UNIVERSITY OF ZULULAND

RESEARCH THESIS

For the degree of Masters of Science

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With the title:

Production of bioflocculant from marine bacteria and its application in the treatment of coal wash plant fines

FACULTY OF SCIENCE AND AGRICULTURE

Candidate: Jade Dafel

Student No. 200902421

Supervisor:

Prof J.J. Simonis

Co-Supervisor:

Prof A.K. Basson

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Abstract

A total of 33 water and sediment samples were randomly selected from the marine environment on the North Coast of KwaZulu-Natal (KZN), South Africa. Strains were isolated and screened for their potential to secrete bioflocculants and tested for flocculating activity with a kaolin suspension. The strains that produced bioflocculants with a high yield of flocculating activity (above 60%) were selected for optimisation and further flocculating tests using coal mine slurry. One strain with good flocculating abilities for both a kaolin suspension and coal mine slurry (74% and 76% respectively) was analysed using its 16S rDNA nucleotide sequence and was identified as *Bacillus cereus*. The bioflocculant bacterium was optimal when glucose (40g/l) and urea, as the carbon and nitrogen sources, a pH of 4 and Ca^{2+} as the cation were utilized. The exceptional flocculating performance of *Bacillus cereus* demonstrated good potential for replacing the chemical flocculants that are currently used in flocculating coal mine slurry generated at Tendele Coal Mine (TCM) located in KZN.

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I acknowledge that I have read and understood the University's policies and rules applicable to postgraduate research, and I certify that I have, to the best of my knowledge and belief, complied with their requirements.

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List of Abbreviations

B. cereus	*Bacillus cereus*
$(NH_4)2SO_4$	Ammonium Sulphate
$CaCl_2$	Calcium chloride
CPP	Coal Processing Plant
DM	Dense Medium
FEL	Front-End-Loaders
HCL	Hydrochloric acid
KZN	Kwazulu-Natal
K_2HPO_4	Dipotassium Phosphate
KH_2PO_4	Monopotassium Phosphate
M. phlei	*Mycobacterium phlei*
$MgSO_4 \cdot 7H_2O$	Magnesium Sulphate Heptahydrate
MoU	Memorandum of Understanding
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
OD_{550}	Optical density at 550nm
P. polymyxa	*Paenibacillus polymyxa*
PDA	Potato Detrox Agar
PM	Particulate matter
ROM	Run-of-mine
RSM	Response Surface Methodology
SDA	Shabouraud Detrotex 4% Agar
TCM	Tendele Coal Mine
YEA	Yeast Extract Agar

Chapter 1: Introduction

1.1 Introduction and background

Water is the most important non-renewable resource on earth. All animals and plants rely on water to survive. Without water there can be no life. Apart from the need for potable water, humans have several other uses for water such as; cooking, washing, cleaning, and recreational purposes. It is crucial that clean water (not necessarily potable water) be used for agricultural purposes, while reusable water is used in industry (SSWM, 2012). South Africa is a water-scarce country and is currently facing its worst drought since 1982, with more than 2.7 million households experiencing water shortages (Water Wise, 2015). It is therefore imperative to use water resources as sparingly as possible as well as to recycle and reuse those resources.

Flocculants are chemical substances that bring about solid-liquid separations by the process of flocculation in a wide variety of industrial processes. Processes such as treating wastewater, downstream processing, treating industrial effluents, waste water sludge disposal, removal of dyes and heavy metals, and in food and fermentation processes because they are inexpensive and readily available (Salehizadeh *et al*., 2000; Mona, 2014; Sanayei *et al.,* 2010). By definition of IUPAC, flocculation is, “a process of contact and adhesion whereby the particles of dispersion form larger clusters” (Hubbard, 2004). Flocculation is synonymous with agglomeration, aggregation and coagulation.

Flocculants are classified into three groups: organic synthetic flocculants, naturally occurring flocculants and inorganic flocculants (Zhang *et al.,* 2007). Inorganic flocculants, such as aluminium sulphate, are not only non-biodegradable in nature but also have been found to induce Alzheimer’s disease and breast cancer (Zaki *et al.,* 2011). Inorganic flocculants are pH sensitive and have the potential for negatively affecting the downstream process (Zaki *et al.,* 2011). Despite the effective flocculating potential and low cost of synthetic flocculants (polyacralamide), their precursors are petroleum-based (non-renewable) with environmental and human health concerns (Zhang *et al.,* 2007).

Due to these concerns when using inorganic flocculants, it is imperative to find alternative substitutes, such as bioflocculants. Bioflocculants are eco-friendly and

can replace inorganic flocculants. It is believed that the use of bioflocculants will increase in the future (Kurane *et al.,* 1986).

Bioflocculation is the removal of colloidal particles from suspension by flocculating sediments (Mona, 2014). Bioflocculants are macromolecule polymers which are secreted by microorganisms (Zhuang *et al.,* 2012). The marine habitat is reported to be responsible for half of the production of all discovered bioactive compounds and is one of the most under-exploited environments (Berdy, 2005). Bioflocculants are said to be produced by bacteria, fungi, actinomycetes and algae microorganisms (Zhuang *et al*., 2012).

Bioflocculants have previously been used in drinking water purification, wastewater treatment and downstream processes in biotechnology (Salehizadeh and Shojaosadati, 2001). The major disadvantage of bioflocculants within large scale production and industrial applications is the high fermentation cost, this is because of the relatively expensive substrates such as glucose, sucrose and fructose which are essential for the production of bioflocculants (He *et al.,* 2009; Kurane and Nohata*,* 1994).

Bioflocculants produced by microorganisms during their growth have attracted scientific and biotechnological attention over the years due to being environmentally friendly, non-toxic, biodegradable, and free of secondary pollution from degradation (Salehizadeh and Shojaosadati, 2001). Because of these characteristics it is of great interest for researchers to isolate and screen new microorganisms for bioflocculants (Dugan, 1975; Ugbenyen *et al.,* 2012). Therefore the search for a low cost substrate and the optimization of the fermentation process to reduce the production costs is essential (Deng *et al.,* 2003).

Industrial processes are potential sources of pollution and require particular treatment for waste water. Waste water treatment raises the running cost of the plant, thus is it imperative to enhance its effectiveness. This is applicable to all industries however it is particularly important for industries with complicated wastes' such as tailing dams, tanneries, mineral processing and clay production industries (Zaki *et al.,* 2011).

The mining industry consumes 3% of the total water utilized in South Africa and is mostly accountable for deteriorating water quality (Haggard *et al.,* 2015). Any reduction in mine water requirements will decrease the demand on the current water resources, thus decreasing the impact on water quality. Mining activities stimulate development but they are often far away from water resources. Therefore reusing water in the coal mining industry has the potential to reduce the cost of water supply and wastewater treatment. It also reduces pressure on the water resources and decreases the quantity of water pollution discharged into the surrounding environment (Haggard *et al.,* 2015). Depending on the type and quality of wastewater, it may either be reused directly (e.g. dust suppressant) or treated before recycling and reuse (coal washing). Synthetic flocculants are normally used to separate the solid from liquid of coal waste water (slurry) for recycling and reuse (Das *et al.,* 2006).

Coal preparation plants (CPP) are the facility where coal is separated from rock, crushed and graded for sorting, stockpiled and prepared for transport. The processing of fine-coal is considered the most complicated and costly operation within the CPP (Asmat *et al.,* 2002). The CPP consists of the following operations; screening, gravity separation, jigs, spiral, dewatering, thickeners and tailing dams. At CPP in coal mines, a considerable amount of wastewater and fine tailings are generated. Tailings contain both organic and inorganic matter.

An appropriate dewatering method, normally by sedimentation in a thickener (Figure 2), results in recycling of large quantities of clean water in a CPP (Sabah and Cengiz, 2004). Flocculants used in the thickener in a coal mine form part of a complex process. To accurately determine the chemical, physical, and electro-kinetic constituents of solid matter in slurry is crucial in successfully destabilising fine particle suspensions (Sabah and Cengiz, 2004). According to Sabah and Cengiz, (2004) there are two processes used to assess the sedimentation and flocculation performance:

1. Clarification of the flow water on top of the thickener (the clarified water on top is usually recycled back into the CPP)

2. Settling rate of the flocculated fines (because it directly affects the capacity of the thickener)

Water is removed from tailings and thickeners for recycling. Thickeners are used for dewatering slurries of either tailings or product. Discarded thickened tailings are generally pumped into the mined-pit or a tailings dam and combined with larger sized rejects.

In this study the culture conditions of *Bacillus cereus* (*B. cereus*) to produce a bioflocculant were investigated. The bioflocculant was then applied to the coal mine slurry (before the addition of chemical flocculants) from TCM to examine its application potential on the solid liquid separation rate directly compared to the flocculant used on the mine (Floxit 5028).

1.2 Research aims

In light of the above concerns, the aim of this research is to produce and evaluate suitable bioflocculants effective in settling coal wash plant fines.

Chapter 2: Literature review

2.1 Introduction

A wide variety of industrial processes use flocculants such as downstream processes, wastewater treatment, drinking water treatment and several fermentation processes (Shih *et al.,* 2001). The use of flocculants in suspended solids is essential due to Brownian movement which keeps them in constant movement and inhibits settling (Lachhwani, 2005).

Flocculants can be divided into three distinct categories; inorganic flocculants (polyaluminium chloride, aluminium sulphate), organic synthetic flocculants (polyacralamide, polyethylene amine) and natural bioflocculants (gelatine, chitosan, guargum and microbial flocculants) (Lachhwani, 2005). Bioflocculants are extracellular polymeric macromolecule components that consist of polysaccharide, protein and nucleic acids produced by bacteria, fungi and actinomycetes during their growth (Sathiyanarayanan *et al.,* 2012). Flocculants are either anionic or cationic therefore the purpose of the flocculant is to neutralize charges in suspension, coagulate and flocculate them into a larger size (the larger the particle size the faster the settling rate) (Lachhwani, 2005; Lugo, 2014).

Inexpensive, naturally occurring polymeric materials that have shown flocculating potential and are currently gaining popularity are microbes (Lachhwani, 2005). Bioflocculants are biodegradable macromolecular flocculant secreted microorganisms (Mona, 2014). Bioflocculation is defined as a dynamic process resulting from the synthesis of extracellular polymers by living cells for clarifying suspended particles in solution (He *et al.,* 2002; Gao *et al.,* 2006; Piyo *et al.,* 2011). The physiochemistry of bioflocculation is determined by the genetic make-up of organisms (Dugan, 1975).

Inorganic flocculants have several advantages such as affordability, availability and flocculation effectiveness. The use of these flocculants is said to be unsafe and harmful to humans (Lachhwani, 2005; Xiong *et al.,* 2010). Inorganic flocculants used in wastewater treatment can compromise the sustainability of the process by producing secondary pollution of metal concentrations in water and sludge resulting in final disposal problems (Lugo, 2014). Aluminium (which can be found in inorganic flocculants) has shown to enhance Alzheimer's disease (Lachhwani, 2005).

Therefore the large quantity of flocculants used worldwide is of great concern because of the health problems caused by flocculants (Lachhwani, 2005). This makes it crucial to find a more environmentally friendly and economic alternative (Lugo, 2014).

According to a study by Dearfield *et al*. (1988) organic synthetic flocculants have proven to be carcinogenic, neurotoxic and recalcitrant to degradation. The monomers of polyacralamide are carcinogenic and neurotoxic towards humans and animals and have a detrimental effect on fauna and flora as they are also non-biodegradable (Lachhwani, 2005; Mona, 2014). Due to the harmful effects of organic synthetic flocculants their application has been limited in the majority of the developed countries (Okaiyeto *et al.,* 2013).

Bioflocculation is a dynamic process which results from the synthesis of extracellular polymers by living cells. Bioflocculation has attracted major scientific and biotechnological attention because of its biodegradability and safety for ecosystems (Okaiyeto *et al.,* 2013). The development of a safe biodegradable flocculant that will minimize environmental and health risks is urgently required. Bioflocculants consist mainly of polysaccharides secreted by microorganisms extracellularly. The exopolymeric substances are commonly produced by bacteria, yeasts and fungi during their development, playing a crucial role in the flocculation process (Vijay and Surendhiran, 2014). Bioflocculants are being considered as an excellent alternative for the use of conventional flocculants and coagulants used in water treatment, waste water, downstream processing, food fermentation processes, the removal of heavy metals, effluent pump, pulp and paper processes as they amplify the preservation of fibres as the filler additives improve drainage and other industrial processes (Salehizadeh *et al.,* 2000; Lachhwani, 2005; Mona, 2014). To enable the efficient and economic operation of both anaerobic and activated sludge wastewater treatment processes, bioflocculation is essential (Morgan *et al.,* 1989). Bioflocculants can destabilize the colloidal particles by means of two main mechanisms:

1) By reducing the zeta potential and increasing the ionic strength of the solution; and/or

2) By interaction of a particular functional group contained in the macromolecule and the particles in bulk (Lugo, 2014)

a) Advantages of bioflocculants

The use of bioflocculants has several advantages (especially when compared with the conventional organic synthetic flocculants) such as:

- Safety
- Strong flocculating effect
- Biodegradability
- Low consumption of reagents when compared with inorganic salts
- Does not lead to secondary pollution
- Reduces the use of other chemical treatments
- Harmless to humans and the environment (Gao *et al.,* 2006)

Bioflocculants are less dependent on the pH of the solution. They also form stronger and bigger flocs which avoid affecting the pH of the working medium allowing for improved settling than those of straightforward coagulating electrolytes (Goa *et al.,* 2006). Bioflocculants have a high molecular weight and a low charge density (Lachhwani, 2005). Besides having a high flocculating ability, they also have a low dosage requirement (Lugo, 2014).Bioflocculants are nontoxic, non-carcinogenic and have not been associated with any medical problems (Luvuyo, 2013 and Organo-floc, 2014). Therefore they could potentially be applied in wastewater treatment plants, drinking water treatment, fermentation and downstream processes (Salehizadeh and Shojaosadati, 2001).

b) Disadvantages of bioflocculants

Although bioflocculants have the advantage of environmental friendliness, they do however have a shorter life span since their active components can also biodegrade with time (Lugo, 2014). The moderate effectiveness of some anionic species (e.g. cellulose and alginate) requires coupling with a cationic coagulant which is often in the form of inorganic metal salts (Lugo, 2014).

Different microbes produce different bioflocculants therefore potentially inhibiting flocculating activity and the result is high production costs which could limit large scale production (Li *et al.,* 2003). Sathiyanarayanan *et al.* (2012) believe that the cost to produce bioflocculants has constrained the commercialisation of bioflocculants for industrial application.

The source of raw material and production technique can significantly influence the physicochemical properties of the final product and its performance. The isolation of some polymers can include extraction with solvents that should be considered as a secondary pollution when assessing the cradle to grave life cycle of the flocculation process (Lugo, 2014).

The methods of extraction and preparation include aggressive physical and chemical treatments such as cell lysis during the process of screening, conservation and culture of the purified microorganisms. These treatments significantly increase the cost of the system, hindering the application of bioflocculants beyond laboratory and pilot scale (Lugo, 2014).

Few bioflocculants have been applied practically in industry due to their miniscule flocculating potential and ample dosage requirements. In order to use bioflocculants extensively in industry, it is advantageous to identify microorganisms with high bioflocculant potential and enhance their flocculating effectiveness (Gao *et al.,* 2006). The uniqueness and environmentally friendliness of bacterium flocculants has encouraged further investigations into screening, isolation, structural classification and characterization of a polymeric flocculant-producing bacterium (Lachhwani, 2005; Gao *et al.,* 2006).

2.2 Isolation, screening and production of bioflocculants

2.2.1 Isolation

The marine environment provides an unexploited resource for new bacteria and the bioflocculants that they can generate. Sathiyanarayanan *et al.* (2012) isolated a wide variety of bioflocculant producing microbes from the aquatic environment.

