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Isolation, identification and characterization of a bioflocculant producing strain, *Bacillus spp*. KC782848.1, from umlalazi catchment, Mtunzini, kwaZulu-Natal.

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A biofloculant- producing bacterium, *Bacillus* spp. KC782848.1, was isolated from the fresh water sample of uMlalazi catchment, Mtunzini area, in the Province of KwaZulu-Natal, Republic of South Africa and identified by 16S rRNA sequencing analysis using universal primers. Medium conditions on the microbial flocculant production reached an optimum as follows: 5% (v/v) inoculum size; glucose as sole carbon source; complex nitrogen source (urea + yeast extract + $(NH_4)_2SO_4$); Ca^{2+} as mediating factor; shaking speed of 160 rpm; temperature of 30 °C and initial pH 4. After 60 hrs of fermentation a bioflocculant yield of 1.522 g/l was recovered. The optimum dose of the purified bioflocculant to clarify 0.4% kaolin clay suspension at neutral pH was 0.4 mg/ml. The bioflocculant seems to be thermostable and more effective over a wide pH range (3–12). Chemical analysis of the purified bioflocculant revealed it to have an amorphous structure (observed with Scanning electron microscope) and composed of carbohydrate (79%), protein (08%) and uronic acid (06%). Fourier transform infrared (FTIR) analysis revealed the presence of amine, hydroxyl and carbonyl group as their main functional groups, N: C: O: elements accounted for about 1.0, 31.4, and 48.9 (% w/t), respectively and whilst the thermogravimetric analysis showed degradation temperature (*Td*) of 150 °C. The produced bioflocculant had good flocculating activity and is thermal stable which renders it suitable for implementation in industries.

Keywords: ***Bacillus*** **spp., flocculating activity, bioflocculant, kaolin clay.**

INTRODUCTION

The Republic of South Africa is one of the water scarce country (Okaiyeto et al., 2016a). Increasing water pollution due to an alarming growth rate of urbanisation and industrialisation poses a serious concern to available water resources and to water quality (Sakar et al., 2006). Major sources of water pollution are untreated, toxic, domestic and industrial effluents and also agricultural by-products. Pollutants in wastewater are often detrimental to aquatic life, rendering it unfit for use (Cosa and Okoh, 2013).

Physicochemical and biological methods have been implemented to treat wastewater and fresh water. Flocculation is among the techniques used in wastewater and fresh water treatment (Xiong et al., 2010). Flocculants are typically effective in aggregating colloids and have been extensively utilized in industrial fields. They are divided into chemical (organic synthetic and inorganic) and biological flocculants (Salehizadeh and Shojaosadati, 2001). Chemical flocculants have been shown to be cost effective but environmentally unfriendly and to impose health threats to humans. They are reported to have carcinogenic and neurotoxic effects (Lee et al., 2014). As a result of these short-comings, natural occurring flocculants such as bioflocculants are viewed as potentially effective alternatives (Okaiyeto et al., 2016a).

Bioflocculants are polymers produced by microorganisms in the course of their growth (Piyo et al., 2011). They are derived from the natural exudations of bacteria and their cell lysis. They are extracellular biopolymers of macromolecular materials such as proteins, glycoproteins, polysaccharides and nucleic acids (Czaczyk and Myszka, 2007). Cosa et al., (2013) stated that the useful functional groups of flocculation in proteinaceous bioflocculants comprise of amino and carboxyl groups, whereas polysaccharide bioflocculants are generally centred on high molecular weights. Thus, their flocculating activities are reliant on their characteristics. Bioflocculants have lately attracted a significant biotechnological attention due to their effectiveness, biodegradability, non-toxicity, benign nature and absence of secondary pollution (Okaiyeto et al., 2016b).

Microbial flocculants applications is hindered at an industrial level due to low production yields, low flocculating capabilities, high production costs, large dosage necessities and lack of knowledge of their characteristics (Cosa et al., 2013). Due to the afore-mentioned challenges, there is a constant increase in scientific research for new and efficient bioflocculants from microorganisms from diverse environments.

This study was focused mainly on the screening for bacterial strain with high flocculation capability from marine sediments from uMlalazi catchment, Mtunzini in KwaZulu-Natal, Republic of South Africa. The study also sought to find low cost substrate, optimal cultivation conditions for the optimal bioflocculant production and characterization of a produced bioflocculant.

