# Characterization of bioflocculant produced by *Proteus mirabilis* PJC12 isolated from Tendele coal mine wastewater and its application


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## DECLARATION

I, Sinothile Nicodim Luthuli, declare that this dissertation entitled “Production and characterization of bioflocculant produced by *Proteus mirabilis* PJC6 isolated from Tendele coal mine wastewater and its application” submitted to the University of Zululand for the degree of Master’s in Microbiology in the Faculty of Science and Agriculture is my original research with exception of the citations and that the work has not been submitted to any other University partially or entirely for the award of any degree or examination purposes.

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## DECLARATION OF PLAGIARISM

I, undersigned, declare that I am fully aware of the University of Zululand's policy on plagiarism and I have taken every precaution to comply with the regulations.

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## CERTIFICATION

This thesis titled “Production and characterisation of bioflocculant produced by *Proteus mirabilis* PJC12 isolated from Tendele coal mine wastewater and its application” meets the regulation governing the award of degree of Masters of the University of Zululand and is approved for its contribution to scientific knowledge.

## DEDICATION

I am dedicating this thesis to four beloved people who have meant and continue to mean so much to me. Although they are no longer of this world, their memories continue to regulate my life. First and foremost, to my mother Miss GCL Luthuli. We never have enough time together, but your love is always in my heart and your memory will never die in me as long as I live, and I will never forget you. A few years after you passed away I finally understood the meaning of the last gift you brought me, and it is a reason I made it so far with my. May your soul find peace.

Next, my maternal grandfather Mr ZA Luthuli. I thank you for all lessons about life you taught me. May your soul rest in peace 'Mkhulu'.

I also remember my paternal grandmother Mrs Dlomo who raised me and taught me my mother tongue.

Last but not least I am dedicating this to my Grade 10 maths teacher Mr T Xaba who believed in me.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| $(NH)_2SO_4$ | Ammonium sulphate |
| BaCl2 | Barium chloride |
| BLAST | Basic Local Alignment Search Tool |
| BOD | Biochemical oxygen demand |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| COD | Chemical oxygen demand |
| Cu(II) | Copper (II) |
| DNA | Deoxyribo nucleic acid |
| FTIR | Fourier transform infrared spectrophotometer |
| G | Grams |
| HCL | Hydrochloric acid |
| HRS | Hours |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| KZN | KwaZulu Natal |
| $MgSO_4$ | Magnesium sulphate |

| | |
|---|---|
| Ml | MililitreMillilitre |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| OD | Optical density |
| PAM | Polyacrylamide |
| RE | Removal efficiency |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| S | Seconds |
| TGA | Thermogravimetric analysis |
| UV | Ultraviolet |

## ABSTRACT

Flocculation has been widely used as one of the most effective methods for removal of suspended particles in water or wastewater treatment. Synthetic flocculants are conventionally used because of their high flocculating activity, low-cost production and availability. However, synthetic flocculants have been reported to have negative impacts on the environment and have caused some serious health problems including neurotoxicity and cancer-genic to humans. Consequently, bioflocculants appear to be an alternative to synthetic flocculants because bioflocculant are eco-friendly, biodegradable, non-toxic and able to function at a low dosage.

In the current study, the potential for bioflocculant production of bacterial isolates recovered from Tendele coal mine wastewater (Kwa-Somkhele) in the KwaZulu-Natal (KZN) Province of the Republic of South Africa (RSA) was evaluated. The isolates were screened for bioflocculant production using kaolin clay suspension (0.4% w/v). The isolate with the best flocculating activity was selected for the study. In the nutrient ager plate, the colony appeared as round and cream white in colour. The isolate was identified by 16S ribosomal ribonucleic acid (rRNA) as *Proteus* genus. The analysis of 16S rRNA nucleotide sequence of the bacterium showed 99% similarity to *Proteus mirabilis* PJC12 with accession number MK 802115.1. The optimisation of nutrients and culture conditions were examined and revealed that optimum inoculum size was 1% (w/v) for maximum bioflocculant production by *P. mirabilis* PJC12. Fructose as carbon source and organic nitrogen source (yeast extract) was the most favoured for the bioflocculant production. The optimal initial pH of production medium was 6. Optimum temperature was 30$^{o}$C and the shaking speed of 140 rpm was optimum. At

72 hrs of incubation period under optimal culture conditions, 2,7 g of pure bioflocculant was recovered from 1 L of bioflocculant production medium of *P. mirabilis* PJC12.

Chemical characterisation of pure bioflocculant was done. Elemental analysis of pure bioflocculant by scanning electron scanning (SEM) showed the morphological images and the presence of the elements C, O, N, Na, Mg, Al, P, S, Cl, K and Ca in the percentage of 17.61%, 45.27%, 0.44%, 4.62%, 0,47%, 14,99%, 0,77%, 1.10%, 1.46% and 13.24% respectively. Functional groups were examined using Fourier Transform Infrared (FTIR) spectrometry and revealed the presence of hydroxyl group, carbonyl group, carboxyl and amide functional group. Optimization of parameters of pure bioflocculant for application plays a critical role on the maximum performance of the pure bioflocculant and these parameters including dosage size, cations, pH and thermo stability were examined. Upon optimization, the optimum dosage size of the pure bioflocculant was 0.6 g/mL with flocculating activity of 89%. The bioflocculant performed best in the presence of BaCl2 (cation) with flocculating activity of 94%. The bioflocculant was stable at a wide pH range 3 -12 with the highest flocculating activity of 96% at pH 7. The bioflocculant produced by *P. mirabilis* PJC12 revealed to be thermostable capable of retaining more than of 70% flocculating activity at 100$^{o}$C after 30 min of exposure.

The bioflocculant showed some good removal efficiency when applied in wastewater for treatment with removal efficiencies of 94% (COD), 97% (BOD), 78%(Ca), 60%(P), and 90%(P) in Vulindlela wastewater.

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# CHAPTER 1: INTRODUCTION

## 1.1 Background of the study

Flocculation is a process whereby suspended particles agglomerate to form bigger flocs in the present of flocculants (Guo *et al*., 2015). Flocculants are chemical substances with the ability to flocculate suspended particles in a solution (Zhang *et al*., 2012; Agunbiade *et al*., 2016). Flocculants are used in different industrial processes including wastewater treatment, downstream processes, food processes and fermentation processes (Buthelezi *et al*., 2009; Salehizadeh and Yan, 2014). Flocculants are categorised into three categories, namely organic synthetic flocculants such as polyethylene amine and polyacrylamide, inorganic flocculants such as poly-aluminium chloride, lime and alum and naturally occurring flocculants such as tannin, cellulose and microbial flocculants (bioflocculants) (Guo *et al*., 2006; Sam *et al*., 2011).

Organic synthetic flocculants and inorganic flocculants are widely used in flocculation processes because of their low production cost and high flocculating activity (Cosa *et al*., 2013). However, chemical flocculants are reported to be non-biodegradable, environmentally unfriendly and associated with health problems such as cancer (Agunbiade *et al*., 2017). Due to said setbacks, there is a need to replace chemical flocculants with the flocculants that are eco-friendly, harmless to the environment and biodegradable.

Bioflocculants have demonstrated to have the ability to be used as a possible replacement for chemical flocculants (Lu *et al*., 2005). Bioflocculants are macromolecular substances produced by the microorganisms during their growth phase (Salehizadeh and Yan, 2014). Their main role is to sediment suspended particles in solution during the process of bioflocculation. Bioflocculation is a process

whereby suspended particles agglomerate to form bigger flocs in the present of bioflocculants (Guo *et al*., 2015). Some bioflocculants are called microbial flocculants because they are produced by microorganisms including algae, actinomycetes, fungi and bacteria (Prasad *et al*., 2013). Bioflocculants are extracellular polymeric substances composed of polysaccharides, protein, glycoproteins and nucleic acid secreted by microorganisms (Salehizadeh and Yan, 2014). Bioflocculants have advantages over inorganic flocculants and organic synthetic flocculants in the numerous applications, as bioflocculants are harmless to the environment and have biodegradable properties (Salehizadeh *et al*., 2000; Wan *et al*., 2013; Liu *et al*., 2015).

However, bioflocculants have major limitations for commercial application, which is high production cost and low yield production (Wang *et al*., 2013), which also limits the progress in bioflocculation research (Salehizadeh and Shojasadati, 2001). Recent studies on bioflocculant production have been focused mainly on isolating and screening of novel microorganisms with high yields and low costs (More *et al*., 2012). The use of agricultural waste that is rich in hydrosates and lignocelluloses as carbon sources to produce bioflocculant has the potential to reduce production costs (Guo *et al*., 2015). Activated sludge was also used as raw material for bioflocculant production (Guo *et al*., 2013; Peng *et al*., 2014) and other waste have also been used as a carbon sources (Agunbiade *et al*., 2017).

Bioflocculant-producing microorganisms have been isolated in many different places or environments. Bioflocculant-producing *Streptomycetes platensis* was isolated from the Sterkfontein Dam outside Harrismith in the Free State province of the Republic of South Africa (Zaki *et al*., 2011). A bioflocculant produced by *Bacillus megaterium* SP1 was used in aquaculture wastewater and applied to reduce the chemical oxygen demand (COD) and accelerate floc formation (Luo *et al*., 2016). This study was mainly

focused on isolation and screening of novel bioflocculant producing bacteria from coal mine wastewater samples of Tendele coal mine located at Mtubatuba, KwaZulu-Natal province of RSA. The screened bioflocculant producing bacteria was being used for the production of bioflocculant. The isolates were identified and used in the production of the bioflocculant.

## 1.2 Problem statement

In the Republic of South Africa (RSA) there is a decrease in potable water availability due to the alarming increase in industries, which has been accepted as an enviable choice due to their contributions to economic growth (Cosa *et al*., 2013). RSA is regarded as one of the countries running short of potable water. The very same little water available is being used not only for domestic purposes but also for agricultural activities. A number of water purification methods including chemical, physical as well as biological methods have been implemented to reduce the degree of water pollution, but these methods have a negative impact on the environment (Cosa and Okoh, 2014).

Chemical flocculants such as inorganic and organic flocculants have been widely used industrially due to their high flocculating activity and low production cost and they have a long shelf life (Aljuboorji *et al.,* 2015). However, these flocculants are reported to be non-biodegradable and harmful when degraded to toxic by-products. Chemical flocculants are also associated with health problems such as Alzheimer's disease (Zaki *et al*., 2011).

Therefore, there is a need to replace these chemical flocculants with flocculants that are eco-friendly and harmless to humans and the environment. Microbial bioflocculants have been seen as an alternative to replace the in-use chemical

flocculants, because they are biodegradable, eco-friendly, harmless to humans and their degradable intermediates do not produce secondary pollution (Salehizadeh and Shojaosadati, 2001). However, bioflocculants have limitations in industrial application and progress in research due to low yield production at a high cost. Therefore, there is a need to search for novel microorganisms from different environments with bioflocculant production potential. Hence this study focused mainly on the isolation of bacteria from coal mine wastewater, and the screening of bacteria with bioflocculant-production potential. This study also focused on optimisation of the culture condition of bacteria to improve both flocculating activity and bioflocculant production.

## 1.3 Hypothesis

*Proteus mirabillis* PJC12 will produce high bioflocculant yield with high flocculating efficiency when applied to coal mine and domestic wastewater.

## 1.4 Main aim of the study

To screen, isolate and identify bioflocculant-producing bacteria, optimise the culture conditions, extract and purify bioflocculant from bacterium, characterise and apply the pure bioflocculant in wastewater treatment.

## 1.5 Objectives of the study

1.5.1 To isolate, screen and identify the bioflocculant producing bacteria;

1.5.2 To evaluate optimum culture condition for bioflocculant production;

1.5.3 To extract, purify and characterise the pure bioflocculant; and

1.5.4 To apply the pure bioflocculant in wastewater treatment.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Introduction

Industrial processes, agriculture and domestic activities are the main cause of the water pollution and specific treatments are needed to deal with these problems (Nwodo and Okoh, 2012). The new wastewater method must improve water quality and be very effective and harmless to humans and the environment compared with the in-use chemical treatment methods (Zhang *et al*., 2007).

Since chemical treatment methods are reported to be harmful to both humans and the environment, effective and eco-friendly methods to treat wastewater are needed to replace chemical treatment methods. The bioflocculants treatment method has potential to replace chemical treatment, because bioflocculants are eco-friendly and biodegradable (Ochieng *et al*., 2010).

Flocculation processes have been seen as the most useful techniques globally for the treatment of wastewater and are regarded as the most effective method of water purification (Ambarsari *et al*., 2011). Flocculation methods involving the use of flocculants, such as organic synthetic flocculants and inorganic flocculants, are frequently used in water treatment industries because of their high flocculating efficiency and low production cost with yield (Zaki *et al*., 2011). These chemical flocculants are made from acrylamide and aluminium sulfate. The acrylamide and aluminium sulfate (alum) have been reported to have a negative impact on the ecosystem (Cracium *et al*., 2013). Chemical flocculants are not eco-friendly and biodegradable in nature.

A lot of research is done to replace the chemical flocculants with bioflocculants. However, bioflocculants are reported to limit the process in research on bioflocculation

due to their low yield production and high-cost production (Sam *et al*., 2011). Thus, this study investigated a novel bioflocculant-producing microorganism with the potential to produce good bioflocculant.

## 2.2 Types of flocculants

In wastewater industries, chemical flocculants such as alum, polyacrylamide and lime are frequently used for wastewater purification (Selepe, 2017). These chemical flocculants used in industrial wastewater treatment are grouped into two categories, namely organic synthetic flocculant and inorganic flocculants (Cosa *et al*., 2013). However, these flocculants are reported to have side effects such as cancer and Alzheimer's disease (Salehizadeh and Yin, 2014). Therefore, flocculating agents such as natural occurring flocculants and grafted flocculants have been introduced in the treatment of wastewater (Dlamini *et al.,* 2019).

### 2.2.1 Organic synthetic flocculants

Many organic synthetic polymers have been frequently used as the flocculants to promote the flocculation (Ahmad *et al*., 2006). Commercialised organic synthetic flocculants are mostly water soluble and derived from acrylamide and acrylic acid (Singh and Sathiyanarayanana*,* 2008). Some organic synthetic flocculants are also produced from oil and non-renewable raw materials (Suopajarvi *et al*., 2013). A few examples of widely used organic synthetic flocculants include polyacrylamide (PAM), poly-(dially dimethyl ammonium chloride) (DADMAC) and polyamine (Singh *et al*., 2000).

Organic synthetic flocculants have different molecular weights, structures, charge types and composition (Nie *et al.,* 2011). Organic synthetic flocculants are grouped as cationic (positively charged), anionic (negatively charged), amphoteric and non-ionic

(Ahmad *et al.,* 2006). The charges of polymer have significant effects on the flocculation as well as molecular weight and density.

The high use of organic synthetic polymers as flocculants is due to their unique characteristic attributes. The organic synthetic polymers are convenient to use at any pH of the wastewater and are soluble in aqueous systems (Cracium *et al*., 2013). These organic synthetic polymers are used on a small scale with high efficiency; large flocs and strong flocs are produced during the flocculation. The polymer increases the floc size, and results in the formation of strong and dense flocs of regular shape and also results in sedimentation (Razali *et al*., 2011). The concentration is reduced from 60% - 40%, if compared to inorganic flocculants (Nie *et al*., 2011).

The majority of organic synthetic flocculants are produced from petroleum based raw material using processing chemistry that is not environmentally friendly. Many organic synthetic flocculants are not biodegradable and those that are biodegradable produce toxic by-products. These toxic by-products could enter into the food chain and cause carcinogenic effects (Nie *et al.,* 2011). Therefore, there is a need for alternative flocculants with no carcinogenic effects or no toxic effects.

### 2.2.2 Inorganic flocculants

Inorganic flocculants such as alum, poly-aluminium chloride, ferric chloride, ferrous sulphate, calcium chloride and magnesium chloride have been applied for many years as flocculants and coagulants in wastewater purification (Joo *et al*., 2007). These flocculants are cheap to produce and sell at low prices as compared to other types of flocculants (Rinaudo, 2006). However, the use of inorganic salts as flocculants in wastewater is limited and the use of inorganic salts is being reduced due to many disadvantages (Ji *et al*., 2010). Many studies have shown that the use of inorganic

flocculants would result in environmental consequences which are the formation of high volumes of metal hydroxide (toxic to environment) sludge (Ji *et al*., 2010). The disposal of inorganic flocculant causes an increase in metal magnesium concentration in the treated water which may cause human complications (Ward *et al*., 2006).

Recent studies on neuropathological, epidemiological and biochemistry have showed the possible relationship between the neurotoxicity of aluminium and the pathogenesis of Alzheimer's disease (Salehizadeh and Yin, 2014). These disadvantages about the use of organic synthetic flocculants and inorganic flocculants are the reason there is a demand for environmentally friendly and effective flocculants (Brostow *et al*., 2009). Scientists are trying to get biopolymer based flocculants from natural sources that can substitute the inorganic and organic synthetic flocculants (Rinaudo, 2006).

### 2.2.3 Naturally occurring flocculants

There is a demand for the environmentally friendly flocculating agents in the treatment of wastewater. Naturally occurring flocculants are promising alternatives to replace environmentally unfriendly flocculants. Naturally occurring flocculants are made from natural polymers, and this may be of great interest because natural polymers are environmentally friendly (Salehizadeh and Yin, 2014). Natural occurring flocculants are biodegradable polymers, shear stable, produce no secondary pollution and are safe for the ecosystem as compared to chemical flocculants (Bolto and Gregory, 2007). Table 2.1 shows some examples of naturally occurring flocculants with their sources. The biopolymers are biodegradable and the sludge they produce during the flocculation process can be degraded by microorganisms easily (Renault *et al*., 2009).

Natural flocculants have the ability to destabilise the colloidal particles by increasing the ionic strength, decreasing the thickness of the diffuse zeta potential and

decreasing the thickness of the diffuse part of the electrical double layer and reducing the zeta potential (Biatskaya *et al*., 2009). They have a macromolecular structure with different functional groups that interact with small particles (Biatskaya *et al*., 2009).