Flocculating bacteria may have predominance in wastewater or sludge samples. Therefore a number of these sources were used for the purpose of isolation. In a

study by Lachhwani (2005), 14 bacterial strains were isolated on agar plates from various industrial wastewaters and sludge's. From the strains the predominant isolates were selected from plates of dilution and re-streaked onto nutrient agar plates. Each isolate was screened on the basis of their flocculating ability prior to growth in both nutrient broth and flocculant isolation broth. The isolates were then tested for their flocculating ability in kaolin clay suspension. Goa *et al.* (2006) isolated over 20 wastewater samples from sewerage samples and industry wastewater, thus in total 206 colonies were isolated. The isolation of polymeric flocculant-producing bacterium's was accomplished with the use of agar plates. One strain showed a kaolin clay suspension which was elected for further testing. Okaiyeto *et al.* (2013) reported that the sediment samples isolated from Algoa Bay, South Africa, demonstrated great bioflocculant production potential.

Further research should be focused on the isolation of bioflocculant producing microbial strains and optimization of the process conditions to improve bioflocculant potential (Sathiyanarayanan *et al.,* 2012).

2.2.2 Screening

According to a study by Xia *et al.* (2008), the screening of microorganisms that yield both large bioflocculant producing ability and an immense flocculating efficiency is crucial for success. Screening is important as it determines whether the microorganism has the ability to produce bioflocculant when grown in the media being investigated (Yang *et al.,* 2015).

In a study by Sathiyanarayanan *et al.* (2012), they collected samples (by scuba diving) from depths of 10-15 meters in marine water on the southeast coast of India. The screening of bioflocculant producing microorganisms was depicted by colony morphology. The bioflocculant screening media was prepared using the method by Zhang *et al.* (2007). In the method used by Zhang *et al.* (2007) the pre-culture media consisted of: 20g glucose, 0.5g urea, 0.5g yeast extract, 0.2g $(NH_4)2SO_4$, 5g K_2HPO_4, 2g KH_2PO_4, 0.1g NaCl, and 0.2g $MgSO_4{\cdot}7H_2O$ per litre of marine water. The culture broths were then tested for flocculating efficiency and the strains with the largest flocculating capabilities were elected for additional studies.

Goa *et al.* (2006) collected 20 samples from several wastewater samples isolated from the Little Moon River, Beijing. After isolation the bioflocculant producing microorganisms were established by screening colony morphology. They were then tested for flocculating activity using a kaolin suspension. One strain yielded a high flocculating activity of 90% for kaolin which was selected for further studies.

After isolation and screening, with regards to the growth rate and the yield of flocculating activity, MSBN 17, species of *Paenibacillus*, *Halmonas* and *Micrococcus* were considered to be the most effective producer of bioflocculants (Oh *et al*., 2001; Sathiyanarayanan *et al.,* 2012; Okaiyeto *et al.,* 2013).

2.2.3 Production of bioflocculants

Bioflocculants have not yet been produced on an industrial scale because of their comparatively minor flocculating efficiency, fermentation costs, reduced yields and large production costs (Xiong *et al.,* 2010). Thus further research is needed to reduce production costs and produce an efficient bioflocculant producing microbe with a high flocculating efficiency and yields (Okaiyeto *et al.,* 2015).

2.3 Identifying the effectiveness of bioflocculants

The production of bioflocculants could be affected by constituents of the culture medium and the cultivation conditions (Zhang *et al.,* 2007).The research by Surendhiran and Vijay (2013), Okaiyeto *et al.* (2013), Ma *et al*. (2003), Oh *et al.* (2001), Sathiyanarayanan *et al.* (2012) and Fujita *et al.* (2000), indicated that there are several independent variables which can affect the effectiveness of bioflocculants during flocculating activity. These variables are factors such as cationic inducers, pH, nitrogen sources, carbon sources and time course assay.

2.3.1 Cationic inducers

The use of cations may enhance the flocculating activity of a bioflocculant microorganism. The calcium ion provokes flocculating efficiency by neutralizing and preserving the residential charge of functional groups of kaolin particles in solution (Goa *et al.,* 2006; Okaiyeto *et al.,* 2013). The bridging mechanism occurs after the absorption of the particles to the bioflocculant chains. However some metal ions are not always required for the flocculating process. Wan *et al.* (2013) produced a

Solibacillus silvestris where no cations was necessary to improve the flocculating efficiency.

A study by Okaiyeto *et al.* (2013) discovered that a purified bioflocculant was cation and pH dependant when using a kaolin suspended solution of 0.1mg.ml^{-1} to test for flocculating activity. The flocculated efficiency of the bioflocculant was greatly accelerated in the presence of Ca^{2+}, Mn^{2+} and Al^{3+}.

Vijay and Surendhiran (2014) used $ZnCl_2$as the cationic inducer. It showed that this cationic inducer significantly affected bioflocculation. The Zn^{2+} cation aids the process as a linker, which neutralizes the residual negative charge of functional groups therefore enhancing the bioflocculant process. A higher concentration of the Zn^{2+} inducer led to the destruction of the compact conformation of the cells resulting in the decrease of flocculating efficiency.

In the study by Oh *et al.* (2001), *Paenibacillus species* showed a flocculating activity of 72% and 78% when using aluminium sulphate and polyacralamide respectively. The highest flocculating efficiency was however obtained when using $CaCl_2$.

Deng *et al.* (2003), Lachhwani (2005), Wu and Ye (2007), Gong *et al.* (2008), Elkady *et al.* (2011) and Aljuboori *et al.* (2014), used various cations to test for flocculating activity. The divalent cations Ca^{2+} and Mg^{2+} showed maximum flocculating activity whilst Na^{+} had a detrimental effect on the various bioflocculation efficiencies.

2.3.2 pH

pH is a very important factor for harvesting microorganisms. The original pH of the pre-culture media determines the oxidation-reduction potential as well as the electric charge of the cells. This could potentially affect the enzymatic reaction as well as nutrient absorption (Salehizadeh and Shojaosadati, 2001). As the pH increases, the negative charge on the microalgae cells increases. The increase in pH often results in an increase in the bioflocculants' flocculating activity (Okaiyeto *et al.,* 2013). This phenomenon could be the main cause of flocculation at a higher pH. This is caused by the difference in protonation conformational changes and structural alterations in flocs (Vijay and Surendhiran, 2014).

Studies by Oh *et al.* (2001), Lachhwani (2005), Lui *et al.* (2013) and Okaiyeto *et al.* (2013) all compared the flocculation efficiency of the bioflocculants with commercial

chemical flocculants. Oh *et al.* (2001) produced a *Paenibacillus* species and found that the flocculating efficiency increased within a pH of 5-11. Lachhwani (2005) produced an *Enterobacter* species bioflocculant, under batch and fed batch cultivation and it showed an initial optimally high yield at a pH of 7, under controlled pH testing. Lui *et al.* (2013) produced a *Klebsiella* sp. utilizing waste discard from food industries and found that the optimal flocculation efficiency was highest at a pH of 9. Okaiyeto *et al.* (2013) found that optimum flocculating efficiency (86%) was obtained with a pH of 8 when using a bioflocculant produced by a consortium of a *Halomonas* sp. and a *Micrococcus* sp.

Studies by Vijay and Surendhiran (2014), Lachhwani (2005), Goa *et al.* (2006) and Okaiyeto *et al.* (2015) all followed similar methodologies where they divided their cultures into a series of test tubes. The pH was then adjusted to fixed values by the addition of HCl or NaOH ranging from 1 to 10. Vijay and Surendhiran (2014) found a pH of 10 showed the highest flocculating efficiency whilst Goa *et al.* (2006) found the pH ranging from 7-10 showed the most efficient flocculating activity. Lachhwani (2005) showed that the flocculating efficiency was most stable and most high at a pH of 7.5 and Okaiyeto *et al*. (2015) showed a flocculating potential of 87% at a pH of 8.

Using Response Surface Methodology (RSM), optimum flocculating efficiency was found when the pH was at 10.4 (Surendhiran and Vijay 2013). However from statistical experimental results from the study by Vijay and Surendhiran (2014) the effect of pH on flocculation efficiency was found to be higher with the increase in pH.

2.3.3 Nitrogen Sources

The production of bioflocculants may be affected by the components found in the culture medium and culture conditions. Therefore, in order to optimize cultivation the nitrogen and carbon sources of the medium must be altered (Zhang *et al.,* 2007). It has been widely documented that nitrogen sources are a crucial factor that enhance bioflocculant production (Ubenyen *et al.,* 2012; Mona, 2014).

A study by Mona (2014) showed that *Nocardiopsis aegyptia*, when compared to various nitrogen sources used in the culture medium, the greatest flocculating potential was evident when peptone and potassium nitrogen sources were used in the medium pre-culture for this microorganism.

Okaiyeto *et al.* (2015) isolated *Bacillus toyonensis* from a sediment sample from the aquatic environment in Algoa Bay, South Africa, which resulted in a flocculating efficiency higher than 60% for a kaolin solution. The effect of inorganic and organic nitrogen sources namely; peptone, tryptone, urea, yeast extract, ammonium sulphate, ammonium nitrate and sodium nitrate, were used to test *Bacillus toyonensis* bioflocculant potential. The organic nitrogen sources resulted in a miniscule bioflocculant production while the inorganic nitrogen soluble sources accelerated the flocculating activity with ammonium nitrate showing a flocculating potential of 73%. Previous studies have shown that a variety of inorganic nitrogen sources are appropriate for the production of bioflocculants (Nwodo *et al.,* 2013). Nwodo *et al.* (2013) demonstrated that ammonium sulphate was the preferred nitrogen source for the bioflocculant production of *Brachybacteria* species.

2.3.4 Carbon Sources

The importance of carbon and nitrogen sources has demonstrated a substantial effect on the growth of the bacteria and the production of bioflocculants. It may however differ with different bioflocculant producing microorganisms (Salehizadeh and Shojaosadati, 2001). A study by Salehizadeh and Shojaosadati (2001) showed that the *Nocardiopsis aegyptia* could grow in all tested carbon sources and could utilize a relatively wide range of tested carbon sources. Mona (2014) also discovered that *Nocardiopsis aegyptia* species could grow on all the tested carbon sources and could utilize a relatively wide range of tested carbon sources (lactose, fructose, rhammnose, glucose, galactose, maltose, sucrose, starch and glycerol). Salehizadeh and Shojaosadati (2001) and Mona (2014) both determined that glucose and rhammnose were the most suitable carbon sources for the microorganism with the flocculating efficiency exceeding 80% after 96 hours cultivation.

Liu and Cheng (2010) isolated a *Penicillium* strain and tested the effects of the cultivation environment on the production of bioflocculant. The flocculating efficiency of the *Penicillium* strain was tested using glucose, sucrose, lactose, maltose, starch and molasses as the sole carbon source. All of the carbon sources had a concentration of 20g/l. While starch and molasses did not produce favourable flocculating results, the remaining carbon sources (glucose, sucrose, lactose and maltose) produced a flocculating activity greater than 80%. However the most

favourable carbon source for cultivation of the *Penicillium* strain was glucose. Glucose yielded the highest flocculating activity with the shortest incubation period in comparison to the other carbon sources.

In general glucose has been reported as the preferred carbon source in most studies for bioflocculant production by various microorganisms (Salehizadeh and Shojaosadati, 2001; Cosa *et al.,* 2013). It also gets readily compared to other carbon sources such as maltose and fructose (Okaiyeto *et al.,* 2015).

2.3.5 Time course interval

The correlation between bioflocculant production and culture time may differ among different microorganisms. Most microorganisms produce bioflocculants during the exponential growth phase of a time course test (Ugbenyen *et al.,* 2012). The flocculating activity should increase gradually with an increase in cultivation time after which time it will reach a peak flocculation potential. After the peak the flocculation potential reaches a plateau and then begins to decrease. The main cause of the decrease in flocculating potential of the microorganisms is a combination of enzyme activity, biosynthesis and cell autolysis (Mabinya *et al.,* 2012).

In the study by Okaiyeto *et al.* (2015) they used a *Bacillus sp*. that had the highest flocculating potential of 83% obtained after 72 hours. The consequential decrease of the flocculating efficiency could be a product of cell autolysis and enzymatic activity. Deng *et al.* (2005) and Wu and Ye (2007) isolated an *Aspergillus parasiticus* and a *Bacillus subtillus* respectively. These microorganisms produced the highest flocculating yield of 98% and 96% of a kaolin suspension after 72 hours as well.

Piyo *et al.* (2011) conducted their time course assay of bioflocculant experimentation using a *Bacillus sp.* with the production medium at a pH of 6.2 over 10 days of cultivation. The peak flocculating activity time was reached after 4 days of fermentation. Subsequently the flocculating efficiency decreased at a constant rate with an increase in culture time.

Mona (2014) found that by using a *Nocardiopsis aegyptia* sp*.,* the production of the flocculant was associated with cell growth. The growth reached its maximum flocculating potential (89%) after 4 days (stationary phase). The steady increase in

cell growth is possibly an indication that the bioflocculant was produced by biosynthesis during growth and not by cell autolysis. Biosynthesis is defined as a production of complex chemical compounds from simpler precursors in a living organism, usually involving enzymes (to catalyse the reaction) and an energy source (Biology Online, 2015). While cell autolysis is more commonly known as "self-digestion" it refers to the enzymatic digestion of cells by enzymes present within them (MedicineNet.com, 2015).

2.4 Coal mining in South Africa

Coal is mined using large earth-moving machines to remove the coal from the ground. There are two basic methods of removing coal: opencast and underground mining. Open cast mining uses either strip mining or open cast mining methodologies while underground mining is generally mined using pillar support methods (Lloyd, 2002).

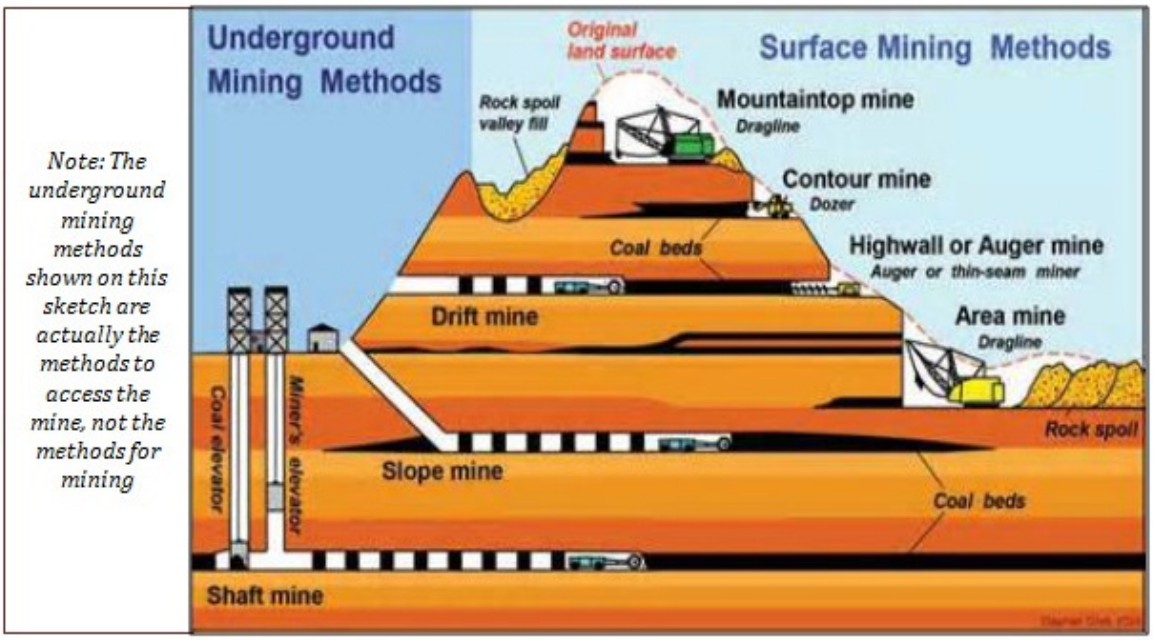

Figure 1: Contrast between underground and surface mining methods (Methods of Mining, 2006)

According to geographic considerations (e.g. the differentiation in the sedimentation, formation, origin, distribution and quality of the coal) there are only 19 functioning coal fields in South Africa (Hancox and Götz, 2014). Approximately 53% of coal production is from opencast mines and the majority of the mines can found in the

Mpumalanga Province (Waterberg, Witbank and Highveld Coalfields) which accounts for 84% of South Africa's coal production. However, the Witbank and Highveld Coalfields are approaching exhaustion (Jeffery, 2005; Mining Weekly, 2010). Coal mines in South Africa are responsible for high levels of indirect and direct employment with approximately 786000 people directly employed in coal mining alone in 2011 (Hall, 2013).

