Production, Characterisation and Application of Bioflocculants from Pure Bacterial Strains and their Consortia Isolated from Sodwana Bay in Kwa-Zulu Natal, South Africa

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Declaration

The research project presented in this dissertation was conducted at the Department of Biochemistry and Microbiology of the University of Zululand (UZ) under the supervision of Prof A. K Basson and Prof J.J. Simonis

I, Tsolanku Sidney Maliehe declare that this work, aside from the supervisory guidance received, is the product of my own original work and effort.

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Abbreviations

%	Percent
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Celsius
µl	Microlitre
µm	Micromililitre
16S-rRNA	16S-ribosomal ribonucleic acid
Al	Aluminium
ANOVA	One-way analysis of variance
BOD	Biological oxygen demand
BSA	Bovine serum albumin
C	Carbon
Ca	Calcium
CaCO2	Human colorectal adenocarcinoma cells
CFU	Colony forming units
Cl	Chlorine
COD	Chemical oxygen demand
DMSO	Dimethyl sulfoxide
DWAF	Department of Water Affairs
EPS	Bioflooculant organic components
FA	Flocculating activity
g	Gram
HEK 293	Human embryonic kidney cells
IR	Fourier transform infrared spectroscope
K	Potassium
l	Litre
MCF7	Breast cancer cells
MDG	Millennium Development Goals
MEM	Minimum essential media
Mg	Magnesium
mg	Miligram
ml	Millilitre

MTT	3-(4,5-dimethylthiazol-2-)-2,5-diphenyl tetrazolium bromide
mV	Millivolts
N	Nitrogen
Na	Sodium
nm	Nanometer
OD	Optical density
P	Phosphorus
PBS	Phosphate buffered saline
Rpm	Revolutions per minute
RSA	Republic of South Arica
S	Sulfur
SD	Standard deviation
SEM	Scanning electron microscopic
SO_2	Sulfur dioxide
*T*d	Degradation temperature
TGA	Thermo gravimetric analyzer
TKT	Bioflocculant from *Alcaligenes faecalis* HCB2
TMT	Bioflocculant from *Bacillus pumilus* JX860616
TPT	Bioflocculant from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2
TST	Bioflocculant from a consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5
TTT	Bioflocculant from *Bacillus subtilis* CSM5
UV	Ultraviolet
v	Volume
w	Weight
WEF	Water Environment Federation
WHO	World Health Organisation

Research output

Published manustripts

- Maliehe TS*, Simonis J, Basson AK, Ngema S, Xaba SP AND Reve M. (2016). Production, characterisation and flocculation mechanism of bioflocculant TMT-1 from marine Bacillus pumilus JX860616. African Journal of Biotechnology. 15 (41): 2352-2367.

- Maliehe TS*, Selepe NT, Ntombela G, Simonis J, Basson AK, Ngema S, Xaba SP and Mpanza F. (2016). Production and characteristics of bioflocculant TPT-1 from a consortium of Bacillus pumilus JX860616 and Alcaligenes faecalis HCB2. African Journal of Microbiology Research. 10 (37): 1561-1575.

Submitted manustripts

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Abstract

Flocculation is the physical purification technique whereby destabilized colloidal particles are enhanced to agglomerate to form large and settleable flocs. Bioflocculants have been the center of attention in bioremediation because of their effectiveness, biodegradability, harmlessness and environmentally inert nature. This study aimed at producing, characterising and applying bioflocculants from pure marine bacteria and their consortia.

The medium composition and culture conditions of previously isolated bioflocculant producing strains: *Bacillus pumilus* JX860616, *Bacillus subtilis* CSM5 and *Alcaligenes faecalis* HCB2 and their consortia were optimised. The produced bioflocculants were obtained through solvent extraction and purification. The physicochemical analyses of the bioflocculants were attained by scanning electron microscopy (SEM), elemental detector, Zetasizer Nano, Fourier transform infrared (IR) spectrophotometry, liquid chromatography-mass spectrophotometry and thermo gravimetric analyzer. The thermal, pH and salinity stabilities of bioflocculants were evaluated spectrophotometrically. Flocculation mechanism was assessed by Zetasizer Nano. The biosafety of the bioflocculants was determined by 3-(4,5-dimethylthiazol-2-)-2,5-diphenyl tetrazolium bromide (MTT) assay. The removal efficiencies of the bioflocculants on biochemical oxygen demand (BOD), chemical oxygen demand (COD), sulphur, nitrogen, calcium and aluminium were determined spectrophotometrically in water from the Nhlabane estuary, effluents from the Umhlathuzi and a local coal mine wastewater treatment plants.

Bioflocculant production by all the three pure bacterial species and two bacterial consortia was obtained when the inoculum sizes were 2% (v/v) or less. Fructose was effectively utilized for production of crude bioflocculant by *B. subtilis* CSM5, while maltose was a good carbon source for the bioflocculant production by *A. faecalis* HCB2. Glucose was preferred for production of crude bioflocculant extracts by *Bacillus pumilus* JX860616, consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2, respectively. Amonium sulphate was efficiently utilized for bioflocculant production by *B. pumilus* JX860616 and consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5. Urea was the best nitrogen source for production of crude bioflocculants by *B. subtilis* CSM5 and *A. faecalis* HCB2, while yeast extract supported production of bioflocculant by consortium of

B. pumilus JX860616 and *A. faecalis* HCB2. The bioflocculants were produced when in optimum culture conditions of: 20-30°C, 55 -165 rpm, after 60-72 hours in different initial medium pH.

The extracted and purified bioflocculant yields from bacterial consortia were higher than those of pure strains. *B. pumilus* JX860616 produced 2.5 g/l, *B. subtilis* CSM5 had 1.5 g/l and *A. faecalis* HCB2 had 2.7 g/l). The consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 yielded 3.1 g/l while that of *B. pumilus* JX860616 and *A. faecalis* HCB2 gave 3.0 g/l. All purified bioflocculants were cation dependent and relatively effective at low dosage bioflocculants (≤ 0.8 mg/ml). The bioflocculants are anionic glycoproteins in nature and are predominantly composed of carbohydrates. They revealed the presence of elements such as: N, C, O, P, Ca, and Na. IR observations showed that biofloculant TMT was indicative of hydroxyl, vinyl, amide and amino groups. All other bioflocculants (TTT, TKT, TST and TPT) had similar functional groups; hydroxyl, amino and amide groups. The bioflocculants were stable in a wide range of pH and also showed saline and thermal stability. They demonstrated a margin of safety as they revealed insignificant cytotoxic effects. The flocculation process could be as a result of double layer compression by Ba^{2+}, chemical reactions and bridging mechanisms. The bioflocculants were comparatively effectively to convesional flocculants in the removal of pollutants. The results of this study advises that the use of bacterial consortia can improve bioflocculant yields and that bioflocculants have potential to serve as an alternatives to conventional chemical flocculants.

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Chapter 1

1.1 Introduction

Recent industrialization and urbanisation have led to high rates of water pollution and water scarcity (Sarkar *et al*., 2006). The disposal of effluents without appropriate treatment can result in long-term undesirable negative impacts on the environment and in humans (Lin and Harichund*,* 2011). Pollutants can be malignant to life in aquatic environments and also greatly contribute to water incompatibility as palatable and potable for consumption (Yang *et al*., 2012). To counter this impact, physico-chemical treatment methods are implemented to remove these pollutants from contaminated sites (McCarthy, 2011). The following treatment methods can be used: chemical precipitation, ion exchange, activated carbon adsorption, reverse osmosis and flocculation (Low *et al*., 2011; Ong *et al*., 2012).

Flocculation is a physical purification technique whereby destabilized particles are allowed to agglomerate and form large flocs (Spellman, 2014). Flocculants are categorised into the following types: (1) inorganic flocculants (aluminium sulphate), (2) organic synthetic flocculants (polyacrylamide derivatives) and (3) natural occurring flocculants (chitons and bioflocculants) (Ji, 2010; Serdar *et al*., 2011). Inorganic and organic synthetic flocculants have been predominantly and effectively applied in various biotechnological areas due to their cost effectiveness and reliability (Yumei *et al*., 2014). However, they have been reported to impose health risks and environmental hazards (Arafa *et al*., 2014). Aluminium sulphate and polyacrylamide derivatives have been reported to be neurotoxic and carcinogenic and resulted in Alzheimer disease (Serdar *et al*., 2011; Ahmad *et al*., 2015). Moreover, the byproducts of these flocculants are environmentally unfriendly as they are toxic and non-degradable (Arafa *et al*., 2014). As a result of these short-comings, natural occurring flocculants such as bioflocculants are viewed as potentially effective alternatives as flocculating agents (Komilis *et al.,* 2016).

Bioflocculants are macromolecules secreted by microbial strains due to interaction with surroundings as a result of substrate metabolism, bacterial growth, cell lysis and degradation of chemical components (Carlos *et al*., 2011). They are mainly found as polysaccharides, lipids, proteins, nucleic acids and other polymeric compounds (Okaiyeto *et al*., 2015). Bioflocculants are produced by algae, actinomyces, bacteria and fungi (He *et al*., 2012). They have high efficiency, are benign, are biodegradable and are innocuous to humans (More *et al*., 2014). They have been effectively used in flocculating inorganic solid suspensions (Zhang *et al.,* 2007), humic acids (Zouboulis *et al.,* 2004), dyes (Olaniran *et al.,* 2012) and in the removal of heavy metals (Lin and Harichund, 2012). However, bioflocculant production has been limited by high production costs (Okoh *et al*., 2012; Liu *et al*., 2015) and low production yields (Duraiarasan and Mani, 2013) at an industrial level. The lack of knowledge concerning the characteristics of active components has also contributed to their restrictions (Liu *et al*., 2015). Nevertheless, researchers have recently focused attention on increasing bioflocculant yields and activities through the following: isolation of novel bioflocculant-producers (Nwodo *et al.,* 2016) and the use of microbial consortium (Zhang *et al*, 2007).

In this study, the previously identified bioflocculant producing bacteria (*Bacillus pumilus* JX860616, *B Bacillus subtilis* CSM5 and *Alcaligenes faecalis* HCB2) from marine sediment of Sodwana Bay in the Kwazulu-Natal Province, South Africa were resuscitated. The construction of bacterial consortia of *B. pumilus* JX860616 and *B. subtilis* CSM5 and of *B. pumilus* JX860616 and *A. faecalis* HCB2 were done. The medium composition and culture conditions of the bacteria were optimised. Lastly, bioflocculants were extracted, characterised and applied as treatment to industrial and domestic wastewater.

1.2 Rational of the study

The Republic of South Africa is a water scarce country. Increasing water pollution due to an alarming growth rate of urbanisation and industrialisation poses a serious concern to available water resources and to water quality. Major sources of water pollution are untreated, toxic, domestic and industrial effluents and also agricultural byproducts. Pollutants in wastewater are often detrimental to aquatic life, rendering it unfit for use. Physicochemical and biological methods have been implemented to treat wastewater and fresh water. Flocculation is among the techniques used in wastewater and fresh water treatment. Convectional chemical (inorganic and organic synthetic) flocculants have shown to be cost effective but environmentally unfriendly and to impose health threats to humans. They are reported to have carcinogenic and neurotoxic effects.

Bioflocculants are biodegradable, biocompatible, lack secondary pollution and thus, are environmentally friendly. They are also innocuous to humans. However, bioflocculant production is limited at an industrial level due to low productivity and high cost. Some of the major disadvantages that hold back the production and industrial application of bioflocculants on a large-scale are: low yields, inconvenience, high cost of production and a lack of knowledge of the characteristics and flocculation mechanism of bioflocculants. The need to explore novel microorganisms with enhanced bioflocculant-producing abilities; to characterise and to determine the bioflocculation mechanism is imperative.

Soil and sludge samples have been the main sources of bioflocculating microorganisms. However, marine water remains an untapped reservoir. It is predicted that bioflocculants from marine microorganisms have high flocculating activities since they possess a different morphological, physiological and metabolic adaptations to adverse marine environments when compared to those found in fresh and terrestrial waters. This study, therefore, focused on the evaluation of bioflocculants from bacteria isolated from marine sediment from Sodwana Bay, Republic of South Africa (RSA). The characteristics of the bioflocculants were evaluated and bioflocculants applied in water and wastewater treatment.

1.3 Study area

The study was conducted in the Department of Biochemistry and Microbiology at the University of Zululand (UZ) main campus. The University is situated at KwaDlangezwa in the north of KwaZulu Natal, South Africa.

1.4 Aims

1. The aimed at producing and improving bioflocculant yields by construction of bacterial consortium
2. To compare the removal effeciencies of produced bioflocculants with those of conventional flocculants in wastewater treatments

1.5 Objectives

a) To resuscitate the previously identified bioflocculating bacteria

b) To optimise medium composition and culture parameters for pure and mixed bacterial species

c) To extract, purify and characterise the bioflocculants

d) To determine the flocculation mechanisms of the bioflocculants

e) To apply the bioflocculants in wastewater treatment

1.6 Hypotheses

a) Optimisation and construction of bacterial consortia improves bioflocculant yields.

b) Bioflocculants can effectively remove colloidal pollutants in industrial and domestic wastewaters.

Chapter 2: Literature

A literature survey presents a relatively large and diverse body of research on bioflocculation. Much of this literature is devoted to areas of water pollution, wastewater treatment, flocculation and bioflocculation processes. Major factors effecting flocculation and bioflocculant production and activities are also discussed. Moreover, extraction, characterisation and application of bioflocculants in different biotechnological fields are reviewed. This chapter also lists a statement, hypothesis, aims and objectives of the study.

2.1 Potable water and its scarcity

Water consists of a dipolar molecule with two hydrogen atoms and oxygen (Boyd, 2015). Water is the most abundant and essential natural resource. Aquatic environments occupy more than 71% of the earth's surface to an average depth of approximately 4 km (Mclntyre, 2010) with approximately 1.3 billion km^3 of water (Boyd, 2015). However, sea water has high salinity (35% of sodium chloride) and is therefore not potable and palatable (Speight and Henderson, 2010). Potable and palatable water is essential to accomplish sustainable development and to fulfil the Millennium Development Goals (MDG) (United Nations Water, 2015). However, only 147 countries have so far met the requirements for sustainable development (Figure 2.1).

Water is important for sustainable development (environmental, economic and social development). It is an essential resource for economic production of most commodities and services. Thus, the rate of water usage has recently doubled the rate of global population increase (Corcoran *et al.,* 2010). Water is utilized for multiple purposes that include domestic (drinking, cooking and bathing), industrial (mining, construction of infrastructure and manufacturing companies) and agricultural (irrigation) (United Nations Water, 2015). Water is important in ecosystem functioning and act as a medium of biochemical reactions.

A fast growing population, especially in cities and towns and growth in industrialization have increased the demand for potable and palatable water. The rate of water demand is currently twice the rate of growth of populace (United States Census Bureau, 2012). Improper agricultural practises such as unmonitored irrigation and waste disposal contribute to water scarcity (United Nations Water, 2015). Moreover, polluted effluents from domestic and industrial areas and a low mean annual global rainfall have also increased water scarcity. Twenty percent of the global population is assumed to be living in countries with water scarcity while 67% of the population will mostly live under water stress by the year 2025 (United Nations Water, 2007). Already, the Republic of South Africa is declared a water scarce country due to its mean annual rainfall of 450 mm, which is far below the world average of 860 mm (National Water Resource Strategy, 2004).

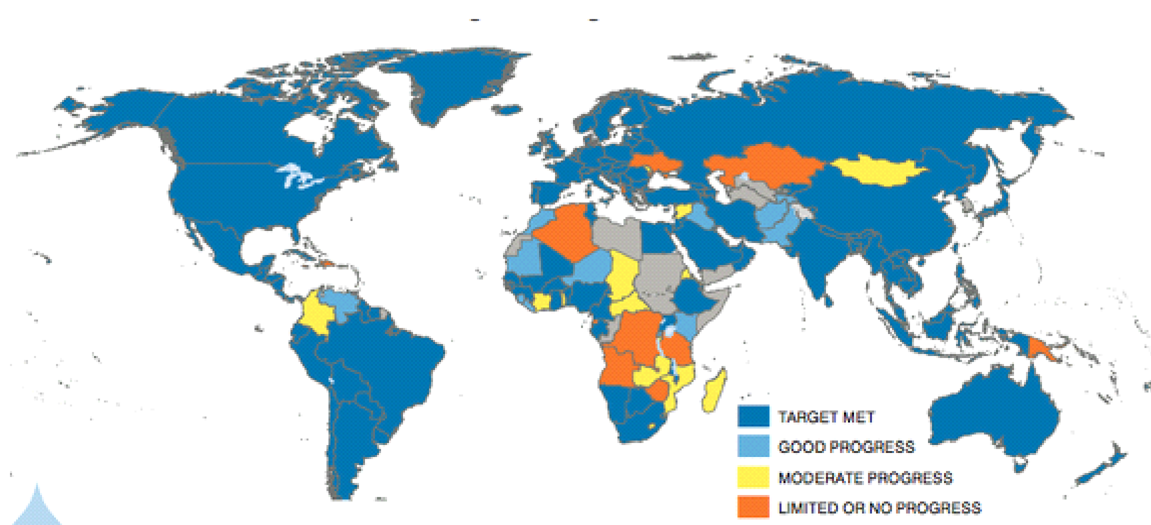


Figure 2.1: Drinking water needs (World Health Organisation, 2015)

2.1.1 Water pollution

A significant environmental crisis currently facing developing countries is the contamination of water resources caused by the disposal of domestic, industrial and mine effluents (Pulles *et al*., 2005). In developing countries, approximately 90% of wastewater is discharged untreated into receiving waterbodies (Corcoran *et al.*, 2010). As a result, water resources which are major receptacles for untreated wastewater are often highly polluted (Osibanjo *et al*., 2011). This water often contains high levels of heavy metals, nutrients, organic matter and microorganisms (Willey *et al.,* 2011). Water pollution degrades the quality of water and has a negative impact on human health, threatens the ecosystem and causes serious environmental pollution (Pulles *et al.,* 2005). In general, water pollutants often result from toxic compounds that have carcinogenic, teratogenic and mutagenic effects on humans and a killing effect on the ecosystem (formation of death zones) (World Health Organisation (WHO), 1997). Control measures against environmental pollution, for removal and for recovery of suspended loads and accumulated nutrients from industrial wastewaters are essential.

2.2 Water pollutants

The most important water quality parameters of concern in wastewater treatment are: (1) chemical oxygen demand (COD), (2) biological oxygen demand (BOD), (3) heavy metals, (4) nitrogenous species, (5) phosphate, (6) microbial load and (7) colloids (Decico, 1979; Brooks, 1996). High concentrations of these pollutants in wastewater are considered unacceptable (Akpor and Muchie, 2011).

2.2.1 Chemical oxygen demand and biological oxygen demand

The main goal in the treatment of wastewater is to reduce organic and inorganic materials (Manahan, 2011). The efficiency of wastewater treatment is often conveyed in terms of reduction in the chemical oxygen demand (COD) and biological oxygen demand (BOD) (Barredo *et al.,* 2005). COD is the amount of oxygen necessary to oxidise completely all organic carbon to carbon dioxide and to water (Weiner, 2013). COD is measured by oxidation of potassium dichromate in the presence of sulfuric acid and silver.

BOD is the relative amount of dissolved oxygen consumed mainly by microorganisms to completely oxidise all matter in wastewater. High levels of oxidisable materials can results in a high BOD (Madigan and Martinko, 2006). BOD is also measured to assess the extent to which effluents could promote anoxic conditions when deposited into waters (van Loon and Duffy, 2005). Normally, the ratio of BOD/COD is approximately 0.5. A ratio of BOD/COD that goes below 0.3, results in large amounts of materials in wastewater that are not easily degradable (Maier *et al*., 2009). High concentrations of oxidisable materials often lead to anaerobic conditions, bad odours and stagnant waters that do not support life (Mihelcic and Zimmerman, 2010).

2.3 Heavy metals

Metal pollution results from human activities such as mining (Mair *et al.,* 2009). Heavy metals are based on cationic-hydroxide formation and on a complex formation of hard-soft acids and bases which form the basis of their relationship to environmental and biological toxicity. Even at low concentrations, heavy metals can affect the diversity of benthic organisms (Hemme *et al.,* 2010). Changes in the physical and chemical parameters of aquatic environments (mostly where effluents are deposited into) often increase the degree of toxicity of heavy metals (Adendorff *et al*., 1996). Thus, heavy metals dissolved in water, may at times be assimilated by aquatic life and consequently affect humans in the food chain. Metal contaminated environments pose serious health threats that include cancer, improper neurological and cardiovascular functioning, encephalopathy and hypophosphatemia (Mair *et al.,* 2009). Metal containing effluents within the environment contribute greatly towards acidification, mineralisation and metal contamination of aquatic habitats (Karthiga and Natarajan, 2015). The mechanism of metal toxicity can be divided into the following three categories; (1) inhibition of the essential biological functional groups of the biomolecules, (2) displacement of the essential metal ion in biomolecules and (3) modification of the active synthesis of biomolecules (Duncan *et al.,* 2003).

2.4 Nutrients

Nutrient pollution results mainly from nitrogen, phosphorus and sulfur (Boyd, 2015). According to United Nations Department of Economic and Social Affairs (2012), the interference of humans in nitrogen and phosphorus cycles is beyond levels of safety. The occurrence of these nutrients in high concentrations in water bodies may have harmful effects to human health and environment (Manahan, 2005).

2.4.1 Nitrogenous species

Nitrogen is an essential component that forms part of the building blocks of life such as amino acids (Hazen *et al*., 2005). However, excessive deposition of nitrogenous species (ammonia and nitrates) in a terrestrial or oceanic ecosystem may result in eutrophication and water pollution (Raboni *et al.,* 2015). The deleterious effects of ammonia include toxicity to aquatic fauna and a reduction of oxygen in water bodies (Weiner, 2013). Nitrates and ammonia have a high affinity for chlorine, as a result, their presence in water hinders the effectiveness of chlorine disinfection in the treatment of drinking water (Raboni *et al*., 2015). Moreover, the presence of nitrates and nitrites inhibit the removal of phosphorus in wastewater treatment (Drysdale *et al*., 2000). Nitric acid could be the main contributor to acid rain (Water Environment Federation (WEF), 2011). Cumulative emissions of nitrous oxide (greenhouse gas) promote global warming (Reilly *et al.,* 2003). Nitrogen often causes some health related issues that include the following: (1) trihalomethane formation in chlorinated drinking water, (2) non Hodgkin lymphoma and (3) methemoglobonemia (Weiner, 2013).

2.4.2 Phosphorus and sulfur

Phosphorus is also a limiting nutrient in freshwater reservoirs, lakes and rivers (Chin, 2013). Phosphorus is mostly the result of drainage from agricultural land, excreta from livestock, municipal and industrial discharge and atmospheric deposition (Gulf of Maine, 2012). Phosphorus greatly contributes to eutrophication (WEF, 2011). Coal contains a substantial amount of sulfur, both in pyrite and organic sulfur compounds (thiophene derivatives (Glazer and Nikaido, 2007). When coal is burned, most of this sulfur is converted to sulfur dioxide (SO_2). SO_2 combines with moisture in the atmosphere to form sulphurous acid. Sulfuric acid is a major component of acid smog and acid rain (Cohen *et al.,* 1981). Thus, it is desirable to

remove as much as possible nitrogen species, phosphorus and sulphur from wastewater before it is discarded into water bodies (WEF, 2011).

2.4.3 Eutrophication

According to Lilley *et al*., (1997), eutrophication is a process that results from the continuous supplementation of excess nutrients in waterbodies. These nutrients are mainly nitrogen, phosphorus and sulfur (WEF, 2011). The major challenges that result from eutrophication are anthropogenic enrichment of the environment and the generation of toxic algae, cyanobacteria and dinoflagellate (Sigee, 2005). These toxins are often concentrated in the food chain during eutrophication. Toxins from algae often result in paralytic shellfish poisoning, liver damage, diarrhea, skin irritation, and amnesia (Chrous and Bartram, 1999; WHO, 2006). In extreme cases, eutrophication promotes the generation of "dead zones" and also erosion of habitats (Bowen *et al*., 2014). Dead zones have serious consequences for ecosystem functionalities. Eutrophication can result in hypoxia and water bodies that do not support life (Solow, 2004).

2.5 Colloids

Natural water often contains solids that can be classified as suspended, colloidal or dissolved (Spellman, 2014). Colloids are often suspensions of nanoparticles that are thermodynamically unstable, kinetically non-labile and which do not settle out under gravity (Atkins and de Paula, 2010). Colloidal particles include colour compounds, clays, microscopic organisms and organic matter (Hodson *et al*., 2004). Effective removal of this colloidal dispersion is greatly impacted by the electrokinetic properties on the surface of the colloids (Davis and Cornwell, 2013). Colloids are stable in water as they possess large surface areas relative to their mass. When the charge is high enough, the colloids often remain discrete and in suspension (Davis, 2011). Colloids do not only result in turbidity but also ~~do~~ shelter microorganisms from being inactivated by disinfectants (Hodson *et al*., 2004).

Colloids are classified into hydrophobic or hydrophilic particles (Atkins and de Paula, 2010). Hydrophilic colloids are stable because their attraction to water molecules often overcomes their surface charge characteristics (Ji, 2008). This attraction makes hydrophilic colloids difficult to remove from suspension (Tripathy and De, 2006). On the other hand, the hydrophobic particles are dependent on the electrical charge for their stability in suspension. In general, the two main phenomena contributing to the stabilization of colloids are hydration and surface charge (Davis and Cornwell, 2013). The layer of water on the surface of hydrated colloids prevents contact, which could result in formation of large units. A surface charge on colloidal particles may prevent aggregation, since like charged particles repel each other (Huang *et al.,* 1995).

2.5.1 Kaolin particles

Colloids are often in suspension as clays such as kaolin particles (Huang *et al.,* 1995). Kaolin ($Al_2SiO_5(OH)_4$) is a product of the weathering of primary minerals (van Loon and Duffy, 2000). Kaolin is a naturally occurring bi-layered alumina-silicate whose composition, crystallographic structure and physiological properties are well defined (Michaels and Morelos, 1995). This pure mineral has a low but measurable cation exchange capacity (van Loon and Duffy, 2000). Kaolin contains a number of negatively charged sites on its surface. The exchange site originates on the polar surface due to the ionisation of the OH group linked to acidic aluminium or silicon (Michaels and Morelos, 1995). Kaolin was utilized as a test for colloidal particles in preparing suspension for this study. It is found in the formation of orthoclase feldspar in the following reaction:

$$2KAlSi_3O_8\ (s) + 2H_3O\ (aq) + 7\ H_2O \Longrightarrow Al_2SiO_5(OH)_4\ (s) + 4H_4SiO_4\ (aq) + 2K$$

2.6 Zeta potential

The primary surface charge on a colloid cannot be measured directly. However, the zeta potential can be computed from measurement of the particle movement within the electric field. The electrical potential between the shear plane and the bulk solution is called zeta potential (Davis, 2011). The zeta potential represents the difference in voltage between the surface of the diffused layer and the water molecules (Dicks, 2011). Zeta potential represents the strength of repulsion between colloidal particles and the distance which must be overcome to bring particles together. Measurement of zeta potential can give an indication of the effectiveness of the added electrolytes in lowering the energy barrier between colloids. This can directly effect the optimisation of flocculant dose in water treatment (Hodson *et al*., 2004). Generally, it can be stated that the greater the magnitude of zeta potential, the greater the stability of the colloidal suspension (Tripathy and De, 2006). Rapid flocculation occurs when the absolute value of zeta potential is reduced to values below 20 mV (Mines, 2014).

2.7 Purification techniques

The challenge of wastewater treatment is to reduce high COD, BOD, nutrients, pathogenic microorganisms and colloids (Glaizer and Nikaido, 2007). A number of physico-chemical treatment methods have been used for the removal of these water pollutants. This includes chemical precipitation, ion exchange, activated carbon adsorption and reverse osmosis and flocculation (McCarthy, 2011; Mines, 2014; Karthiga and Natarajan, 2015). Unfortunately some of these treatment methods impose environmental and human threats (Okaiyeto *et al.,* 2016). Thus, innovative, economically viable and sustainable technical solutions are needed. The use of biological remediants such as bioflocculants has been viewed as effective, eco-friendly and containing less health threatening agents (More *et al*., 2014).

2.8 Flocculation

Flocculation (Figure 2.2) is a physical purification technique whereby destabilised colloidal particles are enhanced to agglomerate to form large flocs (Spellman, 2014). It is mediated by reducing repulsion forces through destabilising the colloidal particles and lowering the energy barrier between them, thus enabling their aggregation (Holt *et al*., 2002; Bahadori, 2014). Flocculation promotes the rate at which suspended particles settle out in suspension (Pandit and Kumar, 2013). It is an operation preceding filtration, flotation and sedimentation (Schenk *et al.,* 2008). The flocs formed during a flocculation process can be effectively and easily removed by sedimentation, flotation and filtration processes (Edward, 2011).

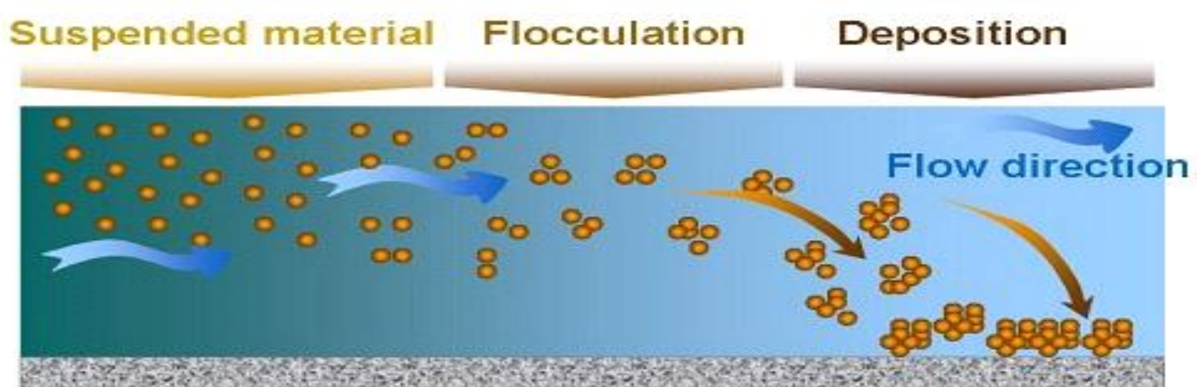


Figure 2.2: Flocculation process (Department of Water Affairs (DWAF), 2002)

2.9 Flocs

Flocs are aggregates of solids that may be organic or inorganic. The characteristics of flocs are important in the subsequent process of settling and filtration (Edward, 2011). The size and density of flocs depend on the colloid size, mineralogy, suspended sediment concentration, fluid shear and salinity of water (Lick, 2009). With regard to size, flocs are referred to often as follows: (1) microflocs, (2) pin point, (3) intermediate and (4) large flocs. These sizes are respectively: microfloc < 10 µm; pin point, 10-50 µm; intermediate, 50-100 µm and large > 100 µm (Hendrik 2006). The net charge of the floc depends on the magnitude and rates of aggregation and destabilisation (Lick, 2009). The strength of the flocs depends on inter-particle bonds within the constituents of the aggregates (Jarvis *et al*., 2005).

The mechanism of floc formation is described by coagulation theory whereby the probability of two particles sticking together depends on the probability of their collision and on the probability of adhesion following their collision (Blackburn *et al.*, 2000; Lick, 2009). Flocculation involves two main aspects: (1) cohesion and (2) collision. Particle collision strongly effects the sizes, settling velocity, strength and densities of flocs (Ji, 2008). When colloidal particles aggregate together, they lose individual kinetic stability. The frequency of collision is often proportional to the concentration of colloidal particles subjected to interaction. As noted, collision processes between floc particles result in an increase in size. However, not every collision results in attachment (Hendricks, 2006). The ratio of attachment to the number of collisions is defined as the agglomeration efficiency (Edward, 2011). Collision enhancers such as van der Waal`s force increase the rate of flocculation. However, hydrodynamic retardation and double-layer repulsion often slow flocculation (Edward, 2011). The three main factors that largely determine particle collision are as follows: (1) velocity gradient, (2) settling velocity difference between particles and (3) Brownian motion (Ji, 2008).

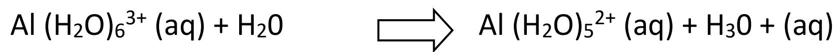
Flocs produced by velocity gradient are strong and dense. However, high velocity gradient tends to pull the flocs apart. This mechanism is a function of the size of the particles or flocs. Normally it can be stated that the greater the difference in size, the faster the rate of flocculation. This mechanism is significant when large flocs have been generated. Cohesion (particles' attraction) is influenced by the electrochemistry of sediment minerals and water (Mines, 2014). Particle cohesion depends primarily on the mineralogical composition, particle size and cation exchange capacity of the sediment (Ji, 2008). Other parameters effecting cohesion include salinity, pH and temperature of the water.

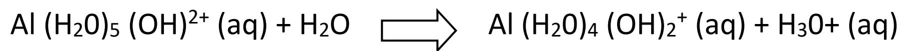
2.10 Flocculants

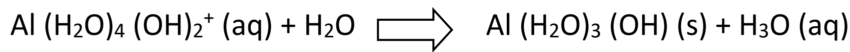
Flocculants have a multifarious biotechnological application in wastewater treatment, drinking water treatment, food processing, dye solutions, inorganic solid suspensions and industrial downstream processing (Cong-Liang *et al*., 2012; Singha 2012; Yumei *et al*., 2014). Flocculants are often added to increase the speed of flocculation and to increase strength and weight of the floc in order to eliminate floc tendency to break under severe fluid flow conditions (Edward, 2011; Spellman, 2014). Flocculants are grouped according to their chemical composition. The three groups of flocculants are as follows: (1) inorganic flocculants, (2) organic synthetic flocculants and (3) biological flocculants (Salehizadeh and Shojaosadati, 2001; Yim *et al.,* 2007).

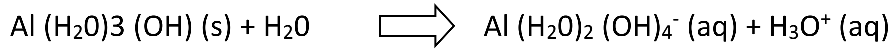
2.10.1 Inorganic flocculants

Inorganic flocculants are used predominately in water treatment and growth industries because of their cost-effectiveness (Piyo *et al*., 2011). In practice, aluminium chloride (Al_3^+) and ferric chloride (Fe_3^+) are the major primary inorganic flocculants used in wastewater treatment (Joo *et al.,* 2007). Both alum and ferric chloride are sources of trivalent metal cations. Their behaviour in wastewater treatment is somehow similar (Tripathy and De, 2006). Since most of the colloidal particles in wastewater have a negative charge (Lee *et al*., 2014), the salt of these metals are usually ionized in wastewater. They create cationic charges with an ability to bind to the negatively charged colloidal particles. The main disadvantage of ferric chloride however, is that its solution is acidic and oxidizing (van Loon, 2000). This means that ferric chloride can be highly corrosive to pumps and other metallic parts of a delivery system (Tripathy and De, 2006). Moreover, inorganic flocculants have inherent utilization limitations due to the fact that large amounts are required. They are also highly sensitive to pH variations and low temperatures (Brostow *et al.,* 2009). Alum undergoes the following series of hydrolysis reaction during wastewater treatment:









2.10.2 Organic synthetic flocculants

Organic synthetic flocculants have been predominately used in industrial processes (Razali *et al.,* 2011). This is due to the fact that they are convenient to utilize, they are highly soluble in aqueous solutions and they have no effect on the pH of media used. Furthermore, they have high molecular weights and possess charges in their molecular chains which facilitate their high efficiency (Lee *et al.* 2014). They include polyacrylic acid, polydiallylmethyl ammonium chloride and polyacrylamide (Tripathy and De, 2006). Although they are costly compared to inorganic flocculants, they have not been reported to be harmful to humans and the environment (Bolto and Gregory, 2007). However, the monomers of these flocculants have been reported to be cytotoxic, neurotoxic and carcinogenic to humans (Brostow *et al.,* 2009; Sun *et al.,* 2015).

Monomers of synthetic products (acrylamide and ethyleneimine) and unreacted chemicals employed during synthesis of the monomer units such as formaldehyde, epichlorohydrin and dimethylamine) are the main contributors of these adverse effects (Wu *et al*., 2012). The monomers of these flocculants have resistance to biodegradability, thereby constituting to environmental pollution (Tripathy and De, 2006). Moreover, most commercial polymers are produced from petroleum-based compounds which have a very low margin of safety to humans and to the environment (Suopajärvi *et al*., 2013). Consequently, the stated demerits have discouraged their use in many industries.

2.10.3 Natural occurring flocculants

The limitations of inorganic and organic flocculants have necessitated more research into biological flocculants such as chitons and bioflocculants (Nwodo *et al.,* 2016). Biopolymers produced by microorganisms (bioflocculants), have gained more scientific attention recently (Mabinya *et al.,* 2012; Sun *et al*., 2015). Bioflocculants are effective at low dosage sizes (Singh *et al.,* 2000), they are inert to pH fluctuations (Tripathy and De, 2006), nontoxic in nature (Chopra and Ruhi, 2016), harmless to humans and animals (Nwodo *et al.,* 2016) and they lack secondary pollution (Mishra, 2016). Bioflocculants are also biodegradable (Agunbiade *et al*., 2016). Nevertheless, this feature (biodegradability) can be a shortcoming as it can reduce their shelf life and consequently reduce efficacy as a result of decomposition (Tripathy and De, 2006). Moreover, flocs lose their stability and strength due to their biodegradable nature (Lee *et al.,* 2014). Bioflocculants have hydrolysable groups which can promote biodegradation via hydrolysis.

2.11 Bioflocculation

Bioflocculation is a natural process whereby bioflocculants are used to flocculate, settle and remove particles, suspended solids and colour in solutions (Cong-Liang *et al.,* 2012). Bacterial flocculants are macromolecules secreted by bacteria due to bacterial interaction with surroundings as a result of substrate metabolism, bacterial growth, cell lysis and degradation of bacteria or bacterial chemical components (Komilis *et al*., 2016). Bioflocculants are found as capsular, slime, loosely bound and tightly bound compounds according to the nature of their association with the cells or the method of extraction (Hendric, 2006).

2.11.1 Chemical composition of bioflocculants

Bioflocculants are composed of organic components such as polysaccharides, lipids, proteins, nucleic acids and other polymeric compounds (Carlos *et al*., 2011). Bioflocculant secretion occurs in prokaryotic as well as in eukaryotic bacteria (Bhaskar and Bhosle, 2006). Carboxyl and hydroxyl are reported as the predominant functional groups in most flocculants (More *et al.,* 2014). Functional groups are needed in flocculation of colloids for provision of adsorption sites for colloids attachment. Functional groups also permit the bioflocculant chain to spread evenly due to electrostatic repulsion forces (Pathak *et al*., 2014). The stretched molecular

chains encourage more efficacious sites for attachment. Bioflocculants, that are mainly proteineous, have lower adsorption capability compared to those of carbohydrates or glycoproteins. According to Okaiyeto *et al*. (2015) most bioflocculants are composed of oxygen, nitrogen, carbon and hydrogen as their main elements. The presence of various elements may attribute to flexibility and stability of bioflocculants. EPS functional groups are generally negatively charged (Mair *et al.,* 2009).

2.11.2 Physiological importance of bioflocculants

Production of extracellular polymeric substances (EPS) is a characteristic of bacteria in natural environments (Bhaskar and Bhosle, 2006). Bacteria produce EPS as a protective mechanism during environmental stress conditions (Chaitany *et al.,* 2013). The main functions of bacterial flocculants are accretions of bacterial cells, cell to cell recognition, attachment to surfaces, formation of a protective barrier for cells and formation of flocs. They are also important to the structural components of biofilms, water retention, sorption of exogenous organic compounds and inorganic ions, adsorption of different metals, enzymatic activities (Hendricks, 2006; More *et al*., 2014). EPS are usually hydrated as they can absorb a large volume of water into their structures by hydrogen bonding. EPS may be hydrophobic, although most are both hydrophilic and hydrophobic. EPS may also vary in their solubility (Donlan, 2002). Generally a bioflocculant enhances microorganisms to establish stable habitats, thereby enabling it to operate as a synergistic consortium.

2.12 Industrial utilization of bioflocculants

Bioflocculants are now widely accepted as products of biotechnology (Li *et al.*, 2009). They have found a niche in biotechnological applications due to their wide chemical and physical structural diversity. Microbial flocculants are used in food, pharmaceutical, biomedical**,** bioremediation, wastewater treatment, textiles and bioleaching fields (Yumei *et al*., 2014). The abbreviation EPS is used for different bioflooculant organic components such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds (Carlos *et al*., 2011).

2.12.1 Food

The uses of EPS compounds in the food industry have been intensively investigated (Babu *et al*., 2013). EPS molecules are good emulsifiers, gelling agents, water-binding agents and food preservers. EPS molecules are used to improve food viscosity, stability, hydration of products and in the production of low calorie food (Singha, 2012). Microbial exopolysaccharides such as xanthan, dextran, curdlan, pullulan and alginate have been extensively used industrially (Banik *et al.,* 2000). Pullulan and gellan are used as food additives (Ferreira *et al*., 2016). Microbial extracellular polysaccharides are used in food production as an edible coating to prevent spoilage (Freitas *et al,* 2014). Nisin from *Lactococcus lactis* and reuterin from *Lactobacillus reuteri* are used in food preservation as they possess antimicrobial properties (Singha, 2012).

2.12.2 Pharmaceutical and biomedical fields

In recent years, EPS compounds have also been used in the pharmaceutical and biomedical fields. EPS compounds are also harvested for probiotics (Singha, 2012). EPS compounds have shown to possess antitumor, antiviral and immune stimulant activities (Okutani, 1984). Clavibacter michiganensis strains produce clavan which is an exopolysaccharide rich in L-fucose. Clavan has shown antitumor properties against tumours cells in lungs and to effectively regulate the generation of white blood cells (Vanhooren and Vandamme, 2000).

2.12.3 Bioremediation (wastewater treatment)

Biofilm-mediated bioremediation has also been found to be a more effective and safer alternative to bioremediation (Czaczyk and Myszka, 2007). EPS have been reported to effectively defecate trona suspension, and to treat dye mixtures (Zhang *et al*., 2005; Olaniran *et al*., 2012). EPS remove heavy metals from the environment due to their involvement in flocculation and capability to bind metal from solutions (Pal and Paul, 2008). In biocorrosion of metals and wastewater treatment, EPS molecules are used to remove and to recover metals and other materials in suspension (Lin and Harichund, 2011).

2.13 History of bioflocculation

Historically, bioflocculation was first reported by Pasteur. (1876) for *Levure casseeuse.* Two years later, it was observed in bacterial strains (Bordet, 1899). *Zoogloea*-forming bacteria, isolated from activated sludge became the first bioflocculant-producing bacterium to be discovered (Butterfield, 1935). At present more than 100 bacterial strains with potential bioflocculant production have been discovered and had their bioflocculants characterised. (Ahmad *et al*., 2015).

2.14 Advantages of bioflocculation

In recent years, as the demand for effective and environmentally friendly wastewater treatment continues to rise, bioflocculants have appeared to be promising alternatives to substitute conventional flocculants. Bioflocculants allow for the maintenance of cell integrity, for opportunities to reuse media and for use of low-cost and readily available substrates by some microbial strains (Lee *et al.*, 2009). They are renewable resources (Ferreira *et al*., 2016). Bioflocculants are biodegradable and their degradation products are typically eco-friendly (Muthulakshmi *et al.*, 2013). This is due to ~~the~~ specific microbial flocculant constituents and a microbial flocculant matrix that shows adsorption abilities, biodegradability and hydrophilicity or hydrophobicity. Bioflocculants can be produced at high rates depending on the culture parameters and on medium compassion and they are easily recovered from the culture (Salehizadeh and Shojaosadati, 2001). Bioflocculants are highly effective even at low concentrations (Sheehan *et al.,* 1998).

2.15 Disadvantages and proposed attempts

The utilisation of bioflocculants in industrial applications has been limited mainly due to high production costs (due to metal dependency of bioflocculant and costly substrates) and low yields (Kurane *et al*., 1994; Christenson and Sims 2011). Cations are often required for charge neutralisation prior to bioflocculant bridge flocculation mechanism (Lee *et al*., 2014). However, there are means that are proposed to enhance economic viability of bioflocculant production. Screening of new microorganisms with the ability to produce high yields of bioflocculants with high activities is still of great importance (Deng *et al*., 2003; He *et al.,*

2004). Moreover, since recently, most researchers are attempting to discover effective and novel metal-independent bioflocculants (Liu *et al*., 2015).