**Table 2.1:** Examples of natural occurring flocculants with their sources

| Natural occurring flocculants | Source | Citation |
|---|---|---|
| Cellulose | Plant | (Das *et al*., 2013) |
| Gum and mucilage | Plant | (Mishra *et al*., 2003) |
| Chitosan | Shellfish | (Szygula *et al*., 2009) |
| Sodium alginate | Alginic acid | (Wu *et al*., 2012) |
| Bioflocculant | Bacterium | (Ahmad *et al*., 2013) |

### 2.2.4 Grafted flocculants

Polysaccharides are shear stable and biodegradable, but polysaccharides are produced on a small scale with a lower flocculating activity compared to chemical flocculants and are needed in high dosage to treat wastewater (Mishra *et al*., 2012). Chemical flocculants are not biodegradable but have a high flocculating activity. Any type of flocculant has its own disadvantage, grafting synthetic polymer on to the backbone of natural polymers is performed to combine the advantages of both synthetic and natural polymers and to overcome the disadvantages of each other (Pal *et al*., 2012). Polyacrylamide is used in most grafting methods. Some examples of grafted flocculants are listed in Table 2.2.

**Table 2.2**: The examples of different grafted flocculants

| Type of grafted flocculants | Citation |
|---|---|
| Hydroxypropyl methyl celloluse grafted with polyacrylamide | (Das *et al*., 2012) |
| Polyacrylamide grafted carboxymethyl guar gum | (Pal *et al*., 2012) |
| Poly(2-hydroxyethylmethylacrylate) grafted ager | (Rani *et al*., 2013) |
| Polymethylmethacrylate grafted psyllium | (Mishra *et al*.,2014) |
| Polyacrylamide grafted starch | (Mishra *et al*., 2011) |
| Polyacrylamide grafted with bioflocculant | (Ngema *et al.,* 2020) |

## 2.3 History of bioflocculation

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

Bioflocculation was first reported by Pasteur in 1876 for *Levure casseeuse*. Years later, it was observed in bacterials (Bordet, 1899). *Zoogloea*-forming bacteria isolated from activated sludge became the first bioflocculant-producing bacterium to be discovered to have potential for bioflocculant production. Currently, more than 100

bacterials with potential bioflocculant production have been isolated and had their bioflocculant characterised (Ahmad *et al*., 2013).

## 2.4 Advantages of bioflocculation

The demand for effective and eco-friendly wastewater treatment techniques has increased. Bioflocculants have appeared to have the potential to replace conventional flocculants (Lee *et al*., 2012). Bioflocculants have high flocculating activity even at low concentrations (Lacchawani, 2005). Bioflocculant are biodegradable and their degradation products are environmentally friendly (Roussy *et al*., 2005). This is due to certain microbial constituents and a microbial flocculant matrix that have adsorption abilities, hydrophilicity and biodegradability. Bioflocculants can be produced at high rates depending on the culture parameters and they are easily recovered from the culture (Salehizadeh and Shojaosadati, 2001).

## 2.5 Disadvantages of bioflocculation

The application of bioflocculants industrially has been limited due to high production costs and low yield production (Rinaudo, 2006), which also resulted in slow progress in terms of research. Most bioflocculants require metal ions for charge neutralisation to the bioflocculant bridge flocculation mechanism since the bioflocculant are negatively charged and even the bioflocculation mechanisms are not clearly documented (Lee *et al*., 2014). Researchers are attempting to discover effective and novel metal-independent bioflocculants because it will reduce the cost (Liu *et al*., 2015).

## 2.6 Growth phases of bioflocculant

Bacteria have been reported to have four growth phases which are lag phase, log phase, stationary phase and death phase (Klumpp *et al.,* 2009).

The lag phase is the less understood growth phase, because of lack of information describing the physiological and molecular processes. It has been assumed that the lag phase is where the microorganism adapts to the new environmental conditions. This process includes the synthesis of cell components for growth and repair of damaged macromoleculars (Klumpp *et al*., 2009). Information about the lag phase remain hypothetical possibilities because the available physiological data only shows that the lag phase is metabolically active (Hemeoka and Kaneko, 2014). Bioflocculation production rate is low in this phase because the bacteria are adapting to the new medium environment and producing new cells (Okaiyeto *et al*., 2016).

The log phase occurs for a short period and requires some factors to be present in the growth medium, including favourable carbon, nitrogen, phosphate sources and trace elements. The production of bioflocculant is high in this phase as the bacteria are utilising all available nutrients for growth. The bioflocculant production rate in this phase increases until the available nutrients are depleted in the production medium (Nwodo *et al*., 2014).

The stationary phase of the growth curve occurs when there is a balance between rate of cell production and the cells dying. In this phase, the bioflocculant production rate has stopped due to depletion of nutrients in the production medium. The bioflocculant produced might decrease if the carbon and nitrogen sources are depleted in the medium (More *et al*., 2012).

The death phase is where the dead cells are dominant in the production medium due to the depletion of nutrients and pH changes in the medium. The microbial growth is depleted and only spore-formers are capable of surviving in this stage. There is no bioflocculant production because no nutrients are available and flocculating activity is lower than in previous phases.

## 2.7 Factors affect that bioflocculant production

Bioflocculation production is affected by a number of factors that play a vital role in their production, factors such as inoculum size, energy sources, metal ions, temperature, agitation speed and time course. These factors are discussed in detail below.

### 2.7.1 The effect of inoculum on bioflocculant production

The inoculum size is the number of cells used to inoculate the production medium for the bioflocculant production (Mohamed and Dagang, 2019). Inoculum size has an impact on the growth rate of the microorganism and on the degree of bioflocculant production (Ugbenyen *et al.,* 2014). The growth patterns and bioflocculant production are affected by different inoculum sizes (Salehizadeh and Yan, 2014). The inoculum size influences the optimum bioflocculant production and varies for different microorganisms. The optimum inoculum size is very crucial in bioflocculant production because a small inoculum size results in the prolonged lag phase period while a large inoculum size will result in niches overlapping excessively, causing the inhibition of bioflocculant production (Salehizadeh and Shajoasadati, 2001). The *Bacillus* produced the highest yield with an inoculum size of 1% (v/v) (Zhang *et al*., 2007), while *Bacillus licheniforms* X14 produced a high yield with the inoculum size of 5% (v/v) (Li *et al*., 2009).

### 2.7.2 The effect of carbon sources on bioflocculant production

There are many different sources of carbon with different effects on bioflocculant production in different microorganisms (Deng *et al*., 2005). Carbon sources in the production medium provide the appropriate biochemical and biophysical environment, and its optimisation is important in the bioflocculant production (Cosa *et al*., 2011).

For example, the bioflocculant produced by *Aspergillus parasiticus* showed that corn starch enhanced its production with flocculating activity of 96% (Pu *et al*., 2018). The effect of different carbon sources on the bioflocculant production showed by *Bacillus sp* indicates more bioflocculant production when sucrose was used as a sole carbon source with high flocculating activity while maltose and ethanol indicate the low flocculating activity (Zhang *et al*., 2007).

### 2.7.3 The effect of a nitrogen source on bioflocculant production

Microorganisms need a nitrogen source in the medium for the production of bioflocculant (Cosa *et al*., 2011). Therefore, it is important to optimise the nitrogen source in the production medium for bioflocculant production. Some microorganisms prefer an organic nitrogen source, such as urea, yeast extract, beef extract and peptone, and some microorganisms prefer inorganic nitrogen sources including $(NH_4)_2SO_2$ and $KNO_3$ (Agunbiade *et al*., 2016). Organic nitrogen sources are more favourable than inorganic nitrogen sources for bioflocculant production which adds additional nutritional value to the bioflocculant production (Ntozonke *et al*., 2017). *Virgibacillus sp* produced a bioflocculant in the presence of peptone as the most preferred nitrogen source (Cosa *et al*., 2011).

Sodium nitrate and peptone were reported as the best nitrogen sources for bioflocculant production by *A. parasiticus* and when ammonium sulphate was provided no bioflocculant production occurred (Deng *et al*., 2005). *Pseudomonas aeruginosa* isolated from activated sludge showed high bioflocculant production in the presence of yeast extract and peptone compared to other nitrogen sources used (Pathak *et al*., 2013).

### 2.7.4 The effect of cations on flocculating activity

Cations are mostly used as stimulating agents to achieve high flocculating activity and neutralise negative charges of the functional groups of the suspended particles. This causes the bioflocculant to reduce the static repulsive force and promote the effectiveness of the bioflocculant (Ntozonke *et al.,* 2017). Cations affect microorganisms differently because microorganisms require different cations for optimum bioflocculant production (Liu *et al*., 2010).

Some microorganisms prefer monovalent, or divalent or trivalent cations. The evaluation of cations used for *Athrobacter sp* bioflocculant production are $FeCl_3$, $MgCl_2$, $FeSO_47H_2O$ and KCl. $MgCl_2$ had the highest flocculating activity compared to other cations $Fe^{2+}$ and $Fe^{3+}$ which had no effect on flocculating activity (Nwodo and Okoh, 2012). Cosa *et al*. (2012) reported $Mg^{2+}$ as the most effective cation for bioflocculant production by *Vingibacillus sp*.

### 2.7.5 The effect of temperature on bioflocculant production

The temperature plays an important role in the growth of bacteria and bioflocculant production since each microorganism requires an optimum temperature for growth and production of the bioflocculant. Different bacteria prefer different temperatures to produce bioflocculants. The bioflocculant production temperature range is 20 $^oC$ - 50 $^oC$ (Zayed *et al*., 2018). *B. licheniformis* was incubated at 37 $^oC$ and the flocculating activity was above 80%. When the temperature decreased to below 37 $^oC$, the flocculating activity dropped drastically. The flocculating activity dropped when the temperature was above 40 ℃ (Liu *et al*., 2017). These results revealed that cultivation temperature plays an important role in bioflocculant production. The most favourable culture temperature of 25 $^oC$ for bioflocculant production by *Klebsiella sp.* was

observed (Liu *et al*., 2014). The optimum temperature for *Bacillus safensis* was 40 $^{o}$C for maximum bioflocculant production (Ntombela *et al.,* 2020).

### 2.7.6 The effect of shaking speed on the bioflocculant production

The shaking speed regulates oxygen absorption and distribution of nutrients which have a great impact on the enzymatic reactions (Garg *et al*., 2014). Different microorganisms prefer different shaking speeds for oxygen absorption and distribution of nutrients during their growth phases (Li *et al.,* 2009). For example, bioflocculant-producing *Klebsiella sp.* ZZ-3 produces the best yield of bioflocculant at an optimal speed of 200 rpm (Yin *et al.,* 2014). *Bacillus sp*. produced the bioflocculant optimally at a speed of 160 rpm (Ntombela *et al.,* 2019).

### 2.7.7 The effect of incubation period on the bioflocculant production

Bioflocculation increases as the incubation period increase on some microorganisms. *A. parasiticus* was incubated for a period of 24 - 60 hrs and the bioflocculant production increased from 12% - 95%, respectively (Deng *et al*., 2005). The fungus reached its stationary phase after 72 hrs of incubation and with a flocculating activity for kaolin suspension of 98,1%. The *P. mirabilis* growth curve showed that the bioflocculant production and cell growth are parallel while the pH of the medium decreased with a flocculating activity of 93,3% in the stationary phase (Feng *et al*., 2008). The bioflocculant production and cell growth of *Chrysebacterium daegue* W6 showed a maximum increase during a short incubation period but the flocculating activity was low. At the death phase the flocculating activity was 90% after 54 hrs when the $OD_{600}$ was less than 0,3. This showed that MBF-W6 was an intracellular bioflocculant (Liu *et al*., 2010).

## 2.8 Mechanisms of flocculation

The flocculation process is taking place though different types of mechanisms including charge neutralization, electrostatic patch and bridging (Lee *et al*., 2012). Some flocculants use a combination of two mechanisms (Mishra *et al*., 2005). According to Blanco *et al.* (2002) the bioflocculation process is mostly involving charge neutralisation mechanisms and bridging mechanisms. These mechanisms are discussed in detail below.

### 2.8.1 Bridging mechanism

Bridging mechanism occurs when charged colloids come together to the surface of the particles to form a bridge between particles. This process allows the particles to gather and results in flocs formation (Mishra *et al*., 2005). High molecular weight polymers with a low charge density attach on the surface in such a way that long loops extend to the second surface. This results in the formation of the bridge between particles because the hanging polymers attach together (Agunbiade *et al*., 2016). This mechanism was reported in wastewater treated using bioflocculants produced by *Plantago psyllium* and *Tamarindus indica* (Mishra *et al*., 2006). This phenomenon is evident of the length of polymers (Rasteiro *et al*., 2008).

### 2.8.2 Charge neutralisation mechanism

Charge neutralisation mechanism occurs whereby positively charged colloids come in contact with negatively charged particles. This results in the disappearance of electrostatic repulsion between the particles and it enhances the process of coagulation. This mechanism occurs when particles and flocculants are of opposite charges and mostly occurring in the presence of inorganic flocculants (Figure 2.1) (Lee *et al*., 2012).

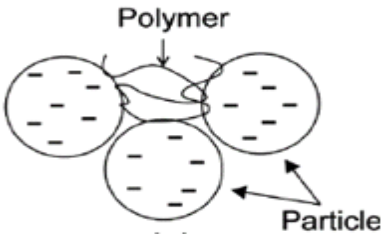


**Figure 2.1:** Charge neutralisation mechanism (Lee *et al*., 2012)

### 2.8.3 Electrostatic patch mechanism

Electrostatic patch mechanism is the process whereby charged polymer bind with a particle of the opposite charge. These particles attract each other through patches of opposite charges resulting in coagulation of the suspensions (Zhou *et al*., 2006). This type of flocculation mechanism can be performed in several ways in the presence of commonly used coagulants including alum and ferric chloride. These metals or coagulants dissociate in water and the metal ions can cause flocculation through charge neutralisation (Rasteiro *et al*., 2007). Figure 2.2 shows the diagram of electrostatic path mechanism.

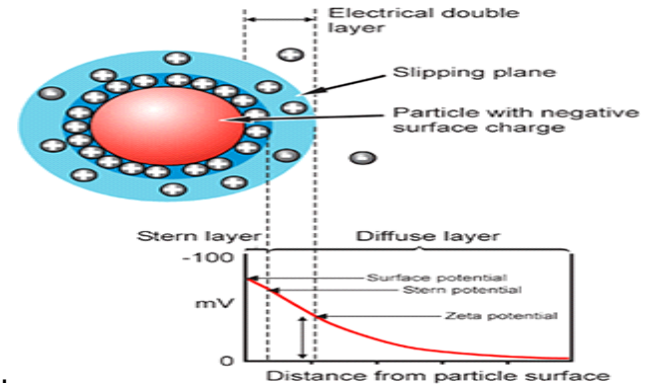


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**Figure 2.2:** Electrostatic path mechanism (Rasteiro *et al*., 2007)

## 2.9 The physiochemical properties of bioflocculants

The physiochemical properties of bioflocculants also enhance the aggregation of pollutants in wastewater treatment systems. The properties include adsorption, biodegradability, hydrophobicity and hydrophilicity, and are discussed in detail below.

### 2.9.1 Adsorption

The flocculation by bioflocculants occurs in the presence of carboxyl, amine and hydroxyl functional groups (Wang *et al.,* 2011). Functional groups provide binding sites for suspended particles and metal ions during the adsorption (He *et al.,* 2011). The carboxyl and amino groups in the bioflocculant structure are reported to provide additional binding sites for metal ions (Pathak *et al*., 2013).

### 2.9.2 Biodegradability

Biodegradability is the breakdown of secondary metabolites by microorganisms. When there is a shortage of nutrients in the medium, microorganisms degrade secondary metabolites produced by them or other microorganisms. Since the bioflocculants are composed mainly of carbohydrates, glycoprotein and proteins, it is easier for the microorganisms to consume them as energy sources for cell growth (More *et al.,* 2014). This biodegradable property caused the eco-friendliness of bioflocculants in wastewater treatment because bioflocculant are used by microorganisms, unlike chemical flocculants including PAM (Shih *et al.,* 2001).

### 2.9.3 Hydrophobicity

Hydrophobicity is an electrostatic interaction of bioflocculant particles promoting the formation of hydrogen bonds and it is the essential characteristic of a bioflocculant (More *et al.,* 2014). Flocculants are mostly hydrophilic polymers. Bioflocculants are

amphoteric polymers containing both hydrophilic and hydrophobic properties (Okaiyeto *et al.,* 2016).

## 2.10 Bioflocculant-producing microorganisms

There are many microorganisms reported to produce bioflocculants. During a study conducted on crude petroleum oil samples, 36 bacterial colonies were isolated and there were only two colonies that could produce bioflocculants (Desouky *et al.,* 2008). Those two colonies were identified as *Bacillus subtilis* and *Pseudomonas* sp. Their flocculating activity was above 90% after three days of cultivation. The analysis of bioflocculants produced showed that all were glycoproteins. These bioflocculants were water soluble and insoluble in any organic solvent tested.

The *Aspergillus niger* produced a bioflocculant (MBFA18) using potato starch wastewater as nutrients (Pu *et al*., 2018). The flocculating activity of MBFA18 was 90.06% with a kaolin suspension under optimal cultivation conditions. Another study was done on *A. parasiticus* and it produced the bioflocculant with high flocculating activity in kaolin suspension and water-soluble dyes. The bioflocculant produced by *Aspergillus parasiticus* composed of 76,3% sugar and 21,6% protein with an average molecular weight of $3,2 \times 10^5$ Da (Salehizadeh and Shojasadati, 2001).

Microalgae have been studied widely to produce biofuel. *Cellulosimirobium cellulans* L804 was isolated and studied for bioflocculant production, using untreated biomass. The cultivation conditions were also investigated such as carbon and nitrogen sources and pH on *C. cellulans* L804 for optimum bioflocculant production (Liu *et al.,* 2015).

## 2.11 Application of bioflocculants

Bioflocculants have been used by researchers on purification of wastewater, heavy metal removal and dye removal. They have also been used as alternative to chemical flocculants because chemical flocculants cause serious environmental and health problems (Lu *et al.,* 2005).

### 2.11.1 Wastewater treatment

Pure bioflocculants have been produced and used in wastewater treatment to remove different materials. The bioflocculant produced by *Serratia ficario* was used to treat wastewater from soy sauce brewing, meat processing and river water. Its promising results on river water was obtained with removal efficiencies of chemical oxygen demand (COD), turbidity and colour to 84.2%, 87.1% and 90.4% respectively. The bioflocculant was more effective on river water (Gong *et al*., 2008). Bioflocculant produced by *Bacillus mucilaginosus* was applied on different wastewater samples and reduced the COD and BOD (Li *et al*., 2009; Li *et al*., 2013). A bioflocculant from the consortium of *Corbetia sp.* and *Bacillus sp.* was used in river water, brewery and diary wastewater and has a high flocculating activity of 99%, 90,2% and 78,8% respectively (Ugbenyen and Okoh, 2014). Bioflocculants have used only on a small scale and gave promising results, encouraging use on a large scale as well.