For the past 150 years coal has played a crucial role in South Africa's economy (Hancox and Götz, 2014). Currently bituminous coal is the main energy source for the generation of household electricity. It is also the main supplier for the production of a significant percentage of the country's liquid fuels (Hancox and Götz, 2014).

Coal plays a crucial role in South Africa's energy-economy as it accounts for 70% of primary energy consumption, 90% of electricity generation and 30% of petroleum liquid fuels (Eberhard, 2011). However coal production and its usage also results in a number of serious environmental impacts. Coal production and usage is one of the world's most 'emission intensive' environmental hazards, emitting approximately 400 million tons of carbon dioxide a year (representing about 1% of global emissions) (Mining Weekly, 2010). Greenhouse gas emissions, water pollution and acid-mine drainage are a result from coal mining.

Therefore a range of interventions, known as clean coal technologies, are being developed in the effort to mitigate environmental pollution. Through beneficiation, clean coal technologies have the potential to optimise coal, and large quantities of coal fines discharge, for economic and energy purposes (Mining Weekly, 2010). The main challenges facing South Africa's primary energy source (coal) are; clean coal technologies, the cost and quality of coal, environmental considerations, sustainable development, the growth of the South African economy and the Governments' regulations for the electricity generation industry (Jeffrey, 2005).

Coal washing is essential as it removes impurities, improves the quality and price of the coal while also reducing eventual emissions. Several methods are available for cleaning coal that is distinguished by the difference in density between coal and other denser rock. The fines have to be cleaned by floatation (International Energy Agency, 2014). The most common method used to clean coal is by dense media separation (commonly magnetite based), where the denser rock falls to the bottom

while the less dense coal floats and is then removed for drying. However, regardless of which method is used to wash the coal, coal washing consumes both energy and water. This adds cost to the producers' cost. For example, China's coal washing contributes to 18% of the entire nation's water usage. It is the second largest source of water usage after agriculture (International Energy Agency, 2014).

Ash is commonly known as the inorganic material or impurity-forming part of the coal (International Energy Agency, 2014), while the other impurities emerge during the mining process. The proportion of ash to coal varies, less than 10% in high quality coal to greater than 40% (International Energy Agency, 2014). Coal ash has several negative aspects such as; it increases the transport cost per energy unit (because the ash is in transit is part of the coal), it cuts the power plant efficiency by hindering heat transmissions due to its difficulty with fly and bottom ash removal. High ash content also results in additional pollutants, while the lower coal burning efficiency increases carbon dioxide emissions.

The removal of the ash through the washing of coal will enhance the quality of the product, thus increasing its value, saving on transportation costs and promoting cleaner coal usage with a positive impact on plant productivity and emissions in the environment. As most of the coal is used for the generation of power, reducing the ash content is crucial. However due to the structural complexity of coal, the washing of coal can be a considerable problem. Bioprocessing is an alternative procedure which can be used adequately for the production of clean coal by floatation or flocculation (Raichur and Vijayalakshmi, 2002).

Both opencast and underground coal mines require washing of the coal in a coal preparation plant. Many sectors in the coal market have very specific requirements with regards to both quality and size. Therefore it has resulted in different levels of preparation and development of specific types of coal preparation plant designs (Peatfield, 2002).

2.4.1 Coal Preparation Plant

Coal preparation is regarded as the processing of raw coal to a suitable size, producing marketable products and waste using methods that do not destroy the physical and chemical identity of the coal. The coal preparation plant (CPP) is a series of operations that are interconnected by a material handling system or a slurry

transport system (Albrecht, 2009). The design and operation of a CPP is governed by the inherent quality of the raw coal that needs to be improved to market specifications and the saleable tonnage requirements.

CPP is necessary because the finely-mined coal contains an assorted mixture of organic (carbonaceous) and inorganic (mineral) matter. The inorganic matter consists of non-combustible resources for example; shale, slate and clay. The existence of surplus surface moisture decreases the heating rate, therefore it can amount to freezing and handling concerns for consumers. CPP makes it important to acquire coal specifications by alleviating impurities from run-of-mine (ROM) coals before shipment to power stations. ROM is coal that is straight from the ground and is produced by mechanized operations that may include particles as minute as powder and as big as hundreds of millimetres (World Coal Institute, 2005). ROM often contains unwanted impurities such as rock and dirt and comes in a mixture of different sized fragments however coal users need coal of consistent quality. CPP treats the ROM coal to ensure consistent quality and to enhance its suitability for end users, however the treatment is dependent on the coal properties and its intended use.

2.4.1.1 CPP Process

The coal washing process differs between plants however it can be divided into four basic phases; preliminary preparation, fine coal processing, coarse coal processing and final preparation (Gluskoter, 2009). Modern CPP's include a complex variety of solid- liquid and solid-solid separation procedures. Such procedures mitigate surplus impurities, for example ash, sulphur and moisture from ROM (unprocessed coal), therefore feed-sticks are needed to enhance the utilization of coal properties (Gluskoter, 2009). Current industries comprise of up to four spate processing circuits for treating the coarse (≥ 10mm), small (1mm ≤ 10mm), fine (1mm ≤ 0.15mm) and ultrafine (≥ 0.15mm) material (Gluskoter, 2009).

There are several stages in a normal CPP, these being; crushing, screening, gravity separation, jigs, spiral, dewatering, thickeners and the waste products pumped to a tailings dam. In the CPP the ROM coal goes through several operations including washing, wet screening, sedimentation and dewatering (Das *et al.,* 2006). The CPP

requires a vast quantity of water from which large volumes of waste water containing a variety of solid particles are generated.

In the preliminary phase of the CPP, the unprocessed coal is offloaded, stored, conveyed, crushed and differentiated by screening into fine and coarse fractions. These sized particles are subsequently dispensed to their relevant cleaning processes (Gluskoter, 2009).

The first process in the CPP is dewatering, from which an important fraction of the water is separated by the usage of thickeners, screens, and cyclones. The final CPP process is removing the moisture from coal, thus reducing freezing and weight problems as well as raising the heating value.

2.4.1.2 Environmental factors

CPP improves the environmental suitability of coal by reducing the contaminations that could be altered into a dangerous gas or particulate pollutants when heated. Impurities normally comprise particulates (fly ash), sulphur dioxide (SO_2) and additional air toxins such as mercury (Gluskoter, 2009). Coal preparation is favorable towards the decreasing of greenhouse gas emissions by increasing the thermal efficiency of coal fired boilers. The existence of impurities could also impact the stability of coal for high end users, therefore CPP needs to reach the high levels of coal purities required by the secondary markets (Hardman and Lind, 2003).

Coal preparation is crucial to reduce the discharge of pollutants associated with the mineral matter found in coal. The emissions from the initial phase of the CPP of either a dry or wet process consists mainly of renegade particulate matter (PM) such as loaded railroad cars, coal dust, refuse areas, stock piles, conveyor belt pour-offs, crushes and clarifiers (Gluskoter, 2009). The primary control method uses water to suppress the mentioned emissions.

Several of the following inorganic harmful air pollutants are established in trace quantities in coal: uranium, thorium, mercury, manganese, lead, copper, chromium, cadmium, beryllium and arsenic. It is probable that most of these are emitted in small dosages from grinding, crushing and drying operations (Albrecht, 2009).

There are several attractive economic and environmental benefits with regards to coal preparation such as enlarged coal reserves, decreasing transportation costs,

improvement in the usage of properties and abatement of pollution (Gluskoter, 2009). Coal preparation will always be crucial to the cost, recovery and quality of the coal produced. Nonetheless advancements in separation technology and practices are essential to enhance additional reduction in waste water generation and downstream environmental impacts. Technology advancement may generate revenue from the recuperation of utilizable coal from waste water streams, which may offer a monetary enticement for the private sector to invest in these activities (Gluskoter, 2009).

2.4.1.3 Coal fines

Coal fines are a result from the crushing (roll crushers) grinding and sizing steps in the CPP. The fine coal streams are traditionally dewatered in equipment such as thickeners, spirals, cyclones, filters and centrifuges (Slottee, 2007).

The cleaning of coal fines was at one stage not practiced extensively in South Africa (England *et al.,* 2011). The mining and processing of coal to provide a product to meet market requirements results in the formation of coal fines (Hardman and Lind, 2003). Coal fines are less than 1mm but greater than 0.15mm in particle size, however some mines have problems with coal up to 6mm in particle size, known as duff coal, while some mines washing coal for export and local markets experience problems with coal size less than 0.15mm known as superfines (Hardman and Lind, 2003). Due to the increase in fine coal production (as a result of mechanised mining), coal mines need to utilise as much of the mined coal as possible as quick as possible. There is an increase in attention towards the beneficiation of this material. The primary problem that arises from the generation of small size coal is the reduction in profits. Coal that cannot be sold because it does not meet customer size requirements had to be discarded yet had already undergone the expense of extraction, coal handling, coal preparation and production (Harman and Lind, 2003). Coal that is too large for customer requirements can always be crushed and the smaller coal created can be sold to another customer. The recreation of larger coal from fines is difficult, although the process of briquetting fine coals is a future plan (Harman and Lind, 2003).

The cost of washing the small sized coal is greater because of the processes used and the product losses that occur resulting in a lower recovery of a saleable product.

Another problem associated with coal fines is the moisture retention, which poses downstream disadvantages due to handling difficulties and the reduction in heating values (Hardman and Lind, 2003). There are several methods that can be used to treat coal fines, such as:

- Spiral beneficiation
- Froth flotation
- Oil agglomeration
- Dense medium beneficiation (England *et al.,* 2011).

The most complicated and costly operations within the CPP are the processing of coal fines (Sabah and Cengiz, 2004). Coal fine cleaning technology improvement has resulted in several commercial procedures that wash coal fines based on size, density or surface features (Harrison and Akers, 1994). There is not a deficit of cleaning technologies for the processing of waste coal fines, however the problem is choosing the most cost-effective technology. The main economic and technical obstacle for the progress of waste coal fines is dewatering. If the slurry waste fines are ultrafine, normal dewatering apparatuses could be incapable to recuperate sufficient marketable product to validate a project investment. For instance thermal drying is theoretically affordable in this instance. However it is usually excessively priced and difficult to permit in many instances. There is also concern that the fine coal can pick up moisture again during transportation and stockpiling (Harrison and Akers, 1994). The price of energy from coal fines is motivated by the price of coal fines, the environmental costs, the quantity of the recovered coal fine materials that is usable and the cost of cleaning and formulation.

The increase in mechanised mining has resulted in an increase of the amount of fine coal generated, many mines report up to 6% of the ROM coal as being in the minus 200µm fraction (Harman and Lind, 2003). Apart from the coal fines being difficult and costly to wash, airborne (less than 0.1mm) and respirational (less than 7µm) coal dust will result in atmospheres that are explosive and dangerous to the employee's health if not sufficiently removed or diluted (Harman and Lind, 2003). Therefore efforts to mitigate coal fines to increase saleable products will automatically reduce airborne and respirational dust fractions and create a safer work environment. Coal fines can accumulate on the floor and becomes sludge in the presence of water

(ground water, rain, or water from a dust suppression system). Sludge reduces friction and miners that rely on the floor for traction are unable to generate maximum forward momentum which results in poor pick penetration during the sumping operation (Hardman and Lind, 2003).

The settling velocity of coal fine particles by interaction with appropriate coagulants and flocculants is crucial in the dewatering process (Das *et al.,* 2006). Microorganisms can be used for the separation of mineral matter from coal as well as for settling or removal of coal fines from the refuse ponds which is a key environmental problem (Raichur and Vijayalakshmi, 2002).

The coal fines generated throughout the mining processes are discarded by a thickener (in which a flocculant is added for a slurry water separation) and the underflow is discarded into a tailings dam.

2.4.1.4 Thickener

The primary objective of a thickener is to produce a clarified overflow and to increase the tailings' concentration/density in the underflow from the feed stream. Thickeners with the addition of flocculants are crucial for solid-liquid separation which is utilized for the treatment of processed water. Once processed it can be recycled and returned back to the industry (Gluskoter, 2009). The density of the tailings slurry underflow will determine the rate in which it is discarded into the tailings dam.

Thickener plant: Thickeners comprise of a large container where particles in suspension are able to settle by gravity, resulting in a clarified over flow and thickened underflow. The clarified over flow can be used for reuse of the processed water. The slurry disposal is normally discarded into an appropriate disposal area or is additionally dewatered before disposal. Thickeners are sized according to the volume of feed slurry to be processed, however all thickeners have a few basic components such as a tank which contains slurry, feed piping and a feed well which will allow the feed stream to enter into the tank, a rotational arm which displaces the concentrated solids to the outlet, an underflow solids withdrawal system, and an overflow (Figure 2) (Metalliferous Mining, 2007; Slottee, 2007).

Several modifications of continuous thickeners have occurred resulting in the development of a vast variety of organic polymeric flocculants. Conventional and

high-rate thickeners are the two basic types of continuous thickeners. They can be distinguished by the way in which they are built.

