Synthesis and application of a grafted flocculant produced from a chemical combination of a bioflocculant TKT and acrylamide (AM)

By

Sicelo Keny Masuku

201200039

MSc (Microbiology)

University of Zululand

Department of Biochemistry and Microbiology

University of Zululand

Private bag x1001

KwaDlangezwa

3886

Supervisor: Prof Albertus Kotze Basson

Co-supervisor: Prof Jean Jacques Simonis

: Dr Tsolanku Sidney Maliehe

2019

Declaration

The research project presented in this dissertation was conducted in the Department of Biochemistry and Microbiology at the University of Zululand (UZ), from March 2017 to March 2018, under the supervision of Prof A.K Basson, Prof J.J Simonis and Dr T.S Maliehe.

I, Sicelo Keny Masuku declare that this work, aside from the supervisory guidance received, is the product of my own original wok and effort.

……

Mr Masuku S.K

……

Dr Maliehe T.S

……

Prof Basson A.K

……

Prof Simonis J.J

Acknowledgements

I would like to give my thanks and acknowledgement to God the creator of nature and its astonishing wonders, for directing my steps towards his promises. Thank you also to my mother for her love and support, my brother for being my inspiration, and to my family and friends for their encouragement.

Thanks to Dr T.S. Maliehe, Prof J.J. Simonis and Prof A.K. Basson for their critical suggestions, discussions, for all assistance, editing, and constructive criticism.

Thanks also to The Department of Biochemistry and Microbiology at the University of Zululand and the University of KwaZulu-Natal, without which I would not have been able to complete my Master's degree in Microbiology.

Abbreviations

%	Percent
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Celsius
µl	Microlitre
µm	Micrometre
AMD	Acid mine drainage
Al	Aluminium
ANOVA	One-way analysis of variance
BOD	Biological oxygen demand
C	Carbon
Ca	Calcium
CaCO2	Human colorectal adenocarcinoma cells
Cl	Chlorine
COD	Chemical oxygen demand
DWS	Department of Water and Sanitation
FA	Flocculating activity
GCMs	Global climate models
HEK 293	Human embryonic kidney cells
IR	Fourier transform infrared spectroscope
K	Potassium
L	Litre
Mg	Magnesium
Mg	Miligram
Min	Minute
mL	Millilitre
MTT	3-(4,5-dimethylthiazol-2-)-2,5-diphenyltetrazolium bromide
mV	Millivolts
N	Nitrogen
Na	Sodium
Nm	Nanometer
OD	Optical density

P	Phosphorus
PAM	Polyacrylamide
Rpm	Revolutions per minute
RSA	Republic of South Arica
S	Sulfur
SD	Standard deviation
SEM	Scanning electron microscopic
SO_2	Sulfur dioxide
*T*d	Degradation temperature
TGA	Thermo gravimetric analyzer
TKT	Bioflocculant from *Alcaligenes faecalis* HCB2
TKT-g-PAM	Bioflocculant grafted polyacrylamide
UN-Water	United Nations Water
UV	Ultraviolet
V	Volume
W	Weight
WEF	Water Environment Federation
WHO	World Health Organisation

Table of Contents

Abstract

An increase in pollution from mining, industries and agriculture has decreased the quality of fresh water in South Africa. Contaminated water is unsafe for animals to drink and for plants to absorb. Synthetic flocculants are extensively used in water and wastewater treatment due to their effectiveness and low cost. These flocculants consist of a flexible chain of high molecular weight to create a bigger surface area for biochemical reactions to take place and are documented to flocculate well in small amounts. However, these flocculants are known to be carcinogenic, neurotoxic and hazardous to the environment. Moreover, natural polymers (bioflocculants) are not toxic and are biodegradable. Nevertheless, these flocculants need to be suitably controlled because biodegradability reduces their shelf-life. Through grafting synthetic polymers onto a backbone of natural polymers, the best properties of synthetic and natural flocculants are merged. When grafted copolymers are successful, the dangling grafted chains have easy access to contaminants in wastewater. In literature it has been reported that grafted polysaccharides provide efficient, shear stable and biodegradable flocculants. This study aimed at synthesizing and applying a grafted bioflocculant produced from marine *Alcaligenes faecalis* HCB2 to wastewater.

Extraction and purification of the bioflocculant was obtained through using the solvent extraction method. Chains of polyacrylamide (PAM) were grafted onto the backbone of bioflocculant TKT extracted from *Alcaligenes faecalis* HCB2 using a microwave initiated method. Various grades of TKT-g-PAM were synthesized and the one with the highest percentage grafting was used throughout this study. Physicochemical analyses of the grafted bioflocculant (TKT-g-PAM) were attained by scanning electron microscopy (SEM), Zetasizer Nano, Thermogravimetric Analyzer (TGA) and Fourier Transform Infrared (IR) Spectrophotometry. A diphenyl tetrazolium bromide (MTT) assay was used to determine the biosafety of the grafted bioflocculant. The effect of dosage size on flocculating activity was evaluated. The pH and thermal stability of TKT-g-PAM were assessed spectrophotometrically. The removal efficiencies of TKT-g-PAM on chemical oxygen demand (COD), biological oxygen demand (BOD), nitrogen and sulfur on wastewater from Tendele Coal Mine were performed using different test kits.

The bioflocculant yield obtained after extraction and purification was 4.0 g/l. TKT-g-PAM6 was the preferred grade with the highest percentage of 62% and a weight of 0.8 g. The images provided by SEM revealed changes in bioflocculant structure from a blocky to a granular structure after grafting. This confirmed that the grafting of PAM chains was successful on the TKT backbone. The zeta potential of grafted bioflocculant (-12.6±0.1) and bioflocculant (-17.1±0.65) had a negative charge. IR observations indicated hydroxyl, carboxyl and amine groups. TKT-g-PAM6 showed less cytotoxic effect on HEK293 cells (retained 82% of cell viability after it was treated in high concentration) while CaCO2 cells (retained 64% of cell viability after it was treated in high concentration). TKT-g-PAM6 was cation and pH dependent and optimally flocculated kaolin clay suspension at a dosage of 0.2 mg/ml with 74% of flocculating activity in the presence of Ba^{2+}. TKT-g-PAM6 was more stable at acidic pH 3-5 with the highest flocculating activity (93%) observed at pH 3. TKT-g-PAM6 was thermostable and retained more than 90% of its flocculating activity after being heated at 100 °C for 1 hour. The pyrolysis properties of the grafted bioflocculant confirmed their thermal stabilities, the IDT has been found to 215 °C (29% weight loss) and FDT to be 571 °C (36% weight loss). Flocculating efficiencies were: 87% on COD, 92% BOD, 91% N and 93% S, respectively. TKT-g-PAM6 was effective and has the potential to be used in wastewater treatment.

List of figures

List of tables

Chapter 1

1.1 Introduction

Industrialization is still increasing due to its contribution to economic growth and it is accepted as desirable based on its contribution to economic activity (Sarkar *et al*., 2006). This causes increased pollution, particularly from industrial sources (Cosa *et al.,* 2014). The disposal of effluents without appropriate management could result in longstanding undesirable negative impacts on the environment and human health (Lin *et al.,* 2011). To combat these challenges, the flocculation process can be employed to reduce the total amount of effluents disposed into the environment. Flocculation is the process in which colloids aggregate together from suspension to form larger particles that can be easily removed either by filtration or sedimentation (Bhunia *et al*., 2012).

Chemical and synthetic flocculants such as iron and polyacrylamide have been widely used industrially because of their effectiveness and low cost (Okaiyeto *et al.,* 2013). However, these flocculants are reported to be linked to cause human neurological disorders such as dialysis encephalopathy (Zheng *et al*., 2008). In addition, they are identified to be hazardous to the environment (Ugbenyen *et al*., 2014).

Biomolecules such as amylase, starch, amyl pectin and xanthan gum are natural polymers which act as good flocculating agents (Adhikary *et al.,* 2004)). They are referred to as bioflocculants. Bioflocculants are inexpensive, fairly shear stable and easily produced. However, high dosage sizes are required for their high removal efficiencies in wastewater treatments (Mishra *et al.,* 2012). Moreover, bioflocculants have a low shelf life and form weak floccules that easily break (Singh *et al.,* 2003). Thus, great attention has been revealed towards the chemical alteration of natural polymers in order to enhance their flocculating properties (Kumar *et al*., 2012).

Recently, many studies have focused on the search for flocculants that are highly effective, ecofriendly and safe as alternatives to conventional (chemical and synthetic) and natural flocculants (Pal *et al.,* 2012). This has led to development of the grafted flocculants. Grafted flocculants combine the desirable properties of natural and synthetic polymers (Lee *et al.,* 2014). They have high efficacies, shear stabilities and intermediate biodegradability. Moreover, they are nontoxic and easily synthesized (Renault *et al.,* 2009).

The increase of the cationic charge and the ratio of effective components of the flocculants during graft polymerization often results in improved flocculation (Wang *et al*., 2008). When the grafting of copolymers is successful, the dangling grafted chains have easy access to wastewater pollutants. At low dosages, grafted bioflocculants are reported to have high efficacy (Bolto and Gregory, 2007; Singh *et al*., 2000). Flocculation efficiency can be improved through the hybridization of natural and synthetic polymers.

In this study, *Alcaligenes faecalis* BCH2 was obtained from the Laboratory stock room in the Department of Biochemistry and Microbiology at the University of Zululand. It was used to produce bioflocculant under the known optimum conditions (Maliehe, 2018). The purified bioflocculant was then grafted to acrylamide. Thereafter the synthesised grafted bioflocculant was characterized and applied for the treatment of Tendele Coal Mine wastewater Mtubatuba (RSA).

1.2 Rationale of the study

Pollution of water bodies has harmful consequences on human well-being such as being neurotoxic, carcinogenic and resulting in Alzheimer disease (Serdar *et al*., 2011; Ahmad *et al*., 2015)**.** The existence of contaminants in aquatic sources can be problematic for aquatic life and to potable water use. Physical, chemical and biological methods have been applied in the reduction of water pollution. Flocculation is one of the most effective methods used in fresh water and wastewater treatment.

Chemical and synthetic flocculants are widely used because of their low cost and effectiveness. However, they are environmentally unfriendly and impose health threats to humans. Natural flocculants-bioflocculants thus are being used as an alternative to chemical and synthetic flocculants. However, natural flocculants have low flocculating activities, short shelf life and form weak flocs that break easily (Singh *et al.,* 2003). These challenges of both conventional and natural flocculants have further necessitated a search for alternative flocculants that are highly effective, safe for humans and eco-friendly (Nwodo and Okoh, 2013).

The hybridization of natural and conversional polymers known as graft polymerization has been seen as a promising way to improve flocculation efficiency and lowering of hazardous effects from chemical and synthetic flocculants. Grafted bioflocculants are the result of the chemical combination of natural and synthetic polymers. By increasing the cationic charge and the ratio of effective component of the flocculants through graft polymerization, improved flocculation (Wang *et al*., 2008). Moreover, these grafted flocculants have high safety and shear stability. Thus, the study aimed to synthesize and characterize grafted bioflocculant using a bioflocculant from *Alcaligenes faecalis* BCH2 and polyacrylamide for treatment of coal mine wastewater.

1.3 Area of study

The study was done at the University of Zululand (UZ) KwaDlangezwa campus, under the Department of Biochemistry and Microbiology. It is located in KwaZulu-Natal, South Africa.

1.4 Aims

To synthesize and applied a grafted bioflocculant from *Alcaligenes faecalis* BCH2 and polyacrylamide in wastewater

1.5 Objectives

1. Extraction and purification of the bioflocculant *Alcaligenes faecalis* BCH2
2. Synthesis of the grafted bioflocculant
3. Characterization of the grafted bioflocculant
4. Application of the grafted bioflocculant on wastewater treatment

1.6 Hypothesis

1. Bioflocculant can be hybridised with polyacrylamide to produce effective grafted bioflocculant.
2. Grafted bioflocculant has improved removal efficiency of colloids in wastewater.

1.7 Value to the Body of Knowledge

This study produced novel grafted bioflocculant (TKT-g-PAM) that can be applied on wastewater treatment.

Chapter 2: Literature

A literature survey presents a relatively large body of research on grafted flocculation. Much of this literature is devoted to areas of water pollution, wastewater treatment, flocculation and grafted flocculation processes, in particular. Major factors effecting flocculation are also discussed.

2.1 Importance of water

In the bionetwork water is a valued commodity for the existence of all life forms (Ogunlana *et al.,* 2010). The search for new water sources has become the key factor (Bhatnagara and Sillanpaa 2010; Rani *et al.* 2013). Freshwater makes up less than 3 percent of earth's water and is the source of virtually all potable waters (Pielou *et al.,* 1998). The aquatic environments occupy more than 71% of the earth's surface. Theoretically water can cover the earth's surface to an average depth of approximately 4 km (Mclntyre, 2010). The aquatic environments include the following: oceans, rivers, lakes, springs, streams, estuaries and aquifers. Over 99% of the hydrosphere is located in oceans. Marine water is characterized by salinity of about 33-37% and is therefore not potable. Twenty percent of the global population is assumed to be living in countries with water scarcity while 67% of the population is predicted to live under water stress by the year 2025 (UN-Water, 2007). Excessive extraction of ground and surface water are contributing to the drying up of aquifers, rivers and wetlands. Water is important for many purposes that include domestic (drinking, cooking, washing and bathing), industrial (mining and other construction), agricultural (irrigation, transport) and waste disposal (Kumar *et al.,* 2005).

Urbanization and growth in industrialization has increased the demand for potable water. The demand for water has exceeded its availability and this has pushed RSA to even import water from other nearby countries such as Lesotho. Therefore, RSA is declared as a water scarce country due to its mean annual rainfall of 450 mm, which is far below the world average of 860 mm (Turton *et al*., 2008). Therefore, water conservation should be the main focus in RSA. Poor agricultural practices such as over irrigation have also contributed to water scarcity. The effluent from industry,

mining and agriculture are life threatening due to the toxic effects imposed by heavy metals and pathogens.

2.2 Water pollution

Pollution is the introduction of foreign substances into the biosphere. Water pollution is caused by substances introduced into the aquatic surroundings. These substances threaten ecosystems, cause environmental pollution and result in poisonous effects on human health (Pulles *et al.,* 2005). The discharge of contaminants from different sources could result in water contamination (Li *et al.,* 2013). Freshwater polluted surroundings have a harmful effect on human well-being (Yang *et al*., 2012). Drinking water contamination can be caused by human contact to heavy metals. Industrialization is one of the main causes of aquatic pollution. The development of industries has led to increased emission of pollutants into ecosystems (Saleh *et al.,* 2010). Diseases, poisoning and even death to biota can be caused by environmental contamination, absorption and the accumulation on different biological tissues.

An important biomedical problem is the absorption of heavy metals and various biological tissues on pollutants (Wan *et al.,* 2013). Any metallic chemical element that is toxic and has a relatively high density can be labeled as a heavy metal. Cadmium (Cd), arsenic (As) and mercury (Hg) are some examples of heavy metals. They cannot be degraded or destroyed. Heavy metals are natural trace components of the aquatic environment, but their levels have been increasing due to industrial waste, agricultural and mining activities (Sprocati *et al.,* 2006).

Uptake of heavy metals through the food chain of aquatic organisms may cause various pathological disorders such as hypertension, sporadic fever, renal damage and cramps in humans (Gabriel *at al.,* 2006). Exposure and eating of polluted seafood can cause health problems in humans and animals including neurological and reproductive problems (Allen *et al.,* 2004). Accumulation of heavy metals in various fish organs may cause structural lesions and functional disturbances (Barbara *et al.,*

2006). Living organisms require variable amounts of heavy metals. For example, the human body requires trace metals such as cobalt, iron and zinc, but excessive levels can cause damage to organs. The fetotoxic, neurotoxic, teratogenic and nephrotoxic effects are systemic toxins caused by heavy metals (Saxena *et al.,* 2005). Neurotransmitter production and utilization can be influenced by heavy metal behavior, impairing and altering numerous mental and metabolic body processes and neurological functions (Nava *et al.,* 2013).

2.3 Coal mine wastewater

A major environmental problem relating to the mining industry is uncontrolled release of contaminated water from abandoned mines (Banks *et al.,* 1997, Pulles *et al.,* 2005). Mine wastes are generated in huge quantities. Waste water from mines contains heavy metals which are capable of contaminating local rivers, streams and groundwater. Coal washing generates similar waste problems (Keating *et al.,* 2001). Raised levels of toxic heavy metals, high levels of salinity, low pH, high concentration of sulphate, manganese, aluminium and iron is the major cause of acid mine drainage (AMD). The generation of AMD from coal mine waste poses a familiar threat to the environment due to high dissolved metal concentrations (Larsen and Mann, 2005). High concentrations of Al^{3+}, Mn^{2+} and Fe^{3+} precipitates have been proposed to be the main cause of plant damage and extinction (Elberling *et al.,* 2007). A major concern of operating mine sites is the prevention and control of AMD. The groundwater effluence can be associated with AMD. Acid mine drainage allows heavy metals to seep into the surroundings leading to alarming environmental conditions for aquatic habitats and soil quality (Adler and Rascher, 2007). Mining plays an important role in South Africa's economy by contributing about 12% of the GDP and about 59% of the total value of exports. The mining industry uses only 4.2% of the total water utilized in RSA. However, the pollution loads carried in mine effluents can impose limitations on the usefulness of the fresh water resources.