Strains of bioflocculant producers with the ability to utilize low cost raw materials are proposed as the greatest means to lower manufacturing costs (Selvin *et al.,* 2013). More *et al*. (2012) used activated sludge as a raw substrate for bioflocculant production. Zheng *et al*. (2005) and Zhi-qiang *et al*. (2007) made use of Agricultural byproducts (molasses) and industrial waste (brewery waste), respectively. These byproducts, however are known to have poor consistency in quality when compared to the pure substrates (Barredo, 2005). In nature, microorganisms do not live in isolation. They coexist and form synergistic and symbiotic relationships that promote bioflocculant production (Chapelle, 2001). Zhu *et al*. (2004) and Zhang *et al*. (2007), have reported that a combination of strains of microbes in consortia produce bioflocculants with better flocculating activity and increased bioflocculant yields than pure microbial strains.

Improved bioflocculant producers can also be attained by employing molecular techniques such as genetic manipulation (He *et al*., 2010). Bioflocculant producers isolated from nature are often used as a starting point in genetic manipulation as an attempt to increase bioflocculant production (Barredo, 2005). The key approach has been to identify the genes that control bioflocculant production and incorporate them to new strains for synthesis of high and pure bioflocculant yields (Ates *et al*. 2013; Finore *et al*. 2014). He *et al*. (2010) successfully used molecular techniques to produce an increased bioflocculant HBF-3 from *Halomonas* sp V3a`.

2.16 Marine bioflocculant producers

Bioflocculant producing microorganisms are found in different ecological niches that include soil and water (fresh and oceanic water) (Singha, 2012). Bioflocculant-producers have been predominately screened and isolated from activated sludge, soil and fresh water samples (Li *et al.,* 2013; Nwodo *et al.,* 2016). However, marine environments remain untapped reservoirs (Mabrouk, 2014; Okaiyeto *et al.,* 2015). Marine sediments have a wide, diversified range of microorganisms that include bacteria, fungi, protozoa and viruses (Boyd, 2015). One ml of sea water has approximately 10^6 bacteria and archaea (Mitchell and Gu, 2010). Even in the most uninhabitable places, marine microbes are found in large numbers (Mclntyre, 2010). With the exception of viruses, the most fundamental element of taxonomic diversity in aquatic environments lies in separations of biota into major domains of Bacteria, mainly Archaea and Eukarya, which belong to mesophilic (*Bacillus* sp and *Streptococcus* sp) and halophilic (*Halomonas* sp) groups (Singha, 2012).

A diverse and large number of microorganisms are suitable to a minimum loss of water, to low temperatures, to solar radiations and to soluble organic and inorganic nutrients that are abundant and readily available (Sigee, 2008). Nevertheless, there are high disturbances in aquatic environments. The ocean exhibits extreme variations that include salinity, depth, temperature, hydrostatic pressure and mixing (Anderson *et al.,* 2009). This can only permit a microbial community with extreme adaptations (Talaro and Chess, 2015). It is predicted that marine microorganisms have high potential to produce bioflocculants with high flocculating activities since they possess different morphological, physiological and metabolic adaptations to adverse environments in seas (He *et al.,* 2010). With a little more research on marine microorganisms, there is a wide chance of obtaining a variety of novel bioflocculant producers with significant EPS molecules.

Therefore in this study, bioflocculant-producing bacterial strains (*Bacillus pumilus* JX860616, *Bacillus subtilis* CSM5 and *Alcaligenes faecalis* HCB2) were isolated from marine sediment of Sodwana Bay, Republic of South Africa.

2.16.1 *Bacillus pumilus* JX860616

The genus *Bacillus* consists of a large number of diverse, Gram positive, rod-shaped, catalase-positive bacterial strains that are motile and aerobic (Taloro and Chess, 2015). Members of the genus produce endospores to resist adverse environment conditions (Morikawa, 2006). *Bacilli sp are* non-fastidious microorganisms (Taloro and Chess, 2015). Their metabolites have been used industrially in pharmaceutical companies (Aunpad *et al.,* 2011), and bioremediation processes (Nwokoro and Dibua, 2014). *Bacillus pumilus* is a spore forming bacilli (Ortigosa *et al.,* 1997). *B. pumilus* belongs to the *Bacillus subtilis* group. *B. pumilus* is one of the major components of marine bacterial communities (Boeye and Aerts, 1976; Ivanova *et al*., 1999). It is resistant to extreme environmental conditions such as: low nutrient availability, aridness, radiation, chemical disinfections and hydrogen peroxide (Nicholson *et al.*, 2000). Based on its capabilities to produce industrially useful products such as, alkaline serine protease (Huang *et al.,* 2003), vanillin (Converti *et al.,* 2010), xylanase (Thomas *et al.*, 2014) and keratinase (Rajput and Gupta, 2014). Thus, *B. pumilus* has received industrial attention.

2.16.2 *Bacillus subtilis* CSM5

Bacillus subtilis is classified as a Gram positive, rod shaped and spore forming bacilli (Taloro and Chess, 2015). It is abundantly found in soil and in plants. *B. subtilis* is a mesophilic bacteria growing optimally between temperatures of 25-35 ˚C (Jitjumroonchokchai *et al*., 2011). During bacterial stress conditions such as heat, alkaline, oxidative and osmotic conditions, *B. subtillis* synthesise endospores in order to survive (Batzing, 2002). *B. subtilis* is not pathogenic and can grow upto 6.0 µm long (Batzing, 2002; Martinko and Madigan 2006). Moreover, *B. subtilis* secretes enzymes which are useful industrially. The enzymes include pollulanase, lipolase, chitinase, protease, amylase and xylanase (Morikawa, 2006).

2.16.3 *Alcaligenes faecalis* HCB2

The genus *Alcaligenes* is formed by saprophytic, motile, oxidase and catalase positive, non-fermentative bacteria. *Alcaligenes* sp are found in soil, water, dairy products and the intestinal tract (Taloro and Chess, 2015). *Alcaligenes* sp have gained more attention industrially. Since recently, *Alcaligenes* strains have been the subject of research due to the production of useful biopolymers (Shabina *et al.,* 2015). They have been utilized in the biodegradation of toxic compounds (Sangkharak and Prasertsan, 2012). The widely known species of this genus is *Alcaligenes faecalis. A. faecalis* is a Gram negative, motile bacilli mostly found in soil and water (Bizet and Bizet, 1997). *A. faecalis* is generally non-pathogenic but can be opportunistic (Kavuncuoglu *et al*., 2010). The bacterium is alkali-tolerant and is capable of degrading urea (Anderson *et al.,* 2003). *A. faecalis* is mostly used in bioremediation of hydrocarbon pollutions (Bharali *et al.,* 2001).

2.17 General growth kinetics of bioflocculating bacterial strains

Production of bioflocculants is growth associated, growth synonymous or growth independent (More *et al.,* 2014). A microbial growth curve (Figure 2.3) has the following four phases: (1) lag, (2) exponential, (3) stationary and (4) death phases (Talaro and Chess, 2015). The kind of the nutrients in the media plays a substantial role in all growth phases with respect to time (Madigan *et al.,* 2012). The initial lag phase is a time of no apparent growth. It shows the metabolic turnover indicating that the cells are in a process of adapting to ~~the~~ environmental conditions (Smith, 2009). Lag phase prolongs with old inoculums present (Willey *et al.,* 2009). This is because the cells are not very viable and are depleted of essential constituents and time is required to resynthesize. A lag phase is prolonged when the inoculum consists of cells that have been damaged (but not killed). It also lengthens when inoculums are transferred from rich culture media to poorer ones. Microbial cells try to synthesise essential nutrients not present in the medium (Madigan *et al*., 2012). Generally the flocculating activities of the bioflocculants are usually lower during the lag phase.

The number of microbial cells increases constantly in the exponential phase because of an abundance of nutrients and lack of growth inhibitors (Madigan *et al*., 2012). In this phase, microbial growth proceeds at the maximum possible rate for microorganisms with nutrients in excess and conducive environmental conditions (Cowan, 2012). Thus, cells in the exponential phase are usually in their healthiest state. Flocculating activities of bioflocculants are usually associated with cell growth at the exponential growth phase (Nwodo and Okoh, 2013). Most researchers have reported the highest bioflocculant production to be at the exponential phase (Peng *et al*., 2014; Nwodo *et al*., 2016).

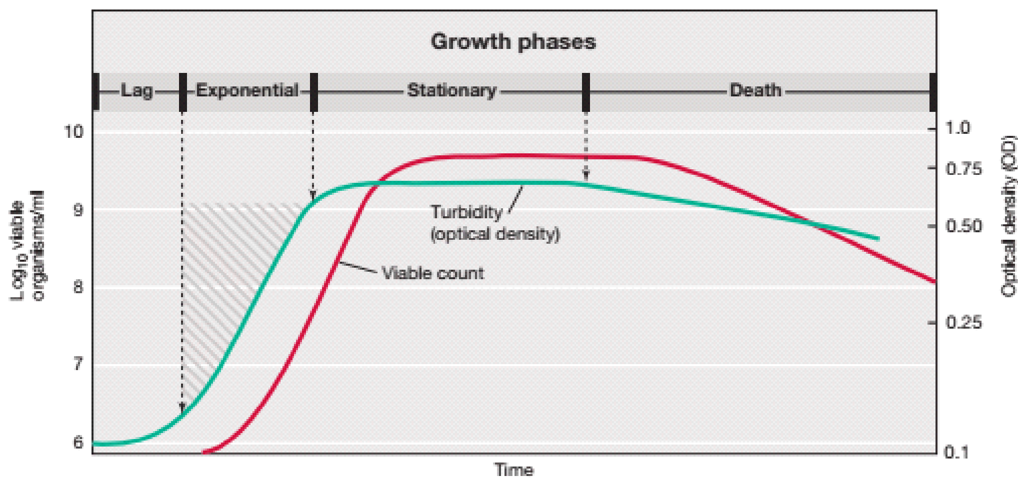


Figure 2.3: Growth kinetics of bacteria (Madigan *et al*., 2012)

In a batch culture, the exponential phase is restricted by nutrient availability (Talaro and Chess, 2015). As nutrients get depleted, the growth rate decreases giving in to the stationary phase (Cowan, 2012). In this phase, there is no net increase or decrease in the number of cells (Madigan and Martinko, 2006). The rate of cell multiplication equals the rate of dying cells (Smith, 2009). This is often due to the depletion of nutrients, a reduction in oxygen availability and metabolic activities of toxic waste products (Willey *et al.,* 2009). At this stage, the flocculating activities of bioflocculants remain static. Bioflocculants produced inside the broth medium can be assimilated by the bacterial strains to serve as a source of energy (More *et al.,* 2014). The death phase emerges just when complete nutrient deficiency is experienced. The

rate at which microbial cells die are much higher than ~~at~~ the rate at which they reproduce (Madigan *et al*., 2012). However most biotechnological batch processes for bioflocculant production are stopped just before the death phase to avoid cell lysis (Smith, 2009).

2.18 Factors effecting bioflocculant production

Bioflocculant secretion from microorganisms in their growth environment is greatly influenced by the factors that govern their metabolism (More *et al*., 2014). Bioflocculant production is influenced by the bacterial growth phase, medium composition and also physicochemical parameters (Singha, 2012; Fang *et al.,* 2013). Growth and bioflocculant production depend on the availability and transport of necessary nutrients to the cell and environmental parameters (Smith 2009). Thus microorganisms must be motivated to produce the desired bioflocculants by means of correct medium composition and environmental growth parameters. The main growth and culture parameters that effect bioflocculant production include the following: (1) inoculum size, (2) carbon and nitrogen sources, (3) cation, (4) initial pH of the culture medium, (5) shaking speed, (6) incubation temperature and (7) culture time (More *et al*., 2014; Okaiyeto *et al*., 2016).

2.18.1 Nutrients and elements

The two most relevant aspects, yields and productivity, are qualities that indicate how the cells convert the substrate into products that are greatly dependant on nutrients (Smith, 2009). The yield presents the amount of products obtained from the substrate. Productivity in turn specifies the rate of product formation. Availability and type of nutrient can exert strong physiological control on growth reaction and product formation. The concentration of nutrients in the vicinity of the organism must be held within a define range since a low value turns to limit the rate of metabolism while an excess concentration ~~turn~~ turns to be toxic. Different microorganisms require different nutrient supplements to make new cell materials and bioflocculants (Madigan and Martinko 2006). Nutrients used by most microorganisms are complexes derived from chemical elements. Normal microbial cells have the chemical formula of $CH_2O_{0.5}N_{0.15}$. This implies that C, H, O, and N components are essential for their growth (Madigan *et al*., 2012).

Microbial cells need carbon to multiply, in order to synthesise metabolite and for energy needed for biochemical reactions (Willey *et al.,* 2009). Nitrogen is the second most abundant element in the cell after carbon. Microorganisms utilize nitrogen for the synthesis of amino acids, proteins and nucleic acids (Madigan *et al*., 2012). Phosphorus in nature is in the form of organic and inorganic phosphates. Phosphorus is required by microorganisms primarily for synthesis of nucleic acids and phospholipids (Black, 2008). Microorganisms require trace elements in small concentrations. Trace elements contribute to cell function as they act as cofactors for enzymatic reactions (Madigan and Martinko 2006).

Magnesium functions to stabilise ribosomes, cell membranes and nucleic acids (Cowan, 2012). It is also essential for enzymatic activities. Calcium, which is not an essential nutrient of many microorganisms, helps to stabilize the cell wall. It also plays a role in the thermal stability of the endospores (Willey *et al.,* 2009). Sodium is required by some but not all microoorganisms. Its need reflects the microbial habitats. For example, marine microorganisms require sodium for growth while the closely related fresh water strains can grow in its absence (Madigan and Martinko 2006). Sulfur is essential for a structural role in the amino acids (cysteine and methionine) (Black, 2008). Potassium is required by all organisms (Talaro and Chess. 2015). Generally, microorganisms utilise essential nutrients and trace elements for their growth and production of byproducts such as bioflocculants.

2.18.2 Inoculum size

Inoculum size is an important factor that has effect on microbial growth and bioflocculant production (Nwodo *et al*., 2013)**.** Optimal inoculum is suitable for a microorganism to adapt to the medium which in turn can shorten the lag phase and promotes bioflocculant production (Li *et al*., 2009). A small inoculum size prolongs the stagnant growth phase. On the other hand, a large inoculum size usually results in the niche of the microorganism overlapping excessively, thereby suppressing bioflocculant production (Salehizadeh and Shojaosadati, 2001).

2.18.3 Cations and initial pH

Cations play a critical role in bioflocculation. Cations enhance the flocculation rate by neutralising and stabilising the residual negative net surface charge of the functional groups of the bioflocculant (Yim *et al*., 2007). In this way, the formation of bridges between colloidal particles and bioflocculants is stimulated (Wu and Ye, 2007). The initial pH of the growth medium greatly influences microbial growth and bioflocculant production (Zheng *et al.,* 2008). Initial pH determines the electrification of the microbial cells and the oxidation–reduction potential (Salehizadeh and Shojaosadati, 2001). The alteration in pH could directly or indirectly affect the absorption of nutrients in the production medium and metabolic reactions. Thus, different microorganisms have different optimal pH for growth and bioflocculant production (Ugbenyen *et al*., 2012).

2.18.4 Culture temperature

Culture temperature is also one of the major parameters that has a great effect on bioflocculant production (Li *et al.,* 2009). This is due to the fact that metabolic reactions usually take place effectively at optimum temperatures for microbial growth and bioflocculant production (Okaiyeto *et al.,* 2016). The rise in cultivation temperature often leads to faster chemical and metabolic reactions which subsequently promote rapid microbial growth and bioflocculant production. Generally, a rise in temperature increases growth, metabolic function and bioflocculant production until the point where denaturation reaction sets in (Madigan and Matinko 2006). Low temperatures turn to a decrease in microbial growth rate and cell wall polymer synthesis (More *et al.,* 2014). Most of the microorganisms are reported to produce bioflocculants in a temperature range of 25-30 °C in a batch culture.

2.18.5 Shaking speed

The agitation speed has a vital effect on bioflocculant production. The primary function of agitation is to suspend the microbial cells and nutrients evenly throughout the medium (Smith, 2009). This ensures that nutrients (including oxygen) are available to all microbial cells. Since oxygen is sparingly soluble in aqueous solutions, aerobic growth is stimulated though continuous, vigorous agitation of the culture medium (Lopez *et al.,* 2003). Generally, agitation speeds up the concentration of dissolved oxygen which can also effect nutrient

absorption, enzyme activity of the microbes and bioflocculant production (Okaiyeto *et al.,* 2016). Agitation also promotes sufficient heat transfer in the culture medium (Smith, 20009). Thus, each microorganism that produces bioflocculants has a preferred optimum agitation speed.

2.19 Factors effecting bioflocculant activities

The effectiveness of the produced bioflocculant is affected by many factors. Dosage size, chemical functionalities (functional groups and chemical components), electrical charge, molecular weight, degree of purity of the bioflocculant, and the structure of bioflocculants are among factors that effect flocculating activity and mechanisms (Badireddy *et al*., 2010; Liu and Cheng, 2010; More *et al*., 2014).

2.19.1 Dosage size

Dosage size is an important factor in the flocculating activity of bioflocculants. An insufficient bioflocculant dosage size has a tendency not to neutralize negative charges on colloidal particles in suspension, leading to a poor flocculation process (Li *et al.,* 2007). On the other hand, excessive amounts of bioflocculant concentration in suspension can lead to a reduction in the settling of flocculated colloidal particles due to high viscosity (Wang *et al*., 2011).Thus, for effective flocculation to occur, an optimum concentration of bioflocculants is required.

2.19.2 Functional groups and chemical components

The functional groups of bioflocculants provide adsorption sites for different colloids in suspension (Xiong *et al*., 2010). Moreover, the binding capability depends on the number of functional groups available in bioflocculant chains. The chemical composition of bioflocculants is an important factor that influences their flocculating activities. Most of the reported bioflocculants in previous studies were predominantly composed of polysaccharides, proteins, fatty acids, and nucleic acids (Salehizadeh and Shojaosadati, 2003).

2.19.3 Electric charge of bioflocculants

The surface electrical charges of bioflocculants are important in flocculating activity. The electric charge of a bioflocculant is greatly influenced by the proportion of its components (Walker and Wilson, 2005). In general, bioflocculants mostly bear a negative charge (Levy *et al.,* 1992; Okaiyeto *et al.,* 2016). This feature enables the binding of bioflocculants to positively charge colloidal pollutants. This is as a result of cation exchange potential offered through electrostatic interaction (Esparza-Soto and Westerhoff, 2003). Bioflocculants that have a negative surface net charge show a higher hydrophobic character. The hydrophobic components form bonds with positively charged colloidal particles such as metal ions and dye in suspension (More *et al.,* 2010).

2.19.4 Molecular weight of bioflocculants

Molecular weight is one of the properties that effect the flocculating ability of bioflocculants (Liu and Cheng, 2010). The molecular weight range of most bioflocculants varies from 10^5 to 2.5×10^6 Da (More *et al*., 2014). The efficiency of the bridging mechanism in flocculation is related to the molecular size of bioflocculants. Bioflocculants with a high molecular weight have more adsorption points, stronger bridging, and higher flocculating activity than those bioflocculants of a low molecular weight (Zhang *et al.,* 2010).

2.19.5 Purity of bioflocculants

Microorganisms do not produce bioflocculants in their pure forms. The crude bioflocculant extracts may be bound to a large range of impurities that include microbial cells. Although it is challenging to purify microbial flocculants, their purity is essential for their effectiveness (Walker and Willian 2005). The degree of purity of the bioflocculant does not only affect its study and exploration of character, but also its functionality in the flocculation process (Smith, 2009). The contaminants may compete on adsorption sites of the bioflocculant with colloidal particles in suspension. This in turn can lead to poor flocculating capability of the bioflocculant (Singha, 2012). Normally it can be stated that the higher the purity, the greater the efficacy in flocculation. Centrifugation, chromatographic methods, heat treatment and precipitation methods are among purification techniques mostly used to remove cells or their debris (Sutherland 1990; Morin, 1998; Zhang *et al,* 2007).

2.19.6 Structure of bioflocculants

The surface morphological structure of a bioflocculant plays a vital role in a flocculation process (Zhang *et al*., 2007). The surface morphological structure may be accountable for effective or poor flocculating activity of bioflocculants (Okaiyeto *et al.,* 2015). Most bioflocculants reported in literature have an amorphous, porous or crystal-like structure (Cosa *et al.,* 2013, Okaiyeto *et al.,* 2015). A scanning electron microscope (SEM) uses electrons in a raster scan pattern to image the sample surface. The electrons react with the atoms to produce signals which contain information about the structures of the sample (He *et al*., 2010).

2.20 Biosafety

Biosafety standards are based on international legislation which aims to prevent risk to human health and the environment resulting from activities that involve the use of products/bioproducts (Smith 2009). Thus safety is of paramount importance in bioflocculation. Although Devi *et al.* (2015) has affirmed bioflocculants as non-toxic compounds, for biosafety reasons, bioflocculants need to be tested for their toxicity before use. This is due to the fact that some bioflocculants, especially from opportunistic and pathogenic bacteria, may impose toxic effects (Spellman, 2014). Bacterial secondary metabolites have the potential to cause harm to people and animals leading to epidemic diseases along with allergenic, neurotoxic and cytotoxic effects (Smith, 2009). Thus, in this study, MTT assay was used to determine cytotoxic effects of a bioflocculant on various cell lines. MTT assay is a colourimetric assay that is based on the conversion of tetrazolium salt (MTT) to formazan by mitochondrial dehydrogenases. This assay is commonly used to determine cell viability (Mosman, 1983).

2.21 Flocculation mechanisms

The mechanism of flocculation by conventional chemical flocculants is well defined and understood. However, the bioflocculation mechanism is still not fully understood (Salehizadeh and Shojaosadati, 2001). This is due to the fact that the characteristics of bioflocculants differ between bioflocculant producers which result in diverse mechanisms (Strand *et al.,* 2002). An understanding of the bioflocculating mechanism is vital not only for assessment of its suitability but also for its efficacy in various biotechnological fields (Lian *et al*., 2008). However, there are two main bioflocculation mechanisms generally proposed: (1) charge neutralisation bridging and (2) bridging mechanisms (Li *et al*., 2009).

2.21.1 Charge neutralisation mechanism

Charge neutralisation mechanism takes place when the flocculants are oppositely charged compared to the suspended particles (Figure 2.4) (Bratby, 2006). In this case, the surface charge density of colloidal particles is reduced by its adsorption onto the bioflocculant (Levy *et al.,* 1992). This action enables colloidal particles to approach each other and agglomerate as attraction forces overpower repulsive forces (More *et al.,* 2014). Thus, charge neutralisation mechanism is induced by a reduction in the electric double layer repulsion. Most bioflocculants and colloidal particles have negative charges; as a result, charge neutralization rarely happens (Levy *et al.,* 1992; Deng *et al.,* 2005).

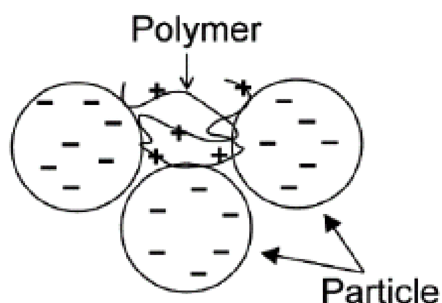


Figure 2.4: Charge neutralisation mechanism (Dobias, 2005)

2.21.2 Bridging flocculation mechanism

Bioflocculation is generally explained by the bridging flocculation mechanism (Zhang *et al*., 2010). The bridging flocculation mechanism describes flocculation by like charged or neutral flocculants (More *et al.,* 2014). Bridging flocculation takes place when segments of the same ion or colloidal particle are attached to more than one particle, consequently linking colloidal particles together (Rose and St John, 1987; Dicks, 2011) (Figure 2.5). It happens when flocculants influence aggregation of colloidal particles and extends from the particles` surfaces into the suspension for a distance greater than the distance which the inter-particle repulsion forces exert (Tripathy and De, 2006). The strength of flocs greatly depends on the bridges formed. Moreover, the availability of adsorption sites on flocculants is the most important factor in the bridging mechanism (Zhang *et al.,* 2010). Generally, the net charge, molecular weight of the flocculants, the suspended colloids, the ionic strength of suspension, and the nature of agitation influence the bridging mechanism (Wang *et al.,* 2011).

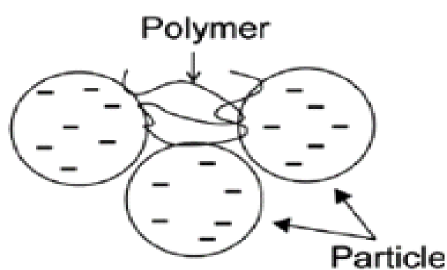


Figure 2.5: Bridging mechanism (Dobias, 2005)

2.22 Electrostatic repulsion forces, van der Waal forces and Brownian motion

The theory of particle to particle interaction is based on the interaction of attraction and repulsion forces (Davis, 2011). Particles in nature remains stable when there is a balance between the electrostatic repulsion forces (repulsive forces) and van der Waals forces (attractive forces) (Ji, 2008). Repulsive forces are associated with electrostatic charges due to the double layer surrounding the colloidal particles. Because the double layer spreads deeper into suspension than van der Waals forces, energy barriers that inhibit particles from aggregation and flocculation are often formed (Bahadori, 2014).

2.22.1 Electrostatic repulsion forces

Agglomeration of colloidal particles in suspension is greatly opposed by electrostatic repulsion of surface charges (Mines, 2014). Electrostatic repulsion is the dominant force controlling stability of hydrophilic and hydrophobic colloidal particles in suspension (Tripathy and De, 2006). Hydrophillic colloidal particles attain their surface charge from dissociation of carboxylic or other organic acid groups on the particle surface. On the other hand, hydrophobic colloidal particles may obtain extra anions or cations at the interface and produce a barrier that can reject particulates with the same surface potential.

Electrostatic repulsion mechanism involves a charged colloidal particle as having a double layer of counter-ions around (Figure 6) (Dicks, 2011). The first layer is known as Stern layer. It is made up of a hard linked layer. The stability of colloids in suspension is influenced by the potential of the Stern layer. The potential can be measured by zeta potential analyser (Zeta-nanosizer) (Tripathy and De, 2006). The second layer (diffuse layer) comprises of less bound counter-ions. The concentration of ions declines with the distance from the surface of the colloidal particle until the concentration of ions is equivalent to the bulk solution. This usually results in an electrical potential that develops around the particle. The ions around a colloidal particle cause repulsion of nearby particles, constrained aggregation and poor flocculation (Edward, 2011).

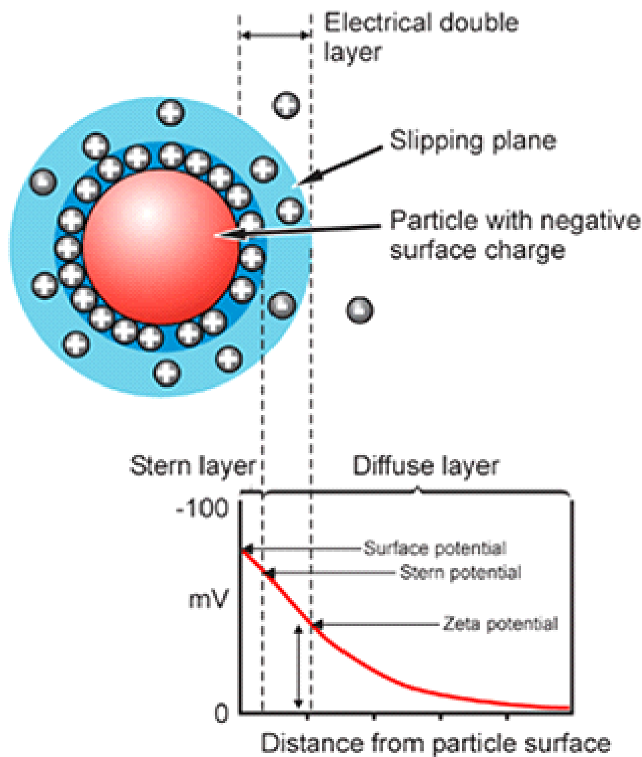


Figure 2.6: Negatively charged colloidal particle (Çabuk, 2016)

2.22.2 Van der Waal's force

An increase in the ionic strength often results in a decrease of the double layer. This in return decreases the repulsion force between particles and promote attraction forces to enhance aggregation and flocculation (Mine, 2014). Van der Waal's force is the attraction force that promotes agglomeration and flocculation of colloidal particles. Van der Waal's attraction between two colloids is actually the result of forces between individual molecules in each colloid (Blackburn *et al*., 2000). Colloidal particles with high zeta potential (negative or positive) are electrically more stable than those with small zeta potentials (Dicks, 2011). Generally, flocculation results from a dominion of van der Waal's forces over electrostatic repulsion forces.

2.22.3 Brownian motion

Blackburn *et al*. (2000), described Brownian movement as the random movement of colloidal particles. It is due to bombardment by water molecules in solution. Brownian movement influences destabilisation of colloids in suspension. It is greatly significant in weak turbulence and in a high concentration of colloidal (submicron) particles as it enhances flocculation (Blackburn *et al*., 2000; Ji, 2008).

Chapter 3: Materials and methods

3.1 Source and identification of bacteria

The bacteria were obtained at the Department of Biochemistry and Microbiology at the University of Zululand, South Africa. The bacteria were previously isolated from a sediment sample from Sodwana Bay in the Province of KwaZulu-Natal in South Africa. They were identified by 16S-rRNA nucleotide sequencing genes with subsequent BLAST analyses as; *Bacillus pumilus* JX860616, *Bacillus subtilis* CSM5 and *Alcaligenes faecalis* HCB2.

3.2 Chemicals and production medium

All chemicals and reagents used were procured from Sigma-Aldrich (St Louis, MO, USA) and water used was glass distilled. The standard production medium by Zhang *et al.* (2007), was used. The medium composed of glucose (20.0 g), KH_2PO_4 (2.0 g), K_2HPO_4 (5.0 g), $(NH_4)_2SO_4$ (0.2 g), NaCl (0.1 g), CH_4N_2O (0.5 g), $MgSO_4$ (0.2 g) and yeast extract (0.5 g) in a litre of filtered seawater and was autoclaved (121°C for 15 minutes).

3.3 Determination of flocculating activities

A loopful of bacterial species was inoculated into a 250 mL flask containing 50 ml of the sterilised production medium. The mixtures were incubated at 30°C in a shaker at 165 rpm for 72 hours. The culture broths were centrifuged at 8000 rpm for 30 minutes at 4°C to remove cells and the flocculating activities were determined spectrophotometrically at an optical density of 550 nm (Kurane *et al.,* 1994). Prior to the determination of the flocculating activities, 100 ml of kaolin suspension (4 g/l) was measured into 250 ml flasks and 3 ml of 1% w/v $CaCl_2$ and 2 ml of the obtained cell free supernatants were added. The mixtures were shaken for 1 minute and then poured into a 100 ml measuring cylinder. The sediment, as well as the control (kaolin solution) were allowed to stand for 5 minutes at room temperature. The formula used for the determination of the flocculating activities was as follows:

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

with A as the optical density of control (kaolin solution) at 550 nm and B as the optical density of a sample at 550 nm.

3.4 Construction of bacterial consortia

A consortium *of B. pumilus* JX860616 and *B. subtilis* CSM5 and of *B. pumilus* JX860616 and *Alcaligenes faecalis* HCB2 were constructed. A loopful of each strain colony was inoculated into 50 ml of the fresh production medium. The medium was incubated at 30°C and at the shaking speed of 165 rpm. After 72 hours, the culture broths were centrifuged at 8000 rpm for 30 minutes at 4°C using a centrifuge machine (Heraeus Fresco 21, Germany). The supernatants were used for the determination of the flocculating activities (Zhang *et al*., 2007).

3.5 Optimisation of the medium composition and culture conditions

The establishment of optimum medium composition and growth conditions for bioflocculant production were obtained through varying parameters such as: the inoculum size (%, v/v), nutrient sources, cations, shaking speed, initial pH of production medium, temperature and time course.

3.5.1 Evaluation of the inoculum sizes and nutrient sources

The effect of inoculum sizes of different bacteria (single species and in consortia) was evaluated. Different inoculum sizes ranging from 1 to 5% (v/v) were used to inoculate the production medium. The effect of each inoculum size on flocculating activities was determined spectrophotometrically as detailed above (Nwodo *et al.,* 2016). The effect of different carbon sources on the flocculating activities was investigated using the procedure by Li *et al.* (2009). Bacterial strains were cultivated on various organic carbon sources (glucose, fructose, sucrose, lactose, maltose, starch and molasses). The flocculating activities were determined, thereafter. The effect of organic nitrogen sources (casein, yeast extract, urea, tryptone and peptone) and inorganic nitrogen ($(NH_4)_2SO_4$) was also evaluated. The nitrogen sources (1.2 g/l) were individually incorporated into the production medium replacing the initial multiple nitrogen (yeast extract (0.5 g), urea (0.5 g) and $(NH_4)_2SO_4$ (0.2 g) in equivalent amounts (Okaiyeto *et al.,* 2016).

3.5.2 Effect of cations, shaking speeds, initial pH and temperatures on flocculating activities

Different cations (NaCl, KCl, LiCl, $MnCl_2$, $BaCl_2$ and $FeCl_3$) were used to assess their effect on flocculating activities of the bioflocculants. Three millilitre of calcium chloride (1%) used as a standard cation was replaced with these cations (Nwodo *et al.* 2016). The control was kaolin solution without addition of cation. The flocculating activities were determined spectrophotometrically at a wavelength of 550 nm, thereafter. The effect of the shaking speeds on flocculating activities was assessed according to Zhi-qiang *et al*. (2007). The shaking speeds were varied in a range of 0-220 rpm. The flocculating activities were evaluated as described previously. The effect of the initial pH of production medium on flocculating activities was determined. Initial pH was adjusted within a range of 3-12 by 1N HCl and 1N NaOH using pH meter (Eutech Instruments pH 700, Singapore). The effect of cultivating temperatures on the flocculating activities was also evaluated. The cultivating temperatures were varied in a range of 20-50°C (Zhi-qiang *et al*., 2007).

3.5.3 Time course assay

The effect of growth time on flocculating activities of the bioflocculants was conducted in accordance to a previous study by Cosa *et al.* (2013). The bacteria *(*single species and in consortia*)* were cultured under obtained optimal growth conditions. From the seed culture, the optimum inoculum size was inoculated into 50 ml of culture medium (prepared in triplicates) and incubated (30°C) on a shaker (165 rpm). Samples (2 ml) were drawn every 12 hours for 120 hours, centrifuged (8000 rpm, 30 minutes) and the supernatant was used to evaluate the flocculating activities. The optical densities (OD_{550}) of the growth broths, which might have represented bacterial growth and the pH were also determined by the spectrophotometer (Pharo 300, Merck KGaA, Germany) and a pH meter (Eutech Instruments-pH 700), respectively.

3.6 Extraction and purification of the bioflocculants

The bioflocculants were extracted and purified in accordance to the method of Chang *et al*. (1998). After 72 hours of incubation, the growth broths were centrifuged (8000 rpm, 4°C, 30 minutes). Distilled water (1 volume) was added to the supernatant phase and centrifuged (8000 rpm, 30 minutes, 4°C) using centrifuge machine (Eppendorf Centrifuge 5804R, Germany) to remove the insoluble substances. Ethanol (2 volumes) was added to the supernatant, agitated and then allowed to precipitate for 12 hours at 4°C. The precipitates were vacuum-dried and the crude products were dissolved in the distilled water (100 ml). One volume of the mixture of chloroform and butanol (5:2 v/v*)* was then added, agitated and left to settle for 12 hours at room temperature. The cell-free supernatants were finally centrifuged (4000 rpm, 4°C, 30 minutes) and vacuum-dried.

3.7 Solubility assay of the purified biofloccuants

Solubility assays were done on the purified bioflocculants. The bioflocculants (0.05 g each) were dissolved in 2 ml of different solvents (water, acetone, chloroform, dichloromethane, benzene, hexane, ethyl acetate, ethanol, methanol and butanol) in accordance with Zaki *et al.* (2011).

3.8 Effect of dosage size and cations on flocculating activities

The different bioflocculant concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were prepared in distilled water and their flocculating activities accessed subsequently for determination of the optimum concentration (Luo *et al.,* 2014). The synergistic effect of different cations (KCl, NaCl, LiCl, $MnCl_2$, $CaCl_2$, $MnCl_2$, $BaCl_2$ and $FeCl_3$) on the flocculating activities of the purified bioflocculants was determined according to Nwodo *et al.* (2016). The control was kaolin solution without addition of cation.

3.9 Physico-chemical characteristics of the purified bioflocculants

The morphological surface structures of the biofloculants, kaolin particles and flocs were examined with a scanning electron microscope (SEM-Sipma-VP-03-67) (Karthiga and Natarajan, 2015). Characterization of the charges of the purified bioflocculants was determined using Malvern Zetasizer Nano (Malvern, UK). The Ninhydrin method was used to determine the amino acid content (Kay *et al.*, 1956). Proteins and nucleic acids were determined qualitatively by UV-vis spectrophotometry (Perkin Elmer Lambda 1050, UV-visible NIR, Germany) (Harrington and Raper, 1968). The quantitative analyses of the total protein content were evaluated by Bradford, (1976), method and bovine serum albumin (BSA) was used as the standard solution. The total carbohydrate contents were assessed by a phenol-sulphuric acid method (Chaplin and Kennedy, 1994). Glucose was used as a standard solution. The elemental analyses were carried out with SEM equipped with an elemental detector (Oxford Instruments-X-MaxN, UK) (Li *et al.,* 2009). The functional groups of the bioflocculants were analysed by a Fourier transform infrared (IR) spectroscopy (Perkin Elmer UATR TWO, 2000, Germany). The wavelength of IR ranged from 400 to 4000 cm^{-1} (Luo *et al.,* 2014).

3.10 Thermal stabilities and pyrolysis profiles of the bioflocculants

Thermal stabilities of the purified bioflocculants were accessed at different temperatures ranging from 50 to 100°C. The bioflocculant solutions were heated for 60 minutes and the flocculating activities were determined thereafter (Okaiyeto *et al.*, 2013). The pyrolysis profiles of the bioflocculants were evaluated by thermogravimetric analyser (Perkin Elmen Pyris 6 TGA, Germany). The purified bioflocculants (10 mg) were heated within a range of 22 to 900°C at a rate constant of 10°C min^{-1} under constant flow of nitrogen gas (Okaiyeto *et al.,* 2016).

3.11 pH and saline stabilities of the bioflocculants

The effect of pH on the flocculating activity of the purified bioflocculants was evaluated according to Nwodo *et al. (*2016). The pH of kaolin solution (4 g/l) was adjusted in a scope of pH 3–10 with 0.1 M HCl and 0.1M KOH. The flocculating activities were determined as described above. The effect of salinity on the flocculating activities of the purified bioflocculants were determined in accordance to Li *et al.* (2008). The bioflocculants and different cations were poured into kaolin solution (4 g/l) with different concentrations of NaCl (5-35 g/l). The flocculating activities were determined spectrophotometrically at 550 nm.

3.12 *In-vitro* cytotoxicity assay of the bioflocculants

The cell cytotoxicity was measured according to Mosman (1983). Human colorectal adenocarcinoma cells (Caco-2), breast cancer cells (MCF7) and human embryonic kidney 293 cells (HEK 293) were all grown to confluency in 25 cm^3 flasks. They were then trypsinised and plated into 48 well plates. Cells were incubated overnight at 37°C. Old medium was supplemented with the fresh medium (MEM + Glutmax + antibiotics). Bioflocculants were then added in triplicate and incubated for 4 hrs. Thereafter, the medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics +10% Fetal bovine serum). After 48 hours, the cells were subjected to 200 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) of the concentration of 5 mg/ml in phosphate buffered saline (PBS) and 200 µl medium was added to each well and incubated at 37˚C for 4 hours. Thereafter, the medium with MTT was aspirated from the wells and the formed formazan crystals were solubilized in 200 µl of dimethyl sulfoxide (DMSO). The optical density of the solutions was read at 570 nm using a micro plate reader. The cell viability was expressed as percentage with control using the equation;

Cell viability (%) = $(F_1/F_0) \times 100$

where F_1 and F_0 are the initial and final values obtained before and after treatment with the bioflocculants, respectively.

3.13 Mechanism of flocculation

The flocculation mechanism of the bioflocculants was proposed after the zeta potential was measured by Zetasizer Nano (Malvern, UK). The zeta potential of the kaolin particles, mixture of kaolin particles and $BaCl_2$, kaolin particles flocculated by the bioflocculants in the presence of $BaCl_2$ and the bioflocculants were measured, respectively at 25°C (Aljuboori *et al.,* 2015).

3.14 Removal efficiencies of the bioflocculants on wastewater

The biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen (N), phosphorus (P) and sulphur (S) in wastewater from the Nhlabane Estuary, Umhlathuzi and the local coal mine wastewater plants (RSA) were measured with spectro-quant (Pharo 300, Merck KGaA, Germany), before and after application of the bioflocculants. The Jar test was done in accordance to the method used by Okaiyeto *et al*. (2016). Three millilitres of 1% (w/v) $BaCl_2$ and 2 ml of the bioflocculant solution were both poured into 100 ml wastewater sample. The mixture was shaken at 200 rpm for 3 min; the speed reduced to 40 rpm and allowed to shake for 5 min. The removal efficiencies of the bioflocculants on BOD, COD, N, P, S, Al, and Ca were measured as follows:

$$\text{Removal efficiency (\%)} = (E_o - D/ E_o) \times 100$$

where E_o and D are the initial and final values obtained before and after treatment with the bioflocculants, respectively. Alum and ferric chloride were used as controls.

3.15 Software and statistical analysis

All the experimentations were done in triplicates and the error bars in the figures shows the standard deviations of the data. Data were subjected to one-way analysis of variance using Graph Pad Prism ™ 6.1. P values ≤ 0.05 were regarded as significant and P values ≤ 0.05 as very significant. Values with different alphabets along the same row are significantly different ($p<0.05$).

Chapter 4: Results

4.1 Introduction

Chapter 4 consists of the results and observations obtained in this study.

4.2 Results for *B. pumilus* JX860616

The results obtained during resuscitation, and optimisation of medium composition and culture conditions of *Bacillus pumilus* JX860616 are shown in this section. The results of the characteristics and application of the bioflocculant are also included.

4.2.1 Optimization of cultivation conditions

Optimization of the cultivation process improves the production of bioproducts. Thus, the varying constituents of the culture medium and growth conditions improved the flocculating action and bioflocculant yield. *B. pumilus* JX860616 demonstrated a potential flocculating activity of 64% in the original pre-culture medium and conditions and with optimisisation could demonstrate 93.3%.

4.2.1.1 Inoculum size

The results of the effect of inoculum size of *B. pumilus* JX860616 on flocculating activity are illustrated in Table 4.2.1. The observations showed that the inoculum size of 2% (v/v) was the best as it revealed the highest flocculating activity of 76.8%. An increase or decrease in the inoculum size did result in a slight decrease in flocculating activities. The inoculum size of 2% (v/v) was then used in all the experiments.

4.2.1.2 Carbon and nitrogen sources

B. pumilus JX860616 utilized various carbon and nitrogen sources, 20 g/l and 1.2 g/l, respectively, when the optimum inoculum size of 2% v/v was used and resulted in bioflocculant production. Glucose, lactose and sucrose were the most preferred carbon sources, showing insignificantly different flocculating activities (Table 4.2.1). Lactose gave the highest flocculating activity of 84.1±4.35%. Lactose was used as the carbon source in all

experiments. Maltose and molasses were poor carbon sources and gave 47.8±0.20% and 53.7±4.38% of flocculating activities, respectively.

The effect of different nitrogen sources was assessed in the presence of lactose and the inoculum size of 2% v/v (Table 4.2.1). Ammonium sulphate was the best nitrogen source, with an outstanding flocculating activity of 90.1±7.89%. Ammonium sulphate was used as a nitrogen source in all experiments. All other nitrogen sources used, (with the exception of casein, which showed the lowest flocculating activity (59.1±2.06%) showed the potential of being good nitrogen sources for bioflocculant production by *B. pumilus* JX860616, giving flocculating activities above 70% (Table 4.2.1).