### 2.11.2 Removal of heavy metals

Heavy metal pollution is being reported to be one of the major environmental problems that pose a threat to human communities. Heavy metals are non-biodegradable and bioaccumulate in organisms causing diseases and disorders (He *et al*., 2013). The consumption of water polluted with heavy metals has led to organ failure such as liver,

kidney and system failure such as nervous system and digestion system in humans (Baby *et al*., 2010). Based on these reports, bioflocculants are considered as a method to treat industrial wastewater containing heavy metals.

Bioflocculants produced by *Bacillus sp* and *Pseudomonas sp* were used as flocculating agents for the removal of heavy metals including Cu (II), Cd (II) and Pb (II) from textile wastewater industries (Azzam and Tawfik, 2015). Both bioflocculants were effective in the removal of the heavy metals mentioned. The removal efficiency of bioflocculating produced by *Bacillus sp* was 99.5% of Pb (II) and 84.0% of Cu (II). High removal efficiency was observed with a bioflocculant produced by *Pseudomonas sp,* 97.3% for Pb (II) and 70.4% for Cu (II). The removal efficiency of Cd with bioflocculants from *Pseudomonas sp* was higher compared with *Bacillus sp.* (Azzam and Tawfik, 2015).

### 2.11.3 Dye removal

Bioflocculants have been used in various industrial processes (Huang *et al.,* 2014). Dye removal in wastewater is a serious challenge since dyes are soluble in aqueous solution (Zhao *et al.,* 2012). The utilisation of dyes in the clothing, plastics and leather industries and paper and pulp manufacturing processes has increased significantly recently, and this has led to the discharge of wastewater contaminated with dyes into the ecosystem (Chouchane *et al.,* 2018).

A bioflocculant produced by *Paenibacillus elgii* B69 contains functional groups that can remove cationic dyes in wastewater (Li *et al.,* 2013). The bioflocculants are able to remove Red X-GRL (72%) and methylene (65%). The acidic bioflocculants are negatively charged and contain a $COO^-$ group in their molecule structures, which

provides adsorption sites for the positively charged cationic dyes resulting in making electrostatic attraction possible (Verma *et al.,* 2012).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Isolation of the microorganisms and culture conditions

The wastewater samples were collected from Tendele coal mine in Mtubatuba, KwaZul- Natal, RSA. Wastewater samples were transported using a cooler box filled with an ice bath and analysed in the laboratory within six hrs after collection. Ten-fold serial dilutions were made up to $10^{-5}$ saline solution (0.85%). One millilitre (mL) of the wastewater sample was added to 9 mL sterile saline solution in a test tube. The mixture was agitated for 30 seconds and further diluted up to $10^{-5}$-fold. 100 µL was spread aseptically using a glass spreader on nutrient agar plates and incubated for 24 - 72 hrs at 30 $^{o}$C (Jensen *et al*., 2005). Pure colonies obtained were used for screening of bioflocculant-producing bacteria (Subudhi *et al.,* 2014).

## 3.2 Screening of bioflocculant-producing bacteria

Screening of the microorganisms for bioflocculant production was done as described by Zhang *et al.* (2007). The production medium was made up of 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 5 g $K_2HPO_4$, 2 g $KH_2PO_4$ and 0.2 g $MgSO_4 \bullet 7H_2O$ was prepared with 1 litre (L) of filtered coal mine wastewater and autoclaved at 121 ℃ for 15 min. Prior to autoclave, 50 mL of the production medium was transferred into 100 mL conical flasks. Single colonies from pure culture were inoculated in 50 mL of production medium in conical flasks. The cultures in the medium were incubated in a rotary shaker at 30 $^{o}$C with a speed of 160 rpm for 72 hrs. After 72 hrs of incubation, 2 mL of culture suspensions were withdrawn and centrifuged at 8000 × g, 4 $^{o}$C for 30 min. The cell free cultures were used to determine flocculating activity.

## 3.3 Determination of the flocculating activity

To determine the flocculating activity of a bioflocculant, the method of Kurane *et al.* (1994) was used with a kaolin clay suspension as test material. About 100 mL of kaolin clay suspension was mixed with 3 mL of 1% (w/v) $CaCl_2$ and 2 mL culture supernatant. The mixture was shaken vigorously in a 250 mL conical flask for 60 sec, and then transferred into a 100 mL graduated cylinder. The mixture was allowed to sediment for 5 mins at room temperature. The control was prepared by replacing 2 mL of the supernatant with freshly prepared production medium. The optical density (OD) of the supernatant was measured at 550 nm with a UV spectrophotometer. The flocculating activity was calculated using the following formula:

Flocculating activity (%) = [(A-B)/A] X 100,

Whereas A is the OD of the kaolin clay suspension and B is the OD of the sample measured at 550 nm wavelength.

## 3.4 Identification of bioflocculant-producing bacteria using 16S rRNA

The identification of bioflocculant-producing bacterium was performed as described by Xiong *et al.* (2010). The bioflocculant producing bacteria isolated from Tendele coalmine wastewater was incubated in 250 mL flasks with 50 mL of fresh LB medium for 16 hrs at 37 ℃ with an agitation speed at 200 rpm. The genomic DNA of the bioflocculant producing bacteria was extracted using the appropriate bacterial DNA kit. PCR amplification was carried out to determine the partial 16S rRNA gene. The PCR primers, forward and reverse primers were designed and used. The PCR programme was 30 cycles of 94 ℃ (1 min), 55 ℃ (30s), and 72 ℃ (1 min and 30s). Purification of the PCR products and the determination of sequence were performed

by GeneCore Bio Technologies Co., Ltd. The 16S rRNA gene sequence of bioflocculant producing bacterium obtained was compared with NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 3.5 Optimisation for bioflocculant production

Optimisation for bioflocculant production has an important role to play in the yield of the bioflocculant to be produced, since optimisation is more about finding the optimum culture conditions which are more favourable for a microorganism to produce maximum bioflocculant yield (Cosa *et al*., 2013). Factors such as inoculum size, nutrients source, initial pH of the production medium, shaking speed, cations and temperature fermentation time were optimised.

### 3.5.1 Effect of inoculum size on bioflocculant production

The effect of inoculum size on bioflocculant production was assessed by inoculating 50 ml of the production medium contained in separate flasks with different concentrations of seed culture broth ranging from 1 to 5% and incubated in a rotary shaker at 160 rpm, 30 ℃ for 72 hrs. The flocculating activity assay was done as previously described (Aljuboori *et al.,* 2015).

### 3.5.2 Effect of carbon and nitrogen sources on bioflocculant production

The effects of various carbon and nitrogen sources on bioflocculant production were studied using the method described by Yim *et al.* (2007). A bioflocculant-producing bacterial was inoculated into the production medium (50 mL) contained in separate flasks supplemented with different carbon and nitrogen sources, and incubated in a rotary shaker at 160 rpm, 30 ℃ for 72 hrs. The carbon sources used include glucose, maltose, fructose, lactose, sucrose and starch. The nitrogen sources were tryptone, peptone, urea, yeast extract, ammonium acetate and ammonium sulphate. The

mentioned carbon sources were replacing 20 g/L glucose in the production medium and 1.2 g/L of multiple nitrogen source (urea, yeast extract and $NH_2SO_4$) was substituted with the mentioned nitrogen sources as nitrogen sources in the production medium. The flocculating activity was determined as 3.3.

### 3.5.3 The effect of cations on flocculating activity

The effect of cations on flocculating activity was evaluated as described by Mabinya *et al*. (2011). To test for the effect of cations, 3 mL 1% $CaCl_2$ (w/v) was replaced by various metal ions salt 1% (w/v) solution including NaCl, KCl, $MgCl_2$, $MnCl_2$, $BaCl_2$, $FeCl_3$, $AlCl_3$ and the flocculating activity was measured as described in 3.3.

### 3.5.4 The effect of initial pH of the production medium on bioflocculant production

The method used by Yim *et al*. (2007) was used to assess the effect of initial pH on bioflocculant production. The production medium was prepared, and 50 mL was transferred in separate flasks. The production medium was then adjusted to different pHs ranging from 3 - 12 using 1N NaOH and 1N HCl prior to autoclaving. The production medium was inoculated with the optimum volume of seed culture and incubated for 72 hrs at 160 rpm and 30 $^oC$. Thereafter the flocculating activity was measured as described in 3.3.

### 3.5.5 The effect of shaking speed on bioflocculant production

The method described by Zhang *et al.* (2007) was used to evaluate the effect of shaking speed on bioflocculant production. Different shaking speeds (60 - 220 rpm) were used. Different flasks with 50 ml production medium and optimum inoculum size were incubated in rotary shaking incubators at different speeds (60, 80, 100, 120, 140,

160, 180, 200, 220 rpm) at temperature 30 ºC for the period of 72 hrs. After 72 hrs the flocculating activities were measured (See 3.3).

### 3.5.6 The effect of cultivation temperature on bioflocculant production

A method used by Mohammed and Dagang (2019) was used to study the effect of cultivation temperature on bioflocculant production. The test bacterium was inoculated in the production medium and incubated at different temperatures including 20, 25, 30, 35, 40, 45, 50, 55 and 60 ℃ for 72 hrs. After incubation for 72 hrs, the flocculating activities were measured as described in 3.3.

### 3.5.7 Time course assay

The optimum conditions obtained were used to evaluate the fermentation time on bioflocculant production. Optimum conditions obtained include inoculum size (1%), carbon (fructose) and nitrogen (yeast extract) sources, and initial pH (6) of the production medium, shaking speed (140 rpm), cation (BaCl2) and temperature (30 ℃). The time course assay was performed for 120 hrs. After every 12 hrs interval of cultivations of the fermented medium was drawn to measure the pH, cell growth ($OD_{600\ nm}$) and flocculating activities were determined (Okaiyeto *et al*., 2016).

## 3.6 Extraction and purification of a bioflocculant

The extraction and purification of the bioflocculant was performed according to the method described by Chang (1998). After 72 hrs of fermentation, the fermented broth was centrifuged at 8000 × g, 40 ℃ for 30 mins to remove bacterial cells and debris. One volume of distilled water was added to the supernatant phase and centrifuged at 8000 × g for 30 min to remove insoluble substances. Two volumes of ethanol were added to the supernatant, mixed and left overnight at 4 ℃ for precipitation. The mixture was vacuum dried to obtain the crude bioflocculant. The crude bioflocculant was then

dissolved in 100 mL distilled water to yield a solution. One volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v) was added to the bioflocculant solution stirred and allowed to stand for 12 hrs at room temperature. The top upper part of the mixture was collected, and vacuum dried to obtain the pure bioflocculant.

## 3.7 Characterisation of the pure bioflocculant

### 3.7.1 Chemical composition analysis of the pure bioflocculant

The total sugar content of a pure bioflocculant was determined by using the phenol-sulphuric acid method with glucose as the standard solution. The total protein content was determined by using the Folin-Lowry method with bovine serum albumin (BSA) as a standard solution (Agunbiade *et al*., 2018). The carbozole method was used to determine the amount of uronic acid present in a pure bioflocculant, and D-glucoronic acid was used as a standard solution (Cesaretti *et al*., 2003).

### 3.7.2 Scanning electron microscopy (SEM) analysis

SEM (SEM-Sipma-VP-03-67) was used to study the morphology of the pure bioflocculant as well as elements present in a bioflocculant. The pure bioflocculant, kaolin clay powder and flocculated dried kaolin clay images were obtained using a SEM at 15 KV. The morphological structure of the pure bioflocculant, kaolin clay powder and the kaolin clay bound to the bioflocculant were observed (Ntsangani *et al.,* 2016).

### 3.7.3 Determination of functional groups of the pure bioflocculant

Fourier transmission infrared (FT-IR) (Perkin Elmer System, 2000, Cambridge, English) was used to determine the functional groups of the pure bioflocculant. Potassium bromide (KBr) powder was used together with fine dried pure bioflocculant

and pressed into pellets for FT-IR spectroscopy analysis over a wavelength range of 4000 to 400 $cm^{-1}$ (He *et al*., 2010).

### 3.7.4 Thermo-gravimetric analysis of the pure bioflocculant

To evaluate the pyrolysis properties of pure bioflocculant, the thermo-gravimetric analyser was used. The temperature of the thermo-gravimetric analyser was adjusted from 30 ℃ to 800 ℃ under nitrogen gas constantly flowing at 20 mL/min. The rate was kept constant at 10 ℃ $min^{-1}$ (Ngema *et al*., 2020).

## 3.8 Optimisation of the pure bioflocculant conditions

### 3.8.1 Optimum dosage determination of the bioflocculant

The optimum dosage concentration of the pure bioflocculant was determined using the jar test as described by Nwodo *et al.* (2014). Different concentrations of a pure bioflocculant solution ranging from 0.2 - 1.0 mg/ml were prepared and their flocculating activities were measured as described previously. Optimum dosage concentration of a pure bioflocculant solution was used in all tests followed.

### 3.8.2 Effect of temperature on the flocculating activity

The thermal stability of a pure bioflocculant was evaluated using the method described by Makapela *et al*. (2016). Tubes containing 2 ml of pure bioflocculant solutions were transferred into various cultivation temperatures ranging between 50 ℃ and 100 ℃ for 30 min, with one tube exposed to 121 ℃ (autoclave with steam under pressure) for 15 mins. The flocculating activities were measured as previously described in 3.3.

### 3.8.3 Effect of pH on the flocculating activity

The effect of pH on the flocculating activity of the pure bioflocculant was assessed using the method described by Liu *et al.* (2015). Kaolin solution (4% w/v) was adjusted

to various pHs ranging from 3 – 12 with 1N NaOH and 1N HCl. The flocculating activities were measured at 550 nm wavelength as described in 3.3.

### 3.8.4 Effect of cations on the flocculating activity of pure bioflocculant

The effect of metal ions on flocculating activity was evaluated using the method by Makapela *et al*. (2016). Salt solutions [1% (w/v)] of NaCl, LiCl, KCl, $BaCl_2$, $MgCl_2$, $MnCl_2$ and $FeCl_3$ were used to substitute 1% (w/v) $CaCl_2$ solution and the control prepared did not contain cations. The flocculating activities were measured (See 3.3).

## 3.9 Application of pure bioflocculant on wastewater treatment

The application of the pure bioflocculant on wastewater treatment was performed using the method described by Li *et al*. (2013). Wastewater samples (2 L) were freshly collected from Tendele coalmine and Vulindlela treatment plant located at KwaDlangezwa area, KwaZulu Natal, RSA. The parameters such as BOD, COD, aluminium, sulfide, calcium, phosphate contents and and pH were determined using spectra-quant (Pharo 300, Merch, SA) and a pH meter before and after flocculation.

100 mL of wastewater samples were poured into 250 mL conical flasks with 2 mL of optimum dosage of the pure bioflocculant solution and 3 mL of $BaCl_2$ solution. The mixture was agitated at 200 rpm for 3 min and the speed was lowered to 45 rpm for 5 min at room temperature. The comparison of chemical flocculants with the bioflocculant was performed by replacing the bioflocculant with commercial flocculants such as $FeCl_3$ and Polyacrylamide (PAM). Removal efficiency of the flocculants was determined using the formula below:

Removal Efficiency (RE) = $[(C_0 - C)/C_0]$.

$C_0$ is the value before the flocculation and C is the final value after the flocculation.

## 3.10 Statistical analysis

Tests were conducted in triplicates; the means and standard deviation were determined by prisms graph pad anova test, where differences were considered significant at 0.5 confidence level.

# CHAPTER 4: RESULTS AND DISCUSSION

## 4.1 Screening, isolation and identification of bioflocculant producing bacterium

In total more than 20 different bacterial isolates were obtained from the coal mine wastewater and five demonstrated the ability to produce bioflocculants. The isolate with the highest flocculating activity of 72% against kaolin clay suspension (0.4%) was selected for further use in this research. The colony morphology of the selected isolate was cream white and the microogarnism was Gram-negative, and bacilli in shape. The 16S rRNA gene sequence of the selected bacterium showed 98% similar with that of *Proteus mirabilis* PJC12. Therefore, the selected bacterium was finally identified as *P. mirabilis* PJC12 according to the morphological and phylogenetic characteristics and 16S rRNA gene sequence of this bacterium. The 16S rRNA gene sequence was blasted to NCBI and the accession number was given as MK 802115.1. *Proteus mirabilis* TJ-1 was isolated and produced a bioflocculant. This shows that *P. mirabilis* has the potential to produce bioflocculant as it was reported by Xia *et al.* (2008).

## 4.2 Optimisation of culture conditions for bioflocculant production

For *P. mirabilis* PJC12 to produce the highest possible yield of bioflocculant, culture conditions for bioflocculant production, such as the inoculum size (% v/v), nutrient sources cations, pH of production medium, shaking speed, temperature and time course were evaluated. According to Akapo *et al.* (2019), these parameters should be optimised to enhance bioflocculant yield product and flocculating activity.

### 4.2.1 The effect of inoculum size on bioflocculant production by *P. mirabilis* PJC12

The effect of inoculum size on bioflocculant production by *P. mirabilis* PJC12 was examined and results are shown in Figure 4.1.

