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Pollutants removal from acidic coal mine wastewater using the non-toxic glycoprotein extracellular polymeric substance from *Bacillus* sp.

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Glycoprotein bioflocculant produced by *Bacillus* sp. isolated from fresh water sample of uMlalazi catchment, Mthunzini, in the Province of KwaZulu-Natal, RSA was used in the removal of different pollutants from coal mine wastewater and dyes from different solutions. The pH stable and heat tolerance bioflocculant with carboxylic, amino and hydroxyl functional groups showed the lowest cell inhibition of 10% at the highest bioflocculant solution concentration (100 µg/µL) for both HEK 293 and MFC 7 cell lines after exposed to different concentrations of bioflocculant solutions. The amorphous in structure bioflocculant with N: C: O: elements accounted for about 1.0, 31.4 and 48.9 (% wt) was revealed with a degradation temperature (T_d) of 150 ^oC. The water soluble bioflocculant which exhibited no antimicrobial activity on both Gram-negative and Gram-positive microorganisms removed various dyes in different solutions effectively in the presence of Ba^{2+} with flocculating activities of 93% (carbol fuchsine and safranin), 90% (methylene blue) and 87% (malachite green) with 0.4 mg/mL optimum dosage concentration. The bioflocculant also showed some remarkable characters of removing biochemical oxygen demand (BOD), chemical oxygen demand (COD), sulphur (S), aluminium (Al) and total nitrogen (N) contents in coal mine wash water with removal efficiencies of 96%, 81%, 100%, 52% and 90%, respectively. The results suggest the potential use of the bioflocculant from *Bacillus* sp. in industrial processes especially in coal mine industries.

Keywords: Pollutants, *Bacillus* sp., flocculating activity, bioflocculant, non-toxic, wastewater

INTRODUCTION

Life on earth is highly dependent in water. Water is a serious concern globally in the 21st century to have access to potable water. One of the basic needs to all living matters is the presence of contaminants-free and pure water (Singh et al. 2018). Water pollution has become a major challenge in underdeveloped countries owing to the discharged of contaminated effluents from civic, agriculture activities, global changes, environmental, and industrial sources (Wu et al. 2012; Connor et al. 2017; Singh et al. 2018). According to Sato et al. (2013), about 8% of the produced contaminated water in the undeveloped nations undergoes treatment of any kind. Less than 1% water from about 71% of water that is embracing the earth surface is consumable as per international standards due to various contaminations. Water contaminated with dyes, microbes, and heavy metals, even in small quantities, have serious health challenges in both humans and animals. The presence of high levels

of nutrients, chemical oxygen demand (COD) and biological oxygen demand (BOD) in wastewater and waters is a major challenge. Therefore, elimination of these water pollutants remains a difficult and huge challenging task in treatment or purification of wastewater (Maliehe et al. 2020). Numerous remediation techniques have been utilized to combat this challenge including flocculation (Dao et al. 2016; Zhu et al. 2016). Flocculation referred to the process of assemblage of microbial cells to produce flocs with different molecules elucidated in fermentation broth (Away et al. 2018). This process involves the use of flocculating agents which assist in flocculation with the bridge formation among suspended particles resulting into the clamping together and deposition of settling colloids. Flocculating agents include synthetic organic polymers (polyethylene amine and polyaluminium chloride), inorganic salts (alum, ferric chloride and ferric sulphate), and biological flocculants (chitosan, chitin and bioflocculants) (Guoa et al. 2018; Salehizadeh et al. 2018).

Owing to their cost-effectiveness, chemical flocculants have been commonly utilized (Lee et al. 2014). However, they are environmentally unfriendly and impose health threats to humans and animals. Reports show that these chemical flocculants are subjected to carcinogenic and neurotoxin effects (Ntombela et al. 2020). In addition, even though these flocculants are highly soluble in aqueous solutions, having well-presented and simplified flocculation mechanisms, but they are sensitive to pH changes and low temperatures, resistance to degradation and constituting resistance to environmental hazards (Brostow et al. 2009; Mishra et al. 2018). Because of these setbacks, their utilization industrially is dejected. Biological flocculants such as bioflocculants are viewed as potentially effective alternative (Okaiyeto et al. 2016). Bioflocculants secreted by microorganisms, such as fungi, bacteria and algae, during their growth have gained a momentum in scientific and biotechnological research of late (Sun et al. 2015; Maliehe et al. 2020). These bioflocculants are macromolecules (such as nucleic acids, proteins, carbohydrates and glycoproteins) derivatives of substrate metabolism, bacterial growth and cell lysis.

These microbial by-products are effective, biodegradable, toxic-free, benign nature and produce no secondary pollution (Ma et al. 2020). Because of these good characteristics, they have been used effectively in various processes including removal of heavy metals from wastewater (Zayed et al. 2019), removal of COD, BOD and phosphate from wastewater (Maliehe et al. 2020), and in the synthesis of nanoparticles as a capping agent (Dlamini et al. 2020). Nevertheless, reports show that their industrial applications as well as scientific researches has been limited by low production yields, low sedimentation rate, expensive in terms of production, huge amount requirements in applications and less information documented in terms of their mechanisms (Ntombela et al. 2019).

The present work focused on the characterization and application of the bioflocculant from *Bacillus* sp. with high flocculation capability obtained from marine sediments of uMlalazi catchment, Mthunzini in KwaZulu-Natal (KZN), Republic of South Africa. The bioflocculant in this study was applied in the treatment of acidic waste water from Tendele coal mine situated at KwaSomkhele area, Mtubatuba, KZN, in the removal of BOD, COD, and some chemicals present in wastewater. The bioflocculant was also used in the removal of dyes in solutions. Its cytotoxicity and solubility effects as well as antimicrobial activity property were evaluated.

MATERIALS AND METHODS

Source of bacteria

The bacteria were isolated from water and sediment samples of uMlalazi catchment, Mthunzini in the Province of KwaZulu-Natal in RSA. Serial dilutions were made using sterile saline solutions. Approximately, 100 µL of the diluted solutions were spread on nutrient agar plates. About 1 mL of the sediment samples were transferred into 9 mL of sterile saline solution and mixed for 60 sec. From these (sediments) solutions, serial dilutions were made. Similarly, 100 µL of the serially diluted samples were spread on the surface of nutrient agar plates, as described by Jensen et al. (1990). The nutrient agar plates were incubated for 2-3 days at 37 ^{o}C. Colonies were randomly picked and sub-cultured on fresh nutrient agar plates overnight at 37 ^{o}C incubation.

Activation of the isolates for fermentation

One litre of the activated medium containing 3 g of beef extract, 10 g of tryptone and 5 g of NaCl was prepared with marine water. Approximately, 5 mL of the activated medium was transferred into different test tubes and incubated at 121 ^{o}C for 15

min. The isolates were inoculated into the tubes and incubated in a rotary shaker for 24 hrs at 30 ^{o}C and 160 rpm.

Evaluation of bioflocculant production

Bioflocculant-producing medium and cultivation

The sample was prepared as described in the method used by Zhang et al. (2007). A cultivation medium made up of urea (0.5 g), glucose (20 g), yeast extract (0.5 g), $(NH)_2SO_4$ (0.2 g), KH_2PO_4 (2 g), K_2HPO_4 (5 g), NaCl (0.1 g) and $MgSO_4$ (0.2 g) was prepared in 1 L of filtered marine water. 1 M NaOH or 1 M HCl was used to adjust the pH to 7. The medium (50 mL) was transferred into 100 mL conical flasks and autoclaved at 121 ^{o}C for 15 min. The media were inoculated with a single colony of bacterium and incubated in a shaking incubator at a speed of 160 rpm and temperature of 30 ^{o}C for 72 hrs. After the incubation period, the broth culture was centrifuged for 15 min at 8, 000 x g, 4 ^{o}C to remove bacterial cells and the supernatant was used for measuring bioflocculating activity (Kurane et al. 1994).