Table 4.2.1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Inoculum size (% v/v)	FA (%)±SD	Carbon source	FA (%)±SD	Nitrogen source	FA (%)±SD	Cations	FA (%)±SD
1	56.5±5.84[a]	Molasses	53.7±4.38[a,b]	Yeast extract	74.4±3.40[a,b]	Control	58.2±9.47[a]
2	76.8±4.03[b]	Sucrose	84.0±1.71[d]	Casein	59.1±2.06[a]	Na^+	60.6±0.32[a]
3	70.6±0.76[b]	Glucose	83.7±3.12[d]	Peptone	73.3±7.72[a,b]	Li^+	67.1±2.52[a]
4	70.6±6.41[b]	Starch	68.5±5.38[c]	$(NH_4)_2SO_4$	90.1±7.89[b]	K^+	60.8±0.15[a]
5	55.9±6.16[a]	Lactose	84.1±4.35[d]	Urea	71.4±1.19[a,c]	Mn^{2+}	69.9±4.43[a,b]
		Fructose	61.8±0.21[b,c]			Ba^{2+}	79.6±3.47[b]
		Maltose	47.8±0.20[a]			Ca^{2+}	79.2±2.54[b]
						Fe^{3+}	58.2±2.62[a]

FA denotes flocculating activity while SD denotes standard deviation.

4.2.1.3 Effect of cations on flocculation activity of crude bioflocculant

Table 4.2.1 also shows the effect of different metal cations on flocculating activity. When the inoculum size of 2% v/v, lactose and ammonium sulphate were utilized for bioflocculant production, Ba^{2+} stimulated flocculating activity. It resulted in the highest flocculating activity of 79.6±3.47%. Ba^{2+} was utilized as the cation in all experiments done on crude bioflocculant. The monovalent (K^+, Na^+ and Li^+) cations did also enhance flocculating activity, giving flocculating activities above 60%. However, the trivalent metal cation (Fe^{3+}) demonstrated the lowest flocculating activity of 58.2±2.62%.

4.2.1.4 Effect of shaking speed

Figure 4.2.1 illustrates the effect of mixing speed on the bioflocculant production. There was no improvement in flocculating activity with the increase in speed from 55 to 220 rpm. Thus, 55 rpm was the optimum mixing speed for bioflocculant production.

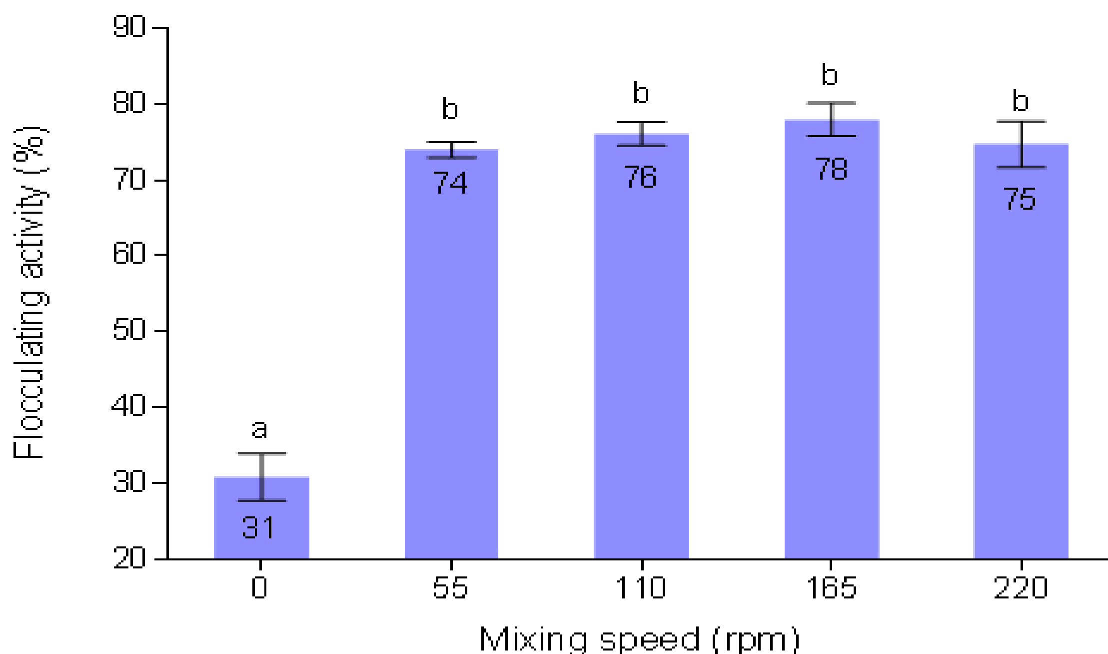


Figure 4.2.1: Effect of shaking speed on flocculating activity

4.2.1.5 Effect of temperature on flocculation activity

Temperature is an environmental factor that greatly effects bioflocculant production. There was no effect observed with an increase in temperature from 20 to 50°C (Figure 4.2.2**)**. Twenty degrees Celcius gave 76% of flocculating activity and was used as the optimum temperature in all experiments.

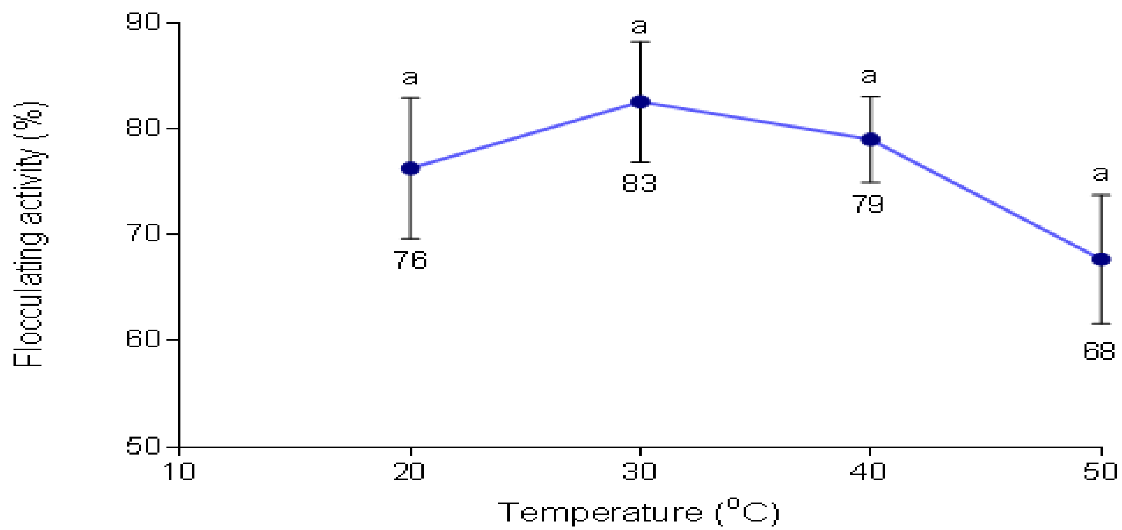


Figure 4.2.2: Effect of temperature on flocculating activity

4.2.1.6 Effect of initial pH on flocculating activity

The effect of the initial pH of the growth medium on flocculating activity was assessed and the result presented in Figure 4.2.3. The flocculating activity was highest (83%) when the initial pH was 7. The observations also showed that *B. pumilus* JX860616 has a potential to survive and produce bioflocculant in a wide range of pH (pH 6-9).

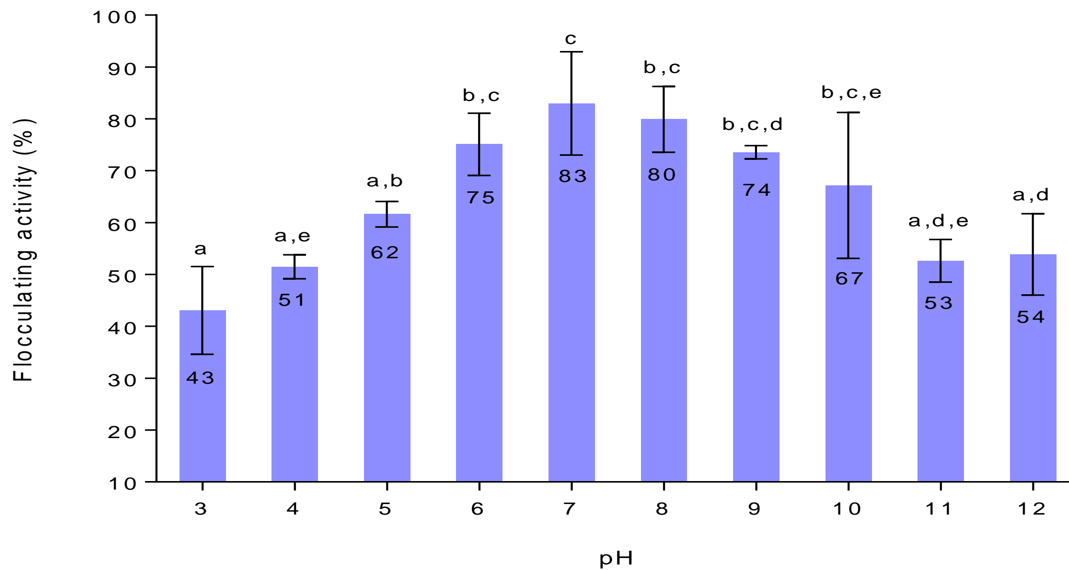


Figure 4.2.3: Effect of initial pH on bioflocculant production

4.2.1.7 Time course

The effect of time course on flocculating activity (FA), on bacterium growth and on pH is shown in Figure 4.2.4. The flocculating activity increased relatively to the *B. pumilus* JX860616 growth until 60 hours, when the maximum flocculation activity (93.3%) was obtained. After 60 hours, the bacterium got into the stationary phase and the flocculation activity and the optical density (OD_{550} nm) (which could present bacterial growth) decreased slightly. On the other hand, the pH of the medium decreased uniformly from the initial pH of 7 to a final pH of 4.7.

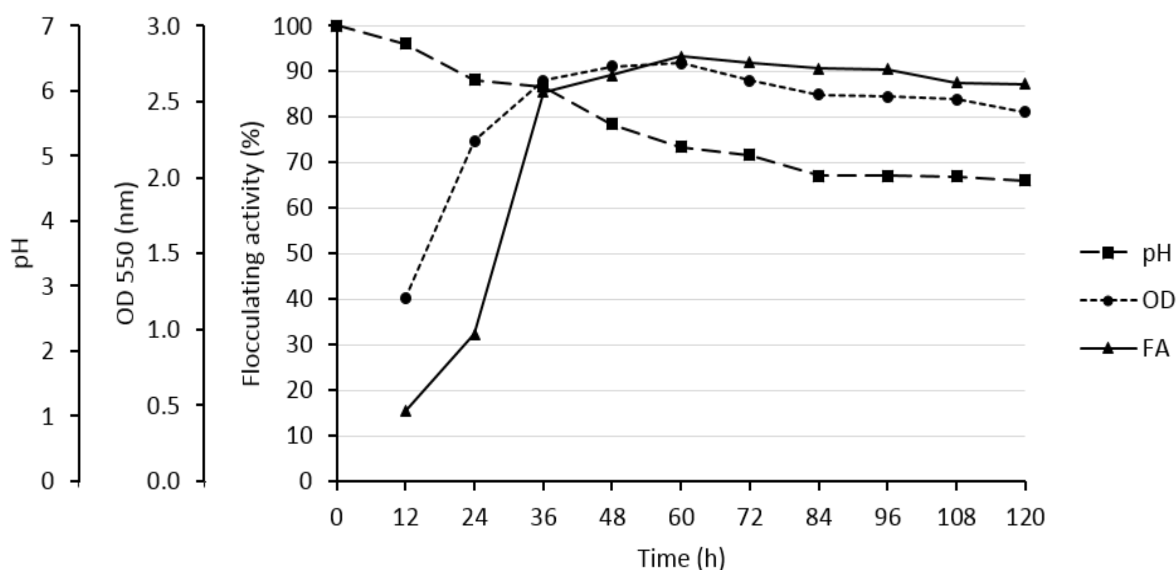


Figure 4.2.4: Effect of time on FA, initial pH and optimum density (OD)

4.2.2 Bioflocculant yield and solubility

One thousand millilitres of fermented broth culture yielded 2.4 g of the purified bioflocculant. The purified bioflocculant was named TMT. The bioflocculant was insoluble in all solvents (acetone, methanol, butanol, ethanol, hexane, benzene, chloroform, ethyl-acetate and dichloromethane), with the exception of water in which it dissolved.

4.2.3 Effect of dosage size and cations on flocculation activity

Table 4.2.2 illustrates the effect of different concentrations of the purified bioflocculant (TMT) on flocculating activity. TMT had the highest flocculation activity (82.77%) at a concentration of 0.6 mg/ml. However, 0.2 mg/ml was the most preferred dosage size since it showed insignificantly different flocculating activity (81.73±1.88) with 82.77% flocculating activity by 0.6 mg/ml.

The effect of various metal cations on the purified TMT was determined. All monovalent cations (with the exception of Li^{+}) and divalent cations significantly enhanced flocculation activities, giving flocculation activities above 80% (Table 4.2.2). K^{+} was the most preferred metal cation with the highest flocculation activity of 96.07±0.35%, while Li^{+} showed the lowest flocculating activity of 51.57±1.72%. Ba^{2+} was used in all experiments done on the purified bioflocculant.

Table 4.2.2: Effect of different bioflocculant concentrations and cations on the flocculating activity of the purified bioflocculant

Dosage (ml/mg)	FA(%) ± SD	Cations	FA(%) ± SD
0.2	81.73±1.88[a,b]	Control	66.87±2.34[a]
0.4	82.0±0.74[a,b]	Li^{+}	51.57±1.72[b]
0.6	82.77±1.0[a]	Na^{+}	95.47±0.42[c]
0.8	78.90±0.53[b]	K^{+}	96.07±0.35[c]
1	79.20±1.92[a,b]	Mn^{2+}	83.27±1.19[d]
		Ba^{2+}	95.23±1.54[c]
		Fe^{3+}	80.63±1.39[d]

FA denotes flocculating activity while SD denotes standard deviation.

4.2.4 Physico-chemical composition of the bioflocculant

4.2.4.1 Surface structure of the bioflocculant

Figure 4.2.5 shows the SEM surface images of the purified bioflocculant, flocculated kaolin particles and kaolin particles. The bioflocculant had a crystal-like structure (Figure 4.2.5a). The clump like structure was observed in the flocculated kaolin particles (Figure 4.2.5b) and kaolin particles revealed a fine and smooth structure (Figure 4.2.5c).

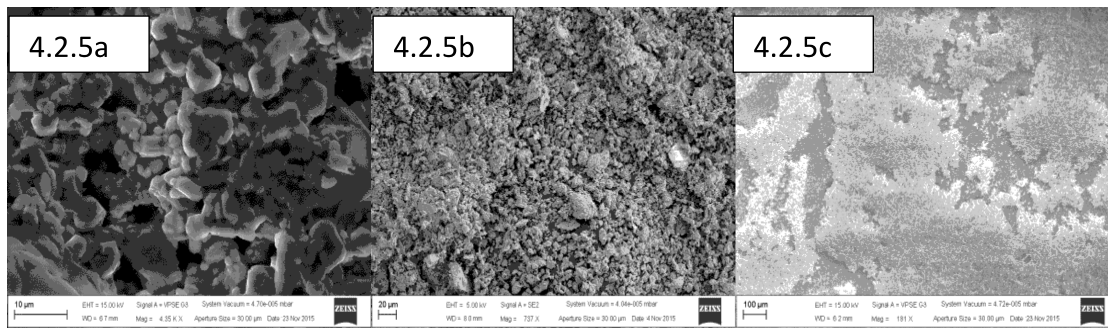


Figure 4.2.5: SEM surface images of the bioflocculant (4.2.5a) flocculated kaolin particles (4.2.5b) and kaolin particles (4.2.5c)

4.2.4.2 Electric charge of the bioflocculant

The surface charge of TMT was determined. The zeta potential of TMT was -9.26±3.00 mV. This implied that the bioflocculant is an anionic biomolecule.

4.2.4.3 Qualitative analyses of proteins and nucleic acids

TMT was Ninhydrin positive. The ultra violet (UV) spectrum of TMT revealed a sharp peak at 290 nm (Figure 4.2.6), which is indicative of protein content. There was no absorptive peak at 260 nm. This implied that the bioflocculant had no nucleic acid content.

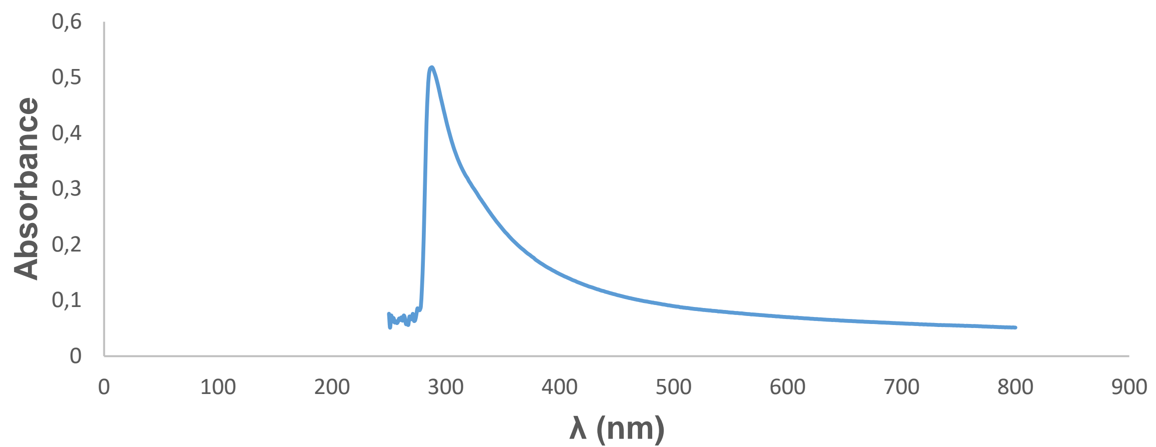


Figure 4.2.6: UV spectrum of TMT

4.2.4.4 Quantitative chemical analysis

The quantitative chemical analysis of the bioflocculant TMT was carried out and the main chemical constituents revealed were polysaccharide (93.1%) and trace protein content (6.0%).

4.2.4.5 Elemental and IR analysis

The elemental analysis of the bioflocculant revealed its elemental composition in mass proportion (% w/w): C (18.7), N (0.3), O (62.1), Na (0.3), Mg (3.0), P (8.1), S (0.6), Cl (0.3), and Ca (6.6) (Figure 4.2.7).

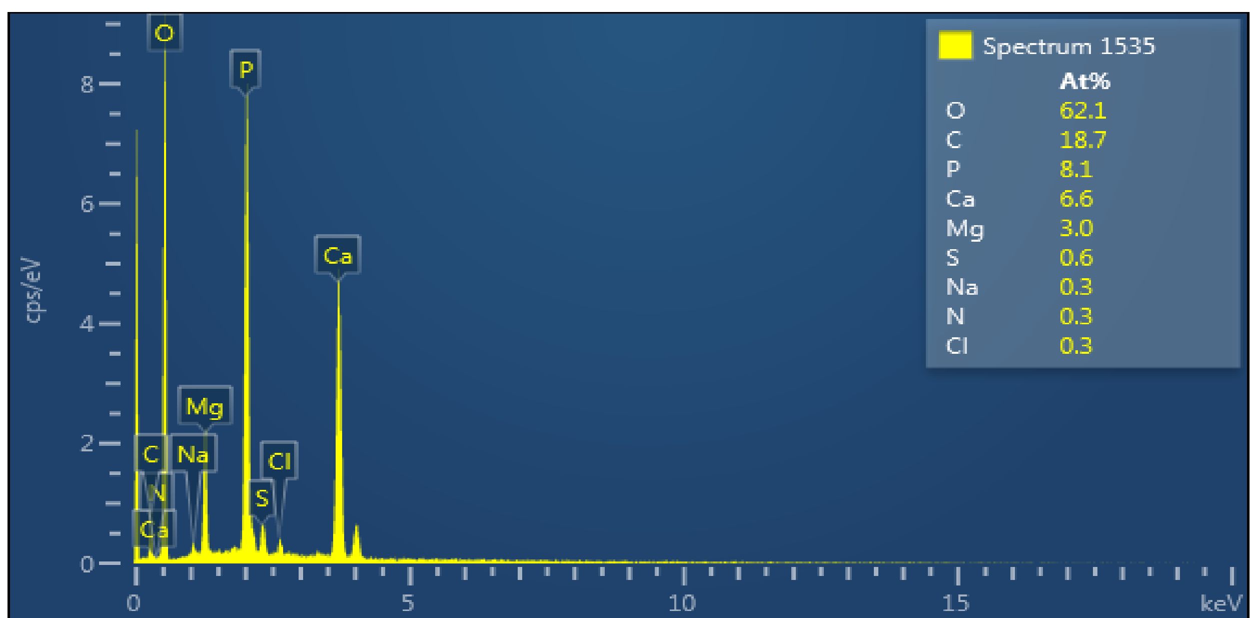


Figure 4.2.7: Elemental analysis of TMT

4.2.4.6 Functional groups of the bioflocculant

The functional groups of TMT were determined and the results illustrated in Figure 4.2.8. The IR spectrophotometric analysis of TMT showed the occurrence of hydroxyl, vinyl, amino and amide groups.

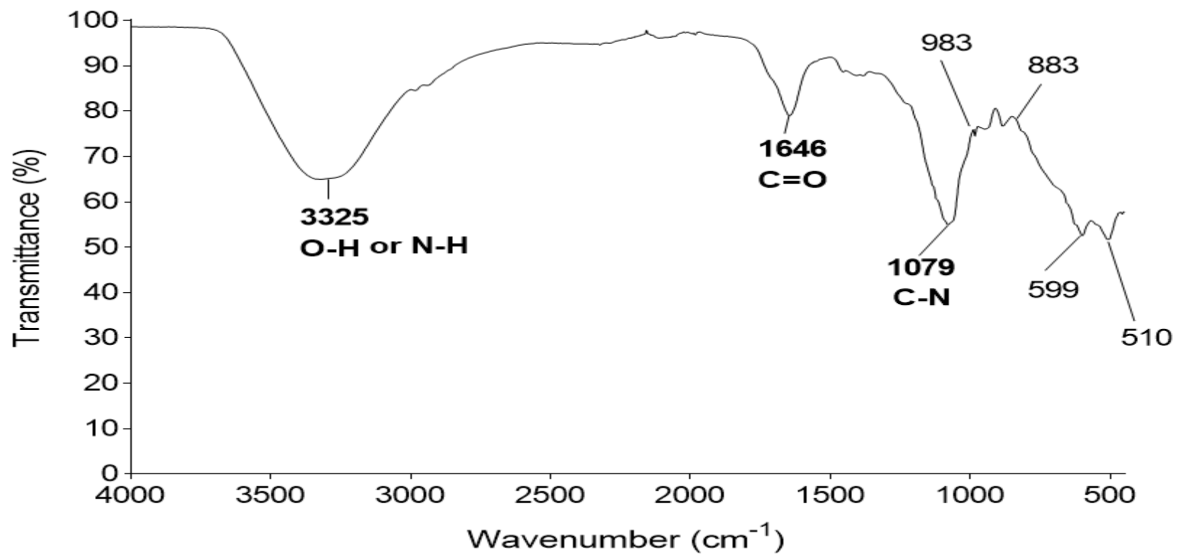


Figure 4.2.8: IR spectrum of TMT

4.2.5 Thermal stability of bioflocculant TMT

Figure 4.2.9 displays the effect of temperature on flocculating activity of the purified bioflocculant TMT. TMT was heat stable showing flocculating activity of 79% at 100°C after an hour of heat exposure to different temperatures.

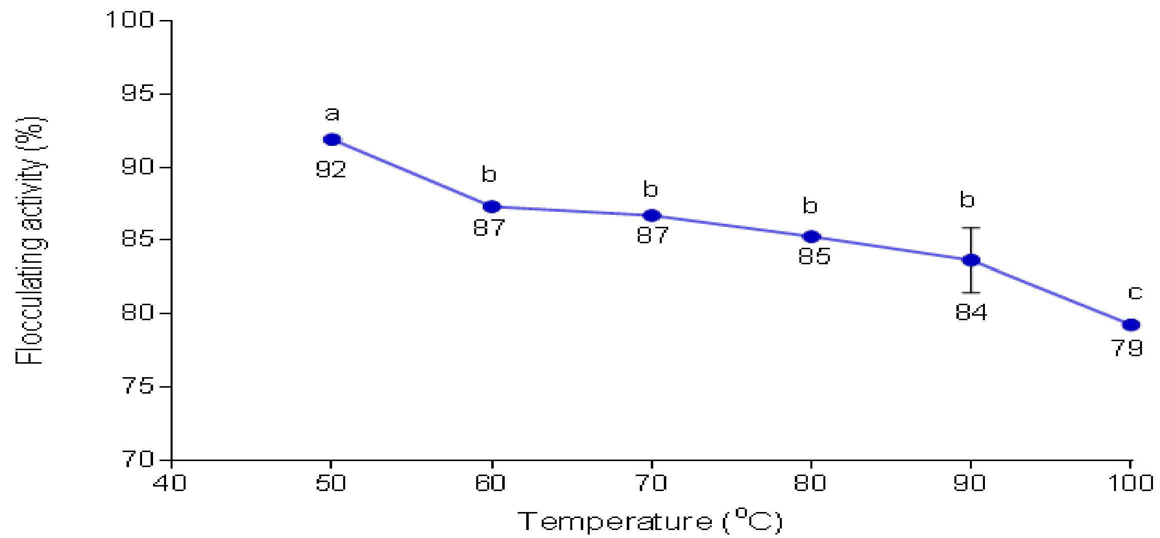


Figure 4.2.9: Thermal stability of TMT

4.2.6 Pyrolysis property of TMT

The pyrolysis property of TMT was also studied and the results are illustrated in Figure 4.2.10. The degradation temperature (*T*d) of 100°C was observed and it validated the thermal stability of TMT.

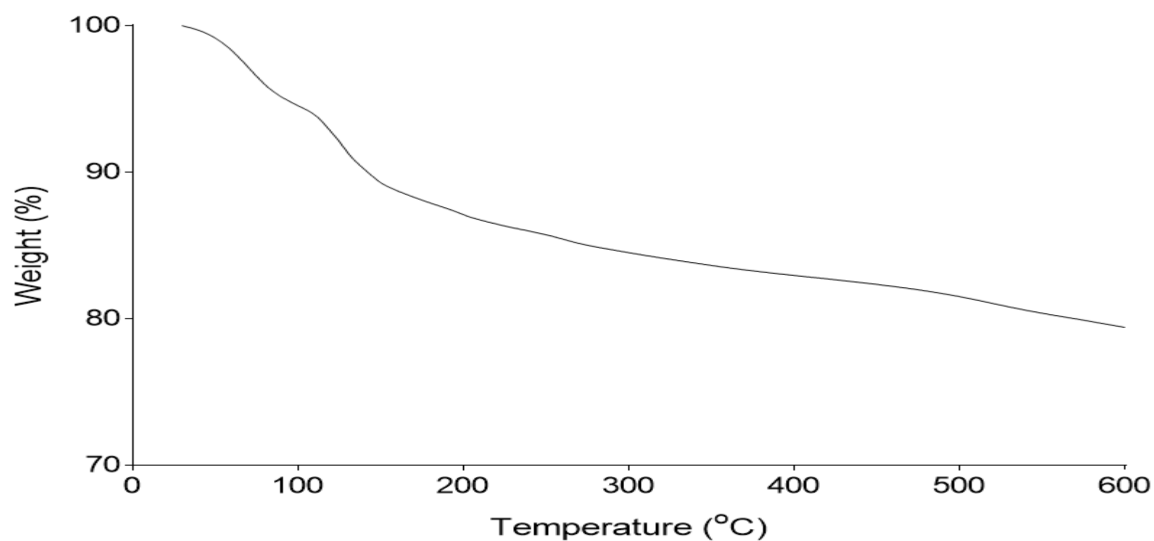


Figure 4.2.10: Pyrolysis property of TMT

4.2.7 pH stability of TMT

The effect of pH on flocculating activity of the purified bioflocculant TMT was assessed and the results are presented in Figure 4.2.11. TMT was more stable in a wide range of pH (pH 3.0-10), giving flocculation activities above 70%. TMT effectively flocculated kaolin particles and gave the maximum flocculation activity (81%) at pH 6.0.

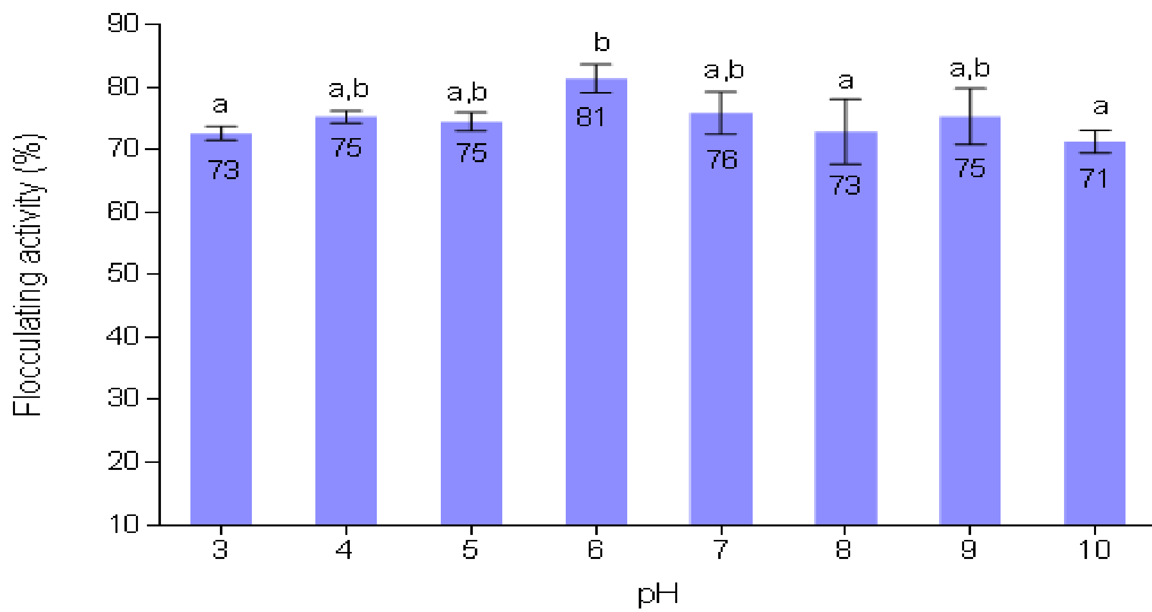


Figure 4.2.11: pH stability of bioflocculant TMT

4.2.8 Saline stability of TMT

Figure 4.2.12 represents the results obtained during the determination of the effect on Na^+ concentration on the flocculating activity of bioflocculant TMT. The flocculating activity of the bioflocculant decreased proportionally with the increase in Na^+ concentration. However, TMT did maintain high flocculation activity (72%) even at high salinity (35 g/l).

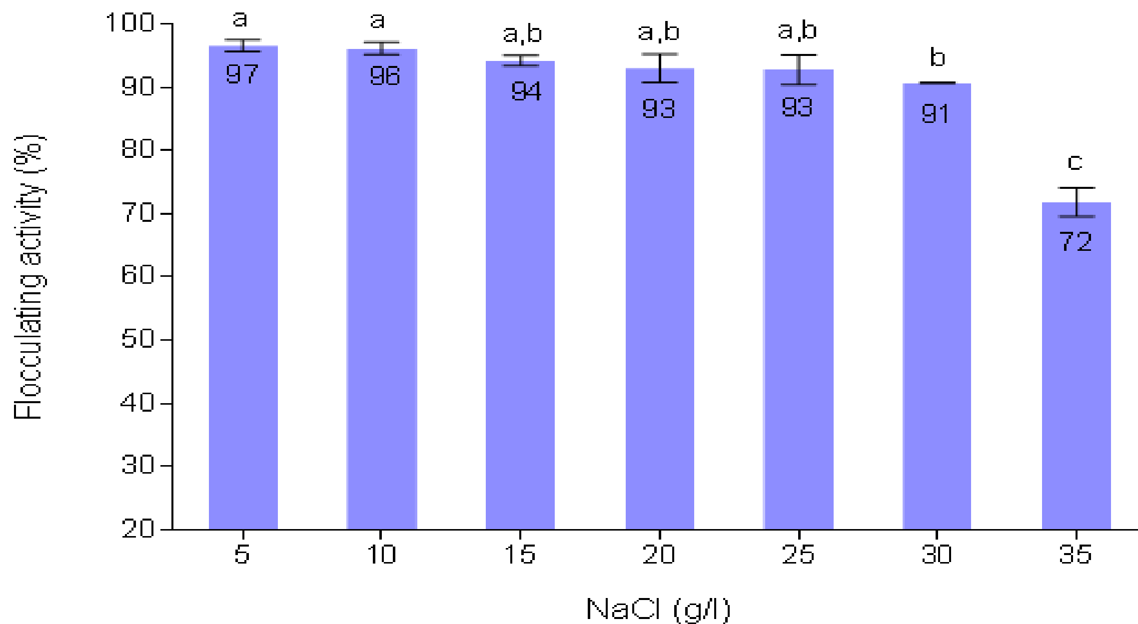


Figure 4.2.12: Effect of salinity on flocculating activity of TMT

Percentage flocculating activities with different letters (a, b and c) are significantly ($p<0.05$) different.

4.2.9 *In-vitro* cytotoxicity of TMT on CaCO2 and HEK293 cells

Figure 4.2.13 and 4.2.14 illustrate the cytotoxicity of the bioflocculant on CaCO2 and HEK293 cell lines, respectively. The bioflocculant showed no toxic effects on both cell lines. The cell lines exhibited 100% viability after treatement.

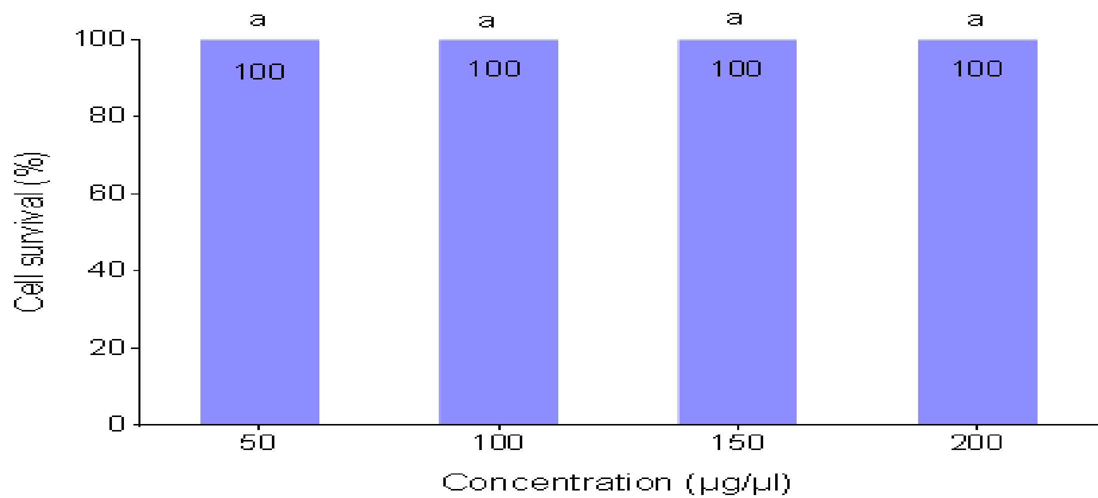


Figure 4.2.13: *In-vitro* cytotoxicity of TMT on CaCO2 cells

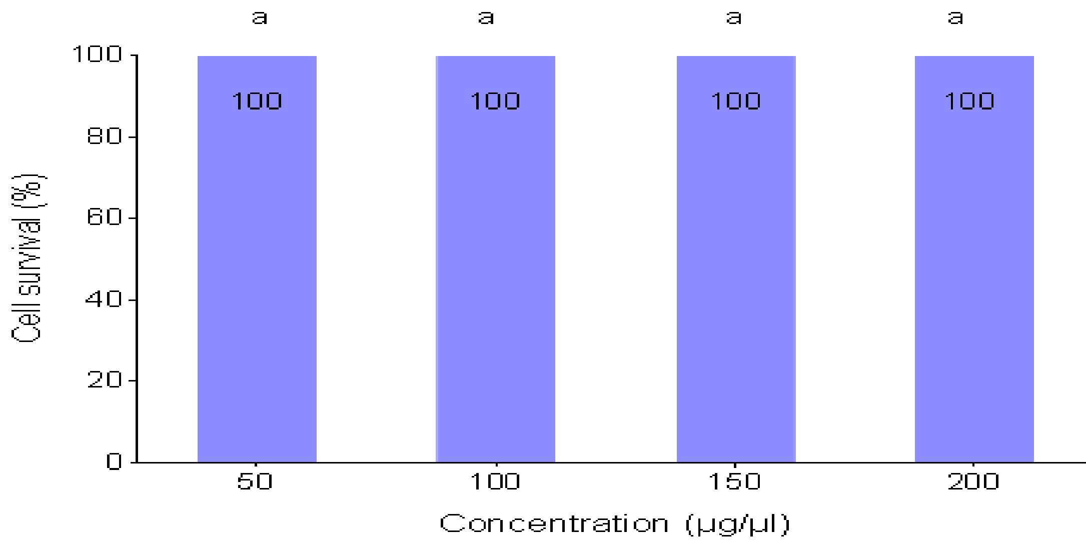


Figure 4.2.14: *In-vitro* cytotoxicity of TMT on HEK293 cells

4.2.10 Proposed flocculation mechanism of TMT

Table 4.2.3 presents zeta potential of the bioflocculant, kaolin suspension, kaolin plus $BaCl_2$ and kaolin suspension flocculated with TMT in the presence of $BaCl_2$. The electrical charges of the bioflocculant and kaolin particles were both negative; -9.26±3.00 and -6.59±3.00, respectively. Addition of Ba^{2+} to kaolin suspension plus bioflocculant TMT resulted in an increase in zeta-potential (-3.51±1.31 mV). Thus, it is suggested that Ba^{2+} mediated a bridging mechanism during flocculation process.

Table 4.2.3: Zeta potential of samples

Samples	Zeta potential (mV)
Bioflocculant	-9.26±3.00
Kaolin particles	-6.59±3.00
Kaolin particles with Ba^{2+}	-7.01±0.99
Kaolin particles flocculated with TMT and in the presence of Ba^{2+}	-3.51±1.31

Values represent mean ± deviation of replicate readings.

4.2.11 Removal efficiency of bioflocculant TMT

Table 4.2.4 and 4.2.5 display the removal efficiencies of TMT on BOD, COD, N, P, S and Ca, respectively of water from the Nhlabane estuary. The bioflocculant had comparatively the same removal efficiencies with alum and ferric chloride on BOD and COD. It also showed good removal efficiencies on N and S. However, TMT had poor removal efficiency on P than the conventional flocculants used.

Table 4.2.4: Removal efficiency of TMT on BOD, COD and N

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD	COD	N
TMT	123.2±0.0	154±0.0	0.93±0.0	0±0.0	0±0.0	0.07±0.0	100[a]	100[a]	92[a]
Alum	123.2±0.0	154±0.0	0.93±0.0	0±0.0	0±0.0	0.09±0.0	100[a]	100[a]	84[a]
$FeCl_3$	123.2±0.0	154±0.0	0.93±0.0	0±0.0	0±0.0	0.32±0.0	100[a]	100[a]	66[b]

Values represent mean standard deviation of triplicate readings.

Table 4.2.5: Removal efficiency of TMT on P and S

Type of flocculants	Water quality beforetreatment		Water quality after treatment		Removal efficiency (%)	
	P (mg/l)	S (mg/l)	P (mg/l)	S (mg/l)	P	S
TMT	2.83±0.0	0.3±0.1	1.5±0.2	0.06±0	48[a]	80[a]
Alum	2.83±0.0	0.3±0.1	0.81±0	0.15±0	71[b]	51[b]
$FeCl_3$	2.83±0.0	0.3±0.1	1.1±0.1	0±0.0	62[c]	100[a]

Values represent mean ± standard deviation of triplicate readings.

4.3 Results for *B. subtilis* CSM5

The results obtained during resuscitation, optimisation of medium composition and culture conditions of *Bacillus subtilis* CSM5 are shown. The observations on the characteristics and removal effeciencies of the bioflocculant on wastewater are also presented.

4.3.1 Optimisation of medium composition and culture conditions

Optimization of the cultivation process improves the production of bioproducts. Thus, the varying of different constituents of the culture medium and growth conditions were done to improve flocculating action and bioflocculant yield. *B. subtilis* CSM5 did demonstrate potential flocculating activity of 62% in the original pre-culture medium and conditions.

4.3.1.1 Inoculum size

Inoculum size is an important parameter in bioflocculant production. Table 4.3.1 illustrates the effect of the inoculum size of *B. subtilis* CSM5 on the flocculating activity. The most preferred inoculum size was 1% (0.5 ml v/v) with a flocculating activity of 75.3±5.5%. The increase or decrease in inoculum size led to a slight decrease in the flocculating activities.

4.3.1.2 Effect of carbon sources on flocculating activity

Carbon and nitrogen sources have been reported to have an important impact on the production of the bioflocculant. Table 4.3.1 shows the flocculating activity (in a Kaolin suspension) after 72 hours of cultivation of *B. subtilis* CSM5, with various carbon sources (glucose, fructose, lactose, starch and glycerol) with glucose at a concentration of 20 g/l. It was evident that fructose, maltose, lactose and sucrose were suitable for bioflocculant production, with flocculating activity exceeding 85%. Fructose yielded the highest flocculating activity (96.1±0.57%), while molasses gave the least flocculating activity (60.4±6.68%).

Table 4.3.1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Values represent mean ± standard deviation of replicate readings of the flocculating activities.

Inoculum size (%)	FA(%) ± SD	Carbon source	FA(%) ± SD	Nitrogen source	FA(%) ± SD	Cations	FA(%) ± SD
1	75.3±5.5[a]	Molasses	60.4±6.68[d]	Casein	31.7±14.7[b]	Na^+	69.5±3.79[a]
2	66.2±0.91[a]	Glucose	73.8±10.28[c,d]	Yeast extract	48.4±2.22[b]	K^+	76.3±4.23[a]
3	69.4±6.98[a]	Starch	77.1±7.25[b,c]	$(NH_4)_2SO_4$	77.0±9.92[a]	Li^+	76.5±5.84[a]
4	73.9±4.0[a]	Sucrose	86.3±2.34[a,b,c]	Peptone	85.3±3.54[a]	Ca^{2+}	74.2±8.16[a]
5	66.3±10.52[a]	Lactose	89.3±5.47[a,b,c]	Urea	91.1±5.22[a]	Ba^{2+}	79.8±4.20[a]
-	-	Maltose	92.4±1.96[a,b]	-	-	Mn^{2+}	81.8±8.35[a]
-	-	Fructose	96.1±0.57[a]	-	-	Fe^{3+}	42.8±12.56[b]
						Control (Without cation)	47.0±3.2[b]

FA denotes flocculating activity while SD denotes standard deviation.

4.3.1.3 Effect of nitrogen sources on flocculating activity

Nitrogen sources are secondary energy sources for microorganisms and play a vital role in microbial growth and bioflocculant production. Table 4.3.1 displays the effect of different nitrogen sources on flocculating activity. *B. subtilis* CSM5 grew well in urea, peptone and ammonium sulphate ($(NH_4)_2SO_4$) (1.2 g/l) giving a flocculating activity above 75%. Urea was the most preferred nitrogen source with a flocculating activity of 91.1±5.22% while casein was poorly utilized and gave a flocculating activity of 31.7±14.7%.

4.3.1.4 Effect of cations on flocculating activity of crude bioflocculant

The effect of cations (3 ml (1%)) on flocculating activity is shown in Table 4.3.1. The most effective cation was Mn^{2+} with a flocculating activity of 85% and the cation with the least flocculating activity of 48% was Fe^{3+}.

4.3.1.5 Effect of shaking speed on flocculating activity

Figure 4.3.1 shows the effect of shaking speed on the flocculating activity. There was asignificant increase in flocculating activity from 0 to 110 rpm. The shaking speed of 110 rpm was preferredand yielded the flocculating activity of 70%. This speed was used in all experiments during bioflocculant production.