2.4 Pollutants of water

In wastewater treatment, the most important water quality parameters are: biological oxygen demand (BOD), chemical oxygen demand (COD), phosphate, nitrogenous species and heavy metals (Torres-Guzman *et al.,* 2010). In wastewater, high concentrations of these parameters are considered unacceptable (Akpor and Muchie, 2011).

2.4.1 Biological oxygen demand (BOD) and chemical oxygen demand (COD)

The reduction of organic and inorganic materials is the main goal in wastewater treatment (Manahan, 2011). The efficiency of wastewater treatment is often calculated in terms of reduction in biological oxygen demand (BOD) and chemical oxygen demand (COD) (Kabuk *et al.,* 2015). The relative amount of dissolved oxygen consumed mainly by microorganisms to completely oxidise all matter in wastewater is called BOD. High BOD can result from high levels of oxidisable materials (Madigan and Martinko, 2006). To assess the extent to which effluents promote anoxic conditions when deposited into water, BOD is used as a measure (van Loon and Duffy, 2005). The amount of oxygen required to oxidise all organic carbon to carbon dioxide and to water is called COD (Weiner, 2013). In the presence of silver and sulfuric acid, COD is measured by oxidation of potassium dichromate. A ratio of BOD/COD with a value of 0.5 in water indicates that a large amount of non-degradable materials are present in wastewater (Choi *et al*., 2017). Anaerobic conditions are often caused by high concentrations of oxidisable materials, stagnant waters and bad odours that do not support life (Mihelcic and Zimmerman, 2010).

2.5 Nutrients

The continuous addition of excess nutrients in water bodies is called eutrophication (Lilley *et al*., 1997). The main nutrients responsible for eutrophication are nitrogen, phosphorus and sulphur (WEF, 2011). The generation of toxic algae, dinoflagellate and cyanobacteria are the main challenges that result from eutrophication (Sigee, 2005). The toxins arising during eutrophication are often concentrated in the food

chain. Skin irritation, amnesia, liver damage, paralytic shellfish poisoning and diarrhoea often result from algae toxins (WHO, 2006). The generation of “dead zones” and erosional habitats are also promoted by eutrophication (Bowen *et al*., 2014). The interference of humans in both phosphorus and nitrogen cycles from fertiliser is in excess of levels of safety, according to UN Department of Economic and Social Affairs (2012).

2.5.1 Phosphorus

In freshwater basins phosphorus is also a limiting nutrient (Chin, 2013). However, excreta from livestock, drainage from fertilised agricultural land, industrial discharge and atmospheric deposition results in excessive phosphorus in freshwater basins (Gulf of Maine, 2012). These high concentrations greatly contribute to eutrophication (WEF, 2011).

2.5.2 Nitrogenous species

Nitrogen forms part of the building blocks of life such as amino acids (Hazen *et al*., 2005). However, water pollution and eutrophication may result from the excessive deposition of nitrogenous species (ammonia and nitrates) in an oceanic or terrestrial ecosystem (Raboni *et al.,* 2015). Furthermore, the effectiveness of chlorine decontamination is hindered by the presence of ammonia and nitrates in drinking water treatment because they have a high affinity for chlorine (Raboni *et al*., 2015). In water bodies, the reduction of oxygen and toxicity to aquatic fauna are the poisonous effect of ammonia (Weiner, 2013). In wastewater treatment the removal of phosphorus is inhibited by the presence of nitrates and nitrites (Drysdale *et al*., 2000). Some health related issues often caused by excessive nitrogen concentrations include the following: methemoglobonemia, trihalomethane formation in chlorinated drinking water and nonHodgkin lymphoma (Weiner, 2013). Global warming is promoted by the increase of emissions of nitrous oxide (greenhouse gas) (Reilly *et al.,* 2003).

2.6 Flocculation

Flocculation is the process in which colloids aggregate or come together from the suspension to form larger particles called flocs (Bhunia *et al*., 2012). During the flocculation process (Figure 1) floccules formed can be efficiently removed by filtration, flotation and sedimentation processes (Edward, 2011).

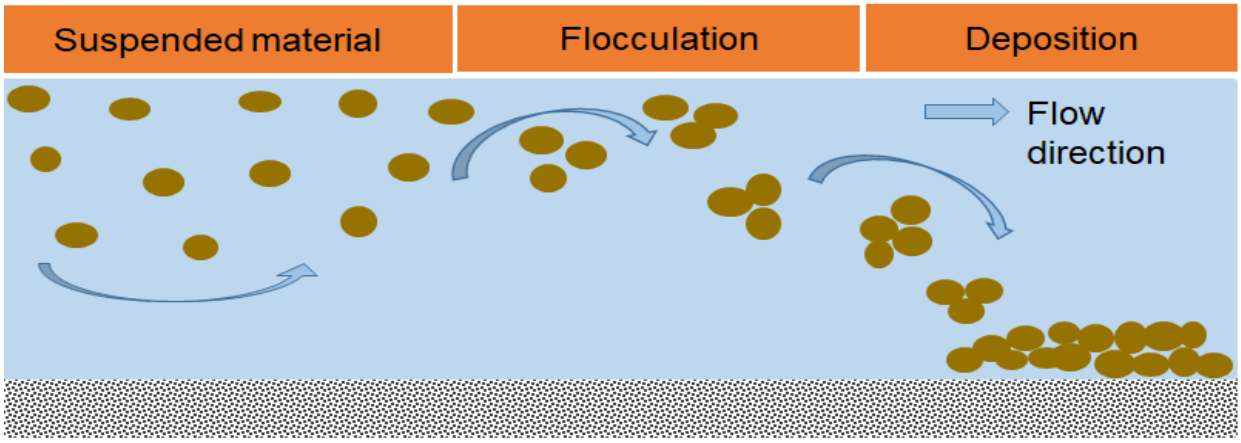

Figure 1: Flocculation process (Department of Water and Sanitation (DWS), 2002)

2.7 Flocculants

Flocculants can be applied to different processes including downstream processing, wastewater treatment and food and fermentation processes (Mabinya *et al*., 2011). The speed of flocculation is often increased by the addition of flocculants. This increases the weight and strength of the floc in order to remove floccules; which improves the floccule propensity for breaking under fluid shear (Edward, 2011; Spellman, 2014). Flocculants are categorized as organic, inorganic, naturally occurring and grafted flocculants (Salehizadeh and Shojaosadati, 2001; Yim *et al.,* 2007).

2.7.1 Inorganic flocculants

Inorganic flocculants such as aluminium sulphate (alum) are normally used because of availability, low price and simplicity of use. Drawbacks to alum include the following

issues: high sensitivity to pH, inefficiency towards very fine particles and applicable to only selected dispersed systems (Sharma *et al*., 2006). In addition aluminium sulphate is reported to be neurotoxic, carcinogenic and result in Alzheimer's disease (Serdar *et al*., 2011; Aljuboori *et al*., 2015). The by-products of conventional flocculants are nondegradable and mostly toxic and thus environmentally unfriendly. Therefore, there is a need for new and safe flocculating agents.

2.7.2 Organic polymeric flocculants

Polyacrylamide is an example of an organic flocculant which is important in treating wastewater. Polyacrylamide has the capability to be effective even when added in small amounts (Sharma *et al*., 2006). Elongated chains with high molecular weight polymers are absorbed on particles with loops and tails spreading into solution. This give the possibility of accessory of these droopy polymer segments onto extra particles, thus bridging particles together (Bolto *et al*., 2007). Therefore, the floccules formed are stronger, larger and heavier with appropriate settling characteristics (Singh *et al*., 2000). They are not sensitive to pH, produce lower sludge volume and immediately soluble in aqueous systems (Renault *et al*., 2009). Organic synthetic flocculants are widely used in biotechnological fields due to their flocculation efficiency and economic friendliness. However, these flocculants monomers have resistance to biodegradability, thus causing environmental pollution (Tripathy and De, 2006). Furthermore, most commercial polymers are produced from petroleum-based compounds which have a very low margin of safety to the environment and humans (Suopajärvi *et al*., 2013). These drawbacks have limit their use in many industries.

2.7.3 Naturally occurring flocculants

The limitations of inorganic and organic flocculants have necessitated more research into biological flocculants (Nwodo *et al.,* 2016). These flocculants are usually assumed to be harmless to humans and animals (Nwodo *et al.,* 2016), nontoxic in nature (Chopra and Ruhi, 2016) and they lack secondary pollution (Mishra, 2016). Microorganisms, plants and animals were used to produce and extract many natural flocculants (García-Fayos *et al.,* 2015). These flocculants are found to be safe, eco-

friendly and environmental friendly. They can be applied in different processes including pharmaceutical, cosmetic, downstream processing, food and fermentation processes and wastewater treatment. Chitosan, cellulose and bioflocculant have been investigated by researchers.

Essential polymers that are produced by microorganisms during growth are defined as microbial bioflocculants (Cosa *et al.,* 2011). Carbohydrates, proteins, lipids, nucleic acid and humic substances are the main microbial flocculant components (Marvasi *et al.,* 2010). Many bioflocculants are produced from different microorganisms such as actinomycetes, bacteria and fungi according to literature reports (Sheng *et al.,* 2006). Bioflocculants are biodegradable and environmentally friendly (Liu *et al.,* 2010). Low flocculating efficiency and low yield can be limiting issues that hamper their industrial application and large scale production (He *et al.,* 2010).

2.7.4 Grafted flocculants

All polymers whether synthetic or natural have disadvantages. Due to these shortcomings, several strategies have been considered for the development of more effective and inexpensive flocculants which contains natural polymers. Many attempts have been made to combine the desirable properties by grafting synthetic polymers onto the backbone of natural polymers (Ghimic *et al.,* 2012).

Grafted flocculants (Figure 2) comprise of a long sequence of one polymer (natural) with one or more branches of another polymer (synthetic) (Odian *et al.,* 2004). Grafted flocculants have appeared as novel tools that constitute remarkable opportunities in wastewater treatment. Grafted flocculants have distinctive properties compared to synthetic flocculants (Lee *et al*., 2012).

Chemical alteration of natural polymers with those of synthetic polymers has been discovered as a technique of integrating the best attributes of both types. Therefore, by improving the aggregating power of flocculants, and by increasing the positive electric charge of flocculants the ratio of effectiveness is improved (Wang *et al*., 2008).

The grafted flocculants consists of fewer and lengthier droopy polymer chains with high molecular weight and a high branched structure. Therefore, such features provide easy accessibility to pollutants in effluent. At small dosages, these flocculants are asserted to be more effective (Bolto and Gregory, 2007; Singh *et al.,* 2000). The simple accessibility model of flocculation revealed that the existence of polyacrylamide grafted chains would improve the hydrodynamic volume (radius of gyration) of a polymer in solution and by that increase its flocculation ability (Brostow *et al.,* 2007; Singh *et al*., 2000).

Several grafted flocculants including poly(2-hydroxyethylmethacrylate) grafted agar (Rani *et al*., 2013), polyacrylamide grafted starch (St-g-PAM) (Mishra *et al*., 2014), hydroxypropyl methyl cellulose grafted with polyacrylamide (HPMC-g-PAM) (Das *et al*., 2013) and polyacrylamide grafted carboxymethyl guar gum (CMG-g-PAM) (Pal *et al.,* 2011) have been successful synthesized. The flocculating properties of the grafted flocculants were tested in kaolin suspension and various types of wastewater treatment (municipal sewage wastewater, pulp mill wastewater, raw mine wastewater and textile effluent wastewater). The positive outcome of these flocculants proves that they can be applied in wastewater treatment.

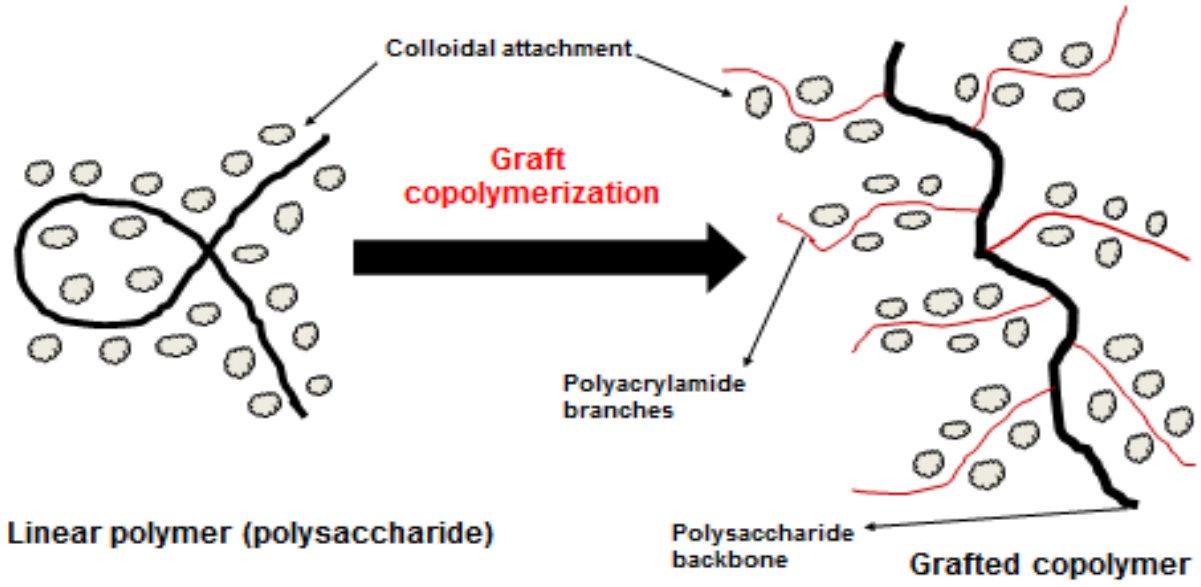

Figure 2: Linear polymer and graft copolymer structural configuration (Singh *et al.,* 2003)

2.8 Bioflocculation

A natural process whereby microbial flocculants (bioflocculants) are used to flocculate, settle and remove particles, suspended solids and dyes in solution is called bioflocculation (Cong-Liang *et al.,* 2012). Microbial flocculants are macromolecules produced by microorganisms through interaction with their environment as a result of substrate metabolism, microbial growth and degradation of microorganisms or microbial chemical components (Komilis *et al*., 2016). According to the nature of their association with the cells or the method of extraction, bioflocculants are defined as capsular, slime, loosely bound and tightly bound compounds (Hendric, 2006).

2.9 Grafting techniques

The most effective technique of modifying the properties of natural polymers is by grafting. This can lead to the production of highly efficient grafted copolymers. The absence of commercial techniques of synthesis is the main problem for developing grafted copolymers (Mishra *et al.,* 2011). Graft co-polymerization can be obtained by conventional and non-conventional techniques, with or without the presence of different initiator systems (Singh *et al.,* 2000). High energy radiations (gamma and X-ray), conventional techniques (chemical free radical initiator) and microwave based techniques are the major methods involved in the synthesis of grafted polysaccharides.

A chemical free radical initiator (ceric ammonium nitrate or CAN) is used in the conventional method of synthesis. On the backbone polymer, this initiator generates free radical sites to allow the monomer to bind and structure the graft chain (da Silva *et al.,* 2007; Sen *et al.,* 2009). Due to low reproducibility, this technique is not appropriate for commercial scale.

High energy radiation (gamma rays or electron beam) is used as a free radical generator. This is a superior technique of synthesizing graft copolymer (Vahdat *et al*., 2007; Wang *et al.,* 2008). However, the polysaccharide backbone can be destructed by this technique.

Microwave radiation is a better technique for graft copolymer synthesis. In this technique, on the polymer backbone, free radical sites are generated (Mishra *et al.,* 2011). However, this technique is expensive for production. More investigations are required to obtain an economic, feasible and eco-friendly technique for synthesis of high grade grafted flocculants.

In this study, a microwave radiation technique was used to synthesize a grafted bioflocculant.

2.10 Factors effecting the activities of a grafted flocculant

The effectiveness of grafted flocculant is affected by many factors. Dosage size, chemical functionalities (functional groups and chemical components), electrical charge, molecular weight, degree of purity and the structure of flocculants are among the factors that affect flocculating activity and mechanisms.

2.10.1 Dosage size

Dosage size is one of the significant factors in the flocculating activity of bioflocculants. A poor flocculation process is often caused by insufficient bioflocculant concentration. This has the tendency of not neutralizing negative charges on colloids in suspension (Li *et al.,* 2007). Over dosage of bioflocculant concentration can lead to a decrease in the settling of the flocculated colloidal particles in a suspension due to high viscosity (Wang *et al*., 2011). Therefore, an optimal bioflocculant concentration is required for effective flocculation.

2.10.2 Functional groups and chemical components

Bioflocculant functional groups provide adsorption sites for multiple colloids in suspension (Xiong et al., 2010). The ability of bioflocculants to bind depends on the number of functional groups present in the chain of the bioflocculant. Flocculating activities can be influenced by chemical composition of the bioflocculant. In previous

studies, it was reported that bioflocculants were predominantly composed of polysaccharides, fatty acids, nucleic acids and proteins (Salehizadeh and Shojaosadati, 2003).

