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Synthesis of TKT-g-PAM 5 copolymer via microwave initiative for treatment of mine wastewater

Tsolanku Sidney Maliehe[1]*, Sicelo Kenny Masuku[1], Albertus Kotze Basson[1] and Moganavelli Singh[2]

[1]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, **South Africa**
[2]Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Private Bag X 54001, Durban 4000, **South Africa**

***Correspondence:** sidttmaliehe@gmail.com, MalieheT@unizulu.ac.za **Received 13-01-2020, Revised: 16-07-2020, Accepted: 18-07-2020 e-Published: 21-07-2020**

Synthesis of natural flocculants with synthetic monomers has been reported to form copolymers with profound efficiencies. This study focused on the production of a bioflocculant from *Alcaligenes faecalis* HCB2 and the synthesis, characterization and application of an acrylamide grafted flocculant. Four grams of a bioflocculant was obtained from a litre of the production medium. A maximum grafting percentage of 62% was achieved with microwave power at 900 W at 70 °C for 3 minutes when 0.5 g of the bioflocculant and 7 g of acrylamide were used. The bioflocculant exhibited a deformation vibration peak around 1646 cm^{-1} and new absorption peaks were formed at 1427 cm^{-1} and 1651 cm^{-1} with the copolymer. The highest flocculating activities of 88% and 94% were obtained at a dosage size of 0.8 mg/ml of the bioflocculant and 0.2 mg/mL of the synthesized copolymer against kaolin solution, respectively. Human embryonic kidney 293 cells displayed above 80% viability when exposed to different concentrations of the copolymer. The copolymer had removal efficiencies of 87%, 92% and 93% on chemical oxygen demand, biochemical oxygen demand and sulphur. The copolymer showed potential for industrial applicability.

Keywords: Flocculation; TKT-g-PAM 5; *Alcaligenes faecalis* HCB2; wastewater

INTRODUCTION

Recent industrialisation and urbanisation in the Republic of South Africa have led to an increase in water pollution. The disposal of effluents without appropriate treatment results in undesirable negative impact on the environment and humans (Lee et al*.,* 2014). Thus, treatment of wastewater to palatable and potable quality remains a concern. Several methods, including coagulation-flocculation, have been employed to remove colloidal pollutants from wastewater (Sun et al*.,* 2019). In the coagulation process, colloids are destabilised by neutralising repulsion forces, consequently resulting in the formation of small, loosely bound flocs (Brostow et al., 2007). A high-energy, rapid-mix to properly disperse coagulant and promote collision of particles is vital for successful coagulation process. Flocculation is a physico-chemical purification technique where the destabilized colloidal particles are gently stirred to agglomerate. The flocs formed are large and strongly bound (Bhunia et al., 2012). The flocculation process is easier to operate and utilises lower energy (Liu et al., 2014). Flocculants are categorized as inorganic, organic or as naturally occurring (Salehizadeh et al., 2018).

Inorganic flocculants such as hydrolysable salts of iron and aluminium are predominantly

used because of their availability and low cost (Liu et al., 2017). Nevertheless, aluminium sulfate and ferric chloride have high sensitivity to pH and demand high concentrations for optimal flocculation (Zeng et al., 2019). Moreover, strong links have been shown between Alzheimer's disease and residual aluminium in treated wastewater (Wang et al., 2014). These drawbacks have led to the use of organic synthetic flocculants. Organic synthetic flocculants (i.e. polyacrylamide and its derivatives) have solubility in water and are effective at low dosage sizes within a wide pH range (Renault et al., 2009). The flocs formed by organic flocculants are bigger and stronger than those formed by inorganic salts. However, there is a shift to use naturally occurring flocculants such as; starch, chitosan and microbial exopolymers, due to poor shear stability, non-degradability and high toxicity nature observed with organic synthetic flocculants (Makapela et al., 2016). In recent years, more attention has been given to microbial bioflocculants from bacteria, fungi, and algae. Microbial bioflocculants are macromolecules (proteins, lipids, carbohydrates and glycoproteins) secreted by microbial strains as a result of substrate metabolism, bacterial growth and cell lysis. They are shear stable, innocuous to humans, biodegradable and thus are eco-friendly (Ben et al., 2018). However, they have poor shelf life and moderate solubility. To combat this, modified microbial flocculants are being synthesised by grafting organic synthetic polymers onto bioflocculants (Chen et al., 2018). The grafting process merges the best features of the bioflocculants with those of synthetic polymers (Wang et al., 2013).

Microwave irradiation is predominately employed in fabrication of modified microbial flocculants (Kaavessina et al., 2017). This method initiates free radical sites on the backbone of the bioflocculants and enhance polymeric synthetic monomers to react with the initiated sites and propagate their functional groups (Parviainen et al., 2014). Usually, the generated modified microbial flocculants have long sequences of the backbone biopolymers with branches (grafts) of synthetic polymers (Sen et al., 2009). The dangling branches enable easier accessibility and adsorption of colloidal particles in wastewater (Huang et al., 2017). Moreover, compared with parental linear polymers, branched copolymers possess lower viscosity, better charge densities, flocculating efficiencies, aqueous solubility, thermal stability and molecular weight ranges (Sun et al., 2013 & Yang et al., 2016). Many synthesised flocculants have been grafted using polyacrylamide as a monomer onto; xanthan (Jain et al., 2014), chitosan (Huang et al., 2013) and starch (El Sayed et al., 2015). This is because polyacrylamide and its monomers are effective, flexible and soluble in solutions and have high molecular weights (Rahul et al., 2014). According to our knowledge, there are no reports on the grafting of the bioflocculant from *Alcaligenes faecalis* HCB2 with polyacrylamide or its derivatives.