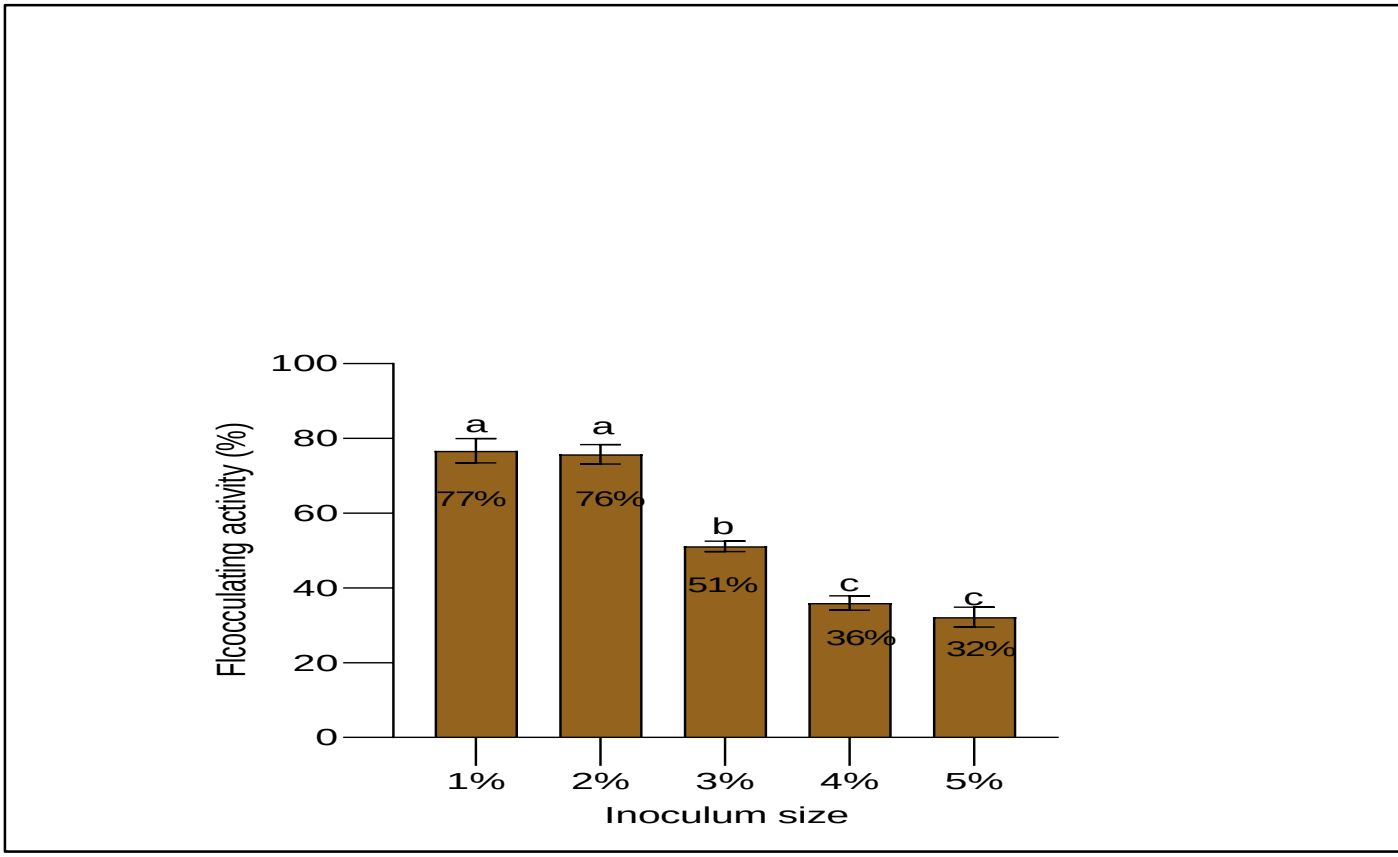



**Figure 4.1:** Effect of inoculum size on bioflocculant production by *P. mirabilis* PJC12.

The inoculum size is a great significant component in bioflocculant production and microbial cell growth (Ntsangani *et al*., 2016). A small inoculum size can prolong the lag phase, while a large inoculum size can create niches to overlap excessively, thus inhibiting bioflocculant production (Salehizadeh and Shojoasadati, 2001). A 1% (v/v) inoculum size gave the highest flocculating activity of 77%. As the inoculum size was increasing there was a decrease in flocculating activity. A 1% (v/v) inoculum size was selected as the optimum inoculum size for bioflocculant production by *P. mirabilis*

PJC12 and was used in the subsequent experiments. A similar result was reported by Guo *et al.* (2018) where 1% inoculum size was optimum for bioflocculant production by *Pseudomonas boreopolis.* Contrary to this study 4% (v/v) inoculum was the optimum inoculum size on the production of bioflocculant by *Aspergillus flavus* (Mohammed and Dagang, 2019). Different bacterials require different inoculum sizes to produce the optimum bioflocculants.

### 4.2.2 The effect of carbon sources on bioflocculant production by *P. mirabilis* PJC12

Different carbohydrates have been used as the carbon sources for microorganisms to grow and produce these microbial products. Carbon sources play a major role in microbial cell growth, since carbon sources provide energy and serve as building blocks for cell components for growth and contribute to bioflocculant production (Shahadat *et al.,* 2017). The sugars mostly used as carbon sources for bioflocculant production include glucose, sucrose, fructose, starch, maltose, lactose and other organic carbon sources (Cosa *et al*., 2012).

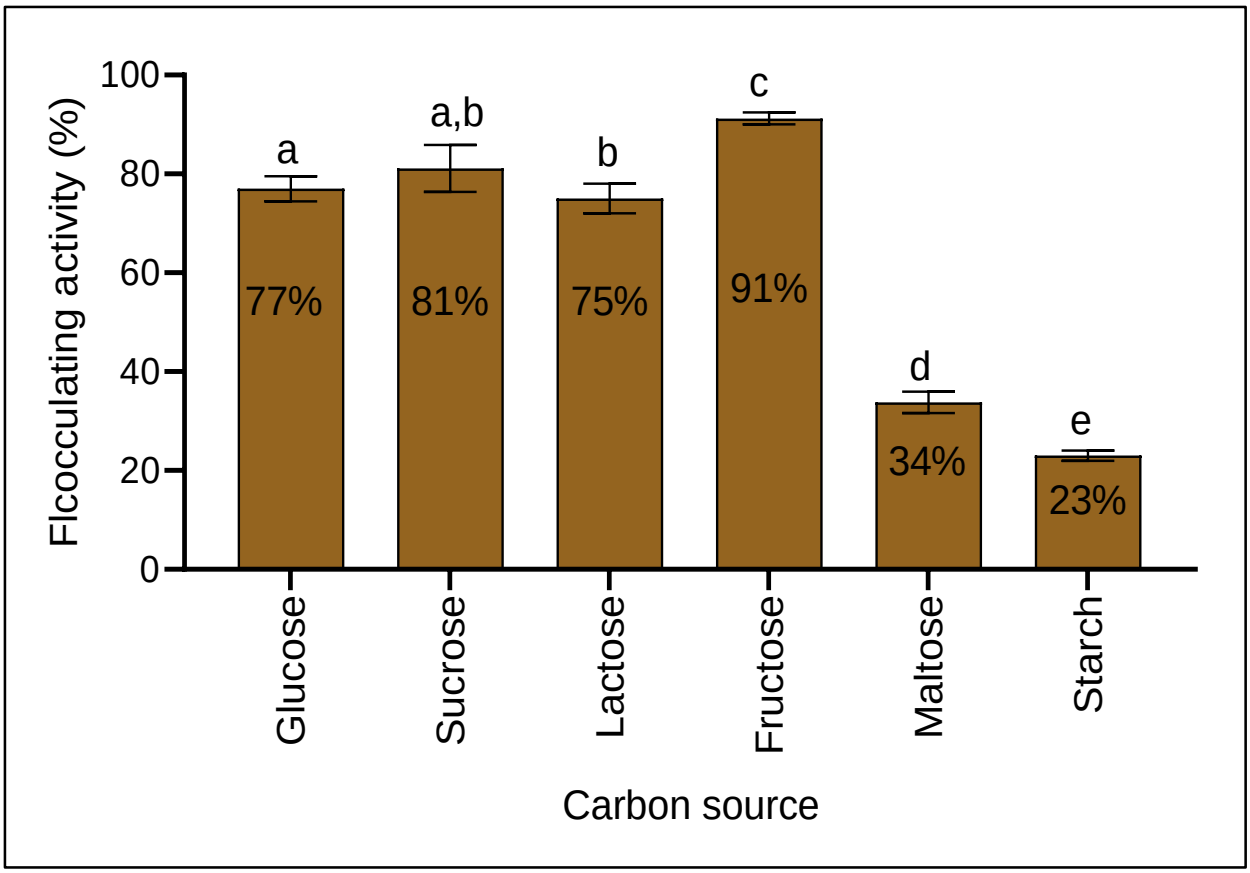



**Figure 4.2:** Effect of carbon source on bioflocculant production by *P. mirabilis* PJC12

In this study, several carbon sources were used to find a favourable carbon source for bioflocculant production by *P. mirabilis* PJC12 (Figure 4.2). Among these carbon sources, sucrose and fructose were found to be the most favourable ones for bioflocculant production by *P. mirabilis P*JC12. Fructose was used for other tests as a carbon source since it gave the highest flocculating activity of 90%, followed by sucrose with 81% (Figure 4.2). Other carbon sources were tested and had the following results: glucose (77%), lactose (30%), starch (23%) and maltose (75%). Reports show that *Paenibacillus jamilae* was found to produce a bioflocculant when sucrose was used as a carbon source (Zhong *et al*., 2018) and *Streptomyces platensis* produced an optimum yield of bioflocculant when glucose was used as sole carbon source. Cosa *et al*. (2013) also reported the glucose was more favourable for

bioflocculant production. Most of the bacterials prefer glucose as carbon source for bioflocculant production and different bacteria use different carbon sources for bioflocculant production.

### 4.2.3 The effect of nitrogen sources on bioflocculant production by *P. mirabilis* PJC12

Nitrogen sources in the medium are used to supply the proteins, nucleic acid and amino acids for microbial growth and bioflocculant production (Ugbenyen *et al*., 2012). Organic and inorganic nitrogen sources were used to prepare the production medium as solo nitrogen source, organic nitrogen source had a high bioflocculant activity compare with inorganic nitrogen sources, as shown in Figure 4.3.

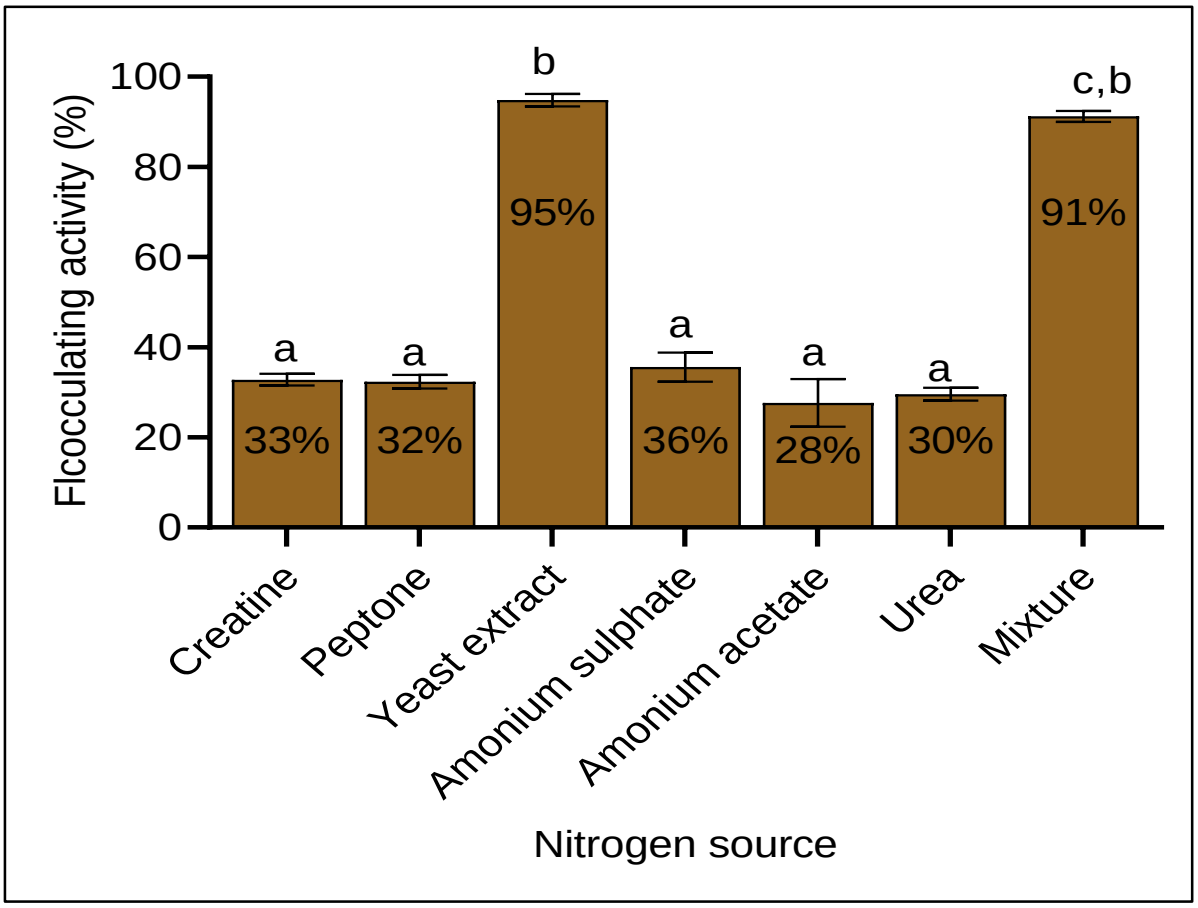


**Figure 4.3:** The effect of nitrogen sources on bioflocculant production by *P. mirabilis* PJC12.

Yeast extract was used as the solo nitrogen source for the bioflocculant production by *P. mirabilis* PJC12, with a flocculating activity of 95%. Other nitrogen sources had the following results: creatine (35%), peptone (32%), urea (36%), ammonium sulphate (28%), ammonium acetate (30%) and a mixture (yeast, urea and ammonium sulphate) (91%). Therefore, yeast was used as the nitrogen source in all subsequent tests. *Proteus mirabilis* TJ-1 isolated from active sludge had yeast extract as its nitrogen source (Xia et *al., 2008)*. In the study by Nwodo and Okoh (2012) it was observed that ammonium nitrate was the optimum nitrogen sources with flocculating activity of 83% for bioflocculant production by *Cellulonas sp* Okoh. In the report by Gong *et al.* (2008), the mixture was the most suitable nitrogen sources (yeast extract, urea and $(NH_4)_2SO_4$) for bioflocculant production.

### 4.2.4 The effect of cations on bioflocculating activity by *P. mirabilis* PJC12

Cations play an important role on the bioflocculating activity of a bioflocculant. Cations promote the bioflocculating activity of bioflocculant by neutralisation and destabilistion of negative charges of functional groups, thereby resulting in a bridge formation that binds kaolin clay particles to each other (Yim *et al*., 2007). The flocculating activities were $MnCl_2$ (54%), $CaCl_2$ (95%), $FeCl_3$ (33%), HgCl (29%), LiCl (39%), KCl (40%), NaCl (40%) and $BaCl_2$ (94%) with $BaCl_2$ had the highest bioflocculating activity (Figure 4.4). Therefore BaCl2 was used subsequently in the next experiments since it was the optimal cation. Maliehe *et al.* (2016) observed the same result on bioflocculating activity (96%) *Bacillus pumilus* JX860616, where the cation BaCl2 greatly improved the bioflocculant production. Salehizadeh *et al.* (2000) observed that the addition of $Al^{3+}$, $FeCl_3$ and $CaCl_2$ elevated flocculating activity of the bioflocculant produced by

*Bacilus* sp.

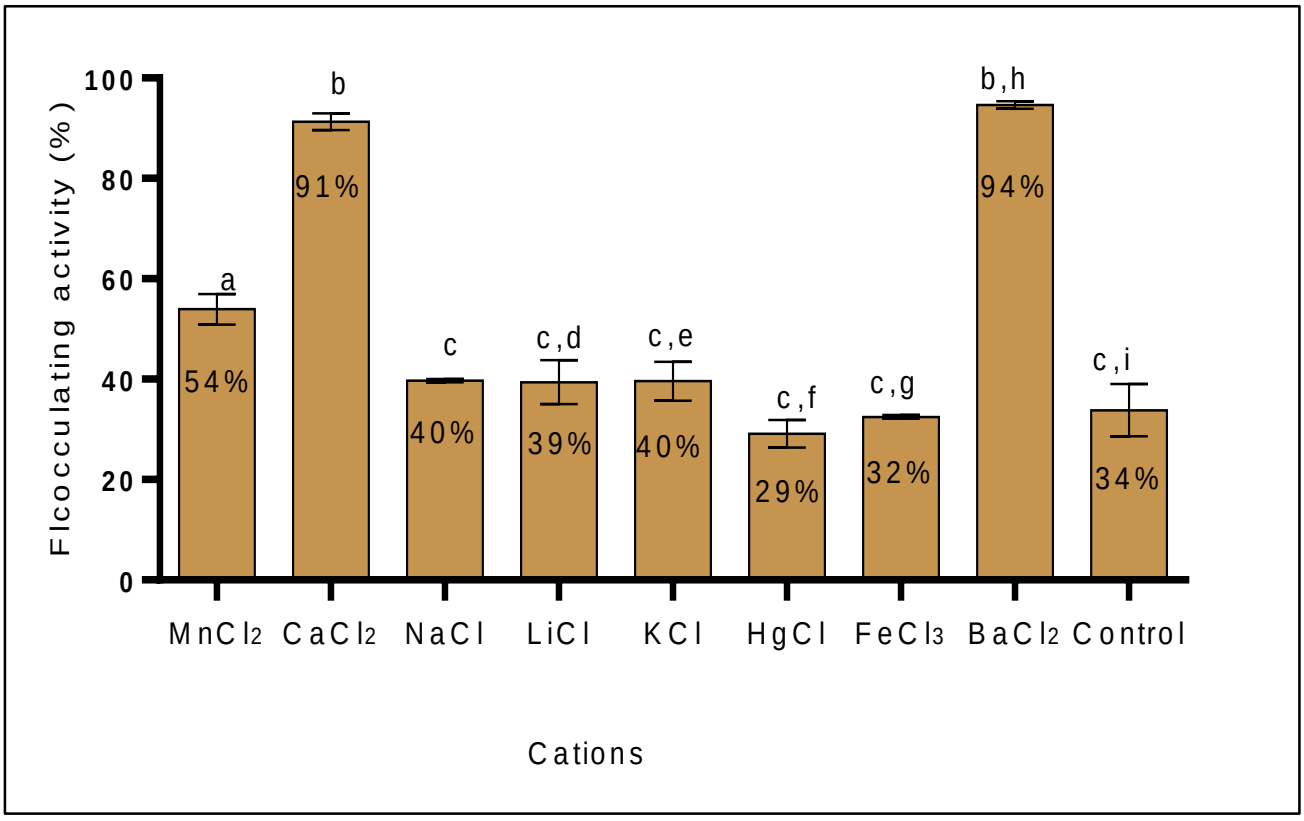



**Figure 4.4:** The effect of cations on bioflocculating activity by *P. mirabilis* PJC12

### 4.2.5 The effect of initial pH of the production medium on bioflocculant production by *P. mirabilis* PJC12

The initial pH of production medium for bioflocculant differs with different microorganisms (Salehizadeh and Yan, 2014). The effect of initial pH of the production medium was examined at a pH range of 3 -12 and the results are depicted in Figure 4.5. In this study there was an increase in bioflocculant production from pH 3 until pH 6. At pH 3 the flocculating activity (44%) started to increase with an increase in pH until the optimum pH 6 with flocculating activity of 87% was reached. Further increase in pH resulted in a decrease in flocculating activity by *P. mirabilis* PJC12. Alkaline pH and strong acidic pH were unfavourable for bioflocculant production by *P. mirabilis* PJC12. Therefore, pH 6 was the optimal pH and was used in the entire following tests.

