ISOLATION, SCREENING, IDENTIFICATION AND OPTIMIZATION OF MICROORGANISMS WITH BIOFLOCCULANT-PRODUCTION POTENTIAL FROM KOMBUCHA TEA SCOBIES AND ITS APPLICATION IN WASTEWATER TREATMENT

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DECLARATION

I, Phakamani Hopewell Tsilo, solemnly declare that this dissertation submitted to the Department of Biochemistry and Microbiology, University of Zululand, for a Master of Science degree in Microbiology, is my original work, unless otherwise where indicated and has not been submitted to any University for any degree award or examination purposes.

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DEDICATION

I dedicate this dissertation to God, my family, colleagues, and my friends who contributed immensely during this research with support.

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ANOVA:	Analysis of variance
BLAST:	Basic Local Alignment Search Tool
BOD:	Biochemical oxygen demand
BSA:	Bovine serum albumin
COD:	Chemical oxygen demand
DCB:	Divalent cation bridging theory
DLVO:	Derjaguin, Landau, Verwey and Overbeek theory
DNA:	Deoxyribo nucleic acid
EPS:	Extracellular polymeric substances
FA:	Flocculating activity
FTIR:	Fourier transform infrared spectrophotometer
OD:	Optical density
PAC:	Polyaluminium chloride
PAM:	Polyacrylamide
PolyDADMAC:	Polydiallyl dimethyl ammonium chloride
RNA:	Ribonucleic acid
RE:	Removal efficiency
RSA:	Republic of South Africa
SEM:	Scanning electron microscope
XRD:	X-Ray diffraction

Abstract

Microorganisms excrete metabolites during growth. Extracellular polysaccharides such as, glycoproteins, proteins, and nucleic acids contributes to the synthesis of bioflocculants. Through formation of bridges between suspended particles in a solution, bioflocculants assist with increase in flocculation, leading to particles precipitation. It is noteworthy that when impurities are flocculated into flocs, they tend to settle down and can easily be removed. In this study, the ability of the yeast *Pichia kudriavzevii* from Kombucha teas Scoby to produce a bioflocculant was investigated. A Kombucha tea Scoby was brought from Pine Town, KwaZulu-Natal Province of South Africa, and bioflocculant-producing microorganisms were isolated from the Scoby. Dilutions of the samples were made and cultivated in nutirents agar plates to obtain pure cultures. Pure cultures were screened for bioflocculant-production potential against kaolin clay suspension (4 g/L) as the test material. The isolate with better bioflocculant production potential was selected for the bioflocculant production. The isolate showed the highest flocculating activity of 84.93% against kaolin clay suspension. The identification of the organism using 16S rRNA showed the organism to have 99% similarities with the yeast *Pichia kudriavzevii* with accession number MH545928.1. The strain was capable of producing a bioflocculant under the optimal production conditions of 1% (v/v) inoculum size, glucose (92%) as carbon source, peptone (94%) as a source of nitrogen, at a temperature of 35 ºC (97%). At an initial pH 7 of the medium, the maximum flocculating activity (91%) was achieved and the shaking speed of 140 rpm (96%) resulted into an optimum production of a bioflocculant by *P. kudriavzevii*. A bioflocculant yield of 2.836 g was produced from 1 L fermentation broth after 60 hours of incubation at 35 ºC. The colour of a produced bioflocculant was milky-white and in a powdered form. The purified bioflocculant obtained had the highest flocculating activity of 80% at a dosage size of 0.4 mg/mL against kaolin suspension. The purified bioflocculant was cation-dependent with Al^{3+} (72%) as the most favourable cation. The purified bioflocculant was able to retain about 70% flocculating activity when exposed to 121 º C temperatures for 15 minutes, which confirmed the thermostability of the bioflocculant. After the Fourier-transform infrared (FT-IR) analysis of the purified bioflocculant, it was revealed that hydroxyl, carboxyl, amine, thiocynates, alkynes, furan functional groups are present in the molecular chain of a bioflocculant and are

responsible for its best flocculation ability. The chemical composition of the purified bioflocculant showed the presence of sugar (69%), protein (11%), and uronic acid (16%) with carbohydrates as main component and responsible for its thermal stability characteristics. A cumulus-like structure of the bioflocculant was revealed using a scanning electron microscope (SEM) and the weight fractions from the elemental analysis of the purified bioflocculant were C, N, O, Na, Mg, Al, P, S, Cl, K, Ca, which accounts for 16.92: 1.03: 43:76: 0.18: 0.40: 0.80: 14.44: 1.48: 0.31: 0.34: 20.35 (%wt), respectively. The XRD analysis of the purified bioflocculant showed that the bioflocculant have bigger particles with diffraction peaks at 10º and 40º indicating the crystallinity of the purified bioflocculant. The produced bioflocculant is highly effective at 5 g/L salt concentration with flocculating activity of 81%. Increasing the salt concentration inhibibed the flocculating activity. The produced bioflocculant showed highest removal efficiencies compared to the conventional chemical flocculants (Fe^{3+} and alum) used in the study. The purified bioflocculant exhibited a remarkable removal efficiency in both domestic and industrial (coal mine) wastewater for COD, BOD, phosphorus, sulfate, nitrate, and total nitrogen with removal efficiency of 49% and 43% (COD), 79% and 64% (BOD), 46% and 48% (phosphorus), 79% and 73% (sulphate), 61% and 71% (nitrate), and 50% (total nitrogen), respectively. The bioflocculant also revealed strong dye removal ability with the removal efficiency of 81% (Congo red), 81% (nigrosine), 73% (methylene blue), and 74% (safranin).

In conclusion, this bioflocculant from *P. kudriavzevii* seems to have a potential in the removal of different impurities from various wastewater especially in the domestic and industrial wastewater.

Key words: Kombucha tea Scoby, flocculating activity, *Pichia kudriavzevii*, bioflocculant, kaolin clay, wastewater, removal efficiency, chemical flocculants

Chapter 1

This chapter contains introduction and brief baground about history of bioflocculants, rational, problem statement and hypothesis of the study.

1.0 Introduction

Water pollution is a serious issue that affects the entire world. If the pollution continues like this, soon the world will suffer due to lack of safe drinkable water to sustain life. Water attributed natural disasters amounts to about 90%, globally (Mwamba, 2009; Grisaffi, 2020). One out of 10 people, approximately on the planet has no access to safe water for drinking to attain energy for life, even though there is about 78% of the water in our surrounding (Rout and Sharma, 2011).

Studies suggest that about a quarter of people by the year 2050 will most probably live in a recurring fresh-water affected countries (Mekonnen and Hoekstra, 2016). The quality and security of water in South Africa have been reported to be greatly affected by pollution (Galvin and Roux, 2019). Most of the pollution of water is as a consequence of agricultural, domestic, and industrial wastes that are being deposited to the water bodies untreated. Resulting from these contaminants, the potential of water to provision for its biotic communities and its human consumption is compromised (Okaiyeto *et al*., 2016). Natural phenomena including earthquakes, volcanos and storms are among the major contributing factors to the water quality and the ecological status of water in the world (Anju *et al*., 2010). Many people around the globe have suffered due to these factors, as most of them fail to attain safe water for drinking (Dlamini *et al*., 2019). Water is a fundamental basis for life, everything that exists needs water to thrive. Therefore, it should be handled with the greatest precision to circumvent its pollution by these anthropogenic contaminants. Water is so vital in a way that the reports suggest a search for it from other planets (Bhatnagar and Sillanpää, 2010; Rani *et al*., 2013).

The Global Oceanic Environmental Survey (GOES) association envisions water pollution attributed issues such as those that bring about the gigantic danger for the

life on earth, in the years to come (Yamashita *et al*., 2005). For counteracting the hurdles of water pollution, the organization (GOES) has proposed several measures such as proper use of disposal of chemicals, control of erosion and sediments from constructions, and non-degradable products should not be disposed in water (Halpaap *et al*., 2019). These proposed measures should be taken promptly for yielding good results. Some countries, including China and India, are more affected than others by elevated water pollution levels. The reason for this could be associated with the fact that India has seen a rise in economic growth (Foster and Rosenzweig, 2003). The economic growth is usually directly proportional to the waste that is produced and liquidated to water bodies which impose a serious pressure to accessible potable water (Murty and Kumer, 2011). Approximately, 90% of the water in China is polluted most probably as a result of a number of industries in the country (Ji *et al.,* 2011).

South Africa also suffers greatly from water pollution as it is one of the 30 driest countries globally (Nkwonta and Ochieng, 2009). Numerous data from the South African weather services indicate that some parts of the country has high precipitations than others. The country has the lowest annual rainfall of 450 mm per year in comparison with the global average of 860 mm per year (Arenas-Sanchez *et al*., 2016). To supply water to consumers, the country relies mostly on rainfall and non-polluted watercourses. The country can therefore, be considered as a water-scarce country (Bwapwa, 2019).

To guarantee and regulate the level or amount of domestic and industrial pollutants liquidated to the environment water bodies, some countries have adhered to stringent code of practice. One of such regulations is to mandate that before disposal of wastewater to water bodies, it must have been appropriately treated utilizing

dissimilar wastewater treatment technologies before release into rivers, streams, and dams (Nouha *et al*., 2018). Solvent extraction, ion exchange, flocculation/coagulation, adsorption, electrolysis, oxidation, and filtration are some of the examples of wastewater treatment technologies frequently used prior to disposal of wastewater to oceans and rivers (Ntsaluba *et al*., 2011; Sivasanker *et al*., 2020). Some of these technologies have a very huge negative impact on the society and new technologies of wastewater treatment need to be developed.

The phenomena of flocculation/coagulation technologies are of paramount importance in wastewater treatment (Zhao *et al*., 2020). These wastewater treatment systems have been used for a very long time. For instance, coagulation was first used by the Egyptians to clarify water from the rivers around 2000 BC. As coagulating agents, they were making use of almond smear around the container. On the other hand, they were plunging an arm into the container for flocculation of water (Faust and Aly, 1998). Romans were using flocculants for wastewater treatment around 77 AD. Some records show that alum was first used in England around 1757, and by that time they were even able to clean public water supplies (Faust and Aly, 1998). This brief history of wastewater treatment highlights how much vital is this process of flocculation/coagulation and how it has been changing over the years.

Flocculants have been utilized for wastewater treatment, and the ideal flocculating agents have been the chemical flocculants (Lee *et al*., 2014). The speed of settling of the particles can be used to demonstrate the flocculation process that is specific to cohesive sediments. This vital parameter has been evaluated with the use of innumerable existing models. Amongst the models, there is low power with or

without degeneracy parameter function and also a constant formulation with or without hindered settling (Spearman and Roberts, 2000). Chemical flocculants have advantages such as competence, cheap, and do not need intensive skills for their operation (Li *et al*., 2013). In general, flocculation process results in the flocs formation in the presence of traditional and biological flocculants. The problem with the chemical flocculants application in wastewater treatment is that, they have a very serious detrimental effect in life and the environment. These chemical agents are responsible for quite innumerable diseases to humans. For example, they are very toxic, carcinogenic, cause neurological diseases and also not biodegradable, thus affecting the ecosystem (Ugbenyen *et al*., 2012). Alum has been mostly used for wastewater treatment because it is very cheap and its application does not have any hurdles (Nguyen *et al*., 2012). However, alum leads to complications such as Alzheimer's disease and various other health-related complications (Wong *et al*., 2012). As an alternative, there has been a search for novel practice or strategies for wastewater treatment processes which involves biological flocculants.

These are alternative (biological methods) means for wastewater treatment involving the use of bioflocculants. Bioflocculants are naturally occurring extracellular biopolymers that result from the exudation of microorganisms including fungi, bacteria, algae, and yeast (Aljuboori *et al*., 2013). Their sustainability and biodegradability are some of the key factors that make them to be more advantageous when compared to the currently in-use chemical flocculants. Bioflocculants are secondary metabolites reported to have no negative effects and they can be reused (Farag *et al*., 2014). These extracellular biopolymers show promising capabilities in flocculation processes as they are circumspect to utilize and are economically vital to replace the in-use chemical flocculants. The major

bottleneck with bioflocculants is the low yield and high cost of production (Salehizadeh and Shajaosadati, 2001). Thus, these factors are the major hindrances for its large scale production such as unpredictable processes of production and delay research progress.

The application of bioflocculants has been observed in various sectors for wastewater treatment such as in wastewater from swine (Guo and Chen, 2017), coal wastewater slurry dewatering (Yang *et al*., 2019), wastewater from textile industries (Buthelezi *et al*., 2012), wastewater from starch (Deng *et al*., 2003), and meat processing wastewater (Pu *et al*., 2014).

The problem with fermentation media for bioflocculant production is that it is very expensive, therefore it is of paramount importance to find bioflocculants from dissimilar environments or sources that will be able to utilize cheap substrates as nutrients supplement. Studies show that the capabilities of the bioflocculant were first investigated from the yeast *Levure casseeuse* by Louis Pasteur (1876) and Bordet (1899) also observed a phenomenon of such nature from the bacterial culture (Shahadat *et al*., 2017). The *Zoogloea ramigera* organism from activated sludge floc forming by Bloch was reported to have flocculation properties (Shahadat *et al*., 2017).

Novel bioflocculant-producing microbes have been searched and screened to determine their bioflocculants production potential for high yield and excellent flocculation efficacies with nominal energy consumption (Day *et al*., 1999). Most of the reported bioflocculants have been obtained from innumerable sources such as marine water, activated sludge, soil samples, domestic drainages, and many other places (Rahman *et al*., 2014). This study, however, was looking for novel

bioflocculant-producing strains from Kombucha tea broth with bioflocculant-producing potential.

Kombucha tea is made up of a Scoby. This Scoby (Symbiotic culture of Bacteria and Yeast), which are produced during a complete fermentation by acetic acid bacteria, lactic acid bacteria and yeast to form several sour beverages and foods, such as, Kimchi and Kombucha tea (Villarreal-Soto *et al*., 2018). The Scoby in its most commonly observable form is a cellulose-based biofilm, gelatinous or a microbial mat suspended air-liquid interfaced container. The word pellicle is the formal known name for this consolidated layer. Its ability to absorb water gives the Scoby a potential to be even able to house a small amount of the previous media and products (Villarreal-Soto *et al*., 2018). A variety of bacterial species and yeasts are utilized for each particular Scoby product. These cultures commonly composed of aerobic, Gram-negative acetic acid bacterial species, including *Gluconobacter, Komagataibacter*, and *Acetobacter*, yeasts including *Zyosaccharomyces* and *Saccharomyces* and lastly aerobic, Gram-positive lactic acid bacteria, such as, *Lactobacillus* (Pandey *et al*., 2000; Yao and Nokes, 2013).

This study focused on the screening and isolation of the novel bioflocculant-producing microorganisms from Kombucha tea Scoby as a source microorganisms. The study also focused on the identification of bacteria with bioflocculating producing-potential, optimization of culture conditions for improved bioflocculant yield and flocculating activity and the characteristics of the produced bioflocculant as well as its application in wastewater treatment.

1.1 Rational of the study

Various flocculating agents have been used in wastewater treatment, dredging, and industrial downstream processes, including inorganic chemical flocculants, organic synthetic flocculants, and naturally occurring flocculants (Yokoi *et al*., 1998). Chemical flocculants such as polyacrylamide has been frequently used as high-polymer organic flocculants because it is both inexpensive and reliable. However, some of chemical flocculants derivatives are difficult to degrade in nature (Bhatia *et al*., 2014). Moreover, some of the monomers and monomer derivatives from these synthetic polymers are hazardous to human and animal health. Because of their harmless degradable intermediates in the atmosphere, microbial flocculants have been one of the solutions to these environmental problems in recent years compared to in-use chemical flocculants (Pan *et al*., 2009). Although microbial flocculants are environmentally friendly and non-toxic, but they are too expensive to be produced and have short shelf-life. They are also very low in their production. .

1.2 Problem statement

Urbanization is viewed as an advantage in South Africa and has been considered as the fundamental basis for the gradual growth of a country because it leads to economic development which is of positive influence. However, urbanization results to the pollution of little water available for consumption. The results of industries and many other factors inherent with urbanization are catastrophic to the ecosystem. Such pollutants from industries need to be treated or removed from water before being utilized again. The chemical flocculants have been frequently utilized for the treatment of polluted water, but chemical flocculants have severe consequences to the life and the environment. Chemical flocculants are very toxic and lead to various health complications (Wauer and Teien, 2010; Surendhiran *et al*., 2014). Chemical flocculants including polyaluminium chloride, aluminium sulphate, and

polyacrylamide to mention the few, are reported to be non-biodegradable and carcinogenic (Maliehe *et al*., 2016). Although chemical flocculants are cheap, but they have a negative effect on the environment. Therefore, novel means for water treatment globally is a crucial need. The Republic of South Africa has been described as one of those countries suffering from water scarcity (Mancosu *et al*., 2015).

Biological methods have been seen as an alternative to traditional flocculants for waste water purification. These methods use by-products of microorganisms and plants extracts called bioflocculants for wastewater treatment. Bioflocculants are environmentally safe, biodegradable in nature and have not been reported as yet to have any negative effect in the environment (Gupta and Diwan, 2017). The extracellular biopolymers are produced during the growth phase of bacteria. Their short-comings are the low yield and high production cost which hinders the large scale production (Salehizadeh and Shajaosadati, 2001). Thus, there is a challenge of finding novel bioflocculant producing strains capable of producing high bioflocculant yield and high flocculating activity using the cheapest substrate available (Selepe, 2017). Thus, the study was aiming to screen, isolate and identify a bioflocculant producing bacteria from Kombucha tea (Scoby broth). The study was also looking for the cheapest ingredient with high production of bioflocculant through optimization of bioflocculant from production conditions. The produced bioflocculant was applied in wastewater treatment and in dye removal from different dye solutions.

1.3 Hypothesis

Kombucha tea Scoby is a potential source of novel bioflocculant producing microorganisms of industrial importance.

1.4 Aim of the study

1.4.1 To screen, isolate and identify bioflocculant-producing bactria or yeast from Kombucha teas Scoby;

1.4.2 To optimize the culture conditions for bioflocculant production, produce, purify, characterize and apply the bioflocculant in wastewater treatment.

1.5 Objectives of the study

1.5.1 To isolate, screen and identify bioflocculant producing microorganisms from the Kombucha tea Scoby;

1.5.2 To optimize culture conditions for bioflocculant production using selected microbial isolate;

1.5.3 To extract, purify, and characterize the bioflocculant;

1.5.4 To determine flocculation properties of the produced bioflocculant;

1.5.5 To apply the produced bioflocculant on wastewater treatment and dye removal.

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Chapter 2

Chapter 2 includes the, introduction about flocculants and their classification, the microbial growth curve, effects of culture condition on production of bioflocculants, mechanism of bioflocculation, microorganisms that produce bioflocculants and their habitats, potential halophilic microbes for bioflocculant production, hydrophobicity and hydrophilicity of bioflocculants, utilization of cheap substrates for bioflocculant production, and lastly application of bioflocculants in the treatment of wastewater.

2.0 Literature review

2.1 Introduction

Microorganisms have a very long history of usage in various vital processes such as downstream processing, medicine, bioprocessing, and many other fields and have extraordinary advantages (Waites *et al*., 2009). Microorganisms have a shorter generation time and are easily adaptable to many environments including soil, water, skin, and rocks (Breibart *et al*., 2002). Extracellular polymeric substances (EPS), enzymes, and several other bioactive compounds as well as antibiotics are some of the excretions of microorganisms during their growth phase (Horner-Devine *et al*., 2004; Mohammed and Dagang, 2019).

Organic and inorganic substances in water, colloids or any other suspended materials are aggregated and removed by the process of flocculation. The aggregations result in suspended solids taking the shape of flocs or flakes during the flocculation process (Bruus *etal.,* 1992; Zulkeflee *et al*., 2016). The flocculation technology is an accustom process with extensive utilization in innumerable places, such as in oil separation, removal of heavy metal, purification of potable water, wastewater recycling, fermentation technology, in down streaming-processing in association with industrial processes and food industries (Xiong *et al*., 2010).

The biological and synthetic substances are all under the category of flocculants and have enormous capabilities in flocculation of impurities from wastewater. Flocculants can be categorically placed into three group organic synthetic flocculants, biological and inorganic chemicals flocculants (Peng *et al*., 2014). The organic and inorganic chemical flocculants are less costly and have shown excellent potential in flocculation of impurities from wastewater but have serious detrimental effects as they are non-biodegradable and toxic to the environment (Kumar *et al*., 2015).

Bioflocculants excreted by the microorganisms during their growth phase have been given attention in research and industrial disciplines to overcome the hurdles that comes with the utilization of these inorganic and organic chemicals flocculants (Sun *et al*., 2015a); Zulkeflee *et al*., 2016). The utilization of microorganisms for bioflocculants production has been given much effort in the field of science and biotechnology owing to their characteristics. Bioflocculants are biodegradable non-toxic and environmentally friendly (Zaki *et al*., 2013). Studies suggest that over 100 microbial species have been screened for the production of bioflocculants, its efficiency and characteristics (Pathak *et al*., 2017). Proteins, glycoproteins and polysaccharides are chemical compounds that are the major macromolecules to which a bioflocculant efficiency and mechanism are dependent (Makapela, 2015). The effects of dissimilar associated parameters have been comprehensively reviewed for bioflocculants efficiency and yield enhancement (Okaiyeto *et al*., 2016). Recently, most of the researches have been dedicated into screening novel microorganisms with bioflocculant-producing potential. The researchers also aid at improving the growth parameters and culture conditions of these novel microbes while still using a low-cost substrate. Polysaccharides have been shown to have contents of uronic acid when extracellular polymeric substances are characterized and these uronic acids such as glucuronic acid and mannuronic acid have carboxyl groups attached to the bioflocculants (Lin *et al*., 2010a). This carboxyl functional group gives the extracellular polymeric substances a neutral pH which affords EPS the negative charge they carry (More *et al*., 2014). The major problem with these macromolecules is their low yield and high costs. These short-falls have delayed the progress in research and industrial application of these by-products (Shaikh *et al.,* 2017; Ntombela *et al*., 2020).

In this study the Kombucha tea Scobies was utilized to isolate, screen, and identify a novel bioflocculant-producing organism. The identified organism with a potential to produce a bioflocculant was optimized to obtain an improved bioflocculant yield. The produced bioflocculant was characterized for its physical and chemical properties essential in flocculation process. Moreover, the bioflocculant was used in pollutant removal from wastewater and dye removal from different dye solutions.

2.2 Classification of flocculants

Utilization of flocculants as supplements in quite a number of industrial applications such as in food and beverages, dyes and textiles, water and wastewater treatments, downstream processing and fermentation, and cosmetics production has been a subject these days (Teh *et al*., 2016; Guo *et al*., 2020). For particles to settle down from unwavering suspension, flocculants have the capability of bringing together the suspended particles in a liquid-solid solution to form large flocs or particles, thus promoting the rate of sedimentation. This ability has resulted in a much wider spectrum utilization of flocculants including suspended and dissolved solids, dye and colour removal, turbidity removal, and chemical oxygen demand (COD) in clarification and sedimentation processes (Salehizadeh and Yan, 2014; Teh *et al*., 2016; Liu *et al*., 2017; Pathek *et al*., 2017). These flocculants have been generally categorized into three groups: organic synthetic flocculants, inorganic chemical flocculants, and naturally occurring flocculants. These flocculants are discussed in detail below.

2.2.1 Organic synthetic flocculants

As a result of their cheapest cost on production, able to be yielded in high quantities and effective rate of flocculation, organic synthetic flocculants are therefore, the most

commonly used flocculants in industries, currently. Although the organic synthetic flocculants have all these excellent advantages, are still having a negative impact on the environment and human life as these organic synthetic flocculants are non-biodegradable and impose detrimental effects on human health (Suopajarvi *et al*., 2013; Okaiyeto *et al.,* 2015a).

Organic flocculants include polyaluminum chloride and alum are water-soluble polymers and usually have molecular weight ranges from 10^3 to above 5 x 10^6 g/mol (Renault *et al*., 2009). Organic flocculants can be classified as either non-ionic or cationic agents (Bergstrom *et al*., 2014). Polyelectrolyte is a term given to charged polymer molecules subunits. Even though water-soluble non-ionic polymers are not in the same definition of polyelectrolyte, in literature these non-ionic polymers are found in the same class (Pergushov *et al*., 2013). In comparison with inorganic flocculants, the organic flocculants are more costly on the unit-weight basis (Gernjak *et al*., 2004). Organic flocculants are more effective when utilized in the separation of solid-liquid suspensions where there is a desire for the generation of sludge reduction and are most commonly used (Nebhani and Jaisingh, 2020).

PolyDADMAC and polyamine are the most commonly used chemicals in flocculation processes. PolyDADMAC and polyamine function by neutralization of charges, as a result, there is no advantage in the sweep-floc mechanism. Raw water of high turbidity (approximately >20 NTU) is mostly treated effectively by polyamines and have good capabilities in wastewater purification (Brostow *et al*., 2009). In the purification of wastewater, PolyDADMAC is utilized as a coagulating agent. This polymer has a high cationic charge density, which is the reason behind it effectively usage in flocculation processes. Polyethylene oxide and polyacrylamide are best known chemical flocculants (Orozco *et al*., 2009). Polyelectrolyte is an anionic group

which is formed in a solution after the addition of functional group and carboxyl (Ntombela, 2017).

Melanin is another example of organic flocculants and it functions as an inorganic flocculant. It also contributes to flocs that have been precipitated besides flocculation of suspended particles in the wastewater. Though melanin is expensive, when disposal and removal of sludge costs are reduced, it can be economical (Sharpe, 1991). Riera-Torres *et al*. (2010) reported that melamine formaldehyde in combination with urea improved colour removal by 98%. To clean wastewater containing a dye (methylene blue) used to mimic leather and textile manufacturing dyes, a melamine-formaldehyde-urea (MFU) resin was used as an adsorbent (Ozdemir *et al*., 2009).

2.2.2 Inorganic chemical flocculants

Inorganic chemical flocculants are both economically reasonable and utilizable for a wide variety of wastewater and water treatment. Inorganic flocculants normally function well where organic chemical flocculant fails as these function effectively well with low turbidity (concentration of total suspended solids) and raw water (Deng *et al*., 2016). Once supplemented in water, the alkalinity of water reacts with these inorganic flocculant chemicals to be hydrated and result in metal (iron or aluminium) formation of hydroxide precipitates through sweep-flocculation mechanism (Sahu and Chaudhari, 2013). Inorganic flocculants can be used to treat colloidal suspensions that are not easily treated effectively as a result of this mechanism.

The mostly used inorganic flocculants are alum, ferric salts and aluminium chloride. Since water mostly have a partial negative charge, these flocculants will make cationic charges in wastewater to cause flocculation of the particles when these two opposite charges come in contact (Aljuboori *et al*., 2014; Mounir *et al*., 2014).

Although the sweep-floc (large aggregates of $Al(OH)_3/Fe(OH)_3$ that are formed when AL/Fe salt is added to wastewater) precipitate for metal hydroxide is a good advantage for wastewater treatment, but are major contributing factors to the volume of an overall sludge which needs to be removed after treatment. The dewaterability and density overall of sludge against precipitates made from organic flocculants is lowered by the metal hydroxide precipitates. Therefore, causes a significant negative impact on the economy (Wei *et al*., 2018). In high temperatures, the inorganic chemicals have been reported to lack productivity and are pH depended (Sengupta *et al*., 2012; Jain *et al*., 2015). Some flocculants such as composite flocculants have their applications limited in wastewater; despite their excellent function due to high cationic charges that these compound flocculants possess (Ovenden and Xiao, 2002). Therefore, there is a need for substitution ofn the compound flocculants in wastewater purification industries with non-toxic and biodegradable natural flocculants.

2.2.3 Naturally occurring flocculants

The environmental friendliness of the naturally occurring flocculants has resulted on naturally occurring flocculants receiving much attention for the treatment of wastewater and are proving to be the potential replacements for oil-based synthetic flocculants (Mishra and Bajpai, 2005; Suopajarvi *et al*., 2012). Most of the natural flocculants are biodegradable, safe, and fairly shear-resistant. Thefore, the fact that tnatural flocculants produce sludge of organic nature can be microbially degraded and recycled to optimize fertility of the soil in agriculture are all the factors that make natural flocculants to be an interesting subject (Lee *et al*., 2014a). Constituted in the natural flocculants are liposaccharides, proteins, glycoproteins and polysaccharides, and lipoproteins, though the dominant constituents are carbohydrates (Sun *et al*.,

2019). Examples of natural flocculants include chitosan, cellulose, alginate, and starch. These natural flocculants are general derivatives of arthropods, plants, seaweeds and microbial-based raw materials (Babu *et al*., 2013; Daza *et al*., 2016). It should be noted that these flocculants can also be modified chemically to augment their flocculation capabilities (Singh *et al*., 2014). Some examples of naturally occurring flocculants are discussed below.

2.2.3.1 Alginate

The residues α- L-guluronate (G-block) and (1→4) - linked □-D- mannuronate (M-block) are the constituents of a linear anionic polysaccharide called alginate. There are 200 dissimilar varieties of alginates available (Lin *et al*., 2010). Alginates are of a dissimilar length and their M and G units of monomers (Homopoly-d-mannuronic acid and homopoly-l-guloronic acid) are not the same. For example, M units are involved with increase in flexibility of polymers while G units are associated with entanglement and zipper mechanism (Lee and Mooney, 2012; Hecht and Srebnik, 2016).

Extraction of alginates was first done by Stanford (1881) from seaweeds in the early twentieth century. Alginate commercial production was observed from genera of *Macrocystis* and *Laminaria*. In plants, alginates play a role similar to cellulose. Brown seaweeds have alginates as structural intracellular components (Remminghorst and Rehm, 2006). Brown seaweeds are extracellularly excreted from bacteria, for example, *Pseudomonas* and *Azobacter* (More *et al*., 2014). These polyelectrolytes are non-toxic, biocompatible, biodegradable, and non-immunogenic biopolymers (Oryan and Sahvieh, 2017). Wide application of alginates has been observed in various fields such as biotechnology, pharmaceutical, and biomedical

fields as a consequence of their versatility, biocompatibility and desirable characteristics (Andersen *et al*., 2012). The average molecular weight range of commercial alginates is between 32 000 and 400 000 g/mol (Sand *et al*., 2010).

Natural alginates have been studied in the past because it showed great potential in flocculation and decolouring processes (Diaz-Barrera *et al*., 2014). The presence of the free carboxyl and hydroxyl groups around polymer chains it gives alginates a strong absorption capability and properties for flocculation (Yang *et al*., 2011). *Monascus purpureus* was incorporated with zinc oxide and calcium alginate as carrires for treatment of monosodium glutamate and soy sauce wastewater. The results revealed that the suspended biomass for MSG wastewater was 228 mg/mg and 74 mg/g, while the suspended and attached biomass for soy sauce was 130 mg/g and 66 mg/g (Zhang *et al*., 2015). The treated MSG wastewater had significantly lower levels of chemical oxygen demand (COD), biological oxygen demand (BOD), SO_4^{2-} and NH_3–N than the raw wastewater (Zhang *et al*., 2015). The gel embedding method was used to prepare immobilized *Pseudomonas aeruginosa* beads with alginate and biochar as composite carriers and anonionic surfactant (TX100) as a degradation promoter (Lu *et al*., 2021). The TX100-facilitated immobilized bacterial beads had a 24% higher acenaphthene removal ratio than the beads without TX100 (Lu *et al*., 2021).