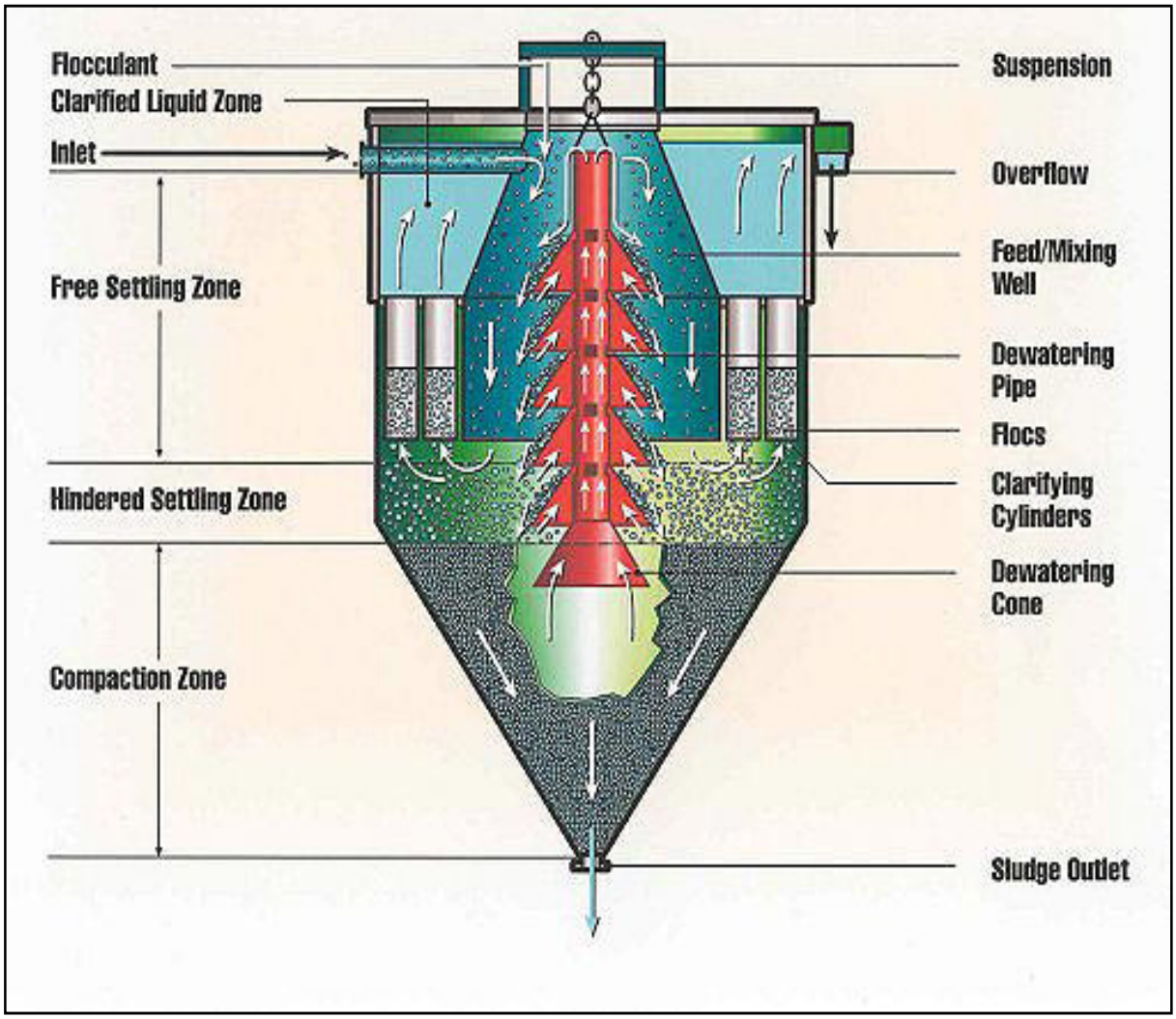

Figure 2: A typical thickener used in the coal mining industry (Mining Technology Market, 2014)

Chemical usage: Chemicals like coagulants and flocculants are normally introduced prior to entering the thickener to enhance the aggregation of coal fines, thus accelerating the settling rate (Gluskoter, 2009). The quantity of flocculant added into the thickener depends on the type of ore (Metalliferous Mining, 2007). The harder the ores, the coarser the size distribution, thus less flocculant is required. Individual particles are heavier and will therefore settle quicker. Soft clay ores often have a

very fine size distribution and because singular fine particles settle extremely slowly, they therefore require larger quantities of flocculants to be added.

Control: The thickener control has several complexities such as varying feed characteristics. The changes in feed concentration, solids' specific gravity, particle size distribution, pH, temperature and the reaction to flocculant are the parameters that can contribute to the variations in a thickener's performance (EIMCO, 2002).

A thickener is run under a controlled environment. A thickener must be operated and controlled within the restricted parameters to obtain the required end result. In order to select the correct operating protocols or control measures it requires a thorough understanding of how each unit functions and the significance of the variables involved (Metalliferous Mining, 2007).

Problems: The majority of thickener operational problems result from a poor understanding or knowledge of the below basic concepts. If the fines accumulate inside the thickener without corrective action, the following is likely to occur (Metalliferous Mining, 2007):

- Together with the overflow, pulp will begin to exit the tank
- Underflow becomes too dense to pump
- A 'doughnut' will form inside the thickener resulting in the feed density being approached by the underflow density
- The rake mechanism will be overloaded and stopped by the control

2.4.1.5 Flocculation

Synthetic or natural flocculants are commonly used in the CPP. Synthetic polymers can be similar to natural polymers when compared to the miniscule quantities utilized for the solid-liquid separation. In order to accomplish the required settling rate and water clarity values the optimization of the process parameters such as suspension, pH, polymer type and dosage are crucial. Various combinations of cationic and non-ionic flocculants have been used to achieve the highest settling rate with the lowest turbidity (Das *et al.,* 2006). The management and discarding of the waste slurry is said to be an extremely complicated challenge that faces the CPP industry (Gluskoter, 2009).

The velocity of settling of coal fines can be increased by increasing the quantity of flocculating agents. The correct quantity of flocculant required for effective flocculation is the most crucial element when operating a high rate thickener however the quantity of flocculant added is dependent on the ore (Metalliferous Mining, 2007). Flocculation may be activated by several methods, such as; the addition of flocculating agents, magnetic flocculation and centrifugal forces.

With regards to water clarification and thickening, there are three conditions that are desirable for effective processes in settling sediments, (England *et al.,* 2011):

- Particle size
- Composition
- Mixing

In a slurry-water separation from CPP tailings, by means of flocculation from the amount and type of flocculant used, the composition of the fines must be known to develop an efficient and feasible sedimentation system within the tailings dam (Sabah and Cengiz, 2004).

2.4.1.6 Waste water effluent

Environmental concerns, resource management and access to water are some of the key national issues for mining industries. The majority of the waste accumulated by the mines is from waste rock, tailings and over burdening.

The accumulation of mine water results in water discharge, regardless of whether the mine is opencast or underground. It is essential that the water be pumped or drained out of the mine to guarantee safety and stability (Doll, 2012). More than 70% of the pollutants emitted from the coal mining industry can be found in the water, the extraction of these contaminants before discharge or reuse has started to receive significant attention by mines and researchers (Doll, 2012). Water and waste water treatment has become a key focal point of mine operations which is altering the landscape of site water management and treatment.

2.4.1.7 Recycling of water

The recycling of water can occur in many different segments within the CPP, such as from the dewatering in the magnetic separator, gravity thickener, cyclones, wet

screens, surface water from a tailings dam (that has been treated) and the overflow from thickeners. An appropriate dewatering method, for fines, usually by sedimentation within a thickener makes the reuse of vast quantities of clarified water in a CPP achievable (Sabah and Cengiz, 2004).

Depending on the accessibility and quality of the water, it could be reused in different sections of the mine. Water is reused for make-up water, dust suppression or mill operations, leaching, steam generation, grinding, magnetite water and used to wash coal (Doll, 2012).

It is crucial that the recirculation of water is of good quality for the smooth functionality of the plant. If suspended and dissolved solids are present in waste water it may result in the reduction in the effectiveness of the washing process (Das *et al.,* 2006).

A minute quantity of fresh water from an external source (river, streams and dams) is normally needed to provide a satisfactory equilibrium between the moisture contents of solids incoming and leaving the plant. Recycling and clarification of processed water provides an efficient solution to mitigate the fresh water demands and decreasing environmental impacts (Gluskoter, 2009).

2.4.1.8 Tailings dam

According to the Sabah and Cengiz, (2004) a tailings dam is a dam made to retain the water-sodden and fine-grained material (tailings) which is the waste product from the mineral processing plant. There are several types of tailings dams such as the valley impoundment, in-pit impoundments, dug pits and ring dikes (Franks *et al.,* 2011). The most common tailings dam used in the mining industry is the Valley pond, which uses a natural topographical depression in the ground to its advantage. Exploited open pit mines can be replenished with tailings; however a lot of consideration must always be taken into account, to avoid impurities in the underlying water table in the midst of other issues (Frank *et al.,* 2011)

Coal fines slurry are complicated to dewater therefore it is discarded into the tailings dam in the form of a slurry. Slurry consists of silt, water, clay, coal fines and other fine particles from the CPP (Luttrell, 2008).

Discard slurry: The underflow from a thickener (tailings slurry) is pumped into a tailings dam and the tailings slurry is left to settle further so that the cleaner water on the surface can be pumped out from the tailings dam into several dams. There the water will be treated and the water quality specifications of the mine will be adhered to before being pumped back and reused in the CPP. The abstraction of water creates a better storage system but it also assists in water recovery which is a great help in light of the current drought conditions (South African Government, 2015).

Environmental concerns: The disposal of the fines waste slurry (tailings) has become a sensitive environmental issue (Kossoff *et al.,* 2014). If the tailings dams aren't constructed correctly, monitored regularly and preserved, it could result in greater safety and environmental concerns. Possible problems are seepage, structural malfunctions, acid drainage, piping, overtopping and inadvertent spillage of processed water that contains particular matter (Gluskoter, 2009). Tailings dams are also hazardous as they attract wildlife, especially waterfowl, because they can appear to be a natural pond, however they are usually extremely lethal and detrimental to the health of these animals (Franks *et al.,* 2011). Tailings dams must be environmentally sound and it is extremely beneficial to maximize water reclamation before pumping the slurry into the tailings dam.

The tailings effluent from coal mining is one of the largest environmental liabilities. Vast quantities of pyrite and pyrotite, which are discarded from the coal ores, are contained in the tailings effluent. Although these minerals are harmless underground, they are highly reactive towards air in the presence of microorganisms, resulting in acid mine drainage (Nehdi and Tariq, 2007).

Size of the tailings dam: The volume of a tailings dam must be big enough to make sure that the fine particles can settle by gravity, so that the clarified water on top can be recycled and reused into the mine. However, chemical coagulates are often added to accelerate the settling and controlling of the pH (Luttrell and Honaker, 2012; Gluskoter, 2009).

2.4.2 Use of bioflocculants to settle fines in a coal mining industry

Due to the high cost of producing bioflocculants on an industrial scale, bioflocculants have not been used extensively in the mining industry. A more effective water slurry

separation can also help promote the reuse of the discarded fines to produce a marketable product, such as bricks.

El-Midany and Abdel-Khalek (2014) used several bacterial species to modify mineral surfaces and enhance their separation in the presence or absence of regular collectors. In most cases of coal treatment, bacteria were used either as a coal flocculant or as an impurity depressant. One of the bacteria used as a flocculant was *Mycobacterium phlei* (*M. phlei*) and it was able to flocculate coal fines because of the hydrophobicity of *M. phlei* and its highly negatively-charged surface. The other bacterium used in the bioflocculation process of high-ash Indian coals was *Paenibacillus polymyxa (P. polymyxa*) which showed a reduction in ash content by 60% (El-Midany and Abdel-Khalek, 2014). Flocculation of coal fines using the bacterium *M. Phlei* was highly effective in the reduction of both ash and pyritic sulphur content (El-Midany and Abdel-Khalek, 2014).

A study by Vijayalakshmi and Raichur (2002) also used *P. polymyxa* to flocculate high-ash Indian coals. The flocculation results using *P. polymyxa* demonstrated that the bacterium flocculated coal effectively and efficiently with 90% of the coal flocculating in approximately a minute at a pH of 2. This study suggests that flocculation of coal using bioflocculants is plausible. The selectivity of bacteria to coal is determined by the differential adhesion and its associated minerals. It was demonstrated that the flocculants from a biological source tend to be more effective than synthetic flocculants (Vijayalakshmi and Raichur, 2002). Flocculation with synthetic flocculants is dependent on the coal type thus affecting the separation of the mineral matter from coal. To-date no studies have been done on how the type or source of coal affects the adhesion of bacteria to its surface, hence enabling bioflocculation to occur (Vijayalakshmi and Raichur, 2002).

2.5 TCM background

TCM has a Memorandum of Understanding (MoU) with the Department of Hydrology at the University of Zululand to conduct this study. TCM is an open cast mine therefore in this study opencast mining was the focus of the mining methodologies utilized.

TCM is one of South Africa's leading producers of metallurgical anthracite. The mine is situated 85km northwest of Richards Bay in Kwazulu-Natal and is operated by Petmin's wholly-owned subsidiary TCM (Petmin, 2015). After commencement of the second coal wash plant in March 2012 worth R162 million, TCM has a 20 year life span for mining with an annual production of more than 1.2 million saleable tones (Petmin, 2015).

TCM is an opencast mine as the geology of the seams are near the surface. Open cast mining recovers a higher proportion of the coal deposit compared to underground mining as all the coal seams are exploited.

Mining operations at TCM, currently, cover approximately 10% of the 23000 hectares of land of which Petmin has the prospecting authority (Petmin, 2015). The applications for the mining license for the remaining land were submitted in 2013. More than 950 jobs were created by Petmin at TCM since the commissioning of the mine from a Greenfield site in 2007 (Petmin, 2015). TCM's yield was further increased in 2013 with the commission of an additional third of the plant to be installed to create an energy product from discard. This plant has the capability to produce 480000 tons of coal per annum (Petmin, 2015).

Chapter 3: Methodology

3.1 Isolation and identification of microorganisms with bioflocculant production potential

3.1.1 Sampling locations

The sample locations were chosen near to or at the river mouths as these areas are believed to have excess suspended particles. Microbes (with bioflocculant properties) thrive in such environments to flocculant suspended materials (Divakaran and Sivasankara, 2001). Water and sediment samples were collected at random along the North Coast of Kwazulu-Natal at the following locations (Figure 3);

a) Richards Bay Harbour; 10 water samples were taken at random selection in the harbour.
b) Richards Bay Pelican Beach; 5 samples were taken off-shore at 1 meter intervals at 0m, 1m, 2m, 3m, 4m.
c) Mtunzini Beach: 3 sediment and 3 water samples were collected at 0m, 10m and 20m intervals from the shore.
d) St Lucia Estuary, 3 water samples and 1 sediment sample were taken at 0m, 10m and 20m intervals from the shore
e) Sodwana Estuary: 4 water samples and 1 sediment sample were collected in the estuary.
f) uMthlatuze River flood plain in close vicinity to the University of Zululand: 2 ponds and 1 river were selected for sampling.

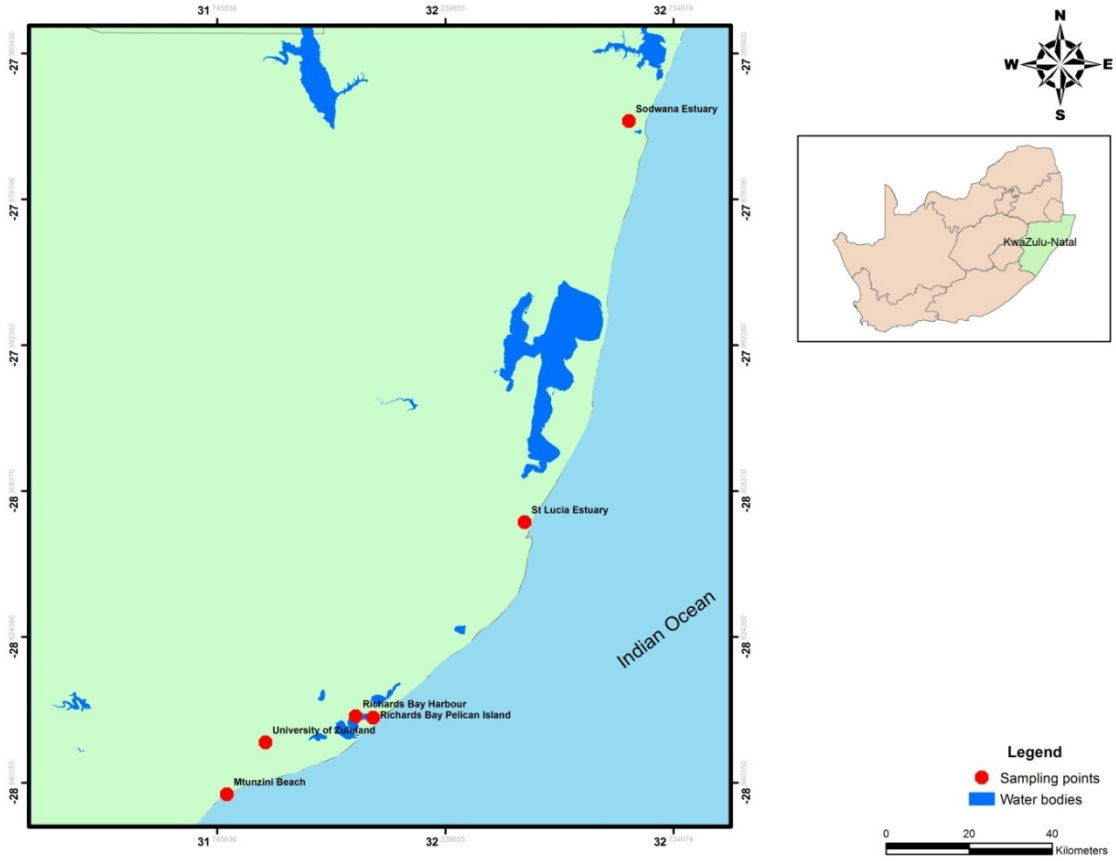

Figure 3: Sampling sites

3.1.2 Sample collection

Samples were collected from the sites using 250ml aseptic plastic bottles. The sample bottles were transported inside a cooler-box within 24hours to the laboratory to the Hydrology Department, University of Zululand.