MATERIALS AND METHODS

Source of bacteria

The bacteria were isolated from water and sediment samples of uMlalazi catchment, Mtunzini in the Province of KwaZulu-Natal in RSA. Serial dilutions were made using sterile saline solution. Approximately 100 µl of the diluted solution 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 half-a-minute. From these 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 activation medium containing 3 g of beef extract, 10 g of tryptone and 5 g of NaCl was prepared in 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 1L of filtered marine water. 1N NaOH or 1N 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 single colony of bacteria. Inoculated media were placed in the shaking incubator at a speed of 160 rpm and temperature of 30 ^{o}C for 72 hrs. After incubation period, the broth culture was centrifuged for 30 min. at 8, 000 x g, 4 ^{o}C to remove bacterial cells and the supernatant was used for measuring a 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 milliliters of broth culture supernatant and 3 ml of 1% $CaCl_2$ (w/v) were mixed with the 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. 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 (1cm beneath the fluid level) was measured using spectrophotometer. The flocculating rate was calculated as;

Flocculating rate (%) = (A-B)/A x 100

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

Optimization of culture conditions for bioflocculant production

Effect of inoculum size on bioflocculant production

To investigate the influence of inoculum size on the production of bioflocculant, the method described by Didar and Ferdosi – Makan (2016) was used. The experiment was conducted using different inoculum size ranging from 1% to 5% (v/v) to inoculate 50 ml of the production medium. All conical flasks with inoculated production media were incubated at 30 ^{o}C for 72 hrs at 160 rpm. The flocculating activity was measured as described previously.

The effect of carbon sources on bioflocculant production

Carbon and nitrogen sources play a vital role in the production of bioflocculant by bacteria (Xia et al., 2008). A method of Ugbenyen and Okoh (2014) was used to assess the effect of carbon and nitrogen sources for the bioflocculant production. The growth media in separated flasks each was supplemented with different carbon sources, including glucose (20 g/l), sucrose (20 g/l), maltose (20 g/l), lactose (20 g/l), fructose 20 g/l) xylose (20 g/l), starch (20 g/l) were inoculated with bacterial strain. Flasks were incubated in a shaking incubator (160 rpm) at 30 ^{o}C for 72 hrs and the flocculating activity was calculated as described previously.

The effect of nitrogen sources on bioflocculant production

To assess the effect of nitrogen sources on the production of bioflocculant, the amount of 1.2 g/l of various nitrogen sources was added into growth medium in separated flasks. Organic nitrogen sources such as yeast extract, urea, peptone and casein: inorganic nitrogen sources including ammonium molybdate, ammonium sulphate and ammonium nitrate and also a mixed nitrogen source made up of yeast extract, urea and ammonium sulphate. Flocculating rate was determined as described previously.

The effect of various metal ions on bioflocculant production

To determine the suitable metal ion on flocculating activity for bioflocculant production, 1% (w/v) solutions of KCl, NaCl, LiCl, $BaCl_2$, $MnCl_2$ and $FeCl_3$ were prepared, and the flocculating rate was assessed as described above by replacing $CaCl_2$ with different metal ions mixtures (Yim et al., 2007).

The effect of initial pH on bioflocculant production

To determine the effect of initial pH on flocculating activity, pH of a production medium was adjusted using 1N NaOH and 1N HCl prior to sterilization and inoculation. The pH values that were used are 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The production medium was then incubated at 30 ^{o}C in a shaking incubator (160 rpm) for 72 hrs and flocculating activity was measured as described previously for each pH (Zaki *et al.*, 2011).

The effect of temperature on bioflocculant production

A method of Adebayo-Tayo and Adebayo G (2017) was used for the assessment of cultivation temperature effect on flocculating activity. A test bacterium was inoculated in the cultivation media and incubated at various temperatures of 20, 25, 30, 35, 40, 45, 50, 55 and 60 ^{o}C. Flocculating activity was determined as previously described.

The effect of shaking speed on bioflocculant production

The assessment of shaking speed effect on bioflocculant production was conducted using the method of Ogunsade et al., (2015). Different agitating speeds were used ranging from 0 to 220 rpm. Various flasks of production media inoculated with bacterium strain were incubated in a shaking incubators at different speeds at the

temperature of 30 °C for the period of 72 hrs, and flocculating activity was measured as previously described.