2.10.3 Electric charge of flocculants

The bioflocculants' surface electric charge plays an important role in flocculating activity. A bioflocculant electric charge is significantly influenced by the proportion of its components (Walker and Wilson, 2005). In most cases, bioflocculants bear a negative charge (Levy *et al.,* 1992; Okaiyeto *et al.,* 2016). This allows the bioflocculant to bind to positively charged colloids in a suspension.

As a result of cation exchange potential, this may occur and be obtainable through electrostatic interaction (Esparza-Soto and Westerhoff, 2003). A higher hydrophobic character is shown in bioflocculants with a net negative surface charge. These hydrophobic constituents form bonds with positively charged colloidal particles such as dyes and metal ions in suspension (More *et al.,* 2010).

2.10.4 Molecular weight of flocculants

The flocculating ability of bioflocculants can be affected by the molecular weight of bioflocculants (Liu and Cheng, 2010). The bioflocculant's molecular weight varies mostly between 10^5 and 2.5 X 10^6 Da (More *et al*., 2014). The molecular size of bioflocculants is related to the efficiency of the bridging mechanism in flocculation. The higher the molecular weight of a bioflocculant, the higher the flocculating activity in contrast to bioflocculants with low molecular weight (Zhang *et al.,* 2010).

2.10.5 Purity of flocculants

Bioflocculants are seldom produced by microorganisms in their pure state. The crude extract may contain a large range of impurities including microbial cells. Even though it is challenging to purify microbial flocculants, purity is important for their effective use (Walker and Wilson, 2005). The degree of purity of a bioflocculant affects its functionality in the flocculation process (Smith, 2009). Poor flocculating ability of the

bioflocculant may be caused by contaminants which may compete with colloidal particles on the adsorption sites in a suspension (Singha, 2012). It is usually stated that the higher the purity, the greater the efficacy in flocculation. Purification techniques that are mostly used to remove cells or their debris are centrifugation, chromatographic methods, heat treatment and precipitation methods (Sutherland 1990; Morin, 1998; Zhang *et al,* 2007).

2.10.6 Structure of flocculants

In flocculation processes, the surface morphological structure of a bioflocculant plays a vital role (Zhang *et al*., 2007). Effectiveness or poor flocculating activity of bioflocculants may be caused by the surface morphological structure (Okaiyeto *et al.,* 2015). In literature most bioflocculants are reported to have an amorphous, porous or crystal-like structure (Cosa *et al.,* 2013, Okaiyeto *et al.,* 2015). A scanning electron microscope (SEM) uses electrons in a raster scanning grid pattern to image the sample surface. The electrons react with the atoms to produce signals containing information regarding the structure of the sample (He *et al*., 2010).

2.11 Biosafety

Biosafety standards are based on international acts and relevant legislation. These aim to prevent risk to human health and the environment which result from activities that involve the use of products or bioproducts (Smith, 2009). Bioflocculants are confirmed to be non-toxic compounds according to Devi *et al.* (2015). As confirmation of this, bioflocculants must be tested for toxicity before any application (Spellman, 2014). Pandemic diseases along with allergenic, cytotoxic and neurotoxic effects can be caused by bacterial secondary metabolites (Smith, 2009).

In this study, an MTT assay was used to test the cytotoxic effects of a grafted bioflocculant on different cell lines. This method uses a colourimetric assay that is based on the change of tetrazolium salt (MTT) to formazan by mitochondrial dehydrogenases. This assay is usually used to ascertain cell viability (Mosman, 1983).

2.12 Flocculation mechanisms of grafted flocculants

An increase in chain extent and molecular weight contributed to an increase in flocculation efficacy for grafted bioflocculants (Agarwal *et al.,* 2002). Both organic and inorganic matter in wastewater convey low positive or negative charges. In nature polyacrylamide grafted polysaccharides are usually anionic or nonionic (Ghosh *et al.,* 2011). In a polymer bridging mechanism, the bridges between adjacent particles are formed when the sectors of a polymer chain are absorbed against different particles surfaces. For this reason grafted flocculants have superior flocculation features compared with pure bioflocculant (ungrafted).

High radius of rotation, high molecular weight and extended polymer chains are characteristics of grafted flocculants. Therefore, the adsorbed polymer molecules have a way of accepting additional extended configuration for interacting with many particles from floccules (Das *et al.,* 2013).

Polymer bridging was accountable for the superior flocculating property in grafted copolymers over a linear polymer (Ghosh *et al.,* 2010). Due to the polymer chain, sectors were adsorbed onto diverse particles, thus connecting together the particles (Mishra *et al.*, 2004).

In other studies, an ideal dosage size was determined for maximum flocculation efficacy. Over dosage of the grafted bioflocculant caused the flocs to redisperse and reduce particle settling in a suspension and resulted in low flocculation. The bridging mechanism involved is responsible for this phenomenon and was reported to link with the behaviour of the flocculation curve (Sen *et al.,* 2012).

Other studies stated that through the bridging effect the colloidal particles come together and form larger net like floccules. This formation develops as a result of the flexibility of polyacrylamide graft chains. The larger floccules with net-like structure can

further grab remaining particles from water. This occurs via a sweeping effect with the help of improved accessibility of the polyacrylamide chains (Yang *et al.,* 2013). Lastly, the compressed aggregated particles are formed and then settle out.

Grafted flocculants are applied in wastewater treatment in most of the reported studies. The study of flocculation mechanism is very limited. The molecular weight and chain length are the significant factors for determining the chief flocculation mechanism of grafted flocculants. To identify the accountable flocculation mechanism, dynamic light scattering techniques with observation of floccule formation and the measurement of molecular weight could be applied for both grafted and ungrafted bioflocculants.

2.13 *Alcaligenes faecalis*

More information is offered for microbial flocculants produced in different habitats by different bacteria. However, rich biodiversity of bacteria remains largely unknown in marine surroundings (Kumari *et al.,* 2014). *Alcaligenes faecalis* is a rod-shaped, gram-negative bacterium and was first discovered in faeces but was later found to be common in soil, water and environments in association with humans (Phung *et al*., 2012). This bacterium is normally non-pathogenic (Kavuncuoglu *et al*., 2010). Due to the production of useful biopolymers, *Alcaligenes* strains have been used in research recently and have gained more attention in industry. It is also commonly used in bioremediation of hydrocarbon pollutions (Bharali *et al.,* 2001). Enzymes that degrade organic contaminants are produced by some of these bacterium strains and can be applicable in wastewater treatment and biodegradation of organic pollutants (Kong *et al*., 2014).

For this study, *Alcaligenes faecalis* HCB2 was used to produce bioflocculant through an extraction method.

2.14 Acrylamide (AM)

Acrylamide is an unsaturated amide used to produce polyacrylamides (Ruiz *et al.,* 2012). Acrylamide is a white or colourless, odourless crystalline solid (Mustatea *et al.,* 2015). It is soluble in water, acetone and alcohol and insoluble in heptanes and benzene (Charoenpanich *et al.,* 2013). Acrylamide is used as a flocculant, in paper production, as an additive for water treatment, as a thickener, in enhanced oil recovery, as a soil conditioning agent, in sewage and waste treatment, for permanent-press fabrics and in ore processing (Edition *et al.,* 2011). In the construction of drinking water reservoirs and wells, acrylamide is also used as a grouting agent (Edition *et al*., 2011). Acrylamide is mostly found in foods that are dense with carbohydrates such as fried potatoes, breads, coffee, and breakfast cereals (US-FDA, 2014). Starchy foods that are cooked at high temperatures also contain acrylamide.

Water utilities tightly control the level of acrylamide in drinking water and it is reported not to be a health hazard (Backe *et al.,* 2014). For potable water, polyacrylamide used should not contain more than 0.05% residual monomer (Edition *et al*., 2011). Bio concentration of acrylamide is unlikely because it is degraded easily by microorganisms (Edition *et al*., 2011). Polyacrylamide (PAM) is efficient in water purification (Lee *et al.,* 2011). PAM also produces dense floccules which are generally helpful for easy sedimentation in water treatment (Yang *et al.,* 2013).

In this study, acrylamide was combined with a bioflocculant to produce a polyacrylamide grafted bioflocculant.

Chapter 3: Materials and methods

3.1 Production medium

Chemicals and reagents bought from Sigma-Aldrich (St Louis, MO, USA) and distilled water were used. The production medium for grafted bioflocculant was prepared using the Zhang *et al*. (2007) method.

Briefly, KH_2PO_4 (2.0 g), glucose (20.0 g), K_2HPO_4 (5.0 g), NaCl (0.1 g), $(NH_4)_2SO_4$ (0.2 g), yeast extract (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g) and urea (0.2 g) were dissolved in a 1L of distilled water and sterilized by autoclaving at 121°C for 15 min. The medium was allowed to cool after autoclaving and inoculated with fresh culture.

3.2 Determination of flocculating activities

A loopful of bacterial species was inoculated into a 250 mL flask containing 50 ml of the sterilized production medium. The mixtures were placed at 30°C in a shaking incubator at 165 rpm for 72 h. At 8000 rpm the culture broths were centrifuged to remove cells for 30 min at 4°C. At an optical density (OD) of 550 nm, the UV-visible spectrophotometer was used to determine the flocculating activity (Kurane *et al.,* 1994). One hundred millilitres (100 ml) of kaolin solution (4 g/l) was measured into 250 ml flasks and was mixed together with 2 ml of the attained cell free supernatant and 3 ml of 1% w/v calcium chloride ($CaCl_2$). The mixtures were agitated for 60 sec and then poured into a 100 ml measuring cylinder. The sediment as well as the control medium, was left to stand for 5 min at 25°C (room temperature). A control was prepared in a comparable way, but TKT-g-PAM was replaced with un-inoculated culture medium. The flocculating activities (FA) were evaluated by using the following formula:

$$FA\ (\%) = [(X - Y/X)] \times 100$$

Where X at 550 nm was the optical density of kaolin suspension and Y at 550 nm was the optical density of the sample.

3.3 Extraction and purification

The bioflocculant was extracted and purified using the method of Chang *et al.,* (1998). The culture medium was centrifuged (8,000 × g, 30 min, 4°C) after incubation for 72 h. To the supernatant, a unit volume of distilled water was added and centrifuged (8,000 × g, 4°C. 30 min) to eliminate insoluble materials. Two 2 L of ethanol were added to the supernatant and agitated. The mixture was allowed to stand to precipitate at 4°C for 12 h. The precipitate was vacuum-dried and 100 ml of distilled water was added to dissolve the bioflocculant. A mixture of chloroform and butanol (5:2 v/v*)* was added (1 volume), agitated and placed at room temperature for 12 h to settle down. The supernatant was centrifuged to remove any debris at 4,000 × g, 30 min, 4°C and vacuum-dried.

3.4 Synthesis of the graft copolymer using the "microwave initiated" method

Microwave irradiation (Figure 3) was used to generate the free radical sites on the polysaccharide backbone (Sen *et al*., 2009). In 40 ml of distilled water, 1.0 g of the bioflocculant was dissolved. Five grams (5.0 g) of acrylamide was dissolved in 10 ml of distilled water and added to the bioflocculant solution. The two solutions were mixed well and transferred to 100 ml borosilicated beaker. The mixture was placed in a microwave turntable oven (25 l LG Microwave oven Model: MG-577B). A temperature probe was also incorporated. Microwave irradiation was set to 900W of power and for a preferred amount of time ranging from 1 to 4 min. The microwave irradiation was stopped periodically to cool the reaction mixture by placing the reaction mixture beaker in cold water to avoid excessive formation of dangerous reaction vapours and to prevent damage to the polymer backbone. The temperature of the reaction mixture was not allowed to exceed 70°C.Once the intended amount of time was completed for microwave irradiation, the reaction beaker and its contents were cooled and kept undisturbed at room temperature for 24 h. This allowed enough time for the grafting reaction to be completed. A saturated solution of hydroquinone (20 mg) was added to terminate the reaction. Excess acetone (25 ml) was added into the gel like mass. A hot air oven was used to dry the mixture resulting in precipitation of the grafted copolymer (60°C, 6 h). Subsequently, it was pulverized and sieved.

3.5 Purification of graft copolymer by solvent extraction method

To remove the homopolymer, the precipitated grafted polymer was dried and then extracted using a formamide acetic acid mixture with a 1:1 ratio (v) (Rani *et al.,* 2012). The percentage grafting was calculated by using the following formula:

% G = (weight of graft copolymer – weight of polysaccharide)/weight of polysaccharide x 100.

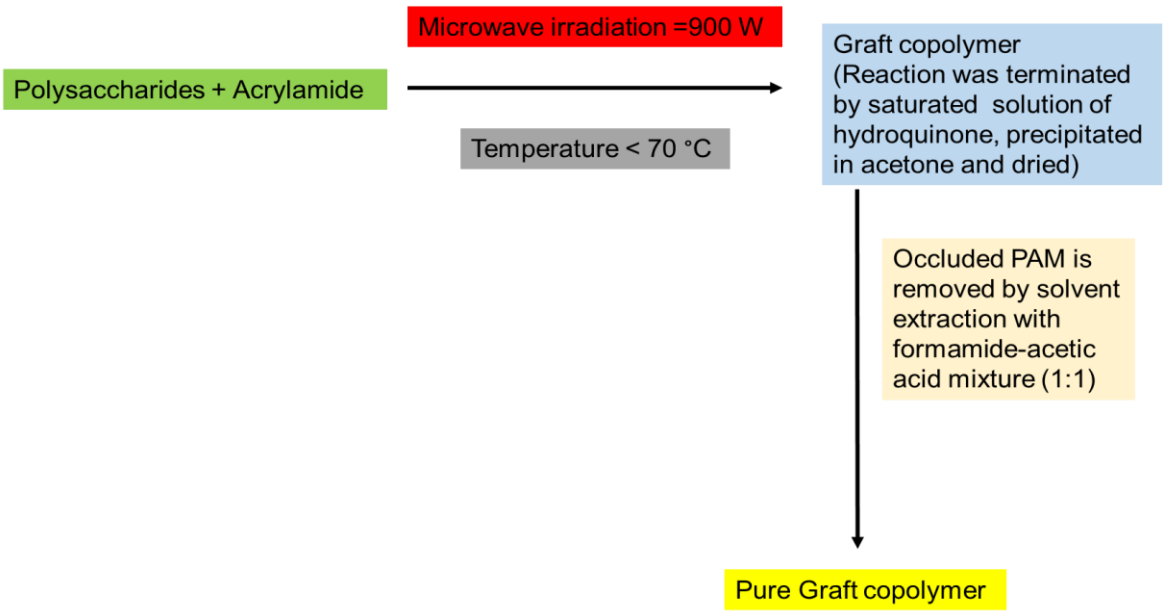

Figure 3: Schematic representation for the synthesis of TKT-g-PAM using microwave-initiated method (Pal *et al*., 2012)

3.6 Physicochemical characteristics of the grafted bioflocculant

3.6.1 Morphological surface of the flocculants

The purified bioflocculant and grafted bioflocculant morphological surface structures were studied using a scanning electron microscope (SEM) (Karthiga devi and Natarajan, 2015). A small amount of the bioflocculant and grafted bioflocculant were

spread and fixed on an iron stub. The fixed specimens were sputter coated with gold and examined.

3.6.2 Charge characterization

The grafted bioflocculant flocculation mechanism was proposed after the zeta potential was measured. The zeta potential of the kaolin particles, mixture of kaolin particles and $BaCl_2$, kaolin particles flocculated by the grafted bioflocculant in the presence of $BaCl_2$ and the grafted bioflocculant were measured, (Aljuboori *et al.,* 2015). Charge characterization of the grafted bioflocculant and the pure bioflocculant was determined. This was done using a Malvern Zetasizer Nano (Malvern, UK), at 25°C (Aljuboori *et al.,* 2015).

3.6.3 Functional groups

The functional groups were assessed using a Fourier Transform Infrared (FTIR) Spectrophotometer (Perkin Elmer System 2000, England). The dried bioflocculant as well as the grafted bioflocculant powder were mixed with potassium bromide salts (KBr), ground at 25°C and compressed into pellets for FTIR spectral measurement. The setting ranged between 4000-370 cm^{-1} (Luo *et al.,* 2014).

3.6.4 Cytotoxicity assay of the grafted bioflocculant

The cell cytotoxicity of the grafted bioflocculant was measured according to the method by Mosman, (1983). Human embryonic kidney 293 cells (HEK 293) and human colorectal adenocarcinoma cells (CaCO2) were both grown to confluency in 25 cm^3 flasks. The cells were then trypsinised and plated into 48 well plates and incubated at 37 °C for 24 h. Then, the old medium was supplemented with a new medium (MEM + Glutmax + antibiotics). The grafted bioflocculant was added in triplicate and incubated for 4 h. Thereafter, the medium was substituted with a new medium (MEM + Glutmax + antibiotics +10% Fetal bovine serum). After 48 h, the cells were subjected to 200 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) medium with a concentration of 5 mg/ml of phosphate buffered saline

(PBS). In addition, 200 µl medium was added to each well and incubated at 37˚C for 4 h. Thereafter, the medium with MTT was aspirated from the wells and the resulting formazan crystals were solubilized in 200 µl of dimethyl sulfoxide (DMSO). The optical density of the solutions was read at 570 nm using a micro plate reader. The cell viability was expressed as a percentage with control using the following equation;

Cell viability (%) = (K1/K0) x 100

Where K1 and K0 are the initial and final values obtained before and after treatment with the bioflocculants, respectively.