Determination of flocculating activity

Using a suspension of kaolin clay as test material, flocculating activity was determined as described by Kurane et al. (1986). A kaolin clay suspension, 0.4% (w/v), was prepared with distilled water. 100 mL of kaolin clay suspension was transferred into 250 mL conical flask. Two millilitres of broth culture supernatant and 3 mL of 1% $CaCl_2$ (w/v) were mixed with kaolin clay suspension. The mixture was vigorously mixed and transferred into a 100 mL measuring cylinder. The sediment was allowed to settle for 5 min at room temperature. A similar procedure was used for a control, except that 2 mL of broth culture supernatant was replaced with a freshly prepared broth medium. The optical density (OD) at 550 nm of the liquid (1 cm beneath the fluid level) was measured using spectrophotometer. The flocculation rate was calculated as;

$$\text{Flocculation rate (\%)} = (A\text{-}B/A) \times 100$$

Where A and B are the optical densities at 550 nm of the blank control and the suspension treated by bioflocculant, respectively.

Extraction and purification

The extraction and purification of the bioflocculant was carried out in accordance with the method of Li et al. (2013), with minor modifications. After 60 hrs of fermentation at 165 rpm, 30 ^{o}C, the culture broth was centrifuged at 8, 000 x g for 15 min at 4 ^{o}C to remove insoluble substances. Two volumes of ethanol were added to the supernatant; the solution was agitated and left at 4 ^{o}C for 12 hrs. The precipitate was then vacuum-dried to obtain the crude bioflocculant and then dissolved in distilled water to obtain a solution (w/v). One volume of a mixture of chloroform and n-butyl alcohol (5:2) (v/v) was added. After agitation, the mixture was left at room temperature for 12 hrs. The supernatant was thereafter centrifuged at 8, 000 x g for 15 min at 4 ^{o}C and vacuum-dried to yield a purified bioflocculant.

Scanning electron microscope analysis

The elemental analysis and structural morphology of a purified bioflocculant were done using the scanning electron microscope (SEM) equipped with elemental analyser (Li et al. 2009). Before the SEM analysis, 5 mg of bioflocculant was added on stubs coated with silicon and fixed by a spin coater at 500 rpm for 60 sec.

Fourier transform infrared spectrophotometer analysis

The functional groups in the bioflocculant were characterized using Fourier transform infrared (FT-IR) spectrophotometer analyser. The dried bioflocculant powder was grounded with potassium bromide (KBr) and pressed into pellets for FT-IR spectral measurement in the wavelength range of 4000 – 40 cm^{-1} (Karthiga and Natarajan, 2015).

Thermogravimetric analysis of the bioflocculant

The pyrolysis analysis of the purified bioflocculant was assessed in the range of 30 ^{o}C - 800 ^{o}C at a constant rate of 10 ^{o}C min^{-1}, under constant flow of nitrogen gas (20.0 mL/min) using thermogravimetric instrument (Muthulakshmi et al. 2017).

Optimization of flocculating activity of a purified bioflocculant

Effect of bioflocculant dosage on flocculating activity

Various concentrations of the bioflocculant solution (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were prepared by diluting the bioflocculant with distilled water (w/v) to achieve the respective

concentrations and then used to obtain the optimum bioflocculant dosage. Two millilitres of the bioflocculant solution was mixed with 100 mL of kaolin suspension (0.4% w/v) containing 1% $CaCl_2$ in 250 mL flasks. The solution was then stirred vigorously and poured into a 100 mL graduated cylinder and left to rest for 5 min at room temperature. After 5 min, the clear top layer of the supernatant was then removed for determination of flocculating activity, as described previously (Lee et al. 2001).

Effect of temperature on flocculating activity

The effect of temperature on bioflocculant was investigated by preparing 0.4 mg/mL bioflocculant solution, which was determined to have optimum flocculating activity. Two millilitres aliquots of the solution was transferred into Eppendorf tubes and heated for 30 min at various temperatures ranging from 50 to 100 ^oC and at 121 ^oC (autoclaved for 15 min). The flocculating activity was measured as described above (Gong et al. 2008).

Effect of pH on flocculating activity

The pH of the kaolin clay suspension (100 mL) was adjusted using either 1 M HCl or 1 M NaOH, to obtain different pH values, ranging between 3 and 12, in 250 mL flasks. The bioflocculant (2 mL), at the predetermined optimal concentration, was added to each flask and the flocculating activity measured using the same method previously used (Akapo et al. 2019).

Effect of cations on flocculating activity

A variety of salts solutions were used to replace 1% (w/v) $CaCl_2$ solution; namely, KCl, NaCl, LiCl, $BaCl_2$, $MnCl_2$ and $FeCl_3$ at the same concentration. The control (without cation) was also prepared. The same method previously used for measuring flocculating activity was employed (Tawila et al. 2018).

Solubility assay of the bioflocculant

The assessment of the bioflocculant solubility was done by dissolving bioflocculant in different solvents such as water, methanol, hexane, acetone, ethyl acetate and benzene (Maliehe et al. 2016).

Cytotoxicity assay of bioflocculant

The cytotoxicity effect of a purified bioflocculant was conducted following the method described by Mosmann (1983). Sangamly human embryonic kidney 293 (HEK 293) cells were cultivated in 25 mL conical flasks. After trypsinization, cells were cultivated into 48 well plates and incubated at 37 ^oC overnight. The old medium was mixed with the freshly prepared medium (MEM+Glutamax+antibiotics). In three folds, the microbial flocculant was introduced and soaked for 4 hrs. The broth medium was then eliminated and replaced by medium made up of MEM+Glatamax+antibiotics+10% Fetal Bovine Serum. After 2 days, the cells were treated with 200 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) in phosphate-buffered saline (PBS) and each well filled with 200 µL medium and stored at 37 ^oC for 4 hrs. The broth was protracted from the wells with resulted formazan crystals dissolved in 200 µL of dimethyl sulfoxide (DMSO). The absorbance for each sample was measured using a microplate reader at 570 nm. The bioflocculant impact on HEK 293 cell viability was determined as the percentage of tetrazolium salts reduction by viable HEK 293 cells counter to the untreated cells. To estimate the cell viability, the following equation was used:

Cell viability (%) = $OD_{\text{treated cells}}$/ $OD_{\text{untreated cells}}$ X 100.