Time course assay of bioflocculant production

The fermentation time experiment was conducted in accordance with the method of Gao *et al*. (2006) with minor modifications. The bacterial strain was grown under optimal conditions obtained from previous tests. To prepare the cultivation medium, the following ingredients were mixed with 1000 ml of filtered marine water: glucose (20 g), urea (0.5 g), yeast extract (0.5 g), $(NH4)_2SO_4$ (0.2 g), KH_2PO_4 (2 g), K_2HPO_4 (5 g), $MgSO_4$-$7H_2O$ (0.2 g) and NaCl (0.1 g). To prepare the inoculum, the bacterial strain was cultured in 50 ml production medium and incubated at 30 °C in a shaking incubator (165 rpm) overnight. After incubation, the optical density ($OD_{660\ nm}$) of the culture medium was adjusted to 0.1 using 0.85% sterile saline solution and used as a seed culture. Seed culture (5% v/v) was used to inoculate 50 ml of production medium in 100 ml Erlenmeyer flasks. Culture samples were withdrawn at 12 hrs intervals for 120 hrs and monitored for growth ($OD_{550\ nm}$), pH and flocculating activity. From each withdrawal, 2 ml of culture broth was centrifuged at 4,000 x g at 4 °C for 30 min and the cell-free supernatant was used to measure flocculating rate of the produced bioflocculant, as described previously. The optical density of the fermented culture was determined with spectrophotometer ($OD_{550\ nm}$) to monitor the bacterial growth.

Extraction and purification of the bioflocculant

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 °C, the culture broth was centrifuged at 8,000 x g at 4 °C for 15 min; this was done to eliminate bacterial cells. One volume of distilled water was added to the supernatant and then centrifuged at 8,000 x g for 15 min at 4 °C to remove insoluble substances. Two volumes of ethanol were added to the supernatant; the solution was agitated and then left at 4 °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). 1 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 °C and vacuum-dried to yield a purified bioflocculant.

Chemical analysis of a purified bioflocculant

The measurement of polysaccharide content was done using the method of Dubois *et al*. (1956) with glucose used as a standard. The total protein content was determined using the Bradford method with bovine serum albumin (BSA) as a standard (Bradford *et al*., 1976). The presence of uronic acid and its content was measured by the carbazole method (Cesaretti *et al*., 2003).

Scanning electron microscopy 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 Spectrophotometer (FTIR) analyser. The dried bioflocculant powder was grounded with potassium bromide (KBr) and pressed into pellets for FTIR spectral measurement in the wavelength range of 4000-400 cm^{-1} (Karthiga and Natarajan, 2015).

Thermo-gravimetric analysis of the bioflocculant

The pyrolysis analysis of the purified bioflocculant was assessed, in the range of 30 °C - 800 °C at a constant rate 10 °C min^{-1}, under constant flow of nitrogen gas (20.0 ml/min) using thermo-gravimetric 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 mg/ml) were prepared by diluting the bioflocculant with distilled water (w/v) to achieve the respective concentrations; and these were 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 millilitre aliquots of the solution was transferred into Eppendorf tubes and heated for 30 min at various temperatures ranging from 50 °C to 100 °C and at 121 °C (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 N HCl or 1 N 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 (Gao et al., 2006).

Effect of cations on flocculating activity

A variety of salt 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 cations) was also prepared. The same method previously used, for measuring flocculating activity was employed (Tawila et al., 2018).

Statistical analyses

All experiments were conducted in triplicates with mean and standard deviation values determined, where differences are considered significant at 0.05 confidence level ($p < 0.05$), by the use of graphpad Prism

RESULTS AND DISCUSSION

Isolation of bioflocculant-producing microorganisms

More than 15 isolates were obtained from fresh water of uMlalazi catchment, Mtunzini in the Province of KwaZulu-Natal, RSA and screened for bioflocculant-production potential. Isolate selected for the study with the initial screening revealed capable of flocculating kaolin clay suspension (0.4 % w/v) at neutral pH. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene analysis of the bacterium produced was conducted. A comparative analysis of the 16S rRNA sequence of the isolate in the GenBank database showed 98% similarity with Bacillus spp. and its repository accession number was KC782848.1. It was then named a Bacillus spp. The Genus *Bacillus* is commonly pervasive in nature including a number of species known to produce extracellular polymeric substances that is very useful industrially, including *B. pumilus* (Makapela et al., 2016), *B. lichermiformis* X14 (Li et al., 2009), *B. clausii* NB2 (Adebayo-Tayo and Adebami, 2014) and *B. velezensis* 40B (Zaki et al., 2013). Handtke et al., (2014) reported that some *Bacillus* species including *B. subtillis*, *B. pumilus* and *B. lichermiformis* are highly resistant to oxidative stress. Since oxidative stress can occur in almost all phases of microbial growth or fermentation processes, these natural features of *Bacillus* species could be a huge boost for the development in any production processes at industrial level (Schweder and Hecker, 2004). An observation of high flocculating rate with the microbial flocculant produced by this strain of *Bacillus* is an added feature over the previously reported strains.