K1= values before treatment

K2= values after treatment

3.7 Evaluation of flocculating activity of the grafted bioflocculant

3.7.1 Effect of grafted bioflocculant concentrations

Grafted bioflocculant concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were set in distilled water. Their flocculating activities were assessed for the determination of the optimum concentration (Luo *et al.*, 2014).

3.7.2 Effect of cations on flocculating activity

Various cations (LiCl, KCl, NaCl, $BaCl_2$, $CaCl_2$, $MnCl_2$, and $FeCl_3$) were used with the grafted bioflocculant to evaluate their effect on flocculating activity. A standard cation (3 ml of 1% w/v $CaCl_2$) was replaced with the above mentioned cations (Nwodo *et al.,* 2016). A kaolin solution was used as a control without addition of a cation. The flocculating activities were determined as shown previously.

3.7.3 Grafted bioflocculant's pH stability

The effect of pH on the flocculating activity of grafted bioflocculant was determined by adjusting the pH of the kaolin clay suspension (4 g/l) in a range between pH 3 to 11

with 0.1 M KOH and 0.1 M HCl (Nwodo *et al.,* 2016). The flocculating activities were determined as indicated in 3.2.

3.7.4 Grafted bioflocculant thermal stability and pyrolysis profile

Temperatures starting from 50 to 100°C were used (Okaiyeto *et al*., 2013). The grafted bioflocculant solution was placed for 60 min in a water bath and the flocculating activities were determined as indicated in 3.2. A thermogravimetric analyser (Perkin Elmen Pyris 6 TGA) was used to evaluate the pyrolysis profiles of both grafted bioflocculant and bioflocculant. The purified flocculants (10 mg) were heated within a range of 22 to 900°C at an increased rate constant of 10°C $mitn^{-1}$ under constant flow of nitrogen gas (Okaiyeto *et al.,* 2016).

3.8 Removal efficiency of the grafted bioflocculant using coal mine wastewater

The chemical oxygen demand (COD), biological oxygen demand (BOD), total nitrogen (N) and sulfur (S) were determined in wastewater from Tendele Coal Mine wastewater plant (RSA). The spectrophotometer with Spectro-quant (Merck Pharo 100) was used to measure these parameters both before and after treatment with the grafted bioflocculant.

The jar test was performed using the method described by Okaiyeto *et al*. (2016). Two millilitres of the optimum dosage size (0.2 mg/ml) of grafted bioflocculant and 3 ml of 1% (w/v) $BaCl_2$ solution were both transferred into a 100 ml wastewater sample. The mixture was shaken for 3 min at high speed (200 rpm). Then the speed was decreased to 40 rpm for 5 min. The depletion efficacies of BOD, COD, N and S by the grafted bioflocculant were determined using the following formula:

Removal efficiency (%) = (B – M/ B) × 100

Where B and M were the original and last values achieved in earlier and later treatment with the grafted bioflocculant, respectively. Bioflocculant, alum and ferric chloride were used as controls.

B= values before treatment

M= values after treatment

3.9 Statistical analysis

Using the one-way analysis of variance (ANOVA) using Graph Pad prism™ 6.1. The information was performed in triplicates and the error bars in the figures that follow show the standard deviations of the data. P values ≤0.05 were considered as significant. Values with different letters along the same row are significantly different ($p<0.05$).

Chapter 4: Results

The results obtained during extraction of the bioflocculant TKT, synthesis of grafted bioflocculant, characteristics and application of the grafted bioflocculant are shown in this section.

4.1 Bioflocculant yield

The purified bioflocculant (4.0 g) was obtained from 1 L of fermentation broth of *Alcaligenes faecalis* HCB2.

4.2 Microwave initiated synthesis (using 900 w microwave radiation)

Table 1 shows a sequence of six graft copolymers that have been synthesized by microwave irradiation. For the first four evaluations of graft copolymers, the concentration of the bioflocculant and acrylamide was fixed with only the exposure time being varied. Only the acrylamide concentration was varied for the second set of three grafted copolymers (3, 5-6) while keeping the other parameters constant. By varying the reaction parameters, various grades of TKT-g-PAM have been developed.

Table 1: Synthetic details of TKT-g-PAM

Grafted bioflocculant	Amount of bioflocculant (g)	Amount of acrylamide (g)	Time in (min)	% Grafting
TKT-g-AM 1	0.5	5.0	1	19
TKT-g-AM 2	0.5	5.0	2	35
TKT-g-AM 3	0.5	5.0	3	51
TKT-g-AM 4	0.5	5.0	4	39
TKT-g-AM 5	0.5	6.0	3	57
TKT-g-AM6	**0.5**	**7.0**	**3**	**62**
TKT-g-AM 7	0.5	9.0	3	59

Note: Bolded values imply optimized grade synthesized by the processes.

Figure 4 shows the effect of irradiation time. The percentage grafting increased up to 3 min (which is optimized) with increases in irradiation time (1-4) after which it was decreased.

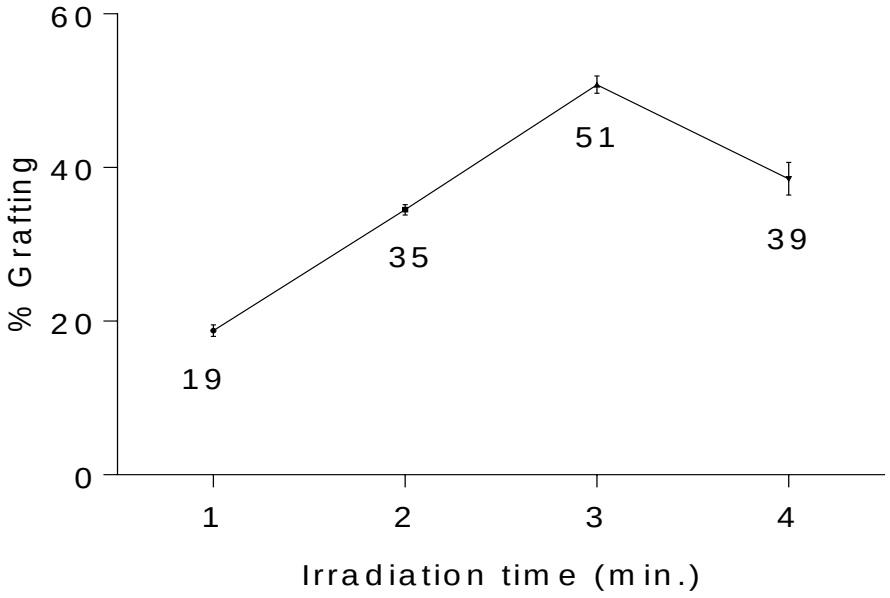

Figure 4: Effect of irradiation time and the percentage grafting (keeping other factors constant)

Figure 5 shows the effect of monomer concentration. With an increase in the monomer concentration of acrylamide from 5.0 to 7.0 g, the grafting ratio also increased. Further increases in monomer concentration beyond 7.0 g, indicates the percentage grafting decreased. Thereby, a homopolymerization reaction was initiated and low graft yield was observed beyond optimum monomer concentration. The highest percentage of the purified grafted bioflocculant was 62% with a weight of 0.812 g.

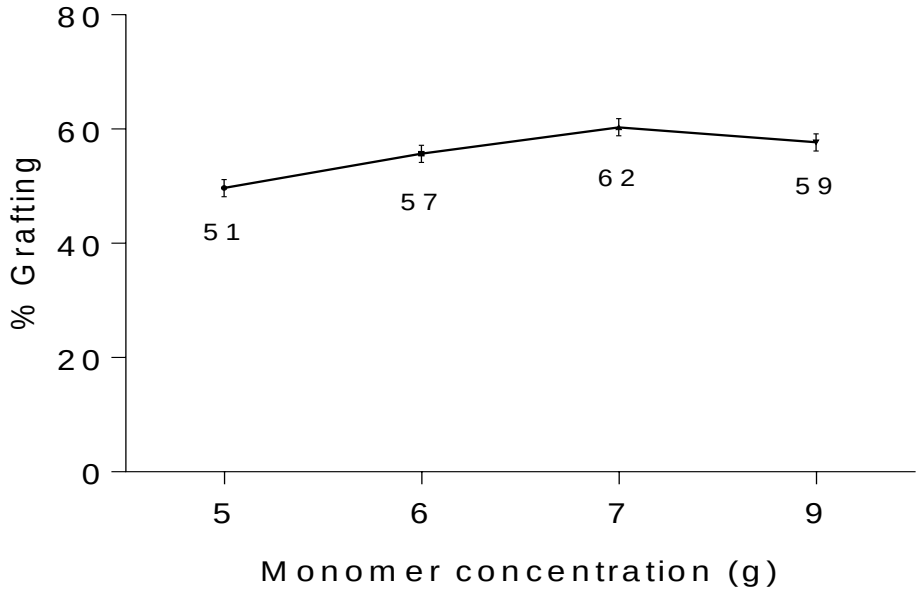

Figure 5: Effect of monomer concentration and the percentage grafting (keeping other factors constant)

4.3 Physico-chemical composition of the bioflocculant

4.3.1 Morphological surface

Figure 6a and 6b show the scanning electron microscopy (SEM) surface image of the bioflocculant (TKT) and grafted bioflocculant (TKT-g-PAM6). The particles were examined in powdered form. It is evident from the SEM micrographs of TKT and its grafted product that morphological changes had taken place because of grafting of PAM chains on TKT. It is clear that the original morphology of the TKT was lost after grafting.

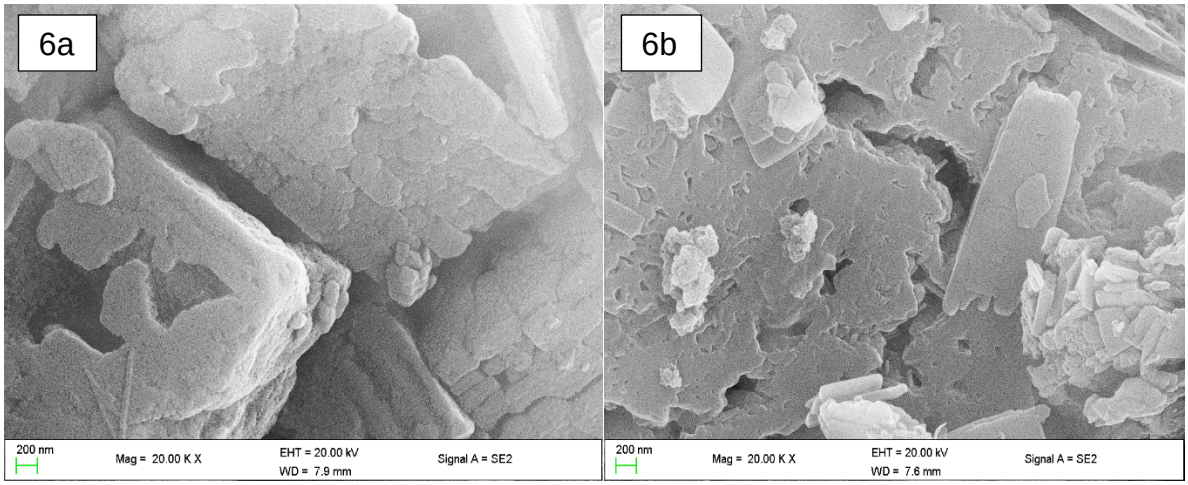

Figure 6: SEM surface images of TKT (6a) and TKT-g-PAM6 (6b)

4.3.2 Zeta potential of samples

Table 2 shows the zeta potential of TKT, kaolin particles, kaolin particles with Ba^{2+}, kaolin particles flocculated with TKT in the presence of Ba^{2+}, TKT-g-PAM6, kaolin particles flocculated with TKT-g-PAM6 in the presence of Ba^{2+} and kaolin particles flocculated with acrylamide in the presence of Ba^{2+}. The electrical charges of the TKT-g-PAM6, TKT and kaolin particles were negative -12.6±0.1, -17.1±0.65 and -6.59±3.0. The grafted bioflocculant used a bridging mechanism facilitated by Ba^{2+} to flocculate kaolin suspension. In accordance, the results show that the chain length contributed to an increase in flocculation efficiency.

Table 2: Zeta potential of various samples

Samples	Zeta potential (mV)
TKT (bioflocculant)	-17.1±0.65
Kaolin particles	-6.59±3.0
Kaolin particles with Ba^{2+}	-7.01±1.0
Kaolin particles flocculated with TKT in the presence of Ba^{2+}	-4.41±0.72
TKT-g-PAM6	-12.6±0.1
Kaolin particles flocculated with TKT-g-PAM6 in the presence of Ba^{2+}	-11.5±0.9
Kaolin particles flocculated with acrylamide in the presence of Ba^{2+}	-10.2±0.7

4.3.3 Functional groups

To confirm that grafting is performed, the presence of different functional groups of TKT (Fig. 7), PAM (Fig. 8), and TKT-g-PAM6 (Fig. 9) were revealed by using IR spectrum. In Figure 7, the broad absorption band seen in bioflocculant (TKT) at 3261 cm^{-1} is due to the stretching frequency of the O-H groups. The bands around 1059 and 1159 cm^{-1} are assigned to C-O stretching as well as bridge-O- stretching respectively.

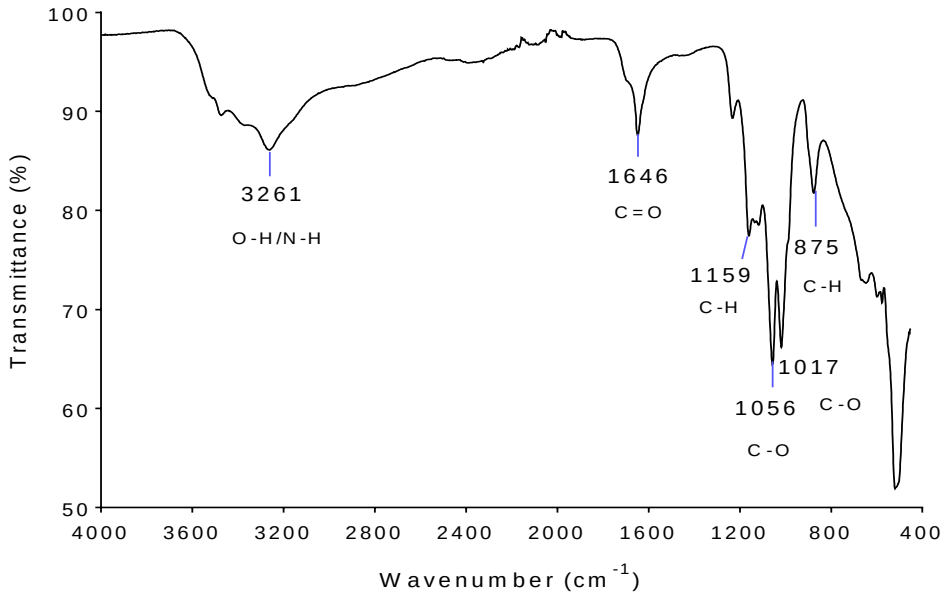

Figure 7: The IR spectrum of TKT

Figure 8 shows the FTIR spectrum of acrylamide (AM). A broad absorption band at 3343 cm–^{1} is for the NH stretching frequency of the NH_2 group. Around 1670 cm–^{1} and 1611 cm–^{1} two strong bands are due to CO stretching and NH bending. The band around 1424 cm–^{1} is for the C-N stretching vibrations.