2.2.3.2 Chitosan

During chitin deacetylation, a most vital natural-based biopolymer called chitosan is produced. Chitin is a glucose derivative and a long chain polymer naturally obtained in many places including exoskeletons of lobsters, and crabs and it is ubiquitous (Cauchie, 2002). The X–ray analysis technique was discovered to be extremely useful in determining the abundance of chitosan in natural resources in 1950

(Sandeep *et al*., 2013). Chitosan is a cationic polysaccharide and linear in composition. It has dissimilar deacetylation degrees with ranges from 40 - 90% and can be found in various molecular weights commercially. There are ampholyte or polyelectrolyte types of chitosan depending on their charged A-units and D-units (Alves and Mano, 2008). Among biopolymer flocculants, chitosan shows the peculiar properties of flocculation. This is due to the presence of the primary amino groups (Guibal *et al*., 2006). Chitosan polycation reveals excellent properties such as biocompatibility, physical and biological activity, biodegradability, ability to flocculate, has non-toxic monomers and antimicrobial properties (Alves and Mano, 2008). Using chitosan and nanochitosan as adsorbents, the removal of arsenate in the presence of competing anions such as sulfate, chloride, and nitrate ions was investigated (Olivera *et al*., 2016). Sulfate ions (initial concentration = 1240 µg/L) were found to have a negative impact on nanochitosan's adsorption potential against arsenate ions (initial concentration = 3000 µg/L), whereas chloride (initial concentration = 4610 µg/L) and nitrate ions (initial concentration = 4050 µg/L) had no influence (Kwok *et al*., 2014).

2.2.3.3 Cellulose

Cellulose can be found on various places including wood, plants, microorganisms and animals (Roy *et al*., 2009) and is a linear polysaccharide. Certain bacteria including *Acetobacter xylinum* are responsible for the production of cellulose (Hu and Catchmark, 2010). Cellulose shows potential as an alternative for the production of functional material that is environmentally friendly (Chauhan and Yan, 2016). It is also capable of biopolymer flocculants production as a result of its chemical reactivity and physical characteristics (Roy *et al*., 2009). A green and high-efficiency flocculant was designed and successfully synthesized using hyperbranched polyethylenimine-

grafted cellulose (hPEI-CE) (Abouzeid *et al*., 2018). In order to treat simulated kaolin wastewater, the flocculation efficiency of the hPEI-CE with different amino group contents was evaluated in terms of turbidity, floc scale, and zeta potential. The hPEI-CE, with an amino group content of 3.97 mmol/g, had the highest removal efficiency for kaolin particles without the use of any coagulants. Under each original pH state, total suspended solid (TSS), chemical oxygen demand (COD), and turbidity reduction reached up to 73.4%, 95.7%, and 87.4% for silk printing and dye wastewater, respectively, and 96.2%, 79.9%, and 93.5%, respectively for machining wastewater (Abouzeid *et al*., 2018).

2.2.3.4 Extracellular polymeric substances (EPS)

Microbial flocculants are extracellular polymeric substances (EPS) which are better alternatives for chemical flocculants currently used as EPSs are sustainable, very cheap, and eco-friendly (More *et al*., 2014).

Extracellular polymeric substances of microbial origin are biopolymers (Wingender *et al.,* 1999). The release of organic matter or products by cell lysis and cellular materials from microbes leads to the formation of biochemicals and these in turn form an EPS matrix. Microorganisms either prokaryotic or eukaryotic can secrete EPS in the natural environment as protective measures against harsh conditions (Donde and Xiao, 2017). The biofilm matrix of EPS is very viscid, with total organic matter ranges from 50 - 90% (Donde and Xiao, 2017). EPSs can be defined as slime, tightly bound or loosely bound, and capsular depending on their association with cells or method of their extraction from the cells.

Some of the functions of EPS are adherence to surfaces, biofilms, flocs formation, cell to cell recognition, bacterial cell aggregations, and exogenous organic compound sorption (Tian, 2008). EPS main components are humic substances,

proteins, carbohydrates, glycoproteins, and proteins. Most of these auspicious components afford the EPS matrix with interesting functions such as hydrophobicity or hydrophilicity, biodegradability, and adsorption capabilities (Czaczyk and Myszka, 2007).

Uronic acids and neutral carbohydrates (need to be converted to ions) are found from the microbial exogenous secretion. These, especially uronic acids and other common substitutes like succinate or formats decide the nature of EPS as cationic, neutral or anionic (Tian, 2008). The presence of many anionic functional groups in the EPS, such as, phosphoryl phenolic, hydroxyl, and carboxyl groups can complex with heavy metals as EPS have the potential for ion exchange. EPSs are of fundamental assistance in the mechanism of flocculation (Feng and Xu, 2008; Ha *et al*., 2010). The bioflocculant structures, chemical functionalities and molecular weights have a huge role in the process of bioflocculation (Bossier and Verstraete, 1996).

It has been suggested that in some instances, EPS in excess might result to some serious consequences to bioflocculation and in turn, leading to lower liquid-solid separation efficiency in the activated sludge process from clarifier or secondary settling tank (Li and Yang, 2007), Gross EPS concentrations alone do not determine flocculation of a microorganism. As a result, it is of fundamental importance that the EPS individual moieties role be determined in controlling the bioflocculation (Geesey and Kloeke, 2004). This is due to the fact that microbes that do not settle results to turbidity effluent increase from the secondary clarifier and removal of biochemical oxygen demand is lowered. Surface characteristics for instance hydrophobicity and surface charge determine aggregation of bacteria before secretion of EPS, suggested by experiments with pure cultures (Zita and Hermansson, 1997).

2.3 Microbial growth curve

The growth of microorganisms has been extensively studied as they are easy to grow in the laboratory. These microorganisms include microalgae, yeasts, protozoa and bacteria. Studies have shown that these microorganisms on a batch culture or closed system follow a predictable pattern of growth. This pattern of growth results leads to a microbial growth curve made up of four different growth phases: namely, lag phase, log or exponential phase, stationary phase, and decline or death phase (Peleg and Corradini, 2011). The nutrients availability and type of nutrients contained in a medium are the fundamental basis of these four stages of microbial growth and bioflocculant production. This means that there can be no observed microbial growth in the absence of nutrients (Ntombela, 2017). Time of incubation and nutrients are of paramount importance in bacterial phases of growth.

2.3.1 Lag phase

Bacterial acclimatization can be considered as a lag phase, as this is where the organism is trying to familiarize itself with its new conditions. The lag phase is not a fixed phase, as it can considerably vary its length based on how the present conditions differ from the previous conditions, from which the microorganism was exposed to as well as the conditions of the bacterial cells themselves (Zaki *et al*., 2011). The shortest lag period is observed from microbes when they have been inoculated from the cells growing actively from one media type to a similar media type, with the same environmental conditions. For some cells however, before they can be engaged in reproduction will need to be repaired and such cells are called damage cells leading to a long period of lag phase. Molecules, for instance, RNA and enzymes start being synthesized by bacteria during this phase of growth. Here

the adaptation degree and microbe type are considered as key factors in this stage of growth (Davey and O'toole, 2000; Okaiyeto *et al*., 2016). The cell growth for microbial bioflocculant is not favoured at this phase of growth as microorganisms are still acclimatizing. *Halomonas sp*. Okoh and *Micrococcus sp*. Leo did not show any visible bioflocculant production in the lag phase (Okaiyeto *et al*., 2013). A study by Abu-Tawila *et al*. (2018) reported that *Bacillus salmalaya* 139SI showed no observable growth within the first 12 hours of cultivation (lag phase). Therefore, no bioflocculant production can be expected at this phase of growth.

2.3.2 Log or exponential phase

Cell division follows once everything needed for cell growth has been accumulated by these cells. Cell multiplication is observed during the exponential phase of microbial growth. Most of the experiments make use of cells at this stage of growth because studies suggest that at this stage cells are most uniform and healthy (Liu *et al*., 2010). The multiplication of cells is accompanied naturally by the bioflocculant production rate during the logarithmic growth phase. This phase of growth has been implicated with quite several productions of bioflocculants optimally. Waste substances accumulation is directly proportional to low flocculation rate when the enzyme deflocculation becomes active together with a nutrient decline in the medium.

The flocculating activity of strain *Citrobacter sp*. TKF04 culture broth increased during the logarithmic phase of growth, then remained stable for about 1 day before showing a significant decrease (Fujita *et al*., 2000). The rate of flocculating efficiency is reduced due to bioflocculant degrading enzyme presence in a system (Mao *et al*., 2011). A decrease in cell number is observed as nutrients are used fully and cell rate of multiplication decreases leading to medium experiencing a gradual decrease in

the number of cells. Dead cells accumulation and other products from the metabolism of microbes are as a result of the above phenomena. Zhu *et al*. (2012) reported that a bioflocculant in significant amounts from *Chlamydomonas reinhardtii* was produced during exponential growth phase. The fungus *Pestalotiopsis sp*. KCTC 8637P produced a novel bioflocculant during its exponential growth phase (Kwon *et al.*, 1996). Kumar *et al*. (2004) recorded on a haloalkalophilic *Bacillus sp*. I-450 which produced the bioflocculant during the late logarithmic growth phase. Similar findings were also reported where the bioflooculants were produced during the logarithmic growth phase of the microorganisms (Shih *et al*., 2001; Yin, 2010).

2.3.3 Stationary phase

In this phase the organism runs out of essential nutrients or its waste products inhibit growth, taking cells to stationary phase of microbial growth. In this stage the number of cells dying off equals the cell numbers produced or has ceased entirely, resulting in a growth curve, and growth flattening out. At this stage, the cells become dissimilar physiologically, as the cells are acclimatizing to their new conditions of starvation. However, the biopolymer flocculating activity that has been produced does not change, and there is maximum bioflocculating activity attained by the produced bioflocculant. For carbon and energy sources, the produced bioflocculant in the medium is utilized by the microbe (Vu *et al*., 2009). This is because when microorganisms are starve, the microbes normally feed on it own by-products (bioflocculants). Bioflocculant TJ-F1 from *Proteus mirabilis* was reported by Xia *et al*. (2008) to reach its maximum flocculation at station phase of growth. A bioflocculant produced by *Enterobacter aerogenes* reached its optimum flocculating activity during the stationary phase of the growth curve (Lu *et al*., 2005). *Klebsiella terrigena* produced bioflocculant during late stationary phase of growth (Buthelezi *et al*., 2010).

Zaki *et al*. (2011) also reported the microorganisms *Bacillus subtilis* and *Pseudomonas spp*. to produce bioflocculants during early and late stationary phases, respectively.

2.3.4 Decline phase

This is the last stage on microbial growth, also known as the death phase. At this phase of growth, the number of viable cells exponentially commences to predictably show a decrease. The way at which how fast viability is lost from cells corresponds to the slope steepness. The cells are irreparably harmed as a result of the conditions of the culture deterioration. Some experiments have shown that the cultivation of cells to another medium from this stage of growth is not possible or takes longer (Yates *et al*., 2007). This could be as a result of the conditions such as insufficient nutrients, variations of temperature, or other conditions that are unaccepted to favour continued growth of the species.

It is noteworthy that in some instances, at this phase of growth only spore formers can survive and still produce their population optimally (Ntombela, 2017). Other microbes to survive are called viable but nonculturable (VBNC) as microbes can be revived given the right conditions. At this stage when one wants to do cell density determination by measuring culture turbidity should take note of the fact that the cells could still be intact and therefore might not show any decrease during this stage. The bioflocculant produced by *Chryseobacterium daaeguense* W6 attained optimum flocculating activity during the decline phase of growth (Liu et al., 2010). So at this stage of growth the chances of bioflocculant production are very limited.

2.4 Merits and demerits of the utilization of organic, inorganic and naturally occurring flocculant. In general, all flocculants (organic, inorganic chemical flocculants, and naturally occurring flocculants) have their ups and downs in their performance. Table

2.1 highlights the advantages and disadantages of flocculants in wastewater purification.

Table 2.1: Merits and demerits of organic flocculants, naturally occurring flocculants and inorganic flocculants (Makapela *et al*., 2016; Mmango-kaseke *et al*., 2016 Okaiyeto *et al*., 2016; Shaikh *et al*., 2017).

Flocculants	Merits	Demerits
Organic synthetic flocculant	• Have high flocculating efficiency. • Compared to bioflocculants they are cost effective. • Polymer structure can be varied, so as the molecular weight, percentage of ionic charge and its nature, and molecular weight distribution. • Not sensitive to pH, able to coagulate fine particles. • Effective on warm and cold water and generate low sludge in comparison to PAC.	• Toxic to the environment as they are non-biodegradable. • Polyacrylamide monomers are neurotoxic and carcinogenic. • Contribute to environmental pollution.
Inorganic chemical flocculants	• Easily found in the markets and of low cost. • Great efficiency of flocculation • Well established flocculating mechanism. • Requires no skilled personnel for production processes	• Polyaluminum chloride (PAC) especially. • They lack ability on coagulation of fine particles. • Neurotoxicity results from aluminum salts. • Ferrite flocculants results to too much ion making unpleasant metallic taste, corrosion of colour, odour, staining, or foaming. • pH sensitive, non-biodegradable thus toxic to environment.
Naturally occurring flocculants	• Biodegradable, harmless, and show no secondary pollution. • Besides bioflocculant production cost that is high, they are cheap. • They have definite molecular constitution and chain length in their molecular weight. • They can be modified on their functional groups to obtain effective flocculants. • Show benign nature, biocompatibility, produce low sludge, and not pH sensitive. • Can coagulate fine particles and they are of both cold and warm water effectiveness.	• Its active ingredients biodegrade with time as they have shorter shelf life. • Compared to organic and inorganic it shows poor activity in flocculating particles. • As a result of their biodegradability the flocs eventually loose strength and stability. • Mechanism of flocculation is not comprehended well in details. • Requirements for large dosage for efficiency in flocculation. • High production cost for bioflocculants.

2.5 Effect of culture medium conditions on the production of bioflocculants

Literature studies have shown that innumerable factors are involved in bioflocculant production. These factors are environmental, chemical, and physical factors, which have major effect in improving bioflocculant production (Wang *et al*., 2007). The production medium composition also has a major effect on bioflocculant production (Feng *et al*., 2013). Culture optimization is of the greatest importance in the production of bioflocculant to ensure an optimum reproducibility of the organism. The key factors on the effect of any bioflocculant production as reported by some researchers are the size of the inoculum, initial pH of a production medium, energy sources, temperature, shaking speed, metal ions and fermentation time (Gao *et al*., 2006; Gong *et al*., 2008; Yin, 2010; Aljuboori *et al*., 2013; Ahmad *et al*., 2015; Chen *et al*., 2017). These factors are discussed in detail below.

2.5.1 Effect of inoculum size on the production of bioflocculant

Inoculum size can be considered as the most fundamental factor for the production of bioflocculants. It is associated with the processes of metabolism where it is a key factor on secondary products excretion and multiplication of cells (Gong *et al*., 2008; Xia *et al*., 2008). For an inoculum size to function effectively an equilibrium, during inoculation optimum inoculum size must be maintained as too much inoculum size will lead to niche overlap of bioflocculant production while a lesser amount will lead to bioflocculant production delays (Salehizadeh and Shojaosadati, 2001). Literature has shown that microorganisms have different preferences for inoculum size which make them yield bioflocculating activities of the highest values (Ntozonke, 2015; Selepe, 2017). Xiong *et al*. (2010) reported that *Bacillus licherniformis* was able to produce an optimum bioflocculant with an inoculum size of 4% (v/v). *Pichia*

membranifaciens and *Bacillus cereus* showed bioflocculant production of above 90% when the inoculum size of the mixture was 10% (Ben Rebah *et al*., 2018). Some of the microorganisms and their preferred inoculum sizes for optimum production of bioflocculants are listed in Table 2.2.

Table 2.2: Microorganisms and their preferred inoculum sizes for bioflocculant production.

Microorganisms	**Inoculum size (%v/v)**	**Citation**
Oceanibacillus sp. Pinky	1	Cosa *et al*. (2013)
Micrococcus sp.	2	Okaiyeto *et al*. (2014)
Bacillus sp.	3	Ugbenyen *et al*. (2014)
Bacillus lecheniformis X14	1	Li *et al*. (2009)
Pseudomonas cepacia	5	Ramadan *et al*. (1990)
Alcaligenes faecalis HCB2 and *Bacillus pumilus* JX860616	2	Maliehe *et al*. (2016)
Bacillus subtilis F9	2	Hassimi *et al*. (2020)
Cobetia and bacillus	4	Ugbenyen and Okoh. (2014)
Aspergillus flavus IH-7	2	Deng *et al*. (2005)

2.5.2 Effect of carbon and nitrogen sources on the production of bioflocculants

Both carbon and the nitrogen sources are of fundamental importance in the bioflocculant production processes (Ramadan *et al*., 1990). Microbes have their differences in terms of nitrogen and carbon sources requirements. There are however, concerns about the utilization of these sources in terms of their cost. These costs have resulted greatly to the unavailability of much bioflocculants in high scale production (Mabinya *et al*., 2012). It has been reported that microorganisms are well

augmented by carbon sources which is a key factor in the bioflocculants production and multiplication of bacteria (Pu *et al*., 2018).

The commonly utilized carbon sources for the production of a bioflocculants include maltose, glucose, sucrose arabinose, mannose, xylose, lactose, starch and ribose (Samal, 2012). Incorporation of nitrogen in the medium for bioflocculant production has been shown to have a great effect. Some of the examples for nitrogen sources are ammonia, ammonium nitrate, peptone, ammonium sulphate, urea, casein, and yeast extract to just mention a few (Maliehe *et al*., 2016).

He *et al*. (2004) reported an effective use of molasses as a carbon source for excretion of bioflocculants. Li-fan *et al*. (2010) reported that molasses could be used to excrete bioflocculant by *Penicillium sp*. HHE-P7 and showed a good flocculating activity of 85% after cultivating for 5 days. *Pseudomonas fluorescence* C-2 and *Pseudomonas alcaligenes* PS-25 also used molasses to produce bioflocculant after cultivation for 3 days (Mao *et al*., 2008; Sajayan *et al*., 2017). Sucrose, glucose, and fructose presence in a medium favoured the excretion of bioflocculant REA-11 by *Corynebacterium glutamicum* CCTCC M201005*,* with sucrose the preferred carbon source. Sucrose showed high yield of bioflocculant production and was used as it is a cheap substrate (He *et al*., 2004).

A study by Nakata and Kurane (1999) showed that alcohol could also be utilized by bacterial strain for the production of a bioflocculant. *Bacillus licheniformis* X14 was reported to be highly favoured by the presence of alcohol, sucrose and starch, with sucrose being the most preferred carbon source and ammonium chloride as the most preferred nitrogen source (Li *et al*., 2009). An organism *Klebsiella pneumoniae* H12 was reported to excrete bioflocculant optimal in a medium

composed of ethanol and galactose as a major carbon source for bioflocculant production (Salehizadeh and Shajaosadati, 2001).

It has been reported that some bacterial strains prefer using lignocellulosic biomasses for bioflocculants production as these are available abundantly in nature and are used as a cheap carbon sources (Salehizadeh *et al,*. 2018). Wang *et al*. (2013) reported that hydrolysates were utilized by the strain *Ochrobactium ceceri* W2 to produce bioflocculant W2. Hydrolysate contains hexoses and pentose, unlike glucose and sucrose which are mostly utilized (Gopinath *et al*., 2011). According to Liu *et al*. (2015a), *Cellulosimicrobium cellulans* L804 strain was able to directly degrade lignocellulosic biomass and produced a bioflocculant, while another study by Liu *et al*. (2019) found *Bacillus agaradhaeren*s C9 (alkaliphilic) with the capability of bioflocculant production utilizing rice bran (untreated) as a carbon source. A carbon source from a wood dust biomass was used solely for bioflocculant production by *Pseudomonas boereopolis* G22 and the produced MBF-G22 bioflocculant (Guo *et al*., 2018a). *Bacillus alvei* NRC-14 produced BPF bioflocculant through the medium consisting of chitosan as a carbon source (Abdel-Aziz *et al*., 2011).

Utilization of cheap carbon and nitrogen sources for the synthesis of bioflocculants is slowly gaining popularity. This will help immensely for successful large scale production of bioflocculants, as high costs is currently associated with bioflocculants production is a major hindrance (Zhang *et al*., 2013). A-GS408 novel bioflocculant from *Klebsiella oxytoca* GS-4-08 was obtained after the bacterium was grown in the presence of acetonitrile as sole nitrogen source (Fan *et al*., 2019). Li *et al*. (2009) reported that *Bacillus licheniformis* X14 preferred ammonium chloride as a nitrogen source for bioflocculant production. The alkaliphilic *bacillus agaradhaerens* C9 was

reported to produce bioflocculant using feather waste as a cheap source of nitrogen (Liu *et al*., 2020). A bioflocculant REA-11 produced by *Corynebacter glutamicum* was reported to use urea and corn steep liquor in combination for improved bioflocculant production (He *et al*., 2004). Ugbenyen *et al*. (2014) reported on a bioflocculant produced by *Bacillus sp*. Gilbert that used potassium nitrate as its nitrogen source for optimum bioflocculant production with flocculating activity of 76.6%.

2.5.3 Effect of initial pH of the medium on bioflocculant production

Initial pH can be considered as a key factor in the bioflocculant production medium. Studies have shown that in acidic conditions some microorganisms have optimum bioflocculants production, but other microorganisms prefer alkaline or neutral pH conditions or values for optimum bioflocculant production (Xia *et al.,* 2008). Besides showing the electric charge effect of the cells and its potential of oxidation-reduction within the cells, pH also has an impact on enzymatic reaction and absorption of nutrients (Salehizadeh and Shojaosadati, 2001). The bacterium *Bacillus sp*. F19 produced a bioflocculant MBFF19 at a pH 7 in the production medium (Zheng *et al*., 2008). Another bioflocculant was also reported to be produced by *Aspergillus niger* at initial pH 6 and showed high flocculating capability (Aljuboori *et al*., 2014). *Bacillus pumilus* ability to produce a bioflocculant within the pH ranges 5 - 11 was reported (Makapela *et al.,* 2016). Keeping pH values within ranges pH 5 -11 was reported to reduce the cost of production (Xia *et al*., 2008).

Mabinya *et al*. (2011) experimented on neutral pH 7 and produced a bioflocculant efficient using *Halomonas sp*. Ahmad *et al*. (2013) reported that *Aspergillus flavus* produced a bioflocculant IH-7 with the optimum flocculating activity of 80% at pH 7. The acidic pH values usually show inconsistencies in production of bioflocculants and this could be the reason why most bioflocculants are not produced

in too much acidic pH ranges 1 - 4. Poor flocculation efficiency has been observed in the produced flocculants at acidic conditions. This could be a consequent of the fact that the surface charge spatial arrangement is pH depended directly proportional to the temperature involved in a solution (Okaiyeto *et al*., 2015b). Ntsangani (2016) reported that *Bacillus subtilis* CPO8 and CPO13 were effective for bioflocculant production at pH 7 and pH range of 3 -9 did not affect the flocculating activity and *Halomonas sp*. V3a was found to be able to produce a bioflocculant of 80% flocculating activity at a range of pH 3 -11. The major reason for all these differences displayed in bioflocculants production could be due to that biopolymers have different electric states from dissimilar pH values, which then negatively impacted efficiency of bridging for biopolymers on clay suspension (Elkady *et al*., 2011).

2.5.4 Effect of temperature on the production of a bioflocculant

Microbial strains have dissimilar preferences in terms of temperature. So bioflocculant production is affected greatly by cultivation temperature. This is due to the fact that all the chemical reactions that take place within the organism needs optimum temperature. The activity of the enzymes and the ability to obtain growth at the highest rate is influenced directly by the cultivation temperature (Li *et al*., 2008). The optimal cultivation temperature has been observed in many occasions (Li *et al*., 2008). For example, Salehizadeh and Shojaosadati (2001) reported that the production of bioflocculants has cultivation temperatures range between 25 °C and 37 ºC. Production at an optimal cultivation temperature of 28 °C was observed for the *Bacillus sp.* Gilbert isolated from Algoa Bay of South Africa (Ugbenyen *et al*., 2017). *Bacillus mojavensis* was reported to exhibit about 96.11% flocculating activity after cultivation at a temperature of 35 ºC (Elkady *et al*., 2011). In a study by Ugbenyen *et al*. (2017), *Pantoea sp*. was able to produce a bioflocculant optimally at

cultivation temperature of 30 °C. Zhang *et al*. (2008) found that *Pichia membranifaciens*, *Bacillus cereus* and *Bacillus atrophaeus* were also able to produce the bioflocculants under optimal cultivation temperature of 30 °C. The cultivation temperature is of paramount importance and should be strictly adhered to ensure the production of the best flocculating bioflocculant. In general, temperature affects the enzymatic processes that occur within the organisms during bioflocculant production. High temperatures might harm or denature the fundamental enzymes responsible for production, whereas too low temperatures might stop the growth of microbes thus affecting the production of secondary metabolites.

2.5.5 Effect of metal ions on flocculating activity for the production of bioflocculant

Metal ions are of vital importance because these ions have the ability to stabilize residual net charge as the result of functional group charges on the bioflocculant. Metal ions also have the ability to neutralize surface charges on a bioflocculant making it to be very effective for its function. Reports suggest that bioflocculants are negatively charged mostly, this is because the bioflocculant structures possess functional groups including carboxylic acids (Wu and Ye, 2007). The charge density of cations supplied can affect the flocculation rate greatly, as these results to inter-particle bridging between particles of kaolin (Takeda *et al*., 1992). Both the valence ions and the concentration of the cations play a major role in flocculation process by enchancing the flocculation process (Watanabe *et al*., 1999). In general, metal ions function to stabilize and neutralize the negative residual charge from functional groups thereby stimulating activity of flocculation via particles bridging (Tekada *et al*., 1991). The cations are associated with the synergistic effect on flocculating activity and are classified as trivalent, bivalent, and monovalent cations. A study by Wu and

Ye (2007)revealed that DYU 500 flocculating activity for monovalent was greater than 10 mM metals (K^+ or Na^+), with 0.10 to 0.90 mM for bivalent metals (Mg^{2+} or Ca^{2+}), and it was less than 0.005 for trivalent metals (Fe^{3+} or Al^{3+}). Bioflocculants have a charge bridging which causes the shear of floc resistance, floc size and the floc density to be increased. Monovalent metals have been associated with the formation of bridges with reduced bonds strength and lead to flocs structure to be loose. This in turn results to floc density decrease and shearing of resistance floc and size (Zhang *et al.,* 2019). The structural components and characteristics of a bioflocculant have been reported to be highly depended on the origin of microorganism (Wu and Ye, 2007).

Liu *et al*. (2010) reported that bioflocculant MBF-W6 production by *Chryseobacterium daeguense* in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+} and K^+ was stimulated whilst in the presence of Ba^{2+}, Al^{3+} and Fe^{3+} was inhibited. *Flavobacterium sp*. was revealed to be well stimulated for bioflocculant production when metal ions such as Mn^{2+}, Ca^{2+}, and Ba^{2+} were used. However, the presence of Cu^{2+} inhibited the production of the bioflocculant (Li *et al*., 2009). Some microorganisms do not require cations, but produce optimally without stimulators. For example, *Chryseobacterium daeguense* W6 was found to be able to produce a bioflocculant without addition of cation, thus preventing secondary pollution that results from cation usage (Liu *et al*., 2015b). *Bacillus sp*. F19 is inhibited by the presence of Fe^{3+} in its production medium as this bacterium is only capable of bioflocculant in the absence of cations (Zheng *et al.,* 2008). Other studies also reported that *Klebsiella pneumoniae* and *Aspergillus flavus* obtained high flocculation of kaolin clay when no cations were added (Aljuboori *et al*., 2013; Luo *et al*., 2014).

2.5.6 Effect of shaking speed on bioflocculant production

Agitation speed during cultivation is very important as it is applied mostly in aerobic fermentation processes for the ease of oxygen distribution. Enzymatic reactions and absorption of nutrients greatly rely on the dissolved oxygen. For microbes to be able to efficiently acquire the sufficient amount of the present concentration of oxygen dissolved there is a preference for each microbial strain for shaking speed and at dissimilar levels for each microorganism (Ntozonke, 2015). Aljuboori *et al*. (2013) found that *Aspergillus flavus* was able to optimally produce bioflocculant using a shaking speed of 180 rpm whereas *Aspergillus parasiticus* was reported to produce a bioflocculant effectively at a shaking speed of 150 rpm (Deng *et al*., (2005). *Bacillus* sp. UPMB13 strain was very effective when a shaking speed of 150 rpm was used for cultivation to completely dissolve oxygen around the medium and produced a bioflocculant with 90% performance (Zulkeflee *et al*., 2012) while Akapo (2019) found that *Bacillus astrophaeus* produced a bioflocculant optimally at an agitation speed of 110 rpm. Another study by Zhang *et al*. (2008) reported that *Pichia membranifaciens* and *Bacillus cereus* preferred shaking speed of 120 rpm and *Proteus mirabilis* had shaking preference of 130 rpm on other studies by Xia *et al*. (2008) for their production of a bioflocculant.

2.5.7 Fermentation time for the production of bioflocculant

Production of bioflocculants happens at dissimilar stages of growth of microorganisms (Salehizadeh and Yan, 2014). Microbial nutrients assimilation mostly results in efficient bioflocculant production during microbial growth. It has been reported that within the cell medium there is a fluctuation of production with dissimilar growth periods. For examples, autolysis of cells, biosynthesis, enzymatic action and metal complexing (Akapo, 2019). The time factor is an important parameter for microorganisms to initiate bioflocculant production. Some microbes

produce bioflocculants for longer periods in the medium while other microorganisms produce bioflocculants after a short period during bacterial growth curve very fast on their production. For example, Luvuyo *et al*. (2013) reported that *Methylobacterium sp*. Obi and *Actinobacterium sp*. were able to yield a bioflocculant with the flocculating activity of 95% after 72 hours of fermentation. Goldern *et al*. (2020) reported the *Bacillus sefensis* bacterium to produce its bioflocculant after 60 hours of incubation. In this study, the bacterium produced its bioflocculant after 60 hours of incubation time. The strain *Virgibacillus sp*. Rob's fermentation time for bioflocculant production was conducted and it was observed that the flocculating activity increased steadily to a peak value of 81.5% after 4 days, followed by a gradual decline in flocculating activity, which was fully lost after 8 days (Cosa *et al*., 2011). Another study reported that the flocculating activity of *streptomycetes griseus* increased with time and peaked on the fourth day of fermentation (Shimofuruya *et al*., 1996). For different species, the culture period for bioflocculant production into the medium can differ. For example, the peak flocculating activity of a bioflocculant by *Citrobacter sp*. TKF04 was attained after 24 hours of cultivation and then declined (Fujita *et al*., 2001). The optimum flocculating activity of a bioflocculant produced by *Vogococcus sp*. W31 was reached after 60 hours fermentation (Gao *et al*., 2006). Maximum cell development was achieved in 24 hours for *Proteus mirabilis* TJ-1 and *Bacillus licheniformis* X14, while maximal flocculating acitivity was achied after 48 hours of fermentation and then declined (Xia *et al*., 2008; Li *et al*., 2009). Differrent factors attributes to bioflocculant production, consequently, on the bioflocculation process (Salehizadeh and Shojaosadati, 2001). Most of the microorganisms are reported to produce bioflocculants during the exponential growth phase through

biosynthesis processes (Kurane and Matsuyama, 1994; Ugbenyen *et al*., 2012; Manheim and Nelson, 2013).

2.6 Bioflocculation mechanism

Aggregation of colloidal substances by microorganisms is termed bioflocculation. This process is very important in the separation of liquid-solid solution. It is noteworthy to know that properties of dewatering and poor settling process are all as the result of poor bioflocculation. Ardern and Lockett (1914) were the first to investigate a sequencing batch reactor (SBR) or fill and draw-type system which compared to the complex mix systems and have proven to produce fantastic settling biomass (Chiesa and Irvine, 1985; Yadav *et al*., 2020). Research has been done to improve and comprehend better mechanisms of the separation of solid-liquid process and bioflocculation (Szogi *et al*., 2018). The separation process starts by the floc particle formation by microbial aggregation. These floc particles can again be removed through a sedimentation process. Finally, water removal by mechanical means and gravity can be accomplished. In dewatering, settling size of the particle is a critical factor.