Optimization of culture conditions for bioflocculant production

The effect of inoculum size on bioflocculant production

The inoculum size is one of the most important factors that influence the production of bioflocculants by bacteria. A small inoculum size delays the lag phase, whereas, a large inoculum size impedes the excessive bacterial growth, thereby inhibiting the production of bioflocculant (Li et al., 2009). The effect of inoculum size on bioflocculant production was determined using different sizes ranging from 1% to 5%. As depicted in Figure 1, small inoculum sizes (1-2%) delayed the growth of bacterium and produced less than 50% flocculating activity. As the inoculum size increases the flocculating activity also increases until it reached its highest flocculating activity (67%) with 5% inoculum size. Similarly, a 5% inoculum size was greatly improved the bioflocculant production by a marine *Bacillus* species (Okaiyeto et al., 2016b). Contrary to this study, Agunbiade et al., (2018) reported 1% to be more effective in the production of

bioflocculant by *Streptomyces platensis* species isolated from Sterkfontein Dam in Harrismith.

The effect of carbon sources on bioflocculant production

Carbon sources are reported to have an essential effect in the growth of extracellular polymeric products (Willey et al., 2009). As a result, several carbohydrates were assessed and the results are depicted in Figure 2. Results show that glucose was the most favoured carbon source with flocculating activity of 82% followed by starch (66%), maltose (61%) and lactose (60%).