This research focused on the fabrication of acrylamide's functional groups onto the novel bioflocculant extracted from *Alcaligenes faecalis* HCB2 to produce a grafted bioflocculant through a microwave-initiated method. The effects of acrylamide concentration and initiator time on grafting efficiency were investigated. Surface structure, functional groups and thermal stability of the copolymer were evaluated by scanning electron microscopy, infrared spectroscopy and thermogravimetric analysis. In addition, the cytotoxicity, flocculation mechanism and removal efficiencies of the modified microbial flocculant were assessed in mine wastewater.

MATERIALS AND METHODS

Chemicals

Acrylamide, hydroquinone, formamide, acetic acid, NaCl, K_2HPO_4, $MgSO_4.7H_2O$, glucose, $(NH_4)_2SO_4$, KH_2PO_4, ethanol, chloroform, butanol, HCl, NaOH and kaolin clay were all obtained from E. Merck Limited, South Africa.

Production media

The production medium was adopted from Maliehe et al. (2016). It consisted of NaCl (0.1 g), K_2HPO_4 (5.0 g), $MgSO_4.7H_2O$ (0.2 g), maltose (20 g), urea (1.2 g) and KH_2PO_4 (2.0 g) in 1000 ml of filtered sterilized seawater.

Extraction and purification of the bioflocculant

Alcaligenes faecalis HCB2 was streaked on nutrient agar and incubated at 37 °C for 18 hours for resuscitation. Thereafter, a loopful of the bacterium colony was inoculated into 50 mL of the production medium and incubated for 72 hours at 30 °C, at a shaking speed of 165 rpm. The fermentation broth was then used as a stock-culture for subsequent inoculations. For fermentation, 10 mL of the stock-culture was inoculated into 1 L of the production medium, indicating a 1% (v/v) inoculum size and incubated at 30 °C at a shaking speed of 165 rpm for 72

hours. The bioflocculant was extracted and purified using the method described by Choi et al. (1998). Briefly, the broth culture was centrifuged (5000 rpm, 30 minutes, 4 °C). To the supernatant phase, 1 volume of distilled water was added and centrifuged (5000 rpm, 4 °C, 30 minutes) to eliminate the substances that are insoluble. Ethanol (2 volumes) was added to the supernatant, agitated and left to precipitate for 12 hours at 4 °C. The precipitate was vacuum-dried and the crude product was dissolved in distilled water (100 mL). One volume of a mixture of chloroform and butanol (5:2 v/v*)* was added, agitated and left to settle for 12 hours at room temperature. The supernatant was again centrifuged (5000 rpm, 30 minutes, 4 °C) and vacuumed-dried.

Synthesis of the graft copolymer

Microwave irradiation was utilized to produce free radical sites on the bioflocculant as illustrated schematically in Figure 1 (Sen et al., 2009). The bioflocculant (0.5 g) was dissolved in 20 mL of distilled water. Different concentrations of acrylamide were added to the bioflocculant solution. The mixture was transferred to a beaker and exposed to microwave radiation using a 25 l LG Microwave oven. Microwave irradiation at 900 W was performed for the desired amount of time ranging from 1 to 4 minutes. The reaction mixture was maintained at 70 °C. Once the microwave irradiations for the intended time period was completed, the reaction mixture was cooled and then left to stand for 24 hours. The reactions were ended by adding a saturated solution of hydroquinone. The gel-like mass left in the reaction beaker was added to 250 mL acetone. The resulting precipitates of grafted polymers were collected and heated to 60 °C for 6 hours to dry. Any obstructed polyacrylamide formed by competing homopolymer formation reaction was removed by solvent extraction using formamide and acetic acid mixture in a ratio of 1:1 (v/v). The percentage grafting of each of the synthesized graft copolymers was evaluated as: %G = (weight of graft copolymer – weight of bioflocculant)/weight of bioflocculant x 100 (Fanta, 1973).