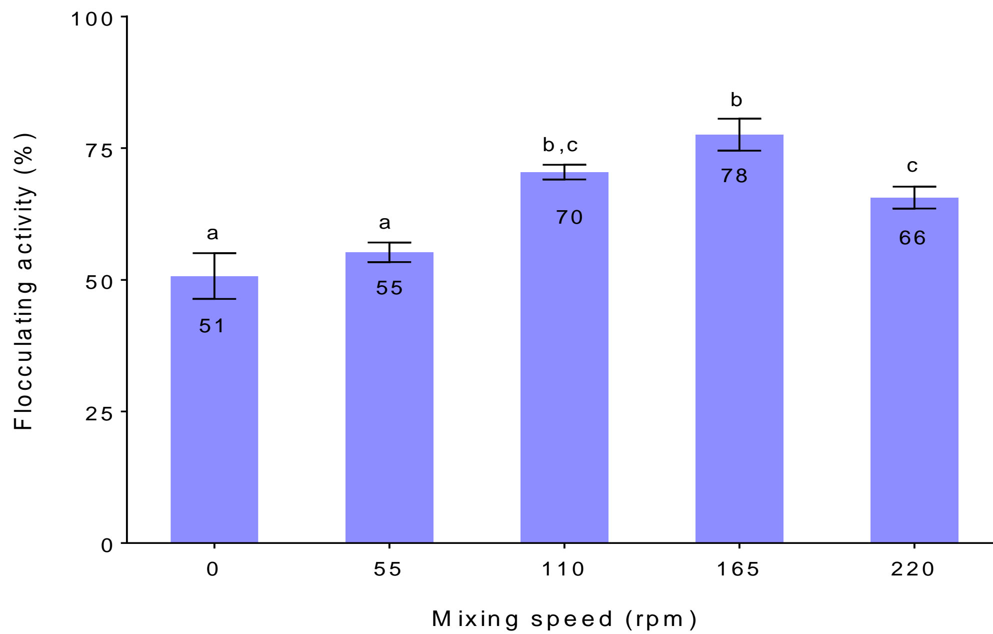


Figure 4.3.1: Effect of shaking speed on flocculating activity

4.3.1.6 Effect of temperature on flocculating activity

Figure 4.3.2 displays the effect of cultivating temperature on flocculating activity. The best flocculating activity occurred at 30°C with a flocculating activity of 83%.Thirty degrees Celcius was therefore used as the optimum temperature for bioflocculant production. Fifty degrees Celsius gave the lowest flocculating activity (41%).

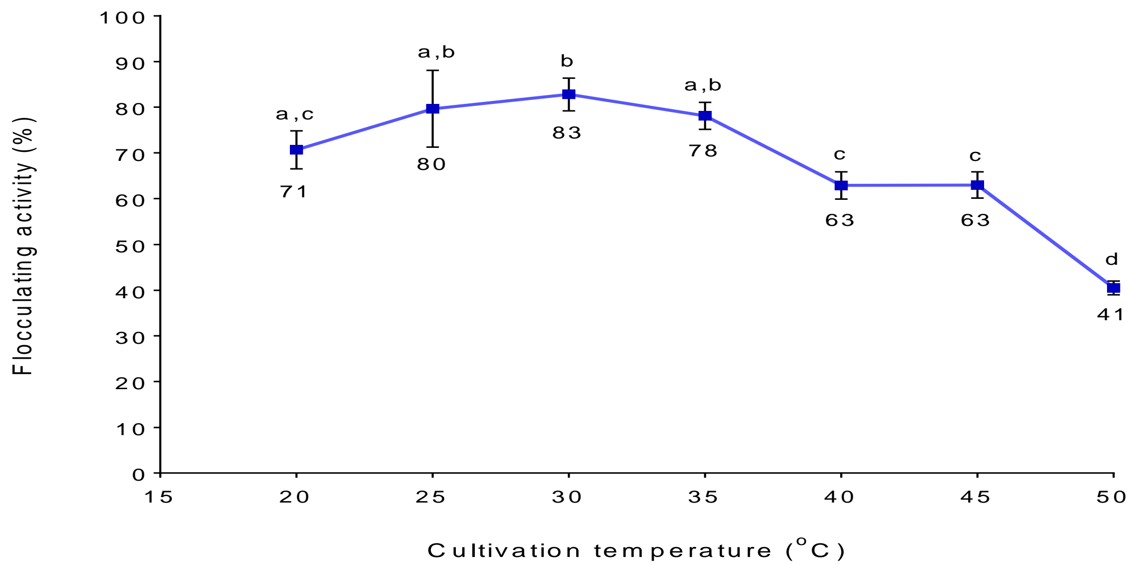


Figure 4.3.2: Effect of temperature on flocculating activity

4.3.1.7 Effect of initial pH on flocculating activity

The effect of the initial pH of the culture medium on the flocculating activity was determined and the results are presented in Figure 4.3.3. The optimal pH for the bioflocculant production was in the range of 7–12, with the highest flocculating activity obtained at pH 10. Poor flocculating activity (57%) was obtained at a pH of 3.

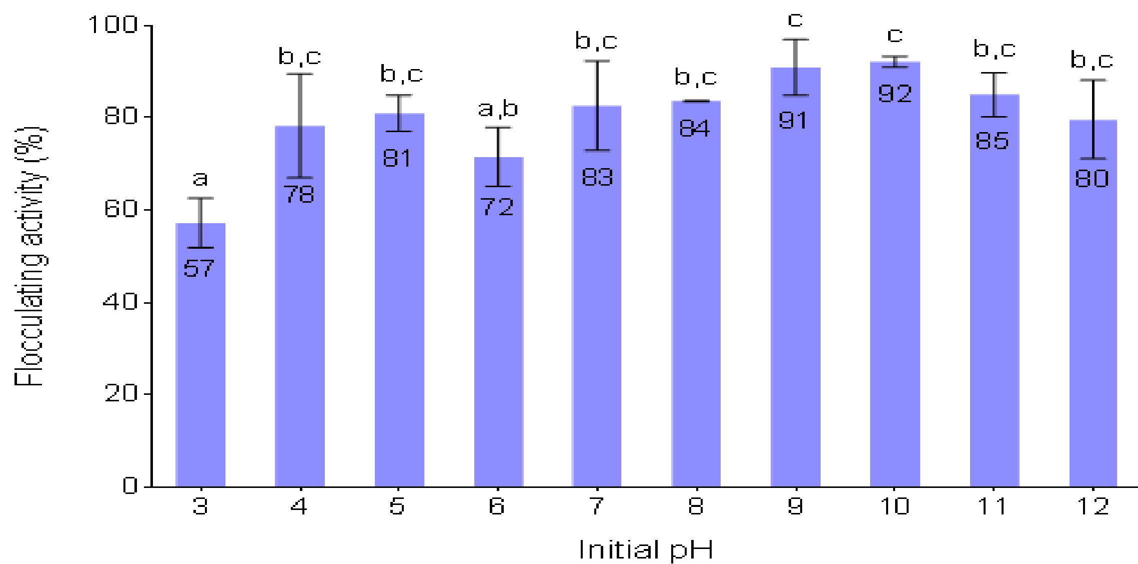


Figure 4.3.3: Effect of initial pH on flocculating activity

4.3.1.8 Time course assay

The relationship between flocculating activity (FA), cell growth (determined at a OD_{550} nm) (OD) and pH over time (108 hours) was assessed and the results illustrated in Figure 4.3.4. The flocculating activity increased with an increase in cell growth. The flocculating activity reached its peak after 72 hours, and slightly decreased thereafter. The pH of the growth broth fluctuated between the linitial pH 10 and the final pH of 8 after 108 hours.

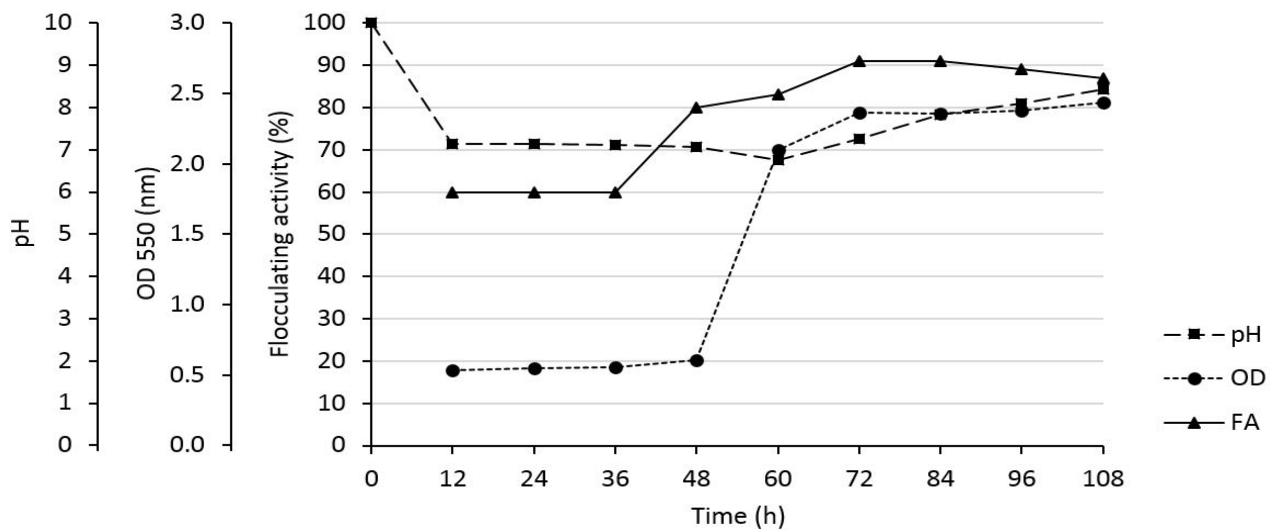


Figure 4.3.4: Effect of time on FA, OD and initial pH of the culture medium

4.3.2 Bioflocculant yield and solubility assay

The extracted and purified bioflocculant was 2.4 g/l. It was named TTT. The bioflocculant demonstrated solubility only in water.

4.3.3 Effect of dosage size on flocculating activity

The effect of the dosage size of the bioflocculant is displayed in Table 4.3.2. The highest and lowest flocculating activities were obtained at a concentration of 0.6 and 0.4 mg/ml, respectively. However, 0.2 mg/ml was used in all other experiments since it is low and gave insignificantly different flocculating activity to 0.6 mg/ml.

Table 4.3.2: Effect of dosage size and cations on flocculating activity

Dosage (mg/ml)	FA(%)±SD	Cations	FA(%)±SD
0.2	83.1±0.51[a,b]	Na^+	67.8±2.15[a]
0.4	82.4±0.96[a]	K^+	70.2±0.35[a]
0.6	85.8±1.0[b]	Li^+	71.6±1.51[a]
0.8	84.5±1.39[a,b]	Ca^{2+}	77.4±0.57[d]
1	82.6±1.51[a]	Mn^{2+}	83.7±1.37[c]
-	-	Ba^{2+}	85.8±1[c]
-	-	Fe^{3+}	44.8±2.06[b]
		Control (Without cation)	51.3±2.14[b]

FA denotes flocculating activity while SD denotes standard deviation.

4.3.4 Effect of cations on flocculating activity of the purified bioflocculant

The synergistic effect of metal cations on the flocculating activity of the purified bioflocculant is depicted in Table 4.3.2. All the cations evaluated, improved the flocculating activity of the bioflocculant and gave activities above 70% except for Na^+ and Fe^{3+}, with flocculating activities of 67.8±2.15 and 44.8±2.06%, respectively. The highest flocculating activity (85.8±1%) was obtained with Ba^{2+}.

4.3.5 Physico-chemical composition of the bioflocculant TTT

4.3.5.1 Surface structures

The surface structures of the bioflocculant, flocs of kaolin pacticles flocculated by the bioflocculant in the presence of $BaCl_2$ and kaolin clay particles were determined by SEM. The results are illustrated in Figure 4.3.5. Figure 4.3.5a displays the SEM image of the bioflocculant, Figure 4.3.5b shows the flocs and Figure 4.3.5c demonstrates the kaolin clay particles before flocculation. The bioflocculant had an amorphous and porous structure, while the flocs appeared clustered together. The kaolin particles were evenly scattered and had fine structures.

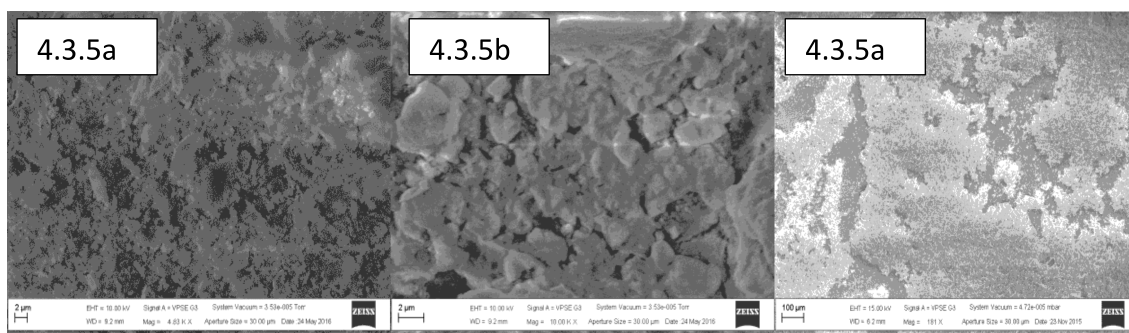


Figure 4.3.5: SEM analysis: SEM surface images of the bioflocculant (4.3.5a), flocculated kaolin particles (4.3.5b) and kaolin particles (4.3.5c)

4.3.5.2 Electric charge and qualitative analyses of components of TTT

The surface charge of the bioflocculant was determined. The zeta potential of the bioflocculant had a negative charge of -16.5±1.07 mV. The bioflocculant was Ninhydrin positive.

The UV spectrum of the bioflocculant demonstrated the qualitative presence of protein, as shown by a sharp absorption peak at 287 nm (Figure 4.3.6). There was no absorptive peak at 260 nm, implying that the bioflocculant had no nucleic acids.

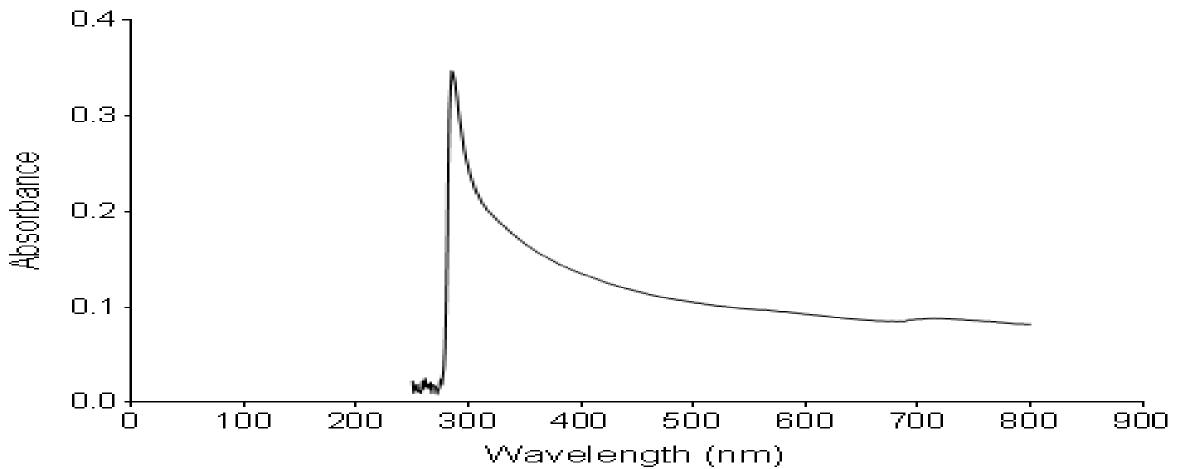


Figure 4.3.6: The UV spectrum of TTT

4.3.5.3 Quantitative analyses of total protein and carbohydrates

The quantitative biochemical analyses of the purified bioflocculant were done. The results reealed that TTT had a total carbohydrate content of 88% and a total protein content of 12%.

4.3.5.4 Elemental analyses of TTT

Figure 4.3.7 shows the results obtained during elemental analysis of TTT. The elemental analysis reveals the weight fractions of the elements; O, C, P, K, Mg, Ca, N, S, Cl, Na and Si were 46.4, 26.3, 5.2, 4.0,3.8, 3.4,3.3,2.7,2.5,2.2 and 0.4 (% w/w), respectively.

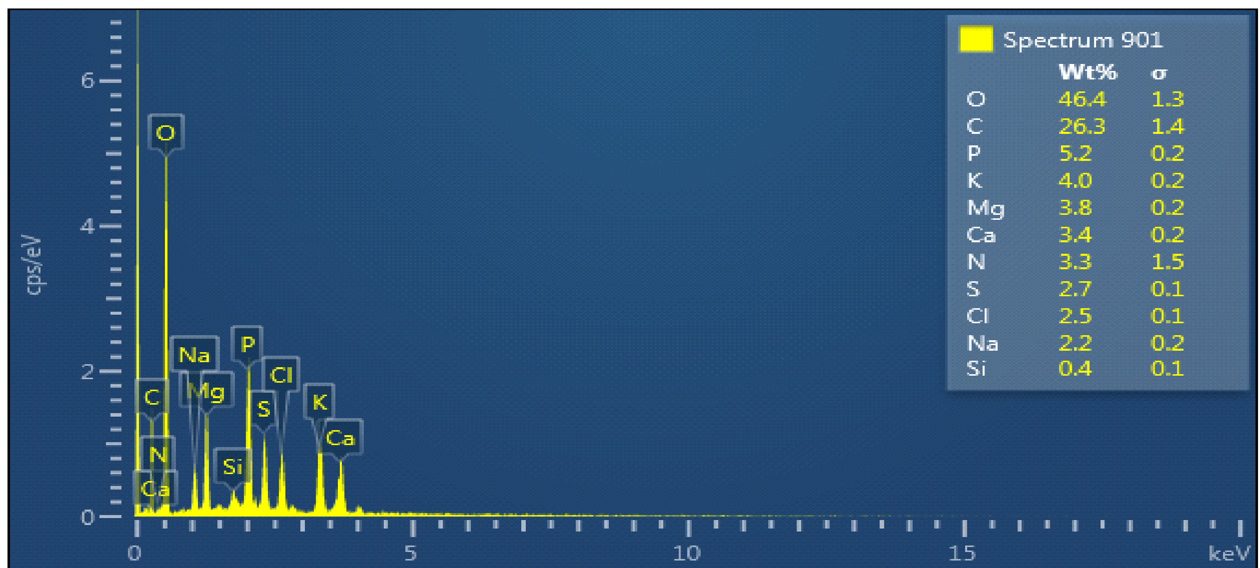


Figure 4.3.7: Elemental analyses of TTT

4.3.5.5 Functional groups of TTT

The flocculating activity is dependent on the characteristics of the bioflocculant, in particular, the functional groups of the bioflocculant. IR spectrum revealed the presence of OH (3372 cm^{-1}), NH_2 (3372 or 1643 cm^{-1}) and OCNH (1056 cm^{-1}) as the main functional groups of the bioflocculant (Figure 4.3.8).

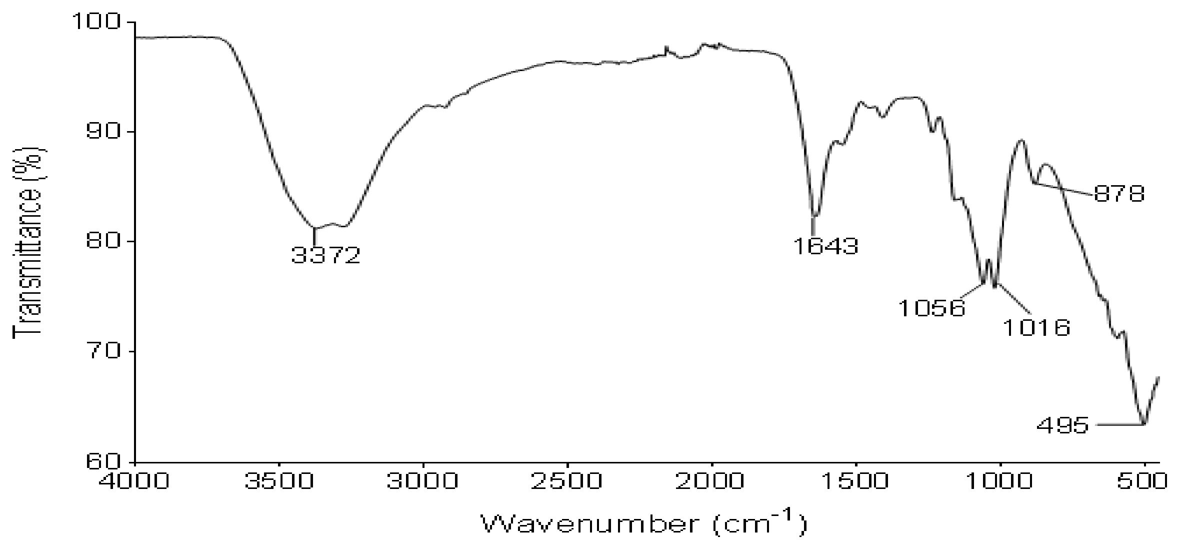


Figure 4.3.8: IR spectrum of TTT

4.3.6 Thermal stability of TTT

Figure 4.3.9 displays the results of the thermal stability of the purified bioflocculant. The decrease in flocculating activity was observed when temperature increased. The bioflocculant was thermostable up to 70°C, whereafter the flocculating activity significantly decreased.

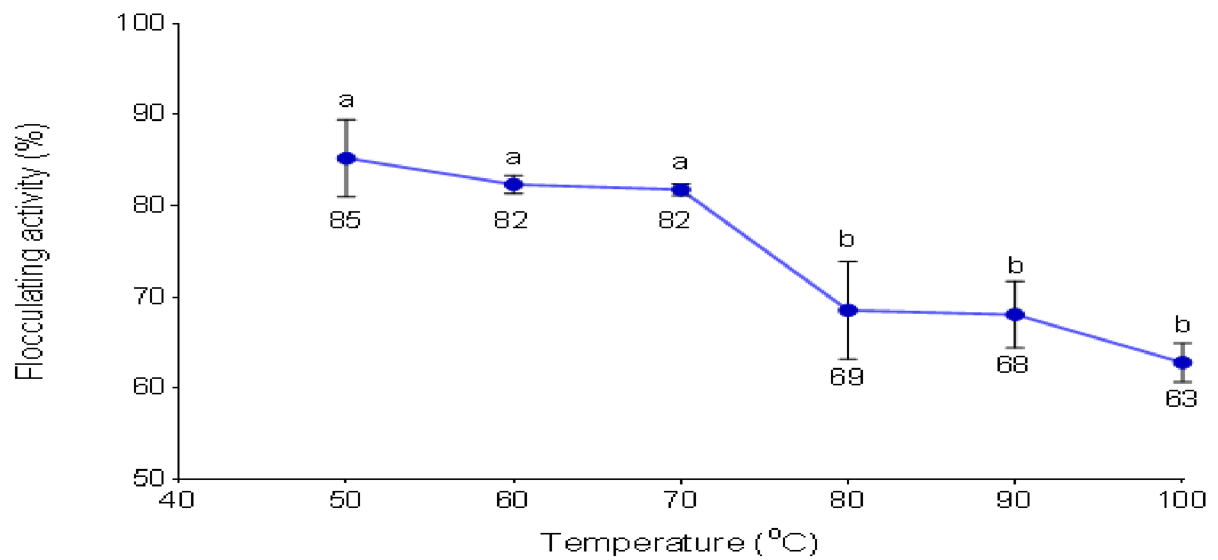


Figure 4.3.9: Thermal stability of TTT

4.3.7 Pyrolysis property of TTT

The pyrolysis property of the bioflocculant was assessed by thermo-gravimetric analysis (TGA) and the results shown in Figure 4.3.10. An initial weight loss of 3% of the bioflocculant was found between 30 and 130°C. A degradation temperature of 150°C was attained. An increase in temperature to 375 and 460 °C also resulted in further weight loss.

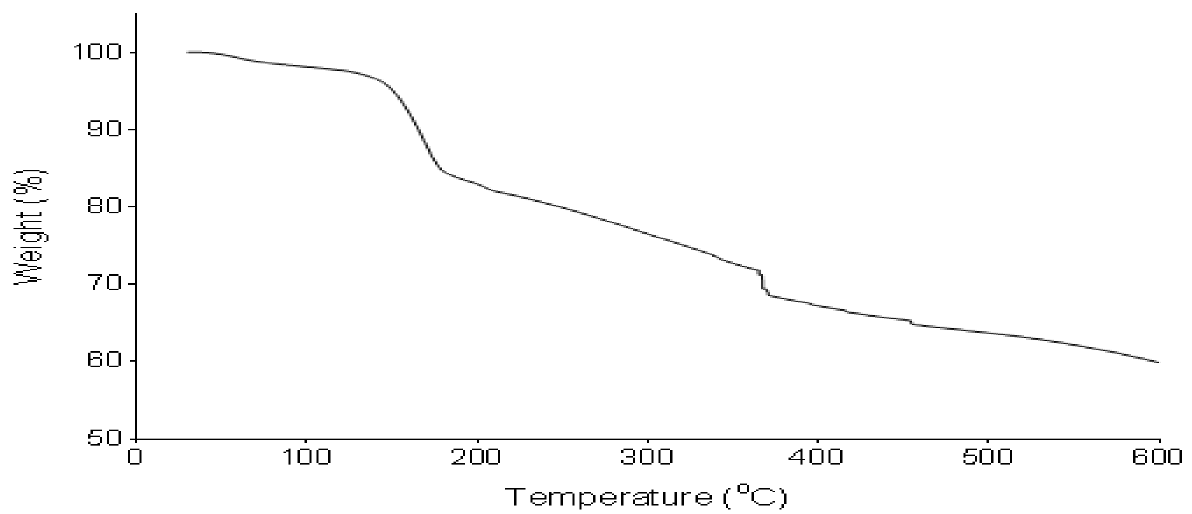


Figure 4.3.10: Pyrolysis property of TTT

4.3.8 pH stability of TTT

The evaluation of the effect of pH on the flocculating activity of the purified bioflocculant was done and the results are represented in Figure 4.3.11. The bioflocculant was stable over a wide range of pH (pH 3-11) and gave flocculating activities above 80%. The lowest and highest flocculating activities of 85% and 94% were attained at pH 3 and 7, respectively.

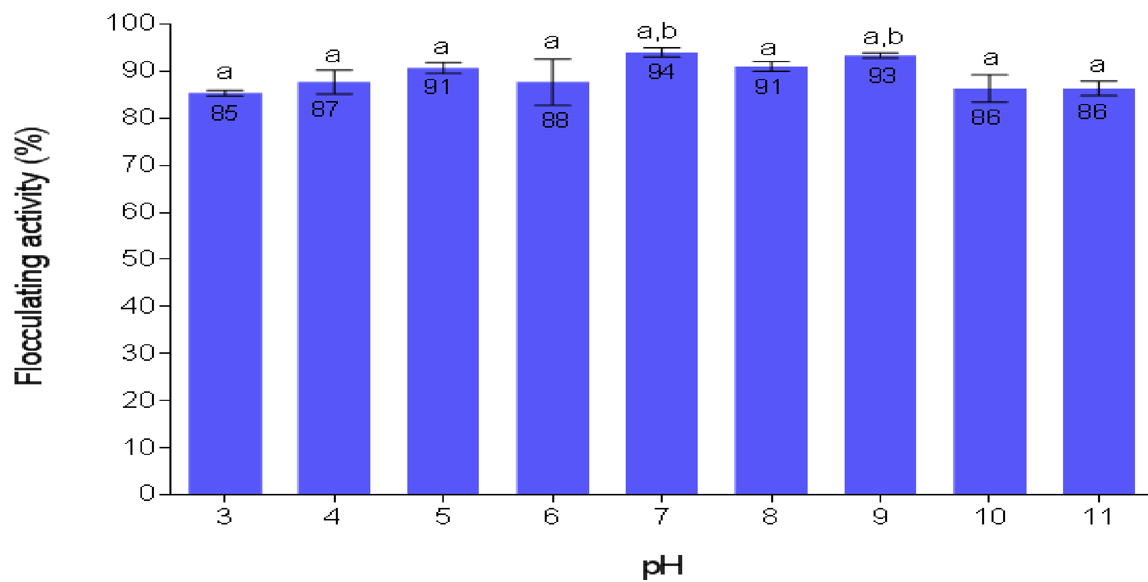


Figure 4.3.11: pH stability of TTT

4.3.9 Saline stability of TTT

The effect of Na^{+} concentration on the flocculating activity of the bioflocculant is illustrated in Table 4.3.3. The flocculating activity of the bioflocculant decreased proportionally with the increase in Na^{+} concentration. TTT demonstrated salinity stability and retained 80.3±4.97% of its flocculating activity even at the highest salinity (35 g/l).

Table 4.3.3: Effect of salinity on flocculating activity

NaCl (g/l)	FA(%)±SD
5	93.8±1.1[a]
10	90.7±1.80[a]
15	87±16.7[a]
20	85.7±2.35[a]
25	84,7±2.82[a]
30	82.3±4.75[a]
35	80.3±4.97[a]

FA denotes flocculating activity while SD denotes standard deviation.

4.3.10 *In-vitro* cytotoxicity of TTT

Figure 4.3.12 illustrates the *in-vitro* cytotoxicity of the bioflocculant on CaCO2 cell line. The bioflocculant showed no toxic effects on the cell lines. The cell line exhibited 90% cell viability after it has been treated with the highest concentration of the bioflocculant (200 µg/µl).

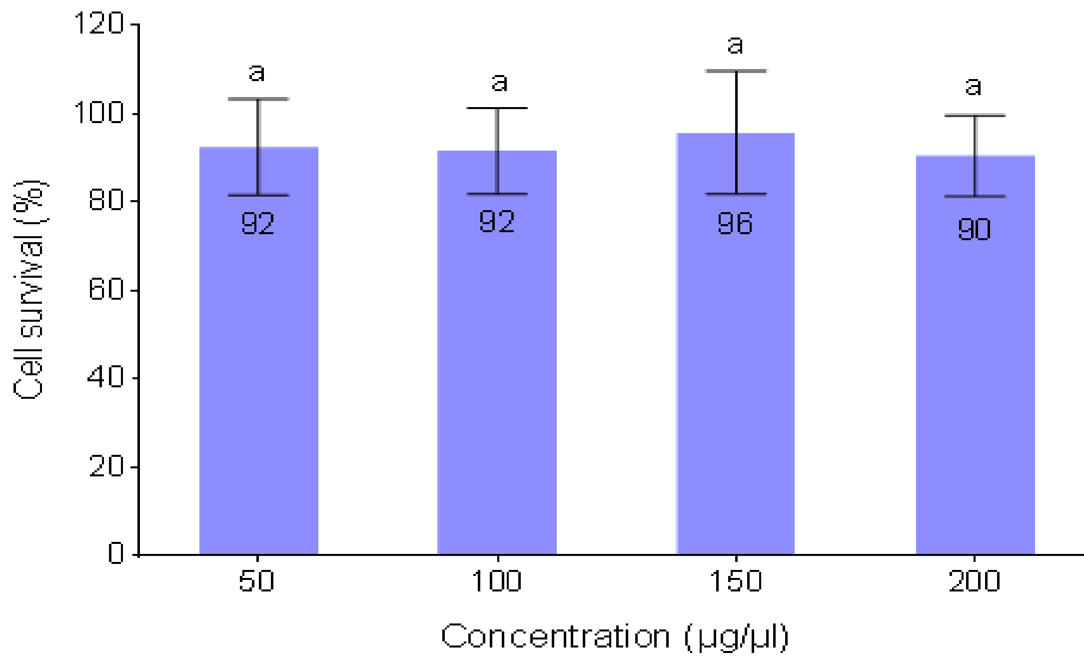


Figure 4.3.12: *In-vitro* cytotoxicity of TTT on CaCO2 cells

4.3.11 Proposed flocculation mechanism of TTT

The zeta potential of the bioflocculant, kaolin suspension, kaolin plus cation suspension and flocculated kaolin suspension with $BaCl_2$ and TTT were as follows: -16.5±1.07, -6.59±3.00, -7.01±0.992 and -5.5±2.07 mV, respectively (Table 4.3.4). Addition of Ba^{2+} to kaolin particles in suspension plus TTT resulted in an increase in zeta-potential (-5.5±2.07 mV). This implied that Ba^{2+} facilitated a bridging mechanism to flocculate kaolin particles in solution.

Table 4.3.4: Zeta potential of samples

Samples	Zeta potential (mV)
Bioflocculant TTT	-16.5±1.07
Kaolin particles	-6.59±3.00
Kaolin particles with Ba^{2+}	-7.01±0.99
Kaolin particles flocculated by TTT in the presence of Ba^{2+}	-5.5±2.07

4.3.12 Removal efficiency of TTT

Table 4.3.5 displays the removal efficiency of the bioflocculant on BOD, COD and S of wastewater from Tendele Coal Mine, RSA. The bioflocculant demonstrated better removal efficiency on COD than alum and ferric chloride.

Table 4.3.5: Removal efficiency of bioflocculant TTT

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/l)	COD (mg/l)	S (mg/l)	BOD (mg/l)	COD (mg/l)	S (mg/l)	BOD	COD	S
TTT	6.4±0.0	1557±0.0	4.1±0.0	2.6±0.5	606±2.5	1.06±0.1	59^{b}	61^{a}	75^{a}
Alum	6.4±0.0	1557±0.0	4.1±0.0	2.5±0.1	1031±2	1.5±0.0	61^{a}	34^{b}	66^{b}
$FeCl_3$	6.4±0.0	1557±0.0	4.1±0.0	2.7±0.0	927±1.5	1.1±0.0	$58^{a,b}$	40^{c}	73^{a}

4.4 Results for *A. faecalis* HCB2

The results obtained during resuscitation, optimisation of medium composition and culture conditions of *Alcaligenes faecalis* HCB2 are shown in this section. The results of the characteristics and application of the bioflocculant from this bacterium are also included.

4.4.1 Optimisation of medium composition and culture conditions

Optimization of the cultivation process improves the production of bioproducts. Thus, the varying of different constituents of the culture medium and growth conditions was done to improve flocculating action and bioflocculant yield. *A. faecalis* HCB2 did demonstrate the potential flocculating activity of 56% in the original pre-culture medium and conditions.

4.4.1.1 Inoculum size

Table 4.4.1 displays the effect of the inoculum size of mixed bacterial isolates on ~~the~~ bioflocculant production. The inoculum size of 5% (v/v) gave the highest flocculating activity (74.7±7.46%). However there was no statistical difference ($p < 0.05$) between 1 and 5% of inoculum sizes. Therefore the inoculum size of 1%, with a flocculating activity of 70.8±5, 50% was preferred.

4.4.1.2 Effect of carbon sources on flocculating activity

The effect of various carbon sources on flocculating activity is shown in Table 4.4.1. Maltose was the preferred carbon source due to its highest flocculating activity of 91%. However *A. faecalis* HCB2 can utilize various carbon sources listed in the table as they all had flocculating activities above 60% except for molasses which yielded the lowest flocculating activity of 39%.

Table 4.4.1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Inoculum size (%)	FA (%)±SD	Carbon source	FA (%)±SD	Nitrogen source	FA (%)±SD	Cations	FA (%)±SD
1	70.8±5,50[a]	Molasses	38.6±23.20[c]	Yeast extract	22.4±7.05[c]	Control	49.5±3.35[a,c]
2	68.5±3.46[a]	Sucrose	61.4±0.78[b,c]	Casein	34.0±4.09[c]	Na^+	62.3±7.28[a,b]
3	66.3±3.52[a]	Glucose	70.7±3.55[a,b]	Peptone	80.4±1.25[b]	Li^+	75.4±2.31[b]
4	71.4±6.78[a]	Starch	79.1±2.81[a,b]	$(NH_4)_2SO_4$	89.2±6.88[a,b]	K^+	78.1±2.52[b]
5	74.7±7.46[a]	Lactose	81.2±1.80[a,b]	Urea	97.4±0.84[a]	Mn^{2+}	63.2±6.78[a,b]
		Fructose	88.1±1.85[a]			Ba^{2+}	63.9±2.08[a,b]
		Maltose	90.6±2.11[a]			Ca^{2+}	71.2±5.42[b]
						Fe^{3+}	31.1±3.15[d]

FA denotes flocculating activity while SD denotes standard deviation.

4.4.1.3 Effect of nitrogen sources on flocculating activity

Nitrogen sources are secondary energy sources for microorganisms and play a vital role in the microbial growth and bioflocculant production. The effect of supplementary nitrogen sources on flocculation activity showed that urea was the better nitrogen source, yielding 97% of flocculation activity (Table 4.4.1). Yeast extract showed the lowest flocculating activity (22%).

4.4.1.4 Effect of cations on flocculating activity of crude bioflocculant

The effect of various metal ions on flocculating activity of the bioflocculant produced by *A. faecalis* HCB2 is shown in Table 4.4.1. Monovalent (K^+, Li^+ and Na^+) and divalent (Ca^{2+} $Fe^{2+}$and Mn^{2+}) cations yielded flocculating activities above 60%. The trivalent cation (Fe^{3+}) showed the lowest flocculating activity of 31.1%. K^+ was the preferred cation as it illustrated the highest flocculation activity (78%).

4.4.1.5 Effect of shaking speed on flocculating activity

Figure 4.4.1 illustrated the effect of mixing speed on flocculating activity. The flocculating activity increased gradually in proportion to the increase in the agitation speed and reached its maximum (87%) at 165 rpm. However, 110 rpm was the preffered speed as it demostrated no statistical difference between speed 165 rpm and in terms of flocculating activities.

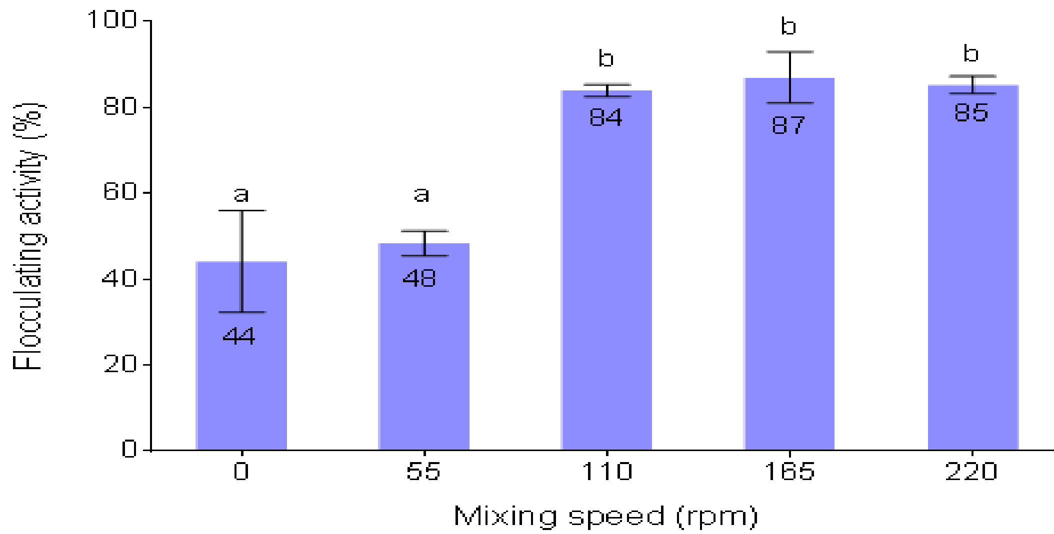


Figure 4.4.1: Effect of shaking speed on flocculating activity

4.4.1.6 Effect of temperature on flocculating activity

The effect of cultivation temperature on flocculating activity is shown in Figure 4.4.2. When the temperature was 25°C, the flocculating activity was 80%. The increase in temperature resuted in the decline in flocculating activity. The temperature at 45°C gave the least flocculating activity of 49% of flocculating activity. Therefore 25°C was used as the optimal temperature for the bioflocculant production.

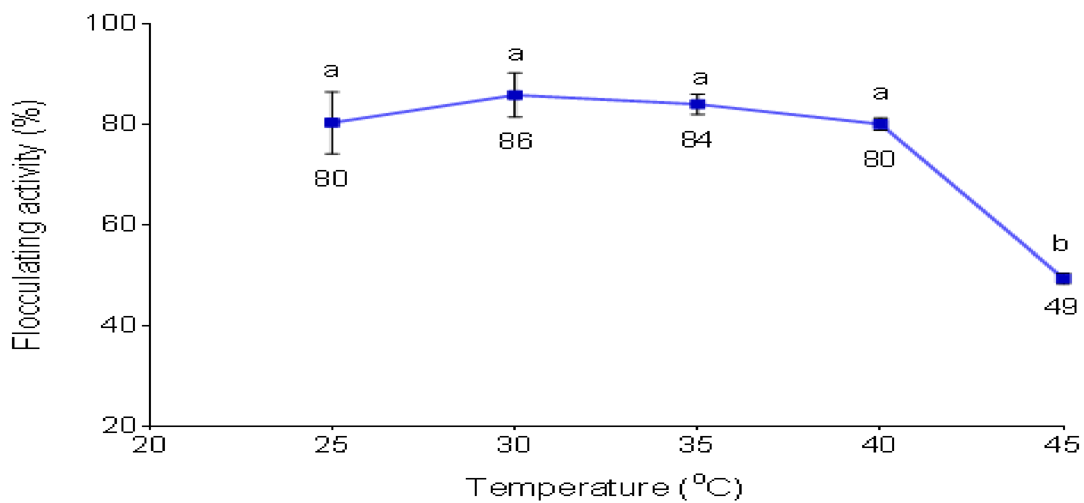


Figure 4.4.2: The effect of temperature on flocculating activity

4.4.1.7 Effect of initial pH on flocculating activity

Figure 4.4.3 shows the effect of initial pH of the medium on the flocculating activity. *A. faecalis* HCB2 produced a bioflocculant with a high flocculating activity of 85% at an initial pH of 9. It was observed that the lowest flocculating activity (53%) was at pH 3.

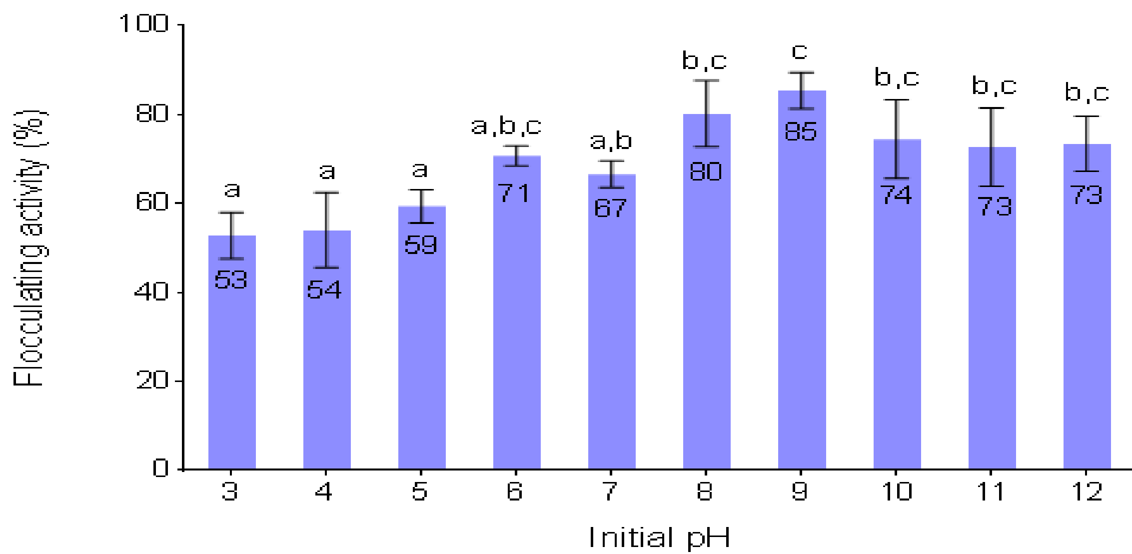


Figure 4.4.3: Effect of initial pH on flocculating activity

4.4.1.8 Time course assay

The effect of time course on flocculating activity (FA), bacterium growth (shown as optical density (OD) and the initial pH are shown in Figure 4.4.4. The flocculating activity increased relatively to the bacterium growth in the first 72 hours of growth. The highest flocculating activity (92%) was obtained after 72 hours. Thereafter, a slight decrease in flocculating activity was observed. There was a fluctuation in the initial pH of the medium between the initial pH of 9 to the final pH of 8 within 108 h of growth.