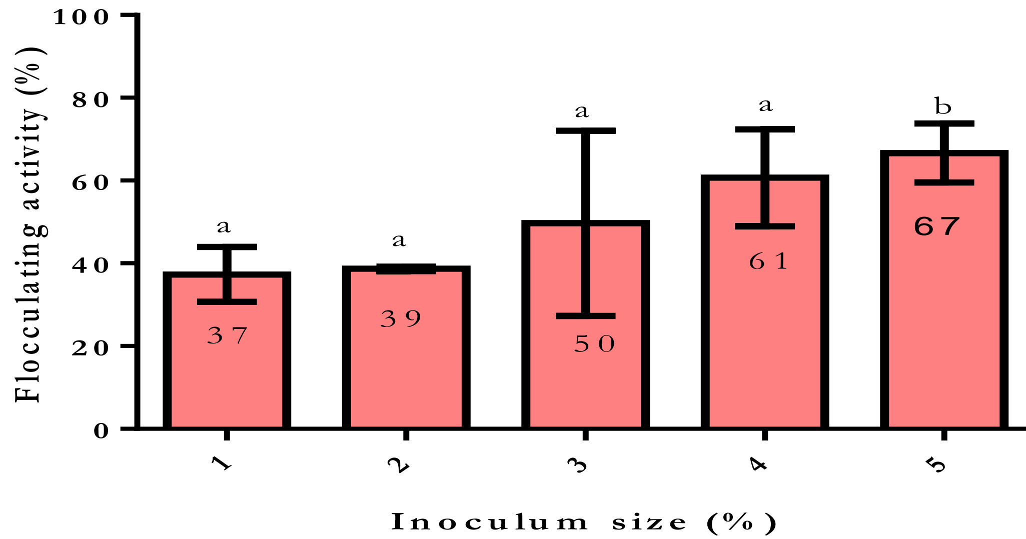

Figure 1: Effect of inoculum size on bioflocculant production

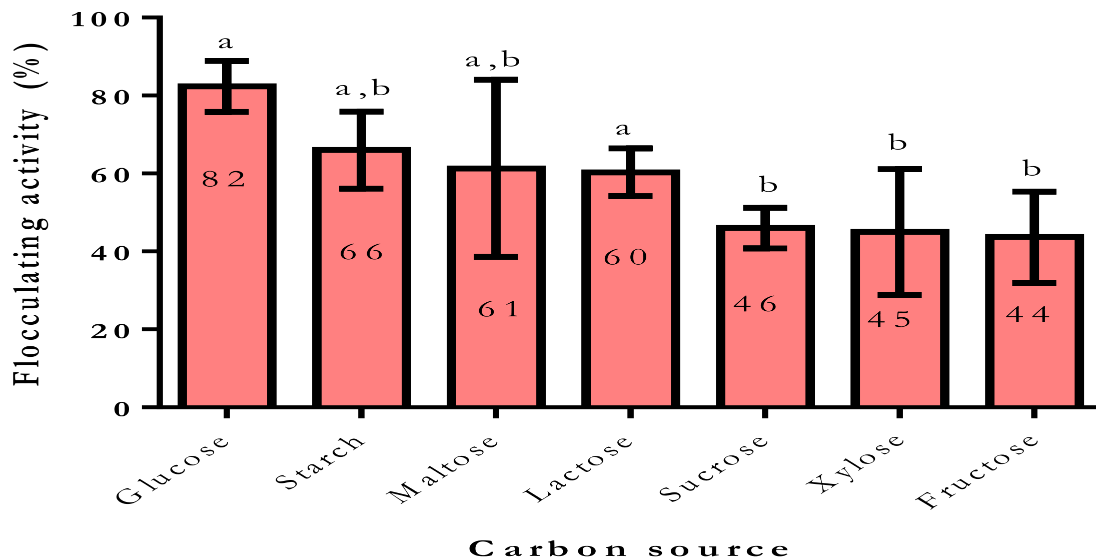

Figure 2: Effect of carbon sources on bioflocculant production

Sucrose, xylose and fructose were the least preferred carbon sources with flocculating activity below 50%. Therefore, glucose was used as a carbon source of choice for all subsequent tests. Literature studies showed that glucose is the most preferred carbon source for bioflocculant production (Chen et al., 2017). For example, Mabinya et al., (2012) reported glucose as a preferred carbon source for bioflocculant production by *Arthrobacacter* sp. Raats, a freshwater bacterium isolated from Tyume river, South Africa and *Aspergillus niger* produced a bioflocculant in the presence of glucose as sole carbon source (Pu et al., 2018). Contrary, Zheng et al., (2008) and Li et al., (2007) reported sucrose to be more effective for bioflocculant production by *Bacillus* sp. F19 and *Aeromonas* sp., respectively. Organic carbon sources have been reported to be the most preferable by microorganisms for optimum bioflocculant production compared to inorganic carbon sources (Cosa *et al*., 2011).

The effect of nitrogen sources on bioflocculant production

Studies show that nitrogen sources also enhance the production of bioflocculant (Ugbenyen et al., 2012). Different nitrogen sources (organic, inorganic and nitrogen complex) have been assessed for their effect on bioflocculant production by *Bacillus* species and the results are shown in Figure 3. From the figure, it was clear that multiple nitrogen sources made up of urea, yeast and ammonium sulphate greatly improved the biofloccucant production compared to single nitrogen sources. Ammonium sulphate and casein as a sole nitrogen sources also enhanced the bioflooculant production (above 70%) when compared with other individual nitrogen sources including peptone (56%), ammonium nitrate, yeast extract, urea and ammonium molybdate (all below 50 %). In line with this study finding, Makapela et al., (2016) reported complex nitrogen source composed of yeast extract, urea and ammonium to greatly improve the bioflocculant production by *Bacillus* pumilus strain. Lixi et al., (2006) also reported mixed nitrogen sources (yeast, ammonium sulphate and urea) as the most suitable source for the optimal bioflocculant production by *Klebsiella* sp. MYC. A multiple nitrogen source consists of beef extract and urea was reported to have high flocculating activity than an individual organic or inorganic nitrogen source (Gong *et al*., 2008). Reports show that organic nitrogen sources are more favourable for microbial flocculant production and have advantage of being more easily absorbed by the cells as opposed to inorganic nitrogen sources (Wang et al., 2011)

The effect of metal ions on bioflocculant production

The main role of metal ions in bioflocculant production is to promote or block the production process (Liu et al., 2010; Ugbenyen *et al*., 2012). Metal ions play a stimulatory mechanism in bioflocculant production thereby neutralizing and stabilizing the residual charge of functional groups on the bioflocculant, and the addition of metal ions resulting in the increase in ionic strength of the suspended particles in a solution thereby reducing Coulomb forces of the suspended solids (Wang et al., 2011). Figure 4 shows bioflocculant production which was stimulated by all divalent metal ions (Ba^{2+}, Mn^{2+} and Ca^{2+}) and Ca^{2+} showed an optimal flocculating activity of 79%. Trivalent ion (Fe^{3+}) used also enhanced the bioflocculant production with flocculating activity above 50%. All monovalent used, with the exception of Li^{+} (54%) showed weak stimulation rate of flocculating activity (below 50%). The bioflocculant produced by *Bacillus* spp. seems to be cation- dependent as less than 30% flocculating activity was observed in the absence of metal ions. Similarly, number of studies in the literature is in line with our findings. For example, Ugbenyen and Okoh (2014) reported the divalent metal ions tested (Ca^{2+}, Mg^{2+}, and Mn^{2+}) to enhance the flocculating activity of the bioflocculant produced by the consortium of *Cobetia* sp. and *Bacillus* sp. A report by Li et al., (2014) revealed *Athrobacter* sp. B4 produced a biflocculant that was Ca^{2+}-dependent and other metal ions used did affect flocculating activity. In contrast to our findings, *Chryseobacterium daeguense* produced a cation-independent bioflocculant MBF-W6, and no notable improvement of flocculation rate was observed from tested metal ions (Liu et al., 2010).