3.1.3 Cultivation media

All samples were cultivated on Nutrient Agar plates except the Mtunzini Beach and Richards Bay Harbour samples where modifications were done. Different methods were used to isolate and cultivate bioflocculant microorganisms from the marine environment. This was to ensure the isolation of a bioflocculant. The cultivation of bacteria microorganisms from the Mtunzini Beach samples were initially isolated on:

- Nutrient Agar
- Yeast Extract Agar (YEA)
- Potato Dextrose Agar (PDA)
- Shabouraud Detrotex 4% Agar (SDA)

From all the above Agars used, Nutrient Agar showed the best results.

The Richards Bay Harbour samples were grown in 5 cultivation carbon broths, namely;

- Glucose
- Maltose
- Galactose
- Fructose
- Sucrose

From which they were all plated on Nutrient Agar plates. The simple formulation of Nutrient Agar provides nutrients such as carbohydrates, vitamins and organic nitrogen compounds, particularly amino acids.

The approximate formula for making up a 1 litre mixture with marine water is shown in the table below:

Table 1: Nutrient Agar composition per litre of marine water

Ingredient	**Mass (g/l)**
Meat extract	1.0
Peptone	5.0
Yeast Extract	2.0
Sodium Chloride	8.0
Agar	15.0
Marine water	1.0 litre

3.1.4 Isolation of the microorganisms

50µl of the water samples were inoculated onto the various agar plates and spread with a flamed alcohol-sterilized glass rod. The sediment samples were inoculated onto various agar plates using the dry stamp method with slight modification (Jensen *et al.,* 2005).

The dry stamp method is the process where the sediments were dried in a laminar flow hood. An autoclaved foam plug (approximately 2cm in diameter) was pressed onto the dried sediment and then pressed onto the different agar plates, repeatedly around the plate in a clockwise direction creating a serial dilution effect.

50µl of the Richards Bay Harbour samples were inoculated into the various carbon broths. 10 Test tubes were used individually for the following carbon sources:

- Galactose
- Maltose
- Fructose
- Glucose
- Sucrose

After inoculation, the test tubes were placed in a rotary incubator and incubated at 30˚C at a shaking speed of 160rpm for 72 hours. The broths were then observed for growth. 50µl of the inoculated broth were pipetted on Nutrient Agar plate, spread using an alcohol sterilized glass rod, parafilmed and incubated at 30˚C for 72 hours. After the incubation period the plates were analysed for growth.

3.1.5 Purification and identification of the bacterial organism

Pure cultures of the organism were prepared by streaking (using the 4-way streaking method by Rosenberg and Weiss (1986). Individual colonies were streaked on fresh Nutrient Agar plates and incubated at 37˚C for 48 hours. Isolation and identification were done on the basis of colony morphological and cultural characteristics. The observations of the morphological characteristics were based on colony colour, form/configuration, margin, elevation, pigmentation colour (if present) and the size of the colony.

3.1.6 Identification using the 16S rDNA gene

Isolates which showed good bioflocculant producing potential were subjected to molecular identification using their 16S rDNA gene. The isolates were then stored in the culture collection of the Department of Biochemistry and Microbiology, University of Zululand.

3.2 Screening for bioflocculant production

3.2.1 Production media and culture conditions

The composition of the production media was composed according to Zhang *et al.* (2007) with a slight modification (distilled water was replaced with marine water). The composition of the media's (1000ml of seawater) are shown in Table 2:

Table 2: Bioflocculant pre-culture composition

Ingredient	Volume (grams/litre)
Glucose	20
Yeast extract	0.5
Urea	0.5
$NH_4 2SO_4$	0.2
K_2HPO_4	5
KH_2PO_4	2
NaCl	0.1
$MgSO_4.7H20$	0.2
Marine water	1 litre

Two loops of bacterial colonies were inoculated in 50ml of the bioflocculant production medium contained in a 100ml conical flask. Fermentation was carried out in an incubator at 30˚C for 72 hours, rotating at a speed of 160rpm. The production medium without the inoculated organisms was used as a control.

3.2.2 Screening of microorganisms for flocculating activity

After the incubation period, 2ml of the fermentation broth was centrifuged (8000g, 30min) to separate the cells and the cell free supernatant, which in turn determined the flocculating activity.

The flocculation activity tests were done according to Kurane *et al.* (1994) with slight modifications. 3ml of 1% (v/v) $CaCl_2$ and 2ml of cell-free supernatant were dispensed into 100ml of kaolin clay solution (4g/l) in a 250ml flask. The mixture was mixed enthusiastically and dispensed into a 100ml cylinder and left at a standpoint for 5 minutes. The control medium of the flocculation activity tests was assayed.

Flocculating activity was calculated with the following formula:

$$\text{Flocculating activity (\%)} = \frac{(A - B)}{A} \times 100$$

A = Optical density at 550nm (OD_{550}) of the control,

B = Optical density at 550nm (OD_{550}) of a sample.

Microorganisms that showed flocculating potential above 60% were selected for optimization and were kept in the freezer at -40˚C to preserve the microorganisms and to prevent contamination.

Once all the selected microorganisms had been identified, the flocculating activity was tested using the mine slurry (instead of kaolin clay),a product of the coal wash mine collected before entering the thickener, to determine which microorganism had the best flocculating potential in the slurry. The microorganisms that showed the greatest flocculating potential were selected and further testing was done to optimize the microorganism using the tests stated below.

3.3 Optimization of bioflocculant producing microorganism

3.3.1 Inoculum sizes

As described by Zhang *et al.* (2007) and Wang *et al.* (2007) inoculum size is a crucial parameter for the production of bioflocculants. The result of various inoculum sizes on the production of bioflocculants is thus fundamental. The inoculum size methodology was done according to Ugbenyen *et al.* (2014) where 50ml of the production medium was dispended into 150ml flasks and separately inoculated with a previously identified microorganism that had shown flocculating potential at 0.5ml, 1.0ml, 1.5ml, 2ml, 2.5ml, 3ml, 3.5ml, 4ml, 4.5ml and 5m (amounting to 1%, 2%, 3%, 4%, 5%, 6%, 7%,. 8%, 9% and 10% (v/v) respectively).

This pre-culture of the bacteria was cultivated at 30°C with a shaking speed of 160rpm for 72 hours. After the incubation period, the fermentation broths were centrifuged (8000g, 30 minutes) to separate the cell and the free cell supernatants and then examined for flocculating efficiency.

3.3.2 Carbon sources

The microorganism that showed the best flocculating potential was tested using various carbon sources to optimize the flocculating potential according to Zhang *et al.* (2007). The Zhang *et al.* (2007) method was used to alter the carbon sources of the bioflocculant producing media. The volume producing the best results from the inoculum tests was inoculated into the 50ml of the various carbon bioflocculant producing media.

The inoculated samples in the various carbon sources were sealed, placed in the incubator and shaken at 30˚C and 160rpm, respectively, for 72 hours. Thereafter the flocculation tests were conducted.

The microorganism identified only ferments certain carbon sources, therefore the carbon sources used to inoculate were identified by Rawhai *et al.* (2014) and de Vreis *et al.* (2005). Thus the carbon sources used were; glucose, maltose, fructose, starch and sucrose

All the various carbon sources used (as stated above) were also tested using various concentrations from 1% to 4% (10g/l to 40g/l respectively). The various concentrations of different carbon sources were substituted in the bioflocculant media as described by Zhang *et al.* (2007). These samples were also placed in the incubator and shaken at 30˚C and 160rpm, respectively, for 72 hours. Thereafter the flocculation tests were done.

3.3.3 Nitrogen sources

The bioflocculant production medium was prepared using Zhang *et al.* (2007), however the nitrogen sources were altered. The soluble nitrogen sources used as a replacement in the bioflocculant production media were; peptone, casein, urea, ammonium sulphate and ammonium molybdate

From the inoculum tests, the volume producing the best flocculating results was inoculated into 50ml of all the various nitrogen bioflocculant production medias. The various inoculated nitrogen pre-cultures were sealed, placed in the incubator and shaken at 30˚C and 160rpm respectively for 72 hours. After the inoculation period, the flocculation activity potential for the various nitrogen sources was tested.

3.3.4 Cation variations

The cation variation effect on bioflocculant was assessed in accordance with Liu *et al.* (2010) and Kurane *et al.* (1994). The flocculating activity tests were assessed using previously grown flocculating potential microorganisms however the $CaCl_2$ was replaced with various cation chlorides, after which the flocculating potential was tested and compared to the original results. The various cations used were; Li^+, Na^+, K^+, Mg^{2+} and Fe^{2+}.

3.3.5 pH

The effect of pH on bioflocculant activity was adhered to according to Liu *et al.* (2010) and Yim *et al.* (2007). The bioflocculant producing culture was prepared using Zhang *et al.* (2007) however the pH of the cultures were changed by adding either sodium hydroxide or hydrochloric acid to either decrease or increase the pH respectively. The pH was measured from 1 to 10 using a pH meter. After which the media was autoclaved, the relevant inoculum size was added to each pH media and taken to the incubator shaker and left for 72 hours at 160rpm. After the inoculation period, the flocculation activity potential for the various pHs was tested

3.3.6 Time course assay

The time course assay was conducted according to Cosa *et al.* (2011), Zhang *et al.* (2007) and Ugbenyen *et al.* (2012). The bioflocculant producing media for the time course assay was determined by utilizing formerly identified determined optimal growth conditions. The bioflocculant producing microorganism was inoculated into the media and was placed in the shaker (160rpm) at 30˚C over 108 hours. This was sufficient time to accommodate the 4 phases of a bacterial growth curve (lag phase, log phase, stationary phase and death phase), the log phase and stationary phase is where the bacteria is being produced.

Samples were taken every 12 hours over a 5 day period and from this 2ml were centrifuged (8000g, 30 minutes) and the cell free supernatant was utilized to calculate the flocculating efficiency at an OD_{550} in the spectrophotometer.

3.4 Application of the produced and purified bioflocculant in settling coal and wash plant fines

The microorganism showing the greatest flocculating potential was tested on the mine slurry. However a site visit to Tendele Coal Mine (TCM) was planned first before any further pilot studies could be carried out.

3.4.1 Site visit

A tour of the mine was conducted by the TCM plant superintendent. This site visit was conducted to understand the thickener processes and to try and simulate these conditions in a laboratory situation. From the site visit the following was observed about TCM:

- It is an opencast mine
- Most of the fines produced are a result of the coal beneficiation process
- The beneficiation process flow has 3 plants that aid the coal making process
- Every plant has its own thickener
- All thickeners follow the same procedure, where premixed Floxit 5028 is dosed into the thickener, the effluent is reused within the plant and all slurry gets pumped to the tailings dam (in a 140mm diameter pipe)

The operation as a whole is versatile and produces a variety of anthracite products that can be ordered to client specifications as well as to meet market requirements. Plant 1 and 2 have the same process, the only difference is Plant 2 is newer. Each one of these plants has the capacity to produce dedicated anthracite products but in addition to this, the TCM 3 plant, is equipped to produce steam coal. From the site visit it was evident that Plant 1 was the only plant that had an access point to the slurry prior to entering the thickener, thus all the TCM slurry utilized in this study was from Plant 1. The thickeners used are the same as Figure 2 where the fines are left to settle by means of gravity.

The Floxit 5028 is stirred in a poly-preparation continuous reagent make-up and dosing plant prior to distribution into the thickener (Figure 4). The concept typically provides for a 3-compartment tank, which creates separates zone for wetting, maturing and dosing. When the dosing zone (the flocculant concentration used in the thickener) reached a minimum level, the feeder increases the flocculant level in

the wetting zone with the reagent emulsion, which overflows into the maturing zone where it displaces the matured reagent into the dosing zone. Mixers 1 and 2 continuously stir the emulsion to optimize dissolution. The reagent and water feed rates are individually adjustable to change the concentration required. Therefore to simulate the mining conditions, a mechanical stirrer was used to continuously stir the flocculants, they were left to stand for 30 seconds then tested for flocculating efficiency.

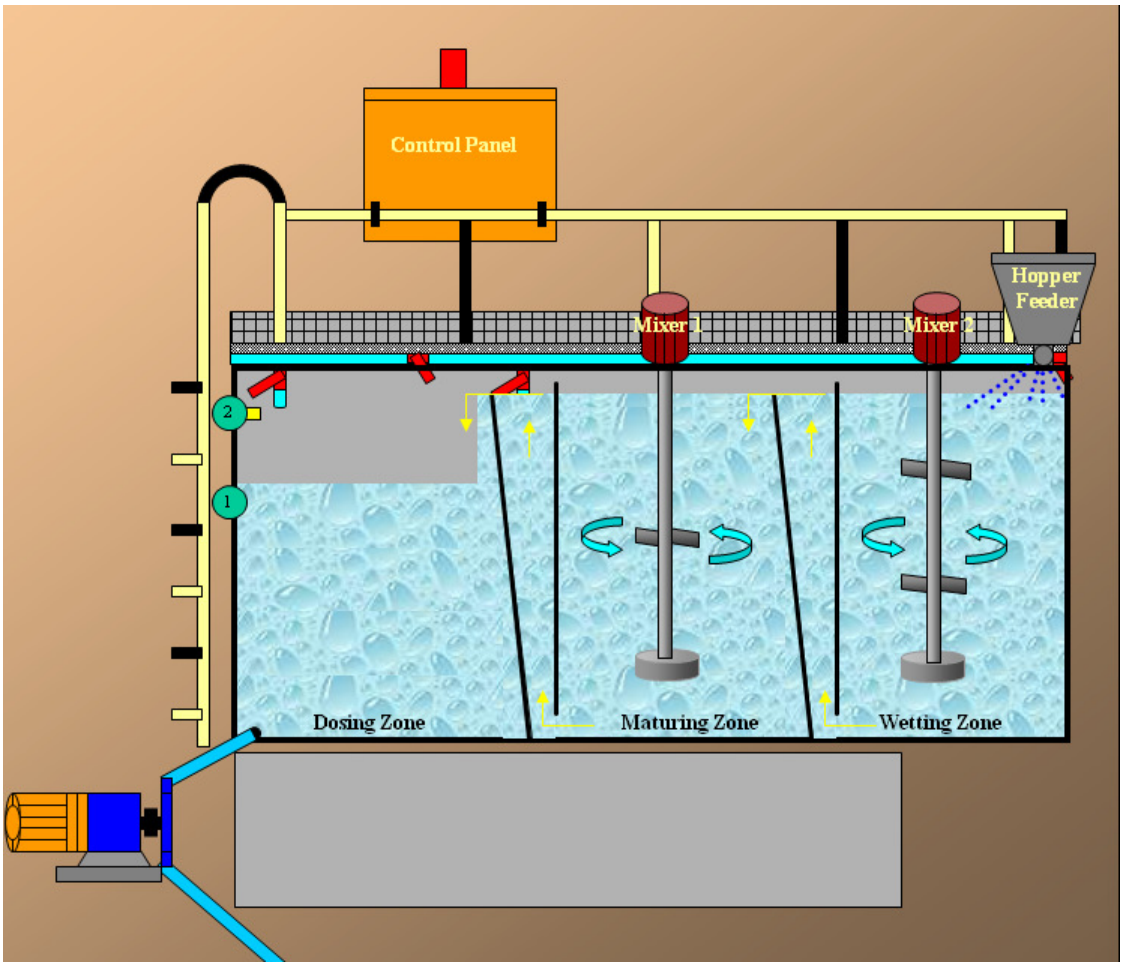

Figure 4: Poly-preparation continuous reagent make-up and dosing plant used at TCM (Somkhele Plant Overview, 2015)

3.4.2 Sampling

Samples of the coal wash plant slurry were collected at a discharge point in the coal processing plant just before the slurry enters the thickener at TCM 1 (Figure 2). These samples were collected so that the various isolated bioflocculants flocculation rate, on the slurry, could be tested. This also allowed for a direct comparison of the flocculation rate of the coal wash plant slurry and the flocculation rate of kaolin clay

suspension flocculation rate both with the use of bioflocculants.5L containers were used to collect the slurry at TCM. The samples were transported from the mine to the laboratory in the Hydrology Department, where the samples were kept under a controlled environment.