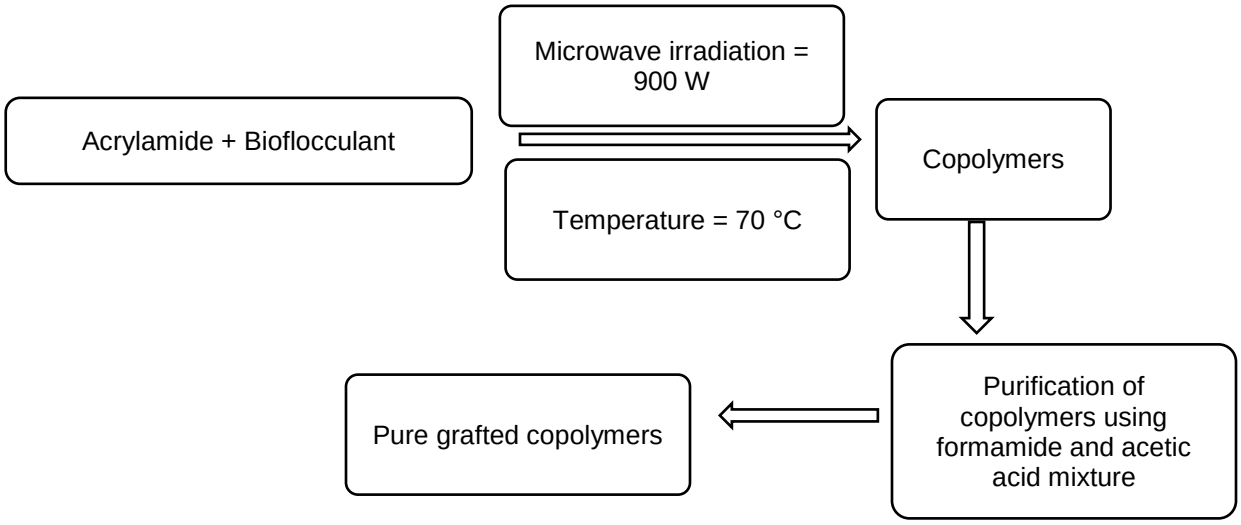

Figure 1: Schematic representation for the synthesis of copolymer.

Characterisation of the flocculants

Morphological surface of the flocculants

The surface topography of the bioflocculant and grafted flocculant (copolymer) were evaluated with a scanning electron microscope (SEM) (SEM-Sipma-VP-03-67). Evaluation was done at a voltage of 15 kV and magnification of 1000-3000X. Film samples were first coated with carbon using sputtering coater.

Functional groups

The functional groups of the bioflocculant and grafted flocculant were assessed by a Fourier transform infrared (FTIR) spectroscopy over a wave length of 400 to 4000 cm^{-1}.

Thermogravimetric analysis

The thermogravimetric analysis (TGA) of the bioflocculant and grafted flocculant were carried out with a TGA instrument (Model: DTG-60; Shimadzu, Japan). Ten milligrams of the flocculants were analysed in an inert atmosphere (nitrogen) from 25 °C to 700 °C. The heating rate was uniform in all cases at 5 °C/minutes.

Effect of bioflocculant and grafted flocculant concentrations on flocculating activity

The different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of the bioflocculant and grafted flocculant were evaluated for their flocculating activity. Hundred millilitres of kaolin clay (4 g/L) was mixed together with 3 mL of 1% (w/v) $CaCl_2$ and 2 mL of different concentrations of the flocculants. The mixtures were shaken at 200 rpm for 3 minutes and the speed was reduced to 40 rpm. After 5 minutes, 3 mL of the upper phases were drawn and read by spectrophotometer (Pharo 300, Merck KGaA, Germany), at the optical density of 550 nm. Flocculating activities (FA) were obtained by using the following formula:

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

where X is the optical density of the control and Y represents the optical density of the sample (Kurane et al., 1986).

Effect of cations on flocculating activity

The synergistic effect of different cations on the flocculating activities of the bioflocculant and the synthesised grafted flocculant were investigated in accordance to Makapela et al. (2016). Briefly, 3 mL of 1% cationic solutions (NaCl, KCl, LiCl, $BaCl_2$ and $FeCl_3$) were used to replace $CaCl_2$, which was initially used. Thereafter, the flocculating activities of both flocculants were determined as described previously.

Effect of pH on flocculating activity

The effect of pH on the flocculating activities of the bioflocculant and grafted flocculant was evaluated. pH of kaolin suspension (4 g/L) was adjusted in a range of pH 3 to 11 with 0.1 M NaOH and 0.1 M HCl prior to application of the flocculants. Flocculating activities were then evaluated as stated previously.

Cytotoxicity assay of the grafted flocculant

The cell toxicity of the synthesised copolymer investigated on Human embryonic kidney 293 (HEK 293) cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay. HEK 293 cells were grown to confluence in 25 cm^3 flasks. The cells were then trypsinised and plated into 48 well plates. Cells were incubated overnight at 37 °C. Old medium was supplemented with the fresh medium (MEM + Glutmax + antibiotics). Grafted flocculant and acrylamide were then added separately into the wells and incubated for 4 hours. Thereafter, the medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics +10% fetal bovine serum). After 48 hours, the cells were subjected to 200 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) with a concentration of 5 mg/ml in phosphate buffered saline (PBS) and 200 µL medium was added to each well and incubated at 37 °C for 4 hours. The medium with MTT was aspirated from the wells and the formed formazan crystals were solubilized in 200 µL of dimethyl sulfoxide (DMSO). Finally, the optical densities of solutions were read at 570 nm using a micro-plate reader. The effect of the grafted flocculant and acrylamide on HEK 293 cell viability were measured as the percentage of tetrazolium salt reduction by viable HEK 293 cells against the untreated cells; Cell viability (%) = $(OD_{untreated} - OD_{treated})/OD_{untreated}$ x 100 (Mosmann, 1983).