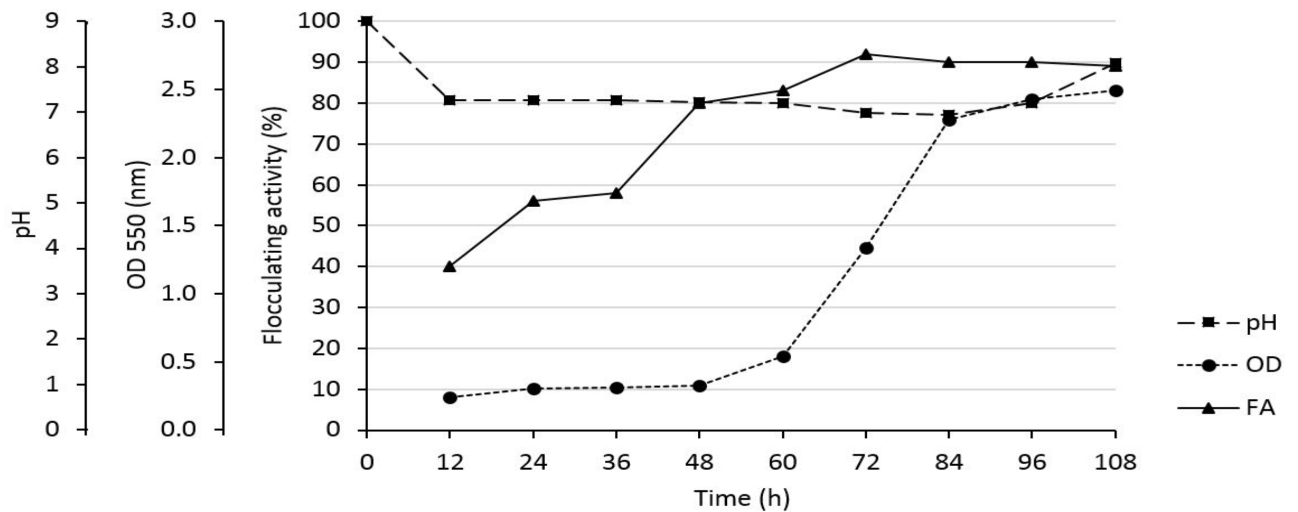


Figure 4.4.4: Effect of time on FA, initial pH and OD

4.4.2 Bioflocculant yield and solubility assay

The extracted and purified bioflocculant was 2.7 g/l. It was named TKT. The bioflocculant was insoluble in all solvents used, but water was an exception.

4.4.3 Effect of dosage on flocculating activity

Table 4.4.3 indicated the effect of different bioflocculant concentrations on the flocculating activity of the purified bioflocculant TKT. The concentration of 0.8 mg/ml, gave the highest flocculating activity of 85.6±1.35.

Table 4.4.3: The effect of dosage size and cations on flocculating activity

Dosage (mg/ml)	FA (%) ± SD	Cations	FA (%) ± SD
0.2	80.4±1.04[a]	Na^{+}	67.8±2.15[a]
0.4	78.7±1.29[a]	K^{+}	70.2±0.35[a]
0.6	78.5±2.72[a]	Li^{+}	71.6±1.51[a]
0.8	85.6±1.35[b]	Ca^{2+}	77.4±0.57[b]
1	84.8±1.04[b]	Mn^{2+}	83.7±1.37[c]
		Ba^{2+}	85.8±1.00[c]
		Fe^{3+}	44.8±2.06[d]
		Control (Without cation)	51.3±0.50[d]

FA denotes flocculating activity while SD denotes standard deviation.

4.4.4 Effect of cations on flocculating activity of the purified bioflocculant

The effect of various cations on the flocculating activity of the bioflocculant TKT was assessed and the results shown in Table 4.4.2. Ba^{2+} was the most preferred cation and gave the highest flocculation activity of 85.8±1.00%. Fe^{3+} showed the least flocculating activity of 44.8±2.06%.

4.4.5 Physico-chemical composition of TKT

4.4.5.1 Surface structures

Figure 4.4.5 shows SEM surface images. TKT had a porous and crystal-like structure (Figure 4.4.5a). The flocculated kaolin particles showed a clump like structure (Figure 4.4.5b) and kaolin particles appeared to be fine and smooth in structure (Figure 4.4.5c)

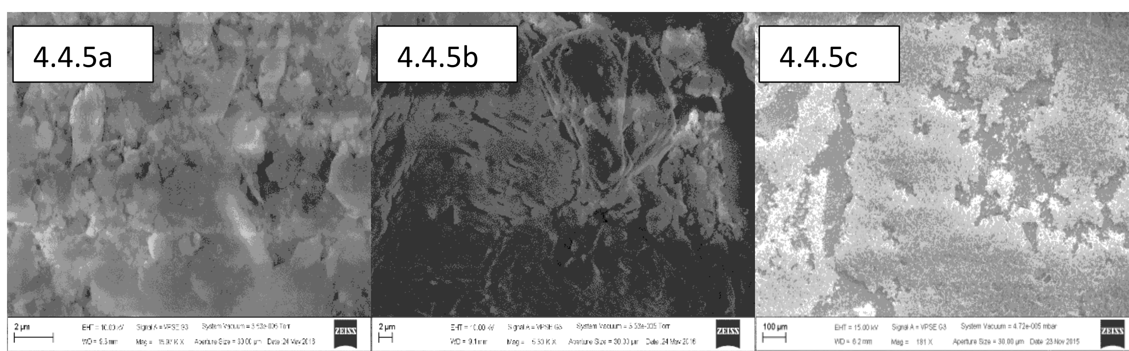


Figure 4.4.5: SEM analysis. SEM surface images of the bioflocculant (4.4.5a), flocculated kaolin particles (4.4.5b) and kaolin particles (4.4.5c)

4.4.5.2 Electric charge and qualitative analyses of the components of TKT

The surface charge of TKT was determined. The bioflocculant TKT had a surface negative charge of -17.1±0.652 mV, as illustrated by the ZetaSizer Nano. TKT was Ninhydrin positive. The UV spectrum of the bioflocculant demonstrated the qualitative presence of protein, as shown by a sharp absorption peak at 289 nm and absence of nucleic acids (Figure 4.4.6).

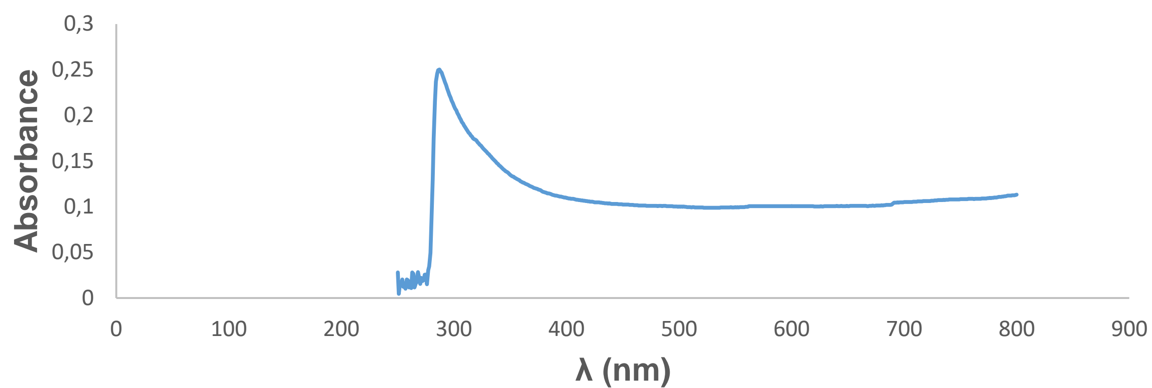


Figure 4.4.6: UV spectrum of TKT

4.4.5.3 Quantitative analyses of total protein and carbohydrates

The quantitative chemical composition of TKT showed the presence of carbohydrates and proteins. The bioflocculant in is predominantly composed of 89% of carbohydrates and 10% of proteins.

4.4.5.4 Elemental analyses of TKT

Figure 4.4.7 illustrates the elemental analysis of bioflocculant TKT. The elemental spectrum shows the absorption peaks indicative of N:C:O:P:Ca:Cl:Mg:S:B:Na accounting for 2.2:24.6:23.6:2.0:1.9:7.2:1.3:0.4:24.3:6.2 (%, w/w), respectively.

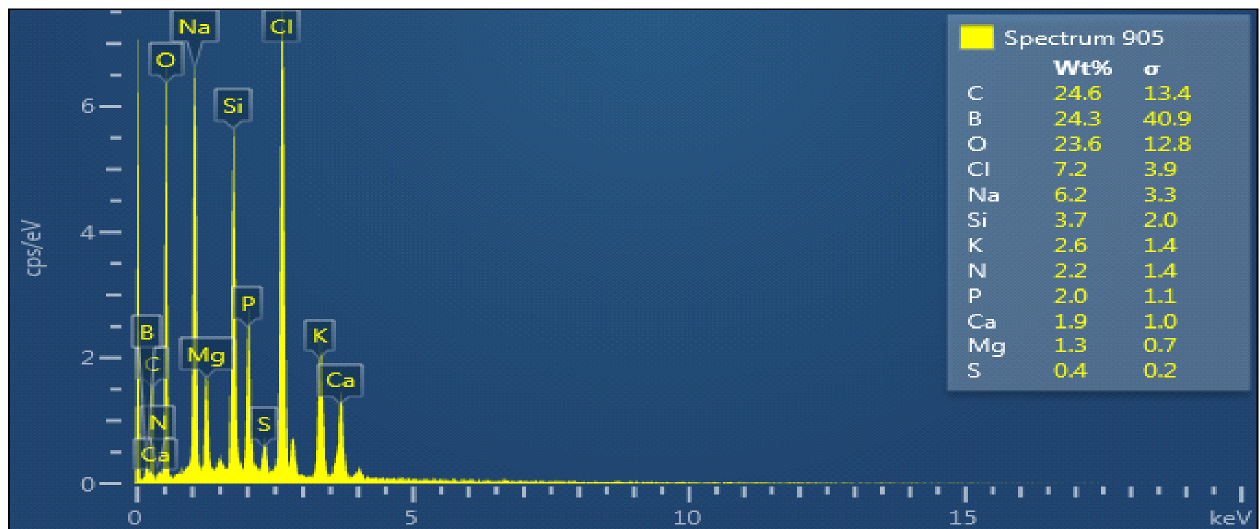


Figure 4.4.7: Elemental analyses of TKT

4.4.5.5 Functional groups of TKT

The functional groups of TKT were determined and the results shown in Figure 4.4.8. The IR spectrum revealed the presence of different functional groups of the bioflocculant. Hydroxyl (3368 cm^{-1}), amide (1641 cm^{-1}) and amino groups (1019 cm^{-1}) were the main functional groups observed.

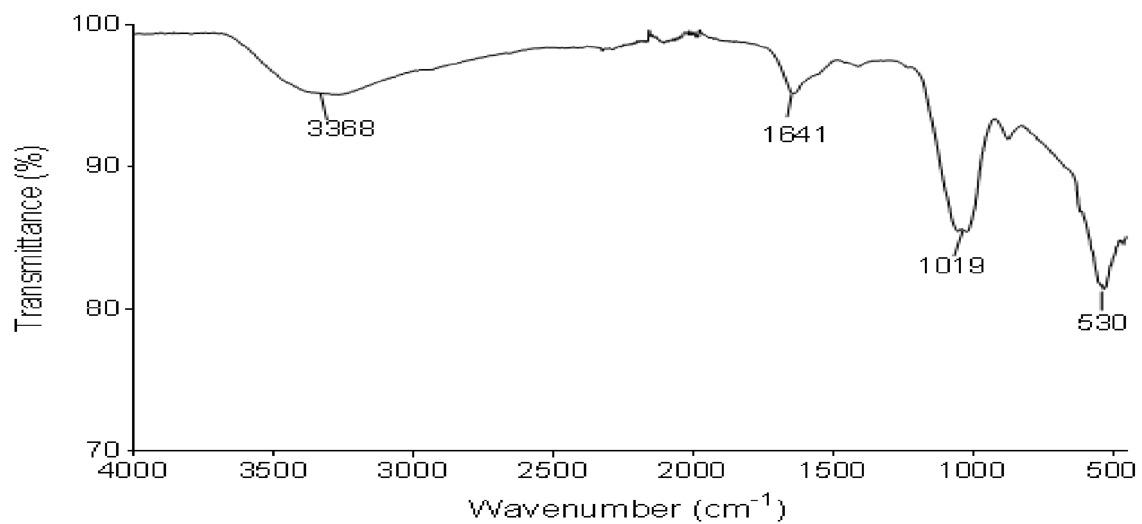


Figure 4.4.8: IR spectrophotometric analyses of TKT

4.4.6 Thermal stability of TKT

The effect of temperature on flocculating activity of bioflocculant TKT is shown in Figure 4.4.9. There was a sharp and significant drop in flocculating activity with an increase in temperature. Between 50 to 100°C, the flocculating activity dropped by 22%. The bioflocculant was thermostable even at 100°C and had a flocculating activity of 70%.

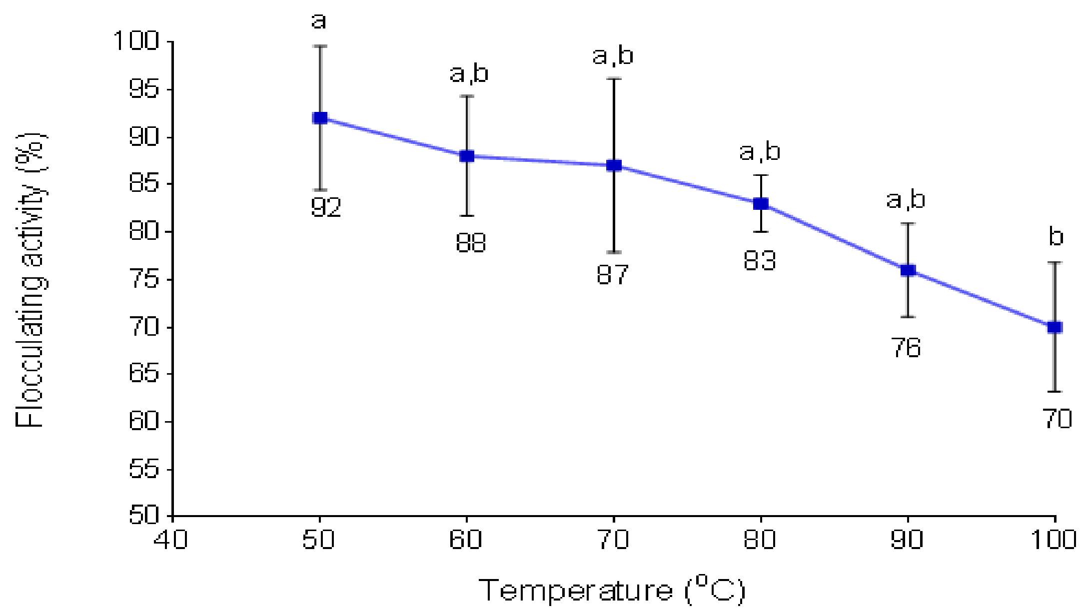


Figure 4.4.9: Thermal stability of TKT

4.4.7 Pyrolysis property of TKT

Figure 4.4.10 shows the pyrolysis properties of bioflocculant TKT. There was an initial weight loss of 9.31% observed between 35 and 151.65°C.

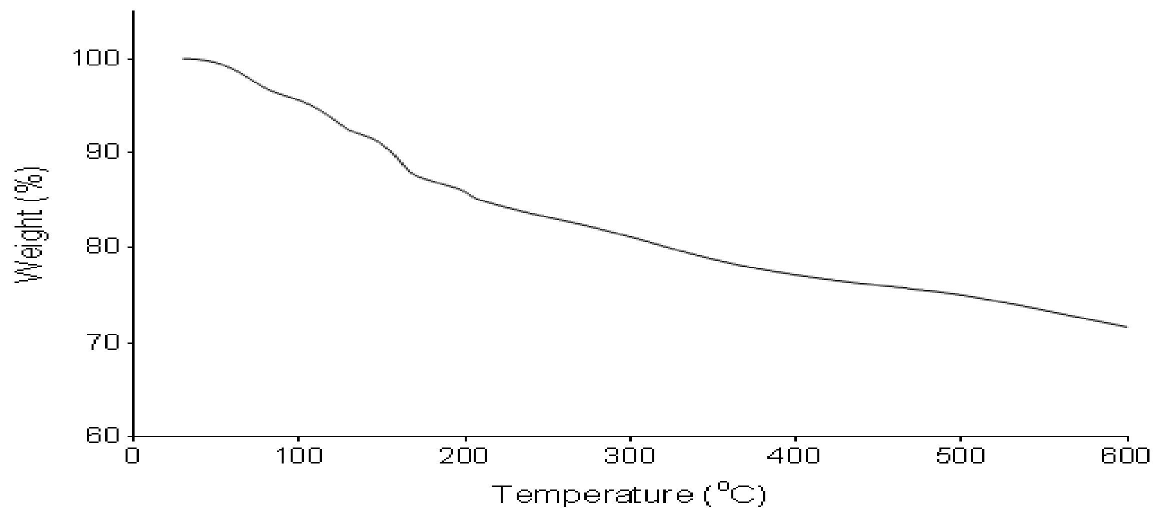


Figure 4.4.10: Pyrolysis property of TKT

4.4.8 pH stability of TKT

Figure 4.4.11 presents the effect of pH on flocculating activity of the bioflocculant. The flocculating activity of TKT was more than 80% in a weak acid (pH 5-6), neutral to akaline conditions. The highest flocculating activity (93%) was observed at pH 7.0 and the least flocculating activity (69%) occurred at pH 3.

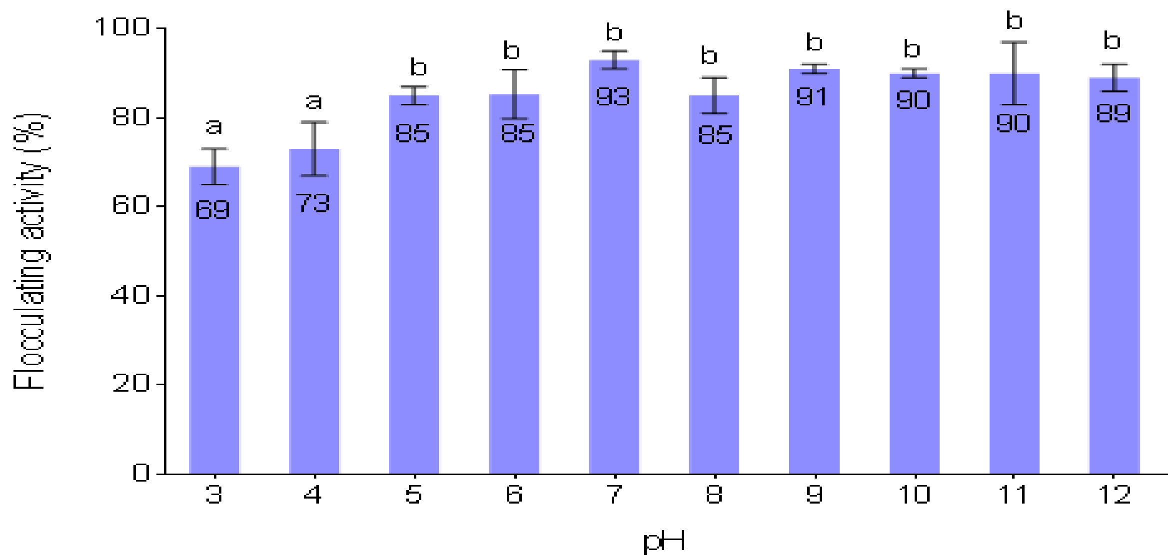


Figure 4.4.11: pH stability of TKT

4.4.9 Saline stability of TKT

The effect of Na^+ concentration on the flocculating activity of bioflocculant TKT is illustrated in Table 4.4.3. The flocculating activity of the bioflocculant decreased proportionally with the increase in the Na^+ concentration. Bioflocculant TKT demonstrated salinity stability and retained 72.4±0.95% of its activity even at the highest salinity (35 g/l).

Table 4.4.3: Saline stability of TKT

NaCl (g/l)	FA (%) ± SD
5	92±4,53^{a}
10	88.2±0.82^{a,b}
15	86±3.05^{a,b}
20	80±3.96^{b,c}
25	79.3±2.44^{b,c}
30	72.8±7.74^{c}
35	72.4±0.95^{c}

FA denotes flocculating activity while SD denotes standard deviation.

4.4.10 *In-vitro* cytotoxicity of TKT

Figure 4.4.12 and 4.4.13 illustrate the *in-vitro* cytotoxicity of the bioflocculant on CaCO2 and HEK293 cells. The bioflocculant showed no toxic effects on the CaCO2 cell line. The cell lines exhibited 89% viability after having been treated with the highest concentration of bioflocculant (200 µg/µl). HEK293 cell line also demonstrated 95% growth after been treated with 200 µg/µl of the bioflocculant.

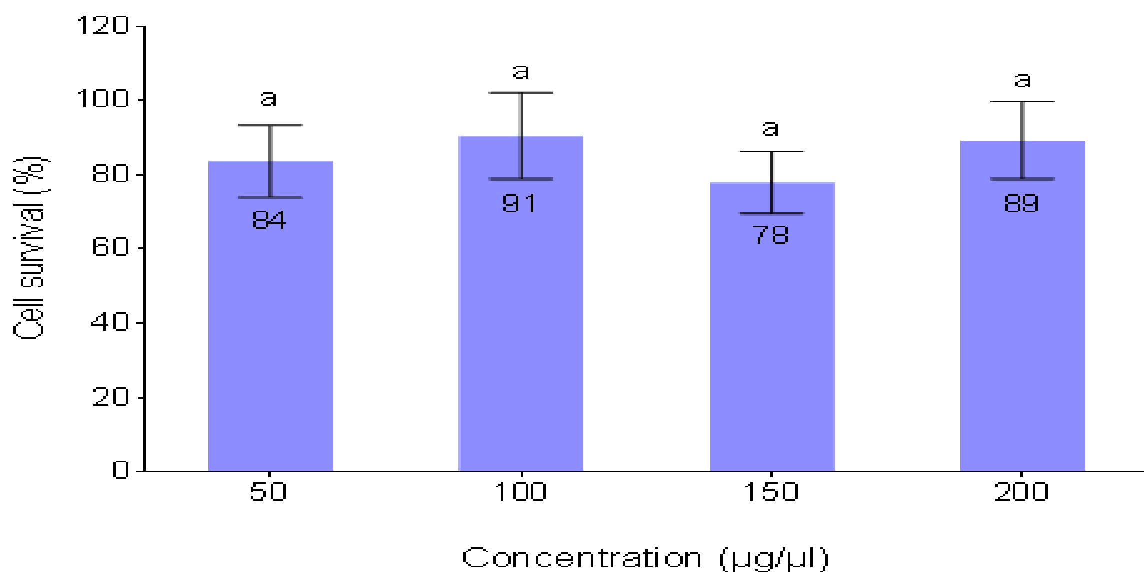


Figure 4.4.12: ***In-vitro*** **cytotoxicity of TKT on CaCO2**

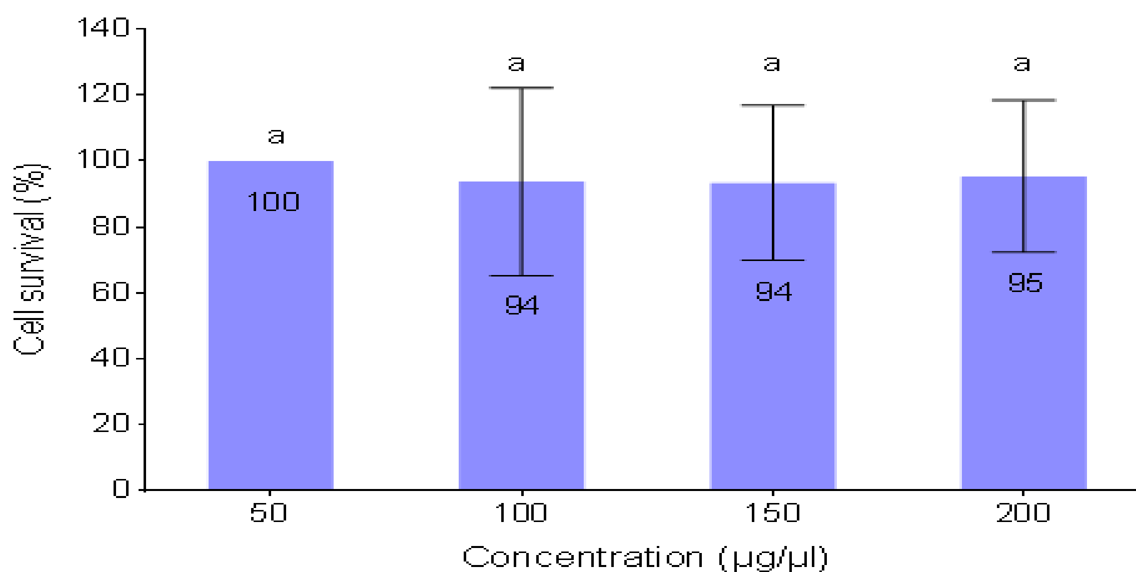


Figure 4.4.13: ***In-vitro*** **cytotoxicity of TKT on HEK293**

4.4.11 Proposed flocculation mechanism of TKT

The zeta potential of the bioflocculant, kaolin suspension, kaolin plus cation suspension and flocculated kaolin suspension with $BaCl_2$ and TKT were as follows: -17.1±0.652, 6.59±3.00, -7.01±0.992 and -4.41±0263 mV, respectively (Table 4.4.4). In accordance to the results, bioflocculant TKT used a bridging mechanism mediated by Ba^{2+} to flocculate kaolin particles

Table 4.4.4: Zeta potential of samples

Samples	Zeta potential (mV)
Bioflocculant TKT	-17.1±0.7
Kaolin particles	-6.59±3.0
Kaolin particles with Ba^{2+}	-7.01±1.0
Kaolin particles flocculated with TKT in the presence of Ba^{2+}	-4.41±0.7

4.4.12 Removal efficiency of TKT

Table 4.4.5 displays the removal efficiency of bioflocculant TKT on BOD and COD in wastewater from Tendele Coal Mine, RSA. The bioflocculant had comparatively higher removal efficiencies than alum and ferric chloride on COD. However, the removal efficiencies on BOD and S were the same with those shown by conventional flocculants.

Table 4.4.5: Removal efficiency of bioflocculant TKT

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/l)	**COD (mg/l)**	**S (mg/l)**	**BOD (mg/l)**	**COD (mg/l)**	**S (mg/l)**	**BOD**	**COD**	**S**
TKT	6.4±0.0	1557±0.0	4.1±0.0	3.2±0.2	436±2.08	1.03±0.13	59^{b}	72^{a}	75^{a}
Alum	6.4±0.0	1557±0.0	4.1±0.0	2.9±0.2	828±1	1.37±0.12	50^{a}	47^{b}	66^{b}
$FeCl_3$	6.4±0.0	1557±0.0	4.1±0.0	2.6±0.58	753±2.65	1.08±0.12	$54^{a,b}$	52^{c}	73^{a}

4.5 Results for a consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5

The results were obtained during optimisation of medium composition and culture conditions of a consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5. The results of the characteristics and application of bioflocculant on wastewater and confirmed coliforms are included.

4.5.1 Optimisation of medium composition and culture conditions

The optimisation of the culture medium and growth conditions was done to improve the flocculating activity and bioflocculant yield of the constructed consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5. The results are presented below.

4.5.1.1 Inoculum size

Table 4.5.1 displays the effect of the inoculum size of mixed bacterial isolates on bioflocculant production. The inoculum size of 2% gave the highest flocculating activity of 92.8±2.73%. Since there was no statistical difference ($p< 0.05$) in flocculating activities by an inoculum size of 1 and 2%, the inoculum size of 1% was preferred. The inoculum size of 1% gave a flocculating activity of 86.3±3.60%.

4.5.1.2 Effect of carbon and nitrogen sources

Bacteria in a consortium utilized various carbon and nitrogen sources in the culture medium. Glucose was the suitable carbon source with a flocculating activity of 92.8±2.73%, while starch was the least preferred carbon source with a flocculating activity of 48.1±8.65% (Table 4.5.1). Ammonia sulphate was the best nitrogen source with a flocculating activity of 98.1±0.45% and yeast extract gave the least flocculating activity (86.8±2.71%) (Table 4.5.1). Generally, all nitrogen sources were good sources for bacteria and showed flocculating activities above 80%.

Table 4.5.1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Inoculum size (%)	FA (%) ± SD	Carbon source	FA (%) ± SD	Nitrogen source	FA (%) ± SD	Cations	FA (%) ± SD
1	86.3±3.60[a]	Fructose	48.3±2.74[a]	Yeast extract	86.8±2.71[a]	Control	87.4±10.93[a]
2	92.8±2.73[a]	Starch	48.1±8.65[a]	Urea	93.5±2.22[b]	Li^{+}	96.5±0.62[a]
3	92.4±2.49[a]	Xylose	48.9±4.93[a]	Peptone	94.1±1.03[b]	Na^{+}	98.8±0.20[a]
4	80.7±12.13[a]	Molasses	76.4±10.12[b]	Casein	95.8±2.28[b]	K^{+}	98.6±0.49[a]
5	76.8±7.09[a]	Lactose	76.8±8.54[b]	$(NH_4)SO_4$	98.1±0.45[b]	Mn^{2+}	98.1±0.40[a]
-	-	Sucrose	82.6±6.01[b]	-	-	Ba^{2+}	98.1±0.21[a]
-	-	Maltose	83.1±10.21[b]	-	-	Ca^{2+}	98.1±0.45[a]
-	-	Glucose	92.8±2.73[b]	-	-	Fe^{3+}	71.4±9.24[b]

FA denotes flocculating activity while SD denotes standard deviation.

4.5.1.3 Effect of cations on flocculating activity of crude bioflocculant

Table 4.5.1 showed the synesthetic effect of metal cations on flocculating activity. In general, all the metal cations and control (with the exception of Fe^{3+}) stimulated flocculation, resulting in flocculating activities above 80%. There was no significant difference observed with all cations (including the control) that gave activity above 80%. However, the maximum activity (98.8±0.20%) was obtained when Na^{+} was used. Fe^{3+} gave the least flocculating activity of 71.4±9.24%.

4.5.1.4 Effect of shaking speed on flocculating activity

The effect of shaking speed on the flocculating activity is shown in Figure 4.5.1. A shaking speed of 165 rpm was the most conducive speed, giving the highest flocculating activity of 95%. The shaking speeds above or below 165 rpm led to a decrease in the flocculating activity.

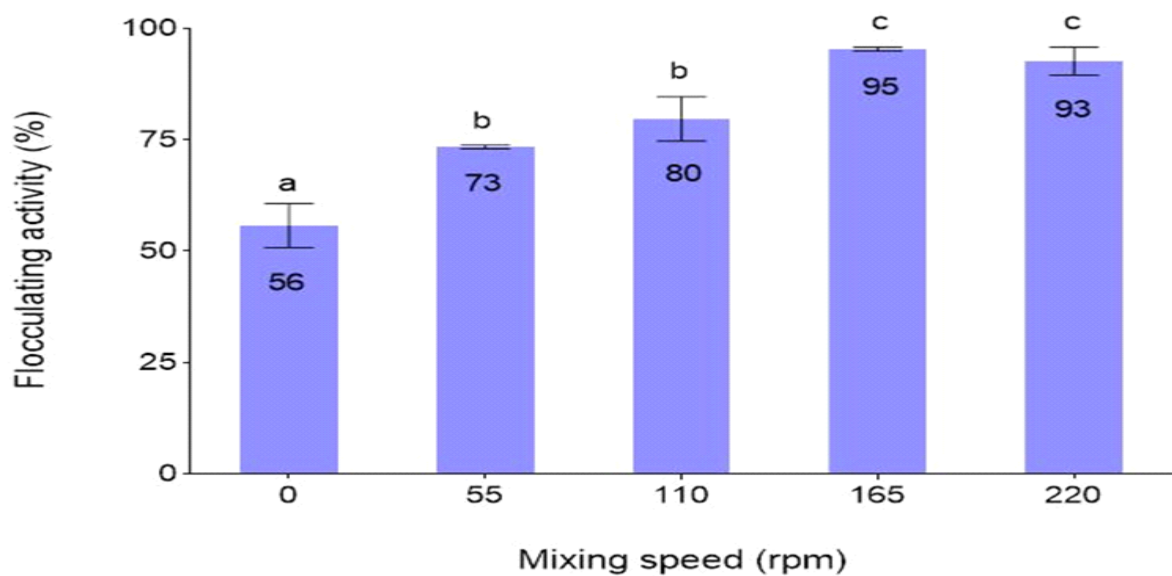


Figure 4.5.1: Effect of shaking speed on flocculating activity

4.5.1.5 Effect of temperature on flocculating activity

Figure 4.5.2 illustrates the effect of culture temperature on the flocculating activity. Maximum flocculating activity (98%) was observed at a culture temperature of 30°C. The culture temperature above or below 30°C led to a decrease in flocculating activities.

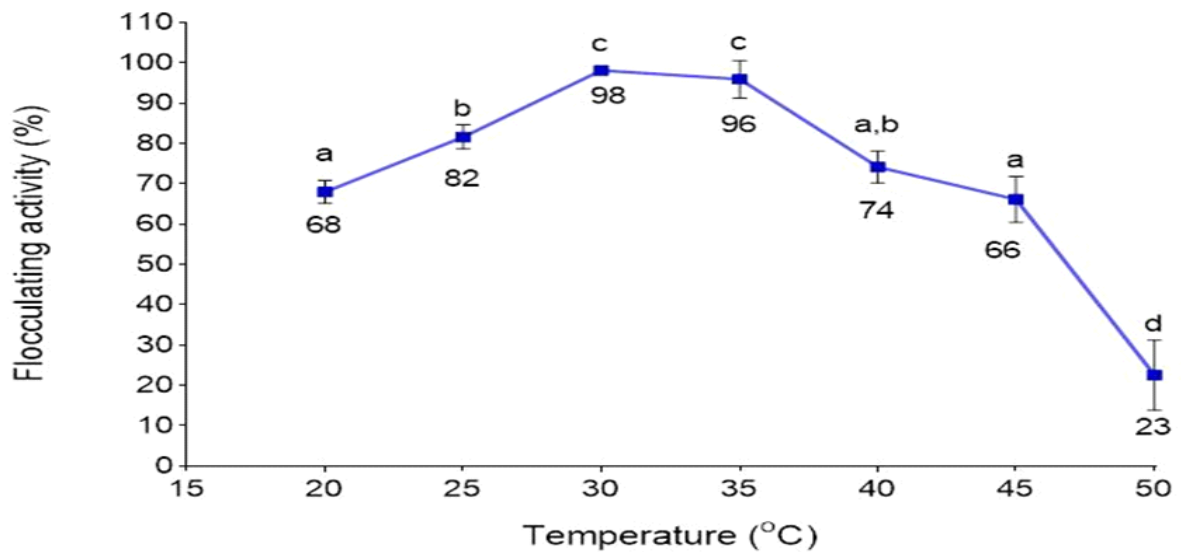


Figure 4.5.2: Effect of temperature on flocculating activity

4.5.1.6 Effect of initial pH on flocculating activity

Figure 4.5.3 presents the effect of initial pH on flocculating activity. The highest flocculating activity (95%) was obtained when the initial pH of the culture medium was 6. However, ~~the~~ flocculating activity was significant in the range of weak acid (initial pH of 4-6), giving flocculating activities above 80%. pH 12 showed the least flocculating activity (8%).

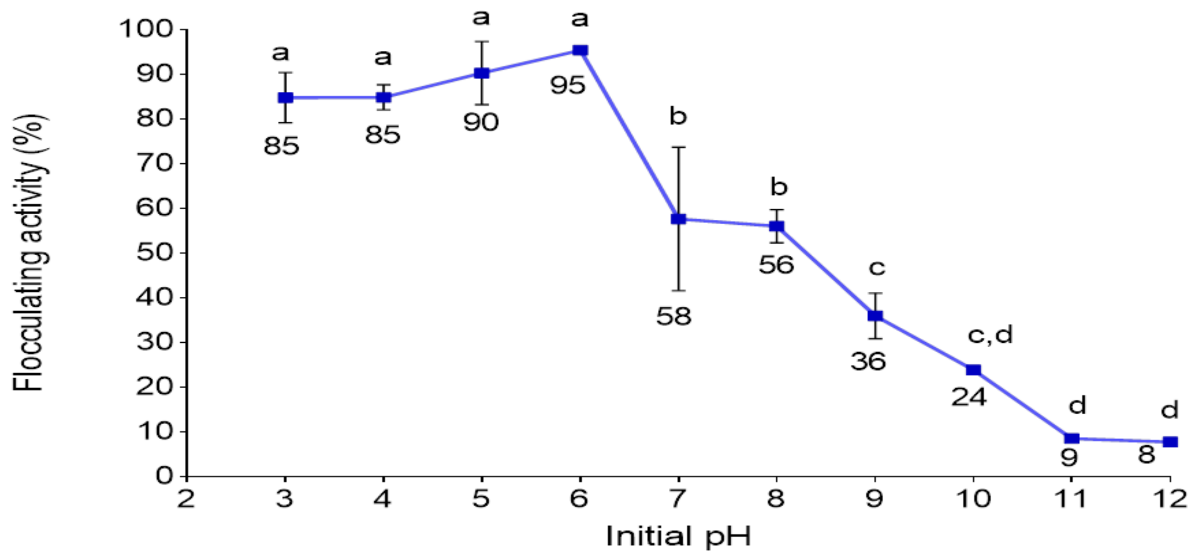


Figure 4.5.3: Effect of initial pH on flocculating activity

4.5.1.7 Time course assay

The effect of time course on flocculating activity (FA), optical density (OD) of culture broth and initial pH are shown in Figure 4.5.4. The flocculating activity increased relatively to the optical density until 72 hours. The flocculating activity of 90% was obtained within 72 hours. After 72 hours, the OD decreased while there was an insignificant increase in flocculating activity. Therefore, 72 hours of growth was preferred. The initial pH of the culture medium dropped uniformly from the initial pH of 6.00 to pH 3.2.

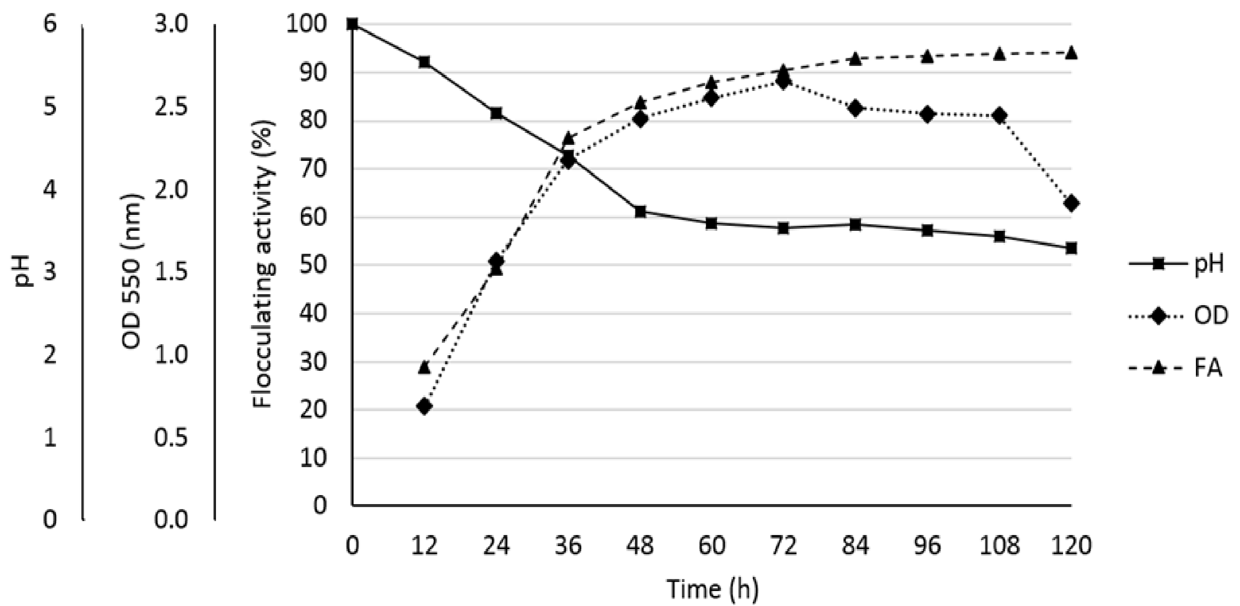


Figure 4.5.4: Effect of time course on FA, OD and initial pH

4.5.2 Bioflocculant yield and solubility

A purified bioflocculant was named TST. The obtained yield of TST was 3.1 g from 1 litre of growth broth of a consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5. The bioflocculant was only soluble in water and insoluble in all other solvents.

4.5.3 Effect of dosage size and cations on flocculating activity of purified bioflocculant

The bioflocculant dosage size assay was done and the results are illustrated in Table 4.5.2. The highest flocculating activity (77.2±2.21%) was observed at a concentration of 0.8 mg/ml. However, there was no statistical difference ($p < 0.05$) observed between 0.2 mg/ml and 0.8 mg/ml, regarding the flocculating activities. Thus, 0.2 mg/ml was preferred, with a flocculating activity of 72.9±6.20%.

The effect of cations on the flocculating activity of the purified bioflocculant is shown in Table 4.5.2. Monovalent cations (Na^{+}, Li^{+} and K^{+}) and divalent (Ca^{2+}, Mn^{2+} and Ba^{2+}) significantly enhanced flocculating activity. The monovalence and divalence gave ~~the~~ flocculating activities above 70%. Ba^{2+} was the best cation with a maximum flocculating activity of 90.1±2.35% and Fe^{3+} had the lowest flocculating activity (47.4±3.59%).

Table 4.5.2: Effect of dosage size and cations on flocculating activity

Dosage size (mg/ml)	FA (%) ± SD	Cations	FA (%) ± SD
0.2	72.9±6.20[a]	Li^{+}	86.5±1.37[a,c]
0.4	71.7±4,70[a]	Na^{+}	80.4±2.36[a,d]
0.6	70.9±2.25[a]	K^{+}	78.1±4.51[b,d]
0.8	77.2±2.21[a]	Ba^{2+}	90.1±2.35[c]
1	72.8±2.34[a]	Mn^{2+}	85.8±3.11[a,b,c]
		Ca^{2+}	77.2±2.21[d]
		Fe^{3+}	47.4±3.59[e]
		Control (Without cation)	71.0 ±1.37[d]

FA denotes flocculating activity while SD denotes standard deviation.

4.5.4 Physico-chemical composition of the bioflocculant

4.5.4.1 Surface structures

Figure 4.5.5 shows SEM surface images. TST had a porous and amorphous surface structure (Figure 4.5.5a), kaolin particles revealed a fine and smooth structure (Figure 4.5.5b) and the clump like structure was observed in the flocculated kaolin particles (Figure 4.5.5c).

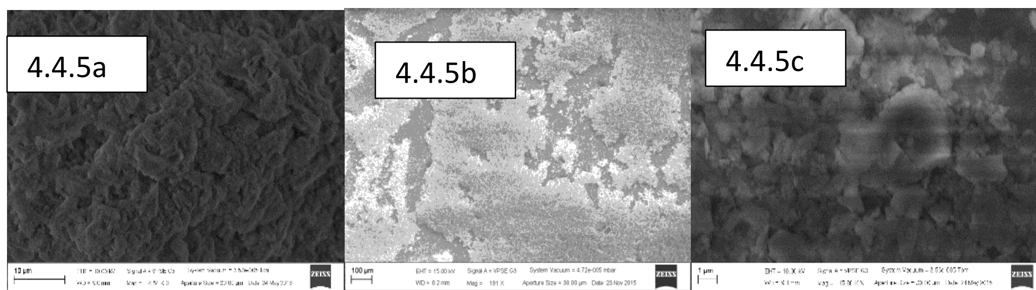


Figure 4.5.5: SEM surface images; bioflocculant TST (Figure 4.5.5a), kaolin particles (Figure 4.5.5b) and flocculated kaolin particles (4.5.5c)

4.5.4.2 Electric charge of TST

The zeta potential of was evaluated to determine the surface charge of bioflocculant TST. The bioflocculant had a negative charge with the zeta-potential of-11.2±1.912 mV.