Breast cancer (MFC 7) cells were also used to test for cytotoxicity effect of the bioflocculant. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (100U), 20 µg/mL penicillin, and 100 µg/mL streptomycin. Incubation was carried out at 37 ^oC with an atmosphere of 5% CO_2. Normal breast (MFC 7) cells were cultured in 1:1 mixture of DMEM and Ham's F12 medium with 20 mg/mL of epidermal growth factor (EGF), 100 µg/mL cholera toxins, 0.01 mg/mL insulin and 500 µg/mL hydrocortisone, and 5% chelex treated horse serum. Purified berberine and tamoxifen were dissolved in DMSO and used for the bioassays. The anticancer activity of samples on MFC 7 cells was determined by the MTT assay that was used to assess the cytotoxicity (Horiuchi et al. 1988). Cells ($1x10^5$/well) were plated in 0.2 mL of medium/well in 96-well plates. For MTT assay, the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS for 2-3 times and 200 µL of MTT (5 mg/mL) was added. The plates were incubated or 6-7 hrs in 5% CO_2 incubator for cytotoxicity. After incubation, 1 mL of DMSO (solubilising chemical) was added to each well and mixed well by micropipette and left for 45 sec. Presence of viable cells was visualized by the development of purple colour due to formation of formazan crystals. The mixture was dispensed to

the glass cuvette of a spectrophotometer and the OD (optical density) values were measured at 595 nm by using DMSO as a blank. Standard graph was plotted by taking concentrations of the drug versus the relative cell viability. To estimate the cell viability, the formula below was used:

Cell viability (%) = $OD_{treated\ cells}$/ $OD_{untreated\ cells}$ X 100.

Antimicrobial activity assay of a purified bioflocculant

Selected Gram-negative and Gram positive microorganisms were first revived by growing them in the sterile nutrient broth tubes and cultivated at 37 °C for 16-24 hrs. After 24 hrs cultivation, one milliliter from each broth culture was mixed with 9 mL of sterile nutrient broth. The inoculated test tubes were incubated at 37 °C overnight. The optical density (OD) at 600 nm for each bacterium was investigated with UV-vis spectrophotometer to ascertain the growth of each bacterium. The $OD_{600\ nm}$ of all tested microorganisms was then adjusted with sterile nutrient broth medium to obtain an optical density of 1– 0.5. This value is within McFarland allowed standard.

Minimum Inhibitory Concentration (MIC)

The similar method utilized by Dlamini et al. (2019) was followed. Determination of the minimum inhibitory concentration of the purified bioflocculant was done. MIC is the minimum concentration of the microbial flocculants required to inhibit microbial growth. Assessment of purified bioflocculant quantitatively was obtained using the 96-well plates. The entire wells of the 96-well plates were filled with 50 µL of sterile nutrient broth medium. About 0.2 g of bioflocculant was mixed with 2 mL of de-ionized water. The bioflocculant solution (50 µL) was poured into the first row of 96-well plates containing nutrient broth, and mixed well. A 3-fold dilution was then done whereby 50 µL from row A was transferred to row B of the 96-micro-well plates, mixed again, and another 50 µL was pipette from row B to other the following rows until all the wells had the different bioflocculant concentrations. The 50 µL in the last column was dispensed so that the final volumes of all the 96 wells are 50 µL. About 50 µL of the bacterial strains of choice were then added into corresponding wells. The antibiotic Ciprofloxacin (40%) was used as a positive control and the negative control was distilled water. All plates were then stored at 37 °C overnight. After incubation, p-iodonitrotetrazolium violet (INT) solution was used as an indicator. 40 µL of 0.2 mg/mL INT solution was transferred to each well and further incubated at 37 °C for 30 min. A reddish colour present indicates the INT reduction to formazan by metabolic active microorganism. The absence of the reddish colour (clear) is an indication of inactivity of microorganisms since INT was not degraded to form formazan.

Dye removal efficiency of bioflocculant

To conduct the dye removal efficiency test of a purified bioflocculant, 2 mL of bioflocculant solution (0.4 mg/mL) and 3 mL of 1% (w/v) Ba^{2+} were mixed with 100 mL of dye solution (4 g/L) and shaken thoroughly for 60 sec. The solution was rested to settle down for 10 min at room temperature. Dyes such as basic fuchsine, malachite green, methylene blue and safranin O were used. Using UV-Vis spectrophotometer at 550 nm, the top clear solution was assessed and the optical densities attained for both treated and untreated dye solutions were utilized to calculate the removal potential of a purified bioflocculant. The dye removal efficiencies were calculated using the following equation:

Removal efficiency (%) = $(B_o\text{-}B/B_o)$ X 100

Where B_o is the initial value and B is the final value after treatment with bioflocculant.

It is important to determine the residual concentration of the dye in the solutions after treatment with the basis of the initial and final dye contents (Dlamini et al. 2019).

Wastewater treatment using bioflocculant

Tendele Coal Mine wastewater samples were freshly collected aseptically from treatment plant situated at KwaSomkhele, Mtubatuba in the Province of KwaZulu-Natal to assess the potential removal effect of contaminants by the bioflocculant from *Bacillus* sp. Using 1.0 M NaOH and 1.0 M HCl, the pH of the wastewater sample was adjusted to pH 7. The jar test was performed following the method described by Ntombela et al. (2020). To 100 mL of wastewater sample adjusted to neutral pH, 3 mL of 1% (w/v) Ba^{2+} and 2 mL of the bioflocculant solution (0.4 mg/mL) were added. The mixture was shaken at 200 rpm for 3 min and the speed was reduced to 40 rpm for 5 min at room temperature. The mixture was then rested for 10 min to precipitate. The supernatant was taken and used to measure chemical oxygen demand (COD), biological oxygen demand (BOD), sulphur (S), calcium (Ca), aluminium (Al) and total nitrogen (N) contents using test kits as described in manufacture's protocol. The untreated

wastewater sample was also assessed for BOD, COD, S, Ca, Aland N. The optical density of both treated (top-layer) and untreated samples were determined using a spectrophotometer (Spectro-quant, Merck Pharo 100) at 680 nm. The removal efficiencies of N, Ca, Al, S, COD and BOD by bioflocculant were calculated using the following equation:

$$\text{Removal efficiency (\%)} = (C_o\text{-}C/C_o) \text{ X } 100$$

Where C_o and C are the initial and final values attained prior and after treatment with microbial flocculant. Both Alum and ferric chloride were used as standards and compared with bioflocculant for their removal efficiencies.

Statistical analysis

All experiments were conducted in triplicates and recorded on one-way analysis of variance (ANOVA). Mean and standard deviation values were determined, where differences are considered significant at 0.05 confidence level ($p<0.05$), by the use of Graph Pad Prism version 6.

RESULTS AND DISCUSSION

Scanning electron microscopy analysis

Scanning electron microscopy analyses were carried out to elucidate the surface morphological structure of the bioflocculant before and after flocculation of kaolin clay suspension. The scanning electron microscope pictures, Figure 1(a) showed an amorphous, white in colour bioflocculant. This structure reveals that the bioflocculant might have a good flocculating ability when in contact with other molecules. The kaolin clay particles appeared to be fine and scattered before flocculation, Figure 1(b). In Figures 1(c), SEM image revealed the big flocs formed as the bioflocculant and the kaolin clay particles come together, which results in the precipitation of the flocs due to gravity. Similarly, Pathak et al. (2017) reported the amorphous shaped bioflocculant.

Elemental analysis of a bioflocculant

The SEM-EDX analysis of the purified bioflocculant was conducted and the findings are depicted in Figure 2. Elemental analysis reveals the elements present in the purified bioflocculant in % wt proportion comprise of C: O: P: Ca: Mg: N: Na: Cl: K: S in the percentage of 31.4%; 48.9%; 7.6%; 4.8%; 4.3%; 1.0%; 0.9%; 0.5%; 0.4%; 0.1%, respectively. This elemental composition indicates the presence of carbohydrate as a main component for the bioflocculant. Hydroxyl, amino and carboxyl groups are also revealed by FT-IR analysis spectrum (Figure 3). Akapo et al. (2019) reported the bioflocculant produced by *Bacillus atrophaeus* with elemental composition similar to the study finding which include (in %wt) C (3.46), O (44.4), Na (5.41), Mg (4.61), P (19.97), Cl (2.33), K (19.34) and Ca (0.75). Pathak et al. (2017) also revealed C (31.20): N (6.12): O (46.39) as the main elemental composition of the bacterial novel bioflocculant.