Proposed flocculation mechanism

The flocculation mechanism of the synthesised copolymer was proposed after the zeta potentials were measured by Zetasizer Nano (Malvern, UK). The zeta potentials of; (1) copolymer solution (0.2 mg/mL), (2) kaolin solution (4 g/L), (3) mixture of kaolin solution in the presence of 1% (v/w) of $CaCl_2$ and (4) kaolin solution flocculated by the copolymer in the presence of $CaCl_2$ were investigated at pH 3 at 25 °C (Aljuboori et al., 2015).

Removal efficiencies of the flocculants on wastewater

Wastewater was collected aseptically from Thandele Coal Mine wastewater plant in Mtubatuba, KwaZulu Natal, South Africa. The biological oxygen demand (BOD), chemical oxygen demand (COD) and sulphur (S) in wastewater were measured with a spectro-quant (Pharo 300, Merck KGaA, Germany), before and after application of the bioflocculant, polyacrylamide, grafted flocculant, ferric chloride ($FeCl_3$) and aluminum sulfate (Alum). Jar test was used to evaluate the removal efficiencies of the flocculants on the selected parameters. After adjusting the pH of the wastewater to 3, 2 mL of

0.2 mg/ml of the grafted flocculant and polyacrylamide and 3 mL of 1% of $CaCl_2$ were added into 100 mL wastewater samples separately. The dosage size of 0.8 mg/mL and 3 mL of 1% KCl were used for TKT, after adjusting the pH of wastewater to 7. The mixtures were shaken at 200 rpm for 3 minutes and the speed was reduced to 40 rpm for 5 minutes. The removal efficiencies of the flocculants on BOD, COD and S were measured as follows;

Removal efficiency (%) = $(E_o – D/ E_o) \times 100$

where E_o and D are the initial and final values obtained before and after treatment with the flocculants (Cosa et al., 2013).

Statistical analysis

The experiments were done in triplicates and recorded on one-way analysis of variance (ANOVA) using Graph Pad Prism™ 6.1. Arrow bars represented the variation of the data and values with different alphabets along the same row represent the significant different ($p<0.005$).

RESULTS AND DISCUSSION

Bioflocculant yield

A. faecalis HCB2 yielded 4 g/L of the bioflocculant. The high yield was due to the ability of the strains to optimally grow and produce bioflocculant in the optimised culture conditions. The polarity of the solvents used during extraction might have also contributed to the high yield obtained. The yield was higher than those mostly obtained from single bacterial strains (Soliman et al., 2014). The high yield suggested the potential use of this strain for bioflocculant production at the industrial level. The bioflocculant was named TKT.

Effect of acrylamide concentration on %grafting

A series of bioflocculant-graft-acrylamide (TKT-g-PAM 5) copolymers were synthesized using bioflocculant as a copolymers' backbone. The grafting reaction was performed via the microwave initiation method and the results are tabulated in Table 1. In all syntheses, the amount of bioflocculant and power were kept constant. TKT-g-PAM 5 indicated the highest grafting percentage of 62. The high grafting percentage was obtained when 0.5 and 7 g of bioflocculant and acrylamide were used, respectively. The observed initial increase in the grafting percentage in relation to the increase of acrylamide was owed to the active sites on bioflocculant molecules, which catered for more acrylamide monomers to chemically propagate. Any increase in acrylamide concentration above the optimum led to a decrease in grafting percentage. Excess acrylamide particles might have accumulated and inhibited electron transfer reactions, consequently reducing reaction efficiency. These findings are similar to those obtained by Ranjbar-Mohammadi et al. (2010) on grafting chitosan onto a fabric of wool.

Effect of time on %grafting

Grafting reactions were carried out at various time intervals (2-4 minutes) while keeping all other parameters constant. There was an increase in grafting percentage with an increase in reaction time from 2 to 3 minutes (Tables 1). The increase was attributed to the prolonged exposure time which allowed more free radicals to be formed on the backbone of TKT. The increase in the reaction time led to an increase in mobility of the acrylamide, consequently enhancing spontaneous collisions of the monomers with the bioflocculant and increased the grafting percentage. However, there was a decrease in the grafting percentage observed beyond 3 minutes of exposure time. The decrease might be due to the degradation of the bioflocculant backbone or the depletion of acrylamide during the grafting progression. Moreover, the decrease may be attributed to the increase in viscosity of the reaction medium, which subsequently might have slowed down the reactants' collision and lowered the grafting ratios. These results are comparable to those obtained by Sen and Pal (2009). They concluded that the optimum time for grafting polyacrylamide onto carboxymethylstarch was 3 minutes.