4.5.4.3 Qualitative analyses of proteins and nucleic acids

Bioflocculant TST was Ninhydrin positive. The UV spectrum showed a sharp peak at 287 nm that characterised the protein content and there was no presence of nucleic acids detected (Figure 4.5.6).

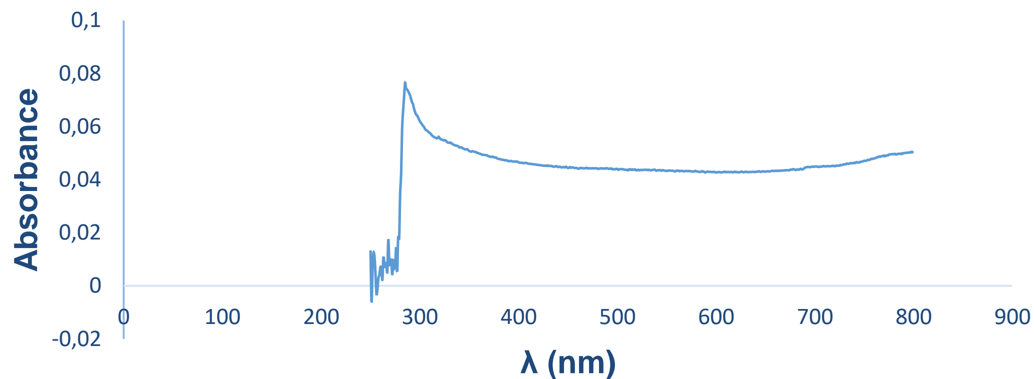


Figure 4.5.6: UV spectrum of TST

4.5.4.4 Quantitative analyses of TST

The chemical assay indicates the major component of TST to be carbohydrates (80% (w/w)) and not protein (15% (w/w)). Thus, TST is a glycoproteing predominately composing of carbohydrates.

4.5.4.5 Elemental analyses of TST

Figure 4.5.7 illustrates the elemental analyses of the bioflocculant. The composition of the elements in mass proportion (% w/t) is: C (44.6), O (24.4), Cl (6.0), Na (4.8), N (4.7), Ca (3.8), P (3.3), Si (2.5), K (2.0), Mg (1.7), S (1.7), Al (0.3) and Pd (0.1).

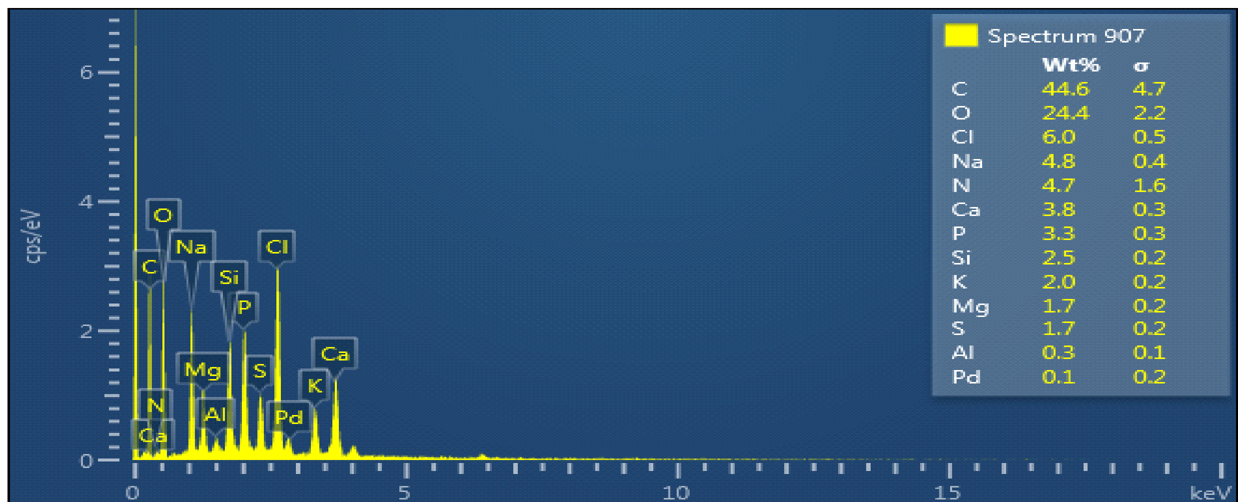


Figure 4.5.7: Elemental analyses of TST

4.5.4.6 Functional groups of TST

Figure 4.5.8 displays the IR spectrum with the obtained functional groups. IR spectrum revealed the presence of hydroxyl (3309 cm^{-1}), amide (1648 cm^{-1}) and amino (1090 cm^{-1}) groups.

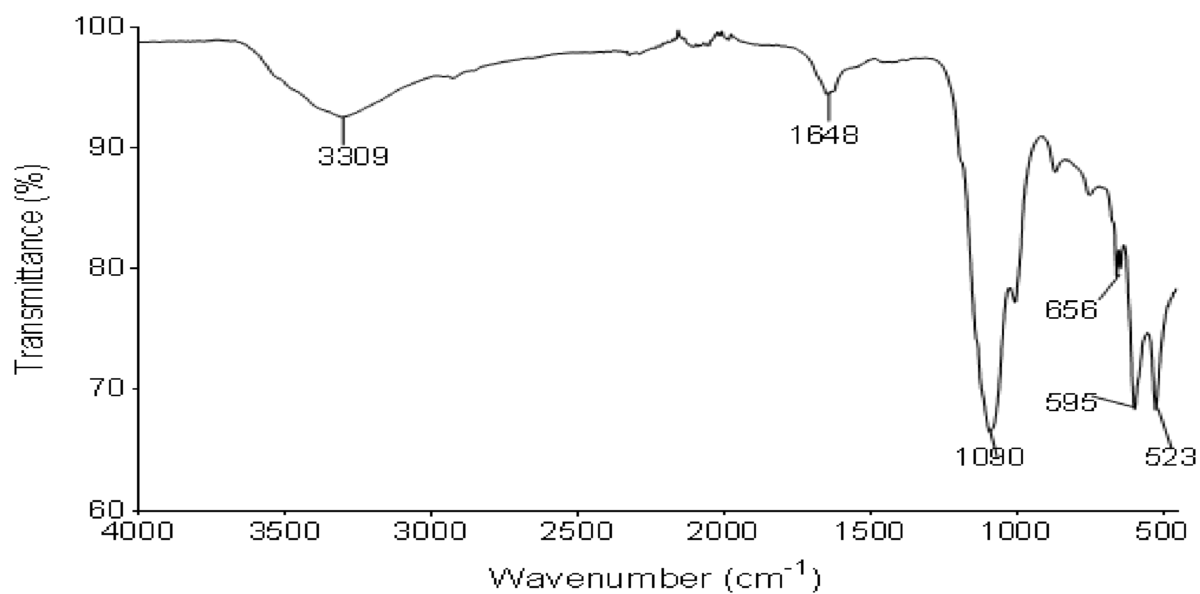


Figure 4.5.8: **IR spectrum of TST**

4.5.5 Thermal stability of TST

The effect of temperature on the flocculating activity of TST is shown in Figure 4.5.9. TST was heat stable showing flocculating activity of 80% at 100°C after an hour of heat exposure.

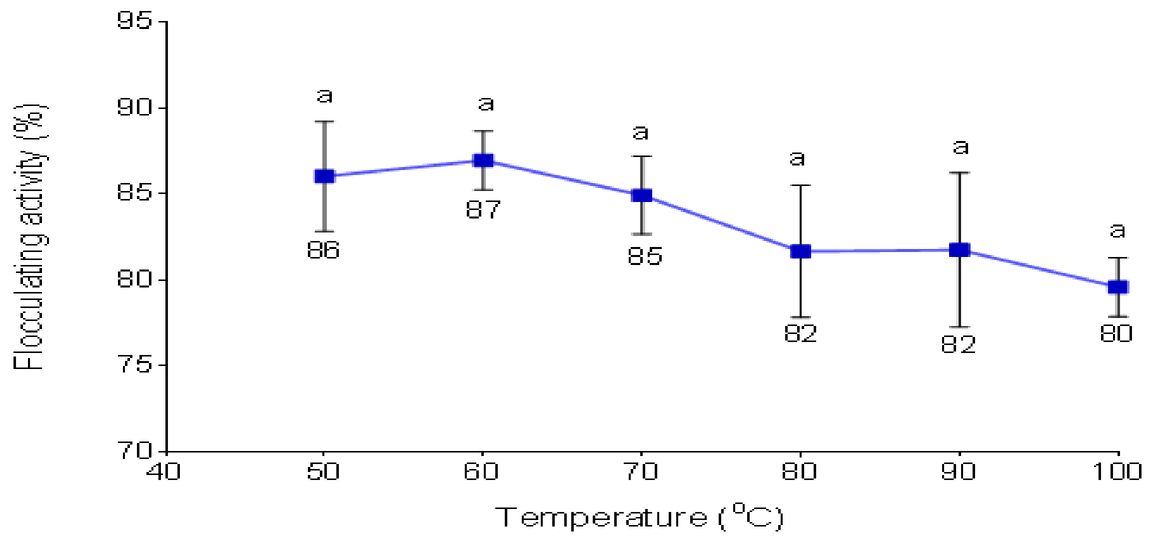


Figure 4.5.9: Thermal stability of TST

4.5.6 Pyrolysis property of TST

The pyrolysis properties of TST were studied and the results shown in Figure 4.5.10. The degradation temperature (*T*d) of the bioflocculant was first observed at 155°C and then at 170, 294 and 384°C.

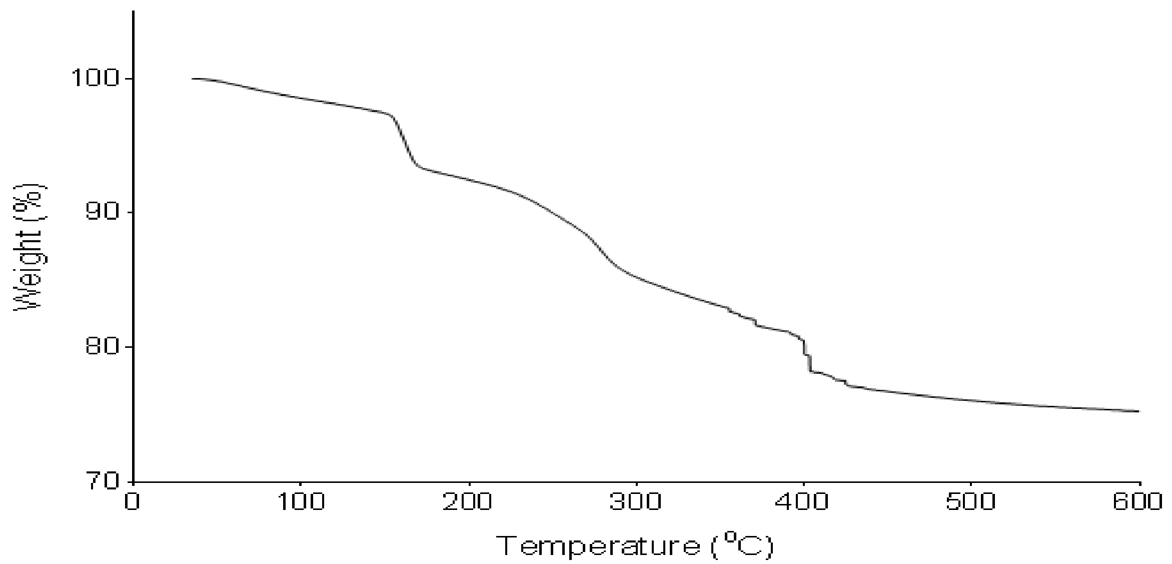


Figure 4.5.10: Pyrolysis property of TST

4.5.7 pH stability of TST

The effect of pH on flocculating activity of bioflocculant TST is displayed in Figure 4.5.11. The flocculating activity of TST was more than 80% in ~~the~~ alkaline conditions and the maximum flocculating activity (95%) occurred at pH 8.0 and 9.0. The flocculating activities were low in acidic conditions and in even, low or weak acidic conditions (pH 6 and 5), with flocculating activities of 54 and 55%, respectively.

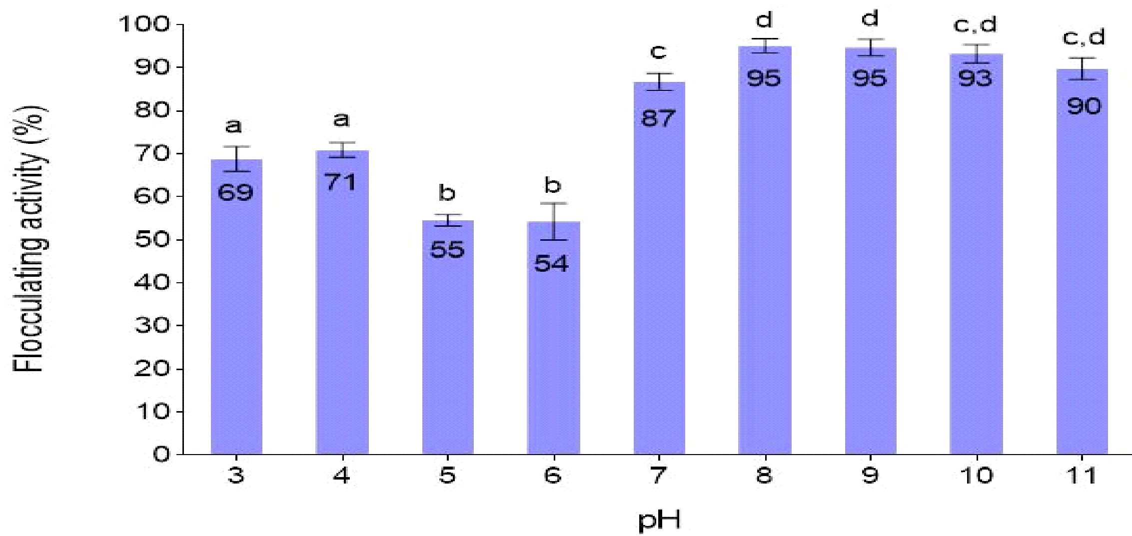


Figure 4.5.11: pH stability of TST

4.5.8 Saline stability of TST

Figure 4.5.12 indicates the effect of salinity on the flocculating activity. The flocculating activity of the bioflocculant decreased proportionally with an increase in Na^+ concentration. Nevertheless, bioflocculant TST retained high flocculating activity (71%) even at the highest salinity (35 g/l).

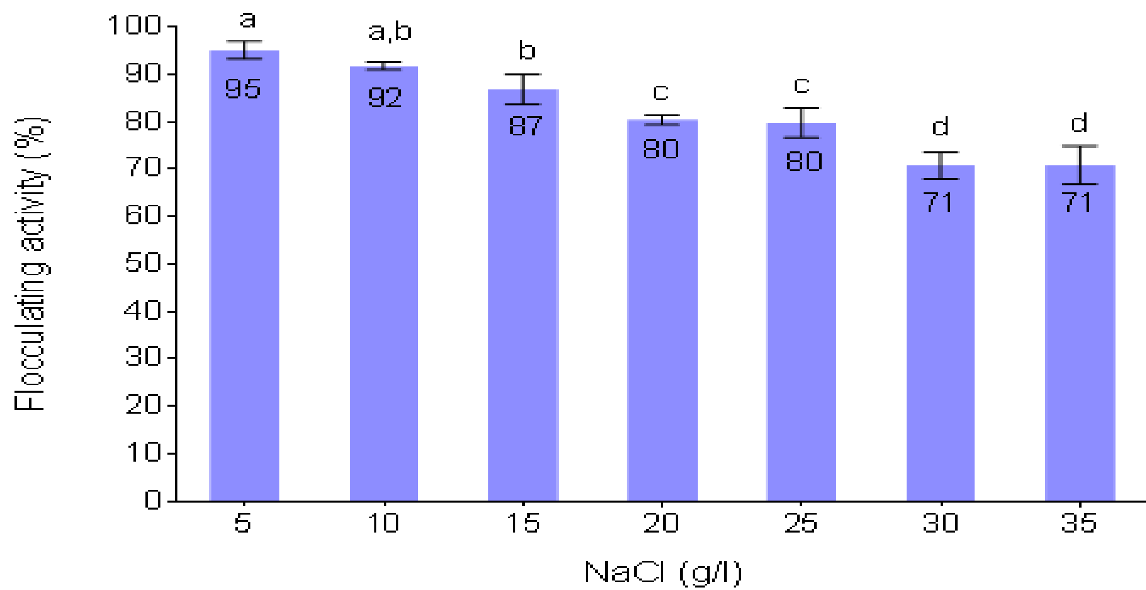


Figure 4.5.12: Saline stability of TST

4.5.9 *In-vitro* cytotoxicity of TST

Figure 4.5.13 and 4.5.14 illustrate the *in-vitro* cytotoxicity of the bioflocculant on MCF7 cell line. The bioflocculant showed no toxic effects on the cell lines. The cell lines exhibited 100% growth in all bioflocculant concentrations. HEK293 cell line also demonstrated 100% cell viability in all bioflocculant concentrations except at 200 µg/µl, where it had 98% cell viability.

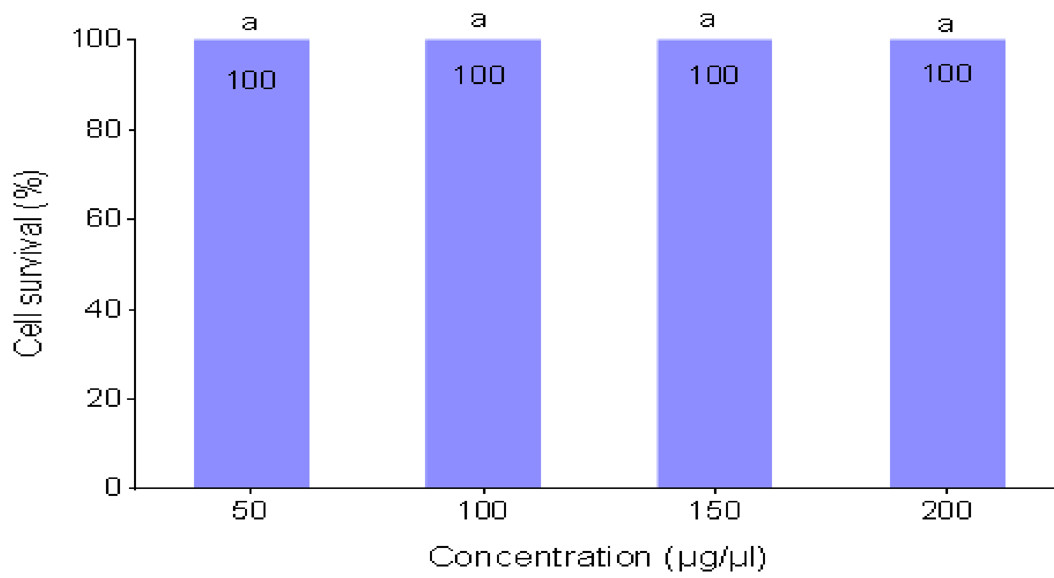


Figure 4.5.13: *In-vitro* cytotoxicity of TST on MCF7 cells

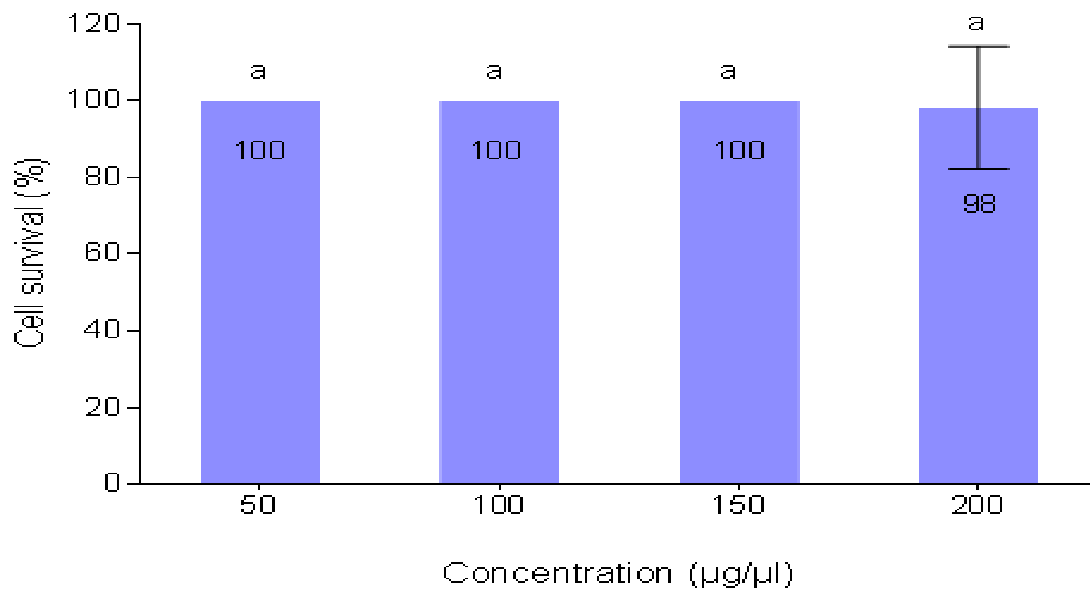


Figure 4.5.14: *In-vitro* cytotoxicity of TST on HEK293 cells

4.5.10 Proposed flocculation mechanism of TST

Table 4.5.3 illustrates the zeta potential of different samples. The zeta potential values of the bioflocculant TST, kaolin suspension, kaolin plus Ba^{2+} and the flocculated kaolin suspension with TST and Ba^{2+} were all negative. However, with addition of Ba^{2+} to the kaolin suspension and the mixture of kaolin particles and bioflocculant TST, there was an increase in zeta potential. Thus, Ba^{2+} mediated a bridge flocculation mechanism between the bioflocculant and kaolin particles in solution.

Table 4.5.3: Zeta potential of the samples

Samples	Zeta potential (mV)
Bioflocculant TST	-11.2±1.912
Kaolin particles	-6.59±3.000
Kaolin particles with Ba^{2+}	-7.01±0.992
Kaolin particles flocculated by TST in the presence of Ba^{2+}	-5.02±2.754

4.5.11 Removal efficiency of TST

Table 4.5.4 and 4.5.5 illustrate the removal efficiency of bioflocculant TST on BOD, COD, N, P and S in water from the Nhalabane Estuary. The bioflocculant showed good removal efficiencies of 99 and 84% on BOD and COD, respectively. It had improved removal efficiencies on COD, N and P than alum and ferric chloride. However, its removal efficiencies on BOD and S were the significantly the same as those of conventional flocculants.

Table 4.5.4: Removal efficiency of bioflocculant TST

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD	COD	N
TST	123.2±0.0	154±0.0	0.93±0.0	0.1±1.7	25±2	0.23±0.0	99^{a}	84^{a}	75a
Alum	123.2±0.0	154±0.0	0.93±0.0	0.2±3.4	55±7.2	0.5±0.2	99^{a}	64^{b}	46^{b}
$FeCl_3$	123.2±0.0	154±0.0	0.93±0.0	0.1±1.7	51±1	0.5±0.0	99^{a}	67^{b}	46^{b}

Table 4.5.5: Removal efficiency of bioflocculant TST

Type of flocculants	Water quality before treatment		Water quality after treatment		Removal efficiency (%)	
	P (mg/l)	S (mg/l)	P (mg/l)	S (mg/l)	P	S
TST	2.83±0.0	0.3±0.1	0.7±0.2	0.1±1.7	75^{a}	67^{a}
Alum	2.83±0.0	0.3±0.1	1.3±0.0	0.1±0.1	54^{b}	68^{a}
$FeCl_3$	2.83±0.0	0.3±0.1	1±0	0.1±1.7	65^{b}	67^{a}

4.6 Results for a consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2

The results obtained during optimisation of medium composition and culture conditions of the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2 are displayed in this section. The observations of the characteristics and removal efficiencies of the purified bioflocculant on wastewater and the identified coliform are also included.

4.6.1 Optimization of cultivating conditions

Optimization of the cultivation process improves the production of bioproducts. Thus the varying of different constituents of the culture medium and growth conditions were done to improve flocculating action and bioflocculant yield. *B. pumilus* JX860616 and *A. faecalis* HCB2 demonstrated potential flocculating activity of 68% in the original pre-culture medium and conditions.

4.6.1.1 Inoculum size

Table 4.6.1 displays the effect of inoculum size of mixed bacterial isolates on bioflocculant production. The inoculum size of 2% (v/v) gave the highest flocculating activity (93.5±3.39%) compared to the other four sizes. The increase or decrease in inoculum size led to a slight decrease in the flocculating activities. However, there was no significant difference between the concentration of 1% and 2%. Thus, an inoculum size of 1% was used in all experiments.

4.6.1.2 Effect of carbon sources on flocculating activity

Bacteria in consortium utilized various carbon and nitrogen sources in the culture medium. Glucose was the most preferred carbon source with the highest flocculating activity of 80.5%, followed by lactose with 70.5% (Table 4.6.1). Fructose, sucrose, maltose, xylose, starch and molasses were poorly utilised by the bacteria for bioflocculant production as shown by the low flocculating activities which were generally less than 70%.

Table 4.6.1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Inoculum size (%)	FA (%) ± SD	Carbon source	FA (%) ± SD	Nitrogen source	FA (%) ± SD	Cations	FA (%) ± SD
1	76.4±6.34[a]	Glucose	80.5±9.07[a]	Yeast extract	97.4±0.57[a]	Control	31.5±9.37[a]
2	93.5±3.39[a]	Molasses	76.6±1.90[a,b]	$(NH_4)SO_4$	92.8±1.71[a,b]	Li^+	87.3±1.11[b]
3	85.1±2.25[a]	Lactose	72.3±4.07[a]	Peptone	84.9±8.87[a]	K^+	89.4±3.82[b]
4	84.3±6.52[a]	Starch	70.1±5.13[a]	Urea	84.7±0.61[a,b]	Na^+	93.3±5.0[b]
5	82.4±14.93[a]	Maltose	64.4±8.92[a]	Casein	79.8±7.53[b]	Ba^{2+}	96.2±1.25[b]
		Xylose	64.2±11.89[a]			Mn^{2+}	89.9±3.59[b]
		Sucrose	63.1±9.54[a]			Fe^{3+}	56.0±1.91[c]
		Fructose	43.1±17.65[b]				

FA denotes flocculating activity while SD denotes standard deviation.

4.6.1.3 Effect of nitrogen sources on flocculating activity

The effect of different nitrogen sources on bioflocculant production was studied and the results are illustrated in Table 4.6.1. All the nitrogen sources showed a potential to be used in bioflocculant production as they resulted in flocculating activities above 80%. Yeast extract was the most preferred nitrogen source with the highest flocculating activity of 97.4% while casein had the least flocculating activity (79.8%).

4.6.1.4 Effect of cations on flocculating activity of crude bioflocculant

Table 4.6.1 demonstrates the effect of metal cations on flocculating activity. All the cations used, including the control (without cation), stimulated the flocculation process and resulted in over 70% of flocculating activities. Ba^{2+} was the most active and preferred metal cation, resulting in the highest flocculating activity of 96%. Fe^{3+} showed the lowest flocculating activity of 77%.

4.6.1.5 Effect of shaking speed on flocculating activity

Figure 4.6.1 illustrates the effect of mixing speed on the bioflocculant production. The mixing speed of 165 rpm was the most conducive, giving the highest flocculating activity of 96%. The decrease in flocculating activity was observed when the mixing speed was below or above 165 rpm.

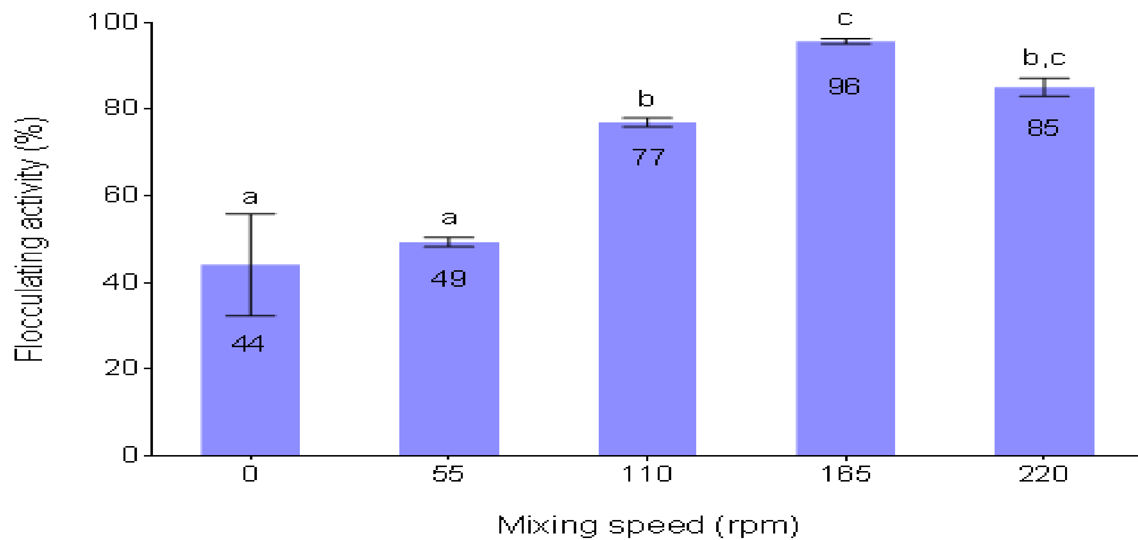


Figure 4.6.1: Effect of shaking speed on flocculating activity

4.6.1.6 Effect of temperature on flocculating activity

Growth temperature has an impact on bioflocculant production. Figure 4.6.2 illustrates the effect of growth temperatures on flocculation activity. The optimum temperature for maximum flocculation was 30°C. The increase or decrease in growth temperature above or below 30°C resulted in a decrease in flocculating activity. Thus, 30°C was used as the optimal temperature for the bioflocculant production.

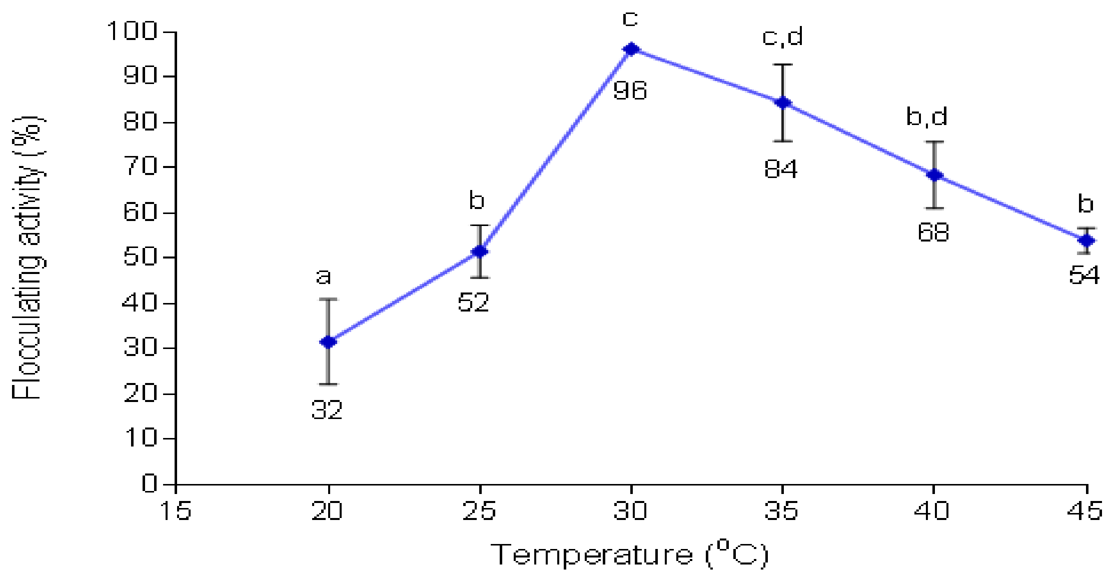


Figure 4.6.2: Effect of temperature on flocculation activity

4.6.1.7 Effect of initial pH on flocculating activity

Figure 4.6.3 shows the effect of initial pH of the growth medium ~~on~~ in the flocculating activity. The bacteria in consortium maintained over 80% of flocculating activity over the weak acidic (pH 5-6) and alkaline (pH 8), with the highest flocculating activity (97%) at pH of 6. It was observed that high alkaline pH 10–12 did not favour bioflocculant production and resulted in poor flocculating activity of less than 70%. pH 12 gave the least flocculating activity of 44%.

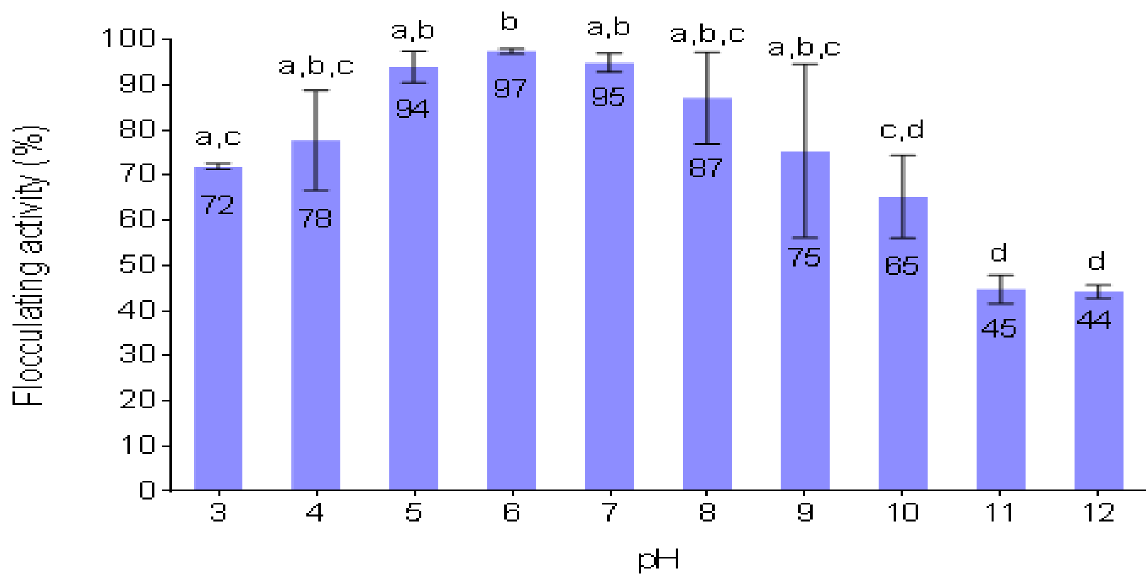


Figure 4.6.3: Effect of initial pH on flocculating activity

4.6.1.8 Time course assay

The effect of time course on flocculating activity (FA), bacterial growth (shown as optical density (OD)) and pH are shown in Figure 4.6.4. The flocculating activity increased relatively to the bacterial growth until 72 hours of growth. The highest flocculating activity (90%) was obtained after 72 hours. A slight decrease in flocculating activity was observed thereafter. The optical density (OD_{550} nm) (which present bacterium growth) increased constantly. The initial pH of the medium dropped constantly from the initial pH of 6.0 to the final pH of 4.3.

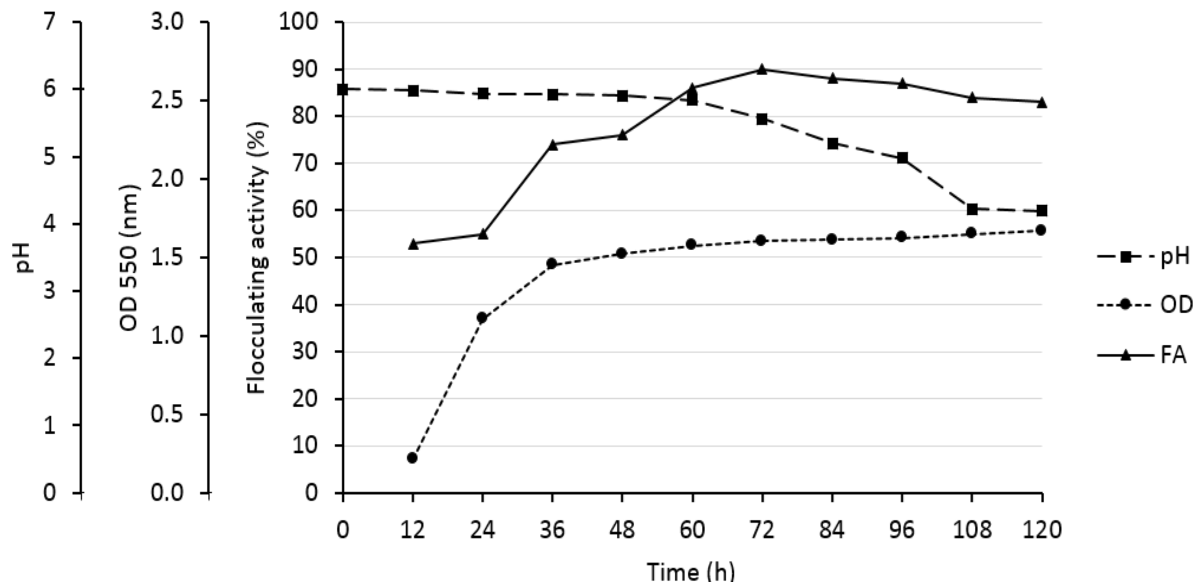


Figure 4.6.4: Effect of time on FA, initial pH and optimum density (OD)

4.6.2 Bioflocculant yield and solubility

Three grams of the bioflocculant was obtained from a litre of growth broth from the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2. The purified bioflocculant was named TPT. TPT was insoluble in all tested solvents, with the exception of water as a solvent.

4.6.3 Effect of dosage size on flocculating activity

Table 4.6.2 displays the results obtained during dosage size assay. The highest flocculating activity of 85.03±2.63% was obtained at a bioflocculant concentration of 0.8 mg/ml. However, there was no statistical difference between the flocculating activity obtained when 0.2 mg/ml and 0.8 mg/ml were used. Thus the concentration of 0.2 mg/ml was preferred to 0.8 mg/ml.

Table 4.6.2: Effect of dosage size and cations on flocculating activity

Dosage (mg/ml)	FA (%) ± SD	Cations	FA (%) ± SD
0.2	78.6±3.33[a]	Na^{+}	59.8±0.96[a,e]
0.4	84.1±1.96[a]	K^{+}	68.5±3.39[a,b]
0.6	82.9±3.08[a]	Ca^{2+}	77.9±0.23[b,c]
0.8	85.03±2.63[a]	Mn^{2+}	82.5±2.93[c,d]
1	84.3±1.60[a]	Ba^{2+}	88.6±4.94[d]
		Fe^{3+}	56.8±6.44[e]
		Control (without cation)	60.3±0.56[a,e]

FA denotes flocculating activity while SD denotes standard deviation.

4.6.4 Effect of cations on flocculating activity of the bioflocculant

Cations enhance flocculating rate by neutralizing and stabilizing the residual negative surface charge of the functional groups on the bioflocculant**.** The flocculating activity of the purified bioflocculant was greatly stimulated by the addition of Li^{+}, Mn^{2+} and Ba^{2+} at concentrations of 1% (Table 4.6.2). These cations showed ~~the~~ flocculating activity above 80%. Ba^{2+} was the most preferred metal cation with flocculating activity of 89%, while Fe^{3+} had a flocculating activity of 56% only.

4.6.5 Physico-chemical analyses of the bioflocculant

4.6.5.1 Surface structures

Figure 4.6.5 shows SEM surface images. TPT had a porous and crystal-like morphology (Figure 4.6.5a). Kaolin particles revealed a fine and smooth structure (Figure 4.6.5b) and a clump like structure was observed in flocculated kaolin particles (Figure 4.6.5c).

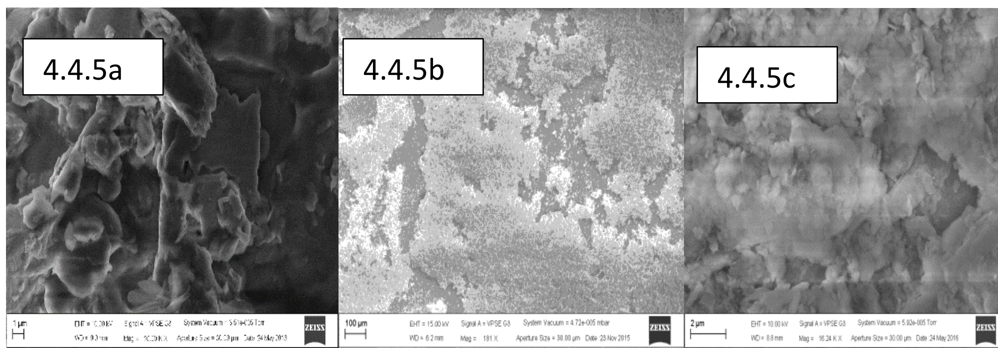


Figure 4.6.5: SEM surface images of flocculated kaolin suspension; bioflocculant TPT (4.6.5a), kaolin particles (4.6.5b) and flocculated kaolin particles (4.6.5c).

4.6.5.2 Electric charge of TPT

The surface charge of TPT was determined. The bioflocculant had a negative surface charge of -14±1.1 mV.

4.6.5.3 Qualitative analyses of TPT

TPT was Ninhydrin positive. The UV spectrum of the bioflocculant demonstrated a sharp absorption peak at 289 nm, which was a characteristic of proteins (Figure 4.6.6).

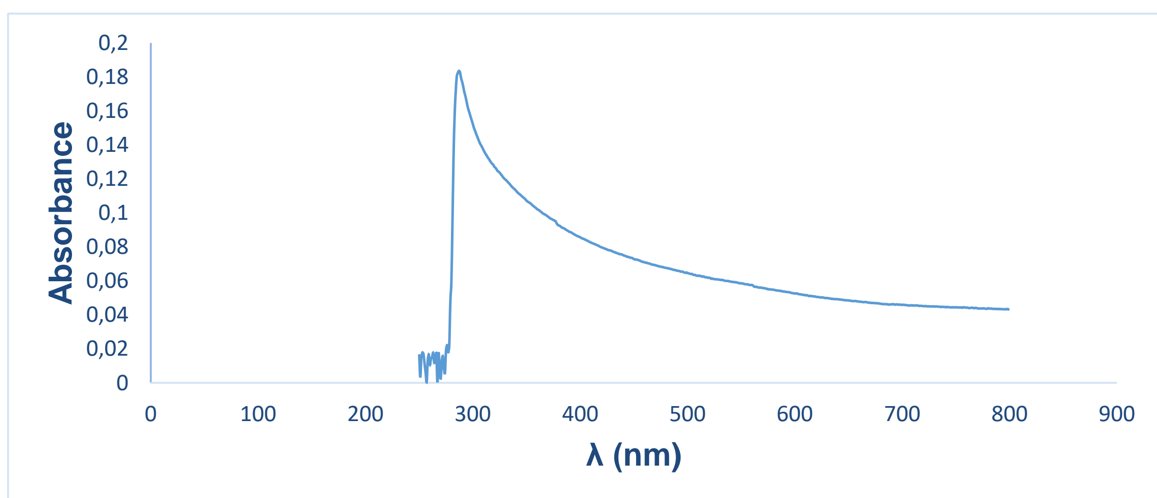


Figure 4.6.6: UV spectrum of TPT

4.6.5.4 Quantitative analyses of TPT

The quantitative chemical composition of TPT was done. The bioflocculant was predominantly composed of carbohydrates (83.1%) and trace protein content (9.7%).

4.6.5.5 Elemental analyses of TPT

Figure 4.6.7 illustrates the elemental analysis of the bioflocculant. The bioflocculant is composed of; N (1.3), C (15.0), O (44.8), P (0.8), Ca (9.0), Cl (2.8), Mg (0.4), S (12.1), K (11.4) and Na (1.9), in mass proportion (% w/t).