Fourier transform infrared spectroscopy analysis

To detect functional groups present on the bioflocculant, the Fourier transform infrared spectroscopy was used. The functional groups serve as attractive sites for metal ions and colloidal particles in solution, resulting in the formation of multiple chemical bonds (Ntsangani et al. 2017). Figure 3 revealed that the bioflocculant is made up of amine, hydroxyl, carbonyl, and carboxylic acid functional groups. The presence of hydroxyl group (3250 cm^{-1}) marks the feasibility of hydrogen bonding between water molecules and the bioflocculant, emanating in notable solubility of the bioflocculant in an aqueous solution (Desouky et al. 2008). According to Pathak et al. (2014), the presence of carbonyl group (1723 cm^{-1}) allows the chain to spread out as a result of electrostatic repulsion, which provides the adsorption site for particle attachment. The absorption peak at 1203 cm^{-1} revealed a C-O bond indicative of the presence of sugar derivates. The sharp peak at 759 cm^{-1} marks the existence of furan sugar (saccharides) and the peak observed at 694 cm^{-1} signifies a halo compound (Desouky et al. 2008). The produced bioflocculant showed the FT-IR spectrum which was consistent with the findings of other microbial flocculants produced by various microorganisms (Feng et al. 2008). The presence of hydroxyl and carbonyl groups in the bioflocculant's functional groups in the bioflocculant is mostly a polysaccharide (Zhao et al. 2013).

Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was used to evaluate the thermal stability of the purified bioflocculant. TG analysis helps to understand the pyrolysis property of a bioflocculant when it exposed to high temperatures. The initial 5 – 16% weight loss observed between 30 – 150 ^oC (Figure 4) was due to moisture lost owing to the increase in temperature. This moisture content

emanated from the presence of carboxyl and hydroxyl groups in the bioflocculant' s molecular chain (Kumar et al. 2004). Further weight loss from 200 to 600 ^{o}C (approximately 30%) was due to the degradation of proteins present in the bioflocculant, and therefore, decomposition of the main molecular chain of the bioflocculant. More than 60% of the bioflocculant weight was retained at 800 ^{o}C; this marks the thermal stability for the bioflocculant due to high carbohydrate content present (Ugbenyen et al. 2014).

Optimization of flocculating activity of a purified bioflocculant

Bioflocculant's dosage effect on flocculating activity

Bioflocculant dosage is the amount of the bioflocculant concentration that is required for optimal flocculation (Okaiyeto et al. 2013). The appropriate bioflocculant concentration used for subsequent experiments was determined by assessing various bioflocculant concentrations ranging from 0.2 – 1.0 mg/mL (Figure 5). An increase in flocculating activity was observed with the increase in dosage concentration until 0.4 mg/mL was reached, thereafter, a slight decrease in flocculating activity was observed with the increase of dosage concentration. According to Wang et al. (2011), inadequate amount of bioflocculant hinders the bridging mechanism of flocs development, and high amount of bioflocculant results in high viscosity which limits sedimentation of colloidal particles. In this study, a bioflocculant dosage of 0.4 mg/mL resulted in an optimum flocculating activity of 76%. Above 0.4 mg/mL concentration there was a slight decrease in flocculating activity. Statistically, there is no significant difference observed, so any concentration can be used for subsequent tests but preferable the lowest concentration for cost effectiveness. This decrease in flocculating activity may be due to high viscosity resulting from the high bioflocculant concentration.

Similar results were reported by Ntozonke (2015), whereby a bioflocculant concentration of 0.4 mg/mL was sufficient to yield the highest flocculating activity. The concentration of 0.2 mg/mL of the bioflocculant produced by a consortium of *Oceanbacillus* and *Halobacillus* resulted in about 90% flocculating activity (Cosa and Okoh, 2014).

Effect of cations on flocculating activity

In various articles the presence of cations has been reported to stimulate the flocculating activity of a purified bioflocculant (Okaiyeto et al. 2016). The role of cations in flocculation is to neutralize and standardize the residual negative charge found in the functional groups, and eventually forms bridges between the bioflocculant and particles (He et al. 2010). In this study, all the tested metal ions enhanced the flocculating activity of the bioflocculant to varying degrees by K^{+} (39%), Li^{+} (44%), Na^{+} (54%), Ca^{2+} (89%), Mn^{2+} (76%), Ba^{2+} (92%), and Fe^{3+} (70%)(Figure 6). The flocculating activity of cation-free bioflocculant was 36%.

The flocculating activity of a bioflocculant was highly improved with divalent cations such as Ba^{2+} and Ca^{2+}. The trivalent cation was also effective (70% flocculating activity), and the monovalent cations (K^{+}, Li^{+} and Na^{+}) were least effective. This bioflocculant seems to be cation-dependent with 36% flocculating activity in the absence of cation. Buthelezi et al. (2012) stated that the role of divalent and trivalent cations is to increase the initial adsorption of biopolymers on suspended particles by decreasing the negative charge on both the polymer and the particle. Several studies are in line with the study findings (Wu and Ye, 2007).

Effect of temperature on flocculating activity

The relationship between temperature and flocculation efficiency of the purified bioflocculant was examined at temperature ranges of 50 – 100 ^{o}C for 30 min and 121 ^{o}C (autoclaved) for 15 min, as depicted in Figure 7. The purified bioflocculant is observed to be thermally stable as it retained about 76% flocculating activity at 100 ^{o}C. Further exposure to high autoclave temperature (121 ^{o}C, 15 min) and pressure resulted in more than 70% flocculating activity attained, but the difference in all tested heat was not significant in terms of statistical analysis. Therefore, it was concluded that the bioflocculant produced by *Bacillus* sp. was thermostable and its flocculating efficiency was not affected by the temperature elevation. By using an autoclaved bioflocculant, it could be a great idea to ensure safety from contaminants especially for industrial applications.

Ntsangani et al. (2017) stated that the existence of protein in the structure of a bioflocculant is commonly related to its sensitivity to heat and high sugar content is mainly for its heat-resistance. Therefore, the bioflocculant produced by *Bacillus* sp. strain was predominately comprised of polysaccharides. The heat resistance of this bioflocculant may be due to the

availability of -OH group liable for hydrogen bond formation in its structure (Ugbenyen and Okoh, 2014). Various studies reported bioflocculants exhibited thermal stability, for example, Li et al. (2007) and Tawila et al. (2018) reported bioflocculants retained high flocculating activities at 100 ^{o}C.