Effect of dosage on flocculating activity

Flocculant dosage is one of the most vital external factor affecting the flocculation effectiveness. Different concentrations of the TKT and TKT-g-PAM 5 were investigated in a range of 0.2-1 mg/mL and results are illustrated in Table 2. The highest flocculating activities of 85.6 and 92.4% were observed at concentrations of 0.8 and 0.2 mg/mL for TKT and TKT-g-PAM 5, respectively. The copolymer was more effective at lower concentrations. Excessive amounts of flocculant concentrations in suspension can lead to a reduction in the settling of flocculated colloidal particles due to high viscosity (Brostow et al., 2007). This phenomenon was observed when the concentration of TKT-g-PAM 5 was further increased above 0.2 mg/ml. Thus, 0.2 mg/ml was

used as the optimum concentration in successive experiments. The high flocculating performance of TKT-g-PAM 5 as compared to TKT at their optimum concentrations may be due to the presence of the long PAM side chains onto the backbone of TKT, which bridge colloids to be flocculated (Ali and Singh, 2009).

Effect of cations on flocculating activity

Cations act as coagulant aids and neutralise and stabilise the residual negative charges of the functional groups of the flocculants (Makapela et al., 2016). The synergistic effects of cations on the flocculating activity of the purified TKT (0.2 mg/mL) and synthesized TKT-g-PAM 5 (0.2 mg/mL) were evaluated and the results are shown in Table 3. K^+ and Ba^{2+} ions were the most preferred cations for the purified bioflocculant, having resulted in the flocculating activities of 85.8 and 85.2%, respectively. These cations gave a significant increase ($P < 0.05$) of about 36% on flocculating activity. On the other hand, divalent cations, namely, Ca^{2+} and Ba^{2+} were more effective cations to aid flocculation by TKT-g-PAM 5 with flocculating activities of 85.8 and 85.2%, respectively. The cations stimulated the formation of bridges between kaolin particles and the copolymer. Ca^{2+} and Ba^{2+} gave a significant increase ($P < 0.05$) in flocculating activity of 24%. Similar findings were observed by Ugbenyen and Okoh. (2014), who reported that cations greatly enhanced the flocculation process. In this study, K^+ and Ca^{2+} were used in all experiments for TKT and TKT-g-PAM 5, respectively.

Effect of pH on flocculation

The alteration of pH may change the flocculant charge status and physiognomies of suspended materials consequently affecting the flocculating activity (Tong et al., 1999). The effect of pH on flocculating activity of TKT and TKT-g-PAM 5 is displayed in Table 4. The highest flocculating activity (93%) of TKT was observed at pH 7. However, TKT-g-PAM 5 performed optimally well in strong acidic conditions (pH 3-5) with the maximum flocculating activity of 93% at pH 3. Connelly and Richardson (1984) reported that copolymers of acrylamide are mostly non-anionic and often demonstrate highest activities at pH ≥ 4. This phenomenon was observed in this study. There was also a significant decrease in flocculating activities of TKT-g-PAM 5 as the pH increased to neutral and alkaline conditions. This was attributed to the fact that hydroxide ions (OH^-) might have interfered with bond formation between TKT-g-PAM 5 and kaolin particles.

Surface structure of the flocculants

The SEM images of the bioflocculant (TKT) and grafted bioflocculant (TKT-g-PAM 5) are displayed in Figure 2. The surface structure of TKT is relatively smooth and amorphous while TKT-g-PAM 5 has a rough, amorphous structure. From the micrographs, it can be deducted that the chemical bonds formed by the interaction of the free radicals and acrylamide radicals led to the roughness in the appearance of TKT-g-PAM 5. Therefore, it is clear that the original structure of TKT was lost after grafting. This affirms the success of the grafting of monomers onto the backbone of the bioflocculant. Wang et al. (2018) observed similar results where the surface structure of CTS-g-P(AM-DMC) was different from that of P(AM-DMC) and CTS.

Functional groups

Functional groups are responsible for adsorption sites of flocculants for colloids in suspension (Xiong et al. 2010). Multiple functional groups are indicative of many adsorption sites for colloidal particles. The FTIR spectra of the TKT and TKT-g-PAM 5 samples are shown in Figure 3. The FTIR spectrum of TKT showed a broad peak at 3261 cm^{-1} that was due to O-H stretching vibrations. TKT exhibited a deformation vibration peak around 1646 cm^{-1}, which might have come from C=C stretching in PAM and exhibited a new absorption peak at 1651 cm^{-1} in TKT-g-PAM 5. Moreover, in TKT-g-PAM 5 spectra, there is an additional peak at 1427 cm^{-1} which is attributed to C-H bending of the grafted PAM chains. Comparing the vibration peaks of C-O stretching at 1200-1000 cm^{-1} with both curves, it could be seen that the intensity of TKT-g-PAM 5 was stronger than that of TKT. Thus, the FTIR spectra verified the success of the grafting process. Zhang et al. (2012) observed similar results, where the success of grafting was indicative of additional functional groups and deformation vibrations in the grafted flocculant.

Pyrolysis properties of TKT and TKT-g-PAM 5

Thermal behaviours of TKT and TKT-g-PAM 5 were studied as a function of percent weight residue with increase in temperature using a thermo-gravimetric analyser. The results are presented in Figure 4. There was a thermal degradation shift from 501 °C for the bioflocculant to 620 °C for the TKT-g-PAM 5. This also affirmed the success of grafting acrylamide onto the

backbone of the bioflocculant. Thus, the findings are in agreement with those observed by Mishra et al. (2006). They discovered that grafting of acrylamide chains to fenugreek mucilage resulted in shifts in degradation temperatures.