Demonstrations through research have been made that illustrates that biopolymers are produced by microbes and these biopolymers are then released into the environment extracellularly, by either active transport or cell lysis (Sutherland, 1972). The produced extracellular polymeric substances also known as extracellular biopolymers (EPS) are synthesized as polysaccharides, humic compounds, proteins, lipids, and nucleic acids (Tenny and Verhoff, 1973; Flemming and Wingender, 2010). Formation of flocs after the microbial aggregation is aided by encapsulated microorganisms from the biopolymer matrix that has been formed. About 80% of the EPS in a floc is represented in activated sludge mass (Frolund *et al*., 1996). As EPS

are primary constituents of bioflocculant, it can therefore be concluded that the interactions within EPS will be of paramount importance in bioflocculation.

Generally, negative charges have been observed on bioflocculants, and this could be due to that EPS have functional groups. For instance, when EPS was extracted for characterization it was revealed that some polysaccharides portions are composed of uronic acids (Horan and Eccles, 1986). The carboxyl functional group is substituted from the C-5 location in the uronic acid structure. At typical pH ranges, carboxyl groups lose protons within the activated sludge system which in turn affects the bioflocculant negative charge. The negative charge of floc is also contributed by the presence of aspartic, glutamic acid, and carboxyl groups. This has been observed from the exocellular characterization (Sobeck and Higgins, 2002).

Since the bioflocculant contains negative charges, this has prompted many researchers around the world to study and show how important is the role of metal ions on bioflocculation (Grady *et al*., 2011). A great deal of research has been focused on the impact of either removal or addition of metal ions on solutions and the properties of bioflocculant subsequence effect.

Some theories have been proposed by researchers which are utilized currently for the mechanism of bioflocculation as influenced by the metal ions and these mechanisms have been backed or supported by many researchers (Chu *et al.,* 2001; Mowla *et al*., 2013). The three promulgated theories are Alginate Theory, Double Layer Theory or DLVO Theory, and Divalent Cation Bridging Theory. These theories are discussed in details below.

2.6.1 Alginate Theory

Bruus *et al*. (1992) was the first to propose the alginate theory and the role displayed by the metal ions on activated sludge during bioflocculation process. Bacteria

produce polysaccharides (alginate), which is formed by the guluronic and mannuronic acids repeating units. This polysaccharide is so unique in such a way that forms alginate gels in the presence of calcium ions (Pawar and Edgar, 2012). Egg-box model is used to refer to the gel that is formed. Figure 2.3 shows the Egg-box model that is involved in gel formation in the presence of calcium ions and alginate (Plazinski, 2011). Some bacteria from an activated sludge including *Pseudomonas aeruginosa* and *Azobacter sp*. have been found to produce alginate and several other bacteria have the potential for alginate production (Grady *et al*., 2011). This therefore, means that alginate is available from the activated sludge.

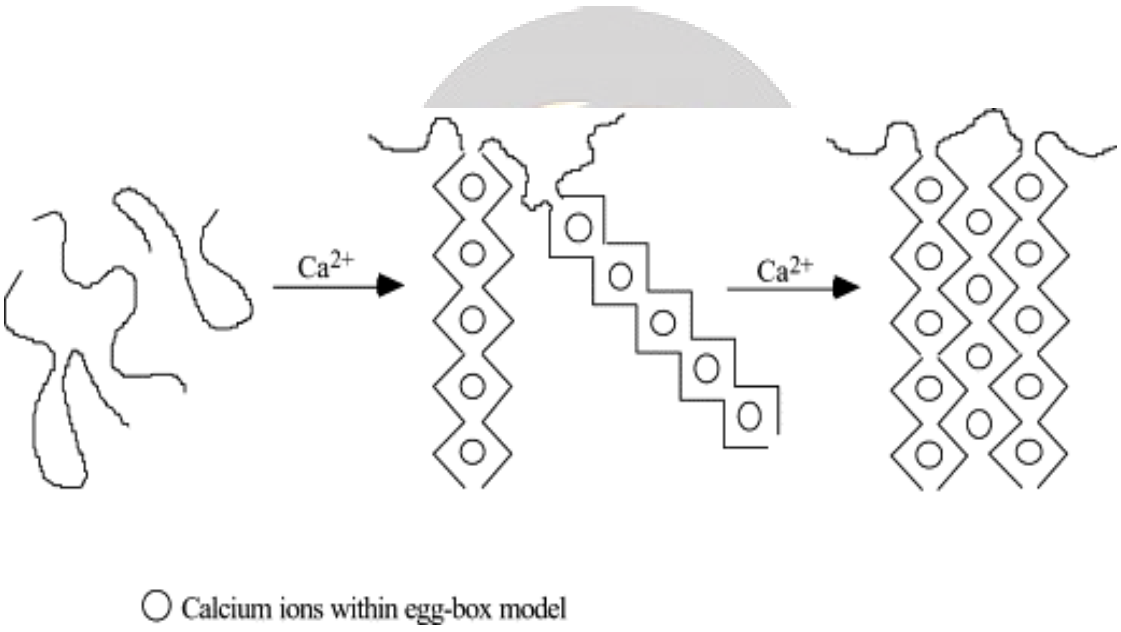

Figure 2.1: Egg-box model and alginate gel formation in the presence of calcium (Plazinski, 2011).

The role observed from several experiments conducted, resulted in the proposal of this model, to explain how calcium affects the microbial aggregation. In the floc, it was observed that calcium ions were displaced by ion-exchange, which was the result of the addition of sodium in high concentrations to activated sludge (Faust *et al*., 2014; Ummalyma *et al.,* 2017). The properties of flocs have deteriorated as a

result of this displacement which can be measured by the turbidity of a supernatant (Neyens and Baeyens, 2003). Researchers have inferred that aggregation induced by calcium is of paramount importance for the process of bioflocculation because aggregation of alginate is only calcium specific (Sobeck and Higgins, 2002). Researchers also found that when magnesium was added, it resulted in a similar exchange of calcium ions between the floc and the properties of floc deterioration (Neyens and Baeyens, 2003). It was therefore, concluded that biopolymer had a greater attraction for calcium than magnesium thus, seconding the alginate role in bioflocculation.

To corroborate the theory further, it was show with the research that synthetic sludge obtained from particles of latex and alginate showed similar characteristics to sludge in terms of properties of dewatering and settling (Ormeci and Vesilind, 2000; Sobeck and Higgins, 2002). It was observed that the properties of dewatering and settling were significantly impacted by calcium concentration while magnesium did not have any impact, which therefore emphasized the specificity of calcium for alginate thus the alginate theory (Ormeci and Vesilind, 2000).

2.6.2 Theory of Divalent Cation Bridging (DCB)

McKinney and Tuzeka (1969) were among the first researchers who proposed divalent cation bridging (DCB) or salt theory (Davey and O'Toole, 2000; Neyens *et al*., 2004). Tezuka (1969) experimented and found that magnesium and calcium were of important in the process of bioflocculation. This was achieved through demonstrations about the role of the divalent cation in the formation of flocs during the monocultures growth. In the EPS, according to the DCB theory, it has been found that the negatively charged functional groups are bridged by the divalent cations. The bridging assists to stabilize and aggregate the biopolymer matrix and

microorganisms and as a result, the bioflocculation is promoted. A divalent cation bridging depiction model is shown in Figure 2.2 (Sobeck and Higgins, 2002). This theory of alginate is a detachment of the DCB theory. The difference amongst the two is that DCB theory is not specific in its divalent cations binding ability while alginate theory involves the formation of gels and specification between alginate and calcium. The DCB theory has been backed by the addition of sodium which resulted in floc properties being deteriorated because of the divalent cation displacement from the floc binding site (Ummalyam *et al*., 2017). A report by some researchers has demonstrated that dividing the concentrations of sum of the monovalent cations Na^+, K^+, and NH_4^+ by the concentrations of the sum of divalent cations $Ca^{2+}$and Mg^{2+} was more than 2, which could lead to deterioration of floc property (Grady *et al*., 2011).

When calculating the ratio of monovalent to divalent (M/D), every obtained concentration is expressed in milliequivalents per Litre (meq/L). Higgins and Novak (1997) commenced proposal for utilization of the M/D ratio as an indicator for instances where there could be problems with properties of floc resulting from increased monovalent cations (sodium) within the system. These findings of Higgins and Novak (1997) could not be satisfactory as thse findings do not explain the mechanism in full, which makes no difference because alginate theory can be utilized to explain these findings.

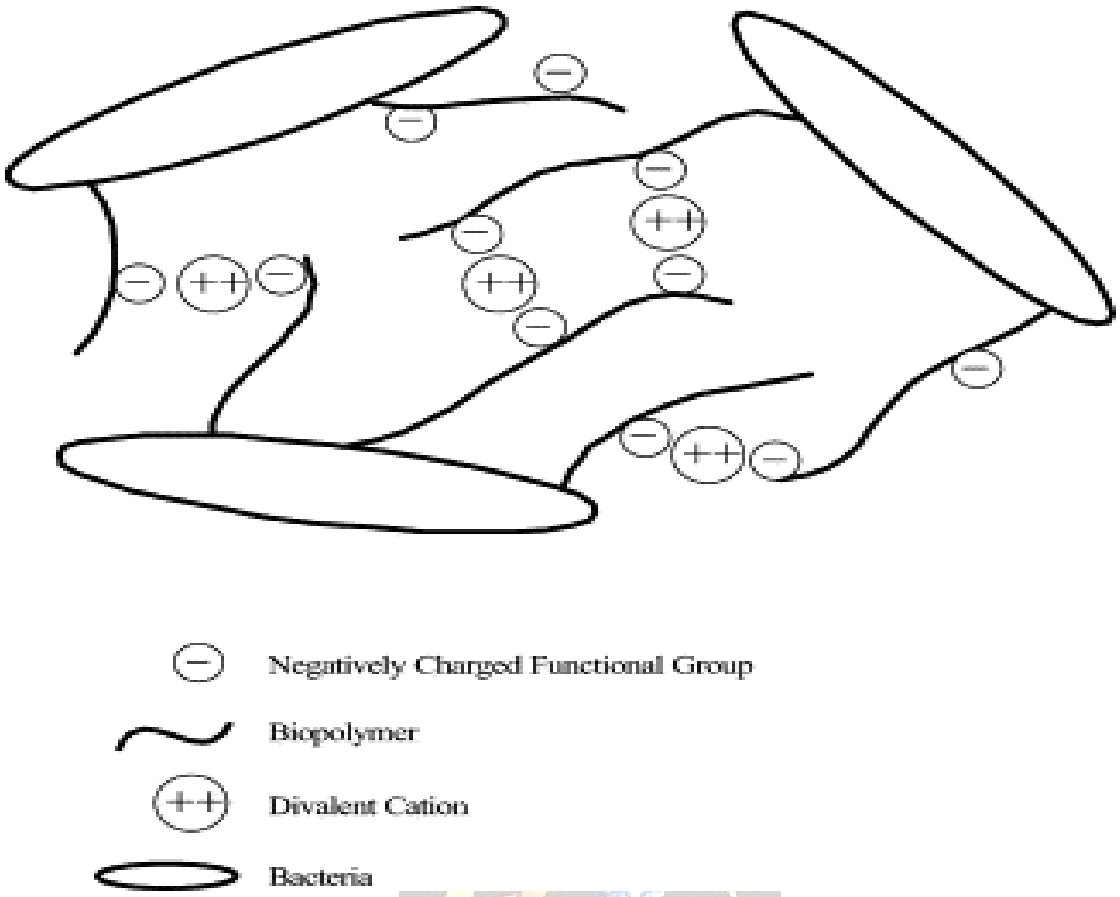

Figure 2.2: A divalent cation bridging depiction model (Sobeck and Higgins, 2002).

2.6.3 Double Layer (DLVO) theory

This theory explains the constituents or particles as doubled layered counter ions which are found around the particle. The Stern layer is considered as the first layer composed of counter ions which are an intact layer of counter ions. The second layer is not tightly associated in its counter ions and is referred to as diffuse layer (Febrega *et al*., 2011). In a diffuse layer, ion concentration decreases from the surface particle about the distance until the bulk solution equals the concentration of ions. As a result, around the particle an electric potential develops. There is a resulting repulsion force around the particles with their neighbouring particles due to this double layer which in turn leads aggregation inhibition. The double-layer size decreases with increase in the ionic strength, thus particle repulsion decreases

within the particles. Finally, aggregation is promoted as the result of the allowed diminutive range of attractive forces (De Schryver *et al*., 2008). This theory suggests that the addition of cations in a solution will augment the process of bioflocculation because there is size decrease in the double layer and the repulsive forces between the particles (Sheng *et al*., 2010).

2.7 Microorganisms that produce bioflocculant and their habitats

Microbes with capabilities for production of bioflocculants have been studied for quite some time and recently, microorganisms are slowly gaining or receiving scientific recognition from around the globe (Wan *et al*., 2013). In the past decades, it has been reported that more than 100 microbial species have been studied and documented to have bioflocculants production potential (He *et al*., 2010). The range of microorganisms from filamentous bacteria of the *Streptomycetes* genus such as actinomycetes, algae and fungi has been reported for bioflocculant production. These microorganisms are of important source for various utilizable microbial exudates and have been extracted from dissimilar environments (Hurst *et al*., 2007). Some of these environments were thought to be unfavourable for the growth of these microorganisms and their utilizable products. However, studies have proven otherwise as these organisms from these environments show beneficial effects for the production of primary and secondary metabolites of useful value on different processes (Xiong *et al*., 2010; Aljuboori *et al*., 2013). From dissimilar sampling sites, samples are collected and brought to laboratories where the microorganisms get grown using different media (selective and enriched). Under appropriate conditions of incubation times, these media get incorporated with antibiotics which then favour the non-fastidious and fastidious growth of microbes. Production of metabolites and cell growth is favoured well in liquid broths. Liquid nitrogen is used to store the

obtained pure colonies or rather freeze-dried in a cryopreservative presence (Akapo, 2019).

Xiong *et al.* (2010) reported a bacterium *Bacillus lecheniformis* to produce a bioflocculant. This bacterium was isolated from Luria Broth medium which was contaminated. Kurane *et al*. (1994) reported the *Rhodococcus erythropolis* to be capable of producing a bioflocculant that is proteinous but when it was exposed to enzyme digestion, it lost its flocculating activity (Subudhi *et al*., 2015). Several reports show that sometimes a conglomerate of bacteria are capable of bioflocculant production including *Bacillus Firmus, Cobetia sp.* OAUIFE, *Bacillus sp*. AS-101, and *Alcaligenes cupidus* KT-201 (Toeda and Kurane, 1991; Ugbenyen *et al*., 2012; Nwodo and Okoh, 2013; Salehizadeh and Yan, 2014). Some organisms with bioflocculant production potential have been found or isolated from the marine sediments including *Bacillus sp*. HXG-C1 (Nontembiso *et al*., 2011) and *Bacillus safensis* (Goldern *et al*., 2020)

Bacillus velezensis reported to produce a bioflocculant with a flocculating activity of above 98% composed of 98% carbohydrates and 2% proteins was isolated from brackish water (Zaki *et al*., 2013). *Bacillus subtilis* F9 isolated from wastewater sludge had an excellent activity for flocculation. The bioflocculant had beneficial utilization on the treatment of industrial drinkable wastewater (Giri *et al*., 2015). Ugbenyen *et al*., (2012) reported that *Cobetia sp*. OAUIFE isolated from Algoa Bay (South Africa) to produce a thermostable bioflocculant. The produced bioflocculant retained a flocculating activity of 78% when exposed to 100 °C for about 25 minutes. *Pantoea sp*. isolated from Mthunzini beach, KwaZulu-Natal, produced a bioflocculant with the excellent flocculating activity of 92.4% with inoculum size of 3% (v//v) (Ugbenyen *et al*., 2017), while by He *et al*. (2010) reported a

bacterium *Halomonas sp*. V3a' from deep sea, to produce a bioflocculant HBF-3 with a flocculating activity of 96.9%. These organisms has been reported to be found from marine and sediment environments while some of them are obtained from other sources (Wang *et al*., 2013).

2.8 Halophilic microbes with potential for bioflocculant production

These microorganisms are sometimes called halotolerant and are capable of living in high saline environments. Halotolerant microorganisms have been found useful in many fields including biotechnology. It has been reported that two ways or strategies could afford an organism to live in a saline environment (Santos and Costa, 2002). These two strategies are osmoprotectants also known as compatible solutes which is employed by most halophilic bacteria, algae, some archaea, yeasts, and fungi and the second one is about absorbing potassium (K^+) in to the cytoplasm (restricted mostly to halophilic bacterial order *Halanaerobiales* and *Halobacteriaceae*, and *Salinibacter ruber*) (Santos and Costa, 2002). Halotolerant Archaea preserves a balance of osmosis in their cytoplasm with the hypersaline environment by increasing the high salt concentrations. For this osmoregulation mechanism to operate efficiently, the intracellular enzymes must have peculiar adaptations so it can function well in the presence of salt (Oren, 1999). These intracellular enzymes have the most amazing metabolic diversity which characterizes the halotolerant eubacteria and make them easily adaptable to the most extreme environments. Halotolerant microbes have less concentration of salt intracellularly, so these microbes maintain cytoplasm osmotic balance with the peripheral medium through the build-up of increased concentrations of organic osmotic solutes. Intracellular enzymes have zero tolerance for salt (Oren, 1999). Among the bacterial isolates from soil samples at Tuzi Gazi, Richards Bay Republic of South Africa was a novel halophilic bacterium

Bacillus atrophaeus with bioflocculant-producing potential (Akapo *et al*., 2019). Sam *et al*. (2011) reported a halophilic bacterial strain *Halomonas sp*. AAD6 to produce a bioflocculant with pollutant removal efficiency of 93%. Cosa *et al.* (2011) reported a bioflocculant produced by a bacterial strain *Virgibacillus sp*. isolated from the bottom sediment of Algoa Bay in the Eastern Cappe, South Africa.

In general, there are three domains of life where the halophilic microorganisms can be found from namely, Bacteria, Eukarya and Archaea (Woese *et al*., 1990). Halophiles are classified as moderate, slightly and extreme halophiles (Sanchez-Porro *et al*., 2003). Literature shows that many marine microbes prefer salt concentrations between 3-15% while some would be optimally favoured by high salt concentrations (25% and above extreme halophiles) (Harding and Simpson, 2018). Various types of biomolecules have been reported to be produced by halophilic microbes (Schiraldi and Rosa, 2002). These are pigments and intracellular polyester polyhydroxyalkanoates, as a result of the nature of their hypersaline which is deleterious (Hernandez-Nunuez *et al*., 2019). Many biotechnologists and scientists around the world have devoted much of their attention to these organisms because these microbes show promising usage from many fields (Katheresan *et al*., 2018).

2.9 Kombucha tea in bioflocculant production

Kombucha tea is made up of a Scoby. This Scoby is an acronym for a Symbiotic Culture of Bacteria and Yeast which are produced during a complete fermentation by acetic acid bacteria, lactic acid bacteria and yeast to form several sour beverages and foods, including, Kimchi and Kombucha tea (Villarreal-Soto *et al*., 2018). The SCOBY in its most commonly observable form is a cellulose-based biofilm, gelatinous or a microbial mat suspended air-liquid interfaced container (Gilbert *et al*., 2021). The word pellicle is the formal known name for this consolidated layer. Its

ability to absorb water gives the Scoby a potential to be even able to house a small amount of the previous media and products (Villarreal-Soto *et al*., 2018). A variety of bacterial species and yeast are utilized for each particular Scoby product. Most of the cultures are commonly aerobic, Gram-negative acetic acid bacteria species, including, *Gluconobacter, Komagataibacter*, and *Acetobacter*, yeasts such as *Zyosaccharomyces* and *Saccharomyces* and aerobic, Gram-positive lactic acid bacteria, including, *Lactobacillus* (Rojan *et al*., 2005; Yao and Nokes, 2013). Kombucha tea contains lot of bacterial microorganisms and this environment has not been explored much for isolation of microbes with bioflocculant-production potential and as a result, there is no literature about Kombucha tea as a source of novel bioflocculant-producing microorganisms.

Figure 2.3: Picture of Kombucha tea with Scobies inside forming layers.

This study isolated the bioflocculant producing organism from Kombucha tea Scoby, and identified the microbe by 16S rRNA as *Pichia kudriavzevii* which falls under the *Saccharomycetes* class and family of *Pichiaceae* and also considered as a *Candida*

krusei teleomorph (Deak and Peter, 2013). The microbes have an oval or ellipsoidal shape of cells and mostly found in the skins of fruits, fermented beverages and soil (Guarro *et al*., 1999). This organism can be active metabolically even at high temperatures and low pH values such as 45 °C and pH 4, respectively (Toivari *et al*., 2013). Some literature studies have reported the microbe as an effective producer of ethanol (Dhaliwal *et al*., 2011). In this study, it showed an excellent flocculating activity of 85% at a temperature of 35 °C with pH 7.

2.10 Factors influencing flocculating activity of a bioflocculant.

Flocculating activity of the purified bioflocculant is reported to be influenced by number of factors including dosage, pH stability, temperature and cation. These factors are discussed in details below.

2.10.1 Dosage size for production of a bioflocculant

The effect of dosage size is very important in the flocculation process of the bioflocculant because it determine the most cost-effective dose for the flocculation process. Studies show that under optimal flocculating conditions, it is reported that the highest flocculating activity is obtained with optimal bioflocculant dose (Wang *et al*., 2011). A decrease in the flocculating activity of a bioflocculant has been suspected to be linked with the excessive addition of negatively charged bioflocculant, which creates strong repulsive forces been the kaolin clay particles and the bioflocculant (Yin *et al*., 2014). These processes restore particle stability by increasing suspension viscosity, blocking adsorption sites, and significantly reducing floc formation (Yuan *et al*., 2011). Therefore, a good dosage size is very important for the produced bioflocculant. Low dose size and high flocculating activity of the bioflocculant reduces water treatment costs. The bioflocculant MBF-UFH showed

flocculating activity of 90% within dosage 0.1 – 0.3 mg/mL (Okaiyeto *et al*., 2015b). The flocculating activies for the bioflocculant CBF-F26 was above 90% for dosage concentration ranges from 8 – 24 mg/L, with optimum dosage of 12 mg/L (Wang *et al*., 2011). Yim *et al*. (2007) reported on a bioflocculant p-KGo3 produced by a marine diflagellate, *Gyrodinium impudicum* KGo3 which was effective at dosage size of 1.0 mg/L. An optimum dosage size of 20 mg/L was reported for PY-90 bioflocculant produced by *Bacillus sp.* (Yokoi *et al*., 1995). Some of the bioflocculants and their dosage sizes that have been reported in Table 2.3.

Table 2.3: Dosage sizes for different bioflocculants

Bioflocculants	**Dosage size (mg/L)**	**Citation**
WF-1 (Enterobacter aerogenes)	90	Lu *et al*. (2005)
MBFF19 (*Bacillus sp*)	2.0	Zheng *et al*. (2008)
MBF-6 (*Klebsiella pneumoniae*)	50	Luo *et al*. (2014)
Bioflocculant by *Rhodococcus erythropolis*	40	Peng *et al*. (2014)
Bioflocculant by *Streptomyces plastensis*	0.2	Agunbiade *et al*. (2018)
Bioflocculant by *Alcaligenes faecalis* HCB2	0.8	Maliehe *et al*. (2019)
MBF-2 (from novel strain SW-2)	20	Zhong *et al*. (2014)
Halomonas sp. Okoh and *Micrococcus sp*. Leo	0.1	Okaiyeto *et al.* (2013)

2.10.2 pH for production of bioflocculant

The pH of the solution is a key parameter in flocculation and has a major impact on flocculation process (Zhang *et al.,* 2007; Kelland, 2014). Both the dispersion stability of suspended particles and the formation of floccules are affected by pH. Thefore, it must be well monitored so that a specific range can be used for a particular

bioflocculant for effective flocculation (Chen *et al*., 2017a). The pH conditions for numerous bioflocculants vary considerably. Prasertsan *et al*. (2006) reported that bioflocculant produced by *Enterobacter cloacae* WD7 had an optimum flocculating activity at pH 6.0. The bioflocculant p-KGo3 had high flocculating activity at acidic conditions with optimum detected at pH 4.0 (Yim *et al*., 2007). The ideal pH range was 5.0 – 7.0, which was slightly acidic or neutral for the bioflocculant SF-1 (Fujita *et al*., 2000; Gong *et al*., 2008). The effect of the pH is usually associated with the presence of the functional groups of the biopolymer (Gong *et al*., 2008). *Bacilllus mucilaginosus* GYO3 produced bioflocculant that was able to flocculate effectively at pH 8.5 (Lian *et al*., 2008).

2.10.3 Temperature for production of a bioflocculant

Different bioflocculants have different preferences for temperature for their optimum flocculating activity (Ayangbenro *et al*., 2019). If a glycoprotein is the primary aspect of a bioflocculant, the relative compositions of protein and polysaccharide will determine its stability (Deng *et al*., 2003). Bioflocculants containing carbohydrates in the structure are thermally stable, while those with protein or peptide backbone are typically heat sensitive (Nwondo *et al*., 2012). The structure of protein bioflocculant will be easily broken by heating; therefore, proteins are classified as thermally unstable. For example, the protein bioflocculant NOC-1 produced by *Rhodococcus erythropolis*, was inactivated by heating at 100 ºC for 30 minutes, resulting in a 50% reduction in flocculation capacity (Natarajan, 2015). Furthermore, the flocculating activity of a homopolymer bioflocculant produced by *Bacillus sp*. PY-90 decreased rapidly at high temperatures and was almost fully inactivated after 40 minutes of heating at 100 ºC (Yokoi *et al*., 1995). The bioflocculant MBF-TG-1 thermal stability was measured for 30 minutes at 30, 40, 50, 60, 70, 80, 90, and 100 ºC and it was

observed that the flocculating rate of the bioflocculant was between 86.7 – 83.8% in the temperature range of 30 – 70 ºC (Liu *et al*., 2013). *Cobetia* and *Bacillus* genera in consortium produced a bioflocculant which after heating at 50 ºC and 80 ºC retained high flocculating activity of 86.7% and 89.3%, respectiviely (Ugbenyen and Okoh, 2014). According to Gong *et al*. (2008), bioflocculant SF-1 retained 85% residual flocculating activity after being heated to 100 ºC for 15 mintues. After heating at 100 ºC, the bioflocculant CBF-F26 showed over 90% of its flocculating activity (Wang *et al*., 2011). A bioflocculant from *Bacillus megaterium* G04 isolated from swine wastewater was investigated for its thermostablity, and it was observed that a flocculating activity of over 85% could be attained in temperature ranges of 10 – 80 ºC, a further 80% could still be obtained after heating the bioflocculant at 90 – 120 ºC for 30 minutes (Guo and Chen, 2017). Giri *et al*. (2015) reported on a bioflocculant produced by *Bacillus subtilis* F9 which retained flocculating activity of 89% when heated for 15 minutes at 100 ºC. A bioflocculant MSI021 proved stable at all temperatures tested from 20 – 120 ºC. A 98% of the flocculating activity was retained when the bioflocculant was heated at 70 ºC, though it lower a little to 80% when further exposed to much higher temperatures (120 ºC) (Sajayan *et al*., 2017).

2.10.4 Cations for bioflocculant production

There are various cations that play a major role in augmenting the bioflocculant so that it can perform it functions effectively. Cations with effect on bioflocculant are divided into monovalent cation (K^+, Li^+ and Na^+), bivalent cations (Zn^{2+}, Fe^{2+}, Ba^{2+} and Ca^{2+}), and trivalent cations ($Al^{3+}$and Fe^{3+}). Bivalent and trivalent cations have large charge capacities compared to monovalent cations due to their to presence of extra valence ions on their last orbitals (Hu and Gao, 2007). Monovalent cations have been preferred unlike trivalent that are very difficult on removal from

wastewater after treatment, which in turn can cause environmental complications (Patil *et al*., 2011). Cations on the purified bioflocculant are often added inorder to attain high flocculating activity (Pal and Paul, 2008). Cations play a role in increasing the initial adsorption of biopolymers on suspended particles by lowering the negative charge of both biopolymers and suspended particles. Zhang *et al*. (2002) documented that a bioflocculant from marine myxobacterium *Nannocystis sp*. NU-2 was highly effective when cations Al^{3+} and Fe^{3+} were added. No flocculation occurred when these cations were not added on the bioflocculant. In the presence of Zn^{2+}, the bioflocculant WF-1 greatly improved the separation of suspension solid from trona suspension (Lu *et al*., 2005). Wang *et al*. (2011) reported that due to a weak electrostatic force between the monovalent metals and the bioflocculant, a monovalent metal such as Na^{+} was unable to effectively enhance the flocculating activity of the bioflocculant from a consortium of *Cobetia* and *Bacillus sp*. Other studies also reported that monovalent showed weak electrostatic forces between their bioflocculant cations and bioflocculant (Salehizadeh *et al.,* 2018; Elkady *et al*., 2011; Yang *et al*., 2012; Okaiyeto *et al*., 2015b). However, some literature have reported on monovalent as proving to have effective flocculating activity for bioflocculant BM2 (Pu *et al*., 2020). Divalent cations are also mostly preferred for addition to augment the bioflocculant flocculation. Ca^{2+} was reported to show flocculating activity of 88.6% for bioflocculant BM2 (Pu *et al*., 2020). Divalent cations (Ca^{2+} and Mg^{2+} increased the flocculating activity of a bioflocculant generated by *Bacillus sp*. As-101. Trivalent cations have also been studied and shown to influence flocculating activity of the bioflocculant. Wang *et al*. (2015) reported on a bioflocculant which was not improved by the addition of Al^{3+} and F^{3+}. The reason behind this could be attributed by that these cations can shield the molecular chains

adsorption characteristics, thus altering the surface potential of kaolin partiles leading in low flocculating activity. Bioflocculant MBF-L03 showed improved flocculating activities when Ca^{2+},Fe^{3+}, and Al^{3+} were utilized while addition of monovalent (K^{+} and Na^{+}) did no have any effect on flocculating activity (Oh *et al*., 2001).

2.11 Hydrophilicity and hydrophobicity

The fundamental property of a bioflocculant is its hydrophobicity. The molecules or rather particles of the bioflocculants have a special behaviour which leads to its hydrophobicity. This is due to these molecules failing to electrostatically join the bonds of hydrogen with water (More *et al.*, 2014). The carbohydrates hydrophobic regions, and the nonpolar groups, such as, aliphatics in proteins, and aromatics and many others charged functional groups including phenolic, sulfhydryl, phosphoric, hydroxyl, and carboxyl are all constituents of the bioflocculants (Leis and Flemming, 2003). The adsorption of an organic pollutant would be well established if the bioflocculant has hydrophobic formation regions around it (Flemming *et al*., 2016). The amphoteric nature of bioflocculant is a proof that within the bioflocculant there is the presence of both hydrophobic and hydrophilic groups (Bisht and Lal, 2019). The importance of bioflocculants is also shown through the presence of these groups as the hydrophilic and hydrophobic groups serve as the sites of sorption for organic pollutants (Lies and Flemming, 2003). Liu and Fang (2002) reported that hydrophobicity of aggregates of microorganisms together with their formation in bioreactors is more likely to be strongly influenced by the hydrophilicity/hydrophobicity of the bioflocculant.

2.12 Production of bioflocculant using the cost-effective substrates

Bioflocculants utilization has gained huge interest from biotechnology and scientific world recently. This is due to the fact that their breakdown products are very harmless and bioflocculants are of prospect application and are also degradable from the environment thus imposing no pollution to the society (Nakata and Kurane, 1999; Nwodo and Okoh, 2013). The problem that has been observed so far with these flocculation agents resulting not to be applied industrially is their ability to flocculate the wastewaters which is very low and high production cost with less production yield (Mabinya *et al*., 2012). The market potential of the bioflocculants has been restricted by the most widely used substrates such as sucrose, maltose, galactose, glucose, and fructose. These substrates bring about the negative influence on the cost of the bioflocculants production. Some literature studies show that one of the major ways to try and counteract this problem is to find cheap substrates for bioflocculant production at an industrial scale (Fujita *et al*., 2000). He *et al*. (2004) and Zhang *et al*. (2012) reported that low-cost substrates have been used for quite sometimes. For example brewery wastewater has been used as a source of carbon for the production of bioflocculant by a conglomerate of microbes (Adebami and Adebayo, 2013).