Effect of pH on flocculating activity

The pH of the bioflocculant has a pronounced effect on its flocculating activity of the bioflocculant (Zhang and Lin, 1999). The flocculating activity of the purified bioflocculant was tested within the pH ranges of 3 – 12, as shown in Figure 8. The tested bioflocculant was highly effective at wide pH ranges of 3-12 with flocculating activities above 70% and an optimum flocculating activity of 86% at pH 9. The bioflocculant is stable and effective at both environments (acidic and basic) and there was no significant differences observed in terms of statistical analysis. The pH stability of the bioflocculant signifies its potential applicability in industrial fields, to treat various waste waters without regulating its pH state, thus minimizing the expenses of treatment (Okaiyeto et al. 2015). A marine dinoflagellate, *Gyrodinium impudicum* KG03 has reported to produce the bioflocculant p-KG03 flocculated best under acidic environments of pH 4 (Yim et al. 2007). A mixed culture of *Bacillus sphaeicus* F6 and *Rhizobium radiobacter* F2 produced a compound biopolymer CBF-F26 with the highest flocculating activity between pH 7-9 (Wang et al. 2011). Similar to the study, results were presented by Ntsaluba et al. (2013), where pH stability was observed at a wide pH ranges of 2-11.

Solubility assay of a bioflocculant

The bioflocculant in this study was investigated for its solubility effect in different solvents and the results are depicted in Table 1. The microbial product only dissolved completely in distilled water not in all other solvent tested. Bisht and Lal (2019) also reported that the bioflocculant BF-VB2 was highly soluble in water but insoluble in organic solvents. This result confirms the soluble principle which states that 'like dissolves like' (Friberg, 1998). If the bioflocculant is mainly composed of sugar, it carries charged and polar groups which are easily solvated by water molecules and transforming the biopolymer to be soluble and hydrophilic (Walker and Wilson, 2005). The presence of hydroxyl groups within the produced bioflocculant by *Bacillus* sp. revealed by FT-IR analysis (Figure 3) promotes the possibility of hydrogen bonding with one or more water molecules, hence intensifying solubility reaction. The insolubility behaviour of the bioflocculant in organic solvents could be due to hydroxyl groups as they introduce more crystallinity to polymer which organic solvents are unable to crack (Kumar et al. 2004).Thus, making them to dissociate the attraction forces resulting to the bioflocculant being insoluble in all of the organic solvents. Number of researchers reported to produce the bioflocculants that are only soluble in water and insoluble in various organic solvents, including Zaki et al. (2011), Maliehe et al. (2016) and Bisht and Lal (2019).

Table 1: Solubility effect of a bioflocculant in various solvents

Solvent	Solubility
Distilled water	+
Ethanol	-
Benzene	-
Acetyl chloride	-
Chloroform	-
Hexane	-
Butanol	-
Acetone	-

Antimicrobial activity assay of the purified bioflocculant

The MIC of microbial flocculant in comparison with antibiotic (Ciprofloxacin) is shown in Table 2. Both Gram-negative and Gram-positive microorganisms were tested against bioflocculant and Ciprofloxacin in terms of growth inhibition and found that no inhibitory properties associated with microbial flocculant revealed towards all tested Gram-positive and Gram-negative microorganisms.

Table 2: Minimal inhibitory concentration in mg/mL for bioflocculant

Test organism	MIC (Bioflocculant)	MIC (Ciprofloxacin)
Escherichia coli	-	6.25 mg/mL
Bacillus cereus	-	3.125 mg/mL
Klebsiella pneumoniae	-	1.56 mg/mL
Bacillus subtilis	-	3.125 mg/mL

This means that bioflocculants cannot inhibit the microbial growth but can only remove microbes with flocs formed during bioflocculation process. Microbes attached to suspended

colloids/particles-kaolin clay complex and eventually removed with formed flocs. Even though the bioflocculants are reported to inhibit microbial growth, but, no clear mechanism is documented based on the removal of pathogenic microorganisms from wastewater.

Ciprofloxacin which was used as a control in this experiment seems to have inhibitory effect towards all microorganisms (E. *coli, B. cereus, K. pneumoniae* and *B. subtilis*) with the lowest concentration of 1.56 mg/mL enough to block the cell growth for *K. pneumoniae.* Muthulakshmi et al. (2017) reported the decrease in bacteria cells by a microbial flocculant produced from *Bacillus* sp. Dlamini et al. (2019) reported the bioflocculant passivated copper nanoparticles to inhibit the growth of both Gram-negative and Gram-positive test microorganisms. Reports show that a bioflocculant MBF-5 produced by *Klebsiella pneumonia* was able to remove *Acanthamoeba cysts* from wastewater (Zhao et al. 2013).

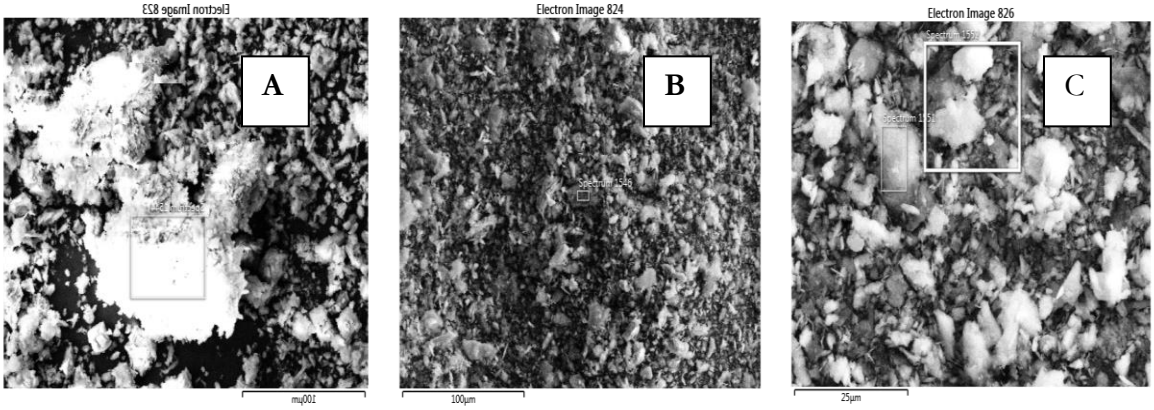

Figure 1: SEM images of kaolin clay (A), bioflocculant (B) and flocculated kaolin clay (C).