Table 1: Synthetic details of TKT-g-PAM 5

Grades	Power (W)	Amount of TKT (g)	Amount of Acrylamide (g)	Time (minutes)	% Grafting using microwave irradiation
TKT-g-PAM 1	900	0.5	5.0	2	35
TKT-g-PAM 2	900	0.5	5.0	3	51
TKT-g-PAM 3	900	0.5	5.0	4	39
TKT-g-PAM 4	900	0.5	6.0	3	57
TKT-g-PAM 5	900	0.5	7.0	3	62
TKT-g-PAM 6	900	0.5	9.0	3	59

Table 2: Effect of dosage size on the flocculating activity of TKT and TKT-g-PAM 5

Dosage size (mg/ml)	Flocculating activity (%)	
	TKT	TKT-g-PAM 5
0.2	80.4 ± 1.04[a]	92.4 ± 1.96[a]
0.4	78.7 ± 1.29[a]	86.2 ± 2.34[a]
0.6	78.5 ± 2.72[a]	81.8 ± 8.35[a]
0.8	85.6 ± 1.35[b]	78.6 ± 1.53[b]
1	84.8 ± 1.04[b]	71.0 ± 1.73[b]

Table 3: Effect of cations on flocculating activity of the TKT and TKT-g-PAM 5

Cations	TKT	TKT-g-PAM 5
Na^+	67.8±2.15[a]	51.3±4.73 [a, c]
K^+	85.8±1.0[c]	47.3±5.12[a]
Li^+	71.6±1.51[a]	50.3±2.31[a, c]
Ca^{2+}	77.4±0.57[b]	92.4 ± 1.96[b]
Ba^{2+}	85.2±0.35[c]	91.1±5.22[b]
Fe^{3+}	44.8±2.1[d]	63.6±1.0[c, d]
Control (without cations)	51.3+0.50[d]	68.6±2.52[d]

Table 4: Effect of pH on flocculating activity of the TKT and TKT-g-PAM 5

pH	Flocculating activity (%)	
	TKT	TKT-g-PAM 5
3	69.1 ± 2.1[a]	93.2 ± 4.3[a]
4	73.2 ± 0.3[a]	90.8 ± 5.2[a]
5	85.6 ± 1.5[b]	83.3 ± 2.1[a]
6	85.4 ± 0.27[b]	69.6 ± 1.4[b]
7	93.1 ± 1.0[b]	66.1 ± 3.0[b]
8	85.1 ± 2.0[b]	71.6 ± 1[b]
9	91.3 ± 0.1[b]	57.6 ± 2.3[c]
10	90.6 ± 0.5[b]	60.3 ± 0.4[b, c]
11	90.6 ± 0.3[b]	61.2 ± 1.1[b, c]

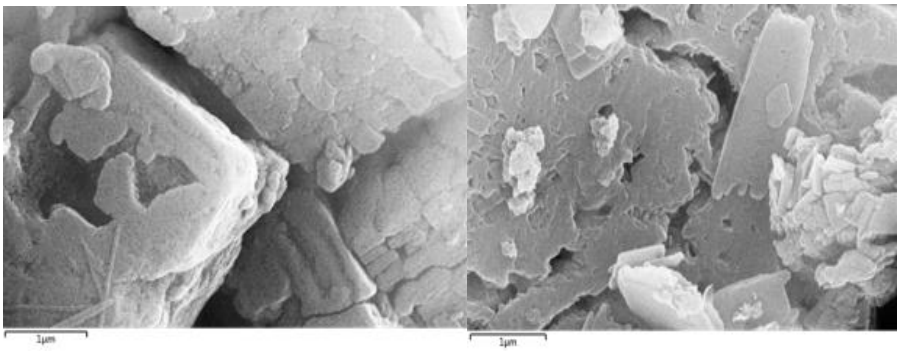

Figure 2. SEM images of TKT (left) and TKT-g-PAM 5 (right).

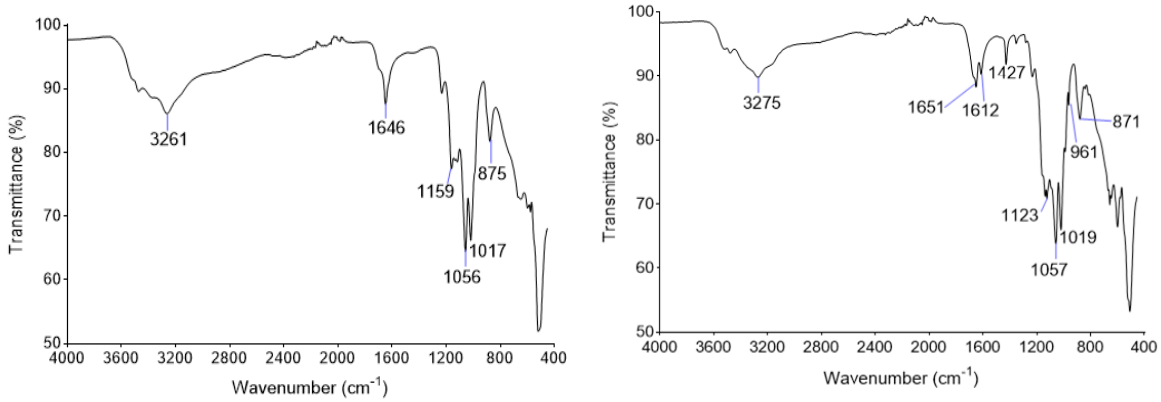

Figure 3: IR spectrophotometry analysis of the bioflocculant (left) and TKT-g-PAM 5 (right).