The effect of initial pH on bioflocculant production

The initial pH of the production medium has an effect on the electric charge and the oxidation - reduction potential of the cells and also affects nutrient absorption and enzymatic reaction within the cells (Agunbiade et al., 2018). Hence, the effect of initial pH of the production medium on bioflocculant production was investigated at pH values ranging from 3-12 and results are depicted in Figure 5. As it can be seen from the figure,

acidic pH range 3-6 seems to support the growth and flocculating activity, with optimum flocculating activity of 75% being observed at pH 4 compared to weak flocculating activity at basic pH ranging from pH 9-10. More basic production medium (pH 11-12) completely inhibited the bioflocculant production. At pH 7 and 8, slightly decrease in flocculating activity was observed. However, the pH requirement for microbial flocculant production varies with different bacterial strains, operational conditions as well as medium composition (Ugbenyen *et al*., 2014). Similarly, the bioflocculant p-KG03 produced by the sea dinoflagellate, *Gyrodinium impudicum* KG03 greatly improved the flocculation rate under acidic conditions of pH 4 (Yim *et al*., 2007). Piyo *et al.* (2011) reported the test bacterium (*Bacillus* sp. Gilbert) to produce a bioflocculant optimally at acidic pH 3. Contrary to the study, *Streptomyces platensis* produced a bioflocculant optimally at neutral pH 7 (Agunbiade *et al*., 2018) and Giri *et* al. (2015)