Much attention has been devoted on bioflocculant-producing microorganisms for isolation and fermentation on cheap substrates. Optimization of fermentation media constituents has been reported to result in a increased production of bioflocculant by microorganisms (Muthulakshmi *et al*., 2013; Xia *et al.,* 2018). Some of the examples where the bioflocculants were produced using cheap energy sources are discussed below. The potential of a bioflocculant produced by *Bacillus licheniformis* using low-cost culture medium and their industrial application was investigated with molasses

being used to replace sucrose as a sole carbon source (Zhang *et al*., 2012). Novel bioflocculants produced from *Rhizobium radiobacter* and F6 *Bacillus sphaericus* (BS-MBF) were produced using wastewater from anaerobic co-digestion of corn straw and molasses (Zhao *et al*., 2017). The bioflocculant produced by the fungus *Mucor rouxii* was reported to use beet-molasses as a low cost substrate under optimized condtions (Li-fan *et al*., 2008). Some of the cheap substrates used for the production of bioflocculants are discussed in details below.

2.12.1 Brewery wastewater as cheap substrate for bioflocculant production

The flocculating behaviour of MM1, a novel bioflocculant developed from MM1 multi-microorganisms consortia, was investigated. These strains were *Staphylococcus sp*. BAFRT4 and *Pseudomonas sp.* CYGS1with flocculating activity of 82.9% obtained using brewery wastewater as an energy source (Zhang *et al*., 2007). *Serratia ficaria*, a bioflocculant-producing bacterium was isolated from soil produced bioflocculant with flocculating activity of 95.4% using brewery wastewater under optimized culture conditions (Gong *et al*., 2008). A strain of *Enterobacter sp*. P3 produced a novel bioflocculant (BW-P3) using brewery wastewater as a substrate, which was then used to extract the dyes from fracturing flow-back water (Ma *et al*., 2020). Cosa and Okoh (2014) reported the potential of the bioflocculant produced by the consortium of two marine bacterial species *Oceanobacillus* and *Halobacillus*, isolated from sediment samples of Algoa Bay in the Eastern Cape Province of South Africa with the highest flocculating activity of 98.3% using brewery wastewater as a source of energy. The characteristics of *Bacillus sp*. BF3-3 which produced the bioflocculant MBF3-3 were investigated by Feng *et al*. (2008) where brewery wastewater was used as energy source. MBF3-3 showed excellent flocculating behaviour in both real

and synthetic wastewaters, and consumed much lower dosage than the commonly used polyaluminium chloride (PAC).

2.12.2 Sludge wastewater for bioflocculant production

Zhang *et al*. (2013) reported four novel bioflocculants (PSB-1-4) obtained using sludge wastewater as a source of energy and the results revealed that PSB-2 strain was the most effective with flocculating activity of 96.0%. *Klebsiella sp*. produced a bioflocculant MBF10 was reported to have a good flocculating activity of 86.5% when wastewater from activated sludge was used (Yang *et al*., 2012). *Rhodococcus erythropolis* used wastewater sludge as a cheap carbon source to produce its bioflocculant with maximum flocculating activity of 87.6% (Peng *et al*., 2014). *Cloacibacterium normanense* was grown in sterilized wastewater sludge fortified with glycerol and produced 25 g/L of extracellular polymeric substance and produced the flocculating activity of 95.3% in a kaolin suspension (Nouha *et al*., 2016).

2.12.3 Starch wastewater for bioflocculant production

The *Paenibacillus polymyxa* was able to produce a bioflocculant optimally using starch wastewater as a cheap substrate and the flocculating activity was 69.7% (Guo *et al*., 2015). *Rhizopus sp*. M9 and M17 produced a complex bioflocculant MBF917 using potato starch wastewater as nutrient source. It showed good flocculation characteristics in treating wastewater (Pu *et al*., 2014). To produce a bioflocculant, starch wastewater was used as a fermentation medium for *Rhodococcus erythropolis* (Guo *et al*., 2018b). Pu *et al*. (2018) reported on bioflocculant MBFA18 produced by *Aspergillus niger* (A18) using starch wastewater as the nutrient source. The bioflocculant-producing potential of *Klebsiella pneumoniae* strain NJ7 was investigated using stach wastewater was used as a source of its nutrients (Joshi *et*

al., 2017). *Candida anglica* F5 was used to produce a bioflocculant utilizing starch wastewater as a substrate for its nutritional condions (Yan and Yun, 2013).

2.13 Application of bioflocculants

The chemical agents that have been used currently for the treatment of wastewater have been reported to pose serious environmental issues (Neyens *et al.,* 2004; Crini, 2005). Therefore, this has resulted in the bioflocculants utilization, because bioflocculants have not been implicated in any health or environmental crisis (Huang *et al*., 2014; More *et al*., 2014). Bioflocculants show reduced secondary impurities and are biodegradable. These properties afford them the ability to be used effectively without causing any effects to the ecosystem (Luvuyo *et al*., 2013; Ugbenyen *et al.,* 2014; Kothari *et al*., 2017). These unique characteristics of bioflocculants make them the alternative to replace the chemical agents used currently. Moreover, bioflocculants have been applied in several fields such as in food and fermentation processes, in pharmacology, mining, and downstream processing (Salehizadeh *et al*., 2018). Bioflocculants are also utilized in wastewater treatment for colour removal, biological oxygen demand as well as chemical oxygen demand removal (Vijayalashmi and Raichur, 2003; Luvuyo *et al*., 2012; Tang *et al*., 2014). Some of the applications of bioflocculants are detailed below.

2.13.1 Wastewater purification

In water purification or the treatment of wastewater, microorganisms as flocculating agents have been utilized to remove numerous impurities that are contained in water. These impurities include biological oxygen demand, chemical oxygen demand, heavy metal ions, pathogens, inorganic solids, humic acids, dyes and other synthetic impurities (Zouboulis *et al*., 2004, Sun *et al.,* 2015b). For example, a

bioflocculant utilization in the treatment of impurities in wastewater was observed when a bioflocculant (S-14) produced by *Serratia ficaria* was able to flocculate suspended particles wastewater, brewery wastewater, river water, and wastewater from meat processing plants (Gong *et al*., 2008). This bioflocculant was also reported to treat pulp effluent by 72.1% removal rate and chemical oxygen demand (COD) with 99.9% which is better when compared to the chemical flocculants frequently used (Gong *et al*., 2008).

Bioflocculant MBFA9 was produced by *Bacillus mucilaginous* isolated from the soil with the excellent flocculating activity of 99.6% with kaolin using a dosage concentration of 0.1 ml/L (Deng *et al*., 2003). This bioflocculant (MBFA9) was applied in starch wastewater treatment and the floc formation were accelerated greatly together with the particles settling in the presence of calcium ions. After settling for about 5 minutes, the rate of removal for chemical oxygen demand and suspended solids were approximately 85.5% and 68.5%, respectively (Deng *et al.,* 2003).

Liu *et al*. (2008) reported a bioflocculant M-1 produced by microbes isolated from backwashing sludge. When all the conditions were well optimized, the bioflocculant had kaolin clay flocculating rate of 92.67%, and its activity was also examined in decolourization of synthetically dyed polluted water. The efficiency of decolourization on fast blue and methylene blue flocculation in aqueous solution was 77.8% and 82.9%, respectively. *Paenibacillus elgii* B69 produced a bioflocculant with a good flocculation activity for removal of pollutants on dyeing pigment, real wastewater, heavy metal ions, and kaolin clay (Li *et al*., 2013).

Manheim and Nelson (2013) have explored the bioflocculation process for algae settling improvement using a bioflocculant. The EPS produced by both algal and

cultures of bacteria in the wastewater environment create spontaneous aggregation of algae by the formation of bridging effect which results in bioflocculation. Oh *et al*. (2001) reported that when the bioflocculant-producing bacterium *Paenibacillus polymyxa* (AM49) was used as a flocculating agent, the removal efficiency of *Chlorella valgaris* in the medium was more than 90%.

Reports on chemical oxygen demand (COD) in water from wastewater meat processing plant was removed by the bioflocculant produced by *Streptomyces platensis* obtained from Sterkfontein dam (46.6%). This bioflocculant also reduced the turbidity of these pollutants by 84.3% and 75.6%, respectively (Agunbiade *et al*., 2018).

2.13.2 Dye removal

The textile wastewater toxicity and the organic load are as the result of the colour change produced by the intractable organic molecules called dyes. The presence of the highly toxic metal dyes and the reduced penetration of light are as the result of untreated effluent sources mostly with dyes (Chen *et al*., 2017b). This has a bad impact on lakes and rivers and are detrimental to aquatic life (Khan and Malik, 2014). Huge sludge production and the induced Alzheimer's disease in humans are both as a result of alum usage in wastewater treatment as a flocculating agent and it is no longer advised to continue using it (Lee *et al*., 2014b). The imperative issue currently in wastewater treatment is the development of biodegradable flocculating agents that will limit the health and environmental risks. Experimentally, textile wastewater dyes are inoculated with a bacterial bioflocculant to assess its ability to remove the dyes. Metal multifaceted dyes are mostly based on chromium, which is carcinogenic (Mishra and Tripathy, 1993; Gupta *et al*., 2020). Disperse dyes do not ionize in aqueous medium and some dissolved dyes have also been shown to be

bioaccumulative (Baughman and Pereich, 1988; Srivastava and Prakash, 1991). Due to chemical stability and low biodegradability of these dyes, convectional biological wastewater treatment systems are incompetent in treating dye wastewater (Srivastav *et al*., 2019). The bioflocculant produced by *Aspergillus parasiticus* was reported to illustrate good flocculating activities depending on the dye and concentration used (90% for AY25 and RB4, 43.5% for AB45) (Deng *et al*., 2005). The ability of bioflocculants produced by bacterial isolates common to wastewater treatment plant in removing dye was investigated in the study by Hassan (2015). The results revealed that all of the produced bioflocculants were able to remove various dyes tested from industrial wastewater. This led to, varying degrees with more than 90% dye removal by inducing particle and cell aggregation due to bridging and charge neutralization mechanisms. Mittal and Gupta (1996) observed bioflocculant produced by the fungus *Fomitopsis carnea* on the removal of three cationic dyes. The Orlamar Red BG, Orlamar Blue G, and Orlamar Red GLT, and reported on the removal decrease of these cationic dyes with decrease in pH. Chen *et al*. (2017b) reported the bioflocculant produced by *Alteromonas sp*. to be able to remove direct black (97.9%), Congo red (98.5%) and Methylene blue (72.3%) dyes.

2.13.3 Removal of metal ions using bioflocculant

The reservoirs most substantially deliberated globally are resources of fresh drinking water. All living organisms on the planet should have free access to potable water for their survival (Okaiyeto *et al*., 2016). The water available is being reduced by world population growth, long droughts, and developing industries (Gourdon *et al*., 1990). The high levels of recalcitrant impurities contaminate natural water resources. This is due to the incompetent treatment of industrial wastewater (Butler *et al*., 2021). In the earth crust, the amplest chemical elements found are metals, while in the food, these

chemical elements are in low concentrations. It is because of their concentrations and types of metals that their toxicological or nutritional hazard varies (Tawila *et al*., 2019). Toxic substances in water from earth quake, storms, volcanoes and any other natural disaster are discharged as a result of lack of the environmentally friendly procedures by anthropogenic industries such as agriculture and mining (Gupta *et al*. 2012). The metals most commonly associated with negative effects are lead, mercury, arsenic, and cadmium (Keil *et al.,* 2011). The negative effects that these metals have include high blood pressure, damage to the reproductive organs, lung damage, brain damage, vision problems, skin changes, neurological problems, cancer, reduced lung and kidney function (Yang *et al*., 2019). Some bioflocculants have been documented to eliminate metal ions from wastewater. For example, a bioflocculant QZ-7 produced by *Bacillus salmalaya* 139SI was found to have the removal efficiencies of 89.8% for As, 77.4% for Zn^{2+}, and 58.4% for Cu^{2+}. The highest mercury and copper removal efficiency of 89.59% and 87.93%, respectively, was recorded for the bioflocculant produced by *Pseudomonas aeruginosa* ATCC-10145 (Gomaa, 2012). The bacterial strain *Rothia sp*. ZHT4-13 produced a bioflocculant MBF4-13 with ability to remove some heavy metal ions such as Nickel (Ni^{2+}) and Dichromate ($Cr_2O_7^{2-}$) (Gao *et al*., 2009). Pathak *et al*. (2017) reported that the carbohydrate monomer constituents of the bioflocculant were investigated, and its functional applicability for removal of various metal ions (Ni^{2+}, Zn^{2+},Cd^{2+}, and Pd^{2+}) from aqueous solutions were made at concentrations of 1- 50 mg/L. The highest flocculating activity (79.29%) of the bioflocculant was obtained for Ni^{2+} removal.

2.13.4 The bioflocculants' role in the removal of pollutants from mine wastewater

The developments of technologies by humans such as metal surfacing industries, mining and metallurgy have contributed to a prompt expansion of industries (Mao *et al*., 2020). The environment has seen high levels of toxic metallic wastes being deposited, making the environment an unpleasant place for its inhabitants. Human beings are affected because this leads to the distortion of the biological functions and health. Once in the environment, these non-biodegradable components become intractable (Batta *et al*., 2013). In South Africa where the nation's economy is immeasurably being contributed by mining, there are some metals considered to be toxic even at low concentrations, including toxic-trio of metals (cadmium, mercury and lead) (Subudhi *et al*., 2016). The methods that are utilized to remove these pollutants are not eco-friendly and also costly. These methods make use of ion-exchange technologies for the incineration and precipitation of metal residues (Green-Ruiz *et al*., 2008). The flocculating efficiency of the chemical flocculants has been used for the treatment of surplus heavy metals and wastewater treatment (Chiu *et al*., 2016). Food insecurity is one of the leading causes of death of fish due to oxygen deprivation. This is due to the cationic polymers that are utilized in the removal of metal residues from water are lethal to aquatic life since it clogs into the surfaces of their gills (Subudhi *et al*., 2015). The environmental clean-up has been a hurdles and as a result, microbial products have been given much devotion than in-use chemical flocculants which have various complications concomitant with them. The environmental standards are very strict in carrying this procedure (Banks *et al*., 2006; Darzins *et al*., 2010). Study shows that some flocculants in wastewater treatment plants have proven to be resistant in metals. These biochemical flocculants are produced by bacteria (Buthelezi *et al.,* 2015; Azzam and Tawfik, 2015; Liu *et al.,* 2015b). For example, in the polymer chains of the biopolymers there

exist innumerable chemical functional groups such as amino groups, acetamido, carboxyl and hydroxyl. Besides their possession, of functional groups have physicochemical properties at which they function as absorbents of particulars (Dobrowolski *et al*., 2017). Patil *et al*. (2011) reported that the bioflocculant produced by *Azatobacter indicus* CAN was able to reduce BOD, COD and SS in wastewater samples in the ranges of 38-80%, 37-79% and 41-68%, respectively, at a dosage of 500 mg/L. *Alcaligenes faecalis* HCB2 isolated from sediment sample of Sodwana Bay produced a bioflocculant with a removal efficiency of 79.2% for COD from coal mine wastewater (Maliehe *et al*., 2019). Dlamini *et al*. (2020) reported that iron nanoparticles synthesized using a polymeric bioflocculant had effectively removed about 76% of COD and 81% of BOD in wastewater samples collected from coal mine water.

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Chapter 3

This chapter describes the methodology of the study as well as the statistical analysis.

3.0 Materials and Methods

3.1 Bioflocculant-producing microorganisms isolation

The Kombucha tea with a SCOBY was purchased from Greenheart Organics Pinetown in Durban KwaZulu-Natal Province, delivered to the University of Zululand at Kwa-Dlangezwa, in the Province of KwaZulu-Natal in South Africa and used for bioflocculant production. The Kombucha tea SCOBY was serially diluted using sterile saline water (0.85%) up to 10^{-5} dilution. For microbial cultivating, potato dextrose agar (PDA), nutrient agar (NA) and Mueller-Hinton agar (MHA) media were used. About 100 microliters of the diluted Kombucha tea broth was spread on agar plates surfaces as described by Ntozonke (2015). All the inoculated plates were incubated up to 3 days at 37 °C to obtain colonies. After 3 days of incubation, the colonies obtained were randomly picked and then subcultured on new agar plates to obtain pure cultures and incubated for 24 hours at 37 °C. Pure cultures were then stored at -80 ºC refrigerator in 80% sterile glycerol broth. Kombucha tea was prepared as follows: The tea was made by boiling distilled water in a clean beaker. After the tea was cooled down, the starter culture (Scoby) was then added in a jar. Lastly, the jar was covered with a few layers of a tightly woven cloth and was wraped around with a rubber band. This Kombucha tea broth was used in the preparation of the production medium for bioflocculant-producing microorganisms.

3.2 Isolates activation for fermentation

A Litre of medium containing 10 g tryptone, 5 g sodium chloride, and 3 g beef extract was prepared in 1 L of distilled water. About 5 mL of the activation medium was transferred into dissimilar test tubes and autoclaved at 121 °C for 15 minutes. After autoclaving, the isolates were inoculated into the media at room temperature in test

tubes and incubated for 24 hours at 37 °C with a shaking speed of 160 rpm in a rotary shaker (Ntozonke, 2015; Ajao *et al.,* 2021).

3.3 Screening for bioflocculant production

The method of Chang *et al.* (1998) and Maliehe *et al*. (2016) was followed for the bioflocculant production of the isolates. Production medium constituted of 20 g glucose, 0.5 g urea, and 0.1 g NaCl, 0.3 g NH_4SO_4, 0.3 g $MgSO_4$, 5 g K_2HPSO_4, 2 g KH_2PSO_4, and 0.5 g yeast extract was prepared in 1 Litre of Kombucha tea broth. Conical flasks (250 mL) were used to pour the production medium (50 mL), and autoclaved at 121 °C for 15 minutes. Each flask was then inoculated with a pure culture. All the inoculated flasks were incubated for 3 days at 30 °C with a shaking speed of 160 rpm. After 3 days of incubation, 2 mL of the fermented medium was pipetted into sterile Eppendorf tubes and centrifuged at 8000 x g for 15 minutes at 4 °C. The supernatant was used to measure the flocculation potential of the organisms while the precipitate was discarded (Ntombela, 2017; Bukhari *et al.,* 2020). The strain with the best flocculating activity for kaolin clay suspension was used for all the experiments in this study.

3.4 Flocculating activity determination

The method of Ugbenyen *et al*. (2012) was followed for the determination of flocculating activity. To determine the flocculating activity, the solution of kaolin was used as test material. To prepare kaolin solution, 4 g of kaolin powder was prepared in a Litre of distilled water and utilized to determine the flocculating activity of the isolates. About 100 mL of kaolin solution was poured in a 250 mL conical flask and mixed with 2 mL of cell-free supernatant and 3 mL of 1% $CaCl_2$ (w/v) solution.The mixture was agitated for 1 minute, poured into a 100 mL measuring cylinder and then

allowed to stand for 5 minutes at room temperature to sediment. A crude bioflocculant was replaced with a sterile production medium for a control. At a wavelength of 550 nm, the optical density of a clarifying solution was measured using a spectrophotometer (Spectro-quant, Pharo 300 Merck USA) (Xia *et al*., 2008). The following equation was utilized to calculate the percentage (%) flocculating activity:

Flocculating activity (FA) = $\left[\frac{A-B}{A}\right]$ x 100%

Where A is the optical density (OD) at 550 nm of kaolin solution and B is the optical density at 550 nm of the sample.

3.5 Identification and purification of a bacterial organism

The morphology of the colony on the different medium were observed. Pure culture was prepared and subjected to agar plate that supported the growth of the obtained isolate by streaking the colonies of that particular isolate and incubated at 37 °C for 24 hours. Pure cultures were then identified based on the morphological, cultural characteristics and biochemical tests using BandB's Yeast classification system. Cultural characteristics and morphology was used to identify pure colonies as described in the method of Christen (2008).

3.6 Identification of the organism using 16S rRNA gene molecular method

Fresh Luria Broth (LB) (50 mL) was used to cultivate pure isolates and incubated for 16 hours at a speed of 200 rpm on a rotary shaker at 37° C. The yeast strain genome DNA was extracted with the use of the DNA kit™ (Zymo Research) (Omega

Bio-Tek, Inc., USA). The method of Xiong *et al*. (2010) was adopted. The extracted DNA was amplified using PCR from DreamTaq ™ DNA polymerase (therno Scientific ™) for 16S rRNA gene sequence. The sequence of the organism was determined using the purified and amplified PCR product which was extracted using Zymo Research, Zymoclean ™ Gel DNA Recovery kit 3500xl genetic Analyser. The universal primers were utitilized for PCR amplification which include, forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer (5'-CGGTTACCTTGTTACGACTT-3'). The findings were then compared with the ones found on the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). Isolates were stored in -80 ºC refrigerator in the Department of Biochemistry and Microbiology, at University of Zululand (Huang *et al.,* 2014; Mohammad and Degang, 2019a).

3.7 Optimization of culture conditions for bioflocculant production

Temperature, fermentation time, initial pH, shaking speed, cations, inoculum size, carbon and nitrogen sources are the major factors which were optimized to enhance and increase production yield and the crude bioflocculant flocculation efficiency (Salehizdeh and Yan, 2014).

3.7.1 Effect of inoculum size on bioflocculant production

To determine the optimum inoculum size for bioflocculant production, the broth culture ranging from 1% (v/v) (0.5 mL) to 5% (v/v) (2.5 mL) was made and inoculated into 100 mL conical flasks with 50 mL sterile production medium. The inoculated flasks were incubated at 30 ºC for 72 hours at 160 rpm. The flocculating activity was measured by mixing 3 mL of 1% (w/v) calcium chloride and 2 mL cell-free

supernatant and 100 mL kaolin suspension (4 g/L) added in 250 mL conical flask. The mixture was shaken thoroughly for 1 minute, transferred into graduated measuring cylinder (100 mL) and allowed to stand at room temperature for 5 minutes for sedimentation. The clear supernatant was utilized to determine flocculating activity at 550 nm as described in 3.4. The inoculum size with the highest flocculating activity was used in all subsequent experiments (Ntozonke *et al.,* 2017).

3.7.2 Effect of carbon and nitrogen sources on the production of bioflocculant

Dissimilar carbon sources such as lactose, galactose, glucose, starch, sucrose, xylose, and maltose were used replacing glucose (20 g/L) with similar amounts. The yeast was inoculated in the production medium and incubated at 30 °C for 72 hours in a shaker at 160 rpm. Flocculating activity determination was done after three days of incubation and the carbon source with the highest flocculating activity was used for all the tests followed (Lee *et al.,* 2007; Cosa *et al*., 2013).

To assess the innumerable inorganic and organic nitrogen sources on flocculating activity, the method of Lachhwani (2005) was used. The nitrogen sources peptone, yeast extract, $(NH_4)_2SO_4$, NH_4Cl, and urea were used in this study. The equivalent amount of 1.2 g/L was used to replace the original pre-culture medium multiple nitrogen sources (urea, yeast extract and $(NH_4)_2SO_4$), in the basal medium. Each nitrogen source was added in different production medium and inoculated with the yeast and then incubated at 30 °C for 72 hours in a rotary shaker with a speed of 160 rpm. After 72 hours of fermentation, the flocculating activity was measured for each of the nitrogen sources. The nitrogen source that showed the highest flocculating activity was used for further research.

3.7.3 The effect of shaking speed on the production of a bioflocculant

The method described by Zhang *et al*. (2007) was followed to assess the dissimilar shaking speed effect on the flocculating activity. The assessed shaking speeds were 60, 80, 100, 120, 140, 160, 180, 200, and 220 rpm. Conical flasks (250 mL) with production media of 50 mL were inoculated with the optimum inoculum size (v/v) of the bioflocculant-producing isolate and incubated at 30 °C for 72 hours at dissimilar shaking speed ranges of 60 - 220 rpm. After 72 hours of fermentation, the flocculating activity was determined for the different shaking speeds and the shaking speed that showed the highest flocculating activity was used for all the experiments followed.

3.7.4 Effect of initial pH on the production of a bioflocculant

The initial pH effect on bioflocculant production was evaluated by varying pH of the production medium (50 mL) in 250 mL conical flask using 0.1 M HCl and 0.1 M NaOH to adjust the pH values ranging from 3 - 12 (Ntozonke *et al*., 2017). The pH adjustment was done before the production medium was sterilized. After 72 hours of fermentation, the flocculating activity was measured for each pH value and the pH with the highest flocculating activity was used in all subsequent experiments (Nguyen *et al*., 2019).

3.7.5 Effect of metal ions on flocculating activity

The metal ions impact on the flocculating activity was evaluated using the method of Nie *et al*. (2011). Kaolin suspension (4 g/L) was mixed with 3 mL of 1 % (w/v) $CaCl_2$ solution and 2 mL broth culture of bioflocculant-producing isolate in a 250 mL conical flasks and the flocculating activity was measured. From the standard method, the 1% (w/v) $CaCl_2$ was replaced with innumerable metal salt solutions (1% w/v) (LiCl, $FeCl_3$, NaCl, $BaCl_2$, $MnCl_2$, KCl and $AlCl_{3)}$. Each of the above metal ions was

prepared and poured into a 250 mL conical flask with 100 mL kaolin solution and 2 mL cell-free culture supernatant, shaken for 60 seconds, transferred to 100 mL measuring cylinder and left to stand at room temperature for 5 minutes. To prepare the control experiment, the mixture of 100 mL kaolin solution and bacterial isolate was made without the addition of cations. The flocculating activity was measured as described in 3.4 and the best cation was then used to all subsequent tests.

3.7.6 Effect of cultivation temperature on bioflocculant production

The method of Xia *et al*. (2018) was used for determination of cultivation temperature effect on flocculating activity. The defferent temperatures (20, 25, 30, 35, 40, 45, 50, 55, and 60 ºC) were used to incubate the inoculated test microorganism in a production medium for a period of 72 hours. After 72 hours of fermentation the flocculating activity was measured and the temperature that showed the highest flocculating activity was used for further research.

3.7.7 Time course assay

The method described by Okaiyeto *et al*. (2016a) was used for the determination of fermentation time. The medium for optimum fermentation was used. To obtain the optimum initial pH, 0.1 M HCl and 0.1 M NaOH were used for the adjustment of the pH of the medium. Production medium was prepared and the pH was adjusted before autoclaving at 121 °C for 15 minutes. The broth culture of 1% (v/v) (optimum inoculum size) was prepared using a saline solution (50 mL of 0.85% NaCl). To standardize, the optical density of the suspension (100 mL) in distilled water was measured with a spectrophotometer at OD_{660nm} and then adjusted gradually to 0.1 absorbance. The production medium (50 mL) in a 250 mL conical flask was inoculated with the standardized optimum inoculum size suspension obtained and

incubated for 5 days at 35 °C in a rotary shaking speed of 120 rpm. Samples were drawn at 12 hours intervals to determine the flocculating activity and the pH of the sample. This was done to illustrate according to the method of Kurane *et al*. (1994). The OD_{660nm} (optical density) was measured using a spectrophotometer at $OD660_{nm}$ to monitor the microbial growth. To determine the flocculating activity, 2 mL of the sample was centrifuged at 8000 x g for 15 minutes at 4 °C and used in flocculating activity measurement described in 3.4.

3.8 Extraction and purification of a bioflocculant

The method by Chang *et al*. (1998), Okaiyeto *et al*. (2016b) and Cosa *et al*. (2012) of extraction and purification of the bioflocculant was followed. After 60 hours of fermentation, the fermented broth was centrifuged at 8000 rpm for 15 minutes at 4 °C to remove bacterial cells. This was done to harvest the bioflocculant from the production medium. About one volume (1000 mL) of distilled water was added into the culture supernatant, mixed properly and re-centrifuged at 8000 rpm for 15 minutes at 4 ºC. About 2 volumes (2000 mL) of ice-cold ethanol were added into the supernatant, mixed properly and stored for 12 hours at 4 °C. To obtain crude bioflocculant, after 12 hours the collected precipitate was vacuum dried. The crude bioflocculant was then re-dissolved in distilled water (100 mL) to form a solution (w/v). Butanol (n-butyl alcohol) and chloroform (5:2 v/v) (100 mL) mixture was added. The mixture was thoroughly shaken and left at room temperature to stand for 12 hours. The precipitate was then centrifuged at 8000 g for 15 minutes at 4 ºC and vacuum-dried to obtain a purified bioflocculant. The weight of the dried bioflocculant was measured and expressed in g/L (Poli *et al*., 2009; Gupta and Diwan, 2017).

3.9 Physicochemical analysis of the purified bioflocculant

3.9.1 Chemical composition analysis of the purified bioflocculant

The phenol-sulfuric acid method was used to determine the total sugar content and D- glucose was utilized to prepare the standard curve (Chaplin and Kennedy, 1994). Bradford assay with bovine serum albumin (BSA) as standard was used to measure the total protein content of the produced bioflocculant (Bradford, 1976). Uronic acid content was analysed as well. The colourimetric methods using carbazole reagent was used in determining uronic acids content and D-Gluconoric acid (Sigma-aldrich, Switzerland) was used to prepare standard curve (Xu *et al*., 2011; Ogunsade *et al.,* 2015).

3.9.2 Bioflocculant surface morphology analysis

A Scanning Electron Microscopy (SEM) (SEM-Sipma-VP03-67, Zeiss, and P-Sigma, Germany) equipped with elemental analyser, was used for the examination of the bioflocculant morphological structure. Silicon-coated slide was used with a purified bioflocculant (5 mg) to determine the structural and elemental analysis of the bioflocculant produced. The silicon-coated slide was fixed with a spin coater at 1000 rpm for 1 minute. The flocculated kaolin clay and kaolin clay particles images were also obtained (Xia *et al*., 2008; Okaiyeto *et al.,* 2014).

3.10 Chemical analysis of a purified bioflocculant

3.10 1 Fourier Transform Infrared spectrophotometer (FT-IR) analysis

The FT-IR spectroscopy (Perkin Elmer System 2000, Cambridge, England) was used to evaluate the functional groups of the purified bioflocculant. The sample of the purified dried bioflocculant infrared spectrum was obtained. This was recorded at room temperature in the wavelength ranges of 4000 - 400 cm^{-1} after being mixed

with potassium bromide (KBr) and constrained into pellets (Xia *et al*., 2008; Cosa *et al*., 2013; Vamila *et al.,* 2020).

3.10.2 X-Ray Diffraction analysis of the bioflocculant

A Bruker D8 Advance diffractometer (Johannesburg, Burker, South Africa) equipped with Cu-Kα radiation (1.5406) at 40 kV, 40 mA at room temperature was utilized to investigate the crystallinity of the produced bioflocculant. Dry samples were put on a sample holder, and the patterns of diffraction were recorded from 0 - 80 degrees (Zhang *et al.,* 2012; Dlamini *et al*., 2020).

3.11 Flocculation characteristics of a purified bioflocculant

3.11.1 Effect of dosage concentration on flocculating activity (Jar test) of a bioflocculant

The method described by Makapela *et al.* (2016) was utilized to determine the purified bioflocculant dosage concentration effect on flocculating activity. Concentration ranges of the bioflocculant solutions were prepared in a range between 0.2 and 1.0 mg/mL (w/v). The bioflocculant solution (2 mL) for each concentration was mixed with the kaolin clay suspension (100 mL) and 3 mL of 1% (w/v) $CaCl_2$ in a conical flask (250 mL) and vigorously shaken. A standing graduated measuring cylinder (100 mL) was used to transfer the thoroughly shaken solution and was allowed to stand for 5 minutes at room temperature to sediment. As previously described, the flocculating activity of the clear supernatant was determined.