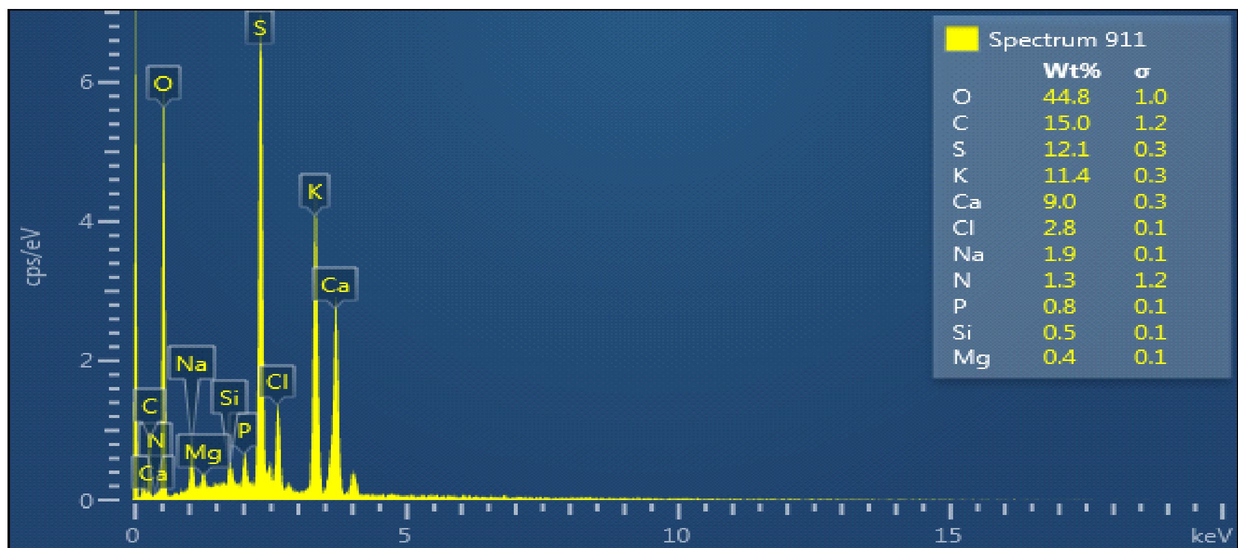


Figure 4.6.7: Elemental analyses of TPT

4.6.5.6 Functional groups of TPT

The functional groups of TPT were determined and the results are shown in Figure 4.6.8. The IR spectrum revealed the presence of different functional groups of the bioflocculant. Hydroxyl (3313 cm^{-1}), amide (1616 cm^{-1}) and amino groups (1080 cm^{-1}) were the main functional groups observed.

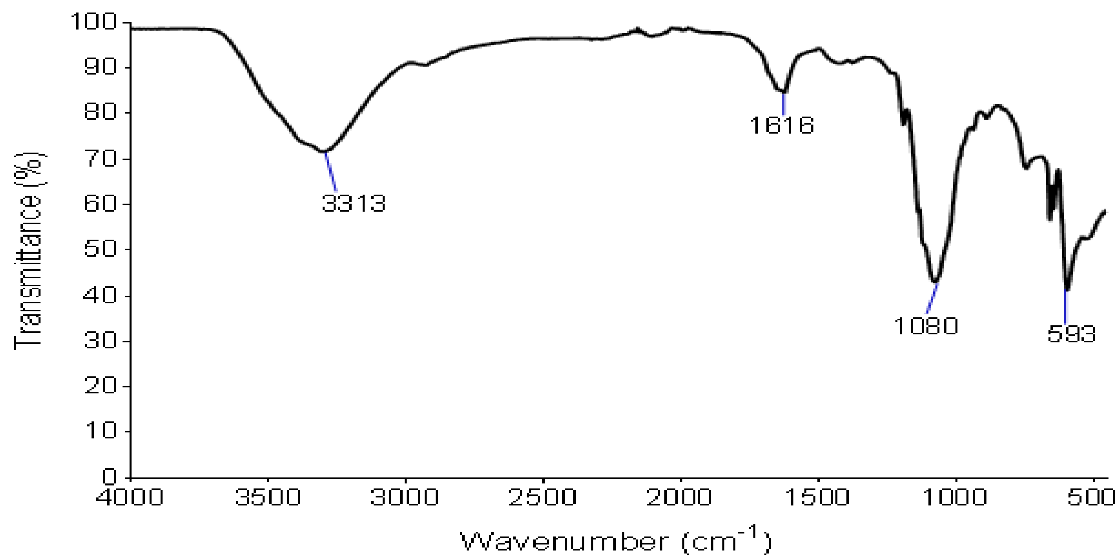


Figure 4.6.8: **IR spectrophotometric analyses of TPT**

4.6.6 Thermal stability of TPT

The effect of temperature on flocculating activity of bioflocculant TPT is shown in Figure 4.6.9. There was a slight decrease in flocculating activity with an increase in temperature. TPT also showed high flocculating activity of 86% even at 100°C after an hour of heat exposure.

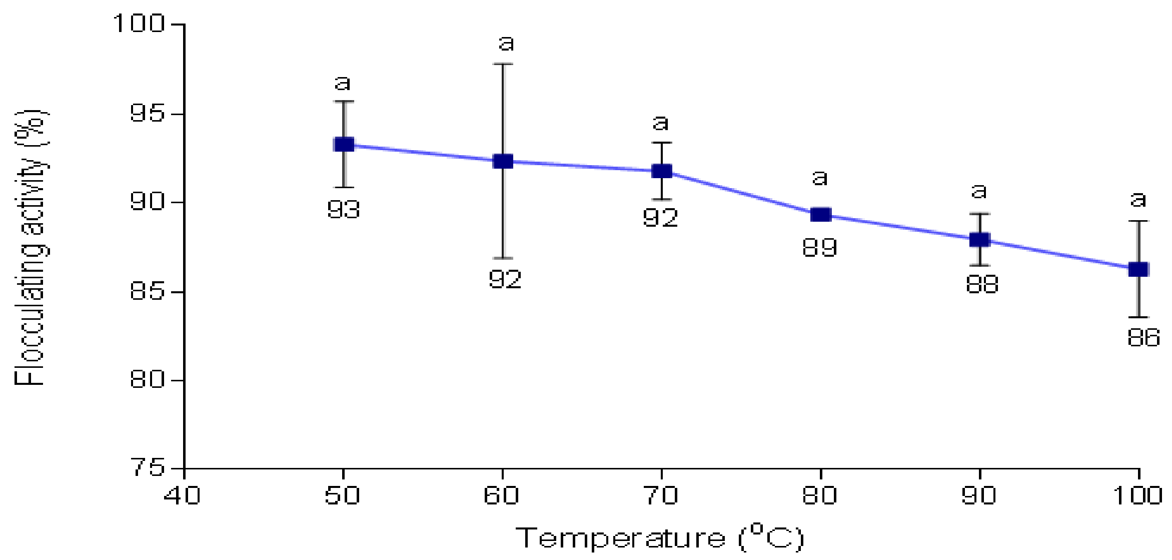


Figure 4.6.9: Thermal stability of TPT

4.6.7 Pyrolysis property of TPT

Figure 4.6.10 shows the pyrolysis properties of bioflocculant TPT. The first degradation was observed at 150°C. The other two degradation temperatures were observed at 270.03 and 402.58°C.

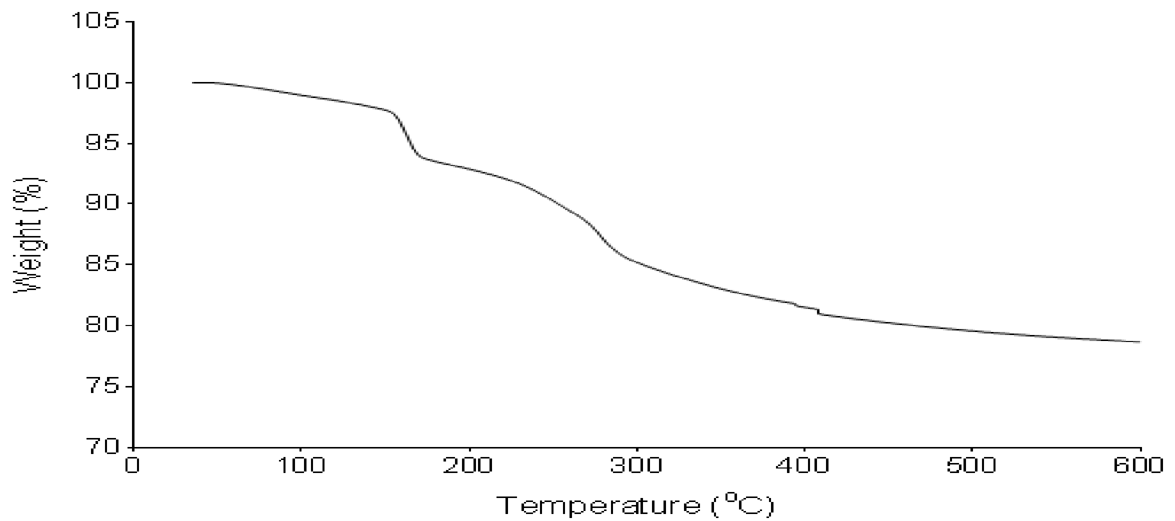


Figure 4.6.10: Pyrolysis property of TPT

4.6.8 pH stability of TPT

Figure 4.6.11 presents the effect of pH on flocculating activity of the bioflocculant. The flocculating activity of TPT was more than 80% in ~~the~~ acidic conditions (pH 3-6) and the maximum flocculating activity (89%) was at pH 3.0. pH 12 demonstrated the least flocculating activity of 58%.

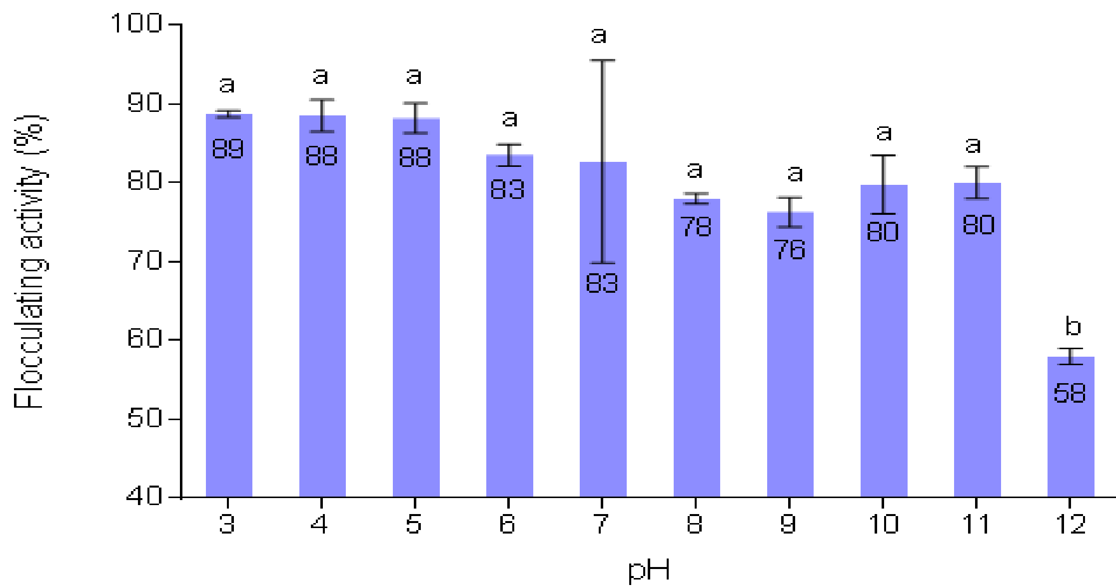


Figure 4.6.11: pH stability of TPT

4.6.9 Saline stability of TPT

The effect of Na^+ concentration on the flocculating activity of ~~the~~ bioflocculant TPT is illustrated in Table 4.6.3. The flocculating activity of the bioflocculant decreased proportionally with an increase in Na^+ concentration. However, TPT maintained high flocculation activity (79.4%) even at ~~the~~ high salinity (35 g/l).

Table 4.6.3: Effect of salinity on flocculating activity

NaCl (g/l)	FA (%) ± SD
5	94.7±2.23^{a}
10	93.5±2.15^{a,b}
15	92,2±1.87^{a,b}
20	92.1±1.04^{a,b}
25	91.2±1.60^{a,b}
30	88.9±1.66^{b}
35	79.4±1.59^{c}

FA denotes flocculating activity while SD denotes standard deviation.

4.6.10 *In-vitro* cytotoxicity of TPT

Figure 4.6.12 and 4.6.13 illustrate the in-vitro cytotoxicity of the bioflocculant on MCF7 cells and HEK293 cells, respectively. The bioflocculant showed no toxic effects on on MCF7 cells. The cell lines exhibited 100% growth in all used bioflocculant concentrations. HEK293 cells demostrated 90% and above cell viability in all concentrations.

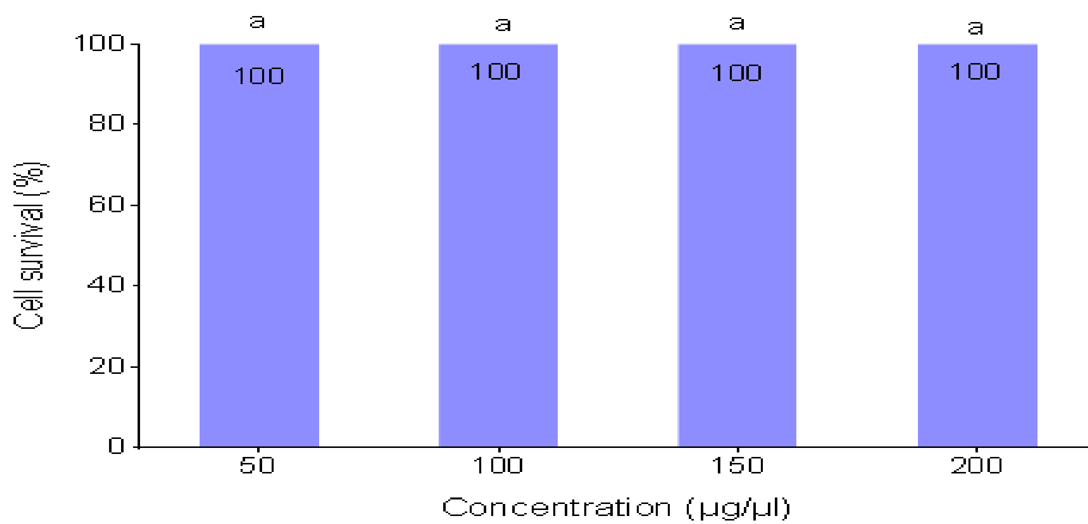


Figure 4.6.12: ***In-vitro*** **cytotoxicity of TPT on MCF7 cells**

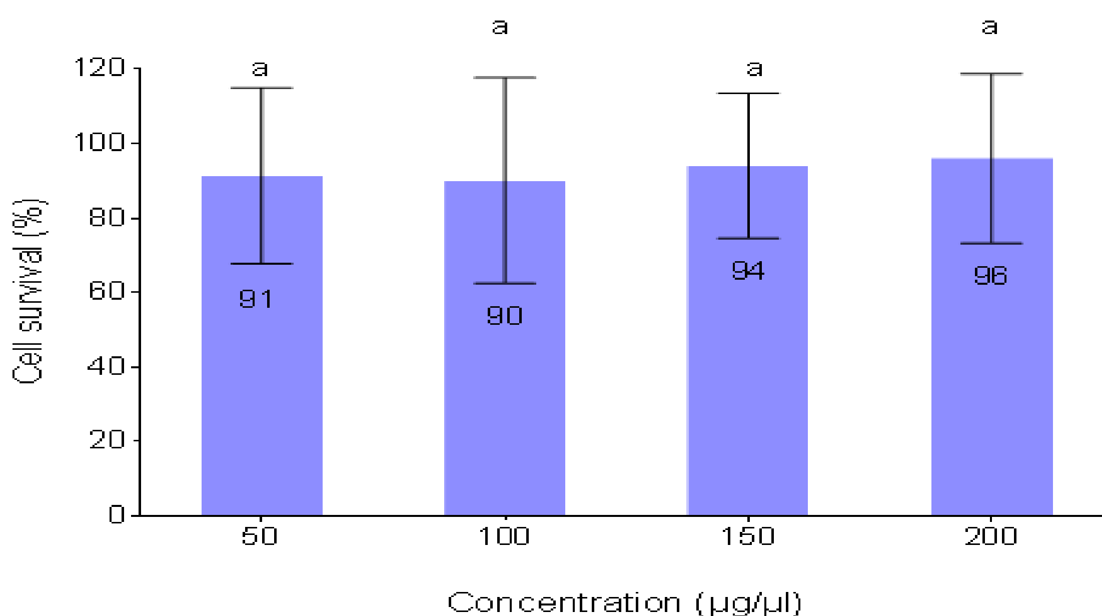


Figure 4.6.13: *In-vitro* cytotoxicity of TPT on HEK293 cells

4.6.11 Proposed flocculation mechanism of TPT

Table 4.6.4 illustrates the zeta potential values of the bioflocculant, kaolin suspension, kaolin plus Ba^{2+} suspension and kaolin suspension flocculated with TPT in the presence of Ba^{2+}. There was a slight increase in the zeta potential with the addition of Ba^{2+}. Thus, it is suggested that Ba^{2+} mediated a bridging mechanism to flocculate kaolin particles in solution.

Table 4.6.4: Zeta potential of different samples

Samples	Zeta potential (mV)
Bioflocculant TPT	-14±1.1
Kaolin particles	-6.59±3.0
Kaolin particles with Ba^{2+}	-7.01±1.0
Kaolin particles flocculated by bioflocculant in presence of Ba^{2+}	-10.4±2.1

4.6.12 Removal efficiency of TPT

Table 4.6.5 displays the removal efficiency of the bioflocculant on BOD, COD and N in wastewater from the Umhlathuzi wastewater plant, RSA. The bioflocculant had comparatively better removal efficiencies on BOD and N than Alum and ferreic chloride.

Table 4.6.5: The removal efficiency of bioflocculant TPT

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal rate (%)		
	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD (mg/l)	COD (mg/l)	N (mg/l)	BOD	COD	N
TPT	0.57±0.0	96±0.6	6.4±0.2	0.1±0.0	69±2	4.4±0.2	83[a]	28[a]	31[a]
Alum	0.57±0.0	96±0.6	6.4±0.2	0.14±0.0	75±4.7	5.1±0.2	75[b]	21[a]	21[b]
$FeCl_3$	0.57±0.0	96. ±0.6	6.4±0.2	0.13±0.0	95±0.0	5.2±0.1	78[b]	0.7[b]	19[c]

Chapter 5

5.1 Discussion

Bioflocculants have in recent times been the focus of attention in biotechnology (Mabinya *et al*., 2012). Soil, activated sludge and fresh water bodies are the predominant reservoirs for isolation of bioflocculant-producers (Salehizadeh and Shojaosadati, 2001; Karthiga and Natarajan, 2015). However, marine environments remain an untapped reservoir for bioflocculant bacteria (Okaiyeto *et al.*, 2015). It is predicted that bioflocculants from marine microorganisms have high flocculating activities since they possess different morphological, physiological and metabolic adaptations to adverse marine environments in comparison to those found in fresh and terrestrial waters (He *et al.,* 2010). Bioflocculants are macro biopolymers produced by microorganisms during their exponential growth (More *et al.,* 2014). Rarely do microbial strains isolated from nature produce bioflocculants at sufficiently high concentrations for commercialisation (Martinko and Madigan, 2006). In order to increase the yields, the culture conditions and medium composition are often optimised. Moreover, microbial species in consortia have also proven to improve bioflocculants when compared to pure strains (Zhu *et al*., 2004). This might be owed to the fact that microbial species coexist in ecological niches in nature and form convoluted relationships which include symbiosis and synergism (Manahan, 2005).

5.2 Optimisation of the medium and culture conditions

Optimization of the growth process is done to improve the yield of microbial byproducts (Pathak *et al.,* 2014). The bioflocculant production is influenced by factors such as medium composition (carbon and nitrogen) and environmental conditions (shaking speed, cation, temperature, pH and time). Moreover, inoculum size also greatly affects bioflocculant production (Nwodo *et al*., 2016). Generally, different microorganisms require different nutritional supplements and optimum fermenting conditions to efficiently synthesise new cell materials and bioflocculants (Madigan and Martinko 2006; Salehizadeh and Yan, 2014). In this study, different factors were optimised in order to increase the bioflocculant yields by both single and mixed bacterial cultures. Good flocculants have flocculanting activity above 90% (Nwodo *et al*., 2016).

5.2.1 Effect of inoculum sizes

Inoculum size effects microbial growth and bioflocculant production (Nwodo *et al*., 2013). Optimal inoculum size is favourable for a microorganism to adapt to the medium which in turn can shorten the lag phase and promotes bioflocculant production (Li *et al*., 2009). A small inoculum size prolongs the stagnant growth phase. A large inoculum size usually results in a niche for the microorganism, overlapping excessively, thereby suppressing bioflocculant production (Salehizadeh and Shojaosadati, 2001). *B. pumilus* JX860616 prefers an inoculum size of 2% (v/v) while *B. subtilis* CSM5, *A. faecalis* HCB2, consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and consortium of *B. pumilus* JX860616 and *Alcaligenes faecalis* HCB2 prefer an inoculum size of 1%. The preferable inoculum sizes range from 1 to 5% (v/v) (Okoh *et al*., 2012). Thus, the bacterial species used in this study were economical.

5.2.2 Effect of nutrients

Nutrients are complexes derived from chemical elements and are utilized by microorganisms for growth, energy and production of byproducts such as bioflocculants. Normally, microbial cells have a chemical formula of $CH_2O_{0.5}N_{0.15}$, implying that C, H, O, and N components are essential for their growth (Madigan *et al*., 2012). The availability and type of nutrient can exert strong physiological control over growth conditions and product formation (Smith, 2009).

5.2.2.1 Effect of carbon sources on flocculating activities

B. pumilus JX860616 utilises lactose as a carbon source while *B. subtilis* CSM5 prefers fructose. *A. faecalis* HCB2 effectively utilises maltose. Similarly, Nwodo *et al*. (2016), found that *Bacillus pumilus* ZAP028 had high flocculating activity when glucose or maltose were used as carbon sources. Zhang *et al*. (2002) reported that glucose inhibited the bioflocculant production by *Sorangium cellulosum*. However in this study glucose was effectively utilized by *B. pumilus* JX860616 and the consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2. Although the flocculating activity was insignificantly higher when sucrose was used instead of glucose by *B. pumilus* JX860616, glucose was the most preferred as it is much cheaper (Smith, 2009; Liu *et al*., 2010). The results were comparable to those of Ogunsade *et al*. (2004) and Bhunia *et al*. (2012) whereby

glucose promoted bioflocculant production by *Bacillus subtilis* and by *Bacillus amyloliquefaciens* ABL 19, respectively. In conclusion, the availability of these nutrients enhanced the bioflocculant production by the bacterial species used which resulted in high flocculating activities.

5.2.2.2 Effect of nitrogen sources on flocculating activities

Nitrogen is the second most abundant element in the cell after carbon. Microorganisms utilise nitrogen for the synthesis of amino acids, proteins and nucleic acids (Madigan *et al*., 2012). *B. pumilus* JX860616, the consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 prefer ammonia sulphate (inorganic nitrogen source) for multiplication and production of bioflocculants. The observations were in contrast to the findings by Hwang *et al*. (2003) and Wang *et al*. (2011). They stated that organic nitrogen sources are preferrable for bioflocculant production and are easier utilised by the cells than the inorganic nitrogen sources. Nevertheless, the observations were in agreement to those of Okaiyeto *et al*. (2016), whereby ammonium sulphate promoted the growth of marine *Bacillus* sp and bioflocculant production. *B. subtilis* CSM5 and *A. faecalis* HCB2 effectively utilised urea while the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2 preferred yeast extract. Kurane and Nohata (1991) reported that *Alcaligenes latus* had maximum flocculating activity when an extract of urea and yeast was used as sole source of carbon. Generally bacterial strains prefer different nitrogen sources to multiply and to produce sufficient bioflocculants.

5.2.3 Effect of cations on flocculating activity of crude bioflocculants

Cations enhance the flocculation rate by neutralising and stabilising the residual negative net surface charge of the bioflocculant functional groups (Yim *et al*., 2007). In this way, the formation of bridges between colloidal particles and bioflocculants is stimulated (Wu and Ye, 2007). The crude and unpurified bioflocculants from *B. pumilus* JX860616 and consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2 were effective when Ba^{2+} and Ca^{2+} was used, while the crude bioflocculant from *B. subtilis* CSM5 was effective in the presence of Mn^{2+}. The results were in accordance to those of Okoh and Ugbenyen (2014), whereby the flocculating

activity was significantly stimulated by all divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}). The flocculating activity of the crude bioflocculant from *A. faecalis* HCB2 was profound in the presence of K^{+}. Na^{+} greatly enhanced the flocculating activity of the crude and unpurified bioflocculant from a consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5. The results were similar to those of Ncedo (2015), whereby the flocculating activity was greatly enhanced by monovalent cations (Na^{+}). Generally, different cations (Ba^{2+} and Mn^{2+}, K^{+} and Na^{+}) effectively neutralised and stabilised the negative charges of the bioflocculants and functional groups of kaolin particle in suspension.

5.2.4 Effect of shaking speed on flocculating activies

The function of agitation is to suspend the microbial cells and nutrients evenly throughout the medium (Smith, 2009). This ensures that nutrients (including oxygen) are available to all microbial cells. Since oxygen is sparingly soluble in aqueous solutions, aerobic growth is stimulated through continuous agitation of the culture medium (Lopez *et al.,* 2003). Agitation also promotes sufficient heat transfer in the culture medium. Each microorganism that produces bioflocculants has its preferred optimum agitation speed. All the bacterial strains used (*B. pumilus* JX860616, *B. subtilis* CSM5, *A. faecalis* HCB2, consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and a consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2) preferred shaking speed ofin a range of 55 to 165 rpm to effectively produce bioflocculants. The results were in close conformity with those reported by Piyo *et al*. (2011) and Cosa and Okoh (2014), whereby the best flocculation was obtained at 160 rpm. The similarities may be due to the same oxygen demand by bacteria at different growth stages (Li *et al.,* 2009).

5.2.5 Effect of temperature on flocculating activities

Metabolic reactions take place effectively at optimum temperatures for microbial growth and bioflocculant production (Okaiyeto *et al.,* 2016). A rise in temperature increases growth, metabolic function and bioflocculant production until the point where denaturation reactions set in (Madigan and Matinko, 2006). Low temperatures may decrease the microbial growth rate and cell wall polymer synthesis (More *et al.,* 2014). In this study, all the bacterial strains (*B. pumilus* JX860616, *B. subtilis* CSM5, *A. faecalis* HCB2, consortium of *B. pumilus* JX860616

and *B. subtilis* CSM5 and a consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2) preferred the optimum growth temperature of between 20 to 30°C to produce bioflocculants. The results were in agreement to other studies (Zhang *et al.,* 2007; Luo *et al*., 2016).

5.2.6 Effect of the initial pH on flocculating activity

Initial pH of the growth medium determines the electrification of the microbial cells and oxidation–reduction potential (Salehizadeh and Shojaosadati, 2001). The alteration in pH could directly or indirectly affect absorption of nutrients in the production medium, metabolic reactions and bioflocculant production. In this study, the effect of the initial pH of the growth medium on flocculating activity was assessed. Bioflocculant from *B. pumilus* JX860616 had the highest flocculating activity when the initial growth pH medium was 7. Sathiyanarayanan *et al*. (2013), reported similar results that the maximum flocculating activity was highest when the initial pH of the medium was adjusted to 7. The optimal initial pH of the medium for bioflocculant production by *Bacillus subtilis* CSM5 and *A. faecalis* HCB2 were 8 and 9. Similar results were observed by Ugbenyen *et al*. (2014), whereby *Bacillus* sp. Gilbert had the highest flocculating activity in weak alkaline conditions (pH 8-9).

The highest flocculating activity was obtained when the initial pH of the culture medium of a consortium of *B. pumilus* JX860616 and *Bacillus subtilis* CSM5 and a consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2 was 6. With this initial pH, bacteria were able to multiply and to produce sufficient bioflocculants. The findings were similar to those reported by Zhang *et al*. (2007), whereby the initial pH of 6 was the best pH for bioflocculant production by strains of BAFRT4, HXCS2, HXTD2, CYGS1 and CYGS4 in consortia.

5.2.7 Time course assay

The relationship between bioflocculant production and culturing time differ among microbial strains (Piyo *et al.,* 2011). The effect of time course on flocculating activity (FA), bacterium growth and pH was investigated for all bacterial strains. The flocculating activity of the bioflocculant from *B. pumilus* JX860616 was optimally produced after 60 hours of cultivation. On the other hand, the initial pH of the medium decreased uniformly. The selection of

bioflocculant producers consider the time interval for bioflocculant production and short time intervals is industrially the most preferable as they are cost effective and less time consuming. Therefore the bioflocculant production by *B. pumilus* JX860616 can be viewed as an economically friendly.

Bioflocculants from other single bacterial species (*B. subtilis* CSM5 and *A. faecalis* HCB2) and constructed consortia (a consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and consortium of *B. subtilis* CSM5 and *A. faecalis* HCB2) had peak flocculating activities within 72 hours of growth. After 72 hours, the flocculating activity of TST only increased insignificantly while the flocculating activities of TMT, TTT, TKT and TPT decreased. These implied that the bioflocculants were produced by biosynthesis during growth and not by cell autolysis (Lu *et al.,* 2005). The decrease in flocculating activities suggested that the bioflocculant producers secreted deflocculating enzymes (Zheng *et al*., 2008). The initial pH of the growth broth decreased from the initial pH value in all the studies. The decrease in the initial pH might have been owed to the fact that organic acidic components were excreted by the bacteria (Okaiyeto *et al.,* 2013). Arafa *et al.* (2014), found similar results whereby the flocculating activity of the bioflocculant from *B. cereus* was maximum within 72 hours of the growth period.

5.3 Factors effecting bioflocculant activities

The effectiveness of the produced bioflocculants is affected by many factors. Dosage size, chemical functionalities (functional groups and chemical components), electrical charge, molecular weight, degree of purity of the bioflocculant, and the structure of bioflocculants are among the factors that effect flocculating activities and mechanisms of flocculation (Badireddy *et al*., 2010; Liu and Cheng, 2010; More *et al*., 2014). Bioflocculant activities and flocculation mechanisms differ from each other, depending on the characteristics of the produced bioflocculants. Thus the characteristics of the biofloccculants were also studied.

5.3.1 Purified bioflocculant yields

Rarely do microorganisms produce bioflocculants in their pure form. The crude extracts are often bound to impurities. The contaminants may compete on adsorption sites of the bioflocculant with colloidal particles in suspension, leading to poor flocculating capabilities (Singha, 2012). *B. pumilus* JX860616 gave 2.4 g/l of the purified bioflocculant (bioflocculant TMT) from the growth broth within 60 hours. *B. subtilis* CSM5 and *A. faecalis* HCB2 yielded 1.5 and 2.7 g/l of the bioflocculants (bioflocculant TTT and TKT, respectively), after purification. TKT and TMT yields were much higher than most of the bioflocculants produced by pure bacterial strains which are often less than 2 g/l (Lin and Harichund, 2011). The high yield might be owed to the ability of the strains to optimally survive and to produce the bioflocculants in the optimised growth conditions and to the polarity of the solvents used during extraction and purification. The yields implied the potential of the bioflocculants to meet the need for wide biotechnological applications (Gomma, 2012).

Low yields of the purified bioflocculants is a major challenge in bioflocculants. However, bioflocculant yields can be improved by using mixed microbial cultures. About 3.1 and 3.0 g of purified bioflocculants (TST and TPT) were obtained from 1 litre of growth broth of the consortium *B. pumilus* JX860616 and *B. subtilis* CSM5 and the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2. Zhang *et al*. (2007), reported the highest bioflocculant production (15 g) ever from a consortium of multiple microorganisms in a litre of growth broth. The obtained yield was five times lower in quantity. Nevertheless, they were higher than the yields mostly obtained from single bacterial strains (including those by single bacterial strains used in this study) (Lin and Harrichurd, 2011). Thus, the results agree with the hypothesis that bioflocculant yields can be increased through the use of mixed microbial strains.

5.3.2 Solubility assays

Bioflocculant biomolecules differ in the balance of the charged, polar and hydrophobic components they possess on their surface. All the purified bioflocculants (TMT, TTT, TKT, TST and TPT) were soluble only in water but insoluble in all other tested organic solvents. Jorand *et al*. (1998), reported that hydrophobic bioflocculants mainly comprised of proteins, whereas the hydrophilic fraction mainly consisted of carbohydrates. All bioflocculants were predominately carbohydrate bioflocculants. Thus the charged and polar groups were solvated by aqueous molecules, making the bioflocculants soluble and hydrophilic (Walker and Wilson, 2005). Moreover, the solvation of these bioflocculants in aqueous solution was attributed to the presence of the hydroxyl groups which might have created hydrogen bonds with water molecules (Okaiyeto *et al.*, 2015).

5.3.3 Effect of dosage sizes on flocculating activities

Dosage size determines the flocculating activities of the bioflocculants. Insufficient bioflocculant dosage sizes have the tendency not to neutralize some negative charges on the colloidal particles in suspension, leading to a poor flocculation process (Li *et al.,* 2007). Excessive amounts of bioflocculant concentrations in suspension can lead to a reduction in the settling of flocculated colloidal particles due to high viscosity (Wang *et al*., 2011). For effective flocculation, optimum concentrations are required.

TMT, TTT, TST and TPT had the highest flocculating activity at 0.6, 0.6, 0.4 and 0.8 mg/ml concentrations, respectively. However, there was no statistical difference shown between these concentrations and the concentration of 0.2 mg/ml of each bioflocculant. Therefore the concentration of 0.2 mg/ml was preferred in all experiments. The concentration of 0.2 mg/ml was optimum enough to flocculate kaolin particles in solution. The effectiveness of these bioflocculants at low concentration translates economic friendliness. The results were comparative to those of Cosa *et al.* (2013) and Cosa and Okoh (2014) whereby the concentration of 0.3 and 0.2 mg/ml of the bioflocculants were optimum for effective flocculation.

A concentration of 0.8 mg/ml of the bioflocculant TKT gave the highest flocculating activity. A concentration between 0.2-0.6 mg/ml may not have permitted the bridging phenomena to happen effectively, hence had low flocculating activities. Moreover, a concentration of TKT above 0.8 mg/ml may have also caused competition and repulsion of the negatively charged kaolin particles, consequently blocking the binding sites available on the surfaces of kaolin particles for the formation of interparticle bridges and leading to the restabilisation of the kaolin particles in solution, resulting in decreased flocculating activity (Guo and Yu, 2014).

5.3.4 Effect of cations on the purified bioflocculants

Cations neutralise and stabilise the negative charge of the functional groups of colloidal kaolin particles in solution and the bioflocculant (He *et al.,* 2010). All the purified bioflocculants (TMT, TTT, TKT, TST and TPT) showed outstanding flocculating activities when Ba^{2+} was used as a cation. Ba^{2+} effectively neutralised the negative surface charge on the bioflocculants and kaolin particles, and thereby shortened the distance between particles and the bioflocculant, which led to high flocculating activities. Several studies agree with this results (Wu and Ye, 2007; Okaiyeto *et al.*, 2015).

5.3.5 Surface structures of the bioflocculants

The surface morphological structure of bioflocculants plays a vital role in the flocculation process (Zhang *et al*., 2007). The surface morphological structure may be accountable for effective or poor flocculating activities of bioflocculants. Most bioflocculants reported in literature do have an amorphous, porous or crystal-like structure (Cosa *et al.,* 2013, Okaiyeto *et al.,* 2015). TMT, TKT and TPT had crystal-like shapes while TTT and TST had amorphous structures. The high level of flocculation by these bioflocculants were attributed to their structures. The flocs in all experiments appeared clustered. This was perceived to be due to the bonds between colloidal particles of kaolin and the functional groups of the bioflocculants. The kaolin clay particles appeared to be fine and evenly scattered.

5.3.6 Electric charges of the bioflocculants

The electric charges of the bioflocculants are greatly influenced by the proportion of their chemical components (Walker and Wilson, 2005). In general, bioflocculants mostly bear a negative charge (Levy *et al.,* 1992; Okaiyeto *et al.,* 2016). This feature enables binding of bioflocculants to positively charge colloidal pollutants due to cation exchange potential, offered through electrostatic interaction (Esparza-Soto and Westerhoff, 2003). The zeta potential of all bioflocculants (TMT, TTT, TKT, TST and TPT) had net negative charge. This implied that all bioflocculants were anionic in nature. The net negative charges were from the carbohydrates and proteins contents, which normally have negatively charged functional groups. Bioflocculants having negative surface net charge show higher hydrophobic character. The hydrophobic components form bonds with positively charged colloidal particles such as metal ions and dye in suspension and leads to high flocculation activities (More *et al.,* 2010). The results were similar to other studies (Liu *et al*., 2015).

5.3.7 Qualitative analyses of the proteins and nucleic acids

The qualitative analyses of proteins and nucleic acids were done for all bioflocculants. All bioflocculants (TMT, TTT, TKT, TST and TPT) revealed to be Ninhydrin positive. The Ninhydrin test is the distinguishing test for all amino acids except proline. The intense purple colour that results is due to a reaction between the alpha amino group and Ninhydrin molecules. The Ninhydrin positive results imply that the bioflocculants possess free amino acids.

Proteins usually show absorption maxima in the range of 275 to 280 nm and between 265 to 295 nm. This absorbance often originates from exposure of the tryptophan residue (Lucas *et al*., 2006). The UV-vis spectrum of all bioflocculants (TMT, TTT, TKT, TST and TPT) displayed intense absorption peaks in a range of 265 to 295, which were distinctive characteristics for a protein content. However, there were no characteristic absorption peaks at 260 nm, implying that the bioflocculant had no nucleic acid components.

5.3.8 Chemical composition of the bioflocculants

The chemical compositions of bioflocculants is an important factor influencing their flocculating activities. Most of the reported bioflocculants in the previous studies were predominantly composed of polysaccharides, proteins, fatty acids, and nucleic acids (Salehizadeh and Shojaosadati, 2003). The chemical assay of TMT, TTT, TKT, TST and TPT revealed the bioflocculants to have high content of carbohydrates and negligible protein content. The composition of both carbohydrates and proteins implied that the bioflocculant might have multiple functional moieties. Multiple functional moieties are indicative of many adsorption sites for colloidal particles. This can lead to high flocculating efficiency as observed by (Verma *et al.,* 2012). The carbohydrate derivatives of the bioflocculants were presumed to be responsible for the stabilities shown by the bioflocculants and the experientially high flocculating activity as they were predominate.

5.3.9 Elemental analyses of the bioflocculants

The elemental analysis of bioflocculants TMT TTT, TKT TST and TPT showed a presence of elements that include: C, N, O, Na, Mg, P, S, Cl and Ca. The elemental composition of the bioflocculants plays a vital role in boflocculant structure and flocculating activity (Cosa *et al.,* 2013). Various elements bring about flexibility and stability of the bioflocculants. The presence of carbon, oxygen and nitrogen elements in all bioflocculants except bioflocculant TMT, further confirmed the bioflocculants as glycoprotein bioflocculants (Devi *et al*., 2015). The absence of a detectable nitrogen element and an abundance of carbon and oxygen elements from TMT affirmed the bioflocculant to be predominately a carbohydrate in nature. Okaiyeto *et al*., (2015), obtained almost similar results with bioflocculant MBF-UFH which possessed different elements such as: C, O, Na, Mg, P, S, Cl, K and Ca.

5.3.10 Functional groups of the bioflocculants

The functional groups of the bioflocculants provide adsorption sites for different colloids in suspension (Xiong *et al*., 2010). The binding capability depends on the number of functional groups available in bioflocculant chains. IR observations done on bioflocculant TMT were indicative of hydroxyl, vinyl, amide and amino groups. All other bioflocculants (TTT, TKT, TST and TPT) had similar functional groups: hydroxyl, amino and amide groups. These functional groups serve as binding sites for cations and colloidal particles in solution, resulting in the formation of diverse chemical bonds (Okaiyeto *et al.*, 2015). The COOH, COO^- and OH groups on the bioflocculants and groups (H^+ and OH^-) on the surface of colloidal particles may interact with the bioflocculant chains to form hydrogen bonds, which permit build up of big flocs. The results were comparative to other studies (ABD-EL-Haleem *et a*l., 2008; Tang *et al.,* 2014).

5.4 Thermal stabilities of the bioflocculants

Bioflocculants have different thermal stabilities as they are sensitive to heat. Bioflocculants with high protein content are generally thermally labile as compared to those rich in carbohydrate content, which are heat stable. When the major component of bioflocculant is glycoprotein, the stability of the bioflocculant depends on the relative contents of protein and carbohydrates (Walker and Wilson, 2005). All bioflocculants (TMT, TTT, TKT, TST and TPT) showed thermal stability. The slight decline in flocculating activities was credited to the denaturation of some protein components of the bioflocculants. The exhibition of thermal stabilities by the bioflocculants was attributed to the nature of the bioflocculants; which were mainly comprised of carbohydrates. The detected carboxyl group in an amide group and hydroxyl groups of the bioflocculants, as shown by IR spectrum, might have permitted the formation of hydrogen bonds, which might be responsible for thermal stability of bioflocculants (Okoh and Ugbenyen, 2014). The results were similar to those of Okaiyeto *et al*. (2015).

5.5 Pyrolysis properties of the bioflocculants

According to Kumari *et al*. (2014) thermal degradation of bioflocculants usually occurs in two steps. The first step involves an increase in temperature to about 150°C resulting in a loss of moisture by bioflocculants (Wang *et al*., 2011). The second step entails depolymerisation of the bioflocculant structure at temperatures above 400°C. There was a decrease in weight in all bioflocculants between 35 and 100°C. The weight loss could be due to a loss of moisture content in the bioflocculants. The moisture content in the bioflocculants was owed to the presence of carboxyl (in amide) and hydroxyl groups. High carboxyl content leads to greater affinity of the carbohydrates' derivatives for water molecules (Kumar and Anand, 1998). The declines in weights of the bioflocculants, observed in temperatures above 100°C were attributed to the degradation of the bioflocculants. The pyrolysis' properties of the bioflocculants affirmed their thermal stabilities.

5.6 pH stabilities of the bioflocculants

The pH of reaction mixtures is a key factor that influences the flocculation process (Zaki *et al.*, 2013). pH may alter bioflocculant charge status and the surface characteristics of colloidal particles in suspension and consequently affecting their flocculating capabilities (Zhang and Lin, 1999). The results vividly showed that TMT, within a wide pH range of 3-12, resulted in flocculation activity above 70%. This implied that TMT can be effectively applied in acidic, neutral and alkaline environments (Adebayo-Tayo and Adebami, 2014). The highest activity (81%) was obtained at pH 6. These observations were similar to those of Zhang *et al.* (2007), whereby the flocculating activity of the obtained purified bioflocculant was the highest at pH 6.

Bioflocculant TTT was stable over a wide range of pH (pH 3-11). However, this bioflocculant had the highest flocculating activity at pH 7. Depending on the functional groups of bioflocculant TTT that are responsible for binding colloids, bioflocculant TTT and kaolin particles absorbed hydrogen ions (H^+) at acidic conditions. This might have slightly weakened the formation of flocs of bioflocculant TTT and kaolin particles. By the same token, hydroxide ions (OH^-) might have also slightly interfered with the combination of the bioflocculant and kaolin particles at alkaline conditions, subsequently leading to a slight but insignificant decrease in flocculating activities (Lin and Harichund, 2012).

The effect of pH on flocculating activity of TKT was also assessed. When the pH of the reaction mixture was a strongly acidic mixture (pH 3-4), the flocculating activity decreased to less than 80%, implying that the bioflocculant and the colloidal kaolin particles did adsorb the H^+, consequently weakening the floc complex formed between the TKT and colloidal kaolin particles, mediated by Ba^{2+}. Moreover, the acidic pH might have denatured some protein components of the bioflocculant, consequently leading to decreased flocculating activity. The results were similar to the observations by Okaiyeto *et al*. (2015), whereby the bioflocculant from *Bacillus toyonensis* strain AEMREG6 showed relative pH stability in the range of 3-11.