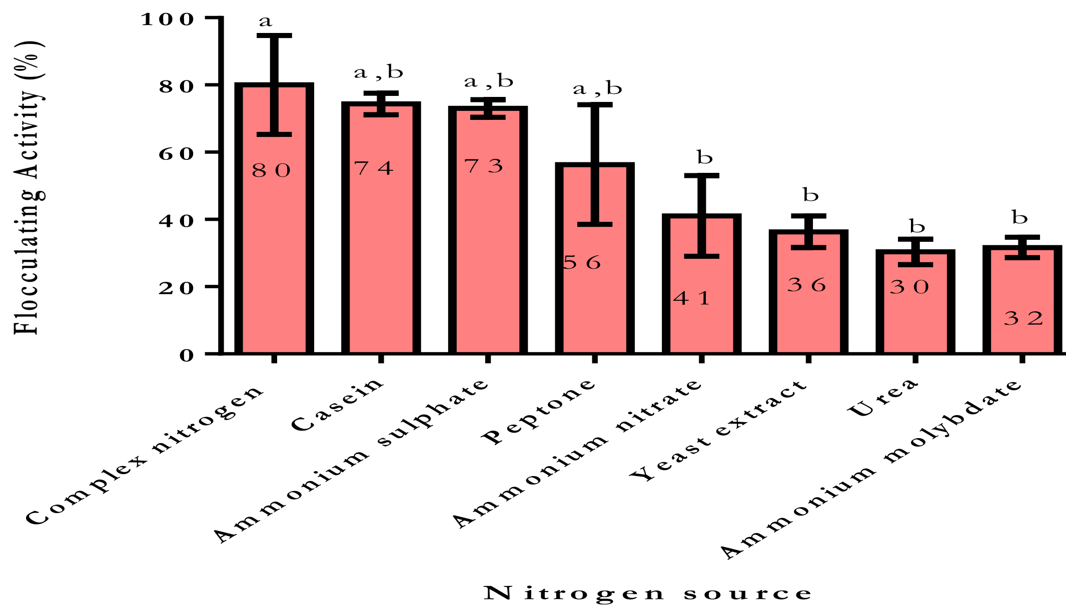

Figure 3: Effect of nitrogen sources on bioflocculant production

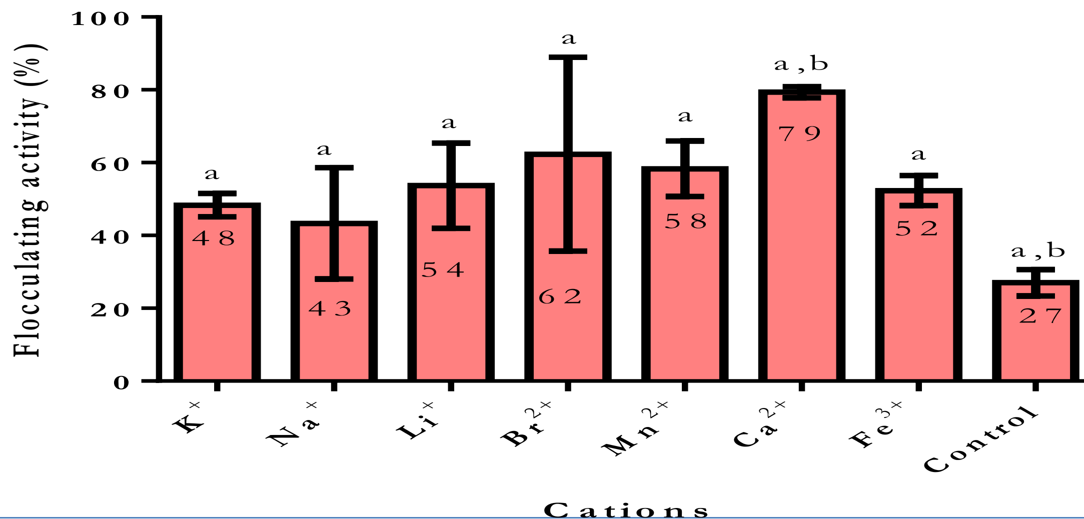

Figure 4: Effect of metal ions on flocculating activity

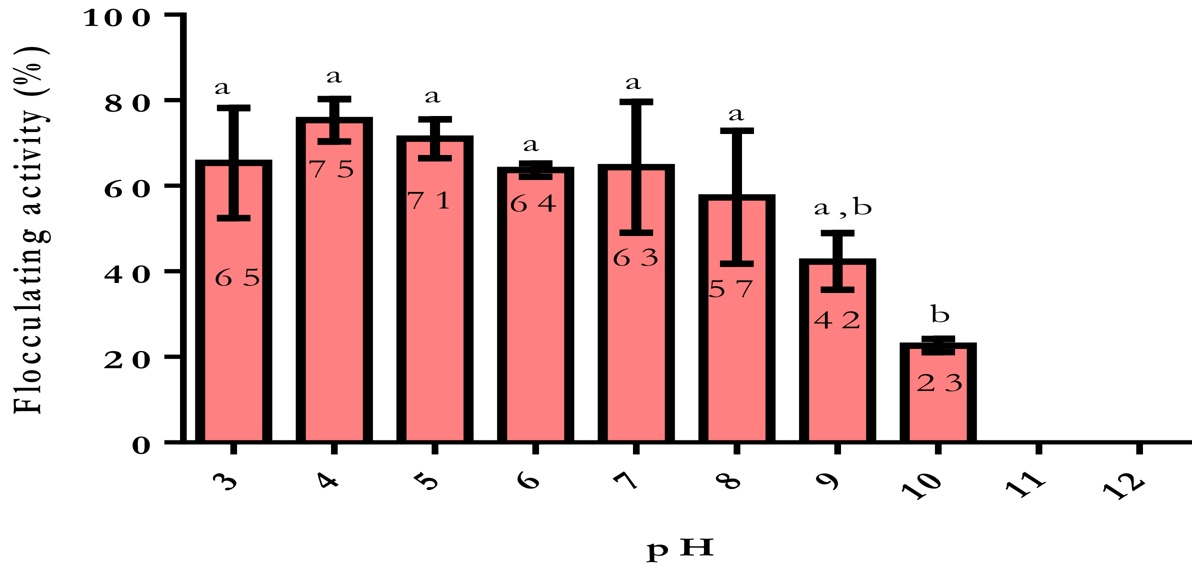

Figure 5: Effect of initial pH of the production medium on bioflocculant production