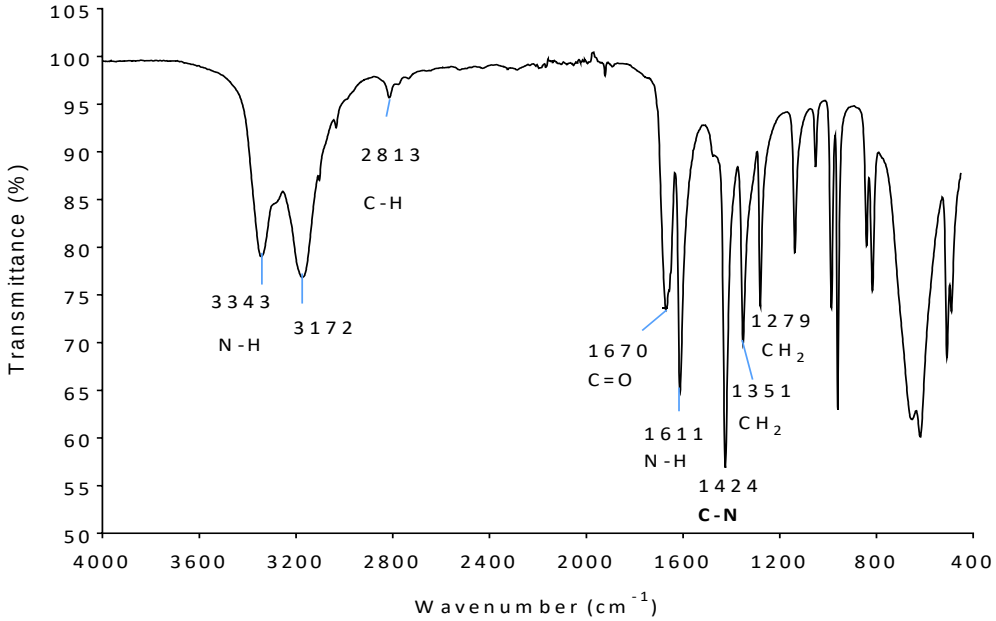

Figure 8: The IR spectrum of AM

Figure 9 shows the optimized grafted TKT. In the spectrum of TKT-g-PAM6 few differences have been observed where O-H stretching band of hydroxyl group of bioflocculant and N-H stretching band of amide group of acrylamide overlap with each other and lead to a broad band, which appear at 3275 cm–^{1} for grafted bioflocculant (TKT-g-PAM6). The CO stretching occurred at 1651 cm–^{1} and NH bending occurred at 1612 cm–^{1}. The C-N stretching of acrylamide appeared at 1427 cm–^{1} and this confirms the grafting of PAM chains onto the polysaccharide backbone was successful.

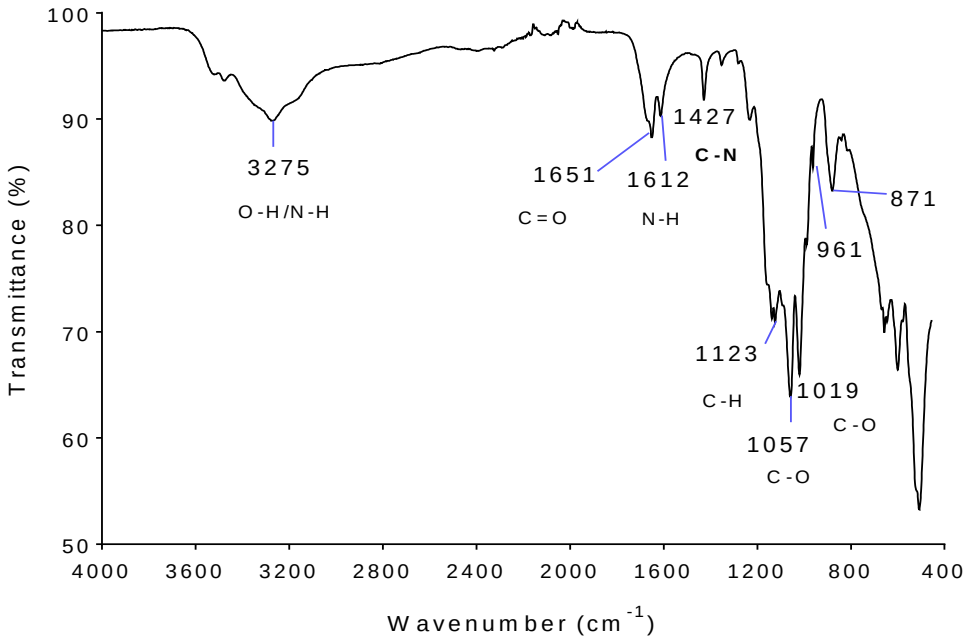

Figure 9: The IR spectrum of TKT-g-PAM6

4.3.4 Biosafety of TKT-g-PAM6

Figure 10 illustrates the cytotoxicity of the grafted bioflocculant on HEK293 cell lines. The grafted bioflocculant showed less toxic effects on HEK293 cell lines. The cell lines on HEK293 exhibited 82% viability after it has been treated with the highest concentration (200 µg/µl) of the grafted bioflocculant.

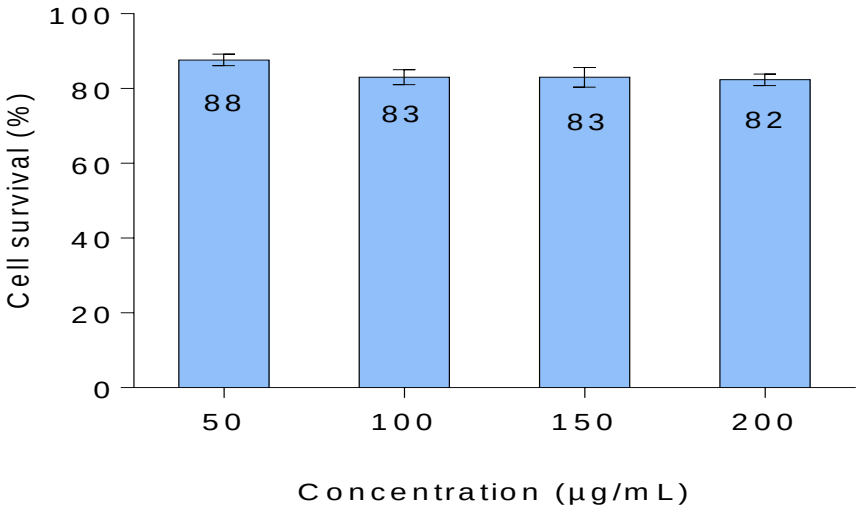

Figure 10: *In-vitro* cytotoxicity of different grafted bioflocculant concentrations on HEK293

Figure 11 illustrates the cytotoxicity of the grafted bioflocculant on CaCO2 cell lines. The grafted bioflocculant showed less toxic effects on CaCO2 cell lines. The cell lines on CaCO2 exhibited 64% viability after it has been treated with the highest concentration (200 µg/µl) of the grafted bioflocculant.

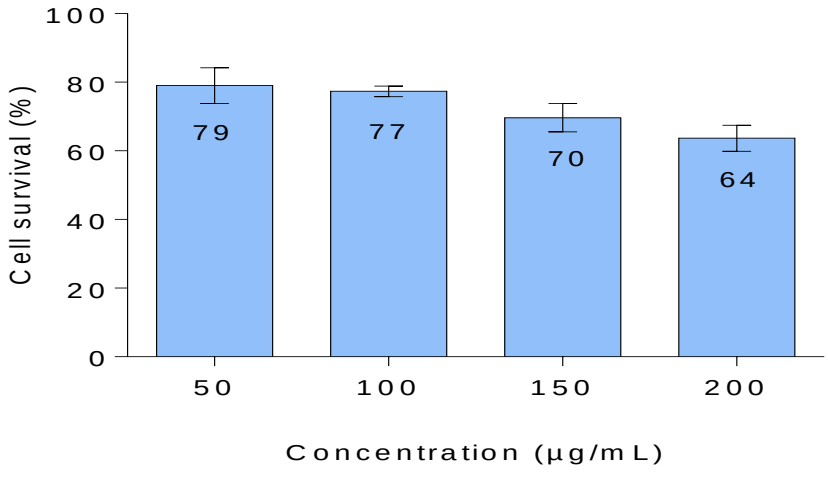

Figure 11: *In-vitro* cytotoxicity of different grafted bioflocculant concentrations on CaCO2

4.4 Effect of grafted bioflocculant dosage on flocculating activity

The highest flocculating activity (79±9.23%) was observed at the concentration of 0.4 mg/ml in Table 3. There was no statistical difference ($p< 0.05$) observed between the lowest concentration of 0.2 mg/ml and 0.4 mg/ml. Thus, 0.2 mg/ml was the most preferred concentration with a flocculating activity of 74±10.01%.

Table 3: Dosage size and cation effect on flocculating activity of the grafted bioflocculant produced *Alcaligenes faecalis* HCB2 and acrylamide

Dosage size (mg/ml)	FA(%)±SD	Cations	FA(%)±SD
0.2	$74.3±2.82^{a}$	Li^{+}	$50.3±2.31^{a,c}$
0.4	$78.6±1.53^{a}$	Na^{+}	$51.3±4.73^{a,c}$
0.6	$71.0±1.73^{a}$	K^{+}	$47.3±5.12^{a}$
0.8	$50.6±1.5^{a}$	Ba^{2+}	$85.0±1.73^{b}$
1	$62.0±5.19^{a}$	Ca^{2+}	$60.6±9.54^{a,c,d}$
		Fe^{3+}	$63.6±1^{c,d}$
		Control	$68.6±2.52^{d}$

Note: Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p<0.05$) different.

4.5 Effect of cations on flocculating activity of grafted bioflocculant

Table 3 displays the cation effect on flocculating activity of grafted bioflocculant. Ba^{2+} was the preferred cation with a flocculating activity of 85.6% and K^{+} showed the lowest flocculating activity of 47%.

4.6 pH stability of the purified grafted bioflocculant

The effect of pH on flocculating activity of grafted bioflocculant is displayed in Figure 12. The flocculating activity in the acidic conditions was above 80% and 93% was

optimum at pH 3.0. The flocculating activities were high for acidic conditions and much lower under alkaline conditions. The grafted bioflocculant will be more effective in acidic mine wastewater.

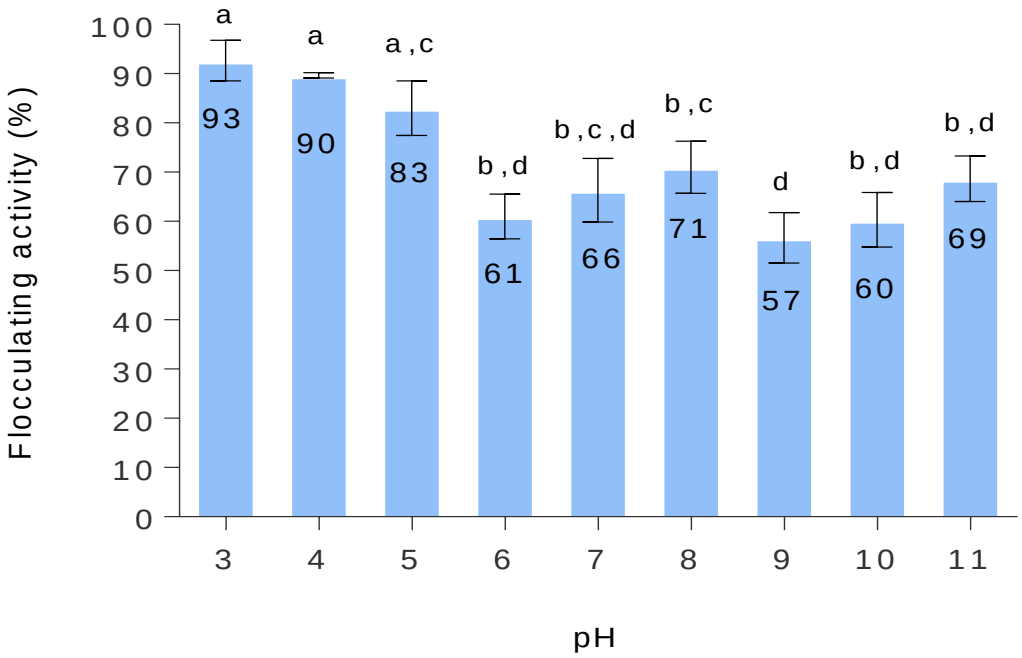

Figure 12: pH stability of TKT-g-PAM6

4.7 Thermal stability of purified grafted bioflocculant

The thermal stability of the grafted bioflocculant is an important property for its commercial exploitation. Thermal stability of the purified grafted bioflocculant was examined at 100°C for a 1 h period. Figure 13 shows the thermal stability of the grafted bioflocculant. The grafted bioflocculant retained 90% flocculating activity after 1 h incubation at 100°C.

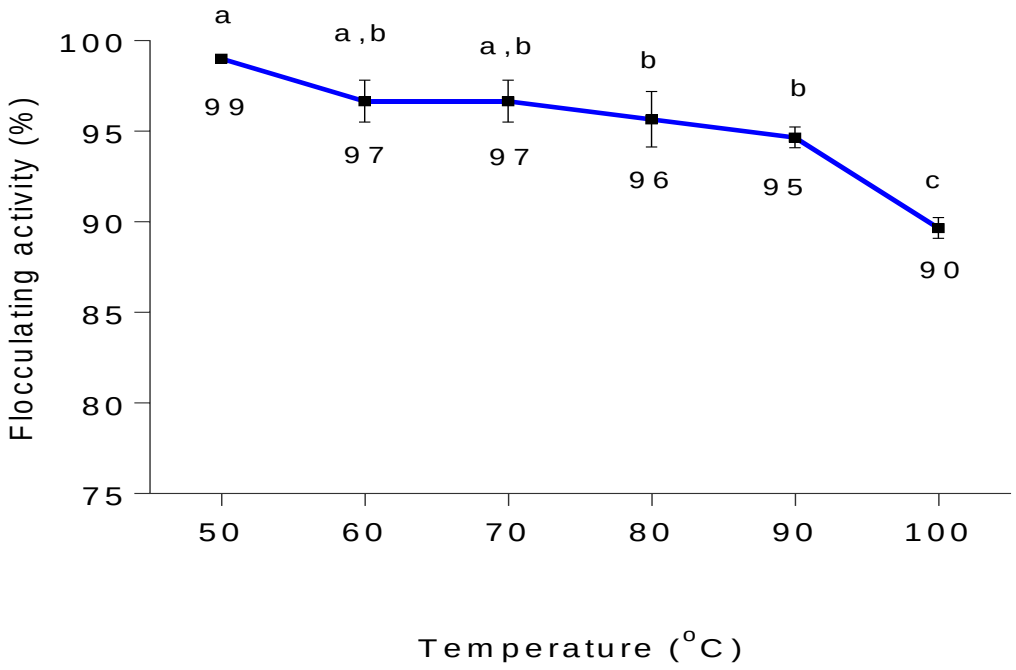

Figure 13: Thermal stability of TKT-g-PAM6

4.8 Pyrolysis' properties of TKT and TKT-g-PAM6

In Figure 14, the thermal degradation of the bioflocculant and grafted bioflocculant is shown. In the case of the bioflocculant TKT, the initial decomposition temperature (IDT) was found to be 50°C (4% weight loss) and final decomposition temperature (FDT) to be 200°C (25% weight loss).

In the case of the grafted bioflocculant, the IDT has been found to be 215°C (29% weight loss) and FDT to be 571°C (36% weight loss). The deterioration temperature (*T*d) of 100°C was detected and it confirmed the thermal strength of TKT and TKT-g-PAM6.

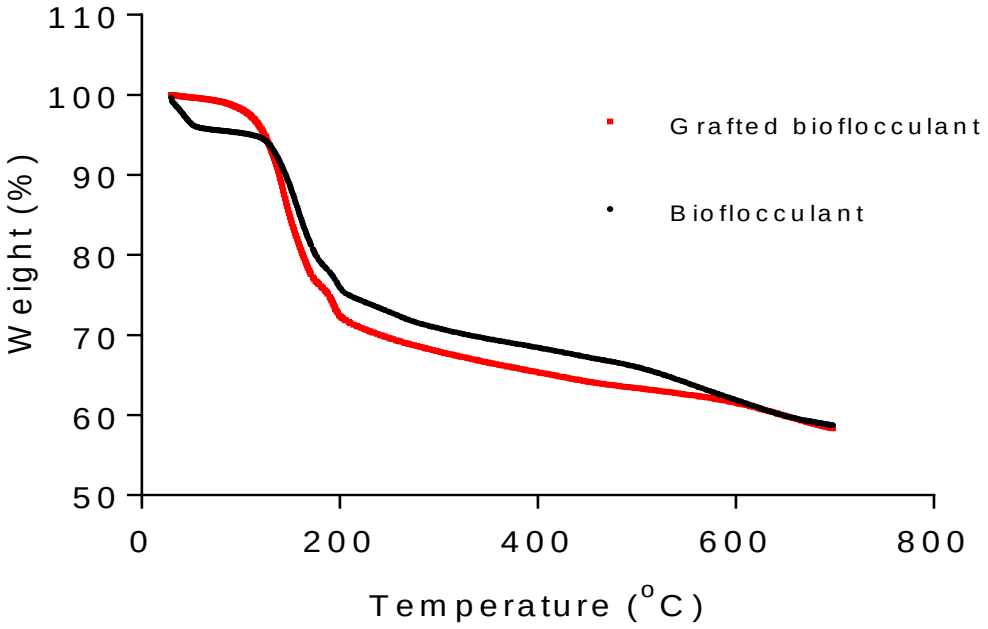

Figure 14: Thermal gravimetric analysis of TKT and TKT-g-PAM6

4.9 Removal efficiency of the grafted bioflocculant on wastewater

The initial characteristics of wastewater were determined before each of the parameters was measured. By using this value, the characteristics before and after treatments were compared. Table 4 displays the initial and final characteristics of wastewater (BOD, COD, N and S) from Tendele Coal Mine. The grafted bioflocculant had removal efficiencies of 92, 87, 91 and 93% on BOD, COD, N and S respectively. TKT-g-PAM6 was effective in removal efficiencies when compared to the bioflocculant and conventional flocculants.