In a study by Ugbenyen *et al.* (2012) the same result was observed on the bioflocculant production by *Cobetia* sp.

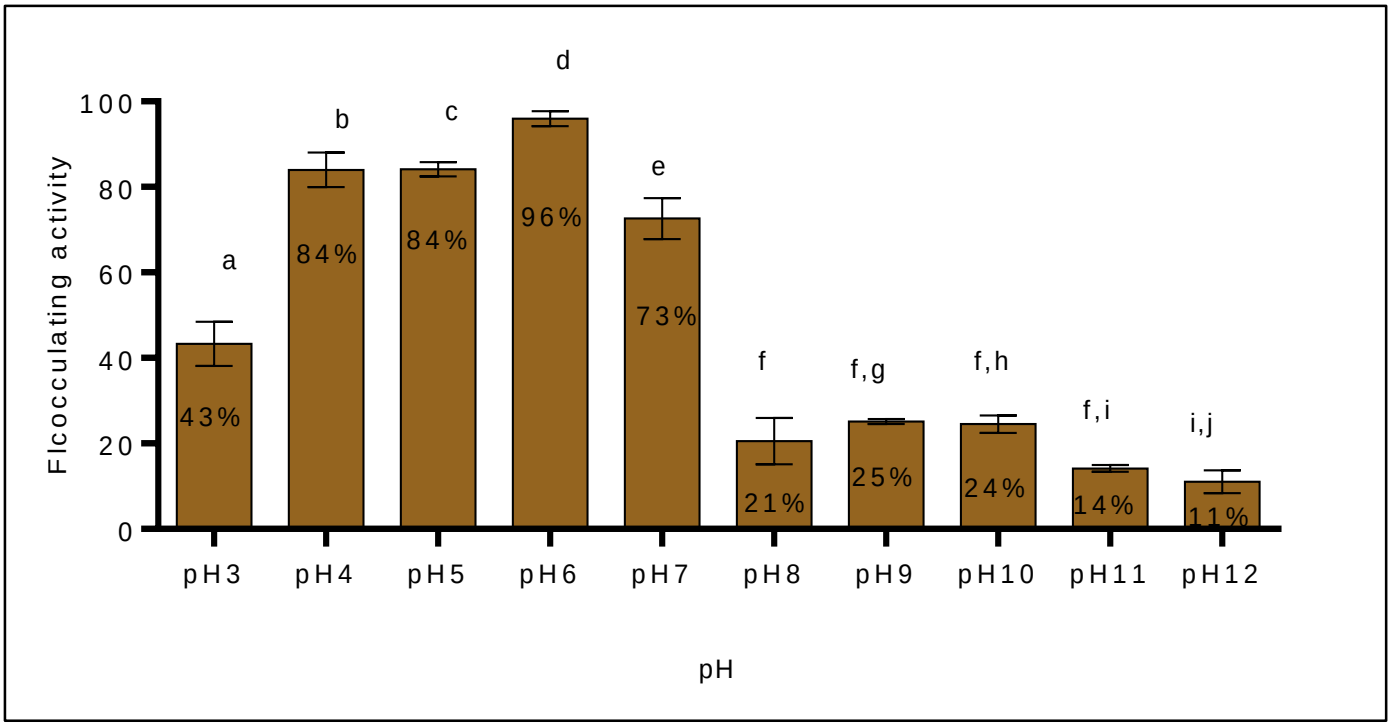



**Figure 4.5:** The effect of initial pH of production medium for bioflocculant production by *P. mirabilis* PJC12.

### 4.2.6 Effect of shaking speed on bioflocculant production by *P. mirabilis* PJC12

To evaluate the effect of shaking speed on the bioflocculant production by *P. mirabilis* PJC12, a shaking speed range of 60 - 220 rpm was used. The effect of agitation on bioflocculant production is shown in Figure 4.6.

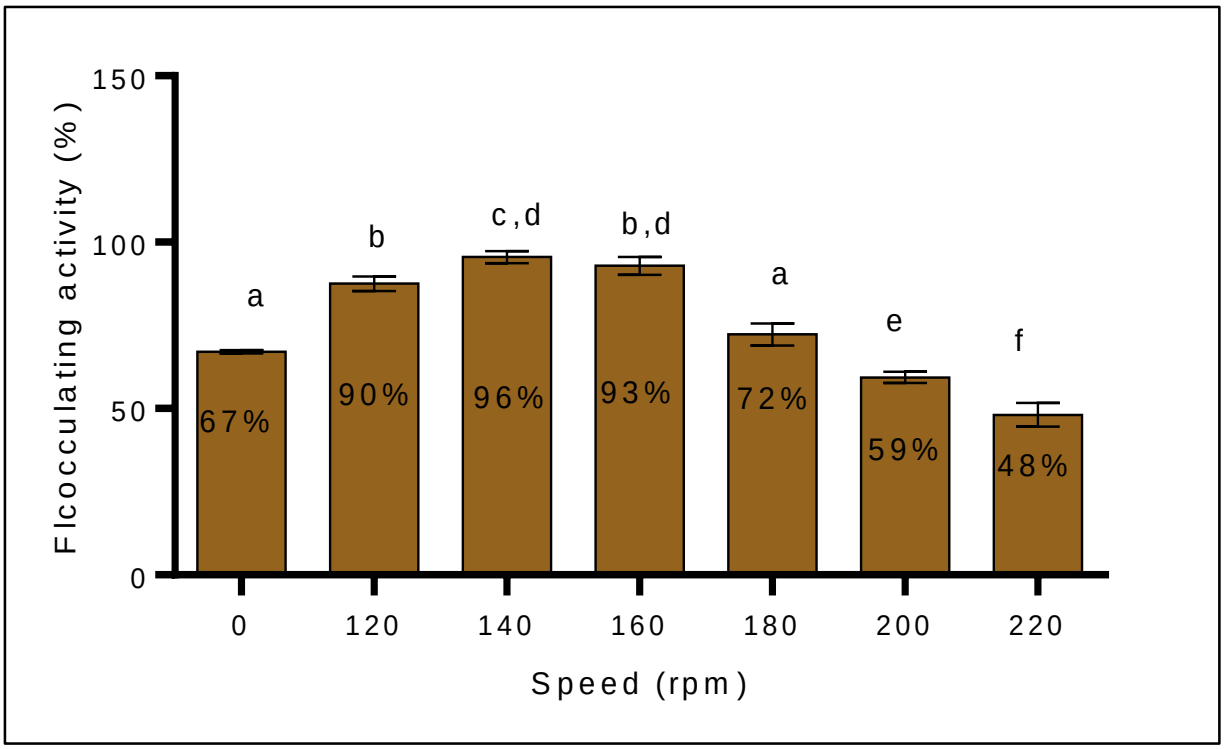



**Figure 4.6:** Effect of agitation on bioflocculant production by *P. mirabilis* PJC12

The optimum shaking speed was found to be 140 rpm, with a bioflocculating activity of 93%. The bioflocculating activity increased from 60 -140 rpm with the higher shaking speeds of 140 rpm, while the shaking speeds above 140 rpm resulted in all subsequent decreases in flocculating activity. The shaking speed of 140 rpm was the optimum shaking speed and was used in all subsequent experiments. Shaking speed has a great influence on dissolving oxygen concentration, which can also have an effect on distribution and absorption of nutrients and enzymatic reactions (Zayed *et al*., 2018). The study done by Adebayo-Tayo and Adebami (2017) showed similar result at a shaking speed of 140 rpm. Li *et al.* (2010) reported that a shaking speed of 140-160 rpm was favourable for bioflocculant production by *Bacillus licheniformis* X14.

### 4.2.7 The effect of incubation temperature on bioflocculant production by *P. mirabilis* PJC12

Several previous studies have indicated the influence of cultivation temperature on the bioflocculant production and also on the flocculation process by microorganisms (Ntombela *et al.,* 2019). The effect of cultivation temperature on bioflocculant production ranging from 25 to 55℃ was evaluated and the resulted are depicted in Figure 4.7.

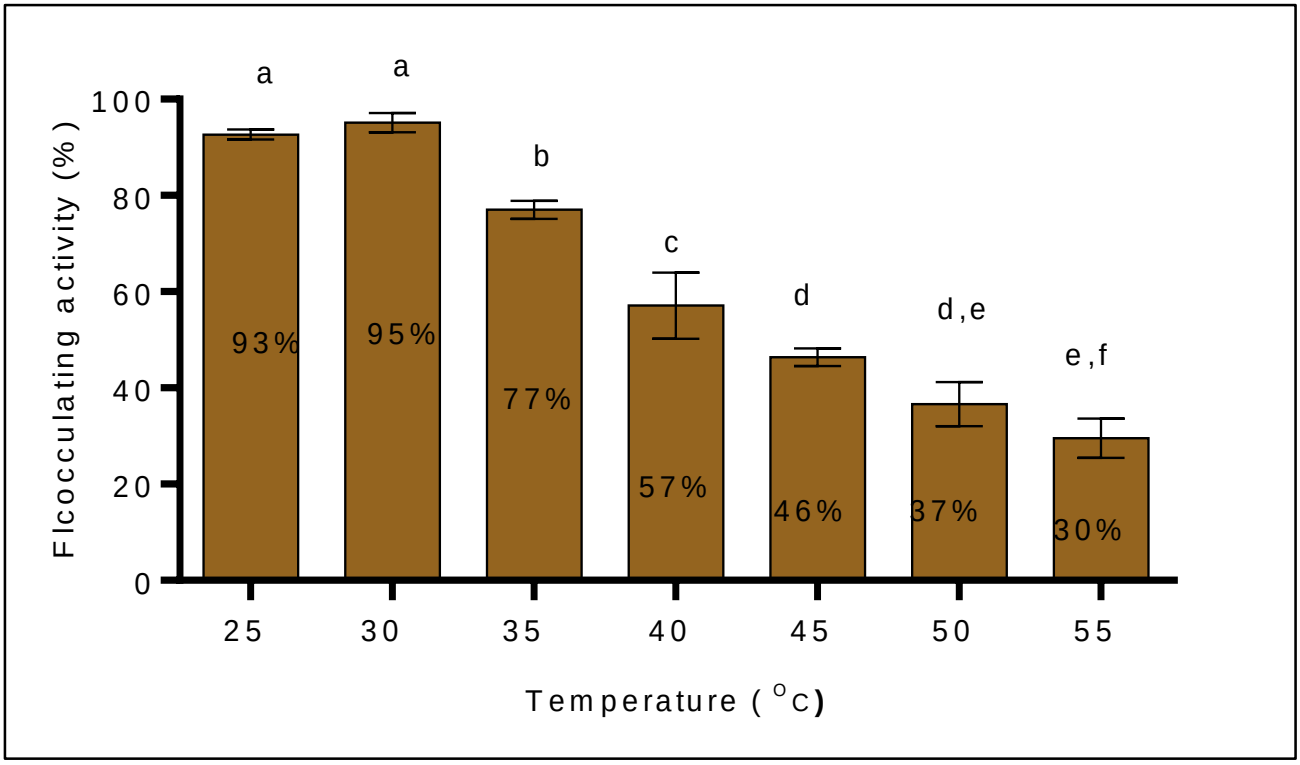



**Figure 4.7:** The effect of incubation temperature on bioflocculant production by *P. mirabilis* PJC12.

The flocculating activity of *P. mirabilis* PJC12 increased from the temperature of 25 (96%) to 30 ℃ (98%) (Figure 4.7). Therefore 30 ℃ was the optimum cultivation temperature with the highest flocculating activity of 98% and was used in the following subsequence of experiments. A further increase of cultivation temperature above 30 ℃ resulted in a decrease of flocculating activity. In the studies by Adebayo-Tayo and

Adebami (2017) and Nakata and Kurane (1994) similar results were obtained where the bioflocculants were optimally produced at 30 ℃ by *Aspergillus aqualitis* and *Citrobacter sp.* TKF04, respectively. Ntombela *et al.* (2020) reported the optimum production of bioflocculant by *Bacillus safensis* at a temperature of 40 ℃.

### 4.2.8 Time course assay of the bioflocculant production by *P. mirabilis* PJC12

The time course assay of bioflocculant produced by *P. mirabilis* PJC12 was studied, where the interrelationship between bioflocculating activity, cell growth and pH were evaluated over a period of 120 hrs (Figure 4.8). The bioflocculating activity, cell growth (optical density) and pH were evaluated at 12 hrs intervals.

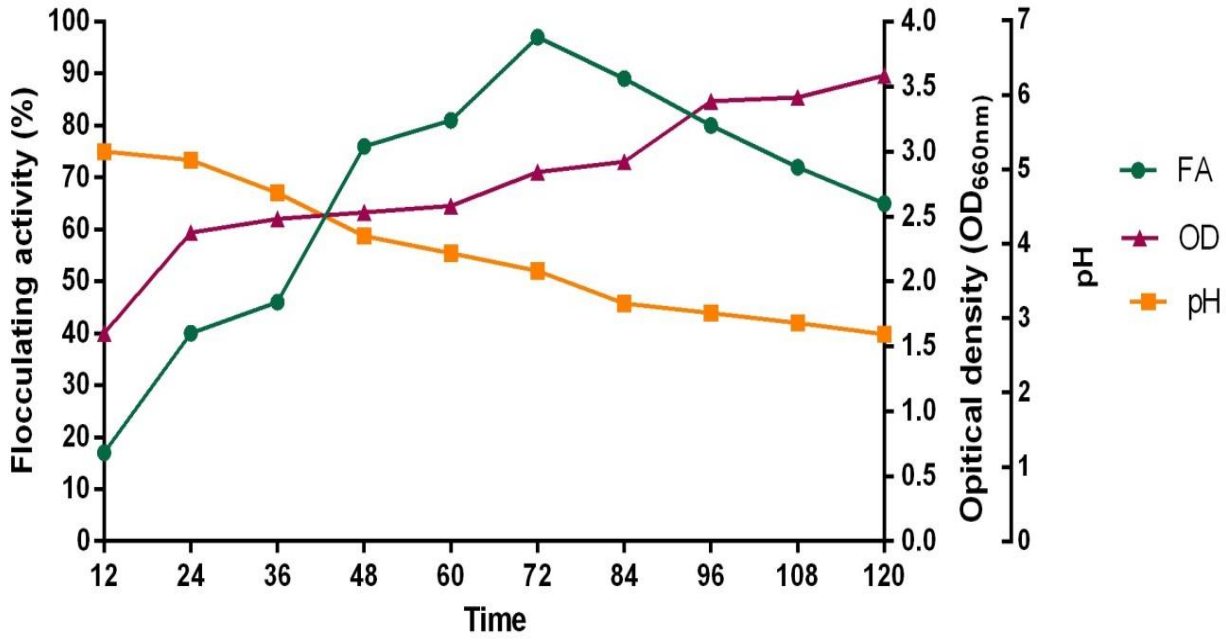



**Figure 4.8:** Time course assay of the bioflocculant production by *P. mirabilis* PJC12

The time course assay for bioflocculant production was evaluated as shown in Figure 4.8. The bioflocculating activity was initially observed to increase gradually from 12 hrs of incubation to 72 hrs. Maximum bioflocculant production was observed at an incubation time of 72 hrs with bioflocculating activity of 97% and, thereafter, a slight decrease and fluctuation in bioflocculating activity was observed. The pH relatively

declined throughout the incubation time. The pH declined from pH 5 to pH 3 over 120 hrs. This indicates that the organism produces acid waste material. The decrease of pH confirms the degradation of the fructose and yeast in the medium by *P. mirabilis* PJC12 (Piyo *et al.,* 2011) or the presence of organic acid components in the bioflocculant being produced (Okaiyeto *et al.,* 2016).

The optical density was used as a measure component for cell multiplication of *P. mirabilis* PJC12 in the production medium. The $OD_{660nm}$ profile showed that the cells rapidly increased for the period of the 120 hrs and optimum bioflocculant production at 72 hrs. The bioflocculant production increased in proportion to the cell concentration increase from 12 to 72 hrs of incubation. This suggests that the bioflocculant was produced by a biosynthesis process (Gong *et al.,* 2008). Flocculating activity after 72 hrs decreased and could be due to cell autolysis. This decrease in flocculating activity could be due to production of bioflocculant degrading enzymes by the bacterium which thought to degrade the bioflocculant (Mabinya *et al.,* 2011). In the study by Zayed *et al*. (2018), maximum bioflocculant was produced at 72 hrs by *Bacillus salmalaya* 139SL. Cosa *et al.* (2011) reported that *Virgibacillus sp*. produced optimum bioflocculant after 96 hrs of incubation.

## 4.3 Extraction and purification of the bioflocculant

The bioflocculant produced by *P. mirabilis* PJC12 was 2.75 g after extraction and purification from 1L of fermentation production medium. This yield seems to be excellent compared to other yields produced by different microorganisms listed in Table 4.1, but is much lower than the 15 g/L produced by a multiple microorganism's consortium reported by Zhang *et al.* (2007).