3.11.2 Effect of heat on flocculating activity of the purified bioflocculant

The variations of temperature such as 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, 100 °C, and 121 °C were used to evaluate the heat stability of the purified bioflocculant

(Ahmad *et al*., 2015). To evaluate the heat stability of the purified bioflocculant, a bioflocculant solution (0.4 mg/mL) was prepared. The bioflocculant solutions (10 mL) were heated for 30 minutes at the different temperatures. One test tube was heated at 121 °C for 15 minutes (autoclaved). The measurement for flocculating activity of the purified bioflocculant was done for all the temperatures (Ugbenyen and Okoh, 2014; Zulkeflee *et al.,* 2016).

3.11.3 Effect of pH on flocculating activity of the purified bioflocculant

The purified bioflocculant optimum concentration solution was made. Before the determination of flocculating activity against pH, in 250 mL conical flasks a prepared kaolin solution (4 g/L) (100 mL) was adjusted to the pH ranges of 3 - 12 using 0.1 M NaOH or 0.1 M HCL. Then, 2 mL of 0.4 mg/mL solution of a bioflocculant, 3 mL of 1% $CaCl_2$ (w/v) and kaolin solution (100 mL) were mixed. The flocculating activity values for each experiment were attained at each pH values as described above (Ugbenyen and Okoh, 2014).

3.11.4 Effect of cations on flocculating activity

The method described by Okaiyeto *et al*. (2013) was followed to evaluate the impact of the cations on flocculating activity of the bioflocculant. The 1% (w/v) $CaCl_2$ solution (3 mL) was substituted by dissimilar metal ions such as $Ba^{2+,}$ Mn^{2+}, Li^{+}, Fe^{3+}, K^{+}, Na^{+} and Al^{3+} during this experiment. The flocculating activity was measured using kaolin solution (see 3.4). To prepare the control, only kaolin solution and bioflocculant were used no cations were added.

3.11.5 Effect of salt concentration on a purified bioflocculant

The method of Maliehe *et al*. (2016) was followed to determine salinity stability of the bioflocculant. By varying different concentrations of NaCl such as 5, 10, 15, 20, 25,

and 30 g/L in kaolin solution (4 g/L), the effect of salinity on bioflocculant flocculating activity was studied using a spectrophotometer @550 nm (Spectro-quant Phero 300, Merck, USA) (see 3.4).

3.12 Application of the purified bioflocculant

3.12.1 Treatment of wastewater

Wastewater samples were collected from KwaDlangezwa wastewater treatment plant wastewater and Tendele coal mine wastewater from KwaSomkhele area, Mtubatuba, KwaZulu-Natal, SA. Parameters such as total nitrogen, biochemical oxygen demand (BOD), phosphorus, sulfate, nitrate, chemical oxygen demand (COD), and pH were measured prior and after flocculation. This was performed using a spectrophotometer (Spectro-quant Phero 300, Merck, USA) and pH meter (Li *et al*., 2013, Sathiyanarayanan *et al*., 2013). About 3 mL of 1% (w/v) NaCl ions preferred and 2 mL of 0.4 mg/mL bioflocculant solution were mixed with 100 mL of wastewater sample adjusted to pH 8 using 0.1 M HCL or 0.1 M NaOH in a 250 mL conical flask. After shaking the solution at 200 rpm for 3 minutes, the mixture was then subjected to a lower shaking speed of 45 rpm for a further 5 minutes. The flasks were left at room temperature to stand for 10 minutes for sedimentation. The residual constraints of the BOD, COD, total nitrogen, sulfate, phosphorus, nitrate, and the flocculating activity were measured utilizing the top clear solution. The absorbances were measured at 680 nm using a spectrophotometer (Spectro-quant Phoro 300, Merck, USA). For comparison, commercial flocculants such as alum and ferric chloride (0.4 mg/mL) were used to substitute the biofllocculant. The following formula was used to calculate the bioflocculant removal efficiency.

Removal efficiency (RE) (%) = $\left[\frac{Co - C}{Co}\right]$ x 100

Where Co and C are the values before and after the flocculation process measured at 680 mn wavelength, respectively (Prasertsan *et al.,* 2006).

3.12.2 The removal of dyes with the bioflocculant

To asses the dye removal potential of the bioflocculant, different dye solutions were used. Various dye solutions such as Congo red, nigrosine, methylene blue, and safranine were prepared (4 g/L). In 100 mL of each dye solution, 2 mL of 0.4 mg/mL bioflocculant solution and 3 mL of 1% (w/v) $AlCl_3$ solution were added. The mixture was shaken vigorously and allowed to sediment at room temperature for 5 minutes. The top clear solution was then used to measure the dye removal potential of the bioflocculant. The following formula was used to ccalculate the removal efficiency of the bioflocculant:

Removal efficiency (%) = $\frac{Do - D}{Do} * 100$

Where D_o and D are values for of dye removal before and after flocculation at 550 nm, respectively (Deng *et al*., 2005, Pathak *et al*., 2015).

3.13 Statistical analysis

All data were collected in triplicates with mean and standard deviation values determined where differences were considered significant at 0.05 at confidence level ($p>0.05$) by the use of Graph Pad Prism version 6. The significance was evaluated by variance analysis (ANOVA).

Chapter 4

This chapter contains results and discussions of the study.

4. 0 Results and discussion

4.1 Screening, isolation and identification of bacteria with bioflocculant production potential

The Kombucha tea SCOBY was used as the source for the isolation of the bioflocculant-producing microorganisms. Dilutions were made to obtain pure cultures and the obtained pure cultures were screened for bioflocculant production potential. Different colonies were picked randomly based on the size, colour, and morphology and screened for bioflocculant production against kaolin clay suspension (4 g/L). About 20 isolates were obtained and screened, and the isolate with the highest flocculating activity was selected. The selected microbe had a flocculating activity of 75% against kaolin clay suspension and was used for further investigation. The isolate on potato dextrose agar plate appeared to have oval colonies with a whitish colour and yeast smell. This isolate was revealed to be Gram-positive and oval-rod shaped with Gram staining technique. The isolate was further identified using 16S rRNA sequencing method. In the GenBank database, the isolates comparative analysis of its 16S rRNA sequence showed 99% similarities with *Pichia kudriavzevii* and the accession number of the isolate was MH545928.1. Therefore, the isolate was named *Pichia kudriavzevii* MH545928.1. To the best of my knowledge, this organism has not been used before for any study as a potential bioflocculant-producing organism. The microbe was optimized for the production of bioflocculant with the improved bioflocculant yield and flocculating activity.

4.2 Optimization of the conditions of culture medium for bioflocculant production by *Pichia kudriavzevii* MH545928.1.

The conditions of production medium was optimized in different major factors such as size of the inoculum, shaking speed, carbon and nitrogen sources, temperature, metal ions, culture medium initial pH, and the time course in order to obtain the improved bioflocculant yield and flocculating activity (Prasertsan *et al.,* 2006, Zhu *et al.,* 2019; Zeng *et al*., 2020). Failure to optimize these conditions could result to poor yield production of the bioflocculant or bioflocculant production with reduced flocculating activity (Zulkeflee *et al*., 2016, Mohammed and Dagang, 2019a).

4.2.1 Effect of inoculum size on bioflocculant production by *Pichia kudriavzevii* MH545928.1

The inoculum size effect on the production of a bioflocculant by *P. kudriavzevii* was evaluated and the inoculum size ranges used was 1- 5% (v/v), as indicated in Figure 4.1. Literature reveals that inoculum size is important for flocculating activity of a bioflocculant and during its production (Renault *et al*., 2009). Muthudineshkumar and Anand (2019) reported that the excessive inoculum size could clump-up the microorganisms and result in the inhibition of bioflocculant production while the insufficient inoculum size might lead to a delay of growth of microbes resulting to the poor biofloculant production. It was observed that the bioflocculant was optimally produced when the inoculum size was 1% (v/v) with the flocculating activity of 95% (Figure 4.1). The inoculum sizes 2, 3 and 4% (v/v) were also effective for the production of bioflocculant with good flocculating activities but less effective than 1% (v/v) inoculum size while the 5% (v/v) inoculum size inhibited the flocculating activity (67%). Therefore, in all the experiments that followed, the inoculum size of 1% (v/v) was used. A study by Zhang *et al.* (2007) also showed that an inoculum size of 1% (v/v) led to the maximum flocculating activity of a bioflocculant. *Klebsiella pneumoniae* YZ-6 was reported with a flocculating activity of 85.5% when the

inoculum size of 1% (v/v) was used and any increase in inoculum size inhibited the flocculating activity (Luo *et al*., 2014). Contrary to these findings, Ntombela *et al*. (2020) reported an optimum bioflocculant production by consortium of the bacteria (*Bacillus sefensis* and *Bacillus sp.*) when using 4% (v/v) inoculum size. *Bacillus licheniformis* CGMCC 2876 produced a bioflocculant with over 90% flocculating activity using 4% (v/v) as inoculum size (Xiong *et al*., 2010). A bioflocculant produced by *Aspergillus flavus* was reported to attain the flocculating activity of 86.6% when 2% (v/v) inoculum size was used (Aljuboori *et al*., 2013). Therefore, each and every microorganism has its preferred inoculum size for optimally production of the bioflocculant. In this study it was observed that between 0.5, 1.0, 1.5, and 2.0 there was no difference statistically therefore, any inoculum size can be used but it should be noted that small inoculum size is an added advantage in the cost reduction factor.

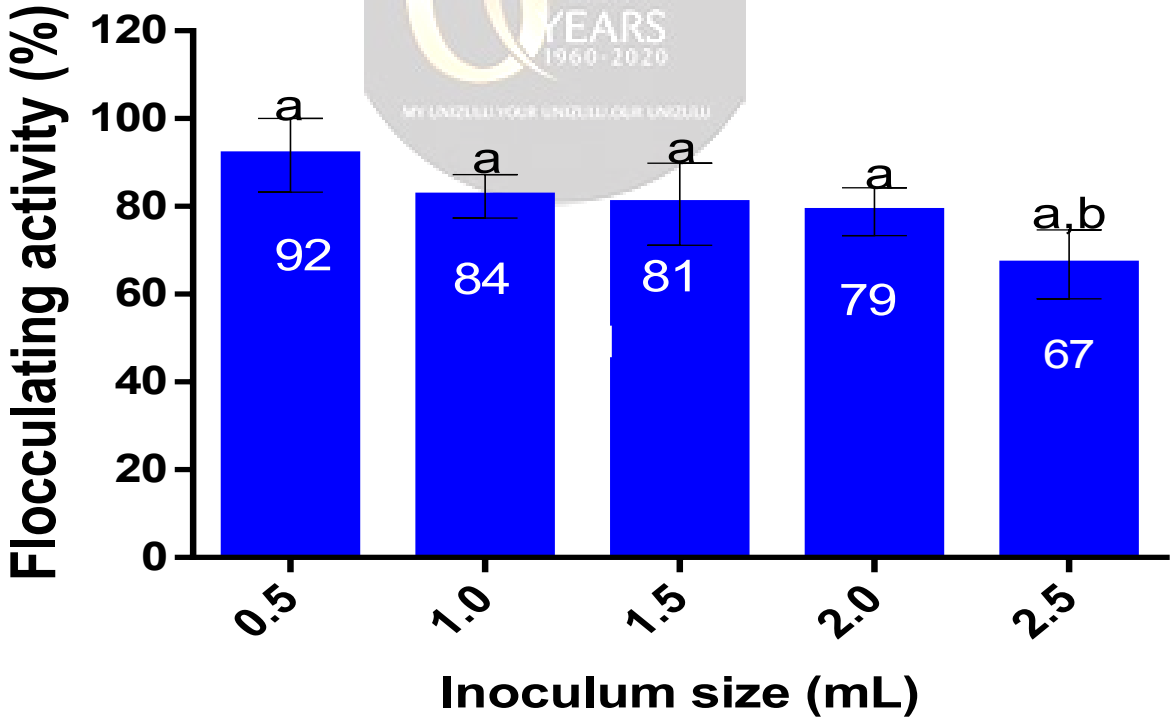


Figure 1.1: The effect of inoculum size on bioflocculant production.

4.2.2 Effect of carbon source on the production of a bioflocculant by *Pichia kudriavzevii* MH545928.1

Carbon sources are considered as a key factor during the bioflocculant production as it enhances the growth and production rate therefore, dissimilar microorganisms have varied preference for carbon sources (Piyo *et al*., 2011). The various carbon sources effect on the production of a bioflocculant by *Pichia kudriavzevii* MH545928.1 was examined and the results are depicted in Figure 4.2. It was observed that among the studied carbon sources glucose was the most preferred carbon source with 94.8% flocculating activity. All tested carbon sources were effective for production of the bioflocculant with more than 70% flocculating activity. Glucose was then used for all subsequent experiments. These results are in accordance with the study conducted by Luo *et al*. (2013) where the production of bioflocculant MBF-6 by *Klebsiella pneumoniae* YZ-6 was reported to be highly favoured by glucose as the carbon source with flocculating activity of 93.87%. Luo *et al*. (2016) also reported that a bacterium *Bacillus megaterium* SP1 was able to produce a bioflocculant with a flocculating activity of 87.9% with glucose as a sole carbon source. Glucose was also used effectively by *Bacillus licheniformis* CGMCC 2876 for the optimum bioflocculant production as well as flocculating activity (Chen *et al*., 2017). A flocculating activity of 95% was observed with glucose as carbon source by *Halomonas* sp. V3a' to produce HBF-3 bioflocculant (He *et al*., 2009). A study by Deng *et al*. (2005) reported that *Aspergillus parasiticus* has been found to prefer glucose as carbon source for bioflocculant processing. Contrarily, Ntombela *et al*. (2021) reported starch as the most preferred carbon source for the production of bioflocculant by the consortium of *Bacillus safensis* and *Bacillus sp*. Bioflocculant BF1 produced by *Klebsiella variicola* BF1 was investigated to use cassava starch as

carbon source for its optimum flocculating activity (97.6%) (Nguyen *et al*., 2019). Okaiyeto *et al*. (2016) reported *bacillus licheniformis* strain W7 which preferred maltose as carbon source for optimum bioflocculant production with highest flocculating activity of 94.9%. Ma *et al*. (2020) reported *Enterobacter sp*. P3 which was shown to be favoured by brewery wastewater as a cheap substrate for its optimum bioflocculant production. In conclusion, different microorganisms have different preferences for their sources of carbon for the efficient production of bioflocculant with excellent potential in flocculation process (Salehizadeh and Yan, 2014).

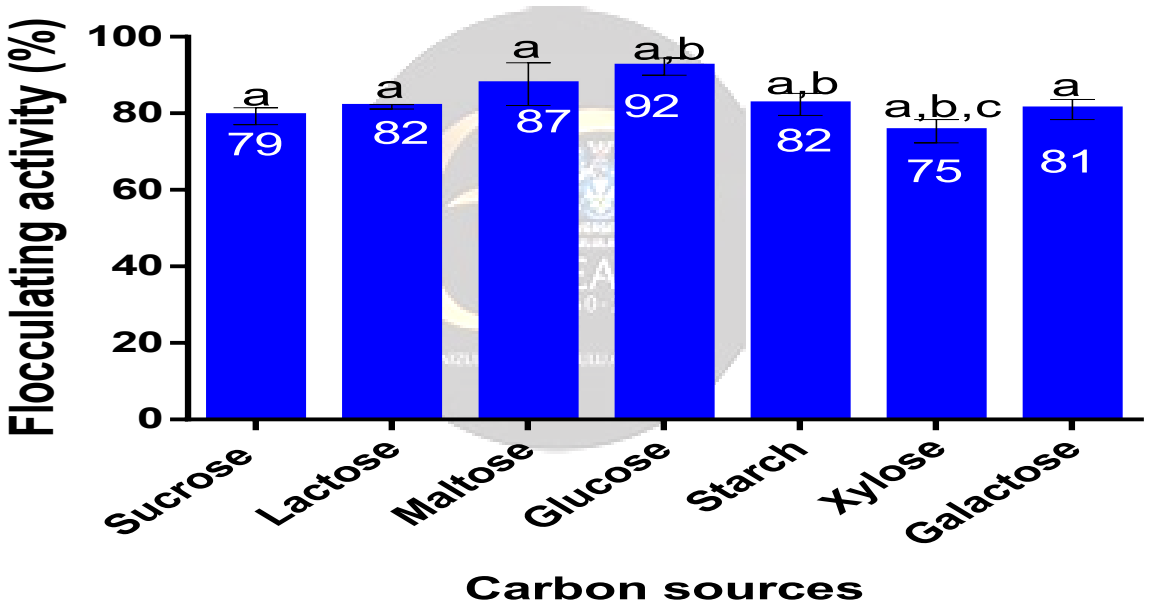


Figure 4.2: The effect of carbon sources on bioflocculant production.

4.2.3 Effect of nitrogen source on bioflocculant production by *Pichia kudriavzevii*

With respect to the requirements for nitrogen sources, it has been found that different microorganisms prefer different nitrogen sources for their efficient growth and production of bioflocculant with effective flocculating activity (Aljuboori *et al*., 2014). Peptone, yeast extract, and urea were the organic nitrogen sources studied

for the production of a bioflocculant by *Pichia kudriavzevii* MH545928.1 (Figure 4.3). The inorganic nitrogen sources used were ammonium sulphate, ammonium nitrate and casein. Figure 4.3, shows that peptone was the most effective nitrogen source compared to the other nitrogen sources examined. Peptone had a flocculating activity of 94% while others were 78% (casein), 80% (ammonium sulphate), 72% (ammonium nitrate), 87% (yeast extract), and 72% (urea). The only source of nitrogen that showed a least flocculating activity was urea with 72% flocculating activity. Although the organism best preferred peptone as its nitrogen source, but the other tested sources of nitrogen were favourable for the effective production of a bioflocculant by *Pichia kudriavzevii* MH545928.1. Threfore, peptone was used in the production of bioflocculant by *P. kudriavzevii* MH545928.1. These results are in accordance with the results reported by Xia *et al*. (2008) where *Proteus mirabilis* TJ-1 produced a bioflocculant in the presence of peptone as nitrogen source with the highest flocculating activity. Giri *et al*. (2015) reported that a *Bacillus subtilis* F9 produced a bioflocculant optimally when peptone was used as a nitrogen source. Contrary to the study findings the combination of nitrogen sources such as urea, ammonium sulphate, and yeast extract improved the bioflocculant production by *Bacillus sp*. (Ntombela *et al*., 2021). When *Klesiella sp.* MYC was cultivated using multiple nitrogen sources such as yeast extract, urea, and $(NH_4)_2SO_4$, an optimum bioflocculant was obtained (Yue *et al*., 2006). Makapela *et al*. (2016) reported the *Bacillus pumilus* to produce a bioflocculant maximally with multiple nitrogen source $(NH_4)_2SO_4$, urea, and yeast extract.

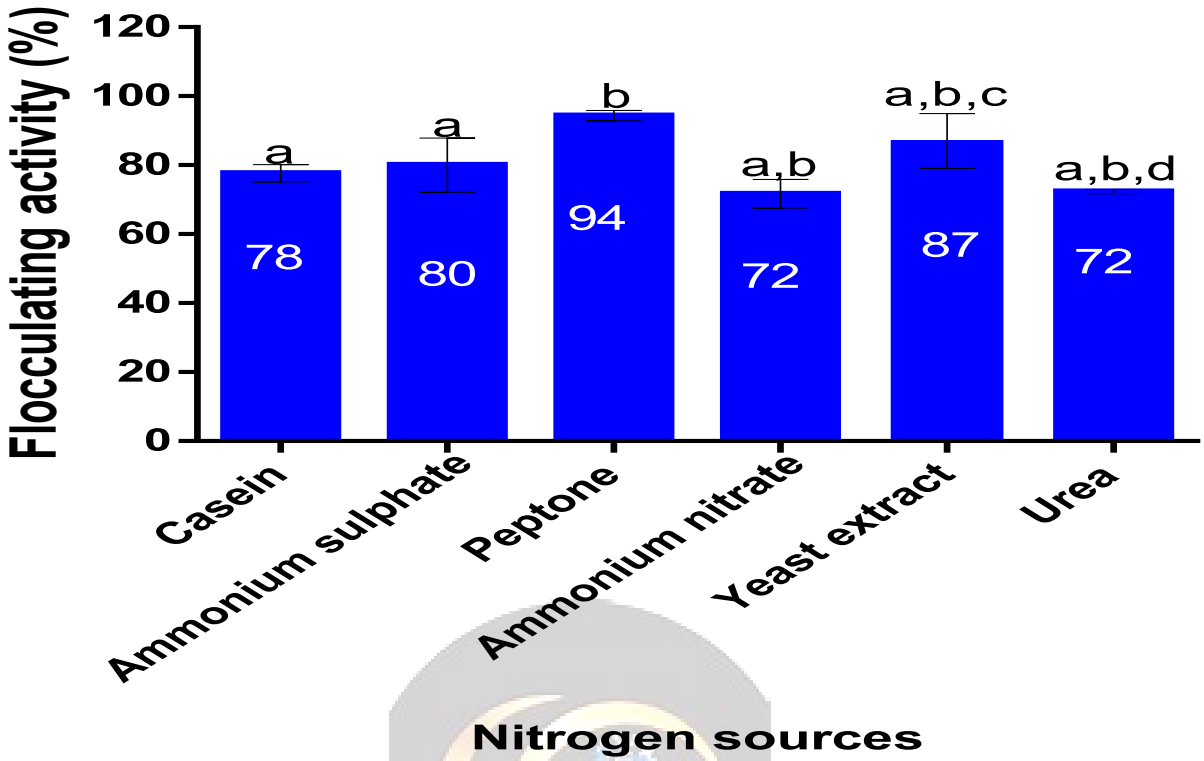


Figure 4.3: Effect of nitrogen sources on bioflocculant production.

4.2.4 Effect of shaking speed on the production of a bioflocculant by *P. kudriavzevii.*

Different microorganisms prefer varied shaking speeds for their effective production of bioflocculants (Salehizadeh and Yan, 2014). Variations in shaking speeds (60, 80, 100, 120, 140, 160, 180, 200, and 220 rpm) for the bioflocculant production by *P. kudriavzevii* were investigated and the results are shown in Figure 4.4. Lopez-Lopez *et al*. (2016) has reported that a continuous shaking of a production medium results in an aerobic growth stimulation in the presence of soluble oxygen. From the graph in Figure 4.4, it can be seen that the low shaking speeds (60, 80 and 100 rpm) did not highly favour the production of bioflocculant by *P. kudriavzevii* MH545928.1. The bioflocculant was favoured by the shaking speeds such as 120, 140, and 160 rpm with flocculating activity of 95%, 96%, and 95%, respectively. Above the shaking

speed of 160 rpm, the flocculating activity declined. The shaking speed of 140 rpm had the highest flocculating activity of 96% and used in the production of bioflocculant by *P. kudriazevii* MH545928.1while 120 and 160 rpm had flocculating activity of 95% each. The differences in shaking speed tolerance could be as a result of the requirements for oxygen by different microorganisms during their growth phases (Lopez-Lopez *et al*., 2016). The study by Li *et al*. (2009) on a bioflocculant ZS-7 produced by *Bacillus licheniformis* reported the shaking speed optima at range of 140 - 160 rpm. Shih *et al*. (2001) reported that the bioflocculant produced by *Bacillus licheniformis* CCRC 12826 was produced optimally with a shaking speed of 150 rpm. *Proteus mirabilis* TJ-1 produced a bioflocculant TJ-F1 which showed good flocculating activity of 93% when shaking speed was 130 rpm (Xia *et al*., 2008). *Bacillus salmalaya* 139SI was reported to produce a bioflocculant optimally at a shaking speed of 160 rpm with flocculating activity of 83.6% (Abu Tawila *et al*., 2018). Ogunsade *et al*. (2015) documented a bioflocculant produced by *Bacillus amyloliquefaciens* utilizing a shaking speed of 120 rpm for its optimum flocculating activity.

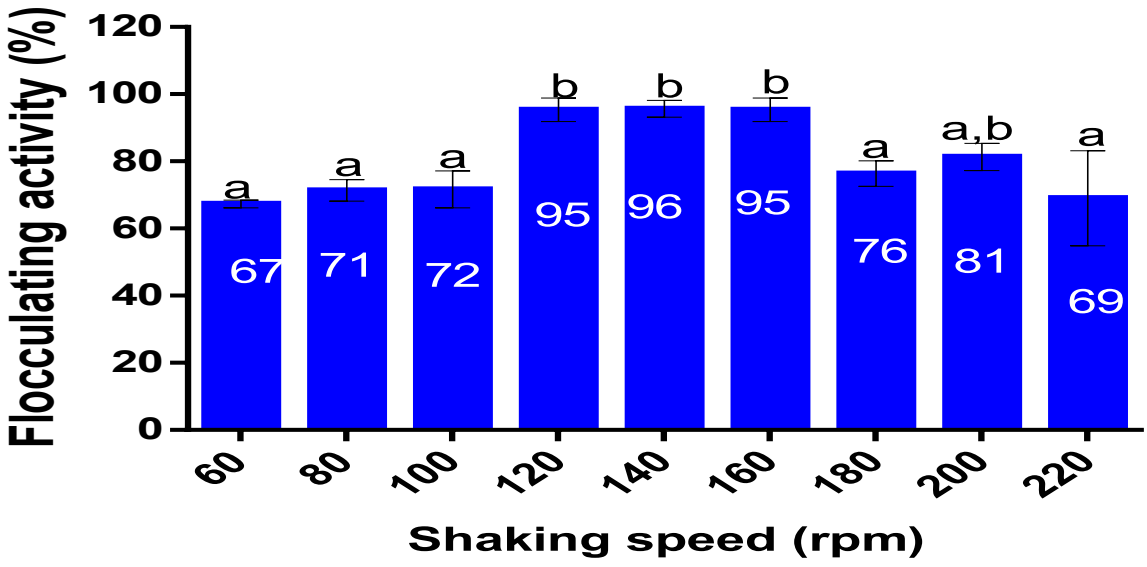


Figure 4.4: Effect of shaking speed on bioflocculant production.

4.2.5 Effect of cultivation temperature on bioflocculant production by *P. kudriavzevii* MH545928.1

The effect of temperature was investigated on bioflocculant production by *Pichia kudriavzevii* MH545928.1 and the most effective temperatures were 30 - 45 ºC as shown on the Figure 4.5. The highest flocculating activity of 97% was observed at temperature of 35 ºC while the lowest temperature (20 ºC) resulted in the flocculating activity of 69%. Microorganism metabolism is inversely proportional to culture temperature and the maximum enzymatic activation can only be obtained at optimum temperatures (Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001; Zhang *et al*., 2007; Xia *et al*., 2008, Ndejiko and Dagang, 2019). This could be as a result of the increase in temperature which leads to the increase in kinetic energy of the cells thus speeding up the molecules that are associated with biochemical reactions, thus increasing chances of collision of the molecules (Salehizadeh and Shojaosadati, 2001). The production of the bioflocculant was not highly favoured by the low temperatures (20 ºC and 25 ºC), while high temperatures also inhibited the production of bioflocculant by *P. kudriavzevii* MH545928.1. The reason for the low yield of production in low temperatures could be attributed to the fact that low temperatures slow down metabolic processes taking place to most organisms. This is because membrane fluidity, the substrates for enzymes affinity is reduced, there is also a reduction in rate of reaction and decreased thermal energy, and the aqueous viscosity is increased (Shih *et al*., 2001). While on the other hand high temperatures result to destruction of microbial structure by denaturing of protein and cellular growth ceases at high temperatures (Robinson, 2015). In terms of statistical significant, there is no difference in all tested cultivation temperatures. Zong *et al*. (2020) reported a novel bioflocculant MBF-9 produced by *Diaphorobacter*

nitroreducens R9 showing flocculating activity of 96.4% when cultured at 30 ºC. Another study by Hassimi *et al*. (2020) reported a bioflocculant from *Bacillus velensis* that was produced at 40 ºC with the highest flocculating activity of 18.5%. The optimum flocculating activity of 90.21% was reported for bioflocculant PL8 produced by *Bacillus megaterium* strain PL8 using 30 ºC as cultivation temperature (Pu *et al*., 2020). Strain RK-23 from *Methanosarcina spelaei* when cultivated at 38 ºC produced bioflocculant MBF-23 that showed the highest flocculating activity of 95.63% (Zhao *et al*., 2020). *Bacillus salmalaya* under optimal culture conditions at temperature 35.5 ºC produced bioflocculant QZ-7 with the highest flocculating activity of 92.6% (Abu-Tawila *et al*., 2018). A bioflocculant TKFO4 with the highest flocculating activity of 98.4% was produced by a bacterial strain of *Citrobacter sp*. when cultured at 30 ºC (Fujita *et al*., 2000).

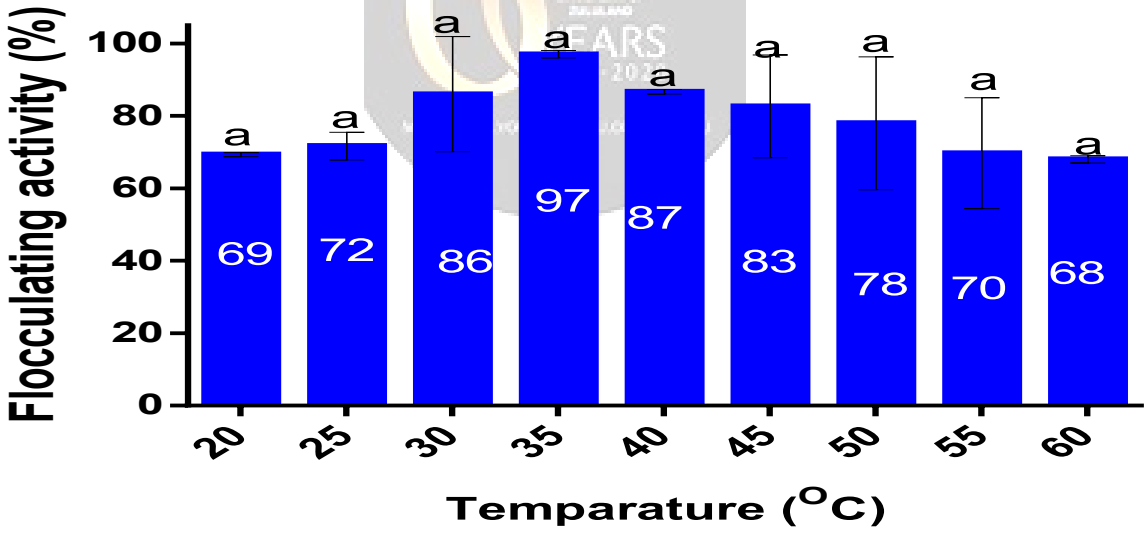


Figure 4.5: Effect of temperature on bioflocculant production.