The flocculating activity of bioflocculant TST was best in alkaline environments, with the highest flocculation activity at pH 8. However, the decrease in flocculating activity at pH 10-11, suggested alkaline degradation of the bioflocculant which could have resulted from changes that included molecular rearrangements of TST polysaccharide chain fragmentation (Patil *et al.,* 2011). It was thus assumed that the slight decrease in flocculation activity was due to OH^- ions which might have inhibited the formation of flocs of TST and kaolin particles in the mixture. TPT was found to be effective at a wide range of pH (pH 3-9), with the highest flocculation activity at pH 3. However, the decrease in flocculating activity at pH 12, suggested alkaline degradation of the bioflocculant TPT, which could have resulted from changes that included molecular rearrangements of the polysaccharide chain fragmentation of the bioflocculant (Patil *et al.,* 2011).

5.7 Saline stabilities of the bioflocculants

High salt concentrations have a tendency to denature bioflocculants, thus effect their flocculation activities (Atlas and Bartha, 1987). High concentrations of Na^+ do interfere with charges of bioflocculants and promote loss of the functional structures. TMT, TTT, TKT, TST and TPT showed salinity stabilities as they did maintain high flocculation activities above 70%, even at the highest concentration of Na^+ (35 g/l). Marine water is characterized by high salinity of about 33-37% (35 g/l) (Abraham and Marteel-Parrish, 2014). Salinity stabilities of these bioflocculants were attributed to the fact that the bioflocculants were obtained from marine bacterial strains (Li *et al.,* 2008).

5.8 Biosafety of the bioflocculants

Biosafety standards are based on an international technical state of act and relevant legislation which aim to prevent risk to human health and the environment, resulting from activities that involve the use of bioproducts (Smith, 2009). Although, Devi *et al.* (2015), have affirmed bioflocculants as non-toxic compounds, for biosafety reasons, bioflocculants need to be tested for their toxicity before use. This is due to the fact that some bioflocculants may impose toxic effects (Spellman, 2014). Secondary bacterial metabolites have can cause epidemic diseases along with allergenic, neurotoxic and cytotoxic effects (Smith, 2009).

MTT cell proliferation assay was used to assess cell viability of CaCO2, MCF7 and HEK293 cells after being treated with different concentrations of the purified bioflocculants (TMT, TTT, TKT, TST and TPT). All bioflocculants showed a margin of safety as they had no significant cytotoxic effects on the cell lines. The toxicity threshold level of the bioflocculants at mean lethal concentration (LC_{50}) was not determined as the cells showed high percentage viability after MTT assay. The results affirmed the probable safe utilization of the bioflocculants in different applications. The results were in agreement with those of Sharma *et al.* (2017), where the exopolymer produced by *Acinetobacter haemolyticus* showed no toxicity on sheep blood cells. The same biopolymer had no effect in an *in-vivo* study done on rats, where no clinical symptoms were observed. Oh *et al*. (2001) also found similar results whereby bioflocculant from *Paenibacillus* sp AM49 showed no clinical toxic effects on rats.

5.9 Proposed flocculation mechanisms of the bioflocculants

The mechanisms of flocculation by conventional chemical flocculants are well defined and understood. However, the bioflocculation mechanisms are still not fully understood (Salehizadeh and Shojaosadati, 2001). This is due to the fact that the characteristics of bioflocculants differ between bioflocculant producers which result in diverse mechanisms (Strand *et al.,* 2002). There are however two main bioflocculation mechanisms generally proposed: (1) charge neutralization bridging and (2) bridging mechanisms (Li *et al*., 2009). Charge neutralization takes place when the bioflocculant is oppositely charged, as compared to the colloids under treatment. Here, the surface charge density is compact by adsorption on the bioflocculants and the colloids, so they can efficiently approach each other. Bridging mechanism dominates when the bioflocculant extends into the mixture in a distance greater than the distance over which the colloids repulsion can act. In this case, the bioflocculant can bind with colloids to form flocs. The zeta potential analyses were done to assist in determining the flocculation mechanism of the bioflocculants.

The zeta potential of the bioflocculants (TMT, TTT, TKT, TST and TPT) and kaolin particles were all negative. The addition of Ba^{2+} to kaolin suspension and kaolin suspension plus bioflocculants resulted in the increase of zeta-potentials. When the negative charge is reduced or totally abolished, the repulsion forces become terminated and particles easily agglomerate (Hadgson *et al*., 2004). Ba^{2+} increased the adsorption of bioflocculants onto the surface of colloidal kaolin particles by decreasing the negative charge on the bioflocculants and kaolin particles. Thus, the attraction forces were capable of weakening and overcoming the electrostatic repulsion force, reducing the distance between kaolin particles and bioflocculants by compressing the double layer of kaolin particles while increasing the adsorption of bioflocculants on the colloidal kaolin particles. Flocculation is speedy when the zeta potential is below 20 mV (Mines, 2014). It was concluded that Ba^{2+} stimulated rapid flocculation by neutralisation and stabilisation of residual negative charges of bioflocculants, forming the bridges that bind~~s~~ kaolin particles to each other as the big flocs were initiated. The bridging mechanism was owed to the surface structures of the bioflocculants, chemical components, functional groups and the weight of the bioflocculants. The results were comparable to other studies (Guo *et al*., 2015; Olatunji, 2016).

5.10 Application of the bioflocculants in water and wastewater treatments

High levels of COD and BOD often lead to anaerobic conditions, bad odours and stagnant waters that do not support aquatic life (Mihelcic and Zimmerman, 2010). High concentrations of N, P and S induce eutrophication (Sigee, 2005). The applications of the bioflocculants (TMT, TTT, TKT, TST and TPT) for removal of these pollutants from water from the Nhlabane Estuary, from wastewater from local coal mine and from the Umhlathuze wastewater plants, were evaluated in comparison to conventional chemical flocculants (alum and ferric chloride). When compared to the conventional chemical flocculants, the bioflocculants showed much better removal efficiencies in a large number of the tested parameters.

Generally, the removal efficiencies of these bioflocculants were owed to the surface structures of the bioflocculants, to the chemical components and to the functional groups. The results were comparable to other studies (Zhang *et al*., 2007; Luo *et al*., 2014; Okoh and Ugbenyen, 2014; Devi *et al*., 2015), whereby the extracted bioflocculants were more capable of efficiently reducing pollutants in wastewater when compared to chemical flocculants. The removal efficiencies showed by the bioflocculants implied that they have potential in industrial applicability. Moreover, the effectiveness of the bioflocculants suggested that they also have potential to reduce the adverse effects of the predominantly used conventional chemical flocculants (Lin and Harichund, 2011).

Chapter 6

6.1 Conclusion

Optimisation of medium composition and culture conditions of bacterial species (single and in consortia) did significantly improve flocculating activities of the bioflocculants. The maximum flocculating activities by the three single bacterial species and two bacterial consortia were obtained at inoculum sizes of 2% (v/v) or less. Glucose was preferred for production of crude bioflocculant TMT, TST and TPT production by *Bacillus pumilus* JX860616, consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5 and consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2, respectively. Fructose was effectively utilized for TTT production by *B. subtilis* CSM5, while maltose was the best carbon source for the production of TKT by *A. faecalis* HCB2. $(NH_4)_2SO_4$ was efficiently utilised for TMT and TST production by *B. pumilus* JX860616 and the consortium of *B. pumilus* JX860616 and *B. subtilis* CSM5. *B. subtilis* CSM5 and *A. faecalis* HCB2 used urea as the best nitrogen source for production of TTT and TKT while yeast extract supported the production of TPT by consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2. The bioflocculants were optimally produced using the following conditions: 20-30°C, 55-165 rpm, after 60-72 hours in different initial medium pH. The bioflocculants were produced by biosynthesis during bacterial growth.

The bioflocculant yields from bacterial consortia were higher than those of single strains. This agreed with the hypothesis that mixed cultures improve bioflocculant production. They were water soluble, cation dependent and relatively effective at low dosage sizes. The chemical composition showed that bioflocculants are anionic glycoproteins by nature and are predominantly composed of carbohydrates. They revealed the presence of different elements that might have contributed to flexibility of the bioflocculants and were indicative of functional groups responsible for flocculation process. All bioflocculants are stable in a wide range of pH, and were thermal and salinity stable. They further showed a margin of safety as they revealed insignificant cytotoxic effects which affirmed their safe utilisation in different biotechnological applications. The flocculation process was a resulted of double layer compression by cations, chemical reactions and bridging mechanisms. The bioflocculants were compared to conventional chemical flocculants. Generally, they showed better removal efficiencies on effluents.

6.2 Recommendations

For further studies, cheap substrates will be investigated on their effect on production and activity of the bioflocculants. The molecular methods will be employed on bacterial strains in order to increase the yield of bioflocculants. Further analyses that include; proton assay, molecular weight, possible components and chemical structures and their shelf lives will be determined. The bioflocculants will also be applied in other bioremediation practises.

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Appendix

Bacillus pumilus JX860616 and TMT

The optical densities of control (kaolin solution) at 550 nm were 2.834 and 3.096 in different experiments.

Inoculum size (%)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
1	1.142	1.418	1.483
2	0.690	0.854	0.610
3	0.938	0.901	0.895
4	0.969	0.690	1.074
5	1.353	1.563	1.183

Carbon source	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Maltose	1.616	1.622	1.610
Molasses	1.341	1.368	1.588
Fructose	1.176	1.189	1.186
galactose	1.068	1.155	1.115
Starch	0.783	1.059	1.084
Glucose	0.616	0.446	0.452
Sucrose	0.489	0.446	0.551
Lactose	0.582	0.337	0.557

Nitrogen source	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Casein	1.341	1.235	1.226
Urea	0.842	0.910	0.901
peptone	1.074	0.805	0.598
y extract	0.675	0.876	0.830
tryptone	0.486	0.406	1.015
$(NH_4)_2SO_4$	0.536	0.334	0.050

Cations	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Control (without cation)	1.607	1.254	1.025
k^+	1.207	1.214	1.217
Na^+	1.232	1.214	1.217
Li^+	1.068	1.059	0.929
Mn^{2+}	0.777	0.975	1.040
Ca^{2+}	0.579	0.731	0.619
Ba^{2+}	0.517	0.644	0.731
Fe^{3+}	1.207	1.303	1.368

Shaking speed (rpm)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0	2.242	2.139	2.050
55	0.839	0.780	0.799
110	0.762	0.777	0.687
165	0.715	0.731	0.607
220	0.811	0.861	0.681

Temperature (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
20	0.508	0.783	0.910
30	0.384	0.505	0.731
40	0.721	0.505	0.721
50	0.892	0.892	1.217

Initial pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	2.034	1.743	1.511
4	1.427	1.570	1.511
5	1.276	1.133	1.158
6	0.560	0.910	0.842
7	0.508	0.845	0.229
8	0.396	0.759	0.709
9	0.864	0.796	0.796
10	1.167	0.526	1.356
11	1.560	1.517	1.322
12	1.480	1.641	1.164

Time (h)	pH			Growth			OD of supernatant at 550 nm		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	7	7	7	-	-	-	-	-	-
12	6.65	6.8	6.72	0.331	0.473	0.228	2.546	2.448	2.202
24	6.25	6.33	5.95	0.71	0.931	1.021	2.005	1.414	2.341
36	6.13	6.15	5.89	2.512	2.572	2.557	0.510	0.342	0.377
48	5.54	5.87	4.98	2.601	2.423	2.813	0.320	0.340	0.266
60	5.02	4.9	5.5	2.645	2.624	2.639	0.248	0.171	0.152
72	5.03	4.8	5.22	2.851	2.868	2.2.03	0.178	0.302	0.199
84	5.01	4.36	4.74	2.838	2.517	2.553	0.211	0.403	0.180
96	4.97	4.38	4.75	2.740	2.494	2.629	0.2	0.399	0.215
108	4.74	4.39	4.99	2.653	2.529	2.660	0.260	0.387	0.411
120	4.75	4.36	4.99	2.631	2.402	2.798	0.301	0.386	0.397

Dosage (mg/ml)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
0.2	0.498	0.604	0.594
0.4	0.533	0.576	0.567
0.6	0.502	0.536	0.563
0.8	0.641	0.672	0.647
1	0.576	0.672	0.684

Cations	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Fe^{3+}	0.646	0.588	0.562
Mn^{2+}	0.528	0.477	0.548
Ba^{2+}	0.181	0.092	0.169
Li^{+}	1.547	1.508	1.444
Na^{+}	0.136	0.131	0.156
K^{+}	0.110	0.121	0.132
Control (without cation)	0.971	0.997	1.109

Temperature (°C)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
50	0.236	0.223	0,230
60	0.365	0.354	0,360
70	0.380	0.375	0.378
80	0.411	0.425	0.425
90	0.508	0.419	0.463
100	0.582	0.594	0.588

pH	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
3	0.889	0.827	0.830
4	0.802	0.746	0.752
5	0.833	0.796	0.743
6	0.638	0.594	0.502
7	0.867	0.678	0.697
8	0.656	0.954	0.910
9	0.731	0.647	0.916
10	0.854	0.692	0.858

NaCl (g/l)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
5	0.080	0.102	0.136
10	0.149	0.121	0.090
15	0.158	0.173	0.207
20	0.167	0.297	0.186
25	0.167	0.307	0.198
30	0.288	0.291	0.288
35	0.879	0.938	0.799

***In-vitro* cytotoxicity**

TMT (μg/ml)	CaCO2		
	Reading 1	Reading 2	Reading 3
50	1.861	1.974	1.601
100	1.896	2.185	2.454
150	1.873	2.407	2.514
200	1.85	2.407	2.514
Control	1.16	1.291	1.649
TMT (μg/ml)	**HEK293**		
	Reading 1	**Reading 2**	**Reading 3**
50	1.957	1.498	1.286
100	1.58	1.84	1.37
150	1.472	2.018	2.463
200	1.46	1.946	2.526
Control	1.186	1.325	1.691

Zeta potential

Samples	mV		
	Reading 1	Reading 2	Reading 3
TMT	-11.6	-10.3	-5.88
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
TMT, kaolin particles with Ba^{2+}	-4.68	-3.76	-2.09

Removal efficiency

Flocculants	parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
TMT	COD	154	154	154	0	0	0
Alum	COD	154	154	154	0	0	0
$FeCl_3$	COD	154	154	154	0	0	0
TMT	BOD	123.2	123.2	123.2	0	0	0
Alum	BOD	123.2	123.2	123.2	0	0	0
$FeCl_3$	BOD	123.2	123.2	123.2	0	0	0
TMT	N	0.93	0.93	0.93	0.07	0.05	0.09
Alum	N	0.93	0.93	0.93	0.1	0.09	0.09
$FeCl_3$	N	0.93	0.93	0.93	0.3	0.32	0.35
TMT	P	2.83	2.83	2.83	1.3	1.5	1.6
Alum	P	2.82	2.83	2.83	0.8	0.84	0.81
$FeCl_3$	P	2.83	2.83	2.83	1	1.1	1.1
TMT	S	0.3	0.3	0.3	0.07	0.09	0.02
Alum	S	0.3	0.3	0.3	0.15	0.17	0.12
$FeCl_3$	S	0.3	0.3	0.3	0	0	0

***B. subtilis* CSM5 and TTT**

Inoculum size (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
1	0.521	0.807	0.769
2	0.981	0.960	0.930
3	0.810	0.705	1.087
4	0.831	0.773	0.612
5	0.681	1.272	0.915

Carbon source	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Fructose	0.117	0.124	0.094
Maltose	0.246	0.151	0.247
Lactose	0.321	0.452	0.141
Sucrose	0.349	0.465	0.351
Starch	0.441	0.651	0.852
Glucose	0.818	0.42	0.987
Molasse	1.001	1.341	1.025

Nitrogen source	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Urea	0.290	0.090	0.380
Peptone	0.501	0.442	0.305
$(NH_4)_2SO_4$	0.942	0.631	0.380
Yeast extract	1.479	1.206	1.700
Casein	1.603	1.800	2.402

Cations	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Li^+	0.478	0.731	0.791
K^+	0.566	0.641	0.802
Na^+	0.961	0.749	0.880
Ba^{2+}	0.700	0.550	0.465
Mn^{2+}	0.309	0.465	0.775
Ca^{2+}	0.531	0.677	0.983
Fe^{3+}	1.415	2.031	1.416
Control (Without cations)	1.400	2.040	1.501

Mixing speed (rpm)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
0	1.510	1.267	1.413
55	1.250	1.328	1.231
110	0.841	0.795	0.875
165	0.543	0.651	0.713
220	1.024	0.911	0.992

Temperature (^oC)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
20	0.854	0.704	0.935
25	0.815	0.341	0.572
30	0.381	0.501	0.582
35	0.637	0.531	0.694
40	0.976	1.143	1.037
45	0.995	1.143	1.009
50	1.651	1.733	1.671

Initial pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	1.230	1.355	1.051
4	0.600	0.302	0.941
5	0.439	0.517	0.658
6	0.608	0.964	0.845
7	0.463	0.780	0.230
8	0.468	0.460	0.463
9	0.117	0.204	0.447
10	0.240	0.237	0.181
11	0.395	0.573	0.305
12	0.315	0.794	0.620

Time (h)	Initial medium pH			Growth			OD of supernatant at 550 nm		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	6	6	6	-	-	-	-	-	-
12	7.140	7.120	7.120	0.511	0.528	0.558	1.320	1.464	0.902
24	7.120	7.120	7.140	0.536	0.542	0.575	1.237	1.460	1.015
36	7.120	7.100	7.100	0.575	0.556	0.534	0.983	1.302	1.428
48	7.060	7.070	7.060	0.648	0.571	0.599	0.636	0.489	0.783
60	6.930	7.00	6.320	2.21	1.856	2.247	0.551	0.607	0.399
72	7.080	7.150	7.560	2.176	2.360	2.551	0.225	0.293	0.33
84	8.260	7.850	7.420	2.445	2.267	2.366	0.224	0.300	0.339
96	8.340	8.080	7.880	2.388	2.382	2.377	0.368	0.320	0.310
108	8.420	8.360	8.470	2.348	2.518	2.447	0.387	0.378	0.401

Dosage (mg/ml)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0.2	0.476	0.496	0.468
0.4	0.479	0.530	0.487
0.6	0.431	0.405	0.374
0.8	0.397	0.473	0.451
1	0.479	0.541	0.459

Cations	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Na^{+}	0.972	0.850	0.915
K^{+}	0.836	0.845	0.856
Fe^{3+}	1.499	1.587	1.610
Mn^{2+}	0.445	0.436	0.507
Ca^{2+}	0.626	0.657	0.635
Li^{+}	0.757	0.839	0.819
Ba^{2+}	0.431	0.405	0.374
Control (Without cation)	1.481	1.500	1.530

Temperature (^oC)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
50	0.334	0.366	0.555
60	0.513	0.519	0.468
70	0.536	0.499	0.513
80	0.989	0.969	0.717
90	1.020	0.819	0.876
100	1.119	1.043	1.01

pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	0.453	0.497	0.492
4	0.508	0.390	0.328
5	0.347	0.260	0.337
6	0.602	0.340	0.294
7	0.184	0.220	0.155
8	0.335	0.274	0.311
9	0.235	0.557	0.384
10	0.390	0.557	0.384
11	0.396	0.479	0.484

NaCl (g/l)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
5	0.227	0.159	0.192
10	0.236	0.346	0.286
15	0.516	0.370	0.438
20	0.579	0.411	0.453
25	0.390	0.682	0.573
30	0.550	0.783	0.491
35	0.999	0.101	0.102

***In-vitro* cytotoxicity**

TTT (µg/ml)	CaCO2		
	Reading 1	Reading 2	Reading 3
50	1.284	1.399	1.106
100	1.28	1.369	1.107
150	1.45	1.381	1.096
200	1.289	1.325	1.093
Control	1.16	1.291	1.649

Zeta potential

Samples	mV		
	Reading 1	Reading 2	Reading 3
TTT	-17.6	-16.8	-15.1
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
TTT, kaolin particles with Ba^{2+}	-3.73	-5.73	-7.00

Removal efficiency

Flocculants	parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
TTT	COD	1557	1557	1557	608	599	610
Alum	COD	1557	1557	1557	1031	1027	1033
$FeCl_3$	COD	1557	1557	1557	927	927	929
TTT	BOD	6.4	6.4	6.4	2.9	2.9	2.11
Alum	BOD	6.4	6.4	6.4	2.5	2.5	2.4
$FeCl_3$	BOD	6.4	6.4	6.4	2.7	2.7	2.7
TTT	S	4.1	4.0	4.1	0.96	1.00	1.22
Alum	S	4.1	4.0	4.1	1.54	1.52	1.46
$FeCl_3$	S	4.1	4.0	4.1	1.11	1.07	1.09

Alcaligenes faecalis **HCB2 and TKT**

Inoculum size (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
1	0.831	0.981	0.670
2	0.995	0.880	0.799
3	0.897	1.071	0.897
4	0.829	0.609	0.991
5	0.557	0.639	0.959

Carbon source	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Maltose	0.260	0.330	0.210
Fructose	0.334	0.390	0.286
Lactose	0.536	0.479	0.581
Starch	0.600	0.669	0.510
Glucose	0.834	0.727	0.930
Sucrose	1.111	1.069	1.101
Molasse	1.755	1.074	2.390
Nitrogen source	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Urea	0.086	0.045	0.087
$(NH_4)_2SO_4$	0.327	0.489	0.102
Peptone	0.558	0.518	0.590
Casein	1.853	1.995	1.765
Yeast extract	2.200	2.002	2.400

Cations	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Control	1.479	1.321	1.490
Na^+	0.911	1.300	0.991
Li^+	0.759	0.702	0.628
K^+	0.56	0.601	0.700
Fe^{2+}	1.271	1.713	1.161
Mn^{2+}	1.260	0.897	0.970
Ba^{2+}	0.993	0.986	1.090
Ca^{2+}	0.918	0.641	0.893
Al^{3+}	1.861	1.593	1.400
Fe^{3+}	1.861	1.593	1.400

Shaking speed (rpm)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
0	1.387	1.395	1.971
55	1.490	1.374	1.531
110	0.473	0.413	0.489
165	0.262	0.567	0.293
220	0.478	0.365	0.421

Cultivating temperature (^oC)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
25	0.374	0.720	0.580
30	0.330	0.331	0.543
35	0.519	0.420	0.422
40	0.595	0.526	0.576
45	1.416	1.471	1.419

Initial pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	1.501	1.216	1.300
4	1.259	1.561	1.090
5	1.031	1.231	1.200
6	0.901	0.780	0.820
7	0.854	1.001	0.995
8	0.797	0.388	0.508
9	0.467	0.500	0.285
10	0.701	0.490	0.989
11	0.981	0.851	0.500
12	0.617	0.700	0.951

Time (h)	Initial medium pH			Growth			OD of supernatant at 550 nm		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	6	6	6	-	-	-	-	-	-
12	7.300	7.260	7.250	0.283	0.323	0.116	2.162	1.648	1.726
24	7.280	7.260	7.240	0.261	0.338	0.314	1.140	1.445	1.537
36	7.250	7.250	7.290	0.287	0.306	0.348	1.345	1.030	1.535
48	7.220	7.220	7.230	0.300	0.363	0.315	0.596	0.545	0.717
60	7.110	7.260	7.200	0.388	0.818	0.414	0.540	0.428	0.572
72	7.120	6.900	6.940	2.077	0.908	1. 380	0.207	0.218	0.314
84	6.860	6.790	7.200	2.06	2.306	2.470	0.330	0.332	0.262
96	7.380	7.620	6.630	2.38	2.465	2.438	0.344	0.273	0.326
108	8.510	7.980	7.780	2.441	2.495	2.530	0.385	0.347	0.329

Dosage (mg/ml)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0.2	0.570	0.633	0.616
0.4	0.645	0.632	0.705
0.6	0.729	0.700	0.571
0.8	0.447	0.486	0.403
1	0.434	0.487	0.493

Cations	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Na^{+}	1.062	0.931	1.00
K^{+}	0.912	0.922	0.936
Fe^{3+}	1.638	1.734	1.757
Mn^{2+}	0.487	0.478	0.553
Ca^{2+}	0.683	0.717	0.692
Li^{+}	0.827	0.915	0.896
Ba^{2+}	0.471	0.444	0.41
Control (Without cation)	1.510	1.370	1.649

Temperature (^{o}C)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
50	0.710	0.716	0.650
60	0.816	0.603	0.682
70	0.649	0.784	0.745
80	0.867	0.943	0.949
90	1.032	0.874	0.915
100	1.188	1.099	1.078

NaCl (g/l)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
5	0.325	0.334	0.086
10	0.339	0.370	0.386
15	0.430	0.529	0.340
20	0.627	0.490	0.734
25	0.667	0.703	0.557
30	0.566	0.958	1.002
35	0.889	0.836	0.840

pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	0.960	1.084	0.836
4	0.836	1.022	0.650
5	0.464	0.526	0.402
6	0.464	0.279	0.619
7	0.217	0.279	0.155
8	0.464	0.341	0.588
9	0.279	0.248	0.310
10	0.310	0.279	0.341
11	0.310	0.526	0.093
12	0.341	0.433	0.248

***In-vitro* cytotoxicity**

TKT (µg/ml)	CaCO2		
	Reading 1	**Reading 2**	**Reading 3**
50	1.368	1.633	1.649
100	0.829	1.481	0.864
150	1.077	1.399	1.017
200	1.062	1.348	1.034
Control	1.16	1.291	1.649
TKT (µg/ml)	**HEK293**		
	Reading 1	**Reading 2**	**Reading 3**
50	2.034	1.985	1.832
100	1.733	0.942	1.263
150	1.672	1.033	1.219
200	1.707	1.159	1.14
Control	1.186	1.325	1.691

Zetapotential

Samples	mV		
	Reading 1	**Reading 2**	**Reading 3**
TKT	-17.7	-17.1	-16.4
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
TKT, kaolin particles with Ba^{2+}	-3.73	-4.34	-5.17

Flocculants	Parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	**Tube 2**	**Tube 3**	**Tube 1**	**Tube 2**	**Tube 3**
TKT	COD	1557	1557	1557	438	437	434
Alum	COD	1557	1557	1557	828	827	829
$FeCl_3$	COD	1557	1557	1557	754	754	755
TKT	BOD	6.4	6.4	6.4	2.6	2.7	2.6
Alum	BOD	6.4	6.4	6.4	3.0	3.2	3.4
$FeCl_3$	BOD	6.4	6.4	6.4	2.9	2.9	3.0
TKT	S	4.1	4.0	4.1	0.88	1.11	1.10
Alum	S	4.1	4.0	4.1	1.38	1.38	1.36
$FeCl_3$	S	4.1	4.0	4.1	1.07	1.10	1.09

***Bacillus pumilus* JX860616 and *B. subtilis* CSM5 and TST**

Inoculum size (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
1	0.533	0.309	0.428
2	0.238	0.133	0.299
3	0.220	0.166	0.320
4	0.705	0.908	0.179
5	0.577	0.610	0.971

Carbon source	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Lactose	0.471	0.998	0.689
Sucrose	0.699	0.334	0.579
Maltose	0.163	0.756	0.652
Fructose	1.501	1.648	1.650
Xylose	1.480	1.510	1.758
Starch	1.333	1.871	1.614
Molasse	0.367	0.929	0.891
Glucose	0.238	0.133	0.299

Nitrogen source	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Urea	0.209	0.127	0.262
Ammonium sulphate	0.047	0.074	0.060
Casein	0.206	0.118	0.067
Peptone	0.174	0.217	0.154
Yeast extract	0.502	0.342	0.382
Urea	0.209	0.127	0.262

Cations	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Lithium	0.093	0.131	0.104
Aluminium	1.008	1.086	0.558
Barium	0.055	0.064	0.052
Potassium	0.036	0.063	0.034
Sodium	0.037	0.031	0.045
Manganese	0.071	0.060	0.046
Control (without cation)	0.026	0.695	0.45

Shaking speed (rpm)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0	1.194	1.444	1.474
55	0.835	0.810	0.822
110	0.788	0.483	0.616
160	0.13	0.143	0.155
220	0.119	0.309	0.254

Temperature (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
20	0.944	1.094	0.938
25	0.572	0.475	0.659
30	0.055	0.064	0.052
35	0.059	0.290	0.029
40	0.753	0.937	0.709
45	1.221	0.871	1.052
50	2.641	2.110	2.441

Initial pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	0.285	0.495	0.633
4	0.368	0.505	0.529
5	0.15	0.197	0.55
6	0.13	0.143	0.155
7	0.961	1.094	1.880
8	1.278	1.491	1.313
9	1.981	1.824	2.139
10	2.352	2.350	2.368
11	2.807	2.851	2.835
12	2.868	2.864	2.839

Time (h)	Initial medium pH			Growth			OD of supernatant at 550 nm		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	6	6	6	-	-	-	-	-	-
12	5.48	5.59	5.52	0.588	0.559	0.722	1.864	2.043	2.687
24	5.12	4.64	4.93	1.539	1.588	1.440	1.386	1.662	1.655
36	4.54	4.44	4.12	2.347	2.138	1.973	0.712	0.854	0.615
48	3.77	3.65	3.61	2.548	2.284	2.409	0.490	0.506	0.510
60	3.54	3.55	3.47	2.713	2.653	2.263	0.410	0.169	0.549
72	3.54	3.51	3.48	2.669	2.609	2.659	0.318	0.148	0.429
84	3.45	3.41	3.51	2.468	2.531	2.440	0.226	0.126	0.308
96	3.46	3.44	3.41	2.44	2.46	2.43	0.176	0.124	0.307
108	3.38	3.38	3.33	2.413	2.395	2.485	0.126	0.130	0.302
120	3.20	3.22	3.20	1.882	1.834	1.942	0.203	0.188	0.160

Dosage size (mg/ml)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0.2	0.89	1.002	0.63
0.4	0.909	0.999	0.716
0.6	0.98	0.85	0.874
0.8	0.777	0.697	0.64
1	0.76	0.882	0.887

Cations	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Li^{+}	0.464	0.381	0.409
Na^{+}	0.659	0.523	0.638
K^{+}	0.517	0.768	0.749
Mn^{2+}	0.418	0.545	0.356
Ba^{2+}	0.232	0.378	0.310
Ca^{2+}	0.777	0.697	0.641
Fe^{3+}	1.548	1.755	1.582
Control (Without cation)	0.871	0.852	0.989

Temperature (°C)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
50	0.331	0.53	0.439
60	0.345	0.439	0.429
70	0.422	0.434	0.548
80	0.705	0.496	0.504
90	0.584	0.419	0.695
100	0.593	0.692	0.609

pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	1.052	0.872	0.976
4	0.867	0.964	0.876
5	1.434	1.358	1.427
6	1.341	1.57	1.345
7	0.354	0.477	0.407
8	0.209	0.139	0.111
9	0.175	0.22	0.102
10	0.273	0.139	0.216
11	0.325	0.234	0.392
3	1.052	0.872	0.976

NaCl (g/l)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
5	0.204	0.157	0.091
10	0.232	0.284	0.244
15	0.339	0.52	0.364
20	0.624	0.629	0.572
25	0.535	0.729	0.615
30	0.82	0.899	1.013
35	1	0.829	0.835

***In-vitro* cytotoxicity**

TST (µg/ml)	**MFC7**		
	Reading 1	**Reading 2**	**Reading 3**
50	0.561	0.265	0.525
100	0.316	0.288	0.672
150	0.191	0.283	0.253
200	0.236	0.269	0.253
Control	0.156	0.157	0.267
TST (µg/ml)	**HEK 293**		
	Reading 1	**Reading 2**	**Reading 3**
50	1.757	1.332	1.248
100	1.796	1.441	1.299
150	1.711	1.267	1.291
200	1.633	1.277	1.22
Control	1.186	1.325	1.691

Zetapotential

Samples	**mV**		
	Reading 1	**Reading 2**	**Reading 3**
TST	-12.6	-11.0	-9.98
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
TST, kaolin particles with Ba^{2+}	-3.53	-4.73	-6.81

Flocculants	parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
TST	COD	154	154	154	25	27	23
Alum	COD	154	154	154	63	53	49
$FeCl_3$	COD	154	154	154	52	50	51
TST	BOD	123.2	123.2	123.2	0.1	0.1	0.2
Alum	BOD	123.2	123.2	123.2	0.2	0.2	0.2
$FeCl_3$	BOD	123.2	123.2	123.2	0.2	0.1	0.1
TST	N	0.93	0.93	0.93	0.20	0.27	0.23
Alum	N	0.93	0.93	0.93	0.5	0.7	0.3
$FeCl_3$	N	0.93	0.93	0.93	0.5	0.5	0.5
TST	P	2.83	2.83	2.83	0.6	0.9	0.5
Alum	P	2.83	2.83	2.83	1.2	1.3	1.3
$FeCl_3$	P	2.83	2.83	2.83	1.2	1.1	1.0
TST	S	0.3	0.3	0.3	0.1	0.1	0.1
Alum	S	0.3	0.3	0.3	0.08	0.09	0.12
$FeCl_3$	S	0.3	0.3	0.3	0.1	0.1	0.1

***Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2**

Inoculum size (%)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
1	0.867	0.818	0.505
2	0.182	0.109	0.316
3	0.392	0.532	0.458
4	0.293	0.698	0.47
5	0.042	0.954	0.637

Carbon source	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
Glucose	0.874	0.623	0.314
Xylose	0.689	1.252	1.384
Maltose	1.082	1.391	0,838
Starch	1.098	0.785	0.895
Lactose	0.76	1.16	0.817
Sucrose	1.225	1.386	0.814
Fructose	1.145	2.185	1.958
Molasse	0.728	0.662	0.78

Nitrogen source	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Casein	0.723	0.358	0.793
Urea	0.463	0.461	0.489
Yeast	0.1	0.065	0.074
Peptone	0.355	0.267	0.78
Ammonium sulphate	0.238	0.163	0.267

Cations	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
Li^+	0.356	0.424	0.396
Mn^{2+}	0.266	0.231	0.439
Na^+	0.315	0.280	0.032
Ba^{2+}	0.115	0.158	0.079
K^+	0.251	0.463	0.270
Fe^{3+}	1.362	1.360	1.364
Control	2.105	2.110	2.100

Mixing speed (rpm)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
0	1.514	1.523	2.152
55	1.548	1.607	1.551
110	0.743	0.681	0.712
165	0.155	0.124	0.124
220	0.526	0.458	0.399

Temperature (%)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
20	1.841	2.421	2.099
25	1.307	1.660	1.540
30	0.115	0.158	0.079
35	0.308	0.785	0.359
40	1.243	0.843	0.850
45	1.408	1.524	1.352

Initial pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	0.853	0.892	0.857
4	0.44	1.081	0.55
5	0.141	0.312	0.108
6	0.1	0.065	0.074
7	0.098	0.149	0.225
8	0.754	0.16	0.284
9	1.414	0.628	0.247
10	1.398	0.969	0.863
11	1.6	1.764	1.77
12	1.737	1.767	1.678

Time (h)	Initial medium pH			Growth			OD of supernatant at 550 nm		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
0	6	6	6	-	-	-	-	-	-
12	5.98	6	5.960	0.214	0.218	0.210	1.455	1.498	1.412
24	5.907	5.953	5.930	1	1.2	1.111	1.381	1.393	1.405
36	5.950	5.890	5.920	1.311	1.452	1.593	0.805	0.816	0.794
48	5.870	5.890	5.900	1.522	1.524	1.520	0.691	0.795	0.743
60	5.810	5.840	5.870	1.575	1.610	1.540	0.468	0.433	0.398
72	5.560	5.509	5.611	1.590	1.620	1.605	0.310	0.297	0.323
84	5.180	5.200	5.220	1.602	1.611	1.620	0.330	0.414	0.372
96	4.980	4.881	5.079	1.626	1.623	1.629	0.402	0.385	0.419
108	4.221	4.321	4.121	1.649	1.721	1.577	0.509	0.495	0.481
120	4.186	4.193	4.200	1.684	1.672	1.660	0.526	0.552	0.500

NaCl (g/l)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
5	0.176	0.091	0.226
10	0.195	0.27	0.137
15	0.218	0.305	0.199
20	0.262	0.206	0.263
25	0.224	0.276	0.322
30	0.307	0.401	0.321
35	0.694	0.607	0.609

Dosage (mg/ml)	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
0.2	0.585	0.780	0.625
0.4	0.548	0.427	0.498
0.6	0.638	0.455	0.498
0.8	0.557	0.415	0.418
1	0.486	0.437	0.536

Cations	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Na^{+}	1.223	1.279	1.232
K^{+}	0.907	0.923	1.096
Ca^{2+}	0.681	0.694	0.681
Mn^{2+}	0.464	0.641	0.517
Ba^{2+}	0.464	0.180	0.418
Fe^{3+}	1.384	1.118	1.508
Control (without cation)	1.092	0.984	1.641

Temperature (°C)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
50	0.235	0.124	0.266
60	0.226	0.412	0.074
70	0.207	0.251	0.307
80	0.331	0.322	0.337
90	0.393	0.406	0.322
100	0.337	0.433	0.505

pH	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
3	0.362	0.353	0.337
4	0.310	0.334	0.427
5	0.307	0.372	0.424
6	0.492	0.483	0.560
7	0.632	0.102	0.879
8	0.669	0.672	0.706
9	0.799	0.684	0.724
10	0.746	0.619	0.517
11	0.619	0.681	0.557
12	1.331	1.269	1.300

***In-vitro* cytotoxicity**

TPT (μg/ml)	MFC7		
	Reading 1	Reading 2	Reading 3
50	0.725	0.25	0.698
100	0.524	0.71	0.674
150	0.744	0.49	0.434
200	0.597	0.358	0.467
Control	0.156	0.157	0.267
TPT (μg/ml)	**HEK293**		
	Reading 1	**Reading 2**	**Reading 3**
50	1.662	1.163	1.073
100	1.689	1.163	0.935
150	1.63	0.921	1.051
200	1.711	1.198	1.126
Control	1.186	1.325	1.691

Zetapotential

Samples	mV		
	Reading 1	Reading 2	Reading 3
TPT	-15.1	-13.8	-13.0
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
TPT, kaolin particles with Ba^{2+}	-9.08	-9.28	-12.8

Flocculants	parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
TPT	COD	96	96	95	71	67	69
Alum	COD	96	96	95	77	76	70
$FeCl_3$	COD	96	96	95	95	95	95
TPT	BOD	0.57	0.57	0.56	0.1	0.1	0.09
Alum	BOD	0.57	0.57	0.56	0.15	0.13	0.15
$FeCl_3$	BOD	0.57	0.57	0.56	0.14	0.14	0.14
TPT	N	6.4	6.3	6.5	4.5	4.5	4.4
Alum	N	6.4	6.3	6.5	5.1	5.1	5
$FeCl_3$	N	6.4	6.3	6.5	5.3	5.3	5.2

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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGD 2016/135							
Project Title	Characterization, biosafety and effectiveness of bioflouccculants by bacterial isolates from Sodwana Bay sediment							
Principal Researcher/ Investigator	TS Maliehe							
Supervisor and Co-supervisor	Prof AK Basson				Prof JJ Simonis			
Department	Biochemistry and Microbiology & Hydrology							
Nature of Project	Honours/4th Year		Master's		Doctoral	x	Departmental	

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions: **(1) This certificate is valid for 3 years from the date of issue.**
(2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-31 August 2017]
(3) Principal researcher must submit a report at the end of project in respect of ethical compliance.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
					X
Low Risk		Medium Risk		High Risk	
X					

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			X
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

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  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion

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Full Length Research Paper

Production, characterisation and flocculation mechanism of bioflocculant TMT^{-1} from marine *Bacillus pumilus* JX860616

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Bioflocculant from marine *Bacillus pumilus* JX860616 was characterised and its flocculation mechanism determined. The bacterium was identified by 16S rRNA and the bioflocculant was obtained through solvent extraction after optimum medium composition and culture conditions were established. The physicochemical analysis of the bioflocculant were obtained by scanning electron microscopic (SEM) equipped with elemental detector, Fourier transform infrared (IR) spectrophotometry. The highest flocculating activity (93.3%) was obtained with optimum medium composition of the energy sources of glucose and $(NH_4)_2SO_4$ and culture conditions of; initial pH 6; and Ba^{2+} after 72 h, at the inoculum size of 2% (v/v). The bioflocculant (2.4 g/L) revealed to have a crystal-like porous structure and had the total carbohydrate of 83.1% w/w and proteins content of 6% w/w. The elemental analysis showed the presence of C (17.0), O (46.0), Na (4.3), Mg (6.8), P (4.1), S (7.0), Cl (5.9), K (7.4) and Ca (0.7) (% w/w). IR observations were indicative of hydroxyl, vinyl, amide and aliphatic amine groups. The bridging mechanism mediated by Ba^{2+} on colloidal Kaolin particles was proposed. The high flocculating activity of TMT^{-1} implied that it has a promise in industrial applications.

Key words: *Bacillus pumilus* JX860616, bioflocculant TMT^{-1}, flocculating activity and flocculation mechanism.

INTRODUCTION

Natural water often consists of thermodynamically unstable and kinetically non-labile colloidal particles that do not even settle out under gravity (Spellman, 2014). Colloidal particles contribute to water turbidity and often shelter some pathogens from inactivation by disinfectants (Mines, 2014). Colloids removal is a paramount goal in wastewater treatment that extensively employs chemo-physico methods such as flocculation (McCarthy, 2011; Karthiga and Natarajan, 2015). Flocculation is a purification technique whereby polymers form bridges with colloids and bind them into large and settled able agglomerates (Davis and Masten, 2014). Inorganic and synthetic organic flocculants are extensively used in vast biotechnological applications due to their cost

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Full Length Research Paper

Production and characteristics of bioflocculant TPT^{-1} from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2

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The combination of microorganisms in consortia enhances high bioflocculant yields. The study aimed at producing and characterising bioflocculant from *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2. Bioflocculant TPT^{-1} was obtained through ethanol extraction after optimum conditions were established. The characteristics of the bioflocculant TPT^{-1} were obtained by scanning electron microscope equipped with elemental detector, Zetasizer nano, ultraviolet-visible spectrophotometry. Fourier transform infrared (IR) spectrophotometry and the thermal decomposition was used to conduct the thermal gravimetric analysis. The flocculation mechanism of TPT^{-1} on Kaolin suspension was obtained by Zetasizer Nano. Glucose and yeast extract were the best energy sources, yielding 3.0 g/L of TPT^{-1} in optimum conditions (30°C, 165 rpm, initial pH 6 and 72h). TPT^{-1} revealed to be an anionic, heat stable glycoprotein, with the total carbohydrate content of 83.1% (w/w) and the total proteins content of 9.7% (w/w). The elemental analysis demonstrated the presence of N (1.3), C (15.0), O (44.8), P (0.8), Ca (9.0), Cl (2.8), Mg (0.4), S (12.1), K (11.4) and Na (1.9) in mass proportion (% w/w), while the IR spectrum showed the presence of hydroxyl, carbonyl and amine groups. Ba^{2+} mediated bridge flocculation mechanism between the bioflocculant TPT^{-1} and Kaolin particles. The high flocculating capability (90%) and characteristics of TPT^{-1} suggested its potentiality in industrial applications.

Key words: Bioflocculant TPT^{-1}, flocculating activity, flocculation mechanism and bacterial consortium.

INTRODUCTION

Flocculation is the natural process whereby flocculants are used to flocculate, settle and remove particles, suspended solids and colour in solutions (Cong-Liang et al., 2012). Flocculating agents are widely used in industrial fields such as dredging, textile dyeing, mining, pharmacology, cosmetology, wastewater treatment, food and fermentation processes (Zhang et al., 1999; Salehizadeh and Shojaosadati, 2001). Flocculants are grouped as inorganic (aluminium salts), synthetic (polyacrylamide) and natural occurring flocculants (bioflocculants and chitosan) (Okaiyeto et al., 2015). Inorganic and synthetic organic flocculants are

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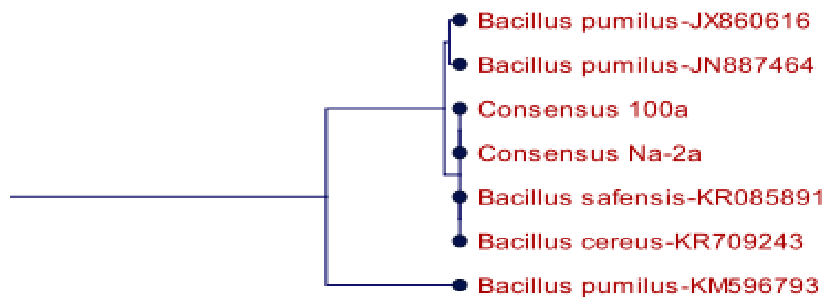
Bioflocculating bacterial strains used



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