Table 4.1: Bioflocculants from different microorganisms

| Microorganism | Yield (g/L) | Citation |
|---|---|---|
| *Micrococcus* sp | 0.738 | (Okaiyeto *et al.,* 2014) |
| *Klebsiella* sp | 1.8 | (Yang *et al*., 2012) |
| *Bacillus* safensis | 2.082 | (Ntombela *et al.,* 2020) |

## 4.4 Chemical composition of the pure bioflocculant by *P. mirabilis* PJC12

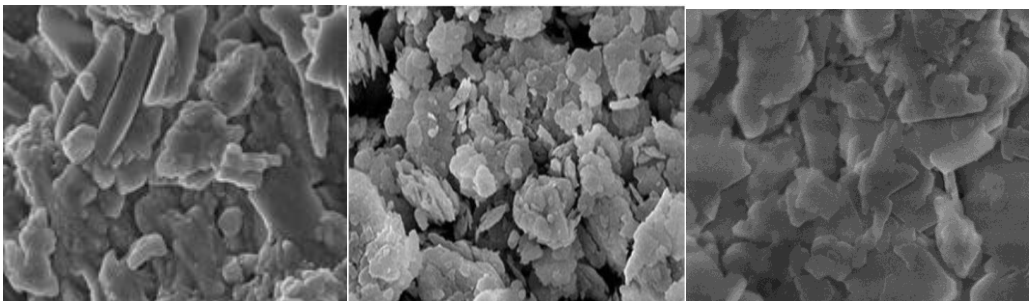
It is important to determine the components of the pure bioflocculant because they determine the flocculation mechanisms (Li *et al.,* 2016). Determination of bioflocculant flocculating mechanisms plays an important role in the improvement of removal efficiency in practical application. In this study, the chemical composition of bioflocculant by *P. mirabilis* PJC12 was evaluated and revealed 69% of total sugar content, 27% of uronic acid content and 10% of protein content. In this study bioflocculant had a high sugar content which could be described as mainly polysaccharides, hence the thermostable. In a study by Maliehe *et al*. (2016), the bioflocculant produced by *Bacillus pumilus* was composed of 5% sugar content, 26% protein content and 69% uronic acid content. Deng *et al.* (2005) reported the bioflocculant that was composed of 66.1% of sugar content and 29.3% of protein content.

## 4.5 Morphology structure and elemental analysis of a bioflocculant

### 4.5.1 SEM analysis

SEM analysis was used to elucidate the surface morphology images of the bioflocculant at high-energy beam of electrons. The signal containing information

about the bioflocculant surface was revealed as the electrons interact with the atoms of the bioflocculant (Akapo *et al.,* 2019). In this study the surface morphology of pure bioflocculant by *P. mirabilis* PJC12 was evaluated with its flocculated kaolin clay by SEM (Figure 4.9). Figure 4.9 shows the surface images of pure bioflocculant (A), kaolin clay particles before flocculation (B) and flocculated kaolin clay particle (C). The SEM image of the pure bioflocculant (Figure 4.9a) showed crystal-like bioflocculant with tubular shape biopolymer-like structure. The kaolin clay particles in Figure 4.9(b) appeared to be scattered tiny molecules. The flocculated kaolin image (Figure 4.9c) revealed the particles becoming bigger compared to Figure 4.9a and 4.9b. This show that bioflocculant and kaolin particles interacted to form big floc, which results in flocculation due to gravity (Akapo *et al*., 2019). These observations were consistent with the findings of Okaiyeto *et al.* (2016) on glycoprotein REG-6 bioflocculant produced from *Bacillus toyonensis* AEMREG6**.**



A B C

**Figure 4.9:** SEM surface image of the pure bioflocculant (A), kaolin particles (B) and flocculated kaolin particles (C).

### 4.5.2 Elemental analysis (SEM-EDX)

The SEM-EDX is used to perform elemental analysis; therefore, in this study it was used to evaluate the element present in the pure bioflocculant produced by *P. mirabilis* PJC12. The SEM-EDX showed the presence of elements such as C, N, O, Na, Mg,

Al, P, S, Cl, K and Ca in the percentage of 17.51%, 0.51, 45.17%, 0.44%, 4.62%, 0,47%, 14,29%, 0,77%, 1.10%, 1.46% and 13.24% (w/t) respectively. The elemental composition reveals the presence of polysaccharides as a backbone in the pure bioflocculant (Pathak *et al.,* 2017). The FTIR analysis spectrum also showed the presence of amine, carboxyl, hydroxyl and carbanol.

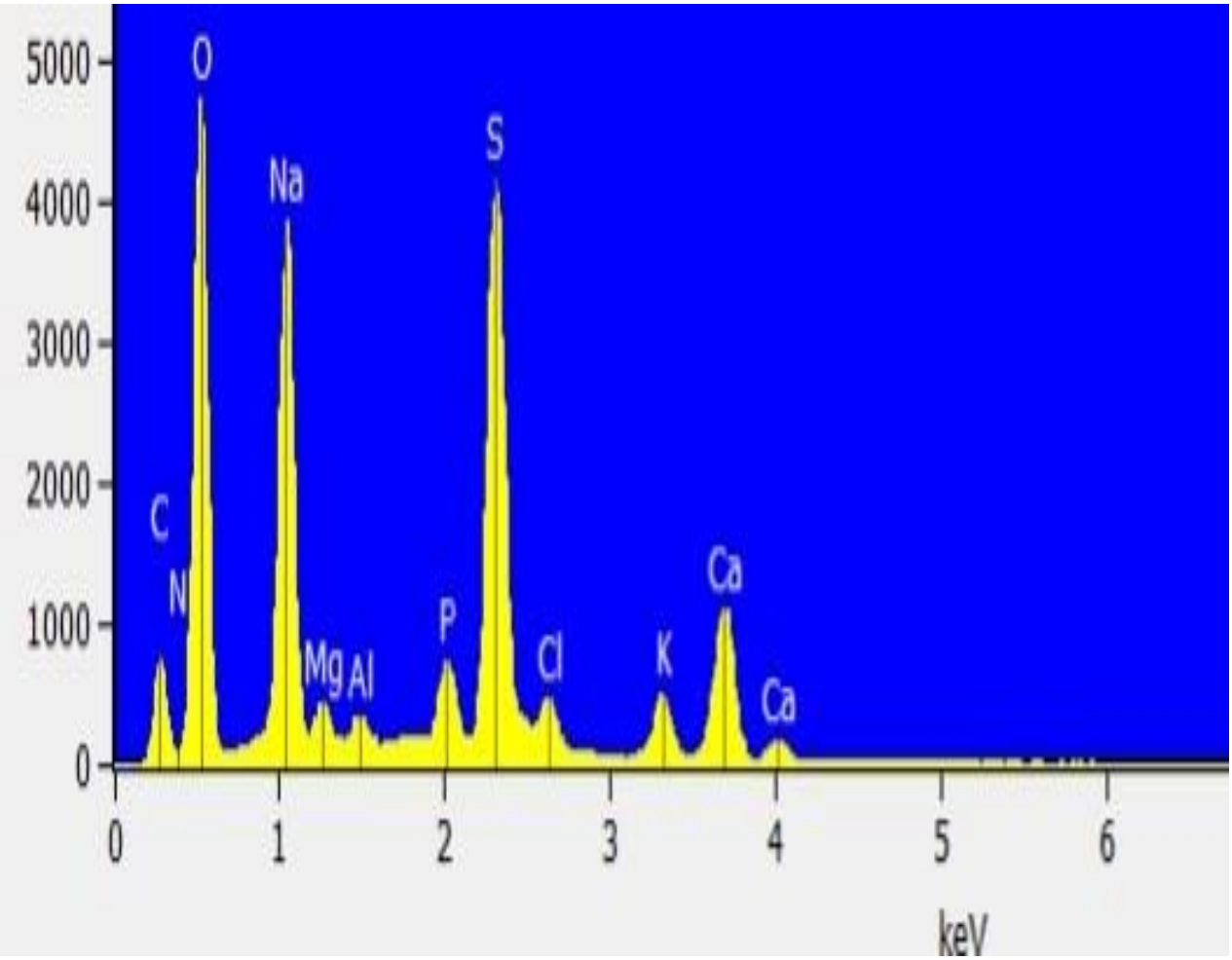



**Figure 4.10:** Elements present in the pure bioflocculant produced by *P. mirabilis* PJC12

Table 4.2: Elements present in the pure bioflocculant with molecular weight

| Elements | Weight (%) | Atom (%) |
|---|---|---|
| C | 17.51 | 27.01 |
| N | 0.51 | 0.49 |

| | | |
|---|---|---|
| O | 45.17 | 52.11 |
| Na | 0.44 | 0.35 |
| Mg | 4.62 | 3.40 |
| Al | 0.47 | 0.32 |
| P | 14.29 | 8.91 |
| S | 0.77 | 0.34 |
| Cl | 1.10 | 0.57 |
| K | 1.31 | 6.40 |
| Ca | 13.24 | 6.09 |
| Total | 100.00 | 100.00 |

## 4.6 Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectrum was used to evaluate the presence of functional groups in the molecular of the pure bioflocculant. The stretching weak peak at 3251 $cm^{-1}$ indicated the hydroxyl group (Li *et al.,* (2009) and the presence of hydroxyl group within the bioflocculant favoured the hydrogen bonding with one or more water molecules. The bioflocculants with the hydroxyl group in its molecular chain exhibits high solubility in an aqueous solution (Desouky *et al.,* 2008). The stretching peak noted at 1645 $cm^{-1}$ indicated a carbonyl group and amide group (Cosa *et al.,* 2011; Yin *et al*., 2014). The presence of the absorption peak at the range of 1000- 1200 $cm^{-1}$ is the characteristic of all sugar derivates (Li *et al.,* 2008). The presence of the peak at the 500 $cm^{-1}$ indicates the functional group alkyl halide. The FT-IR analysis of pure bioflocculant of *P. mirabilis* PJC12 revealed the presence of carbonyl, amine, carboxyl and hydroxyl functional groups which might be responsible for the bioflocculation. (See Figure 4.11) These functional groups result in the floc formation, since these functional groups serve as the binding site for suspended particles (Freitas *et al.,* 2009). Other

researchers also presented similar results Elkady *et al.* (2011), Nwodo and Okoh, (2012) and Guo *et al.* (2015) and also similar to this study.

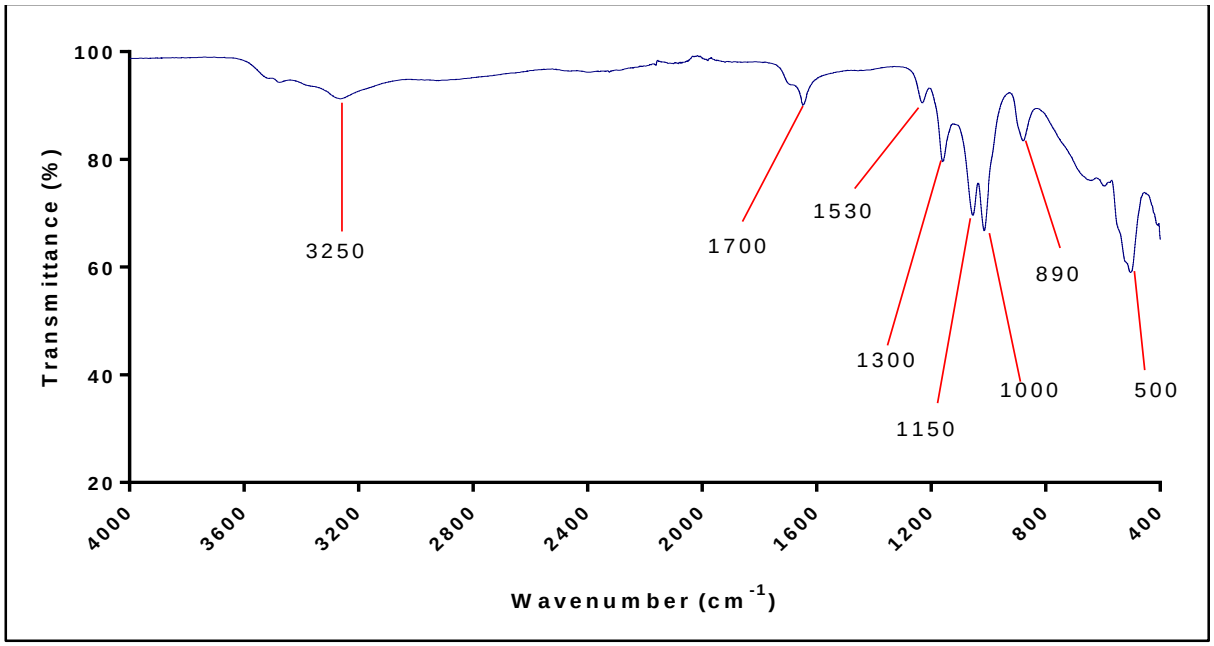



**Figure 4.11:** FT-IR analysis for pure bioflocculant produced by *P. mirabilis* PJC12

## 4.7 Thermo-gravimetric analysis of pure bioflocculant

To study the pyrolysis property of the pure bioflocculant produced by *P. mirabilis* PJC12, the thermogravimetric analyser was used, and the results are shown in Figure 4.11. The bioflocculant was heated from 30 °C to 900 °C at the constant rate of 10 °C $min^{-1}$ under constant flow of nitrogen gas. The curve in Figure 4.11 showed that between 30 $^{o}$C and 100 $^{o}$C the bioflocculant lost 2.5% of its weight. At 50 $^{o}$C the bioflocculant had 99.80% weight and was still intact. Interestingly, the bioflocculant lost 13% of its total weight when exposed up to 200 $^{o}$C. This shows that the bioflocculant was very stable even at high temperatures. The bioflocculant has shown high flocculating activity of 70% at 100 $^{o}$C (See Figure 4.15).

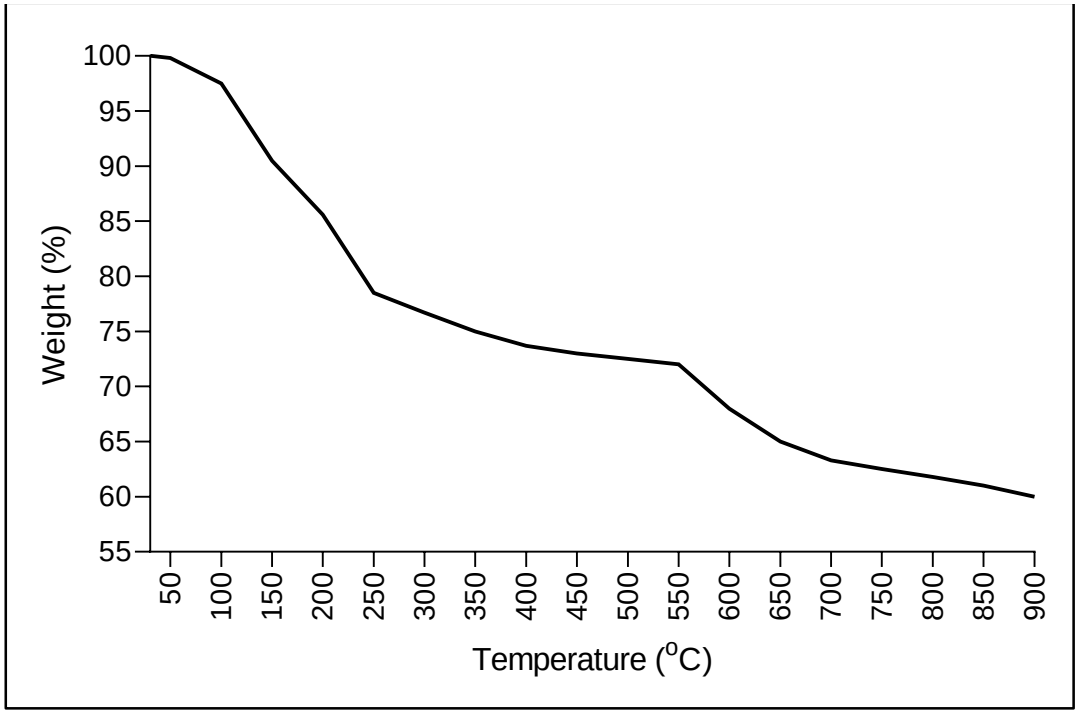



**Figure 4.12:** Thermogravimetric analysis of pure bioflocculant produced by *P. mirabilis* PJC12

## 4.8 Optimisation of conditions for flocculating activity of the pure bioflocculant

Optimisation of conditions for flocculating activity plays an important role in the application of the pure bioflocculant industrially. This helps investigate the most suitable conditions for the bioflocculants which plays an important role in achieving the great flocculation process and great removal efficiency of pollutants by the bioflocculant.

### 4.8.1 Effect of a dosage size on bioflocculating activity of pure bioflocculant by *P. mirabilis* PJC12

The optimum dosage size of the bioflocculant plays an important role in flocculating activity and minimising the cost (Ogunsade *et al.,* 2015). The effect of a dosage size

on the bioflocculating activity of the pure bioflocculant was evaluated. The optimum dosage size was determined by testing different bioflocculant concentrations within a range of 0.2-1.0 mg/mL (Figure 4.12).

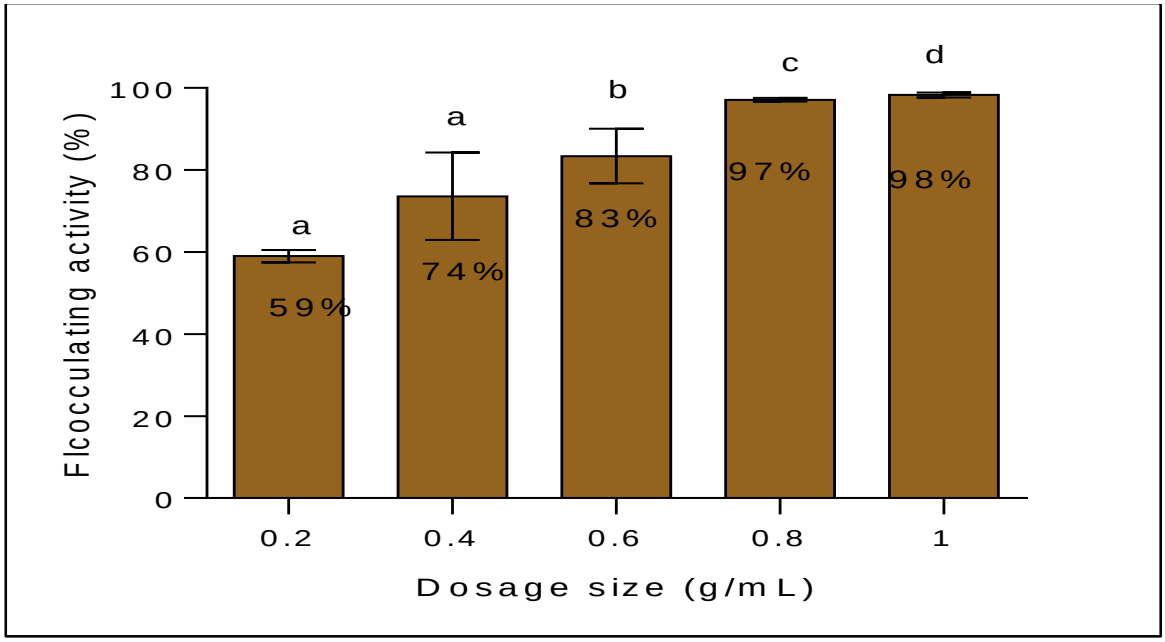



**Figure 4.13:** Effect of dosage size on flocculating activity of pure bioflocculant by *P. mirabilis* PJC12

If the bioflocculant dosage size increases the flocculating activity also increases. Above 0.8 mg/mL dosage concentration was reached. The lower or higher dosages decreased the bioflocculant efficiencies (Liu *et al*., 2014). Due to less dosage size, complete saturation between metal ion, bioflocculant and kaolin clay particles does not occur to produce maximal performance of the bioflocculant. Higher dosage size results in the blockage of adsorption sites and this reduces the flocs' formation (Zufarzaana *et al*., 2012). In this study, the optimal dosage size was observed at the concentration of 0.8 mg/mL with flocculating activity of 89%. The low flocculating activity below 0.8 mg/mL may be due to less flocs' formation and decreased

flocculating activity after 0.8 mg/mL may be caused by flocculation deterioration (Liang *et al.,* 2011).