Table 4: Removal efficiency of the grafted bioflocculant and inorganic flocculants in coal mine wastewater

Type of flocculants	Water quality before treatment (mg/l)				Water quality after treatment (mg/l)				Removal efficiency (%)			
	BOD	**COD**	**N**	**S**	**BOD**	**COD**	**N**	**S**	**BOD**	**COD**	**N**	**S**
TKT-g-PAM	6.4±0.0	1557±0.0	5.6±0.0	4.1±0.0	0.5±3	206±0.0	0.5±0.0	0.3±1.2	92	87	91	93
TKT	6.4±0.0	1557±0.0	5.6±0.0	4.1±0.0	2.5±1.2	589±1	1.3±1.2	1.2±4	61	62	77	71
Alum	6.4±0.0	1557±0.0	5.6±0.0	4.1±0.0	2.4±35.2	934±1	1.9±2	1.7±4.5	63	40	66	59
$FeCl_3$	86.4±0	1557±0.0	5.6±0.0	4.1±0.0	1.9±1.2	896±0.6	2.0±3.6	1.4±7.1	70	42	64	66

Values represent mean ± standard deviation of triplicate readings.

Chapter 5: Discussion

5.1 Yield of the bioflocculant

A bioflocculant total yield of 4 g after purification was obtained from 1 L culture broth. This yield is higher than the yields obtained mostly from single bacterial strains. Maliehe *et al*. (2016) obtained 2.4 g of refined bioflocculant TMT^{-1} from single bacterial strains.

5.2 Microwave initiated synthesis (using 900 w microwave radiation)

The optimized grade (TKT-g-PAM6) has been determined through its higher percentage grafting from Table 1 (P. 28). It is obvious that the grafting is optimized at a monomer concentration of 7.0 g and at an irradiation time of 3 min, when the microwave power is fixed at 900W.

In Figure 4 (P. 29), the decrease in percentage grafting may be because of the prolonged exposure of microwave irradiation which may have degraded the polysaccharide backbone beyond exposure time of 3 min. The results were similar to those of Sen *et al.* (2009), whereby CMS-g-PAM V had a maximum of 50% grafting for 3 min.

In Figure 5 (P. 30), the availability of more monomer may be responsible for the increase in grafting. This increase may be caused by the formation of more homopolymer. The accumulation of monomer free radicals in close proximity to the backbone gave rise to graft copolymerization. However, the primary radicals attacked the monomer instead of reacting with the backbone polymer at higher monomer concentration. In a similar report Pal *et al*. (2010) found that with an increase in acrylamide concentration from 0.14 to 0.21 mol, percentage grafting efficiency increased and reached the maximum concentration of 0.17 mol. Afterwards, the percentage grafting efficiency decreased.

5.3 Morphological surface

The bioflocculant surface morphological structure plays a vital role in the process of flocculation (Zhang *et al*., 2007). The effectiveness or poor flocculating activities of bioflocculants may be due to surface morphological structure. Bioflocculants with porous, amorphous or crystal-like structure have a high level of flocculation (Cosa *et al.,* 2013, Okaiyeto *et al.,* 2015). The information obtained from the structures of the samples occurred when the electrons react with the atoms to produce signals (He *et al*., 2010). In Figure 6 (P. 31), differences can be observed among the bioflocculant and grafted bioflocculant samples. In Figure 6, SEM revealed the physical change from a blocky structure of a bioflocculant to a granular structure of a grafted bioflocculant because of grafting of PAM chains on TKT. Therefore, it is clear that the original morphology of TKT is lost after grafting indicating that the grafting was successful.

5.4 Zeta potential of grafted bioflocculant

Flocculant charge properties exerts great influence on the flocculation efficiency (Yang *et al.,* 2012). In Table 2 (P. 32), the zeta potential of grafted bioflocculant and bioflocculant had a negative charge. This facilitates binding to positively charged colloidal pollutants. This may be due to cation exchange potential offered via electrostatic interaction (Esparza-Soto and Westerhoff, 2003).

5.5 Functional groups

The functional groups of grafted bioflocculants were responsible for adsorption sites of different colloids in suspension (Xiong *et al.,* 2010). Multiple functional groups in TKT-g-PAM6 were indicative of many adsorption sites available for colloidal particles. Figure 9 (P. 34), IR observation was done on TKT-g-PAM6 and showed the presence of hydroxyl, carboxyl and amine groups. These groups were the main adsorptive forces of TKT-g-PAM6 and were important for the flocculation process (Wang *et al*., 2011). Furthermore, the carboxyl groups were responsible for binding sites of the cations to improve flocculation. The OH, COO^- and COOH groups on the bioflocculant

interacted with the hydrogen ions and hydroxyl ions available on the surface of the particles. This forms hydrogen bonds which permit build-up of big floccules (ABD-EL-Haleem *et al*., 2008; Tang *et al.,* 2014).

5.6 Biosafety of grafted bioflocculant

In Figures 10 and 11 (P. 35), MTT cell proliferation assay was used to assess cell viability of HEK293 cells and CaCO2 cells after being treated with different concentrations of the grafted bioflocculant. The grafted bioflocculant showed less cytotoxic effects. Few adjustments must be made to affirm their safe utilization in different industrial applications.

5.7 Effect of grafted bioflocculant dosage on flocculating activity

Table 3 (P. 36) showed the effect of dosage and cations on flocculating activity of grafted bioflocculant. Dosing and mixing conditions achieved can affect the degree of flocculation (Yu *et al.,* 2010). It was reported that flocculation occurs quickly for low polymer doses and high solids concentrations (Okaiyeto *et al.,* 2016). The flocs can be broken at a moderate settling rate because they are not stable. When half the area of solids is covered with polyelectrolytes, optimum flocculation occurs (Harif *et al.,* 2012). From Table 3, it was observed that the degree of flocculation decreases at high concentrations. This may be due to the absorbed polymer layer which completely covers the particles. Mostly, insufficient dosage or overdosing would result in low flocculation (Cosa *et al.,* 2013). Consequently, it turns out to be vital to institute the optimum grafted bioflocculant dose. Thus, could help to lessen costs and achieve superior performance in the treatment processes. Liang *et al*. (2010) suggested that the flocculation deterioration phenomenon may be accountable for a decline in flocculating activity. This occurs when some colloidal particles were enclosed by the concentrated flocculant and a colloid protection function occurred, leading to low flocculating activity. The TKT-g-PAM6 can therefore be economically viable in industrial applications as it gave high flocculating activity at low concentrations.

5.8 Effect of cations on flocculating activity

Cations have a significant effect on flocculation (Liu *et al*., 2010). Commonly, cations are used as a coagulant aid in achieving high flocculating activity. It neutralizes both negatively charged functional groups of bioflocculant and colloidal kaolin particles in a mixture (He *et al.,* 2010). The grafted bioflocculant without cation addition flocculated better than the monovalent cations used in the study in Table 3 (P. 36). Both divalent and trivalent cations stimulated flocculating activity to a greater degree compared to the monovalent cations. Ba^{2+} effectively neutralized the negative surface charge on the grafted bioflocculant and kaolin suspension. The shortened distance between particles and the grafted bioflocculant, led to high flocculating activities. Similarly Okaiyeto *et al.* (2015) reported that Ba^{2+} was the most active cation, with the highest flocculating activity of 96%.

5.9 pH stability of the purified grafted bioflocculant

The effective species in inorganic flocculants can be solvated metal ion which disturbs flocculation through Schulze-Hardy effects and double-layer compression (Borchate *et al.,* 2014). The variation of pH might change the flocculant charge status and physiognomies of dissolved materials consequently affecting the flocculating activity (Li *et al.,* 2016). Figure 12 (P. 37), illustrates that the flocculating activity of grafted bioflocculant was above 80% in acidic conditions with a maximum of 93% flocculating activity at pH 3. The decrease in flocculating activity for the alkaline conditions, may be due to OH^- ions which might have inhibited the formation of floccules of kaolin particles and grafted bioflocculant in the mixture. Furthermore, decrease in flocculating activity in alkaline condition suggested the alkaline degradation of the bioflocculant. This could have resulted from changes that included molecular rearrangements of the polysaccharide chain fragmentation of the bioflocculant (Bathe *et al.,* 2010).

5.10 Thermal stability of purified grafted bioflocculant

The thermal stability of the grafted bioflocculant is an important property for its commercial exploitation. Thermal stability of the purified grafted bioflocculant produced by *Alcaligenes faecalis* HCB2 and acrylamide was examined at 100°C for 1

h period. In Figure 13 (P. 38), there is a notable decline in flocculating activity with time. This may be due to the partial denaturation of the protein component in bioflocculant structures. The high flocculating activity and thermal stability may be attributed to the nature of the bioflocculant, which is mainly polysaccharides and the interaction within. The grafted bioflocculant was more stable.

5.11 Pyrolysis' properties of TKT and TKT-g-PAM6

Thermogravimetric analysis (TGA) was used to study the pyrolysis properties of the TKT and TKT-g-PAM6. Thermogravimetric analysis of TKT and TKT-g-PAM6 is shown in Figure 14 (P. 39). Thermal behaviour of both TKT and TKT-g-PAM6 was studied as a function of the percent weight of the residue with the increase in temperature.

In the case of bioflocculant TKT the initial decomposition temperature (IDT) may correspond to the loss of adsorbed and bound water while the final decomposition temperature (FDT) may be due to the degradation of the bioflocculant.

In case of the grafted bioflocculant (TKT-g-PAM) the initial decomposition temperature (IDT) may be caused by the degradation of part of the ungrafted bioflocculant final decomposition temperature (FDT) attributed to the degradation of grafted polymer. Both TKT and TKT-g-PAM6 were thermal stable as they retained more than 60% of weight loss even after exposed to high temperatures above 500°C.

5.12 Flocculation mechanism of the grafted bioflocculant

Charge neutralization bridging and bridging mechanisms are the two main bioflocculation mechanisms normally proposed (Li *et al*., 2009). When the bioflocculant is oppositely charged as compared to the colloids under treatment, then charge neutralization takes place. In this mechanism the surface charge density is condensed by adsorption on the bioflocculant and the colloids, so they can efficiently approach each other. When the bioflocculant extends into the mixture in a distance greater than the distance over which the colloids repulsion can act, then a bridging

mechanism occurs. To form floccules, the bioflocculant can bind with colloids in this case. To assist in determining the flocculation mechanism of the grafted bioflocculant zeta potential analyses were done.

The zeta potential of TKT-g-PAM and kaolin particles were all negative. The addition of Ba^{2+} to kaolin suspension and kaolin suspension plus grafted bioflocculants resulted in the increase of zeta-potentials. The repulsion forces become terminated when the negative charge is reduced and particles easily agglomerate (Hadgson *et al*., 2004). By decreasing the negative charge on the grafted bioflocculant, Ba^{2+} increased the adsorption of grafted bioflocculants onto the surface of colloidal particles and kaolin particles. Therefore, the attraction forces were capable of weakening and overcoming the electrostatic repulsion force, reducing the distance between grafted bioflocculant and kaolin particles by increasing the adsorption of grafted bioflocculant while compacting the double layer of kaolin particles on the colloidal kaolin particles. When the zeta potential is below 20 mV, flocculation increases (Mines, 2014). Therefore, it was concluded that Ba^{2+} stimulated flocculation rapidly by neutralizing and stabilizing residual negative charges of the grafted bioflocculant, forming the bridges that bind kaolin particles to each other as the big floccules were initiated.

A combination of polymer bridging and charge neutralization is the mechanism of flocculation used by the grafted flocculants in wastewater treatment (Pal *et al*., 2011; Song *et al*., 2011; Yang *et al*., 2012). At the beginning of the flocculation process, charge neutralization predominates and quickly produces numbers of insoluble complexes. The insoluble complexes aggregate and form larger net like floccules via a bridging effect owing to the versatile polymeric graft chains. Lastly, the condensed floccules are formed and quickly settle down (Yang *et al*., 2013). A bridging mechanism was discovered as the leading flocculation mechanism (Pal *et al*., 2011; Das *et al*., 2013). Owing to polymer bridging mechanism, the grafted bioflocculant flocculate better than linear polymers (bioflocculant, alum and $FeCl_3$) in this study. Sections of the polymer chain adsorbed onto different particles surfaces creating bridges amongst adjacent particles and lastly connected all the particles together. Grafted flocculants have high radius gyration and longer polymer chains. Therefore,

the adsorbed polymer molecules gravitate to receive more elongated configuration for interacting with many particles.

5.13 Removal efficiency of the grafted bioflocculant on wastewater

The grafted bioflocculant (TKT-g-PAM6) was applied to wastewater collected from Tendele Coal Mine. As expected, the grafted bioflocculant showed much better removal efficiencies in all of the tested parameters compared to other flocculants in Table 4 (P. 40). The improved flocculation efficacies of TKT-g-PAM6 compared to TKT is attributed to their structural differences. TKT consists of a linear structure while TKT-g-PAM6 consists of a comb like structure. Generally, the removal efficiencies of the grafted bioflocculant were owed to the functional groups and to the surface structures of the grafted bioflocculant.

The results were similar to those obtained by Brostow *et al.* (2007), whereby the optimized 'microwave irradiation' based CMS-g-PAM renders into higher flocculation efficacy. Also, the optimized microwave-assisted based TKP-g-PAM was reported to be superior to TKP in flocculation tests (Ghosh *et al.,* 2010). The grafted bioflocculant removal efficiencies implied that they have great potential in industrial applications.

Chapter 6

6.1 Conclusions

The bioflocculant yield was higher than other single strains. Grafted copolymers of a bioflocculant and polyacrylamide were successfully synthesized using a microwave initiated method as hypothesized. The reaction parameters such as power, monomer concentration and reaction time were optimized to obtain the maximum grafting. TKT-g-PAM6 was water soluble, effective at low dosage sizes and cation dependent. The functional groups that were responsible for flocculation process were indicated. TKT-g-PAM6 was effective in acidic pH and was thermal stable. TKT-g-PAM6 further showed a side of safety as they exposed less cytotoxic effects. Few adjustments must be made to affirm their safe utilization in different industrial applications. The flocculation process resulted in the presence of the dangling longer branches of PAM onto the rigid bioflocculant backbone and bridging mechanisms which leads to better approachability towards contaminants. Again the optimized best grade of the microwave irradiation based TKT-g-PAM6 is found to have superior flocculation efficacy over conventional flocculants as hypothesized. The grafted bioflocculant is a suitable alternative to substitute conventional flocculants.

6.2 Current challenges and future direction

The complexity of the synthesis process could be improved and simplified. Firstly the process of synthesizing grafted bioflocculant is energy intensive if compared with ungrafted bioflocculant production. Therefore, the development of suitable synthesis techniques with a high efficacy must be pursued. The study would be conducted at the wastewater plant and not at the laboratory. Further characterization of the flocculant would need to be done.

References

ABD-EL-Haleem DAM, Al –Thani RF, Al-Mokemi T, Al-Marii S and Hassan F. (2008). Isolation and characterisation of extracellular bioflocculants produced by bacteria isolated from Qatari Ecosystem. Pol J Microbiol. 57 (3): 231-239.

Adhikary P and Singh RP. (2004). Synthesis, characterization, and flocculation characteristics of hydrolyzed and unhydrolyzed polyacrylamide grafted xanthan gum. *Journal of applied polymer science*, *94*(4), pp.1411-1419.

Adler R and Rascher J. (2007). A Strategy for the Management of Acid Mine Drainage from Gold Mines in Gauteng. Report. No. CSIR/NRE/PW/ER/2007/0053/C. CSIR, Pretoria.

Agarwal M, Srinivasan R, Mishra A. (2002). Synthesis of Plantago psyllium mucilage grafted polyacrylamide and its flocculation efficiency in tannery and domestic wastewater. J. Polym. Res. 9 (1), 69−73.

Ahmad HRA, Azni I, Hamid HRA, Yoshimitsu U and Abubakar BSUI. (2015). Flocculation behavior and mechanism of bioflocculant produced by *Aspergillusflavus*. J Envirol Manage. 150: 466-471.

Akpor B and Muchie M. (2011). Environmental and public health implications of wastewater quality. Afr J Biotech. 10 (13): 2379-2387.

Aljuboori AHR, Idris A, Al-joubory HHR, Uemura Y, Abubakar BSUI. (2015). Flocculation behavior and mechanism of bioflocculant produced by *Aspergillus flavus*. Journal of Environmental Management. 150: 466-471.

Allen J and Moore M. (2004). Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation? Marine Environmental Research, 58: 227-232.

Backe W, Yingling V, and Johnson T. (2014). The determination of acrylamide in environmental and drinking waters by large-volume injection – hydrophilic-interaction liquid chromatography and tandem mass spectrometry. Journal of Chromatography A 1334: 72-78.

Banks D, Younger PL, Arnesen R-T, Iversen ER, and Banks SB. (1997). Mine-water chemistry: the good, the bad and the ugly. Environmental Geology 32 (3), 157-174.

Barbara J and Malgorzata W. (2006). The metal uptake and accumulation in fish living in polluted waters. Soil and Water Pollution Monitoring Protection and Remediation, 69: 107-114.

Bathe GA, Patil DM. (2010). Studies on characterization of bioflocculant exopolysaccharide of Azotobacter indicus and its potential for wastewater treatment. Appl. Biochem. Biotechnol. DIO 10.1007/s12010-010-9054-5.

