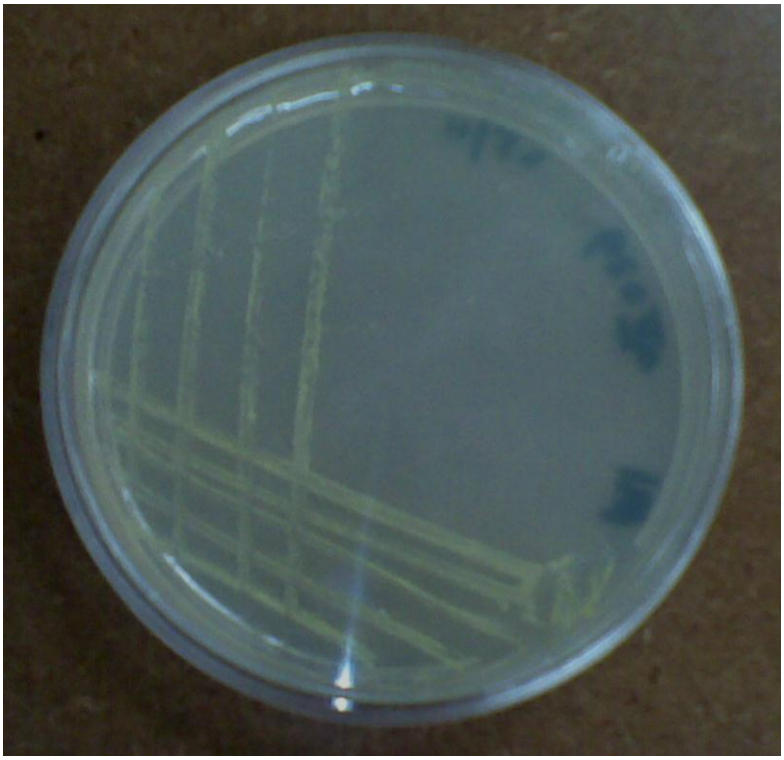
Picture 1: *Halobacillus* sp. Mvuyo on M1 agar

APPENDIX B



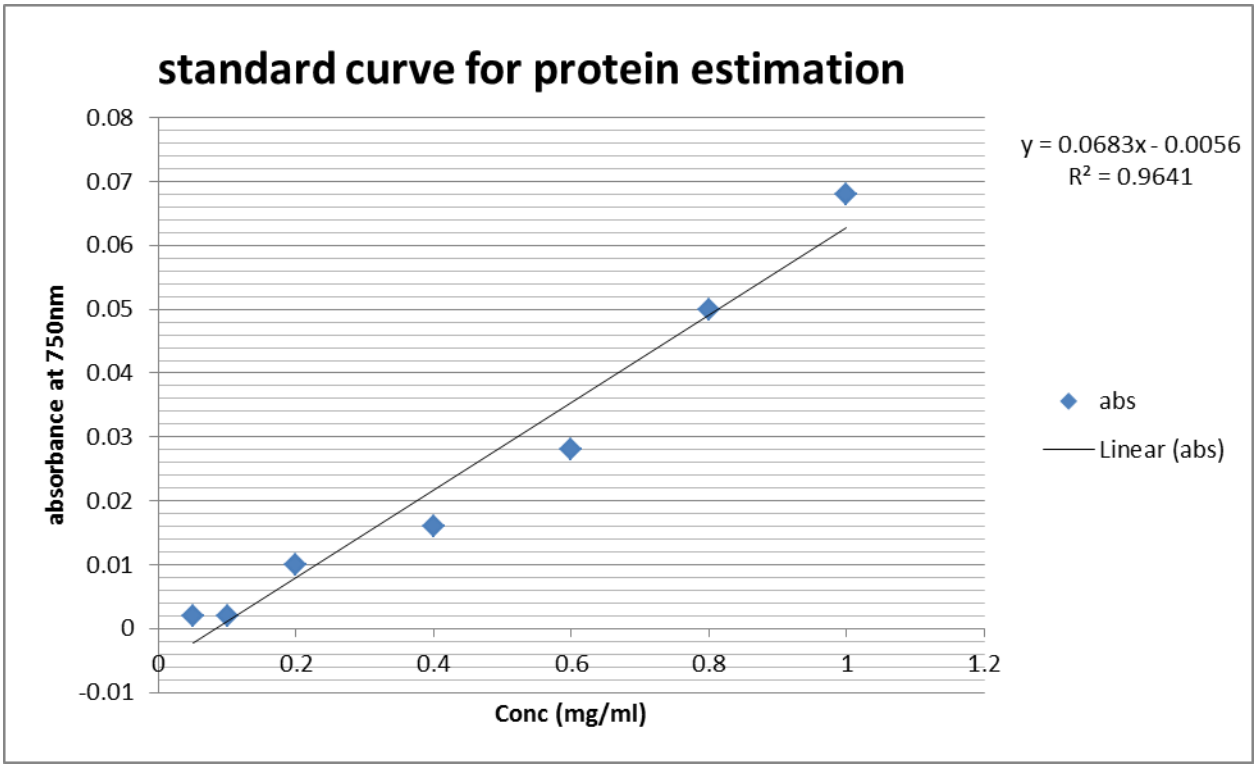
Picture 2: *Virgibacillus* sp. Rob on M1 agar