Similar results were observed by Agunbiade *et al*. (2017), where the optimum dosage size 0.8 mg/mL with flocculating activity of 98% on the bioflocculant was produced by bioflocculant *Arthrobacter humicola.* In a study by Abu-Elreesh *et al.* (2011) the optimum dosage size of 0.13 mg/mL was observed. Bioflocculants by different microorganisms have different optimum dosage sizes to give maximum flocculating activities.

### 4.8.2 Effect of cations on the flocculating activity of the pure bioflocculant

Bioflocculant is a polymer which is usually negatively charged; this property limits the bioflocculant application in water treatment because water pollutants are usually negatively charged as well (Huang *et al.,* 2014). Therefore, in order to apply bioflocculants, researchers have used a combination of bioflocculant and metal ions to promote bioflocculating activity (Elkady *et al*., 2011). The cations are used as coagulant aid to achieve high bioflocculating activity by neutralising and stabilising the negatively charged functional groups on the bioflocculant and suspended particles thereby increasing the adsorption of the bioflocculant to the suspended particles (Mabinya *et al.,* 2011). In this study the following cations were used: $BaCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$, LiCl, NaCl and KCl. They were used to examine the effect of cations on the flocculating activity of the pure bioflocculant by *P. mirabilis* PJC12. $BaCl_2$ was the most effective cations for bioflocculation (98%). Similar results were reported by Ntombela (2019) on bioflocculant produced by *Bacilus* sp.

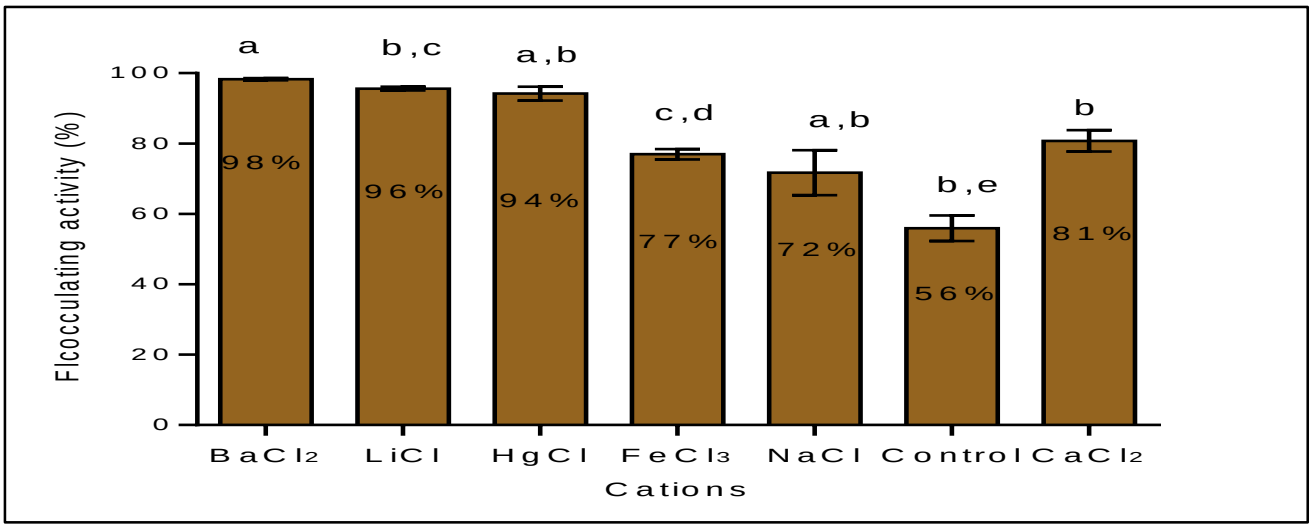



**Figure 4.14:** Effect of cation on flocculating activity pure bioflocculant produced by *P. mirabilis* PJC12.

### 4.8.3 Effect of pH on the flocculating activity of the pure bioflocculant

The pH plays a critical role in the bioflocculating activity of the bioflocculants. It affects the flocs' formation and stability of the suspended particles (Maliehe *et al*., 2016).

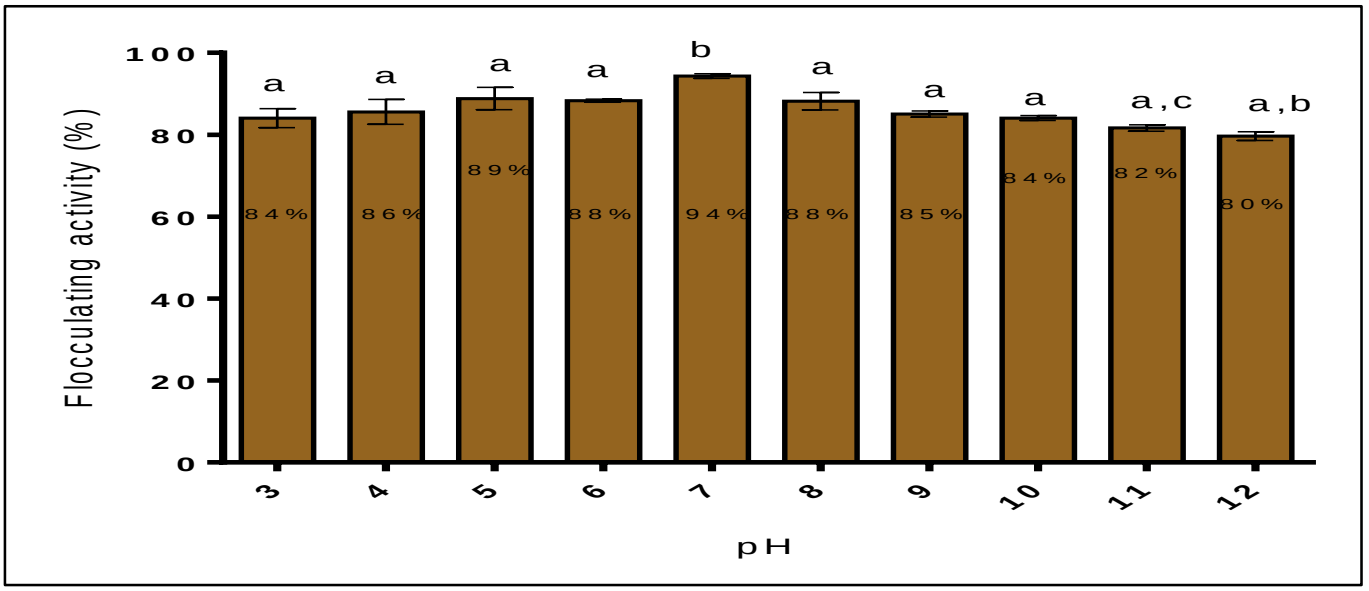



**Figure 4.15:** Effect of pH on flocculating activity of the pure bioflocculant produced *P. mirabilis* PJC12.

The results in Figure 4.15 show stability in pH of the bioflocculant from 3 - 7 with the bioflocculating activity ranging from 80 to 96%. Additionally, the results show a slight decrease in bioflocculating activity in an alkaline in pH range of 8 - 12. Therefore, bioflocculant by *P. mirabilis* PJC12 functioned optimally over a wide pH range of 3-12 with the maximum bioflocculating activity of 96% observed at pH 7. Similar results were observed by He *et al*. (2010) on a bioflocculant from *Halomonas sp.,* where the maximum bioflocculating activity was achieved at a neutral pH (7). In the study by Abd EI-Salam *et al.* (2017), the maximum flocculating activity was observed at acidic pH (2) by the bioflocculant produced by *Bacillus aryabhattai*. In contrast, in a study done by Li *et al.* (2016) it was observed that the bioflocculant produced by *Shigella albus* had an optimal flocculating activity at a pH of 10.

### 4.8.4 Effect of temperature on pure bioflocculant by *P. mirabilis* PJC12

In the study Wu and Ye (2007) reports show that the bioflocculants with a high concentration of polysaccharides cause thermal stability, as compared to the bioflocculants containing high protein concentrations, which are usually heat labile. If the bioflocculant is composed of glycoprotein, its thermal stability depends on the content of protein and polysaccharides. The bioflocculant by *P. mirabilis* PJC12 had high thermal stability within a range of 50 - 90 °C with flocculating activity > 75% (Figure 4.16). It was observed that the bioflocculant was thermo-stable as it retained high flocculating activity (80%) at 100 °C. The thermal stability of the bioflocculant might be due to the main backbone of the bioflocculant polysaccharides. Similar results were reported by Makapela *et al*. (2018) and Maliehe *et al*. (2016) where the bioflocculant retained more than 80% flocculating activities at 100 °C.

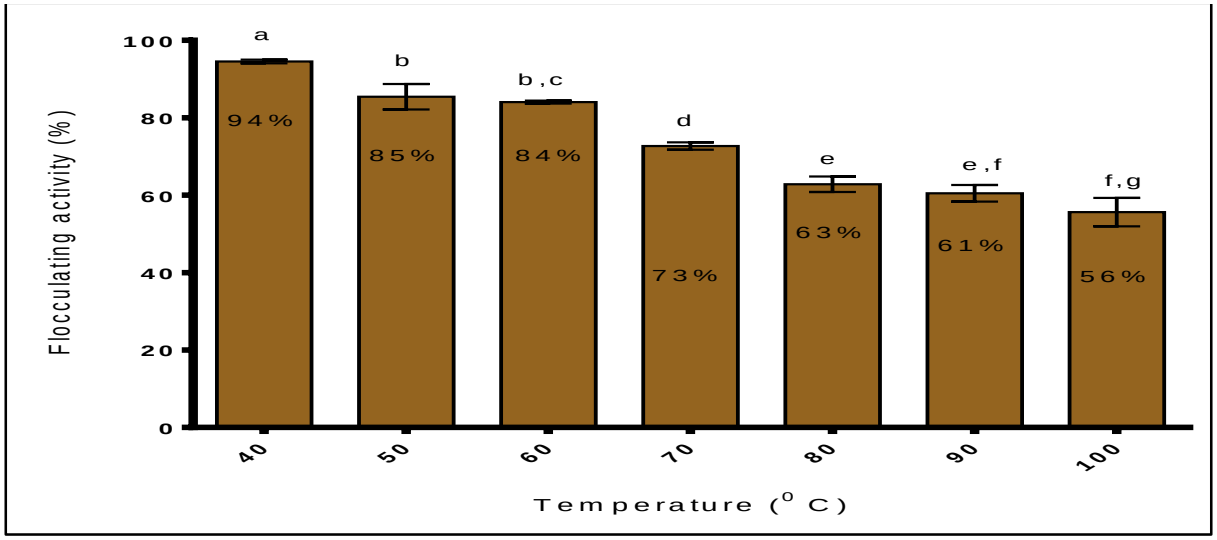



**Figure 4.16:** Effect of temperature on pure bioflocculant produced by *P. mirabilis* PJC12

## 4.9 Application of pure bioflocculant in wastewater treatment in comparison with chemical flocculants

The removal efficiencies of pure bioflocculant were compared with those of conventional flocculants (ferric chloride and polyacrylamide). Both bioflocculant and conventional flocculants were tested against Tendele coal mine (mine industrial) wastewater and Vulindlela (domestic) wastewater.

The wastewaters were tested separately with the following parameters=chemical oxygen demand (COD), biological oxygen demand (BOD), sulphur(S), aluminium (Al), phosphate (P) and calcium (Ca) and their removal efficiencies were also calculated.

Table 4.3: Removal efficiency of pure bioflocculant produced by *P. mirabilis* PJC12 in the treatment of Vulindlela (domestic) wastewater

| **Flocculant** | | **COD** | **BOD** | **Ca** | **P** | **S** | **Al** |
|---|---|---|---|---|---|---|---|
| Bioflocculant | Before | 1250 | 91,8 | 9.30 | 0.98 | 26.67 | 0.14 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | After | 175 | 8.3 | 2.4 | 0.31 | 5.6 | - |
| | Removal efficiency (%) | 86 | 91 | 78 | 82 | 79 | - |
| Ferric chloride | Before | 1250 | 91,8 | 9.30 | 0.98 | 26.67 | 0.14 |
| | After | 263 | 15.6 | 1.55 | 0.26 | 8.54 | - |
| | Removal efficiency (%) | 79 | 83 | 66 | 73 | 68 | - |
| Polyacrylamide | Before | 1250 | 91.8 | 9.30 | 0.98 | 26.67 | - |
| | After | 200 | 11.9 | 1.4 | 0.12 | 7.21 | 0.14 |
| | Removal efficiency (%) | 84 | 87 | 85 | 88 | 73 | - |

Table 4.4: Removal efficiency of pure bioflocculant produced by *P. mirabilis* PJC12

| Flocculants | | **COD** | **BOD** | **Ca** | **P** | **S** | **Al** |
|---|---|---|---|---|---|---|---|
| Bioflocculant | Before | 2155 | 87.3 | 9.93 | 1.54 | 36 | 0.173 |
| | After | 215.5 | 6.11 | 1.99 | 0.17 | 5.4 | - |
| | Removal efficiency (%) | 90 | 93 | 80 | 89 | 85 | - |
| Ferric chloride | Before | 2155 | 87.3 | 9.93 | 1.54 | 36 | 0.173 |
| | After | 366 | 13.10 | 2.38 | 0.45 | 2.52 | - |
| | Removal efficiency (%) | 83 | 85 | 76 | 71 | 93 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Polyacrylamide | Before | 2155 | 87.3 | 9.93 | 1.54 | 36 | 0.173 |
| | After | 345 | 11.34 | 1.89 | 0.23 | 2.16 | - |
| | Removal efficiency (%) | 84 | 87 | 81 | 85 | 94 | - |

Table 4.4 shows the removal efficiency of bioflocculant produced by *P. mirabilis* PJC12 on COD, BOD, Ca, P, S and Al in comparison to polyacrylamide and ferric chloride. Both bioflocculant and chemical flocculants showed comparable COD removal efficiency of above 75% on Tendele coal mine wastewater and Vulindlela wastewater. The BOD removal efficiencies for bioflocculant and chemical flocculants were all above 80% on Tendele coal mine and Vulindlela wastewater. The above results of COD and BOD removal efficiency of bioflocculant had significance differences when compared to those of the chemical flocculant.

The calcium, phosphate and sulphate contents showed high removal efficiency above 65% irrespective of the flocculants used on Tendele coal mine and Vulindlela wastewater. Aluminium concentration after treatment was too low to be determined for both Tendele coal mine and Vulindlela treatment.

# CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

The use of chemical flocculants in wastewater treatments have been reported to cause environmental and human health problems. Chemical flocculants are also not biodegradable. The bioflocculants have been reported to be alternatives for chemical flocculants because of their biodegradability properties. The bioflocculants have gained wide attention in the water treatment fields because they are eco-friendly and harmless to humans and the environment. Bioflocculants are produced by microorganisms during their growth phases as slime layers.

In the present study the bacterium isolated from Tendele coal mine was screened for bioflocculant potential against 4 g/L of kaolin clay suspension. The bacterium showed a flocculating activity of 72% and the culture conditions required for its optimal bioflocculant production were evaluated. The inoculum size of 1% (v/v) was optimum for bioflocculant production. This result proved that large inoculum sizes create the niches of the overlap and reduce bioflocculant production. The *Proteus mirabilis* PJC12 preferred fructose and yeast extract as carbon and nitrogen sources respectively. Therefore, evaluation of optimal culture conditions using inoculum size, carbon and nitrogen sources were essential for bioflocculant production and bacterial growth.

Cations play an important role in flocculating activity because they neutralise the negative charges of bioflocculant and suspended particles. Cations stimulate the process of flocculation by promoting adsorption of bioflocculant. In the present study $BaCl_2$ enhanced the bioflocculant production of *Proteus mirabilis* PJC12. The examination of pH was essential, because microorganisms required a different pH

range to produce bioflocculant and for growth *Proteus mirabilis* PJC12 preferred the slightly acidic pH of 6 and that improved flocculating activity.

Temperatures affect the growth of microorganisms and bioflocculant production. The evaluation of optimal temperature was important for improving the bioflocculant production. *Proteus mirabillis* PJC12 preferred a temperature of 30 °C as the optimal temperature for growth and bioflocculant production. Shaking speed allows the even distribution of oxygen and nutrients to microorganisms; hence evaluation of speed of agitation was essential and the optimal speed of agitation of *P. mirabillis* PJC12 was 120 rpm. These results proved that microorganisms require different parameters to grow and to produce a bioflocculant. The results also supported the fact that microorganisms from different environments have evolved genomic and metabolic diversity resulting in high bioflocculant production ability. Therefore, evaluation of optimal growth and bioflocculant production parameters improve bioflocculant production and could overcome the limiting factors of bioflocculants with regard to their application.

After extraction and purification, the pure bioflocculant yield was 2.75g from 1L of fermented production medium. Dosage size, cations, pH, and temperature were evaluated on the pure bioflocculant as this plays a crucial role in improving the bioflocculating efficiency. The optimal dosage size was 0.8 mg/mL. The cation $BaCl_2$ remained as the optimal cation on the pure bioflocculant. The pure bioflocculant was stable in pH ranges from 3 – 12, resulting in flocculating activity above 80%. The pure bioflocculant was stable to high temperatures between 25 - 35 $^{o}$C with flocculating activity above 75%.

The pure bioflocculant was characterised and the bioflocculant revealed to be composed of carbohydrate, uronic acid and protein, and carbohydrates were

dominant. The pure bioflocculant has functional groups such as amide, carboxyl and hydroxyl groups. These functional groups make up the carbohydrates, proteins and uronic acids and play an important role in flocculation.