Bharali P, Das S, Konwar BK and Thakur AJ. (2001) Crude biosurfactant from thermophilic *Alcaligenes faecalis*: Feasibility in petro-spill bioremediation. Internation Biodeterioration and Biodegradation 65: 682-690.

Bhatnagara A and Sillanpaa M. (2010). Utilization of agro-industrial and municipal waste materials as potential adsorbents for water treatment—a review. Chem. Eng. J. 157:277 296.

Bhunia B, Mukhopadhy D, Goswami S, Mandal T, Dey A. (2012). Improved production, characterization and flocculation properties of poly (*γ*)-glutamic acid produced from *Bacillus subtilis* . J. Biochem. Tech. 3:389–394.

Bolto B, Gregory J. (2007). Organic polyelectrolytes in water treatment. Water Res. 41 (11), 2301−2324.

Borchate SS, Kullkarni GS, Kore VS and Kore SV. (2014). A review on applications of coagulation-flocculation and ballast flocculation for water and wastewater. *International Journal of Innovations in Engineering and Technology*, *4*(4), pp.216-222.

Bowen RE, Depledge MH, Carlarne CP and Fleming LE. (2014). Oceans and human health-Implications for society and well-being. Willey-Black-well. pp. 96-98.

Brostow W, Pal S and Singh RP. (2007). A model of flocculation. Materials Letters, 61, 4381–4384.

Chang WC, Soon AY, In HO, Sang HP. (1998). Characterization of an extracellular flocculating substance produced by a planktonic cyanobacterium, *Anabaena sp*. Biotechnol Lett. 20(12): 643–646.

Charoenpanich J. (2013). Removal of acrylamide by microorganisms. *Applied bioremediation-active and passive approaches. InTech Open Science Online Publishers, Croatia*, pp.99-119.

Chin DA. (2013). Water quality-Engineering in natural systems-Fate and transport process in waste environment. Wiley. pp. 1-5.

Choi YY, Baek SR, Kim JI, Choi JW, Hur J, Lee TU, Park CJ and Lee BJ. (2017). Characteristics and biodegradability of wastewater organic matter in municipal wastewater treatment plants collecting domestic wastewater and industrial discharge. *Water*, *9*(6), p.409.

Chopra H and Ruhi G. (2016). Eco friendly chitosan: An efficient material for water purification. Pharma Innov J. 5 (1): 92-95.

Cosa S and Okoh A. (2014). Bioflocculant Production by a Consortium of Two Bacterial Species and Its Potential Application in Industrial Wastewater and River Water Treatment. *Polish Journal of Environmental Studies*, *23*(3).

Cosa S, Mabinya LV, Olaniran AO, Okoh OO, Okoh AI. (2011). Bioflocculant production by *Virgibacillus.*sp. Rob Isolated from the bottom sediment of Algoa Bay in the Eastern Cape, South Africa. *Molecules*, *16*, 2431–2442.

Cong-Liang Z, Ya-Nan C and Yan W. (2012). Bioflocculant produced from bacteria for decolorization, Cr removal and swine wastewater application. Sustain Environ Res. 22(2): 129-134.

Cosa S, Ugbenyen MA, Mabinya LV and Okoh IA. (2013). Characterization of a thermostable polysaccharide bioflocculant produced by *Virgibacillus species* isolated from Algoa Bay. Afr J Microbiol Res. 7 (23): 2925-2938.

da Silva DA, de Paula RCM, and Feitosa JPA. (2007). Graft copolymerization of acrylamide onto cashew gum. European Polymer Journal, 43, 2620–2629.

Das R, Ghorai S, Pal S. (2013). Flocculation characteristics of polyacrylamide grafted hydroxypropyl methyl cellulose: An efficient biodegradable flocculant. Chem. Eng. J. 229 (0), 144−152.

Department of Water and Sanitation. (2002). Quality of domestic water supplies. Vol 4: Treatment Guide. WRC No. TT 1181/02. pp. 22-23.

Devi K, Pathak M, Bhattacharyya H, Sarma HK, Subudhi S and Lal B. (2015). Production of non-cytotoxic bioflocculant by a bacterium utilizing a petroleum hydrocarbon source and its application in heavy metal removal. RSC Adv. 5: 66037-66046.

Drysdale GD, Atkinson BW, Mudaly DD, Kasan HC and Bux F. (2000). Investigation of the microbial contribution to nutrient removal in activated sludge-Wastewater treatment process. WRC Report No: 822/1/00. pp. 1-22.

Edition F. (2011). Guidelines for drinking-water quality. *WHO chronicle*, *38*(4), pp.104-108.

Edward JK. (2011). Water quality and treatment-A handbook of drinking water.6th edition. American Water Works Association. McGraw-Hill. pp. 5.1-5.9, 8.1-8.72.

Elberling B, Søndergaard J, Jensen LA, Schmidt LB, Hansen BU, Asmund G, Balic-Zunic T, Hollesen J, Hanson S, Jansson PE, Friborg T. (2007). Arctic vegetation damage by winter-generated coal mining pollution released upon thawing. Environ. Sci. Technol. 41, 2407–2413.

Esparza-Soto M and Westerhoff P. (2003). Biosorption of humic and fulvic acids to live activated sludge biomass. Water Res. 37: 2301–2310.

Gabriel OM, Rita O, Clifford A and Kennedy O. (2006). Heavy metal pollution of fish of Qua-Iboe River Estuary: Possible implications for neurotoxicity. The International Journal of Toxicology, 3(1): 1-6.

García-Fayos B, Arnal JM, Monforte-Monleon L and Sancho M. (2015). Alternatives to the use of synthetic organic coagulant aids in drinking water treatment: improvements in the application of the crude extract of Moringa oleifera seed. *Desalination and Water Treatment*, *55*(13), pp.3635-3645.

Ghimici L and Nichifor M. (2012). Flocculation by cationic amphiphilic polyelectrolyte: Relating efficiency with the association of polyelectrolyte in the initial solution. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 415, 142–147.

Ghosh S, Jha U and Pal S. (2011). High performance polymeric flocculant based on hydrolyzed polyacrylamide grafted tamarind kernel polysaccharide (Hyd. TKP-g-PAM). Bioresour. Technol. 102 (2), 2137−2139.

Ghosh S, Sen G, Jha U and Pal S. (2010). Novel biodegradable polymeric flocculant based on polyacrylamide-grafted tamarind kernel polysaccharide. Bioresour. Technol. 101 (24), 9638−9644.

Gulf of Maine. (2012). Eutrophication. http://www.gulfofmaine.org.

Hadgson KG, Freese SD, Nozaic DJ and Borain G. (2004) Quantification of factors affecting coagulation of water with cation polymers and laboratory methods for determining these effects. WRC Report. No 1225/1/04. pp. 5-25.

Harif T, Khai M and Adin A. (2012). Electrocoagulation versus chemical coagulation: coagulation/flocculation mechanisms and resulting floc characteristics. *Water Research*, *46*(10), pp.3177-3188.

Hazen RM. (2005). Genesis: Rocks, Minerals and the Geochemical Origin of Life. Elements. 1: 135–137.

He J, Zou J, Shao Z and Zhang J. (2010). Characteristics and flocculating mechanism of a novel bioflocculant HBF-3 produced by deep-sea bacterium mutant *Halomona* sp. V3a. World J Microbiol Biotechnol. 26: 1135–1141.

Hendric D. (2006). Application treatment unit process-physical and chemical. CRC-Taylor and Francis. pp. 481-17.

Kabuk HA, Avsar Y, Kuzu SL, Ilhan F and Ulucan K. (2015). Determination of biological treatability processes of textile wastewater and implementation of a fuzzy logic model. *International Journal of Photoenergy*, *2015*.

Karthiga D, Natarajan KA. (2015). Production and characterization of bioflocculants for mineral processing applications. Int. J. Miner. Process. 137: 15–25.

Kavuncuoglu F, Unal A, Oguzhan N, Tokgoz B, Oymak O and Udas C. (2010). Outbreak of *Alcaligenes faecalis pseudobacteremia* in neontology and pediatric units. J Hosp Infect. 74 (4): 397-399.

Keating M. (2001) Cradle to the Grave: The Environmental Impacts from Coal, www.catf.us/publications/reports/Cradle_to_Grave.pdf, viewed 9/12/04.

Komilis D, Sánchez A, Yusoff MK, Aris AZ, Shamsuddin ZH and Zulkeflee Z. (2016). Performance, yield and characteristics of bioflocculants (UPMBF13) produced by *Bacillus subtilis* UPMB13. Global Nest Journal. 18(3): 611-620.

Kong L, Zhu S, Zhu L, Xie H, Wei K, Yan T, Wang J, Wang J, Wang F, Sun F. (2014). Colonization of *Alcaligenes faecalis* strain JBW4 in natural soils and its detoxification of endosulfan. Appl Microbiol Biotechnol 98: 1407–1416. http://dx.doi.org/10.1007/s00253 013-5033-4.

Kumar K, Kaith BS, Mittal H. (2012). A study on effect of different reaction conditions on grafting of psyllium and acrylic acid-based hydrogels. J. Appl. Polym. Sci. 5(123):1874–1883. doi: 10.1002/app.346.

Kumar R, Singh RD and Sharma KD. (2005). Water Resources of India. Curr. Sci. 89:794 811.

Kumari K, Vijay KS, Avinash M, Bhavanath J. (2014). Characterisation and anti-biofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*. Carbohydr. Polym. 101:29–35.

Kurane R and Matsuyama H. (1994). Production of a bioflocculant by mixed culture. Biosci Biotechnol Biochem. 58: 1589–1594.

Larsen D, Mann R. (2005). Origin of high manganese concentrations in coal mine drainage, eastern Tennessee. J. Geochem. Explor. 86, 143–163.

Lee CS, Robinson J and Chong MF. (2014). A review on application of flocculants in wastewater treatment. *Process Safety and Environmental Protection*, *92*(6), pp.489-508.

Lee KE, Morad N, Teng TT, Poh BT. (2012). Development, characterization and the application of hybrid materials in coagulation/ flocculation of wastewater: A review. Chem. Eng. J. 203, 370− 386.

Lee KE, Teng TT, Morad N, Poh BT, Mahalingam M. (2011). Flocculation activity of novel ferric chloride-polyacrylamide (FeCl (3)-PAM) hybrid polymer, Desalination. 266: 108–113.

Levy N, Magdassi S and Bar-Or Y. (1992). Physico-chemical aspects in flocculation of bentonite suspensions by a cyanobacterial bioflocculant. Water Res. 26: 249–254.

Li O, Lu C, Liu A, Zhu L, Wang P, Qian C. (2013). Optimization and characterization of polysaccharide-based bioflocculant produced by *Paenibacillus elgii* B69 and its application in wastewater treatment. Bioresour. Technol. 134:87–93.

Li S, Gao L, Cao Y, Gui X and Li Z. (2016). Effect of pH on the flocculation behaviors of kaolin using a pH-sensitive copolymer. *Water Science and Technology*, p.wst2016266.

Li X, Yang Q, Huang K, Zeng G, Liao D and Liu J. (2007). Screening and characterization of a bioflocculant produced by *Aeromonas* sp. Biomed Environ Sci. 20: 274–278.

Li Z, Zhong S, Lei H, Chen R, Yu Q and Li H. (2009). Production of a novel bioflocculant by *Bacillus licheniformis* X14 and its application to low temperature drinking water treatment. Bioresour Technol. 100: 3650–3656.

Liang Z, Han B, Liu H. (2010). Optimum conditions to treat high concentration microplate slime water with bioflocculants. Mining Sci. Technol. 20:0478-0484.

Lilley ID, Pybus PJ and Power SPB. (1997). Operating manual for biological nutrient removal in wastewater treatment. WRC Report No.TT 83/97.

Lin J, Harichund C (2011). Industrial effluent treatments using heavy-metal removing bacterial bioflocculants. Water SA. 37, 265.

Liu L and Cheng W. (2010). Characteristics and culture conditions of a bioflocculant produced by *Penicillium* sp. Biomed Environ Sci. 23: 213-218.

Liu W, Wang K, Li B, Yuan H, Yang J. (2010). Production and characterization of an intracellular biofloculant by *Chryseobacterium daeguense* W6 cultured in low nutrition medium. *Bioresour. Technol*. *101*, 1044–1048.

Luo Z, Chen L, Chen C, Zhang W, Liu M, Han Y, Zhou J. (2014). Production and characteristics of a bioflocculant by *Klebsiella pneumoniae* YZ-6 isolated from human saliva. Appl Biochem Biotechnol. 172: 1282-1292.

Mabinya LV, Cosa S, Mkwetshana N, Okoh AI. (2011). *Halomonas* sp. OKOH—A marine bacterium isolated from the bottom sediment of Algoa Bay—produces a

polysaccharide bioflocculant: partial characterization and biochemical analysis of its properties. *Molecules* 16 4358–4370.

Madigan MT and Martinko JM. (2006). Brock, Biology of Microorganisms. 11th ed. Pearson Prentice Hall. pp. 80,102-108, 136-164,702-708, 949.

Maliehe TS. (2018). Production, Characterisation and Application of Bioflocculants from Pure Bacterial Strains and their Consortia Isolated from Sodwana Bay in Kwa-Zulu Natal, South Africa (Doctoral dissertation, University of Zululand).

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

Manahan S. (2011). Water Chemistry-Green Science and technology of nature`s most reneable resource. CRC Press. pp. 223-265.

Marvasi M, Visscher PT, Casillas Martinez L. (2010). Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. FEMS Microbiol. Lett. 313, 1e9.

McIntyre AD. (2010). Life in the world`s oceans-Diversity, Distribution and Abundance. Willey-Blackwell (UK): 223-243.

Mihelcic JR and Zimmerman JB. (2010). Environmental Engineering –Fundamentals, sustainability, design. Wiley. pp. 397-417.

Mines RO. (2014). Environmental engineering-principles and practice. Wiley-Blackwell. Oxford-UK. pp. 239-330.

Mishra A, Yadav A, Agarwal M, Bajpai M. (2004). Fenugreekmucilage for solid removal from tannery effluent. React.Funct. Polym. 59, 99–104.

Mishra AK. (2016). Smart materials for waste water applications. Chapter 8. Wiley-Scrivene https://books.google.co.za/books?id.

Mishra S, Sinha S, Dey KP, Sen G. (2014). Synthesis, characterization and applications of polymethylmethacrylate grafted psyllium as flocculant. Carbohydr. Polym. 99,462–468.

Mishra S, Usha Rani G, Sen G. (2012). Microwave initiated synthesis and application of polyacrylic acid grafted carboxymethyl cellulose. Carbohydr. Polym. 87 (3), 2255−2262.

Mishra S, Mukul A, Sen G, Jha U. (2011). Microwave assistedsynthesis of polyacrylamide grafted starch (St-g-PAM) and itsapplicability as flocculant for water treatment. Int. J. Biol.Macromol. 48, 106–111.

More TT, Yadav JSS, Yan S, Tyagi RD and Surampalli RY. (2014). Extracellular polymeric substances of bacteria and their potential environmental applications. J Environ Manag. 144: 1–25.

More TT, Yan S, Tyagi RD and Surampalli RY. (2010). Potential use of filamentous fungi for wastewater sludge treatment. Bioresour Technol. 101: 7691-7700.

Morin A. (1998). Screening of polysaccharide-producing microorganisms, factors influencing the production and recovery of microbial polysaccharides. In: Polysaccharides-Structural diversity and functional versatility. Dumitriu, S. (Ed.), Marcel Dekker Inc. Publication. New York. USA. 275–296.

Mosman T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 65 (1-2): 55-63.

Mustatea G, Popa ME and Negoita M. (2015). A case study on mitigation strategies of acrylamide in bakery products. *Scientific Bulletin. Series F. Biotechnologies*, *11*.

Nava-Ruíz C, Méndez-Armenta M. (2013). Cadmium, lead, thallium: Occurrence, neurotoxicity and histopathological changes of the nervous system. In *Pollutant Diseases, Remedition and Recycling*; Lichtfouse, E., Schwarzbauer, J., Robert, D., Eds.; Springer International Publishing: Cham, Switzerland, Chapter 6, p. 323.

Nwodo UU and Okoh AI. (2013). Characterization and flocculation properties of biopolymeric flocculant (glycosaminoglycan) produced by *Cellulomonas* sp. Okoh. J Appl Microbiol. 114: 1325–1337.

Nwodo UU, Makapela B, Okaiyeto K, Ntozonke N, Green E, Mabinya LV, Okoh AI. (2016). Assessment of *Bacillus pumilus* Isolated from Fresh Water Milieu for Bioflocculant Production. Appl. Sci. 6(8), 211; doi: 10.3390/app6080211.

Odian G. (2004). Principles of Polymerization, 4th ed.; John Wiley & Sons, Inc.: Hoboken, NJ, March 2004; p 789.

Ogunlana OO, Ajayi A, Ogunlana OE, Keleko AA and Olasehinde GI. (2010). Microbiological and Physicochemical Properties of Drinking Water at Ota, Southwest, Nigeria. *International Journal of Tropical Agriculture and Food Systems*, *4*(4), pp.358-361.