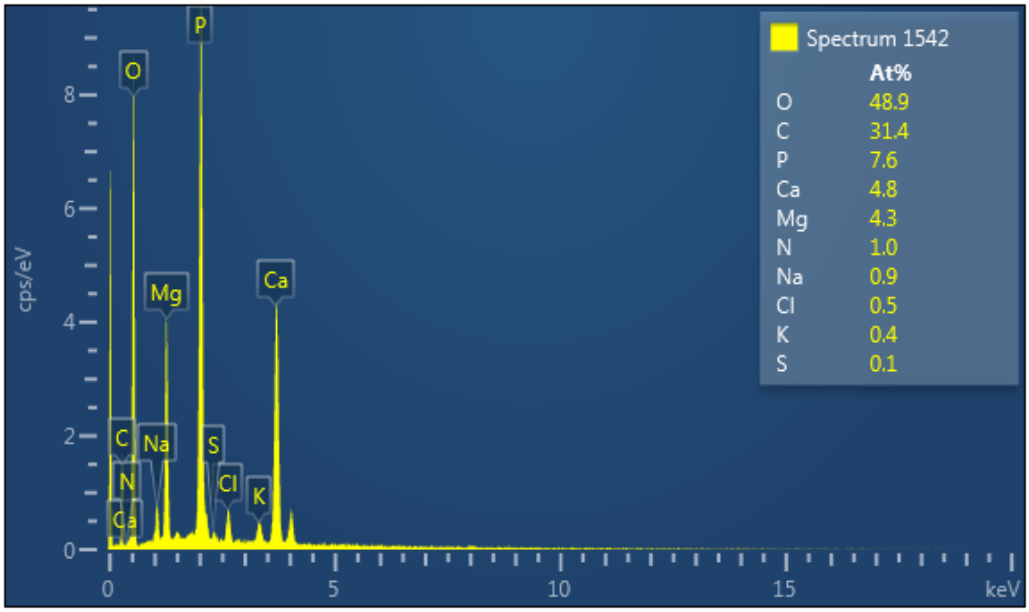

Figure 2: Elemental analysis of a purified bioflocculant

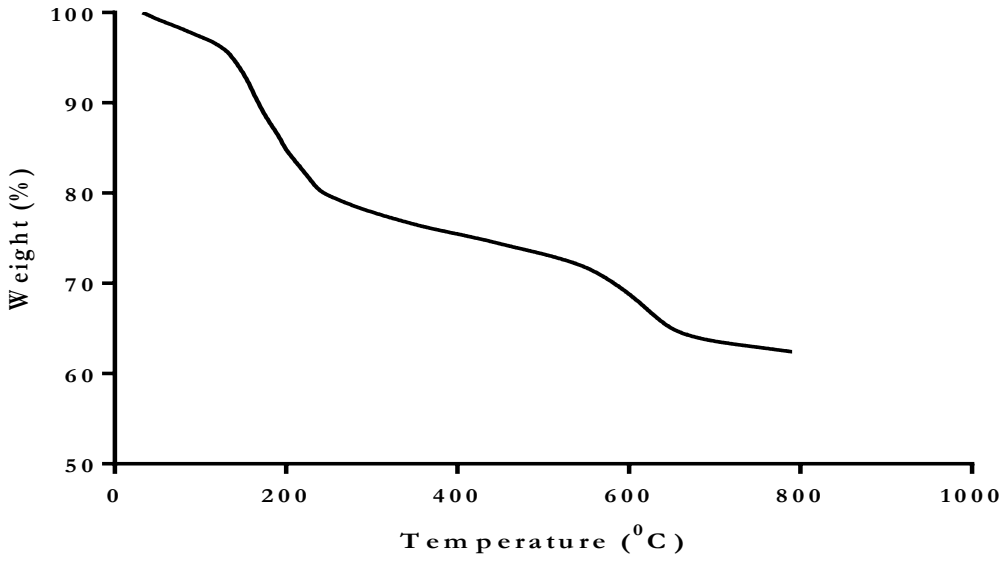

Figure 4: Thermogravimetric analysis on bioflocculant

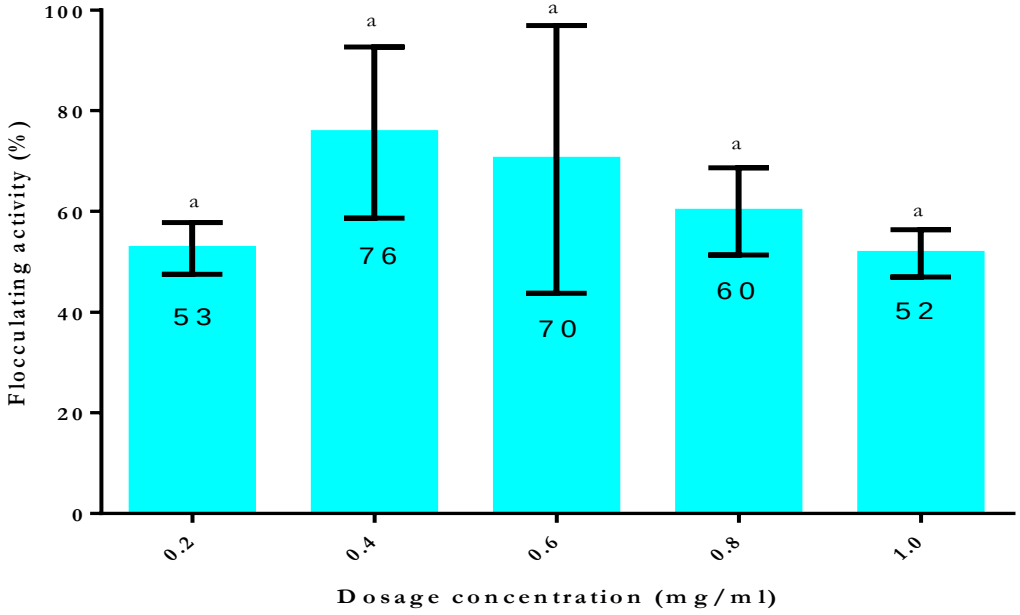

Figure 5: Effect of dosage concentration on flocculating activity

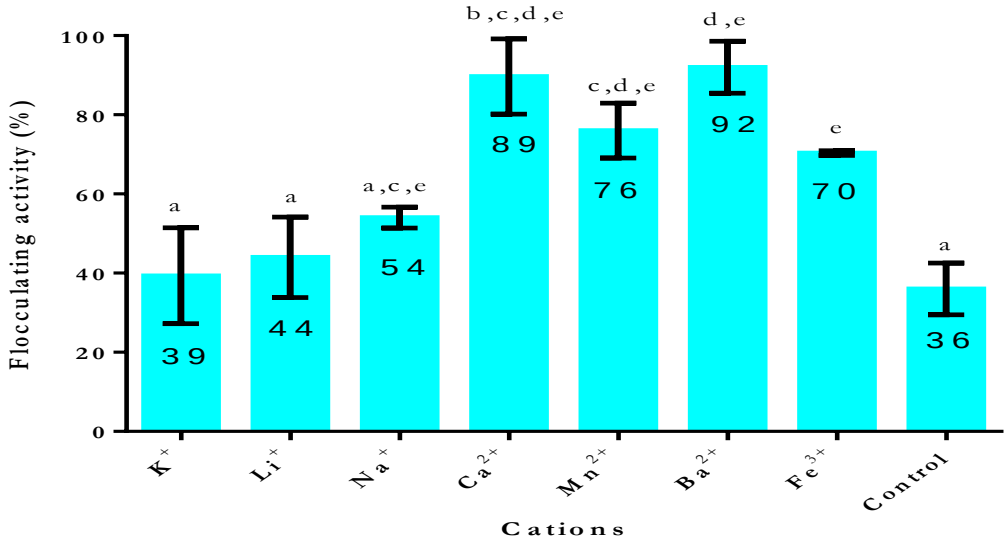

Figure 6: Effect of cations on flocculating activity

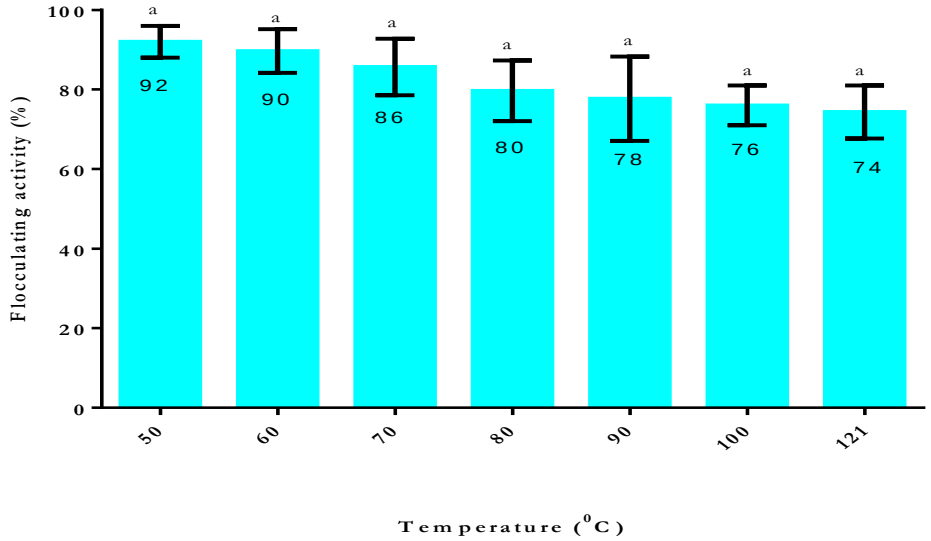

Figure 7: Effect of temperarute on flocculating activity of a bioflocculant