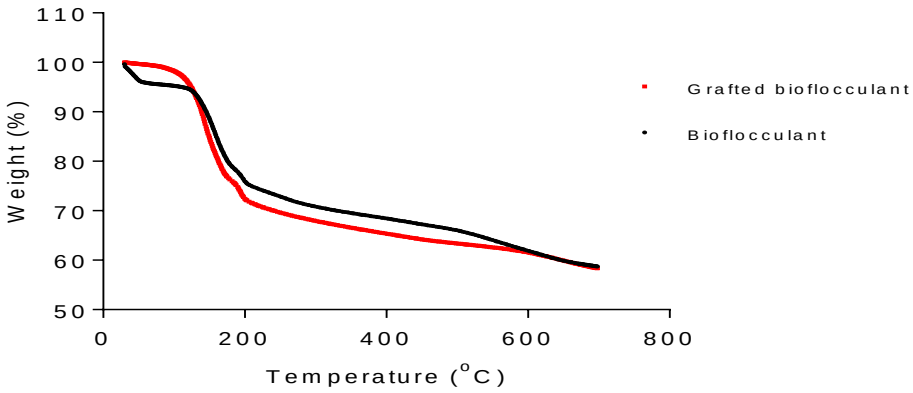

Figure 4: Thermo-gravimetric analysis of the bioflocculant and TKT-g-PAM 5.

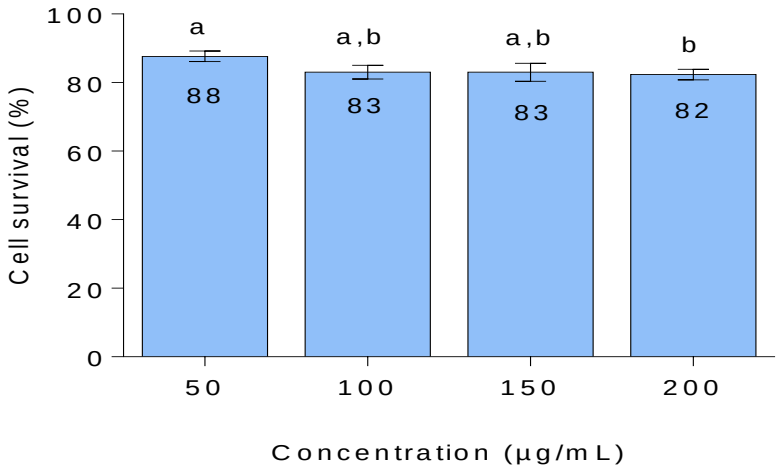

Figure 5: In-vitro cytotoxicity of TKT-g-PAM 5 on HEK293 cells.

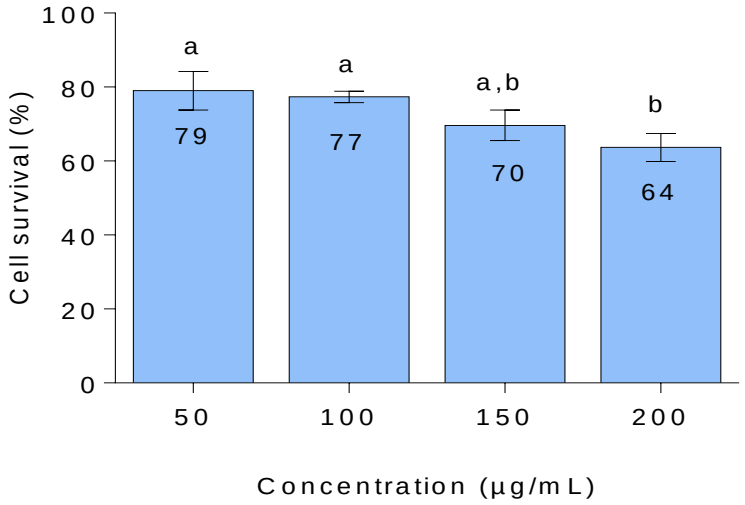

Figure 6: In-vitro cytotoxicity of acrylamide on HEK293 cells.

Biosafety assay of TKT-g-PAM 5

Although copolymers are generally reported to be safe, it is still important to investigate their toxicity before use. The results for the cytotoxicity study of the TKT-g-PAM 5 and acrylamide are presented in Figures 5 and 6, respectively. There was 82% and 64% HEK 293 cell viability when exposed to high concentrations (200 µg/mL) of TKT-g-PAM 5 and acrylamide, respectively. According to Li et al. (2005), the cell viability percentages obtained in this study means that the acrylamide grafted flocculant has a cytotoxicity index of 1. Thus, it can be concluded that it is much safer to use compared with acrylamide. These results were comparable to those obtained by Giri et al. (2015). They observed no clinical signs of toxicity or gross behavioural changes after oral administration of the graft copolymer to mice. Moreover, mortality was not encountered within the 14 days of treatment.