APPENDIX C

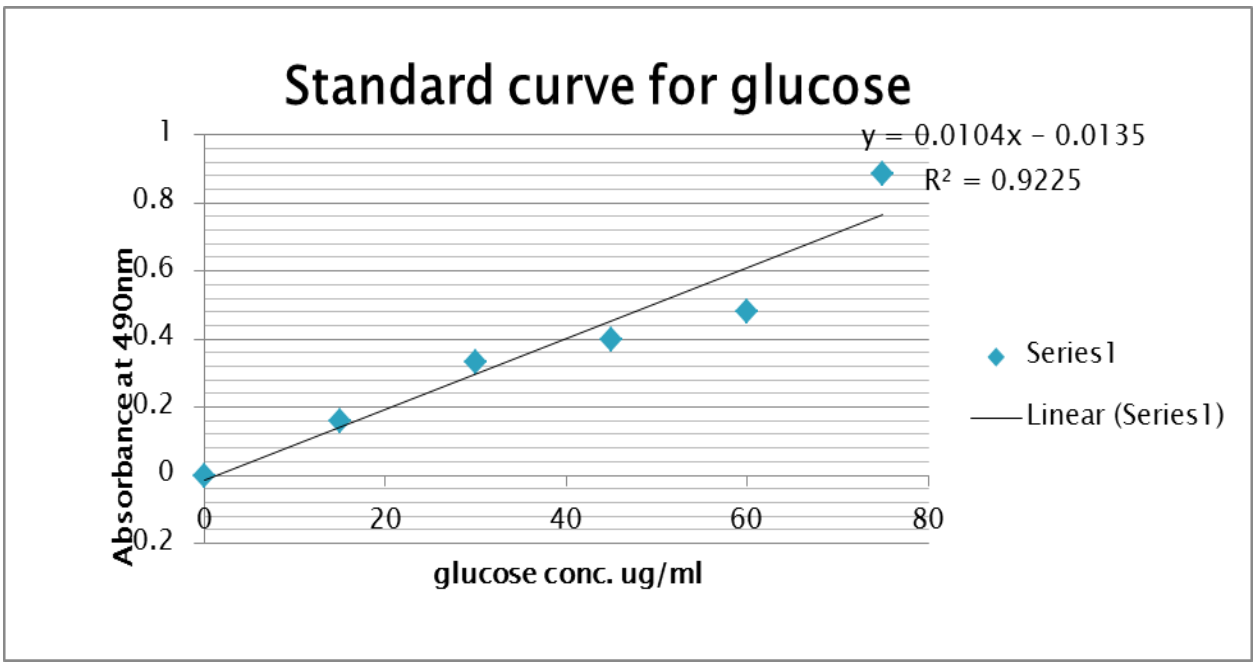


Picture 3: *Oceanobacillus* sp. Rob on M1 agar

APPENDIX D


standard curve for protein estimation
absorbance at 750nm
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
0
0.2
0.4
0.6
0.8
1
1.2
Conc (mg/ml)
y = 0.0683x - 0.0056
R² = 0.9641
abs
Linear (abs)

APPENDIX E


Standard curve for glucose
y = 0.0104x - 0.0135
R² = 0.9225
Absorbance at 490nm
1
0.8
0.6
0.4
0.2
0
0.2
0
20
40
60
80
glucose conc. ug/ml
Series1
Linear (Series1)