4.2.6 The effect of metal ions on bioflocculant production by *P. kudriavzevii* MH545928.1

Most of the studies have shown the addition of metal ions in the culture medium has a beneficial effect in efficiency of bioflocculation production through the formation of

bridges between particle and charge neutralization (Salehizadeh and Shajaosadati, 2001). In addition to the efficiency of bioflocculant posed by the concentration and presence of metal ions. The ions valency also contribute immensely to the production of effective bioflocculants (Zulkeflee *et al*., 2012). These metal ions have been found to either inhibit or stimulate the production of bioflocculants (Feng *et al*., 2008; Li *et al*., 2009; Liu *et al*., 2010). Mechanisms involved in stimulation are stabilization and neutralization of the residual charge of functional groups on the bioflocculant by the metal ions (Kwon *et al*., 1996). The suspension solution of metal ions result in the increase in the ionic strength because of the addition of the metal ion. Thus, reduce electrostatic forces of the suspended impurities (Wang *et al*., 2011). The various metal ions effect on flocculating activity of the production of the bioflocculant by *Pichia kudriavzevii* MH545928.1 was studied and the results are shown Figure 4.6. The different cations ions including monovalents (Na^+, K^+, and Li^+), divalents (Mn^{2+} and Ba^{2+}), and trivalents (Al^{3+} and Fe^{3+}) and the control were examined. The monovalent NaCl showed the highest flocculating activity of 95%, followed by divalent $MnCl_2$ and $BaCl_2$ with flocculating activity of 78% and 84%, respectively. Other metal ions such as $AlCl_3$, $FeCl_3$, KCl, and LiCl were also effective on bioflocculant production by the yeast with flocculating activity above 70%. However, the bioflocculant seems to be cation dependent with the control showing less than 40% flocculating activity when no cations were added. Therefore, Na^+ cation was used in all subsequent experiments. Similar findings were reported by Ugbenyen and Okoh (2014), where flocculating activity of the bioflocculant OKOH produced by *Halomonas sp*. was stimulated by divalent cation addition (Mn^{2+}). This could be attributed to the rigid bonds of its structure, while Li^+ was responsible for the inhibition of the production of a bioflocculant by the microorganism. Lian (2008)

reported that some microorganisms do not require addition of any ions for their optimum bioflocculant production including *Citrobacter sp*. TKF04. Contrary, to the study findings, a bioflocculant produced by *Bacillus sp*. AEMREG4 was reported which showed low flocculating activity of 67.7% for Na^{+} and Al^{3+} with the highest (83.3%) flocculating activity (Ntsangane, 2016). On the other studies, the Mn^{2+} and Al^{3+} were reported to increase the flocculating activity of the bioflocculant produced by *Halomonas sp*., *Micrococcus sp*., xn11 and xn7, *Bacillus sphaeicus* F6 and *Rhizobium radiobacter* F2 (Zheng *et al.,* 2008; Wang *et al*., 2011; Okaiyeto *et al*., 2013). A consortium of two bacterial isolates *Bacillus* and *Cobetia* was evaluated and the produced bioflocculant showed flocculating activity of 90% in the presence of Ca^{2+} (Ugbenyen and Okoh, 2014). The bioflocculant (MBF-W6) production by *Chryseobacterium daeguense* was inhibited by Ba^{2+}, Al^{3+}, and Fe^{3+}, while stimulated by the addition of K^{+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} (Liu *et al*., 2010). The complex metal ions such as Mg^{2+}, Ca^{2+}, and Fe^{3+} on bioflocculant production (PSBs) produced by *Paenibacillus mucilaginosus* GIM1.16, their addition attributed with vital effects in flocculating activity and production of metabolites (Tang *et al*., 2014).

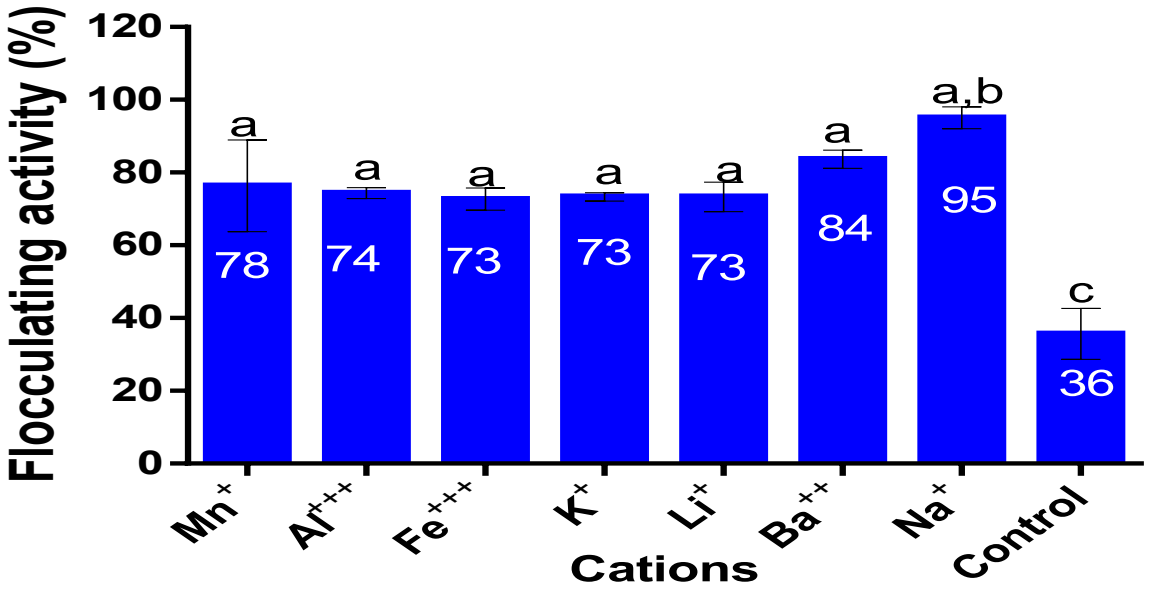


Figure 4.6: Effect of cations on flocculating activity.

4.2.7 Effect of initial pH on bioflocculant production from *P. kudriavzevii* MH545928.1

Dissimilar microbes prefer varied pH ranges for the production of bioflocculants (Salehizadeh and Yan, 2014). Figure 4.7 depicts the initial pH on culture medium that was examined for bioflocculant production with range from pH 3 - 12. It was observed that *Pichia kudriavzevii* mostly preferred pH 7 to pH 9 with flocculating activity of 91%. Therefore, pH 7 was used for all subsequent tests. These findings are in accordance with the study by Shimofuruya *et al*. (1996) and Yokoi *et al*. (1995) where the organisms *Virgibacillus sp*. Rob and *Bacillus lichemiformis* X14 that produced a bioflocculant optimally under alkaline pH values (8-14), and *Holomonas sp*. OKOH and *Paecilomyces sp*. optimally produced bioflocculant under neutral pH (Ntsangani, 2016). Another study by Ugbenyen *et al*. (2012) reported that alkaline pH 8, 9,10, 11 and 12 were unfavourable for the production of a bioflocculant by the microorganisms *Klebsiella mobilis*, *Serratia ficaria*, and *Chryseobacterium*

daeguense W6. The range of pH 3-6 was not favourable as this might be too acidic for the microbe tested thus yielded poor results. Contrary, the findings by Yokoi *et al.* (1995) revealed that *Bacillus sp*. PY 90 and *Streptomycetes griseus* produced bioflocculant under acidic conditions. All these findings confirm that microorganisms have dissimilar preferences for their initial pH conditions for bioflocculant production. According to the literature, both the kaolin particles and bioflocculant are likely to absorb hydrogen ion (H^+) at low pH. As a consequence, the bioflocculant-kaolin complex, which is regulated by Ca^{2+}, is weakened and the same effect is experienced at high pH values due to hydroxyl ions (OH^-) (He *et al*., 2010).

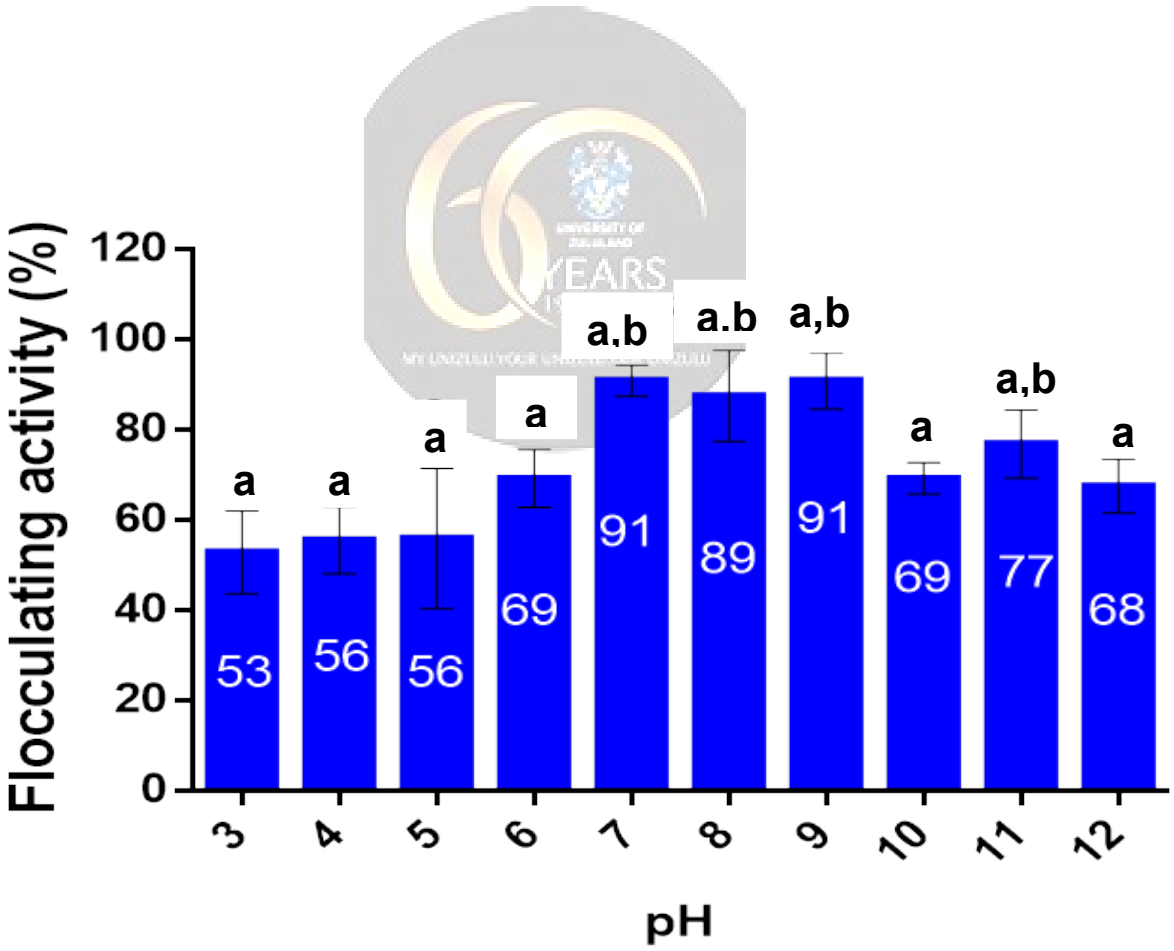


Figure 4.7:2 Effect of initial pH on bioflocculant production.

4.2.8 Time course of production for a bioflocculant by *P. kudriavzevzii* MH545928.1

The growth curve for the yeast according to flocculating activity, optical density (OD_{660nm}), and the pH is depicted in Figure 4.8. The optimal flocculation conditions that were obtained previously from the experiments were utilized for the growth curve. The yeast *P. kudriavzevii* MH545928.1 was examined for the production of a bioflocculant during the growth curve (120 hours). The microbe was able to optimally produce the bioflocculant at 60 hours of cultivation with flocculating activity of 99.1%. After 60 hours of fermentation, flocculating activity of the bioflocculant began to decline. It has been observed that nutrients deficiency is in direct proportion to the decline of the cell growth from the production medium of the microorganism. This this explains the reason for a reduction in flocculating activity of the bioflocculant production after 12 hours. In general, the reduction may also be in correlation to the bioflocculant degrading enzyme that are produced and released, in turn the produced bioflocculant is utilized as a source of carbon or this depletion may be the result of autolysis (Zaki *et al*., 2011; Abd El-Salam *et al*., 2017).

A study conducted with *Enterobacter aerogenes* seems to support the study findings during this research as the bioflocculant produced by this microbe reached its optimal flocculating activity in the early stationary phase. Then it peaked after 60 hours of cultivation which indicated that the bioflocculant was produced through biosynthesis process during growth of the microorganism (Ntsangani *et al*., 2017). *Alcaligenes latus* produced bioflocculant with a maximum flocculating activity after 2 to 3 days and the flocculation thereafter declined due to degrading enzymes (Kurane and Nohata, 1991).

This confirms that some microorganisms are able to produce bioflocculant with maximum flocculating activity during the early stationary phase of their cultivation. The bioflocculant producing bacterium *Serratia ficaria* reached a flocculating activity optima in the early stationary phase of 72 hours, with a steady decline 12 hours later (Gong *et al*., 2008). *Aspergillus flavus* produced a bioflocculant with maximum flocculating activity of 87.2% at cultivation period of 60 hours. Deng *et al*. (2005) reported similar findings when *Aspergillus parasiticus* produced bioflocculant with maximum flocculating activity (98.1%) after 60 hours. The *Bacillus subtilis* DYU1, *Citrobacter sp*. TKF04, and *Bacillus mucilaginosus* also showed similar phenomenon (Aljuboori *et al*., 2013).

The production of bioflocculant by microorganisms takes place at dissimilar growth phases of these microbes. In the production medium, the autolysis of cells results to decrease in bioflocculant production, and there is also mixing of cations or a reduced activity of enzymes with respect to the cell culture of a particular microbe (Lu *et al*., 2005; Ntsangani, 2016). Depending on the secretion period of bioflocculants from the broth culture, bioflocculants can either be primary metabolites or secondary metabolites (Salehizadeh *et al*., 2018). A study by Nwodo *et al*. (2012) reports that *Streptomyces sp.* produces a bioflocculant during the logarithmic phase of growth and reached highest flocculating activity during this phase. This therefore, implied that biosynthetic processes were responsible for the process of bioflocculant production.

Contrary to these study findings, other studies have shown that some bioflocculants produced an optimum flocculating activity at late stationary phase of cultivation. For example*Virgibacillus sp*. Rob produced bioflocculant with highest flocculating activity at 96 hours (Cosa *et al*., 2011). *Bacillus sp*. HXG-C1 optimally produced a

bioflocculant when it reached the 96 hours of cultivation in the stationary phase and then declined (Nontembiso *et al*., 2011). Studies suggest that a decrease in the flocculation activity could also be attributed to the molecular weight decrease of the polymer when subjected to protease hydrolysis. This was observed in bioflocculant produced by *Corynebacterium glutamicum* (He *et al*., 2002). A direct proportionality was also observed in the study between optical density and flocculation activity almost ran in the same direction on the growth curve. The similarities shown by the growth and activity is an indication that bioflocculant was not produced by autolysis of cell rather it was produced by a biosynthesis process (Taniguchi *et al*., 2005).

The pH of the production medium decreased from pH 7.05 to pH 6 during the exponential production of the bioflocculant. There after, it started to increase from pH 6 up to pH 7.0 at which it remains constant for sometime during the maximum production of the bioflocculant and decreased to 5.5 pH eventually. The sudden decrease might be attributed to organic acids secreted by the organism *Pichia kudriavzevii* MH545928.1 during metabolic reactions (Okaiyeto *et al*., 2013). A bioflocculant IH-7 produced by *A. flavus* presented similar findings to the current study where pH was seen to decrease from pH 7.0 to 5.3 within 48 hours of fermentation, then little bit increased again after 96 hours to pH 5.6 (Aljuboori *et al*., 2013). Contrary to the findings of the present study, Gong *et al*. (2008) documented the bioflocculant with flocculating activity profile of pH decreased from pH 7.0 to 5.3. During the cultivation time from 0 to 96 hours, the pH of the culture broth declined from 6.5 to 4.9, which could be attributable to the excreation of organic acids from glucose metabolism or the formation of the organic acid components of the polymers generated for bioflocculant MBFW31 (Gao *et al*., 2006).

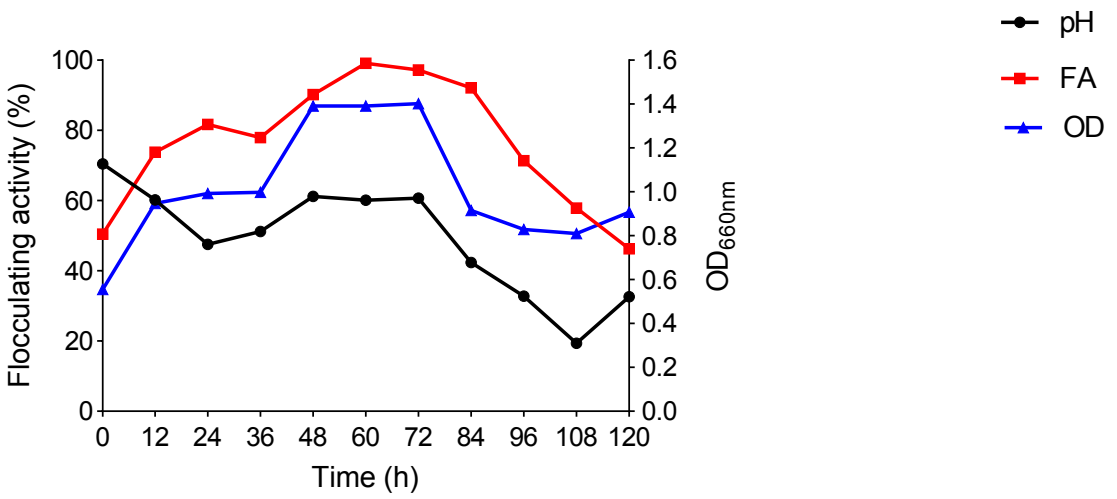


Figure 4.8: Time course of the production for a bioflocculant.

4.3 Extraction and purification of a bioflocculant for the yeast *P. kudriavzevii* MH545928.1

The crude bioflocculant produced by *Pichia kudriavzevii* MH545928.1 strain after extraction was 3.6 g/L. The colour substance of the bioflocculant was a milky white. This substance was dried to obtain a purified bioflocculant after the addition of chloroform and butanol (5:2 v/v) mixture which was a white powdered substance weighing about 2.836 g/L from a fermentation broth. This yield is better when compared to the one reported by Okaiyeto *et al.* (2016a) where by *Bacillus sp*. AEMRG7 produced 1.6 g/L bioflocculant. Cosa *et al*. (2011) reported a bioflocculant yield of 0.264 g/L obtained from the broth culture of *Virgibacillus sp*. Rob. Low yields and high costs present significant practical application constraints in the production of bioflocculants. The performance of the production of bioflocculants was among the critical factors considered for its application (Kurane *et al*., 1994).

The produced bioflocculant by *P. kudriavzevii* is low compared to the yield of 5.916 g/L produced by *Bacillus atrophaeus* (Akapo *et al*., 2019). Another high yield (4.52 g/L) was observed from the study of He *et al*. (2009) produced by *Halomonas sp*.V3a

and Wang *et al*. (2013) also reported a yield of 3.8 g/L produced by *Ochrabactium cicero* W2. All these bioflocculants were higher than the produced bioflocculant in this study. Zhang *et al*. (2002) reported a yield of 14.8 g/L bioflocculant from the organism *Myxobacterium nannocystics sp*. NU-2, which is 4 times to the yield obtained in this study. Li *et al*. (2013) reported that *Paenibacillus elgii* B69 produced a bioflocculant of 25.63 g/L utilizing sucrose as a carbon source.

Studies show that a method of extraction plays a huge role in obtaining a high yield of bioflocculant, as some methods tends to be more preferred than others. For example, chemical extraction methods are more efficient compared to physical methods (Comte *et al*., 2007). Normally, the extracting reagents from chemical methods contaminate EPS during extraction and this often prevents the configuration of EPS from being determined and better understood (Comte *et al*., 2006, Mohammed and Dagang, 2019a).

4.4 Characterization of the purified bioflocculant

4.4.1 FT-IR analysis of the purified bioflocculant

Figure 4.9 depicts Fourier transform infrared spectroscopy analysis of the bioflocculant produced by *Pichia kudriavzevii* MH545928.1 that was undertaken to detect the presence of functional groups, which may contribute to its flocculating activity. The IR spectrum of the bioflocculant showed the presence of sharp band O-H stretching bond at 3723 cm^{-1}, which indicated the availability of alcohol. The hydroxyl presence in the polymer favours hydrogen bonding possibilities with one or more molecules of water, which therefore, means in an aqueous solution, all bioflocculants are highly soluble (Kasan *et al*., 2015). A band stretching at 3304 cm^{-1} indicated the presence of secondary amine. The peaks at 2996 cm^{-1} and 2925 cm^{-1}

were attributed to the functional group C-H of stretching vibration of sugar residues constituents. Pathak *et al*. (2014) and Yu *et al*. (2020) reported that a stretched molecular chain of a bioflocculant presents the sites of absorption for the attachment of the particles. The presence of a strong vibration peak was observed at 2354 cm^{-1} which indicated the presence of C-O and at peaks 2193 cm^{-1} and 2131 cm^{-1} vibration were observed which indicated the presence of weak alkyne and strong thiocynate, respectively. Alkynes are unsaturated hydrocarbons and have been found to have a higher boiling point which could be the reason for the observed flocculating activity at higher temperatures of the bioflocculant (Subudhi *et al*., 2016). The thiocynate functional group shows high solubility in water (177 g/100 mL (0 °C)) and has a high boiling point (500 ºC) which could be the attributory effect to the bioflocculant being able to dissolve in water and show good flocculating activity even when autoclaved (121 ºC) (Zhan and Zhang, 2011). Sharp absorption vibrations were observed at 1660 cm^{-1} and 1582 cm^{-1} indicating the presence of carbonyl functional groups, which have been said to allow the spreading out of the chain due to electrostatic repulsion (Joshi *et al*., 2019). A strong peak at 1416 cm^{-1} indicated the presence of sulfonyl functional group which is an asymmetrical stretching vibrations of S=O (Li *et al*., 2021a). The presence of sugar was indicated by a small absorption peak at 1244 cm^{-1} and 1169 cm^{-1} which showed C-O bonds. The aliphatic amine was revealed by the present of strong peaks showed by the spectrum at 1060 cm^{-1} and 1045 cm^{-1}. The presence of furan sugar (saccharide) was indicated by a sharp peak at 894 cm^{-1} and a strong peak stretching at 607 cm^{-1} and 520 cm^{-1} represented a halo compound. Similar IR spectrum for bioflocculant produced by *Bacillus sp*. was reported by Ntombela (2017). The strong absorption peak present at 1100 cm^{-1} and 1200 cm^{-1} and the functional groups (carboxyl and hydroxyl) that are available in the

molecular chain of a bioflocculant are known to be general characteristics of all sugar deravatives (Gong *et al*., 2008). From these observations it can be deduced that the bioflocculant consist of a polysaccharide as its main constituent. Studies have shown that the main adsorptive forces of a bioflocculant are described by the functional groups. These functional groups were thought to be responsible in the bioflocculant's flocculation process (Kurane and Matsuyama, 1994; Lu *et al*., 2005; Yim *et al*., 2007; More *et al*., 2014, Mohammed and Dagang, 2019b). In order to have an optimum flocculating activity, cations must be used and these require a biding site for their action, therefore, the carboxyl groups found in the bioflocculant fulfils this purpose (Maliehe *et al*., 2016). Putra *et al*. (2014) reported the infrared spectrum similar to this study finding. The observed findings were also in line with the findings of other researchers for the FT-IR produced for variety of bioflocculants from dissimilar microorganisms (Kavita *et al*., 2014; Cosa and Okoh, 2014; Rasulov *et al*., 2017(a); Sarang and Nerurkar, 2017; Mohammed *et al*., 2019b).

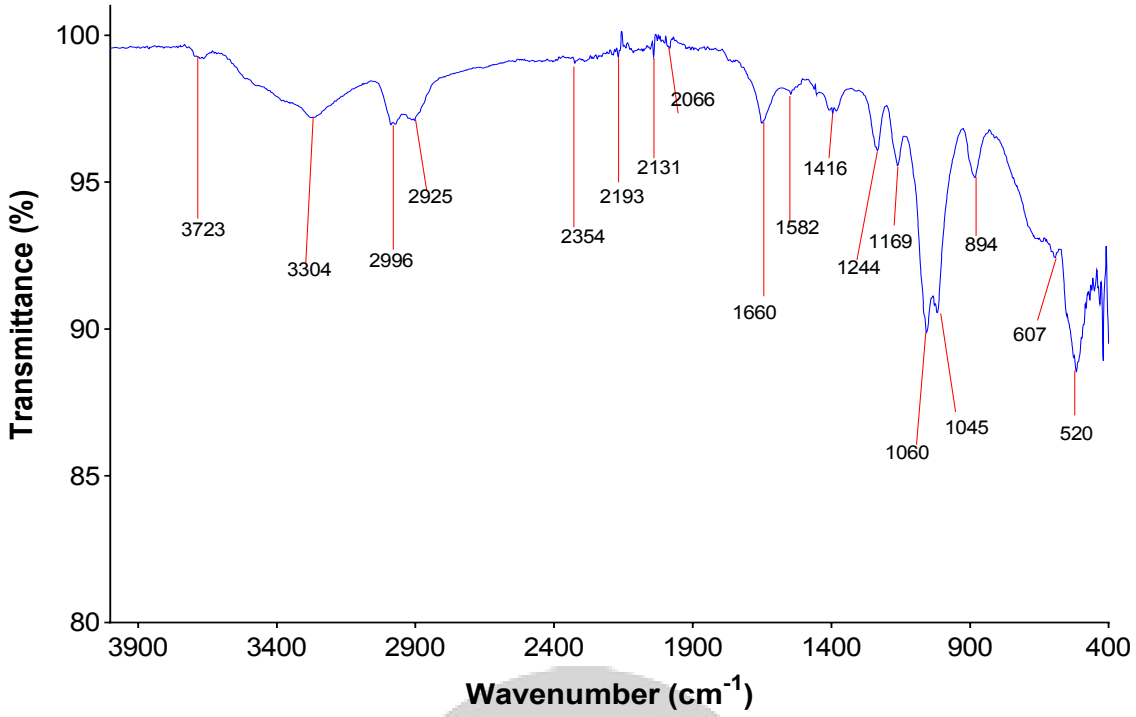


Figure 4.9: Infrared spectrum of the purified bioflocculant by *Pichia kudriavzevii*.

4.4.2 X-Ray Diffraction pattern of the bioflocculant

The X-ray pattern illustration of the bioflocculant at angle (2θ) is depicted in Figure 4.10 with deep peaks observed between 10º and 40º angles. The strong peaks shown on the bioflocculant are very intense which could mean that the bioflocculant may have some impurities (Dlamini *et al*., 2019). The broadening of peaks in solid XRD pattern is usually due to particle size effect (Muthulakshmi *et al*., 2019). Broader peaks signifies smaller particle size, and this means the bioflocculant produced in this study has bigger particles as the bioflocculant does not show any broader peaks.

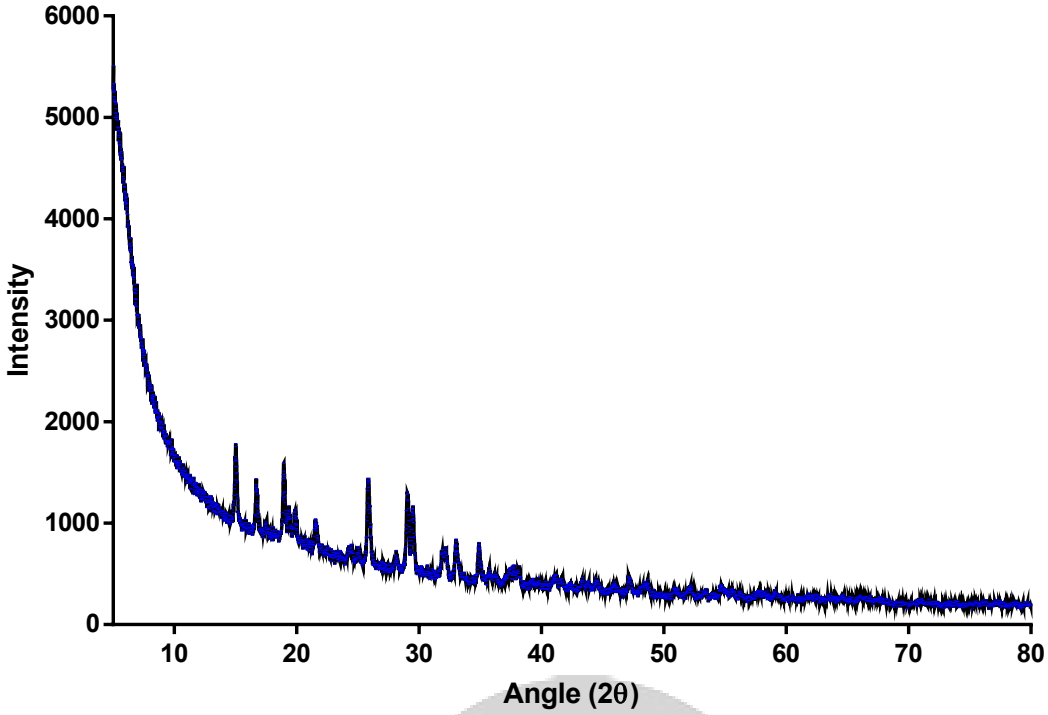


Figure 4.10: X-Ray Difraction of the purified bioflocculant produced by *Pichia kudriavzevii* MH545928.1

4.4.3 SEM analysis of a bioflocculant

As high-energy beam of electrons scans in a faster scan pattern, SEM analysis displays the surface images of the samples. In the flocculation process, the surface morphological structure of the bioflocculant is of great importance (Zhao *et al*., 2012). It determines whether bioflocculants are effective or ineffective at flocculation process. Figure 4.11 shows images obtained using scaning electron microscope. The cumulus-like form of bioflocculant was shown using a scanning electron microscope emaging (Figure 4.11a). The polymer of the bioflocculant produced by *Bacillus licheniformis* CGMCC 2876, has an uneven, coarse-grained structure coupled in netted textures, obtained with SEM measurements (Xiong *et al*., 2010). The SEM surface morphology of the bioflocculant p-KG03 had fibrous type of structure (Yim *et al*., 2007). The bioflocculant high flocculating activity could be due

to its configuration. The structure of the kaolin particles seemed to be fine and smooth (Figure 4.11b). The floc appeared clumped together after flocculation with bioflocculant (Figure 4.11c). It appeared to be interconnected as the result of the flocculation process, which allowed particles to be adsorbed onto the bioflocculant, resulting in larger flocs.

Figure 4.12 depicts the findings of elemental analysis of the bioflocculant. Bioflocculant structure and flocculating activity are influenced by the elemental content of the bioflocculants (Cosa *et al*., 2013). The bioflocculants' flexibility and stability are aided for by various elements. The adsorption peaks are showed by the elemental spectrum which indicates the presence of elements such as C, N, O, Na, Mg, Al, P, S, Cl, K, Ca which accounts for 16.92: 1.03: 43.76: 0.18: 0.40: 0.80: 14.44: 1.48: 0.31: 0.34: 20.35 (%wt), respectively. The presence of carbon, nitrogen and oxygen in the biofloflocculant affirms that the bioflocculant in this study was indeed a glycoprotein polymer. The observed results were similar to those of *Halobacillus* produced bioflocculant (Cosa *et al*., 2013). The bioflocculant elemental analysis reported the following percentages: 42.03: 5.31: 21.44: 5.83: 0.85 (%wt) for C, N, O, P, and S, respectively for *Halobacillus*. A study by Lu *et al*. (2005), it was reported that a bioflocculant produced by *Enterobacter aerogenes* had elements such as C: O: N: S: with 39.8: 52.6: 0.8: 0.2 (%wt), respectively. Acidic polysaccharide elemental analysis were conducted for bioflocculant from *Bacillus sp*. As-101 and recorded as C: H: O: N: S with 40.2: 5.9: 52.8: 0.9: 0.3 (%wt), respectively (Salehizadeh *et al*., 2000). Aljuboori *et al*. (2013) reported on a bioflocculant IH-7 elemental analysis C, H, O, N, and S with 29.9%, 4.8%, 34.7%, 3.3%, and 2.0%, respectively.