Chapter 4: Results and discussions

4.1 Introduction

The production of bioflocculants is affected by several parameters, such as inoculum size, carbon and nitrogen sources, cations and pH. Optimization of these parameters is crucial since productivity and distribution is dependent on the culture conditions (Piyo *et al.,* 2011). The effects of these parameters on the production of *Bacillus cereus* (*B. cereus*) bioflocculant from this current study are discussed bellow.

4.2 Isolation and identification of microorganisms with bioflocculant producing abilities

A total of 33 water and sediment samples were selected at random from the North Coast. After incubation of the 33 samples on nutrient agar plates, only 10 showed signs of growth of flocculating microorganisms (Table 4). These microorganisms were further streaked to obtain pure cultures of the strains, which were screened for the production of bioflocculants on the basis of kaolin suspension flocculation.

The strains that showed better production and great flocculating potential were further tested against mine slurry (Table 4). The mine slurry was obtained from the TCM coal wash plant discharge point before the slurry enters the thickener. One strain flocculated both the kaolin suspension as well as the coal mine slurry with a relatively constant and high yield with flocculating activities of 74% and 76% respectively.

The successful strain was originally isolated from the estuary at Sodwana Bay. Based on morphological characteristics and 16S rDNA sequence data analysis the strain was identified as *B. cereus.*

Table 3: Average flocculating activity for kaolin suspension and mine slurry by previously isolated microorganisms

Name of plate	Microorganism	Kaolin average flocculating potential (%)	Standard deviation (±)	Coal mine slurry average flocculating potential (%)	Standard deviation (±)
Mtunzini Plate 5	*Pantoea*	43	6.9	56	1.8
Mtunzini Plate 4	*Pseudoalteromonas sp.*	44	3.2	4	4.4
Richards Bay Harbour 5	*Bacillus pumilus*	69	6.1	37	5.5
Sodwana 10	*Alcaligenes faecalis*	31	5.2	26	7.6
Sodwana 12	*Alcaligenes faecalis*	30	6.9	22	4.2
Sodwana 26	*Bacillus subtilis*	55	3.6	21	5.3
Sodwana 27	*Bacillus cereus*	71	0.3	43	7.4
Sodwana 28	*Bacillus cereus*	34	5.8	22	0.7
Sodwana 32	*Bacillus cereus*	74	4.9	76	1.4
Sodwana 34	*Bacillus stratosphericus*	78	4.9	17	4.3

4.3 Effect of inoculum size on bioflocculant production

Table 4: Literature review for inoculum size

Author	Inoculum size (%)	Microorganism
Zheng et al. (2008)	1	*Serratia ficaria*
Gong *et al.* (2008)	1	*Serratia ficaria*
Aljuboori *et al.* (2014)	2	*Aspergillus flavus*
Cosa *et al.* (2013)	2	*Virgibacillus* sp
Okaiyeto *et al.* (2015)	4	*Bacillus toyonensis*

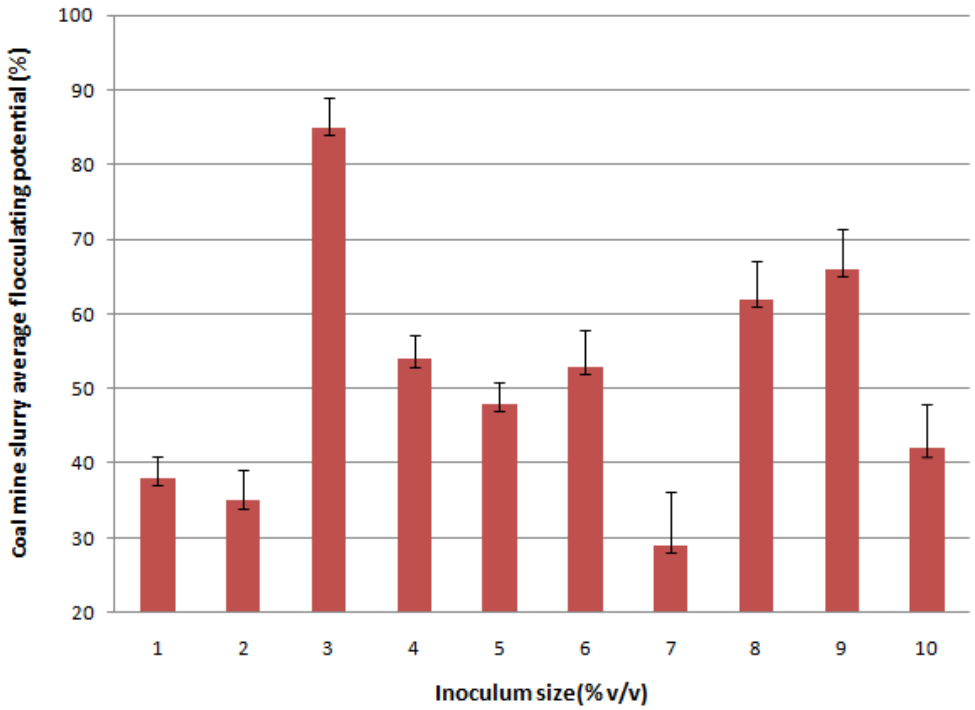

Figure 5: Effect of *Bacillus cereus* on inoculum size on the average flocculating potential of coal mine slurry

The preferred inoculum size of *B. cereus* bioflocculant production is shown in Figure 5. The greatest flocculating activity was observed for an inoculum size of 1.5ml per 50ml (3% v/v) of *B. cereus*. The flocculating potential averaged at 85%.The inoculum size results from *B. cereus,* in the current study, are similar to that from previous studies.

4.4 Effect of carbon source on flocculation efficiency

Table 5: Literature review for various carbon sources

Author	Carbon sources utilized	Preferred carbon source	Microorganism
Liu and Cheng (2010)	Glucose, sucrose, maltose, starch, lactose and molasses	Glucose	*Penicillium* sp
Piyo *et al.* (2011)	Glucose, sucrose, fructose and starch	Sucrose and glucose	*Bacillus* sp
Okaiyeto *et al.* (2015)	Glucose, fructose, sucrose, maltose, starch, sodium carbonate and phthalate	Glucose	*Bacillus toyonensis*

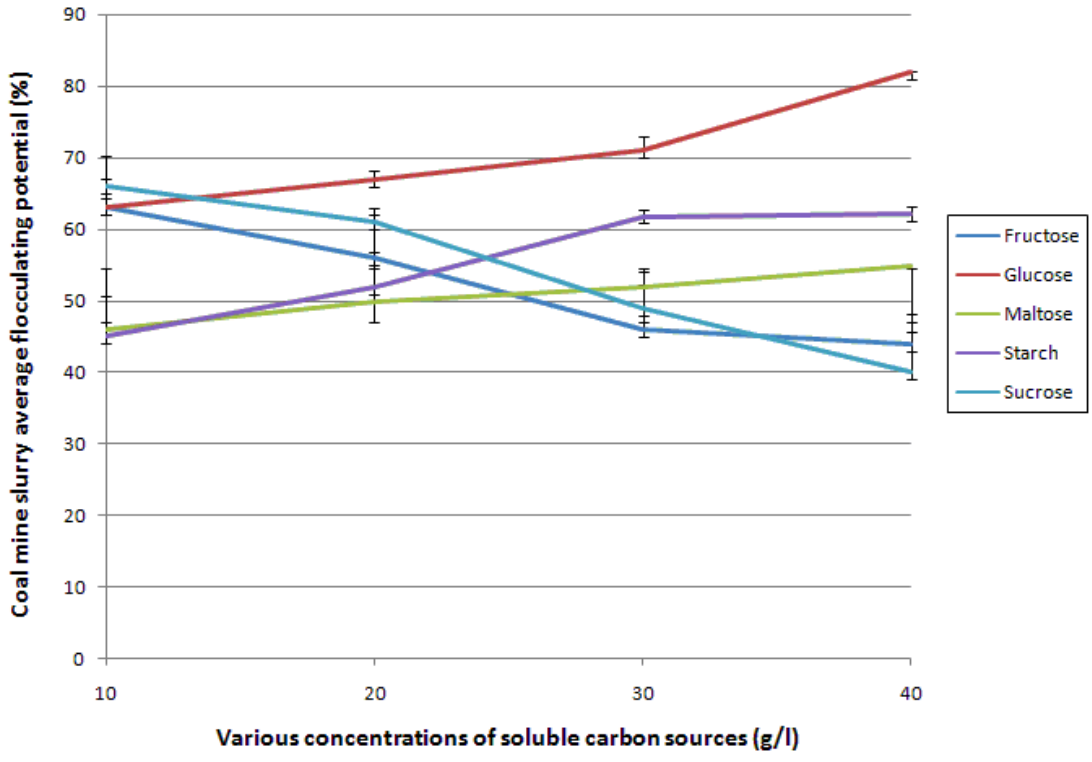

Figure 6: Impact of various carbon source types and concentration of the flocculating activity of *Bacillus cereus* on coal mine slurry

The carbon sources used in this study for the optimization of *B. cereus* were distinguished by literature. The flocculation efficiency of a *B. cereus* extract was tested using glucose, maltose, fructose, starch and sucrose as carbon sources. The various carbon source concentrations were varied from 10g/l to 40g/l. Figure 6 shows that *B. cereus* could effectively utilize all of the carbon sources. The highest flocculating efficiency was achieved when using glucose as the carbon source. A concentration of 40g/l glucose resulted in the best flocculating activity of 82%.

From literature, it is evident that glucose is the preferred carbon source for the production of the *Bacillus* bioflocculant strain. The results from this study directly correlate with that from literature.

4.5 Effect of nitrogen sources on flocculating activity

Table 6: Literature review for various nitrogen sources

Author	Nitrogen Source	Microorganism
Piyo *et al.* (2011)	Ammonium chloride	*Bacillus* sp
Zhuang *et al.* (2012)	Urea	*Bacillus licheniformis*

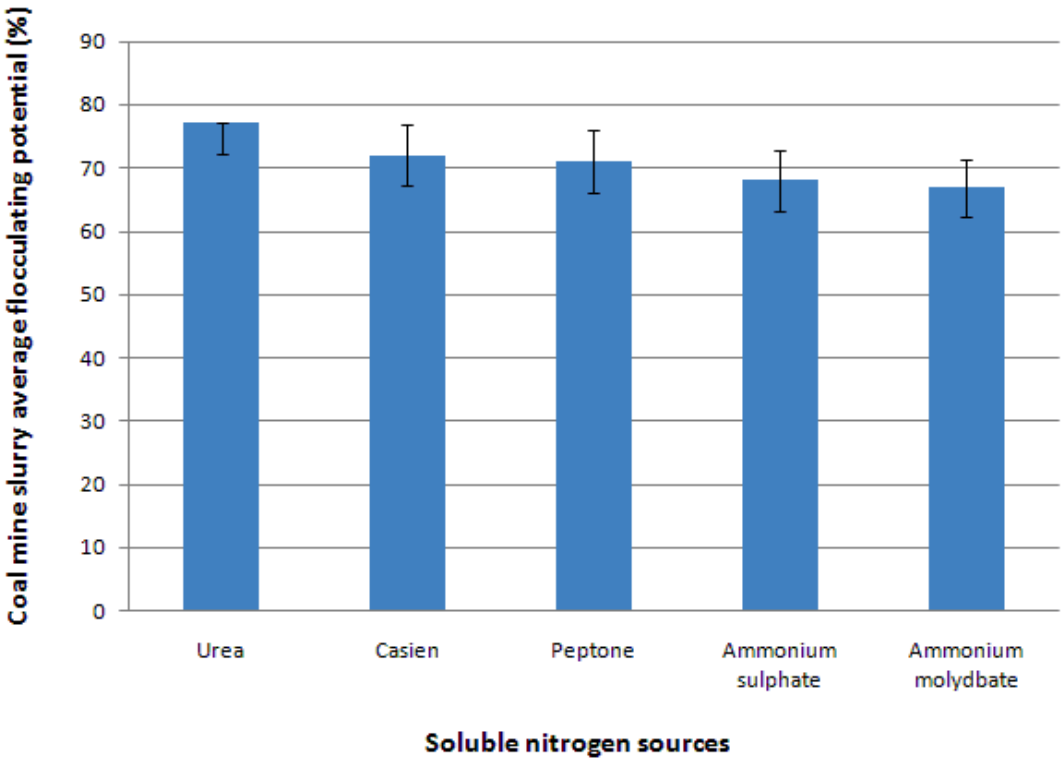

Figure 7: Effect of various soluble nitrogen sources on the flocculating activity of *Bacillus cereus* for coal mine slurry.

The effects of various nitrogen sources on the flocculating activity (with glucose as the carbon source) are presented in Figure 7. Various soluble nitrogen sources were used to optimize the flocculating efficiency. The nitrogen sources used were peptone, casein, urea, ammonium sulphate and ammonium molybdate. Urea was discovered to be the favorable nitrogen source for *B. cereus.* It produced a flocculating activity of 77% as shown in Figure 7.

The use of urea has previously been used for *Bacillus* strains. From the literature it is evident that different *Bacillus* microbes can tolerate various nitrogen sources for optimal flocculating production.