Proposed flocculation mechanisms

Among the coagulation/flocculation mechanisms, charge neutralization and bridging

mechanisms have been generally accepted in explaining the chemical bonding modes between colloidal particles and coagulants/flocculants. Charge neutralization occurs when flocculant is oppositely charged in comparison to the colloids in solution. Bridging occurs when flocculant extends from the colloids' surface for a distance greater than the distance over which the repulsion forces act and flocculant adsorbs colloids to form flocs (Liu et al., 2014). The flocculation mechanism of TKT-g-PAM 5 was predicted by measuring different zeta potentials of different samples. The zeta potentials of TKT-g-PAM 5, kaolin suspension, kaolin plus Ca^{2+}, kaolin suspension flocculated by TKT-g-PAM 5 in the presence of Ba^{2+} were all negative (Table 5).

Table 5: Zeta potential of different samples

Samples	Zeta potential (mV)
TKT-g-PAM 5	-13.2±5.95
Kaolin particles	-6.59±3.00
Kaolin particles with Ca^{2+}	-7.01±1.0
Kaolin particles flocculated by TKT-g-PAM 5 in the presence of Ca^{2+}	-4.41±0.72

However, it was observed that the addition of Ca^{2+} to kaolin suspension plus TKT-g-PAM 5 resulted in the slight increase in zeta-potential. When the negative charge is reduced, the repulsion forces decrease and particles are easily aggregated (Maliehe et al., 2016). Thus, Ca^{2+} acted as a coagulant aid and decreased the negative charges on the copolymer and kaolin particles. This enabled bridging mechanism between TKT-g-PAM 5 and kaolin particles. The bridging mechanism between TKT-g-PAM 5 and kaolin particles was due to the surface structures of the copolymer, chemical components and functional groups of TKT-g-PAM 5.

Removal efficiencies of the flocculant

The removal efficiencies of TKT-g-PAM 5 in comparison to TKT, alum and ferric chloride on mine wastewater were investigated. It is observed that although the removal efficiencies of TKT and the inorganic flocculants were good in some parameters, the copolymer had an outstanding removal efficiency in all tested parameters (Table 6). However, polyacrylamide performed better than TKT-g-PAM 5 in the removal of COD. The synthesised copolymer, because of its structure-branching nature and multiple functional groups, the approachability of contaminants towards it was enhanced. This was characterised by increased flocculation activities. The results were similar to those obtained by Das et al. (2019), whereby the copolymer-St-g-(PAAm-co-PMETAC) showed improved flocculating activities over its backbone polymer. The high removal efficiencies of TKT-g-PAM 5 implied its potential industrial applicability.

CONCLUSION

TKT-g-PAM 5 copolymer was successfully synthesized through a microwave-initiated method. A maximum grafting percentage of 62% was obtained when the microwave power and temperature were maintained at 900 W and 70 °C for 3 minutes; the mass of bioflocculant and acrylamide were 0.5 g and 7 g, respectively. FTIR spectrum revealed new vibration peaks at 1427 cm^{-1} and 1651 cm^{-1} on the copolymer. TKT-g-PAM 5 is safe to use at low concentrations. HEK 293 cells showed about 82% viability at the highest concentrations of 200 µg/mL of the synthesised copolymer. Flocculation mechanisms involved charge neutralisation and bridging mechanism between colloids and the copolymer mediated by Ca^{2+}. TKT-g-PAM 5 demonstrated removal efficiencies of 92%, 87% and 93% on BOD, COD and S in mine wastewater, respectively. For further studies, the copolymer will be used in treatment of other waste such as dye removal and metal recovery.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Basson A.K. and Maliehe T.S. designed the project. Maliehe T.S Masuku S.K and Singh M performed the experiments. Maliehe T.S and Masuku S.K wrote the manuscript. All authors read and approved the final version.

Table 6: Removal efficiency of the flocculants on wastewater

Flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/L)	COD (mg/L)	S (mg/L)	BOD (mg/L)	COD (mg/L)	S (mg/L)	BOD	COD	S
TKT-g-PAM 5	6.4±0	1557±0	4.1±0	0.5±3	206±0.	0.3±1.2	92[a]	87[a]	93[a]
TKT	6.4±0	1557±0	4.1±0	3.2±0	436±2	1.03±0	59[b]	72[b]	75[b]
Alum	6.4±0	1557±0	4.1±0	2.9±0	828±1	1.37±0	50[b]	47[c]	66[c]
$FeCl_3$	6.4±0	1557±0	4.1±0	2.6±0	753±2	1.08±0	54[c]	52[c]	73[b]
Polyacrylamide	6.4±0	1557±0	4.1±0	0.3±0	20±1	0.48±7	95[a]	99[d]	88[a]

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