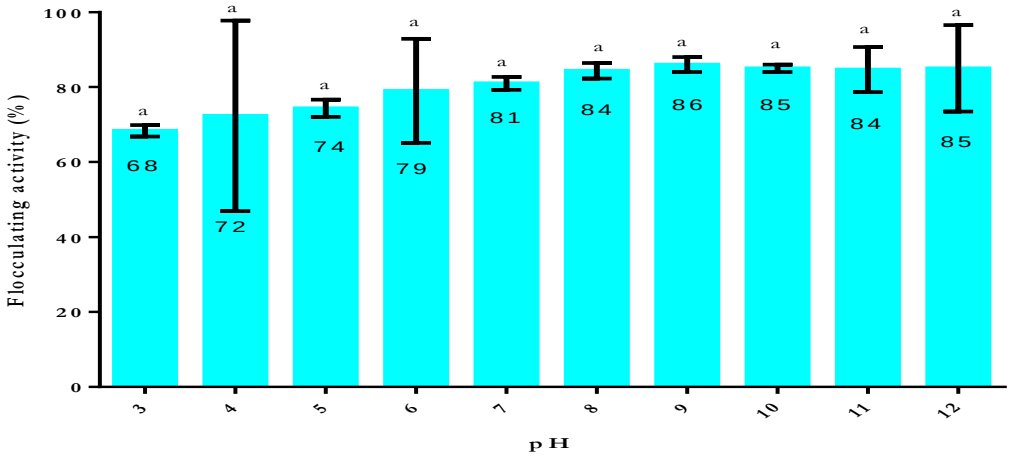

Figure 8: pH stability of a purified bioflocculant

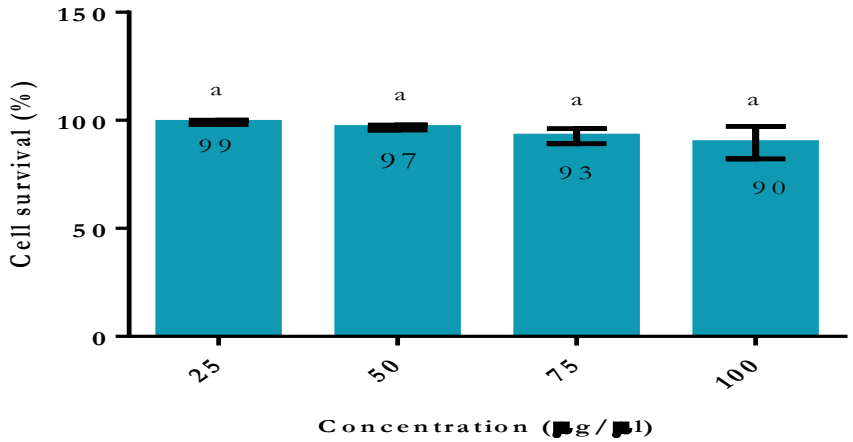

Figure 9: *In vitro* cytotoxicity of different concentrations of bioflocculant on HEK 293 cell lines.

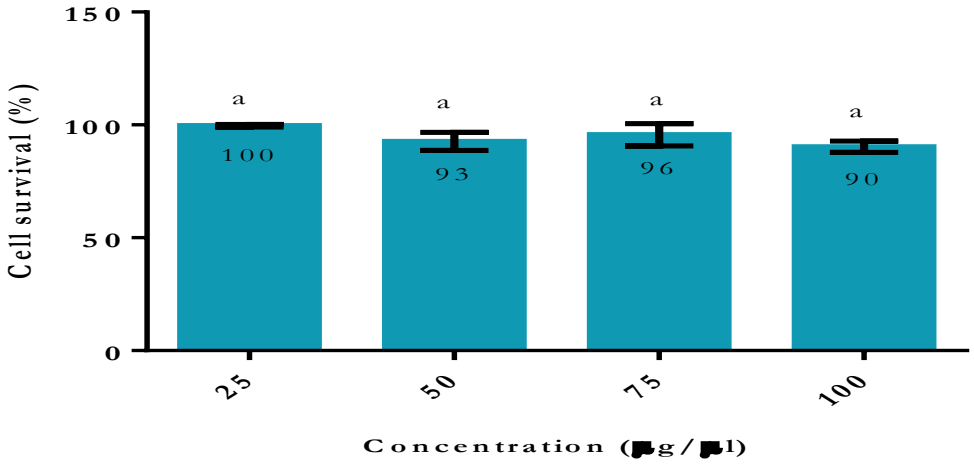

Figure 10: MFC7 Cell survival on various amounts of bioflocculant.

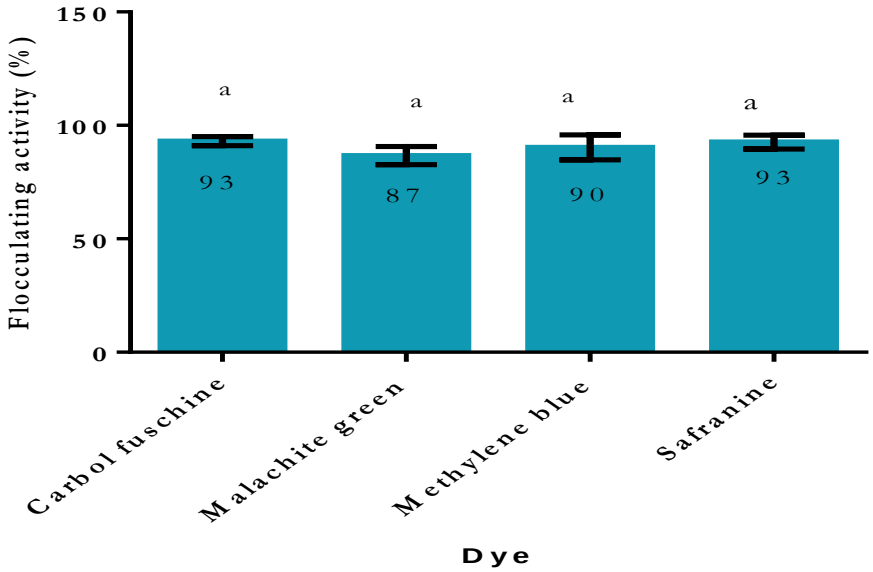

Figure 11: Dye removal by bioflocculant from different dye solutions.