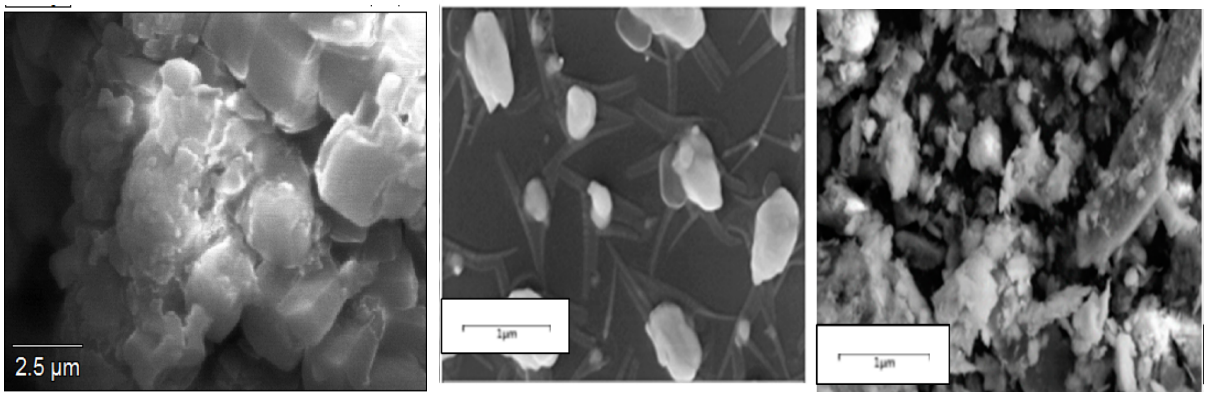
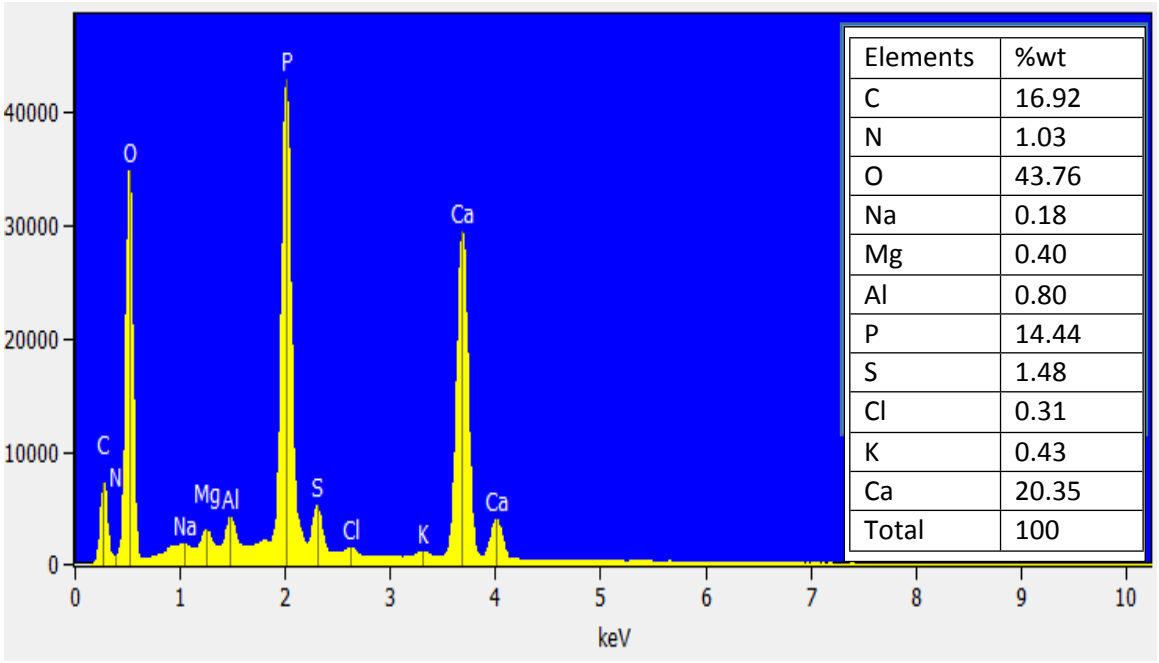


Figure 4.11: Scaning micrograph of the bioflocculant (a), kaolin particles (b), and flocculated kaolin particles (c).



Elements	%wt
C	16.92
N	1.03
O	43.76
Na	0.18
Mg	0.40
Al	0.80
P	14.44
S	1.48
Cl	0.31
K	0.43
Ca	20.35
Total	100

Figure 4.32: SEM - EDX analysis of the purified bioflocculant.

4.4. 4 Chemical composition of the bioflocculant

Chemical analysis of the bioflocculant was done to determine the presence of carbohydrates, protein, uronic acid and other compounds. The components of the bioflocculant must be identified in order to comprehend their flocculation mechanisms (Guibaud *et al*., 2003; Okaiyeto *et al*., 2016b). This would aid in the enhancement of the flocculating parameters, resulting in an increase in the

performance during applications. In this study, chemical analysis of the pure bioflocculant from *Pichia kudriavzevii* showed that the bioflocculant was made up of sugar, uronic acid, and protein, with sugar as a main component. The chemical proportions of the bioflocculant from analysis were shown that total sugar content was 69%, uronic acid content 16%, and protein content was 11%. This indicates that the bioflocculant is dominated by carbohydrates. The ability of the bioflocculants to be heat stable is affirmed by these results along with the hypothesis of Ntombela (2017) that carbohydrates dominate bioflocculants. As a consequence, carbohydrates are thought to be the most active components in the flocculation process. A total carbohydrate content of 23 mg/mL was reported by Mabinya *et al*. (2011) with proteins absent from the bioflocculant. Xiong *et al*. (2010) reported a bioflocculant with carbohydrates (89%) and protein contents (11%). The crude biopolymer flocculant produced by *Bacuillus coagulants* had 83% and 17 % (w/w) total carbohydrtate and total protein, respectively (Salehizadeh *et al*., 2000). *Agrobacterium sp*. M-503 produced a bioflocculant with optimum flocculating activity and contained neutral suger, uronic acid, and protein in weight ratios of 85.0: 0.9: 3.0 (Li *et al*., 2010). *Klebsiella terrigena* produced bioflocculant with total sugar content of 66.8% (w/w) and protein 2.4% (w/w) (Ghosh *et al*., 2009). Zheng *et al*. (2008) reported on a bioflocculant from *Bacillus sp*. that had a total sugar content of 3.6% (w/w), uronic acid 37.0% (w/w), and protein 16.4% (w/w) which contradicts the findings of this study. A study by Liu *et al*. (2010) reported on a bioflocculant produced by *Chryseobacterium daeguense* W6 which showed a high protein percentage of 32.4% while polysaccharide and uronic acid were 13.3% and 6.8%, respectively. If the bioflocculant is dominated by protein content its heat stability is

very weak because proteins denature easily at high temperatures (Bukhari *et al*., 2020).

4.5 Flocculation properties of the purified bioflocculant

4.5.1 Dosage size effect on flocculating activity

The impact of bioflocculant dosage in the dosage ranges of 0.2 - 1 mg/mL, and the impact of bioflocculant dosage on flocculating efficiency was examined (Figure 4.13). In deciding bioflocculant efficiency, dosage concentration is a critical factor (Akapo, 2019). Inadequate or excessive dosage can lead to inhibition or decrease in performance (Cosa and Okoh, 2014). Appropriate dose size helps to minimize expenses and increase flocculation efficiency in industrial processes as well as other applications (Ntsangani, 2017). A solution of a bioflocculant (0.2 mg/mL) showed the flocculating activity of 54.4%, 0.4 mg/mL (80.2%), 0.6 mg/mL (59.4%), 0.8 mg/mL (50.4%), and 1 mg/mL (28.3%), as shown in Figure 4.13. The dosage concentration of 0.4 mg/mL was used for all the experiments that followed as it showed the best flocculating activity. It is very important to find the lowest dosage size since low dosage can minimize costs for industrial applications. Similar results were reported where 0.4 mg/mL dosage size was found to have high flocculating activity for bioflocculant produced by *Bacillus pumilus* JX860616 (Maliehe *et al*., 2016).

Contrary to this study, a conglomerate of *Micrococcus sp.* Leo and *Halomonas sp*. OKOH produced a bioflocculant with optimum flocculating activity with 0.1 mg/mL dosage size (Okaiyeto *et al*., 2013).

However, the flocculation rate was poor at a 0.2 mg/mL concentration, which could be due to the negative charges on kaolin particles that neutralized due to inadequate bioflocculant concentration. At high bioflocculant concentrations, the charges are

excessively restored, leading to reduction in kaolin particles and reduce the flocculation rate (Gong *et al*., 2008). Deng *et al*. (2005) has reported that higher or lower doses have also been shown to cause lower performance on a bioflocculant. Competition and repulsion of negatively charged kaolin particles are attributed to the formation of negatively charged bioflocculants, and decreased effective volume often contributes to poor settability (Gong *et al*., 2008).

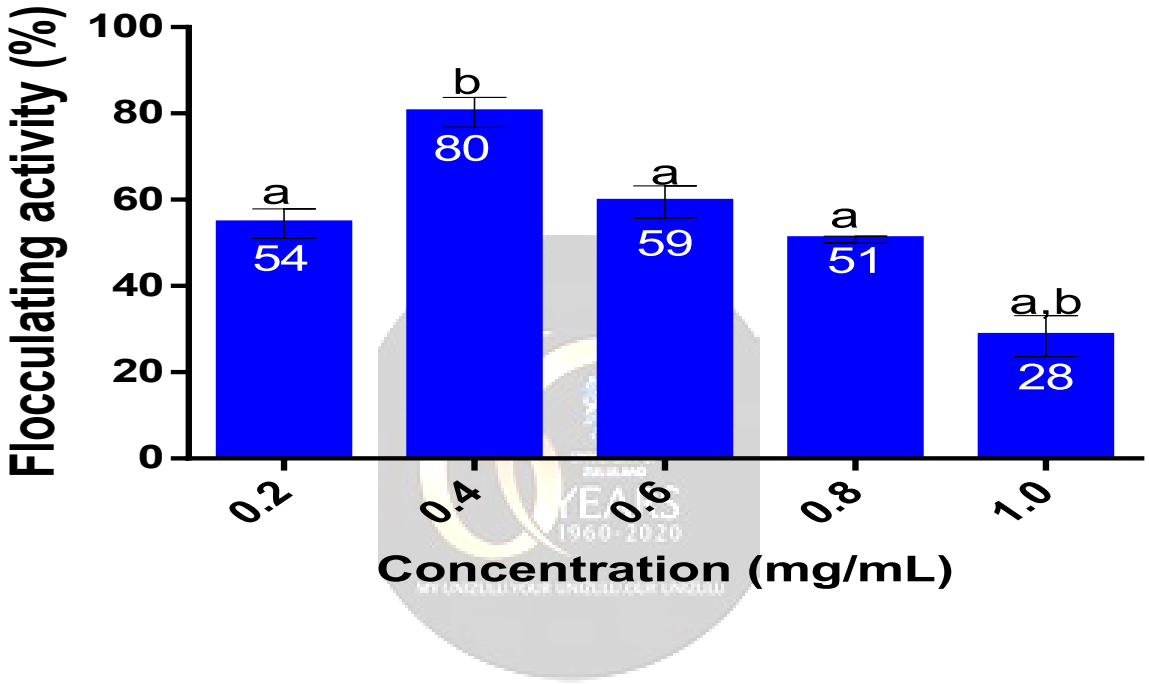


Figure 4.13: Effect of bioflocculant concentration on flocculating activity.

4.5.2 Effect of cations on flocculating activity of a purified bioflocculant

The effect of cations on flocculating activity of a purified bioflocculant was investigated and the results are shown in Figure 4.14. The flocculating activity of the bioflocculant produced by *Pichia kudraivzevii* MH545928.1 was highly enhanced by the addition of Al^{3+} (72%) and other tested cations showed little effects on flocculating activity as follows 51% (K^{+}), and 61% (Mn^{2+}), 50% (Fe^{3+}), 53% (Na^{+}), and 35% (Ba^{2+}), 44% (Li^{+}), and control with the lowest flocculation rate of 16%. The

lowered effect on flocculation of the produced bioflocculant in the presence of Ba^{2+} compared to Mn^{2+} could be linked to the synergistic effect of Mn^{2+} with the bioflocculant and the antagonistic effect of Ba^{2+} with the bioflocculant, which results in the lowered flocculating activity of Ba^{2+} and increased flocculating activity of Mn^{2+} (Okaiyeto *et al*., 2015). These observations are similar to the findings of Ugbenyen *et al*. (2014) where Mn^{2+} cation addition resulted to a flocculating activity of 71.63%. Divalent and trivalent metal ions have been shown to have the effect of inducing bioflocculant adsorption on the particulate matter by reducing both the polymer and the particle's negative charge (Levy *et al.*, 1992). Luo *et al*. (2014) reported that bioflocculant MBF-6 produced by *Klebsiella pneumoniae* YZ-6 could not flocculant kaolin with the addition of any metal ions.

The flocculation rate could be stimulated by metal ions through destabilization and neutralization of residual negative charges of uronic acid carboxyl groups in an acidic polysaccharide, resulting to bridges formation that bind the kaolin particles together (Luo *et al*., 2014). The bioflocculant p-KG03 produced by *Gyrodinium impudicum* KG03 was not enhanced by the addition of any metal ions, which indicates that some bioflocculants are cation-independent, and the addition of metal ions has no positive impact (Yim *et al*., 2007). A bioflocculant 40B produced by *Bacillus velezensis*, improved significantly kaolin particle flocculation in the presence of calcium ions (Ca^{2+}) (Zaki *et al*., 2013). The addition of Ca^{2+} in the bioflocculant produced by *Klebsiella sp*. S11 was found to induce effective flocculation activity (Demlim *et al*., 1999).

A study by Ugbenyen *et al*. (2014) documented that Fe^{3+} inhibited or resulted to a low flocculating activity of 24.7% for bioflocculant produced by *Bacillus sp.* Gilbert. Less flocculating activity (50%) was also observed in this study for Fe^{3+}. The

flocculating activity of 84.1% was reported for the bioflocculant produced by *Chryseobacterium daeguense* W6 when Al^{3+} was added as a metal ion. While a study by Xia *et al.* (2008) showed that Al^{3+} did not have any effect on flocculating activity when it was added to the bioflocculant produced by *Proteus mirabilis*. The control used in this study for bioflocculant produced by *P. kudriavzevii* MH545928.1 showed low flocculating activity, which means that this bioflocculant is cation-dependent.

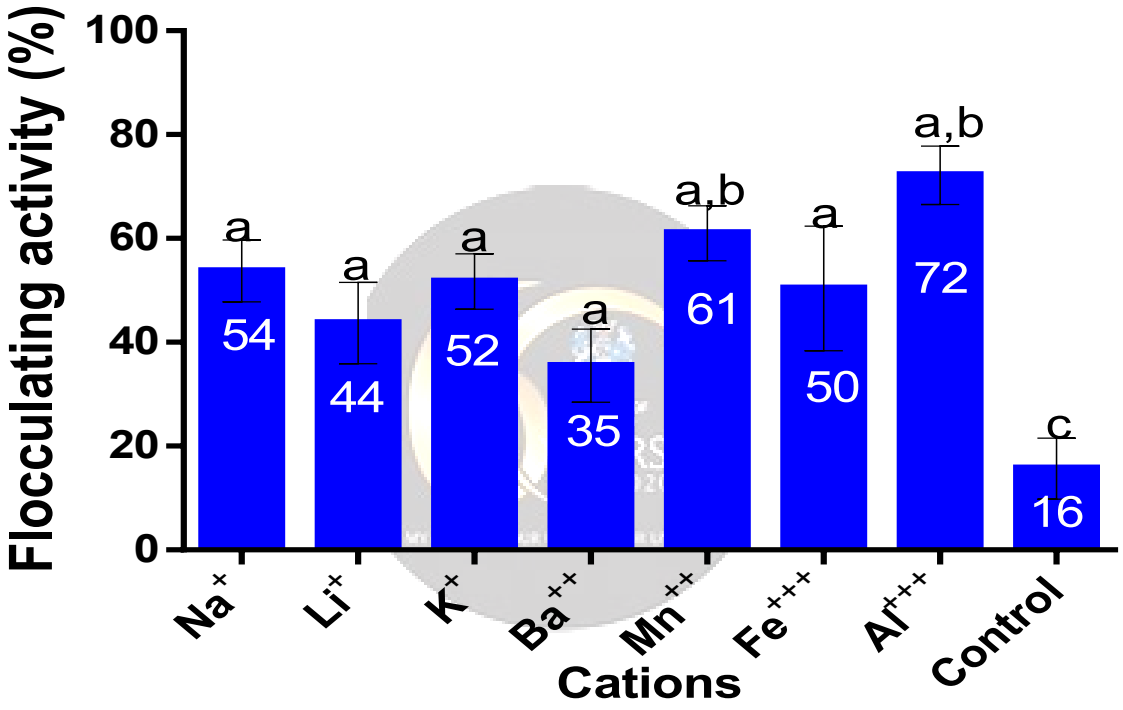


Figure 4.14: Effect of cations on flocculating activity.

4.4.3 Effect of pH on flocculating activity of a purified bioflocculant

The flocculation process is mainly influenced by the pH, which can be considered as the key factor in reaction mixture (Zhao *et al*., 2013). Studies have suggested that pH influences the flocculating activity by imposing an effect to the stability of formation of floc and suspended particles (Ugbenyen *et al*., 2014). In this study the

effect of pH on flocculating activity of the purified bioflocculant, and the results are shown in Figure 4.15. The pH ranges of 3 - 12 were tested using 0.4 mg/mL optimum dosage concentration. The optimum flocculating activity was observed at pH 7 (69%) and high flocculating activities at pH 3 (68%), pH 8 (69%), and pH 9 (65%). This could be attributed to the fact that bioflocculant exhibits dissimilar electric states at dissimilar pH values, thereby bridging efficiency for kaolin clay is affected (Yong *et al*., 2009). At pH 4 (48%), 5 (36%) and 12 (43%) there was a sharp decline in flocculating activity observed. This could be as the result of the surface charge arrangement of the bioflocculant which is both temperature and pH dependent (Abd El-Salam *et al*., 2017). The reason for such a sudden decrease could be due to the arrangement of the spatial charge was not ambient for flocculation process (Zhang *et al*., 2013). The bioflocculant by *P. kudriavzevii* MH545928.1 had good flocculating activity at neutral, and slightly alkaline pH conditions. Similar findings were reported by Zaki *et al*. (2012) where the bioflocculant produced by *Bacillus velezensis* 404 showed high flocculating activity at pH 3, 7, 8, and 9 with the optimum flocculating activity observed at pH 7. Wang *et al*. (2011) reported that dissimilar pH values have been always preferred by dissimilar microorganisms for production of bioflocculant with optimum flocculating activity. Ntsangani (2016) reported that bioflocculant produced by *Bacillus sp*. AEMREG4 had maximum flocculating activity at wide pH range of 3, 7, 8, 9 and 10 with decrease in flocculating activity at pH 5. Contrary to these findings, Liu *et al*. (2009) reported that a bioflocculant M-1 obtained from the activated sludge had optima flocculating activity at pH 5 (93%), with good high flocculating activity in the pH ranges of 3 – 11.

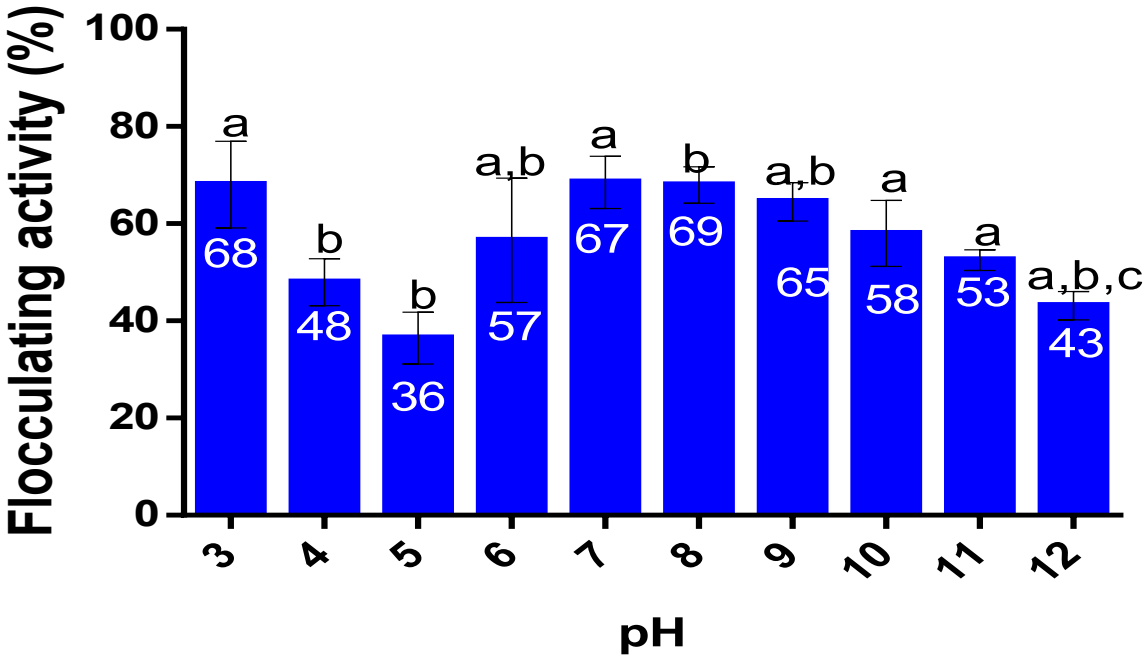


Figure 4.154: Effect of pH on flocculating activity of the purified bioflocculant.

4.5.4 Effect of temperature on flocculating activity of the purified bioflocculant

Various temperatures were evaluated for their effect on the bioflocculant's flocculating activity and the results are depicted in Figure 4.16. It was observed that the bioflocculant had strong thermal stability at temperatures from 50 - 121 ºC, with the highest flocculating activity of 79% observed at 50 ºC. The other temperatures showed also good flocculating activity, temperature such as 60 ºC, 70 °C, 80 ºC, 90 ºC, 100 ºC, and 121 ºC have flocculation efficiencies of 77%, 68%, 68%, 69%, 67%, and 70%, respectively. This could be that the increase in temperature also increase the rate of flocculation, thus collision of particle is enhanced (Gong *et al*., 2008). At 70 °C, 80 °C, 90 °C, 100 °C, and 121 ºC a moderate flocculating activity of 68%, 68%, 69%, and 67%, respectively was observed. A slight increase in flocculating activity (70%) was observed when the bioflocculant was placed in an autoclave at

121 °C for 15 minutes. There was no statistical difference observed in terms of analysis from these temperatures. Akapo (2019) observed similar findings where bioflocculant BA-CGB retained the flocculating activity above 60% at 121 °C. This bioflocculant is thermal stable since 70% flocculating activity was retained after exposure at 121 ºC for 15 minutes. This signifies that the bioflocculant is stable in high temperatures, which could indicate that the bioflocculant have a polysaccharide backbone, which is confirmed with chemical composition analysis. The polysaccharide backbone helps by extending when exposed to high temperatures as a result the binding sites are exposed for formation of flocs and increase flocculating activity (Giri *et al*., 2015). Nucleic acids and protein rich bioflocculants has poor thermal resistance than those of polysaccharides. This is because nucleic acids and protein lack heat resistance properties, this could mean that this bioflocculant is composed manly of polysaccharides (Zhang *et al*., 2012). Some bioflocculants with sugars as their main flocculation components have been reported to be heat stable and can retain more than fifty percent of their flocculating activity when heated in water (Akapo *et al*., 2019). Zaki *et al*. (2011) reported that strains CPO8 and CPO13 produced bioflocculants with flocculating activity which was not affected by increasing the temperature from 5 - 70 ºC, although strain CPO14 had flocculating activity reduced when reaction temperature was raised above 50 ºC.

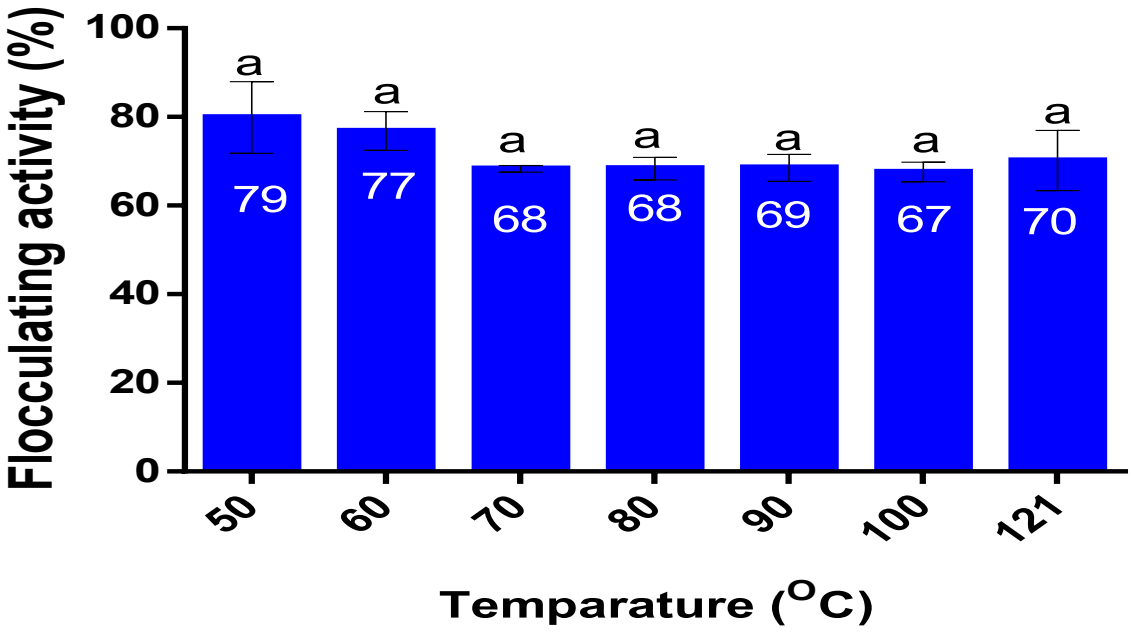


Figure 4.16: Effect of temperature on flocculating activity of the bioflocculant.

4.4.5 Salinity effect on the flocculating activity of the purified bioflocculant

Depicted in Figure 4.17 are the results of the effect of salt concentrations on the flocculating activity of the purified bioflocculant. The rate of flocculating activity showed that various NaCl concentrations on the bioflocculant was observed to be 81% (5 g/L), 75% (10 g/L), 61% (15 g/L), 57% (20 g/L), 50% (25 g/L), and 40% (30 g/L). The maximum flocculating activity was obtained at salt concentration of 5 g/L, and decreased as the salt concentration incease. This means that the bioflocculant was not favoured by high NaCl concentrations. Contrary to these findings, Satyanarayana *et al.* (2012) reported the bioflocculant MSB17 produced by *Bacillus subtilis* MSBFN17 that when this bioflocculant was exposed to high salinity (25 g/L) environment it retained a flocculating activity of 94.26%. This study found that the increase in NaCl concentration have negative impact or resulted to poor flocculating

activity of the purified bioflocculant. Such effect could be attributed to the excessive Na^+ interfering with the reaction between kaolin particles and the bioflocculants. Alternatively, the bioflocculants physical properties being changed due to increase in Na^+ concentrations (Bilanovic *et al*., 1988, Li *et al*., 2021a). A biopolymer with 10 mg/L concentration from *Bacillus mojavensis* strain 32A in the presence of 50 ml/L $CaCl_2$, a peak flocculating activity of 92.2% was observed (Elkady *et al*., 2011). From these findings, it can be deduced that the bioflocculant produced from yeast *P. kudriavzevii* MH545928.1 in this study can be used to clean turbid water with salinity range from 5 - 20 g/L NaCl concentration. At a concentration of 20 g/L NaCl, the highest flocculating activity was reached for bioflocculant MBF-C9 produced by *Bacillus agaradhaerens* C9, however, when the the NaCl concentration increased above 60 g/L the flocculating activity decreased (Liu *et al*., 2014). Aljuboori *et al*. (2015) reported that at 0% (w/w) NaCl, the flocculating activities of PAC and IH-7 were 88.4% and 94.9%, respectively. This means that some bioflocculants do not need the presence of NaCl for their efficient functioning. A bioflocculant MBF-HG6 produced by *Oceanobacillus polygoni* HG6 was reported to show high flocculation rate of 90.25% at 19.24 g/L NaCl dosage (Li *et al*., 2017). A 6% NaCl concentration in bioflocculant XF-56 from *Bacillus sp*. XF-56 resulted in a 93.5% flocculating activity of the bioflocculant (Liu *et al*., 2015).

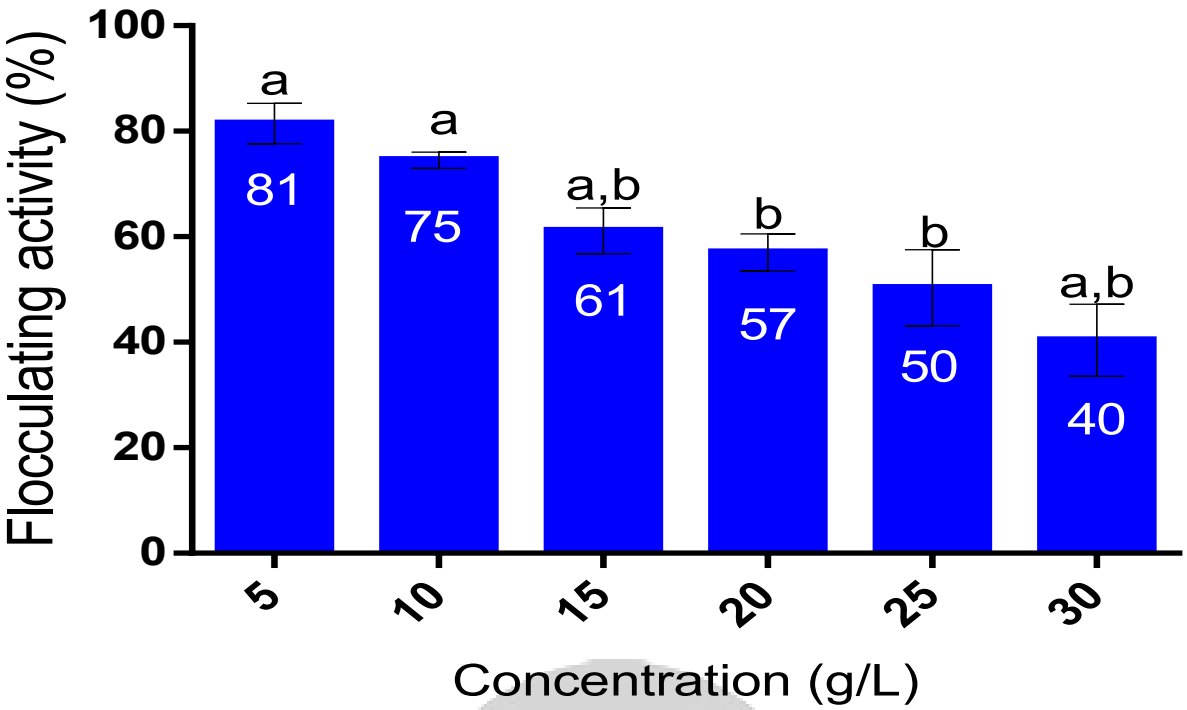


Figure 4.175: Effect of salinity on flocculating activity of the bioflocculant.