The pure bioflocculant had high bioflocculant efficiency at 0.8 mg/mL and the most favourable cation was $BaCl_2$. The effective pH ranges were 3 to 12 with flocculating activity above 70% and the pure bioflocculant was thermostable at temperatures between 40 and 100 $^{o}C$.

The pure bioflocculant was applied to domestic and coalmine wastewater and removal efficiency of COD, BOD, Ca, P, S and Al. It showed excellent results for COD, BOD, Ca, P, S and Al. Based on the observed results this bioflocculant can be used as an alternative for chemical flocculants.

## 5.2 Recommendations

As a sequel to the findings of this study, the following recommendations are made:

a. Further characterisation of the bioflocculant.
b. Large scale production conditions should be developed.
c. Biosynthesis of nano-particles using the bioflucclant.

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## APPENDICES

### RAW DATA

**Appendix 1: Effect of inoculum size on bioflocculant produced by *Proteus mirabilis PJC12***

| | Absorbance(550nm) | | |
|---|---|---|---|
| Inoculum size (%) | Tube 1 | Tube 2 | Tube 3 |
| 1% | 0.688 | 0.655 | 0.652 |
| 2% | 0.700 | 0.669 | 0.701 |
| 3% | 1.428 | 1.404 | 1.406 |
| 4% | 1.863 | 1.850 | 1.838 |
| 5% | 1.916 | 2.010 | 1.950 |

**Appendix 2: Effect of carbon source on bioflocculant produced by *P. mirabilis PJC12***

| | Absorbance (550nm) | | |
|---|---|---|---|
| Carbon source | Tube 1 | Tube 2 | Tube 3 |
| Sucrose | 0.679 | 0.399 | 0.562 |
| Starch | 2.250 | 2.125 | 2.204 |
| Fructose | 0.262 | 0.251 | 0.252 |
| Lactose | 0.753 | 0.690 | 0.873 |
| Maltose | 2.416 | 2.103 | 1.976 |
| Glucose | 0.688 | 0.655 | 0.652 |

**Appendix 3: Effect of nitrogen source on bioflocculant produced by *P. mirabilis PJC12***

| | Absorbance (550nm) | | |
|---|---|---|---|
| Nitrogen source | Tebe 1 | Tube 2 | Tube 3 |
| Mixture | 0.262 | 0.251 | 0.252 |
| Yeast | 0.151 | 0.126 | 0.209 |
| Creatine | 2.062 | 2.095 | 2.144 |
| Peptone | 2.095 | 2.146 | 2.107 |
| Urea | 2.183 | 2.198 | 2.208 |
| Ammonium | 1.964 | 1.949 | 0.123 |
| Ammonium | 2.446 | 2.161 | 2.296 |

**Appendix 4: Effect of cations on flocculating activity.**

| | Absorbance (550nm) | | |
|---|---|---|---|
| Cations | Tube 1 | Tube 2 | Tube 3 |
| $MnCl_2$ | 1.505 | 1.345 | 1.346 |
| $BaCl_2$ | 0.190 | 0.186 | 0.215 |
| NaCl | 1.819 | 1.835 | 1.831 |
| LiCl | 1.990 | 1.918 | 1.790 |
| KCl | 1.918 | 1.884 | 1.700 |
| HgCl | 2.080 | 2.244 | 2.131 |
| $FeCl_3$ | 2.060 | 2.050 | 2.039 |
| $CaCl_2$ | 0.151 | 0.126 | 0.209 |
| Control | 2.152 | 2.158 | 2.170 |

**Appendix 5: Effect of initial pH on bioflocculant production by *P. mirabilis PJC12***

| | Absorbance (550nm) | | |
|---|---|---|---|
| pH | Tube 1 | Tube 2 | Tube 3 |
| 3 | 1.896 | 1.590 | 1.831 |
| 4 | 0.646 | 0.442 | 0.413 |
| 5 | 0.557 | 0.463 | 0.470 |
| 6 | 0.147 | 0.124 | 0.127 |
| 7 | 0.979 | 0.699 | 0.872 |
| 8 | 2.476 | 2.319 | 2.540 |
| 9 | 2.358 | 2.322 | 2.307 |
| 10 | 2.287 | 2.383 | 2.396 |
| 11 | 2.657 | 2.687 | 2.706 |
| 12 | 2.708 | 2.760 | 2.870 |

**Appendix 6: Effect of shaking speed on bioflocculant production by *P. mirabilis PJC12***

| | Absorption (550nm) | | |
|---|---|---|---|
| Speed (rpm) | Tube 1 | Tube 2 | Tube 3 |
| 60 | 1.038 | 1.015 | 1.042 |
| 80 | 1.007 | 1.023 | 0.983 |
| 100 | 0.695 | 0.820 | 0,835 |
| 120 | 0.444 | 0.411 | 0.309 |
| 140 | 0.200 | 0.110 | 0.102 |
| 160 | 0.148 | 0.190 | 0.321 |
| 180 | 0.784 | 0.987 | 0.847 |
| 200 | 1.271 | 1.230 | 1.271 |
| 220 | 1.676 | 1.462 | 1.606 |

**Appendix 7: Effect of temperature on bioflocculant production by *P. mirabilis PJC12***

| | Absorption (550nm) | | |
|---|---|---|---|
| Temperature | Tube 1 | Tube 2 | Tube 3 |
| 25 | 0.219 | 0.195 | 0.259 |
| 30 | 0.149 | 0.130 | 0.147 |
| 35 | 0.677 | 0.660 | 0.769 |
| 40 | 1.090 | 1.509 | 1.326 |
| 45 | 1.609 | 1.701 | 1.601 |
| 50 | 1.816 | 1.901 | 2.090 |
| 55 | 32.82 | 30.92 | 28.99 |

**Appendix 8: Time course of bioflocculant production by *P. mirabilis PJC12***

| 12 hrs | | | 24 hrs | | | 36 hrs | | |
|---|---|---|---|---|---|---|---|---|
| pH | OD (620nm) | FA | pH | OD (620nm) | FA | pH | OD (620nm) | FA (550nm) |
| 6.0 | 1.849 | 2.611 | 5.4 | 2.639 | 1.668 | 4.9 | 2.720 | 1.886 |
| 5.7 | 1.740 | 2.560 | 5.6 | 2.667 | 1.600 | 4.8 | 2.650 | 1.845 |
| 5.9 | 1.932 | 2.600 | 5.1 | 2.574 | 1.750 | 4.5 | 2.639 | 1.902 |
| 48 hrs | | | 60 hrs | | | 72 hrs | | |
| pH | OD | FA | pH | OD | FA | pH | OD | FA |
| 3.9 | 2.751 | 0.986 | 5.5 | 0.461 | 0.461 | 3.4 | 2.856 | 0.096 |
| 3.7 | 2.650 | 0.500 | 3.5 | 0.144 | 0.144 | 3.4 | 2.866 | 0.098 |
| 3.8 | 2.698 | 0.717 | 3.4 | 0.190 | 0.190 | 3.4 | 2.831 | 0.094 |
| 84 hrs | | | 96 hrs | | | 108 hrs | | |
| pH | OD (620nm) | FA | pH | OD (620nm) | FA | pH | OD (620nm) | FA (550nm) |
| 3.1 | 2.703 | 0.336 | 3.2 | 3.082 | 0.561 | 3.0 | 3.087 | 0.801 |
| 3.3 | 3.125 | 0.315 | 3.0 | 2.895 | 0.601 | 3.1 | 3.192 | 0.840 |
| 3.6 | 3.032 | 0.318 | 3.5 | 3.183 | 0.599 | 3.1 | 3.091 | 0.825 |
| 120 hrs | | | | | | | | |
| pH | OD (620nm) | FA | | | | | | |
| 2.7 | 3.630 | 1.052 | | | | | | |
| 2.9 | 3.614 | 1.070 | | | | | | |
| 2.4 | 3.501 | 1.049 | | | | | | |

**Appendix 9: Determination of optimum dosage of pure bioflocculant produced.**

| Absorption (550nm) | | | |
|---|---|---|---|
| Dosage size | Tube 1 | Tube 2 | Tube 3 |
| 0.2 | 1.210 | 1.205 | 1.895 |
| 0.4 | 1.090 | 0.853 | 0.452 |

| | | | |
|---|---|---|---|
| 0.6 | 0.564 | 0.667 | 0.325 |
| 0.8 | 0.076 | 0.082 | 0.109 |
| 1 | 0.050 | 0.090 | 0.33 |

**Appendix 10: Effect of cations on flocculating activity of the pure bioflocculant produced**

| Absorption (550nm) | | | |
|---|---|---|---|
| Cations | Tube 1 | Tube 2 | Tube 3 |
| $BaCl_2$ | 0.061 | 0.043 | 0.050 |
| LiCl | 0.060 | 0.121 | 0.036 |
| $HgCl_2$ | 0.244 | 0.087 | 0.120 |
| $FeCl_3$ | 0.110 | 0.781 | 0.154 |
| NaCl | 1.108 | 0.566 | 0.705 |
| $CaCl_2$ | 0.076 | 0.082 | 0.109 |
| Control | 0.160 | 0.235 | 0.190 |

**Appendix 11: Determination of pH of pure bioflocculant produced**

| Absorption (550nm) | | | |
|---|---|---|---|
| Ph | Tube 1 | Tube 2 | Tube 3 |
| 3 | 0.596 | 0.487 | 0.453 |
| 4 | 0.571 | 0.438 | 0.382 |
| 5 | 0.441 | 0.267 | 0.370 |
| 6 | 0.366 | 0.389 | 0.372 |
| 7 | 0.165 | 0.181 | 0.201 |
| 8 | 0.327 | 0.354 | 0.458 |
| 9 | 0.475 | 0.495 | 0.459 |
| 10 | 0.497 | 0.506 | 0.527 |
| 11 | 0.561 | 0.509 | 0.603 |
| 12 | 0.633 | 0.616 | 0.624 |

**Appendix 12: Determination of thermal stability of the pure bioflocculant**

| | | | |
|---|---|---|---|
| Temperature | Tube 1 | Tube 2 | Tube 3 |
| 40 | 0.164 | 0.170 | 0.162 |
| 50 | 0.171 | 0.169 | 0.174 |
| 60 | 0.193 | 0.186 | 0.188 |
| 70 | 0.230 | 0.225 | 0.227 |
| 80 | 0.254 | 0.261 | 0.270 |
| 90 | 0.354 | 0.358 | 0.361 |
| 100 | 0.459 | 0.461 | 0.457 |

**Appendix 13: 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.

Bradford Test Kit Includes, Bradford Reagent – 1 L, Albumin, Bovine – 0.5 mg/ml Standard, 0,15N Sodium chloride

Method

1. Different concentrations of BSA was added to microfuge and sufficient 0,15N NaCl were added to make a final volume of 100µl

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 mins incubation.

**Appendix 13-1: Determination of the protein content using assay at $OD_{595nm}$**

| | | | |
|---|---|---|---|
| Concentration (mM) | Tube 1 | Tube 2 | Tube 3 |
| Blank | 0.013 | 0.017 | 0.011 |
| 0.2 | 0.849 | 0.834 | 0.844 |
| 0.4 | 0.958 | 0.897 | 0.979 |
| 0.6 | 1.100 | 1.124 | 1.127 |
| 0.8 | 1.044 | 1.032 | 1.039 |
| 1.0 | 1.156 | 1.201 | 1.221 |
| 1.5 | 1.315 | 1.313 | 1.318 |
| Bioflocculant | 0.160 | 0.161 | 0.157 |
| | | | |

**Appendix 14: Phenol-sulfuric acid assay for the determination of carbohydrates**

Sensitivity: glucose (0.05−2mM)

Final volume: 1.4 ml

**Reagents:**

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

(b) Concentrated sulphuric acid.

**Method**

(i) Mix samples, standards and control solutions (200 µl containing up to 100 µg carbohydrate) with 200 µl of reagent A.

(ii) Add 1.0 ml of reagent B rapidly and directly to the solution surface without touching the sides of the tube.

(iii) Leave the solutions undisturbed for 10 minutes before shaking vigorously.

(iv) Determine the absorbance at 490 nm after a further 30 minutes

**Appendix 14-1: Determination of the total sugar content using Phenol Sulphuric Acid assay at $OD_{490nm}$**

| | | | |
|---|---|---|---|
| Concentration (mM) | Tube 1 | Tube 2 | Tube 3 |
| Blank | 0.023 | 0.021 | 0.024 |
| 0.05 | 0.034 | 0.031 | 0.035 |
| 0.1 | 0.075 | 0.073 | 0.078 |
| 0.2 | 0.160 | 0.161 | 0.165 |
| 0.4 | 0.174 | 0.166 | 0.181 |
| 0.6 | 0.179 | 0.182 | 0.175 |
| 0.8 | 0.200 | 0.205 | 0.197 |
| 1.0 | 0.206 | 0.201 | 0.203 |
| 1.5 | 0.203 | 0.207 | 0.205 |
| 2.0 | 0.240 | 0.239 | 0.235 |
| Bioflocculant | 0.086 | 0.088 | 0.084 |

**Appendix 15: Carbazole assay for the determination of uronic acid**

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

Final volume: 1.8 ml

**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**

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

(ii) Add ice-cold reagent A (1.5 ml) with mixing and cooling in the ice bath.

(iii) Heat the mixtures at 100 $^{o}$C for 10 minutes.

(iv) Cool rapidly in the ice-bath.

(v) Add 50 µl of reagent B and mix well.

(vi) Reheat at 100 $^{o}$C for 15 minutes.

(vii) Cool rapidly at room temperature and determine the absorbance at 525 nm.

**Appendix 15-1: Determination of the uronic acid content using Carbazole assay at $OD_{525nm}$**

| | Abs@490 | | |
|---|---|---|---|
| Concentration (mM) | Tube 1 | Tube 2 | Tube 3 |
| Blank | 0.262 | 0.264 | 0.258 |
| 0.001 | 0.282 | 0.284 | 0.280 |
| 0.002 | 0.301 | 0.300 | 0.305 |
| 0.003 | 0.303 | 0.300 | 0.307 |
| 0.004 | 0.312 | 0.310 | 0.315 |
| 0.005 | 0.351 | 0.348 | 0.346 |
| 0.010 | 0.436 | 0.430 | 0.433 |
| 0.050 | 0.521 | 0.526 | 0.519 |
| Bioflocculant | 0.433 | 0.431 | 0.429 |

## Appendix 16: Application of bioflocculant in the Vulindlela domestic wastewater

### Appendix 16-1: Flocculating activity- Abs@550nm

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 1.319 | 1.047 | 1.240 |
| Bioflocculant | 0.042 | 0.038 | 0.053 |
| Ferric chloride | 0.083 | 0.089 | 0.075 |
| Polyacrylamide | 0.073 | 0.061 | 0.066 |

### Appendix 16-2: COD (mg/L)

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 1243 | 1253 | 1254 |
| Bioflocculant | 172 | 166 | 187 |
| Ferric chloride | 265 | 257 | 270 |
| Polyacrylamide | 200 | 191 | 209 |

### Appendix 16-3: BOD (mg/L)

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 92.5 | 92.4 | 90,5 |
| Bioflocculant | 7.9 | 8.0 | 8.9 |
| Ferric chloride | 15.1 | 15.9 | 15.8 |
| Polyacrylamide | 10.3 | 14.2 | 10.8 |

**Appendix 16-4: Ca (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 9.32 | 9.21 | 9.39 |
| Bioflocculant | 2.11 | 1.83 | 3.32 |
| Ferric chloride | 1.05 | 1.43 | 2.17 |
| Polyacrylamide | 1.4 | 1.6 | 1.2 |

**Appendix 16-5: P (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 0.95 | 0.90 | 1.06 |
| Bioflocculant | 0.31 | 0.29 | 0.33 |
| Ferric chloride | 0.22 | 0.31 | 0.24 |
| Polyacrylamide | 0.084 | 0.089 | 0.188 |

**Appendix 16-6: S (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 26.68 | 25.63 | 27.72 |
| Bioflocculant | 5.43 | 5.56 | 5.84 |
| Ferric chloride | 8.49 | 8.52 | 8.61 |
| Polyacrylamide | 7.15 | 7.31 | 7.32 |

**Appendix 16-7: Al (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 0.12 | 0.16 | 0.14 |
| Bioflocculant | - | - | - |
| Ferric chloride | - | - | - |
| Polyacrylamide | - | - | - |

## Appendix 17: Application of bioflocculant in the Tendele coalmine wastewater

### Appendix 17-1: Flocculating activity- Abs@550$_{nm}$

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 2.413 | 2.197 | 2.329 |
| Bioflocculant | 0.117 | 0.132 | 0.109 |
| Ferric chloride | 0.147 | 0.169 | 0.157 |
| Polyacrylamide | 0.141 | 0.136 | 0.133 |

### Appendix 17-2: COD (mg/L)

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 2160 | 2146 | 2159 |
| Bioflocculant | 203 | 225 | 220 |
| Ferric chloride | 360 | 377 | 361 |
| Polyacrylamide | 348 | 337 | 350 |

### Appendix 17-3: BOD (mg/L)

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 77.3 | 92.6 | 91.9 |
| Bioflocculant | 9.46 | 4.65 | 4.23 |
| Ferric chloride | 9.61 | 11.43 | 18.30 |
| Polyacrylamide | 12.26 | 10.81 | 9.95 |

### Appendix 17-4: Ca (mg/L)

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 8.83 | 11.53 | 9.43 |
| Bioflocculant | 2.08 | 1.98 | 1.92 |
| Ferric chloride | 3.03 | 1.69 | 2.43 |

| | | | |
|---|---|---|---|
| Polyacrylamide | 1.75 | 2.09 | 1.84 |

**Appendix 17-5: P (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 1.66 | 1.37 | 1.59 |
| Bioflocculant | 0.21 | 0.13 | 0.17 |
| Ferric chloride | 0.49 | 0.42 | 0.46 |
| Polyacrylamide | 0.15 | 0.29 | 0.25 |

**Appendix 17-6: S (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 29.0 | 41 | 38.1 |
| Bioflocculant | 5.8 | 6.1 | 4.3 |
| Ferric chloride | 1.7 | 2.8 | 3.1 |
| Polyacrylamide | 2.2 | 2,7 | 1.6 |

**Appendix 17-7: Al (mg/L)**

| | Tube 1 | Tube 2 | Tube 3 |
|---|---|---|---|
| Original | 0.173 | 0.159 | 0.186 |
| Bioflocculant | - | - | - |
| Ferric chloride | - | - | - |
| Polyacrylamide | - | - | - |