Table 3: Pollutants removal from coal mine wastewater by bioflocculant in comparison with Alum and $FeCl_3$

Flocculants	Treatment	COD	BOD_5	S	Ca	Al	N	Flocculating activity at $OD_{680\ nm}$
Microbial	Before (mg/mL)	154	123.2	0.55	56	24	7.2	0.437
	After (mg/mL)	29	5.0	0.00	56	11.5	0.69	0.032
	Removal rate / Flocculation efficiency (%)	**81**	**96**	**100**	**0**	**52**	**90**	**93**
$FeCl_3$	Before (mg/mL)	154	123.2	0.55	56	24	7.2	0.437
	After mg/mL)	35	6.2	0.49	7	0.00	0.74	0.109
	Removal rate / Flocculation efficiency (%)	**77**	**95**	**11**	**88**	**100**	**90**	**75**
Alum	Before (mg/mL)	154	123.2	0.55	56	24	7.2	0.437
	After (mg/mL)	29	6.8	0.40	19	0.19	0.80	0.083
	Removal rate / Flocculation efficiency (%)	**81**	**95**	**27**	**56**	**99**	**89**	**81**

Note: Values are means of triplicates data.

In vitro cytotoxicity assay of the bioflocculant on HEK 293 and MFC7 cell lines

Although microbial flocculants have been documented as toxic-free polymeric substances, but for biosafety reasons, it is mandatory to investigate their toxicity effect prior to their applications (Pathak et al. 2015; Czemierska et al. 2017). According to Abdullah et al. (2017), not all bioflocculants are toxic-free; the source of bioflocculants decides their biosafety property. The fact is that, other microbial flocculants may impose toxic effects (Spellman, 2014). Bacterial by-products may have negative effects including causing epidemic diseases together with allergenic, cytotoxic and neurotoxic effects (Smith, 2009).Therefore, the cell viability for both HEK 293 and MFC7 cells were assessed using MTT cell proliferation assay after exposed to various concentrations of pure bioflocculant. The bioflocculant shows a margin of safety as no significant cytotoxicity effects observed in all tested cell lines (Figure 9 and 10). Higher percentage of cell survival has been shown on

both cells after MTT test; therefore, the toxicity threshold level of the product at mean lethal concentration was not investigated. The observations asserted the safe usage of the bioflocculant in various implementations. The bioflocculant produced by *Acinobacter haemolyticus* was also reported to be toxic-free on sheep blood cells and on the *in vivo* studies done on rats (no clinical manifestation was witnessed (Sharma et al. 2017). No clinical symptoms on the rats were also shown when treated with the bioflocculant from *Paenibacillus* sp. AM49 (Oh et al. 2001). Also no toxic effect on the clinical signs, mortality, body weight changes or necropsy was reported caused by bioflocculant MBF-5 from LZ5 strain on mice (test organism) (Zhao et al. 2013).

Application of a bioflocculant

Decolourization of different solutions

Chronic and acute toxicity as well as coloured water bodies are as a result of disposing of unprocessed pigmented effluvium to the surroundings. Unprocessed effluvium can reduce the re-oxygenation capacity of the receiving water. This could also block the sunlight resulting in the distraction of photosynthetic processes in the aquatic environment. Therefore, it is mandatory to treat dyes effluents in wastewater before discarding them into water bodies or re-use them (Tim et al. 2001; Elkady et al. 2017). In this study, the effect of bioflocculant on staining dyes removal was investigated and the findings are depicted in Figure 11. The bioflocculant from *Bacillus* sp. seems to be effective in the reduction of stains in wastewater from different industrial processes using or producing dye, such as garments factories. This is witnessed by the good and high affinity showed by the bioflocculant for the entire treated dyes with more than 80% removal proficiency. According to Salehizadeh and Shojaosadati (2001), microbial flocculant's ability to reduce different dyes in solutions feasibly through forcing aggregation of cells and particles owing to bridging and charge neutralization mechanisms.

The bioflocculant solution of 0.4 mg/mL was optimal to the removal of all dyes treated effectively with activities from 87 to 97% when barium chloride salt (1% w/v) was used to stimulate the flocculation process at neutral pH. Number of authors has reported the effective usage of microbial flocculants in the removal of dyes from different wastewater. For example, Bisht and Lal (2019) reported the bioflocculant BF-VB2 to effectively reduce the dye colour with 82.78%. Buthelezi et al. (2012) reported about the bio flocculants from indigenous bacteria to be very effective in the decolourization of various dyes treated, with a removal efficiency of 97.04%.

Pollutants removal from coal mine wastewater

The bioflocculant produced by *Bacillus* sp. was used to flocculate various suspended particles in coal mine wastewater collected from Tendele coal mine located at KwaSomkhele area (Mtubatuba, KZN, RSA) and the results of the tests conducted are depicted in Table 3. The bioflocculant was compared with two traditional flocculants (Alum and iron(III) chloride). Both bioflocculant and traditional flocculants showed comparable COD reduction efficiency above 75% on Tendele mine wash water with both Alum and bioflocculant showed the highest efficiency of 81%. For BOD removal efficiency, all flocculants tested showed good removal potential with 95% removal rate showed by the chemical flocculants and bioflocculant have a removal rate of 96%. The bioflocculant seems to flocculated wastewater better than both traditional flocculant with 93% flocculating activity and both conventional flocculants resulted in 75% (iron chloride) and 81% (Alum) flocculating activities. The total nitrogen content removal by all flocculants revealed more than 88% removal rate with bioflocculant leading the race (90%). Very good removal rate of sulphur (100%) was obtained with the bioflocculant compared to poor removal rate by both chemical flocculants (below 30%). Chemical flocculants had a better removing potential for both calcium and aluminium when compared to microbial flocculants with more than 56 % and 45% differences, respectively. Zhang et al. (2015) and Ngema et al. (2020) reported the similar results whereby the bioflocculants were able to remove different pollutants from various wastewaters. Pham and Bui (2020) reported the effective removal of ammonium, phosphate, nitrate, COD, BOD_5, and nitrogen from the fertilizer plant wastewater by *Scenedesmus* sp. This algal species was able to remove ammonium, nitrate, phosphate, total phosphorus, COD, and BOD_5 with removal rates up to 93%, 84%, 97%, 96%, 93%, and 84%, respectively.

CONCLUSION

The glycoprotein bioflocculant from *Bacillus* sp. revealed an excellent flocculating characteristic with 0.4 mg/mL dosage in the presence of Ba^{2+} cation. The non-toxic

bioflocculant with 90% cell survival rate showed in both HEK 293 and MFC 7 cell lines at the highest concentration tested (100 µg/µL) had some great flocculation characteristics with functional groups including amino, carboxylic, and hydroxyl groups playing a major role in the flocculation process. The bioflocculant exhibited no antimicrobial activity against different microorganisms (both Gram-negative and Gram-positive) tested and showed the best flocculation character in the removal of different pollutants from Tendele coal mine wash water. The pH stable microbial flocculant was also able to remove various dyes from different dye media. More than 80% of COD and BOD_5 removal efficiency with flocculating activity of 93% were observed and some remarkable characters showed in the removal of some tested chemicals from coal mine wash water such as total nitrogen, sulphur and aluminium. This water soluble *Bacillus* sp. by-product showed great potential in the removal of dyes from solutions with a minimum of 87% removal rate for all treated dye solutions. The removal efficiencies showed by the bioflocculant implied that it has potential in industrial applicability.

CONFLICT OF INTEREST

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

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AUTHOR CONTRIBUTIONS

Conceptualization: BAK, ME, and PVSR; formal analysis: NZG and AKB; investigation: NZG; supervision: BAK, ME and PVSR; writing (original draft): NZG; writing (review and editing): all authors.

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