4.6 Effect of cations on flocculating activity

Table 7: Literature review for cations

Author	Cations	Microorganism
Nwodo *et al.* (2013)	Ca^{2+}, Mg^{2+} and Mn^{2+}	*Brachybacteria* sp
Elkady *et al.* (2011)	Ca^{2+}, Na^{+} and K^{+}	*Bacillus mojavensis*
Piyo *et al.* (2011)	Ca^{2+}, Mg^{2+},Fe^{2+} and K^{+}	*Bacillus* sp
Zulkeflee *et al.* (2012)	Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Al^{3+}	*Bacillus* spp
Fujita *et al.* (2000)	Cation independent	*Citrobacter* sp
Zheng *et al.* (2008)	Cation independent	*Bacillus* sp
Wan *et al.* (2013)	Cation independent	*Solibacillus silvestris*
Liu *et al.* (2010)	Cation independent	*Corynebacterium daeguense*

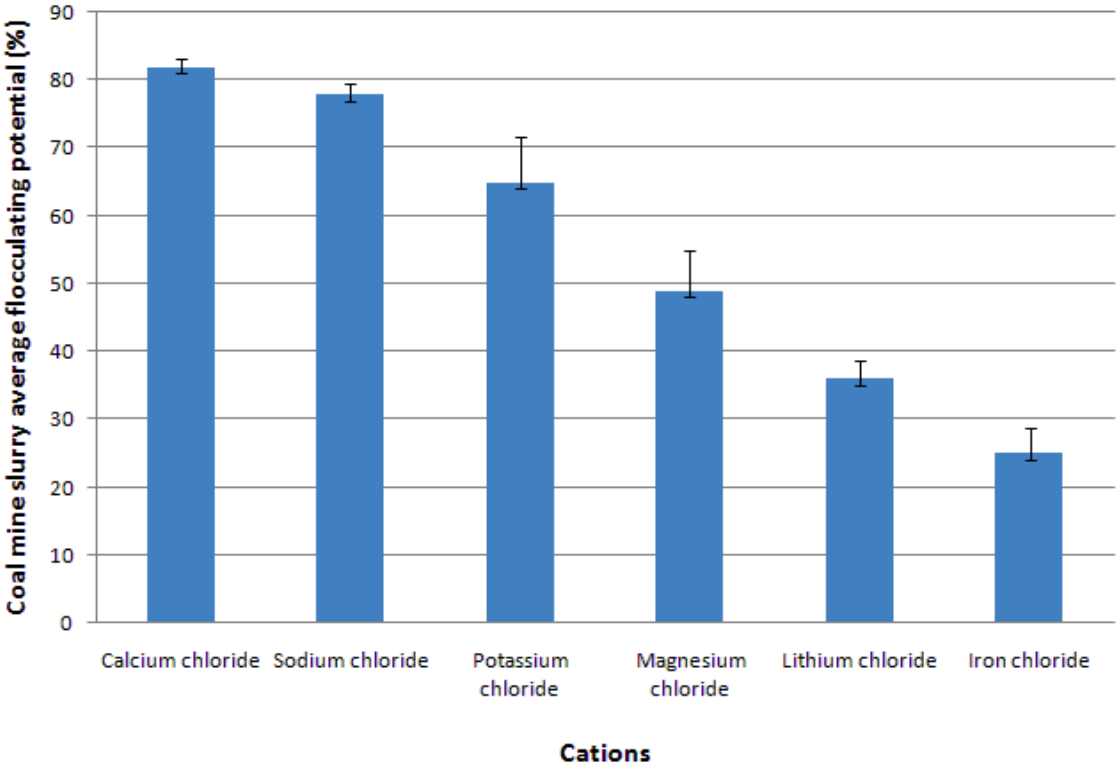

Figure 8: Effect of cations on bioflocculation when using *Bacillus cereus* on coal mine slurry

The effect of various selected cations on the flocculating efficiency is shown in Figure 8 (glucose and urea were used as the carbon and nitrogen sources in the pre-culture medium). The bacterium was stimulated by the addition of 3ml of a 1% (v/v) concentration of the following cations: Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} and Fe^{2+}. Ca^{2+} was found to be the most effective cation for the bioflocculant produced by *B. cereus* with a flocculating efficiency of 82%. The cations used for the optimization of *B. cereus* were determined by literature.

From the literature it is evident that Ca^{2+} is commonly used for bioflocculant production of the *Bacillus* strain, however it can be cation independent which is preferable for cost effectiveness.

4.7 Effect of pH on flocculating activity

Table 8: Literature review for pH

Author	pH	Microorganism
Liu *et al.* (2010)	5	*Chryseobacteria daeguense*
Zulkeflee *et al.* (2012)	5	*Bacillus* spp
Ugbenyen *et al.* (2012)	6	*Cobetia* sp
Liu *et al.* (2013)	8	*Klebsiella* sp.
Zheng *et al.* (2008)	9	*Bacillus* sp.

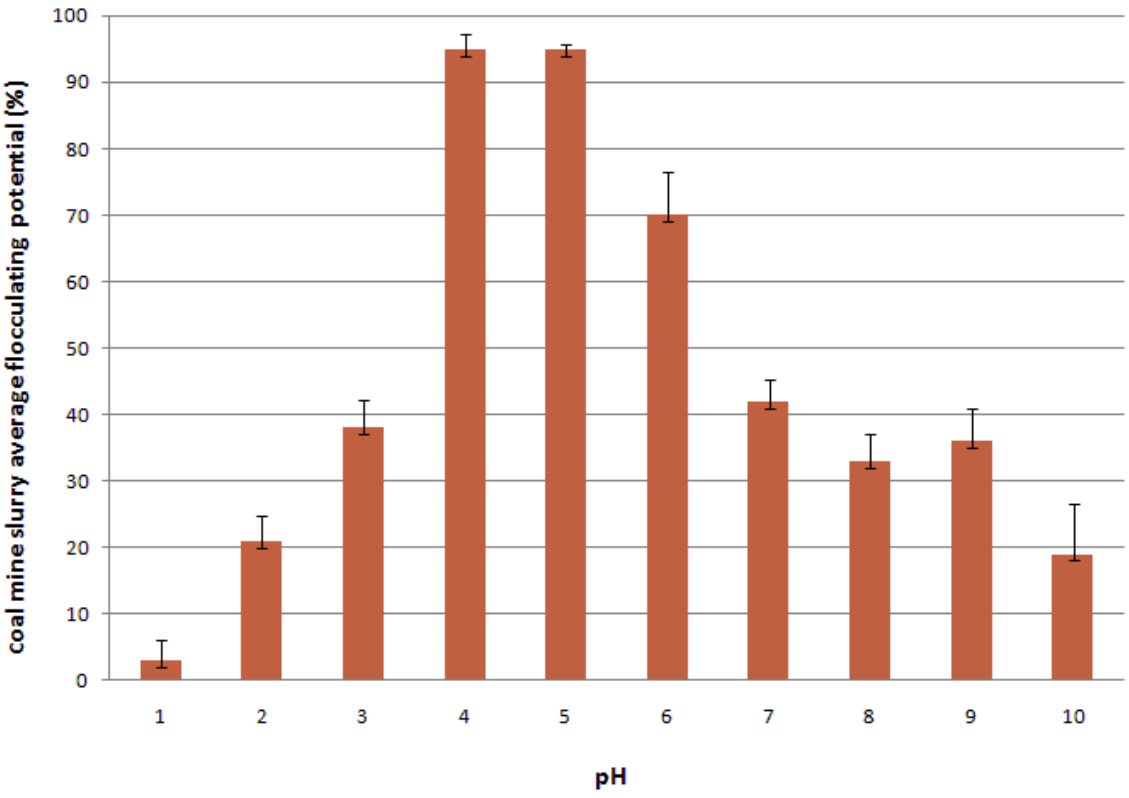

Figure 9: Effect of pH on the flocculating activity of *Bacillus cereus* for coal mine slurry

The effect of pH on flocculating activity was examined at pH values ranging from 1 to 10 (Figure 9). The bioflocculant production improved in acidic conditions. The greatest flocculating yield of *B. cereus* of 95% was obtained between pH 4 and 5. Neutral and alkaline conditions inadequately supported the bioflocculant production of *B. cereus.* At a pH of 10 the lowest flocculating efficiency was observed.

From previous studies it has been discovered that the original pH of the growth medium required differs between organisms with bioflocculant production. The *Bacillus* strain has shown to flocculate at both acidic and alkaline pH which compares to this study.

4.8 Time course assay

Table 9: Literature review of time course assays

Author	Time course (hours)	Microorganism
Elkady *et al.* (2011)	20	*Bacillus licheniformis*
Cosa *et al.* (2013)	28	*Klebsiella* sp
Wu and Ye (2007)	72	*Bacillus subtilis*
Fujita *et al.* (2000)	72	*Aspergillus parasiticus*
Deng *et al.* (2003)	72	*Bacillus mucilaginosus*
Deng *et al.* (2005)	72	*Aspergillus parasiticus*

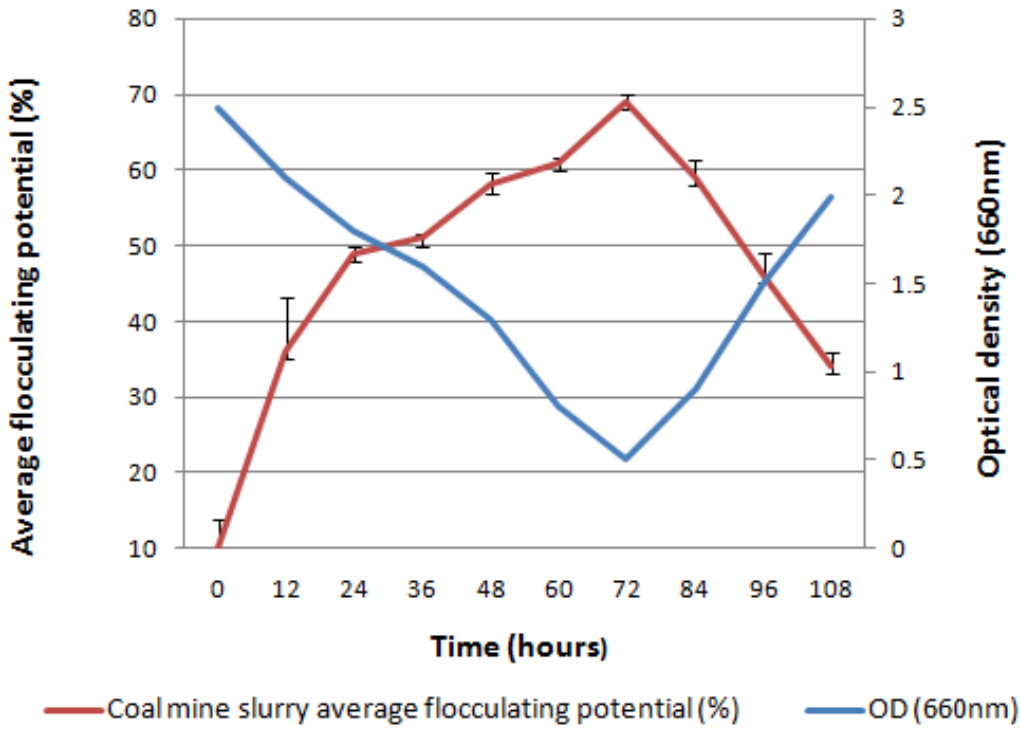

Figure 10: Time course assay for *Bacillus cereus* flocculation potential for a period of 108 hours tested with coal mine slurry.

In this research 40g/l of glucose and 0.7g/l of urea were used as the carbon and nitrogen sources in the culture medium of *B. cereus*. The time course results of bioflocculant production of *B. cereus* in the production medium over a period of 108

hours are presented in Figure 10. The flocculating yield increased steadily with the increase in incubation period and produced a high flocculating activity of 69% in the early stationary phase (72 hours) of fermentation. This suggests bioflocculant production produced biosynthesis during its growth. Following fermentation the flocculating activity decreased steadily with time. The subsequent decrease in flocculating yield could be an outcome of cell autolysis and enzymatic activity.

Previous studies have demonstrated that different microorganisms require different culture times to produce bioflocculants. From the literature it is evident that some bioflocculants are capable of producing bioflocculants during a shorter cultivation period than various other reported strains thus reducing the production costs. The time course results from this study collaborates with previously isolated microorganisms.

4.9 Application of the produced and purified bioflocculant in settling coal and wash plant fines

4.9.1 Site visit

From the site visit conducted at TCM, the slurry used for testing flocculation of the wash plant fines capabilities was taken from Plant 1.Plant 1 was the only plant with an access point to the slurry prior to the thickener (Figure 11).

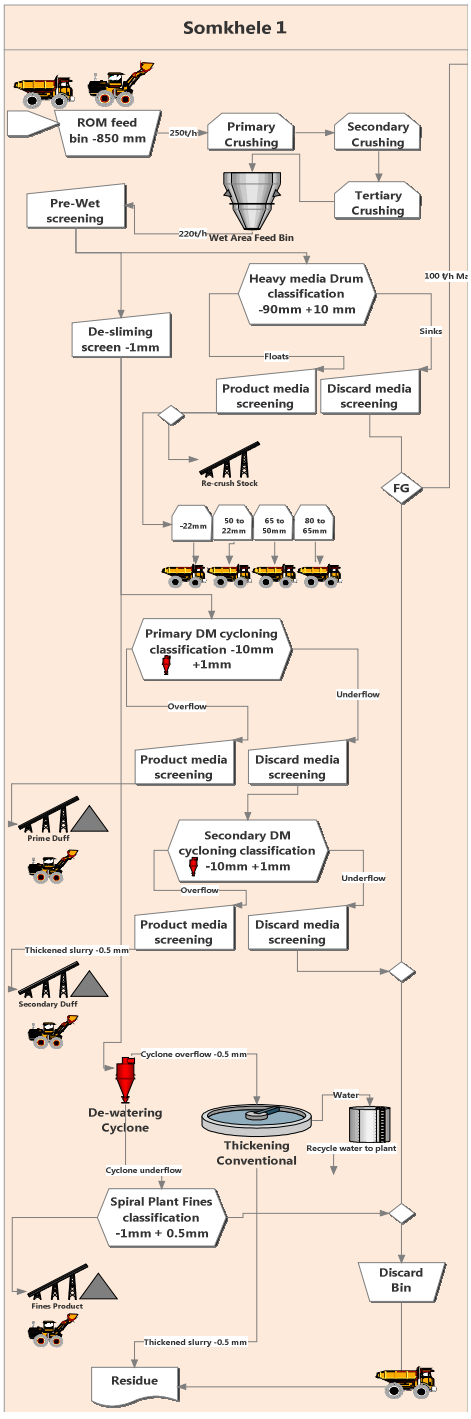

Figure 11: TCM CPP Plant 1 (Somkhele Plant Overview, 2015)

4.9.1.1 TCM Plant 1 (Figure 11)

The coal washing plants are designed to process raw coal into the following sections:

- Dense Medium (DM) drum plant (+10mm)
- Primary DM cyclone plant (-10 x 1mm)
- Secondary DM cyclone plant (-10 x 1mm)
- Magnetite medium make-up plant
- Spirals plant (-1 x 0.1mm)
- Thickener, filter press (Plant 1) and slimes disposal system (-0.1mm)
- Flocculent make-up and dosing plant. Water circuits

Each coal separation plant is equipped to beneficiate the tonnage variations from the different top sizes of feed and the natural variations in size from the mining and raw coal handling operations.

The fines that are ≥–1mm and ≤+0.1mm in size are conveyed to the fines stockpile where the excess surface moisture is allowed to drain off before loading it for the customers.

Discard from the plant is conveyed by a common discard conveyor to a discard bin. Discard is loaded from the bin onto dump trucks and shuffled to the discard dump where it is spread and compacted for final disposal.

For TCM Plant 1, the discard from the drum section is either fed to the common discard conveyor that feeds the S1 discard bin, or it is diverted to the feed conveyor that transports the discard to TCM Plant 3 for further processing. In the same manner, the total discard of TCM Plant 2 can be diverted via the S2 belt to TCM Plant 3.

4.9.1.2 Generation of the fines

At TCM some of the fines dispersed around the mine and found in the outlying areas are fines that have been generated by the drilling and blasting used to find the coal seam. The remaining fines are created by crushing and milling of the rock to extract the coal.

To reduce the size of the materials most crushers employ a combination of the following crushing methods:

- Impact
- Attrition
- Shear
- Compression

The three primary objectives of crushing coal are:

- Reducing the size of run-of-mine coal, shale and/or middlings to size suitable for treatment or retreatment in a coal processing plant
- Producing coal that meets market requirements in terms of size
- Attempting to generate a minimal amount of fines

All fines created in the CPP are settled in the thickener. The ultimate objective is to remove as much water from the slurry before disposal and dispose coarse and fines together as a single product (as a slurry to the tailings) and recycle the recovered water back into the coal beneficiation plant.