Okaiyeto K, Nwodo UU, Mabinya LV, Okoli AS, Okoh AI. (2016). Evaluation of flocculating performance of a thermostable bioflocculant produced by marine *Bacillus* sp. Environmental technology. 37(14): 1829–1842.

Okaiyeto K, Nwodo UU, Mabinya LV, Okoli AS and Okoh AI. (2015). Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7. Int J Mol Sci. 6: 12986–13003.

Okaiyeto K, Nwodo UU, Mabinya LV. (2013). Characterization of a Bioflocculant Produced by a Consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. Int J Environ Res Public Health. 10(10): 5097–5110.

Pal S, Sen G, Ghosh S and Singh RP. (2012). "High performance polymeric flocculants based on modified polysaccharide microwave assisted synthesis," Carbohydrates polymers, vol. 87, pp. 336-342.

Pal S, Ghorai S, Dash MK, Ghosh S, Udayabhanu G. (2011). Flocculation properties of polyacrylamide grafted carboxymethyl guar gum (CMG-g-PAM) synthesised by conventional and microwave assisted method. J. Hazard. Mater. 192, 1580–1588.

Pal S, Nasim T, Patra A, Ghosh S and Panda AB. (2010). "Microwave assisted synthesis of polyacrylamide grafted dextrin (Dxt-g-PAM): Development and application of a novel polymeric flocculant," International Journal of Biological Macromolecules, vol. 47, pp. 623-631.

Phung LT, Trimble WL, Meyer F, Gilbert JA, Silver S. (2012). "Draft genome sequence of *Alcaligenes faecalis* subsp. *faecalis*NCIB 8687 (CCUG 2071)," *Journal of Bacteriology*, vol. 194, no. 18, p. 5153.

Pielou EC. (1998). Freshwater (University of Chicago Press) pp. 286.

Pulles W, Banister S and Van Biljon M. (2005). The development of appropriate procedures towards and after closure of underground gold mines from a water management perspective. Report No. 1215/1/05. Water Research Commission, Pretoria.

Raboni M, Capodaglio AS and Hlavínek P. (2015). Physico-chemical technologies for nitrogen removal from wastewaters: a review. Rev Ambient Água. 10 (3). doi: 10.4136/ambi-agua.1618.

Rani GU, Mishra S, Sen G, Jha U. (2012). Polyacrylamidegrafted agar: synthesis and applications of conventional andmicrowave assisted technique. Carbohydr. Polym. 90,784–791.

Rani P, Sumit M, and Gautam S. (2013). Microwave based synthesis of polymethyl methacrylate grafted sodium alginate: its application as flocculant. Carbohydr. Polym. 91:686–692.

Reilly JM, Jacoby HD and Prinn RG. (2003). Climate Impacts and Mitigation Costs of Non-CO_2 Gases- Multi-gas contributors to global climate change. Pew Center on Global Climate Change. 4-45.

Renault F, Sancey B, Badot PM, Crini G. (2009). Chitosan for coagulation/flocculation processes-An eco-friendly approach. Eur. Polym. J. 45 (5), 1337−1348.

Ruiz P, Faroon O, Mumtaz M and Wohlers D. (2012). Toxicological profile for acrylamide.

Saleh T, Seyed SS, Gholamali AB, Atena D, Mohammed S. (2010). Heavy metals (Zn, Pb, Cd and Cr) in fish, water and sediments sampled from southern Caspian Sea. Iranian Toxicology and Industrial Health, 26(10): 649-656.

Salehizadeh H and Shojaosadati SA. (2001). “Extracellular biopolymer Flocculant recent trends and biotechnological importance”J. Biotechnol.Adv, 19, pp.371 - 385.

Salehizadeh H and Shojaosadati SA. (2003). Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. Water Res. 37: 4231–4235.

Sarkar B, Chakrabarti PP, Vijaykumar A, Kale V. (2006). Wastewater treatment in dairy industries – possibility of reuse. Desalination, 195, 141.

Saxena G, Dube SN, Flora SJS. (2005). Heavy metals induced central cholinergic system disorders and their possible mechanisms. In *Recent Trends in the Acetylcholinesterase System*; Parveen, M., Kumar, S., Eds.; IOS Press: Amsterdam, The Netherlands, Chapter 3, p. 28.

Sen G, Mishra S, Rani GU, Rani P and Prasad R. (2012). Microwave initiated synthesis of polyacrylamide grafted Psyllium and its application as a flocculant. Int. J. Biol. Macromol. 50 (2), 369−375.

Sen G and PAL S. (2009). Microwave initiated synthesis of polyacrylamide grafted carboxymethylstarch (CMS-g-PAM): Application as a novel matrix for sustained drug release. *International Journal of Biological Macromolecules*. 45: 48-55.

Serdar S, Faruk K, Orhan Y, Barbara N, Ebru TO, Mehmet AY. (2011). Flocculating performances of exopolysaccharides produced by a halophilic bacterial strain cultivated on agro-industrial waste. Bioresource Technology. 102: 1788–1794.

Sharma BR, Dhuldhoya NC. (2006). Merchant UC Flocculants-An ecofriendly approach. J. Polym. Environ. 14 (2), 195−202.

Sheng Y, Zhang Q, Sheng Y, Li C, Wang H. (2006). Screening and flocculating properties of bioflocculant-producing microorganisms. Sci. Technol., 13, 289.

Sigee DC. (2005). Freshwater Microbiology. Wiley. pp. 1-3, 443-457.

Singh RP, Karmakar GP, Rath SK, Karmakar NC, Pandey SR, Tripathy T, Panda J, Kanan K, Jain SK, Lan NT. (2000). Biodegradable drag reducing agents and flocculants based on polysaccharides: Materials and applications. Polym. Eng. Sci. 40 (1), 46−60.

Singh RP, Nayak BR, Biswal DR, Tripathy T, Banik K. (2003). *Mater. Res. Innovat.* 7, 331.

Singha TK. (2012). Microbial extracellular polymeric substances: Production, isolation and application. IOSR Journal of Pharmacy. 2 (2): 276-281.

Smith JC. (2009). Biotechnology, 5th ed, Cambridge, UK, Cambridge University Press. pp. 19- 25, 49-71.

Song Y, Gan W, Li Q, Guo Y, Zhou J, Zhang L. (2011). Alkalinehydrolysis and flocculation properties of acrylamide-modifiedcellulose polyelectrolytes. Carbohydr. Polym. 86, 171–176.

Spellman FR. (2014). Handbook of water and wastewater treatment plant operation. 3rd ed. CRC Press-Taylor and Francis. pp. 453-489, 630-638,729.

Sprocati AR, Alisi C, Segre L and Cremisini C. (2006). Investigating heavy metal resistance, bioaccumulation and metabolic profile of a metallophile microbial consortium native to an abandoned mine. Science of the Total Environment, 366(2-3): 649-658.

Suopajärvi T, Liimatainen H, Hormi O, Niinimäki J. (2013). Coagulation–flocculation treatment of municipal wastewater based on anionized nanocelluloses. Chem. Eng. J. 231, 59–67.

Sutherland IW. (1990). Biotechnology of microbial exopolysaccharides. In: Cambridge Studies in Biotechnology 9. Cambridge University Press, Cambridge: 1–163.

Tang W, Song L, Li D, Qiao J, Zhao T and Zhao H. (2014). Production, Characterization, and Flocculation Mechanism of Cation Independent, pH Tolerant,

and Thermally Stable Bioflocculant from *Enterobacter* sp. ETH-2. PLoS ONE 9 (12): e114591. doi:10.1371/journal.pone.0114591.

Torres-Guzman F, Avelar-Gonzalez FJ and Rico-Martinez R. (2010). An assessment of chemical and physical parameters, several contaminants including metals, and toxicity in the seven major wastewater treatment plants in the state of Aguascalientes, Mexico. *Journal of Environmental Science and Health, Part A*, *45*(1), pp.2-13.

Tripathy T and De BR. (2006). Flocculation: A New Way to Treat the Waste Water. Journal of Physical Sciences. 10: 93-127.

Turton AR, Patrick MJ and Rascher J. (2008). Setting the Scene—Hydropolitics and the Development of the South African Economy.

U.S. Food and Drug Administration. (2014). Acrylamide Questions and Answers. http://www.fda.gov/Food/FoodborneIllnessContaminants/ChemicalContaminants/ucm053569.htm

Ugbenyen AM and Okoh AI. (2014). Characteristics of a bioflocculant produced by a consortium of Cobetia and *Bacillus species* and its application in the treatment of wastewaters. *Water SA*, *40*(1), pp.140-144.

United Nations Department of Economic and Social Affairs. (2012). Back to our Common Future: Sustainable Development in the 21st Century (SD21) project. New York, United Nations (UN). https://sustainabledevelopment.un.org/content/pdf

UN-Water, FAO, Coping with Water Scarcity. (2007). Challenge of the Twenty-First Century.

Vahdat A, Bahrami H, Ansari N, and Ziaie F. (2007). Radiation grafting of styrene onto polypropylene fibres by a 10 MeV electron beam. Radiation Physics and Chemistry, 76, 787–793.

Van Loon GW and Duffy SJ. (2005). Environmental Chemistry. A global perspective. Oxford University Press. Pp. 81, 313-315.

Walker J and Wilson K. (2005). Principles and Techniques of Practical Biochemistry. 6th ed. Cambridge University Press. pp. 349-404.

Wan X, Wang JY, Ye JH, Wang P, Zhan ZM. (2013). Analysis of distribution and contents of heavy metals pollution in fish body with laser-induced breakdown Spectroscopy. NCBI, 33(1): 206-209.

Wang L, Ma F, Qu Y, Sun D, Li A and Guo J *et al.* (2011). Characterization of a compound bioflocculant produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6. World J Microbiol Biotechnol. 27: 2559–2565.

Wang Y, Gao B, Yue Q, Wei J, Li Q. (2008). The characterization and flocculation efficiency of compositeflocculant iron salts–polydimethyldiallylammonium chloride.Chem. Eng. J. 142, 175–181.

Water Environment Federation (WEF). (2011). Nutrient removal-WEF Manual of Practice. No34.WEF Press. pp. 103-142.

Weiner ER. (2013). Application of environmental aquatic Chemistry-A practical guide. 3rd ed. CRC Press. pp. 98-134.

World Health Organisation (WHO). (2006). Guidelines for the Safe Use of Wastewater, Excreta and Greater, vol. 3. World Health Organisation Press, Geneva, Switzerland.

Xiong Y, Wang Y, Yu Y, Li Q, Wang H, Chen R and He N. (2010). Production and characterization of a novel bioflocculant from Bacillus licheniformis. Appl Environ Microbiol. 76: 2778–2782.

Yang Q, Luo K, Liao D, Li X, Wang D, Liu X. (2012). A novel bioflocculant produced by *Klebsiella* sp. and its application to sludge dewatering. Water Environ. J. 26:560–566.

Yang Z, Yang H, Jiang Z, Cai T, Li H, Li H, Li A, Cheng R. (2013). Flocculation of both anionic and cationic dyes inaqueous solutions by the amphoteric grafting flocculant carboxymethyl chitosan-graft-polyacrylamide. J. Hazard.Mater. 254–255, 36–45.

Yang Z, Shang Y, Huang X, Chen Y, Lu Y, Chen A, Jiang Y, Gu W, Qian X, Yang H, Cheng R. (2012). Cationic content effects of biodegradable amphoteric chitosan-based flocculants on the flocculation properties. J. Environ. Sci. 24, 1378–1385.

Yang Z, Yuan B, Huang X, Zhou J, Cai J, Yang H, Li A, Cheng R. (2012). Evaluation of the flocculation performance of carboxymethyl chitosan-graft-polyacrylamide, a novel amphoteric chemically bonded composite flocculant, Water Res. 46, 107–114.

Yim JH, Kim SJ, Ahn SH and Lee HK. (2007). Characterization of a novel bio-flocculant, p-KG03, from a marine dinoflagellate, *Gyrodinium impudicum* KG03. Bioresour Technol. 98: 361-367.

Yu WZ, Gregory J and Campos L. (2010). Breakage and regrowth of Al-humic flocs-effect of additional coagulant dosage. *Environmental science & technology*, *44*(16), pp.6371-6376.

Zhang Z, Xia S and Zhang J. (2010). Enhanced dewatering of waste sludge with microbial flocculant TJ-F1 as a novel conditioner. Water Res. 44, 3087-3092.

Zhang ZQ, Lin B, Xia SQ, Wang XJ and Yang AM. (2007). Production and application of a novel bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source. J Environ Sci. 19 (6): 667-673.

Zheng Y, Ye ZL, Fang XL, Li YH and Cai WM. (2008). Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. Bioresour Technol. 99: 7686–7691.

Appendix

Appendix 1: Effect of dosage size on flocculating activity of TKT-g-PAM6

Dosage (ml)	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
0.2	0.876	0.767	0.748
0.4	0.630	0.640	0.697
0.6	0.867	0.964	0.876
0.8	1.469	1.378	1.686
1	0.999	1.258	1.265

Appendix 2: Effect of cations on flocculating activity of TKT-g-PAM6

Cations	**Absorbance at 550 nm**		
	Tube 1	**Tube 2**	**Tube 3**
Li^{+}	1.468	1.577	1.586
Na^{+}	1.469	1.686	1.378
K^{+}	1.733	1.640	1.531
Ba^{2+}	0.422	0.434	0.531
Ca^{2+}	0.902	1.406	1.419
Fe^{3+}	0.918	0.641	0.893
Control	0.974	0.890	1.049

Appendix 3: Zeta potential of the samples

Samples	Mv		
	Reading 1	Reading 2	Reading 3
TKT-g-PAM	-11.98	-12.88	-12.79
TKT	-17.7	-17.1	-16.4
Kaolin particles	-9.24	-7.21	-3.33
Kaolin particles with Ba^{2+}	-6.04	-8.03	-6.96
Kaolin particles flocculated with TKT in the presence of Ba^{2+}	-3.73	-4.34	-5.17
Kaolin particles flocculated with TKT-g-PAM in the presence of Ba^{2+}	-11.15	-10.89	-12.4
Kaolin particles flocculated with acrylamide in the presence of Ba^{2+}	-9.06	-9.19	-12.4

Appendix 4: pH stability of TKT-g-PAM6

pH	Absorbance at 550 nm		
	Tube 1	Tube 2	Tube 3
3	0.245	0.139	0.329
4	0.232	0.379	0.309
5	0.345	0.504	0.692
6	1.136	1.226	1.253
7	0.872	0.964	1.358
8	0.867	0.964	0.876
9	1.013	1.498	1.492
10	0.902	1.406	1.419
11	0.974	0.890	1.049

Appendix 5: Thermal stability of TKT-g-PAM6

Temperature (°C)	Absorbance at 550 nm		
	Tube 1	**Tube 2**	**Tube 3**
50	0.016	0.017	0.017
60	0.101	0.103	0.120
70	0.074	0.065	0.1
80	0.116	0.132	0.109
90	0.209	0.139	0.111
100	0.379	0.232	0.309

Appendix 6: *In-vitro* cytotoxicity of TKT-g-PAM6

TKT-g-PAM (µg/ml)	HEK293		
	Reading 1	**Reading 2**	**Reading 3**
50	1.085	1.077	1.108
100	1.028	1.049	1.004
150	1.021	1.063	1.003
200	1.001	1.037	1.013
Control	1.163	1.228	1.324
TKT-g-PAM (µg/ml)	**CaCO2**		
	Reading 1	**Reading 2**	**Reading 3**
50	0.901	1.003	1.001
100	0.975	0.937	0.947
150	0.897	0.868	0.798
200	0.749	0.761	0.833
Control	1.274	1.183	1.241

Appendix 7: Removal efficiency of the four (4) flocculants

Flocculants	parameters	Water quality before treatment			Water quality after Treatment		
		Tube 1	**Tube 2**	**Tube 3**	**Tube 1**	**Tube 2**	**Tube 3**
TKT-g-PAM	BOD	6.4	6.4	6.4	0.5	0.3	0.7
TKT	BOD	6.4	6.4	6.4	2.5	2.4	2.5
Alum	BOD	6.4	6.4	6.4	1.1	1.1	5.0
$FeCl_3$	BOD	6.4	6.4	6.4	1.9	1.8	1.9
TKT-g-PAM	COD	1557	1557	1557	203	201	198
TKT	COD	1557	1557	1557	586	575	602
Alum	COD	1557	1557	1557	952	933	918
$FeCl_3$	COD	1557	1557	1557	896	891	902
TKT-g-PAM	N	5.6	5.6	5.6	0.5	0.5	0.5
TKT	N	5.6	5.6	5.6	1.3	1.2	1.3
Alum	N	5.6	5.6	5.6	1.9	2.0	1.8
$FeCl_3$	N	5.6	5.6	5.6	2.1	2.2	1.8
TKT-g-PAM	S	4.1	4.0	4.1	0.3	0.3	0.2
TKT	S	4.1	4.0	4.1	1.1	1.1	1.4
Alum	S	4.1	4.0	4.1	1.9	1.7	1.5
$FeCl_3$	S	4.1	4.0	4.1	1.1	1.7	1.3