4.5 Application of the purified bioflocculant

4.5.1 In wastewater treatment

The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in stagnant waters that do not sustain aquatic life result in foul odours and anaerobic conditions (Masuku, 2019, Li *et al*., 2021a). Turbidity in wastewater is caused by suspended solids, while BOD and COD levels in wastewaters are caused by organic matter (Sarkar *et al*., 2006). In wastewater treatment, flocculants or coagulants destabilize suspended matter resulting in the formation of coagulants or flocs (Wolf *et al*., 2015). A bioflocculant produced by *Pichia kudriavzevii* was used to flocculate a variety of impurities in domestic wastewater from Vulindlela township KwaDlangezwa area, KZN, RSA and Tendele coal mine from Smkhele area, Mthubatuba, KZN, RSA wastewater. The results of the removing potential of the bioflocculant are whown in Table 4.1 and Table 4.2, respectively. The bioflocculant produced was compared with two conventional flocculants (Alum and $FeCl_3$). The removal rate of COD, BOD, phosphorus, sulfate, NO^-_3, and total nitrogen were tested from the domestic wastewater and the removal rate was 49%, 73%, 46%, 79% 61%, and 50%,

respectively. The flocculation efficiency (92%) of the bioflocculant on wastewater was also examined. The bioflocculant showed the highest removal effiencies than the conventional flocculants tested ($FeCl_3$ and alum) with low removal rate of COD (36%), BOD (27%), phosphorus (47%), sulfate (68%), NO_3^- (39%), and total nitrogen (40%), and COD (33%), BOD (47%), phosphorus (29%), sulfate (53%), NO_3^- (44%), and total nitrogen (40%), respectively. The tested conventional flocculants ($FeCl_3$ and alum) had excellent flocculating activies of 83% and 88%, respectively, but the bioflocculant; had much higher flocculation activity (92%).

Coal mine wastewater was also treated with the produced bioflocculant and was also compared with the two conventional flocculants ($FeCl_3$ and alum) and the results are shown in Table 4.2. The bioflocculant had better removal effiencies for COD (43%), BOD (64%), P (38%), sulfate (73%), NO_3^- (71%), and total nitrogen (50%) from coal mine wastewater compared to conventional flocculants with similar flocculation rate (85%) for all tested flocculants. The removal effiencies for $FeCl_3$ and alum recorded were COD (53%), BOD (57%), phosphorus (50%), sulfate (50%), NO_3^- (48%), and total nitrogen (38%), and COD (40%), BOD (36%), P (63%), sulfate (64%), NO_3^- (62%), and total nitrogen (50%), respectively. The produced bioflocculant was competative for the removal of impurities from both domestic wastewater and coal mine wastewater, and therefore, the bioflocculant can be used to treat various wastewaters, especially industrial and domestic wastewater.

The findings of this study are comparable with the rsults from Dlamini (2017), where the removal effiencies for NO_3^-, total nitrogen and phosphate were 37% and 56%, 35%, respectively from Tendele coal mine wastewater for a bioflocculant produced from *Alcaligenes faecalis*. Contrary to the findings of this study, Gong *et al*. (2008) reported much higher removal efficiencies for the bioflocculant SF-1 COD (60 - 80%). A COD removal efficiency of the bioflocculant produced by *Chryseomonas luteola* was recorded to be 32.87% (Syafalni *et al*., 2012). Bioflocculant PG-a21 Ca made from polygutamic acid, Ca and other minerals had over 50% removal efficiency for total phosphorus and 33.2% for COD (Pan *et al*., 2009), while Campos *et al.* (2016) reported the COD removal efficiency of 79.5% with the bioflocculant PGα21 Ca. Kaur *et al*. (2019) presented a bioflocculant capable of removing the impurities from raw composting leachate such as phosphorus, COD, and nitrate with removal rate of, 92%, 69%, and 95%, respectively. COD, BOD, suspended solids,

and nitrates were all removed from sewage wastewater with the removal efficiencies of 65.7%, 63.5%, 55.7%, and 71.4%, respectively (Agunbiade *et al*., 2017). These impurities were removed using a bioflocculant produced by *Arthrobacter humicola*. *Bacillus salmalaya* 139SL-7 was utilized to treat industrial wastewater with the initial BOD content of 418 mg/L. The final BOD content was 302 mg/L, with the removal efficiency of 28% (Abu Tawila *et al*., 2018). Agunbiade *et al.* (2019) reported a bioflocculant from *Terrabacter sp*. with a BOD removal efficiency of 63.3% and 60.9% for COD were better compared to convenctional flocculants (PAC and alum). Maliehe *et al*. (2019) examined a bioflocculant produced by *Alcaligenes faecalis* that had the BOD removal rate of 59% which was higher compared to alum (50%) and $FeCl_3$ (54%). Hydrogel-based bioflocculant was used for the treatment of wastewater, and it showed a removal efficiency of 65% for BOD (Fosso-Kankeu *et al*., 2017). On another study it was reported a bioflocculant from strain MSI021 of *Dendrilla nigra* that was able to remove 55% of BOD from a distillery effluent (Sajayan *et al*., 2017).

Table 4.2: Removal efficiency of impurities from Vulindlela wastewater treatment plant**.**

Flocculants	Water quality	COD (mg/L)	BOD (mg/L)	Phosphorus (mg/L)	Sulphate (mg/L)	Nitrate (mg/L)	Total Nitrogen (mg/mL)	Flocculating Activity @OD_{680nm}
Bioflocculant	Before Treatment	320	1.5	7	19	18	10	0.550
	After Treatment	162	0.4	4	4	7	5	0.044
	Removal rate/flocculating activity (%)	**49**	**73**	**46**	**79**	**61**	**50**	**92**
$FeCl_3$	Before Treatment	320	1.5	7	19	18	10	0.550
	After Treatment	204	1.1	4	6	11	6	0.086
	Removal rate/flocculating activity (%)	**36**	**27**	**47**	**68**	**39**	**40**	**83**
Alum	Before Treatment	320	1.5	7	19	18	10	0.550
	After Treatment	214	0.8	5	9	8	6	0.066
	Removal rate/flocculating activity (%)	**33**	**47**	**29**	**53**	**44**	**40**	**88**

Table 4.2: The removal efficiency of the bioflocculant in coal wastewater from Tendele coal mine**.**

Flocculants	Water quality	COD (mg/L)	BOD (mg/L)	Phosphorus (mg/L)	Sulfate (mg/mL)	Nitrate (mg/L)	Total Nitrogen (mg/mL)	Flocculating activity @OD_{680nm}
Bioflocculant	Before Treatment	435	4.2	8	22	21	8	0.542
	After Treatment	247	1.5	5	6	6	4	0.081
	Removal efficiency/flocculating activity %	**43**	**64**	**38**	**73**	**71**	**50**	**85**
$FeCl_3$	Before Treatment	435	4.2	8	22	21	8	0.542
	After Treatment	205	1.8	4	11	11	5	0.083
	Removal efficiency/flocculating activity%	**53**	**57**	**50**	**50**	**48**	**38**	**85**
Alum	Before Treatment	435	4.2	8	22	21	8	0.542
	After Treatment	249	2.7	3	8	8	4	0.081
	Removal efficiency/ flocculating activity%	**40**	**36**	**63**	**64**	**62**	**50**	**85**

4.5.2 In dye removal from various dye solutions

Two anionic dyes (Congo red and nigrosin) and two cationic dyes (methylene blue and safranin) were used in the dye removal with the bioflocculant. The decolorization ability of the bioflocculant varied depending on the dye used. According to the findings depicted in Figure 4.18, both anionic dyes had a removal rate of 81% and for the cationic dyes showed removal ability of 73% (methylene blue) and 74% (safranin). The availability and strength of positive charges in the solution, which

were fixed by the confirmation and cationicity of the bioflocculant, had a direct effect on the removal of anionic dyes (Ngulube *et al*., 2017). The pH affects the electrochemistry of the dyes and the dissociation of the polyelectrolytes and thus, the conformation in the solution (Somasundran and Runkana, 2005). Because of the cationic property of the bioflocculant, the effect of pH on dye removal is not apparent for cationic dyes, therefore the low removal activities. Overall, the bioflocculant had the modest capacity to remove anionic dyes, such as Congo red and nigrosin in the solution. These fidings indicated that the bioflocculant worked better with anionic dyes than with cationic dyes. Contrary, *Kocuria rosea* BU22S produced a bioflocculant which was also effective in anionic dye removal (Chouchane *et al*., 2018). Chen *et al*. (2017) also reported in a bioflocculant produced by *Alteromonas sp*. CGMCC 10612 with the highest removal rate for the anionic dyes Congo red and direct black with removal rate of 98.5% and 97.9%, respectively. In another study, a bioflocculant produced from a bacterium (*Bacillus megaterium)* isolated from slaughter house wastewater was able to remove methylene blue with removal efficiency of 64.9% under optimal conditions of the bioflocculant (Guo and Chen, 2017).

Li *et al*. (2013) reported in a bioflocculant produced by *Paenibacillus elgii* B69 which showed low removal rates for anionic dyes with less than 50% removal efficiencies, while there was moderate removal efficiency for cationic dyes methylene blue with 72% removal rate. This adsorption of dye molecules by *Paenibacillus elgii* B69 occurred through two mechanisms. One of which being for the long chained strucutures of EPS, which provides a large number of usable adsorption sites, and is primarily based on electrostatic attraction of two oppositely charged ions (Verma *et al*., 2012, Li *et al*., 2021b). Secondly, *P. elgii* EPS contains a high degree of

mannose, which may lead to further van der Waals forces and hydrogen bonding, as discussed by Blackburn (2004). The decolorization efficiency level for flocculating Methylene blue in aqueous solution was 83% for bioflocculant M-1 (Liu *et al*., 2009). A study by Kristanti *et al*. (2016) used seed of red chilli (*Capsicum annuum*) and bark of cengal tree (*Neobalanocarpus hepmii*) as absorbents for Methylen blue removal, and obtained 99.84% removal efficiency for Methylene blue. In conclusion, the bioflocculant produced by *P. kudriavzevii* MH545928.1 can be used in various dye-producing textile wastewater industries for the removal of different dyes wastewater.

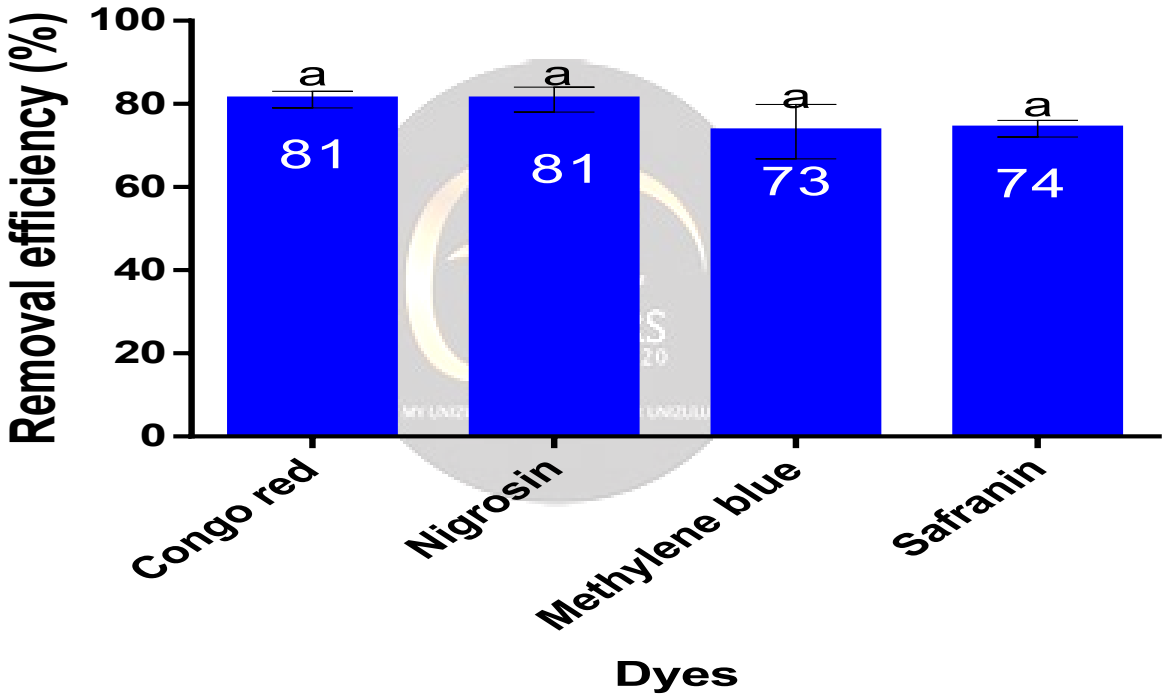


Figure 4.18: On dye removal efficiency in various solutions.

Chapter 5

This chapter contains a conclusion based on the study findings as well as the recommendations for further analysis.

5.0 Conclusion and recommendation

5.1 Conclusion

Biopolymers have shown significant promises in improving wastewater treatment productivity and quality, since biopolymers are innocuous for the environment and human utilization when compared to the conventional chemical flocculants frequently used, which have been associated with health complications. The application of bioflocculants is limited, however, due to high production costs and difficult production procedures. It has been discovered that optimizing culture conditions in dissimilar major factors such as inoculum size, energy sources, initial shaking speed, pH, cations, temperature, and fermentation time helps to improve both flocculating efficiency and production yield.

In this study a bioflocculant-producing yeast isolated from Kombucha tea broth was used the bioflocculant production. The isolate on agar plate appeared to possess oval cells in shape with a whitish colour and yeast smell. The microorganism showed an excellent flocculating activity of 84.93% against kaolin clay during the screening. The isolate was further identified using 16S rRNA sequencing method and deposited in the Gen Bank. It was found that in the GenBank database comparative analysis, the isolate had 99% similarities with *Pichia kudriavzevii* and the accession number of the isolate was MH545928.1. Therefore, the isolate was named *Pichia kudriavzevii* MH545928.1. To obtain the best bioflocculant production from *P. kudriavzevii*, the growth medium parameters were optimized. The production of a bioflocculant was seen to be proportional to the growth of the microorganism with time. A bioflocculant of 2.836 g was recovered from a Liter of fermentation broth with an inoculum size of 1% (w/v) and glucose and peptone were utilized as energy sources for optimum production. The bioflocculant production medium was optimum when incubated at 35 ºC and adjusted to pH 7 with NaCl as a stimulating agent at a shaking speed of 140 rpm for 60 hours, and the optimal bioflocculant of 2.836 g/L was obtained.

The bioflocculant was further characterized to obtain its flocculation properties. The adsorption peaks of a bioflocculant was shown by the elemental spectrum which indicated the presence of C, N, O, Na, Mg, Al, P, S, Cl, K, Ca accounts for 16.92: 1.03: 43.76: 0.18: 0.40: 0.80: 14.44: 1.48: 0.31: 0.34: 20.35 (%wt), respectively. The bioflocculant was composed mainly of carbohydrates (69%), with uronic acid (16%)

acid and protein (11%) in small amounts which revealed it to be a glycoprotein. The FT-IR analysis of the purified bioflocculant showed carboxyl, hydroxyl, amines, furans, sulfonyl, and thiocynate as its functional groups responsible for the flocculation process. The thermal stability of a bioflocculant was observed within the ranges 50 – 121 ºC and retained the flocculating activity of 70% after exposed at 121 ºC for 15 minutes. The XRD analysis of the bioflocculant revealed it to possess bigger particles with diffraction peaks at 10º and 40º and its crystallinity which is highly purified. For optimum flocculation of the purified bioflocculant, a dosage size of 0.4 mg/mL was used. The bioflocculant flocculated effectively when Al^{3+} was used as a stimulating agent. The bioflocculant seems to be effective at wide pH ranges (6 – 11) and revealed to flocculant efficiently at 5 g/L salt concentration. The bioflocculant was further used to treat Vulindlela domestic and Tendele coal mine wastewater and showed good removal efficiencies for BOD (79% and 64%), COD (49% and 43%), phosphours (46% and 38%), sulfate (79% and 73%), total nitrogen (50%), and nitrate (61% and 71%), respectively. These results were compared with chemical flocculants Fe^{3+} BOD (27% and 57%), COD (36% and 53%), phosphorus (47% and 50%), sulphate (68% and 50%), nitrate (39% and 48%), total nitrogen (40% and 38%) and alum COD (33% and 40%), BOD (47% and 36%), phosphorus (29% and 63%), sulfate (53% and 64%), nitrate (44% and 62%), total nitrogen (40% and 50%) used in the study. The purified bioflocculant also revealed good dye removal capability with removal efficiencies of 81% (Congo red), 81% (nigrosine), 73% (Methylene blue), and 74% (safranin). The results obtained in this study indicate that the bioflocculant can be used as an alternative to the currently used chemical flocculants, especially in domestic and industrial wastewater treatment.

5.2 Recommendations

The following investigations are recommendations for the further study.

I. Utilization of cheaper substrates such as molasses, bagasse, sugarcane waste, brewery wastewater, industrial wastewater or chicken waste for optimization production of the bioflocculant.

II. The bioflocculant mechanism analysis and more characterization (e.g. TGA, UV, etc.) should be done.

III. The production of the bioflocculant in a large scale should be investigated for industrial application of the bioflocculant.

IV. The antimicrobial activity and cytotoxicity effect assay of the produced bioflocculant should be conducted iin future.

V. Grafting of the bioflocculant to improve its shelf-life and flocculation rate.

VI. Synthesis of nanoparticles utilizing a bioflocculant as a cappying and stabilizing agent in order to advance the quality of water.

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Appendix

Raw data

Table 1: Inoculum size effect on bioflocculant production by *Pichia kudriavzevii*

	Absorbance at 550 nm			
Inoculum size (v/v)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
1	0.365	0.045	0.071	0.160
2	0.245	0.436	0.433	0.371
3	0.636	0.352	0.251	0.413
4	0.318	0.501	0.535	0.451
5	0.520	0.845	0.733	0.700
Control	1.956	1.793	1.859	1.869

Table 2: Carbon sources effect on the bioflocculant production by *Pichia kudriazvezii*

	Absorbance at 550 nm			
Carbon sources (g/L)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
Sucrose	0.503	0.430	0.403	0.445
Lactose	0.400	0.381	0.404	0.395
Maltose	0.212	0.183	0.232	0.209
Glucose	0.113	0.203	0.190	0.169
Starch	0.350	0.453	0.356	0.386
Xylose	0.467	0.602	0.526	0.532
Galactose	0.449	0.337	0.427	0.404

Table 3: Effect of nitrogen sources on bioflocculant production by *Pichia kudrazvezii*

	Absorbance at 550 nm			
Nitrogen sources	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
Casein	0.301	0.420	0.236	0.319
Ammonium sulphate	0.306	0.129	0.282	0.239
Peptone	0.043	0.075	0.104	0.074
Ammonium nitrate	0.319	0.296	0.401	0.339
Yeast extract	0.047	0.189	0.225	0.154
Urea	0.342	0.320	0.337	0.333

Table 4: Effect of shaking speed on bioflocculant production by *P. kudriavzevii*.

	Absorbance at 550nm			
Shaking speed (rpm)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
60	0.815	0.934	0.881	0.880
80	0.764	0.616	0.734	0.705
100	0.850	0.889	0.602	0.780
120	0.224	0.026	0.137	0.129
140	0.064	0.201	0.102	0.122
160	0.230	0.124	0.014	0.123
180	0.612	0.780	0.584	0.659
200	0.483	0.410	0.627	0.507
220	0.722	0.541	1.299	0.854

Table 5: Effect of temperature on bioflocculant production by *P. kudriavzevii.*

	Absorbance at 550 nm-			
Temperature (°C)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
20	0.904	0.883	0.900	0.896
25	0.894	0.887	0.713	0.831
30	0.237	0.948	0.051	0.412
35	0.127	0.100	0.066	0.098
40	0.368	0.372	0.410	0.383
45	0.882	0.586	0.073	0.514
50	0.064	0.199	1.511	0.591
55	1.400	0.630	0.617	0.882
50	0.922	0.300	0.954	0.725

Table 6: Effect of metal ions on flocculating activity.

	Absorbance at 550 nm			
Metal ions sources	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
Manganese chloride ($MnCl_2$)	1.065	0.642	0.349	0.685
Aluminium chloride ($AlCl_3$)	0.675	0.772	0.831	0.759
Iron (iii) chloride ($FeCl_3$)	0.807	0.698	0.868	0.791
Potassium chloride (KCl)	0.736	0.810	0.745	0.764
Lithium chloride (LiCl)	0.646	0.750	0.886	0.761
Barium chloride ($BaCl_2$)	0.452	0.545	0.414	0.470
Sodium chloride (NaCl)	0.045	0.140	0.227	0.137
Control	1.642	2.021	1.934	1.867

Table 7: Effect of initial pH on bioflocculant production.

	Absorbance at 550 nm			
Initial pH	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
3	0.927	1.452	1.115	1.165
4	1.248	1.153	0.885	1.095
5	1.306	0.639	1.323	1.089
6	0.640	0.953	0.692	0.762
7	0.269	0.132	0.265	0.222
8	0.172	0.073	0.576	0.274
9	0.178	0.396	0.089	0.221
10	0.773	0.673	0.840	0.762
11	0.406	0.539	0.769	0.571
12	0.656	0.942	0.814	0.804

Table 8: Time course of the production for a bioflocculant.

	Flocculating activity at 550 nm				Optical Density at 660 nm			
Time (hr)	**Tube 1**	**Tube 2**	**Tube 3**	**Ave**	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
0	0.994	0.821	0.894	0.903	0.710	0.452	0.503	0.555
12	0.479	0.556	0.400	0.478	0.994	0.897	0.953	0.948
24	0.339	0.340	0.324	0.334	0.996	0.998	0.989	0.998
36	0.430	0.380	0.393	0.401	1.133	1.139	1.914	1.395
48	0.178	0.192	0.168	0.179	1.142	1.931	1.132	1.491
60	0.014	0.023	0.010	0.016	0.210	0.191	0.171	1.402
72	0.061	0.058	0.051	0.051	0.924	0.911	0.938	0.924
84	0.119	0.110	0.203	0.144	0.881	0.853	0.799	0.844
96	0.626	0.534	0.402	0.521	0.764	0.847	0.875	0.829
108	0.821	0.689	0.792	0.767	0.803	0.873	0.754	0.810
120	0.976	1.00	0.963	0.979	0.931	0.898	0.892	0.907

Table 9: Final pH on time course for bioflocculant production

	Final pH			
Time (hr)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
0	7.00	7.01	7.24	7.08
12	7.11	6.88	7.06	7.02
24	6.52	6.02	6.11	6.22
36	7.04	7.20	7.18	7.14
48	7.41	7.32	6.69	7.14
60	7.90	9.01	9.20	8.70
72	9.00	7.82	8.31	8.38
84	5.35	6.54	7.21	6.34
96	5.43	6.24	5.25	5.63
108	4.17	5.12	5.10	4.80
120	5.54	5.38	5.91	5.61

Table 10: Dosage concentration on flocculating activity of the purified bioflocculant.

	Flocculating activity at 550 nm			
Concentration (mg/mL)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
0.2	0.533	0.445	0.510	0.496
0.4	0.221	0.290	0.214	0.242
0.6	0.578	0.509	0.560	0.549
0.8	0.723	0.602	0.592	0.639
1.0	0.816	0.932	0.880	0.873

Table 11: Effect of cations on flocculating activity of a purified bioflocculant.

	Flocculating activity at 550 nm			
Cations	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
Na^{+}	0.438	0.562	0.539	0.513
Li^{+}	0.629	0.531	0.702	0.621
K^{+}	0.531	0.597	0.479	0.536
Ba^{2+}	0.645	0.701	0.800	0.715
Mn^{2+}	0.251	0.474	0.391	0.372
Fe^{3+}	0.693	0.430	0.527	0.550
Al^{3+}	0.261	0.379	0.285	0.308
Control	0.954	0.982	1.089	1.008

Table 12: Effect of pH on flocculating activity of a purified bioflocculant.

	Flocculating activity at 550 nm			
pH	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
3	0.404	0.645	0.414	0.488
4	0.841	0.709	0.656	0.735
5	1.056	0.893	0.960	0.970
6	0.603	0.505	0.881	0.663
7	0.395	0.488	0.558	0.480
8	0.524	0.520	0.423	0.489
9	0.539	0.603	0.483	0.542
10	0.521	0.696	0.706	0.641
11	0.726	0.691	0.756	0.724
12	0.818	0.904	0.882	0.868

Table 13: Temperature effect on flocculating activity of a purified bioflocculant.

	Flocculating activity at 550 nm			
Temperature (ºC)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
50	0.720	0.649	0.701	0.690
60	0.714	0.760	0.523	0.666
70	0.890	0.931	0.911	0.910
80	0.914	0.899	0.996	0.936
90	0.961	0.798	0.946	0.902
100	0.991	0.887	1.01	0.963
121	0.970	1.21	0.961	1.044

Table 14: Salinity effect on flocculating activity of a purified bioflocculant**.**

	Flocculating activity at 550 nm			
Salt concentration (g/L)	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
5	0.242	0.320	0.217	0.260
10	0.345	0.381	0.344	0.357
15	0.524	0.612	0.495	0.543
20	0.587	0.656	0.561	0.601
25	0.743	0.763	0.580	0.695
30	0.920	0.731	0.854	0.835

Table 15: Phenol-sulfuric acid assay for the determination of total sugar @490 nm.

Sensitivity of gulocse concentration (0.05 – 2 mM)

Concentration (mM)	Tube 1	Tube 2	Tube 3	Average Absorbance	Adjusted OD
Blank	0.149	0.151	0.152	0.151	0.000
0.05	0.207	0.211	0.198	0.207	0.056
0.1	0.349	0.351	0.350	0.350	0.199
0.2	0.292	0.280	0.296	0.292	0.141
0.4	0.410	0.410	0.411	0.410	0.259
0.6	0.670	0.590	0.660	0.66	0.150
0.8	0.586	0.584	0.581	0.585	0.434
1.0	0.262	0.260	0.263	0.262	0.111
2	0.526	0.524	0.526	0.526	0.375
Bioflocculant	0.456	0.455	0.449	0.455	0.304

Reagents:

(a) Phenol dissolved in water (5% w/v).

(b) Concentrated sulphuric acid

Procedure:

1. Mix the standards, samples, and control solutions (0.2 mL containing up to 100 µg Carbohydrate) with 0.2 mL of dissolved phenol.
2. Add 1 mL of concentrated sulphuric acid rapidly and directly to the solution without touching the surfaces of the tubes.
3. Allow the tubes to state for 10 minutes before shaking thoroughly.

4. Determine the absorbance at 490 nm after further 30 minutes.

Table 16: Carbazole assay for the determination of uronic acid @525

Sensitivity: D-glucurono-6, 3-lactone (0.001−0.050mM)

Concentration (mM)	Tube 1	Tube 2	Tube 3	Average Absorbance	Adjusted OD
Blank	0.299	0.369	0.380	0.359	0.000
0.001	0.399	0.491	0.4780	0.460	0.101
0.002	0.506	0.604	0.526	0.526	0.167
0.003	0.706	0.73	0.703	0.706	0.347
0.004	0.954	0.957	0.975	0.957	0.598
0.005	0.741	0.736	0.729	0.736	0.377
0.010	0.702	0.710	0.699	0.701	0.342
0.050	0.751	0.749	0.753	0.752	0.393
Bioflocculant	0.732	0.731	0.728	0.732	0.373

Reagents:

(a) Dissolve 0.95 g of sodium tetraborate decahydrates in 2.0 mL of hot water and add 98 ml of ice-cold concentrated sulphuric acid carefully with stirring.

(b) Dissolve 125 mg of carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.

Method

1. Cool the samples, standards and controls (250 µl) in an ice bath.

2. Add ice-cold reagent A (1.5 ml) with mixing and cooling in the ice bath.
3. Heat the mixtures at 100 oC for 10 minutes.
4. Cool rapidly in the ice-bath. (v) Add 50 µl of reagent B and mix well.
5. Reheat at 100 oC for 15 minutes.
6. Cool rapidly at room temperature and determine the absorbance at 525 nm.

(C) Bradford Method for the determination of protein

The Bradford Protein Assay is the preferred colorimetric assay for quantifying total protein concentration. Bradford method is based upon the complex formation between basic and aromatic amino acid residues with Coomassie® Brilliant Blue G250 dye; the microassay is used for measuring 1-10 µg of protein. For maximum convenience, Bradford Reagent and 0.5 mg/ml BSA standard solution are offered individually or as components of a kit.

Table 17: Determination of protein content of the bioflocculant using Bradford method.

Concentration (mM)	Tube 1	Tube 2	Tube 3	Average Absorbance	Adjusted OD
Blank	0.210	0.211	0.213	0.211	0.000
5µL	0.635	0.633	0.630	0.633	0.422
10µL	0.694	0.695	0.691	0.693	0.482
15 µL	0.891	0.890	0.891	0.891	0.680
20 µL	1.116	0.998	1.159	1.116	0.905
25 µL	1.149	1.206	1.209	1.206	0.996
Bioflocculant	0.421	0.411	0.411	0.414	0.203

Bradford Test Kit Includes:

- Bradford Reagent – 1 litre
- Albumin
- Bovine – 0.5 mg/mL Standard,
- 0.15 N Sodium chloride

Method

1. Different concentrations of BSA was added to microfuge and sufficient 0.15 N NaCl were added to make a final volume of 100 µl

2. 1 mL of aliquot of Bradford reagents was added to each standard/sample and the absorbance was determined with a spectrophotometer at 595 nm after a 2 minutes incubation.

15.0 Application of bioflocculant in Vulindlela domestic wastewater

Table 18: Flocculating activity –Absorbance at 680nm

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	0.523	0.601	0.522	0.550
Bioflocculant	0.048	0.033	0.051	0.044
Alum	0.081	0.058	0.058	0.066
$FeCl_3$	0.097	0.067	0.093	0.086

Table 19: Nitrate (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	18	17	18	18
Bioflocculant	6	7	7	7
Alum	9	9	8	9
$FeCl_3$	11	11	11	11

Table 20: Total nitrogen (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	10	10	11	10
Bioflocculant	4	5	5	5
Alum	6	6	5	6
$FeCl_3$	6	7	6	6

Table 21: COD (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	320	321	324	322
Bioflocculant	162	161	161	161
Alum	204	205	205	205
$FeCl_3$	214	215	214	214

Table 22: Phosphorus (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	7	6	7	7
Bioflocculant	4	5	4	4
Alum	5	4	4	4
$FeCl_3$	7	7	7	7

Table 23: Sulfate (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	19	18	19	19
Bioflocculant	4	3	4	4
Alum	6	7	6	6
$FeCl_3$	9	9	8	9

Table 24: BOD (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	1.3	1.5	1.8	1.5
Bioflocculant	0.3	0.5	0.3	0.4
Alum	0.8	0.6	0.9	0.8
$FeCl_3$	1.2	0.9	1.3	1.1

Application of bioflocculant in the Tendele coal mine wastewater

Table 25: Flocculating activity- Absorbance at 680nm.

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	0.503	0.514	0.611	0.542
Bioflocculant	0.028	0.042	0.032	0.081
Alum	0.092	0.088	0.063	0.081
$FeCl_3$	0.086	0.078	0.085	0.083

Table 26: Nitrate (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	21	20	21	21
Bioflocculant	6	6	5	6
Alum	8	7	8	8
$FeCl_3$	11	10	10	10

Table 27: Total nitrogen (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	8	7	8	8
Bioflocculant	4	3	4	4
Alum	6	6	6	6
$FeCl_3$	5	4	5	5

Table 28: COD (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	435	434	434	434
Bioflocculant	247	246	247	247
Alum	259	259	260	259
$FeCl_3$	205	204	204	204

Table 29: Phosphorus (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	8	7	8	8
Bioflocculant	5	5	4	5
Alum	3	4	3	4
$FeCl_3$	4	4	4	4

Table 30: Sulfate (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	20	23	22	22
Bioflocculant	6	5	6	6
Alum	8	9	8	8
$FeCl_3$	11	13	10	11

Table 31: BOD removal (mg/L).

	Tube 1	**Tube 2**	**Tube 3**	**Average**
Original	3.5	4	5	4.2
Bioflocculant	1.3	1.0	2.1	1.5
Alum	1.7	3	3.6	2.7
$FeCl_3$	2.6	1.9	0.9	1.8

Dye removal from different dye solutions.

Table 32: Dye removal @550 nm.

Dyes	**Tube 1**	**Tube 2**	**Tube 3**	**Average**
Congo red	0.618	0.551	0.693	0.620
Safranin	0.819	0.762	0.894	0.825
Methylene blue	0.902	1.098	0.665	0.888
Nigrosin	0.621	0.513	0.709	0.614