4.9.1.3 Thickener specification for Plant 1

The following thickener specifications are specifically designed to the machine present at TCM (Table 10).

Table 10: Thickener specifications for TCM

Description	Amount (Maximum capacity)
Maximum capacity	20 tons
Flocculant dosage rate	300g/h @0.1% concentration = 300 litres per hour
Flocculant handling capacity	75Kg. Hopper
Flocculant flow rate	300 litre per hour (600 maximum via VSD)
Potable water rate	60 litres/min @ 4bar
Feed density	1.015 – 5% solids
Feed rate	75 - 80m^3/hr can go up to 100m^3/hr
Discard density	1.15 – 1.18
Discard slurry discharge rate	65m^3/hr for 1.6km through a 140mm pipe
Dry tonnage	37T/hr
Solids w/w	25% to 30%
Water recovery	16.96 m^3/t solids
Water overflow rate	60 – 70m^3/hr
Water lost	187l/t solids (1.09%)
Highest viscosity produced in thickener underflow	SG of 1.35

4.9.1.4 Flocculant used by TCM

The settling velocity of coal of slimes without a catalyst to boost flocculation is low, as the effective settling requires long residence time. The settling velocity can be increased by the addition of flocculating agents. TCM uses a chemical flocculant called Floxit 5028, which is a Patlochem brand product. Floxit 5028 is a fine, white, free flowing powder that is medium in molecular weight and a non-ionic polyacralamide flocculant. Floxit 5028 currently priced at R36/kg. In general coal slurry in thickeners, is a concentration of coal fines and other associated waste materials in the size range of ≥0mm to ≤ 2mm.

TCM's fines have a particle size range of ≤0.5mm. The chemical flocculant (Floxit 5028) loses its full potential when pumped long distances to the tailings dam through a 140mm diameter pipe. Thus it is important to develop a flocculant that continues to flocculate even after transportation. This will result in continuous settling in the

tailings dam and enable clarified water to be recycled back into the plant. This could result in major savings and advantages for the mine and the environment.

4.9.2 Effect of various concentrations of Floxit 5028 using coal mine slurry

TCM uses a concentration of 0.1% when inserted into the thickener. However the concentration may vary according to the size of the fines and the velocity of the flocculating rate required. From Figure 11 it is evident that the flocculating potential of Floxit 5028 is suitable for a broad spectrum of concentrations. However, the smaller the concentration used the better.

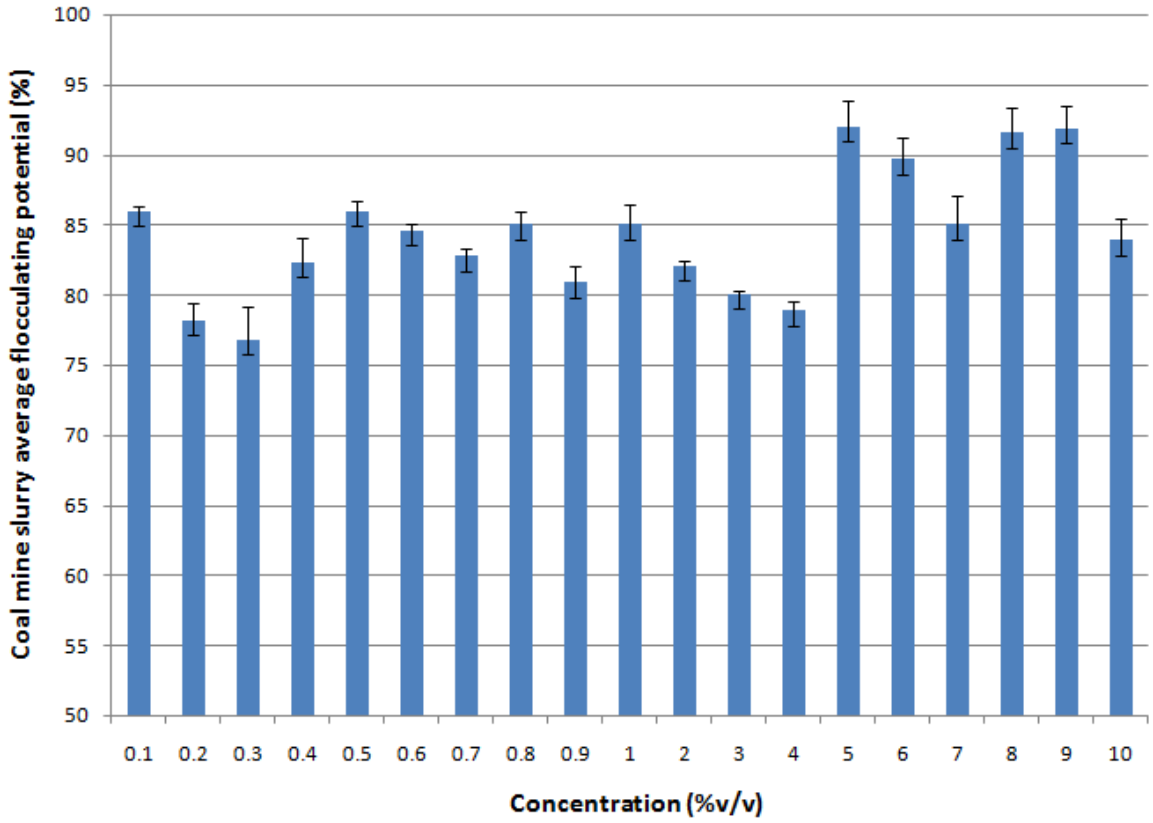

Figure 12: Floxit 5028 concentrations tested with TCM slurry

4.9.3 Effect of various cations on Floxit 5028 tested with coal mine slurry

The cations were used during the flocculating activity of Floxit 5028 to determine if they would have any effect/impact on the flocculating potential of the non-ionic polyacralamide flocculant. In Figure 12 it is evident that all of the cations tested could be used when flocculating the coal mine slurry with Floxit 5028. Lithium chloride showed the best flocculating activity. However it is not feasible and viable to use other cations.

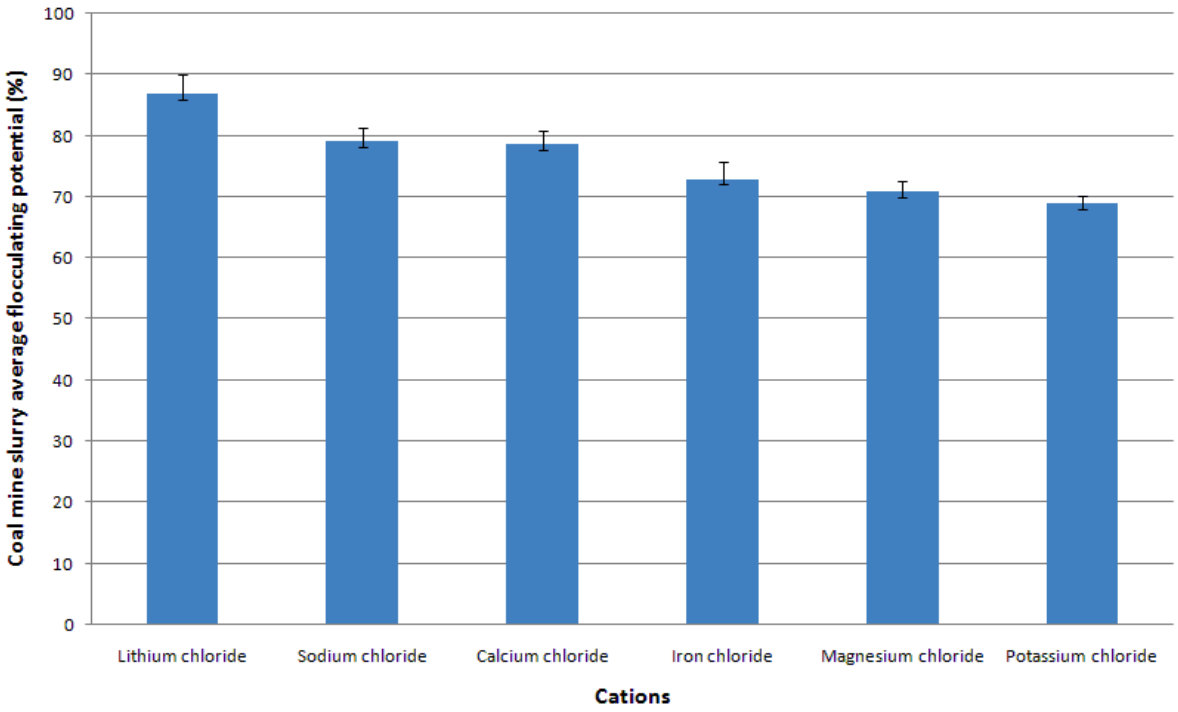

Figure 13: Cations used together with Floxit 5028 tested with TCM slurry

4.9.4 Pilot scale test (1l) using coal mine slurry

The TCM conditions were simulated using a 1l pilot study. The 1L pilot study was adhered to after completing optimization for *B. cereus* flocculating potential against coal mine slurry from the 100ml cylinders. 1l of coal mine slurry was tested against 20ml of the pre-culture medium simulating the methodology used at TCM in a laboratory situation. A mechanical stirrer was used to mix the flocculants and left to stand for 30 seconds prior to addition to the TCM slurry. In this 1L pilot study the TCM slurry flocculating potential was tested against the following:

a) Chemical flocculant (Floxit 5028) at a 0.1% concentration.
b) A bioflocculant media where the optimized inoculum size, glucose, nitrogen sources and cations were used in the medium while the pH was not altered (B. cereus).
c) An optimization of the *B. cereus* bioflocculant medium at a pH of 4
d) An optimization of the *B. cereus* bioflocculant medium at a pH of 5

The flocculating activity was measured at both 5 minutes and 10 minutes simultaneously to compare the results over a longer period of time. The bulk density of the discard slurry was determined at 10 minutes. From the results observed in

Table 5, the *B. cereus* showed the best results when compared with Floxit 5028. The *B. cereus* optimized bioflocculant flocculated at 90% and 94% while the Floxit 5028 had a flocculating potential of 93% and 96% at 5 and 10 minutes respectively. The slurry water displacement (after 10 minutes) and the density discard slurry were 82% and 1.14g/ml respectively for the Floxit 5028 while the *B. cereus* was 87% and 1.12mg/l respectively.

Table 11: Pilot study of *Bacillus cereus* and Floxit 5028 for TCM slurry

1000ml scale up using coal mine slurry	Coal mine slurry average flocculating potential at 5 min (%)	Standard deviation (±)	Coal mine slurry average flocculating potential at 10 min (%)	Standard deviation (±)	Slurry water displacement after 10 min (%)	Density of discard slurry after 10 min (g/ml)
Floxit 5028 (@0.1%)	93	1.9	96	1.9	82	1.14
B. cereus	90	2.0	94	2.1	78	1.12
B. cereus pH 4	87	1.4	96	1.6	80	1.06
B. cereus pH 5	74	1.8	91	1.6	70	1.05

Chapter 5: Conclusion

Flocculation is the most vastly utilized process for the treatment of wastewater in industries with difficult waste such as tanneries and clay process industries. Separation by flocculation is an effective method utilized for a quick separation of solid from liquids. Inorganic flocculants such as aluminium (commonly combined with lime) is traditionally utilized for the removal of suspended particles from unprocessed water. The sludge produced from such treatments could have discarding issues because of the aluminium contents which tends to accumulate within the environment.

5.1 Isolation and identification of microorganisms for bioflocculant production

From the 33 water and sediment samples isolated from the marine environment only 10 pure culture strains showed signs of growth of flocculating microorganisms (Table 4). These 10 pure culture strains that showed potential signs of flocculating microorganisms were subjected to molecular identification using their 16S rDNA gene.

5.2 Screening the selected microorganisms for bioflocculant production

The 10 pure culture strains were screened for bioflocculant production based on kaolin suspension flocculation activity. These strains were further tested against TCM slurry (Table 4). Among them, one strain flocculated both the kaolin suspension and the TCM slurry with a relatively constant and high flocculating potential and was selected for further testing. This strain was isolated from the Sodwana Bay Estuary and according to their 16S rDNA sequence analysis the strain was identified as *Bacillus cereus.* This result relates to several preceding studies which reported that various bacterial strains closely related to the genus *Bacillus* are able to produce bioflocculants (Shih *et al.,* 2001; Deng *et al.,* 2003; de Vreis *et al.,* 2005; Wu *et al.,* 2007; Zheng *et al.,* 2008; Elkady *et al.,* 2011; Zulkeflee *et al.,* 2012; El-Midany and Abdel-Khalek, 2014; Okaiyeto *et al.,* 2015).

The *B. cereus* microorganism produced optimal bioflocculant potential when the inoculum size was (3% v/v), glucose (40g/l), urea (0.7g/l), calcium chloride (1% v/v) and ph4 in a period of 72 hours. This was evident when the favourable inoculum size (Figure 5) carbon (Figure 6), nitrogen source (Figure 7), cation of choice (Figure 8), initial growth medium pH (Figure 9) and time course (Figure 10) were used respectively for a 100ml experiment.

5.3 The application of the produced bioflocculant compared to the coal mine synthetic flocculant

Initially the optimization of the *B. cereus* was optimized at 100ml using TCM slurry. An inoculum size of 3%, 40g/l of glucose, 0.7g/l of urea, $CaCl_2$ as the cation, at a pH of 4 and/or 5 with a fermentation time of 72 hours was when the production of *B. cereus* was optimal using 100ml tests.

The produced bioflocculant was then directly compared to the synthetic flocculant (Floxit 5028) used at TCM in a pilot study simulation to 1L. The synthetic flocculant (Floxit 5028) used at the mine was used at 0.1% concentration (Figure 11) with no cation (Figure 12) additives in the pilot study to simulate the conditions at TCM.

During the pilot study of 1L (Table 5), 3 different optimized pre-cultures media's were used to test against the TCM slurry simulating mining conditions in the laboratory. Where the premixed synthetic flocculant was mixed with a mechanical stirrer, left to stand, added to the thickener at 0.1% concentration and left to settle. The settling time in the laboratory was tested at both 5minutes and 10minutes. Both pH4 and 5 did not have a detrimental impact on the flocculating activity on the coal mine slurry over 10minutes. The *B. cereus* (Table 5) showed the best flocculating potential (95% after 10minutes) while the Floxit 5028 had a flocculating potential of 96% after 10 minutes. From the results for the 1litre pilot study, it is very plausible to use this *B. cereus* bioflocculant instead of the harmful chemical flocculant used in the thickener at TCM. However using large concentrations of glucose as the sole carbon source will have a high impact on the final costs of the production of bioflocculants in a mass scale, therefore making the use of bioflocculant in an industrial scale, unfeasible.

5.4 Recommendations

Further pilot studies are required to determine the *B. cereus* flocculating potential at a larger scale and eventually in the CPP itself using a smaller concentration of glucose as the carbon source. The produced bioflocculant needs to be extracted, purified and chemically analysed using Ugbenyen *et al.* (2014). The chemical analysis of the purified bioflocculant should be determined using Scanning Electron Microscopy (SEM), High performance Liquid Chromatography (HPLC) and Fourier Transform Infra-red spectrophotometer (FTIR).

Furthermore, the *B. cereus* bioflocculant will need to be dried into a powder form, this will determine the quantity (thus the cost), that will be required by the mine to substitute the chemical flocculant with the *B. cereus* bioflocculant. This powder will also allow for a direct comparison with TCM Floxit 5028 (simulating the mining conditions at TCM), to determine if the *B. cereus* bioflocculant is a viable alternative to their synthetic flocculant in its purest form.

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