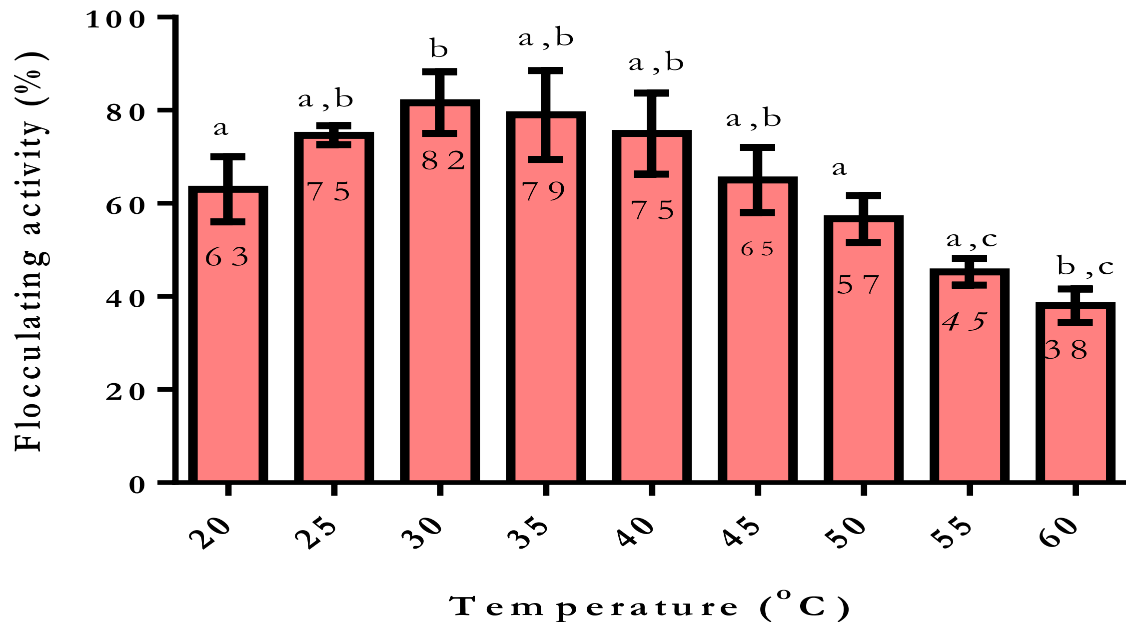

Figure 6: Effect of cultivation temperature on bioflocculant production

also reported neutral pH 7 as an optimum for the production of bioflocculant from *Bacillus subtilis* F9. Basic pH 12 was reported to have highest (optimum) flocculating activity for the production of bioflocculant by *Arthrobacter* sp. B4 (Li et al., 2014).

The effect of temperature on bioflocculant production

Cultivation temperature plays a vital role in metabolic reactions to occur for both bacterial growth and microbial flocculant production (Okaiyeto et al., 2016b). The increase in temperature results in the increase of growth, microbial flocculant production and metabolic function until it starts the denaturation processes (Madigan and Matinko, 2006). The degree of bacterial growth and bioflocculant production decreases at lower temperatures (More *et al.*, 2014). From the literature survey, for production of bioflocculant the optimal temperature range is between 25 °C and 37 °C (Salehizadeh and Shojaosadati, 2001; Luo et al., 2014). In this study, the optimum flocculating activity of 82% was observed at the cultivation temperature of 30 °C (Figure 6). Below or above the temperature of 30 °C there is a decrease in flocculating activity. *Bacillus amyloliquefaciens* ABL 19 isolated from Adeti Stream in Nigeria produced an optimum bioflocculant at 25 °C (Ogunsade *et al.*, 2015). A thermostable polysaccharide bioflocculant was produced at 30 °C by *Virgibacillus* species isolated from Algoa Bay (Cosa et al., 2013), whereas, Muthulakshmi and Nellaiah (2013) reported a novel bioflocculant produced at 37 °C by *Klebsiella* sp.

The effect of shaking speed on bioflocculant production

The main role of shaking/ agitation is to distribute nutrients, bacterial cells and heat evenly throughout the solution (Smith, 2009). The shaking speed of 165 rpm was the most productive speed resulted in the highest flocculating activity of 78% (Figure 7). The shaking speed below/ above 165 rpm led to the decrease in flocculating activity. The results were in the close range with those reported by Cosa and Okoh (2014), whereby the highest flocculating activity was obtained at 160 rpm. These similarities may be due to the same degree of oxygen demand posed by microorganisms at various growth phases (Maliehe et al., 2016).

Fermentation time of bioflocculant production

Figure 8 shows the fermentation time of bioflocculant production by *Bacillus* spp. in relation to optical density (OD_{660}) representing cell growth, pH and flocculating activity over a period of 5 days. During fermentation process, the flocculating activity increased in parallel with

microbial growth and reaching a maximum flocculating activity of 80% in 60 hrs of fermentation. After 60 hrs of incubation, a slightly decreased in flocculating activity over the remaining period of fermentation was noted. This decrease in flocculating activity might be due to the activity of exoenzyme, which deflocculates its own flocs, in the stationery phase of the bacterial growth (Mabinya *et al*., 2012). Thus this increase in both cell growth and flocculating activity implies that the bioflocculant was produced through biosynthesis during microbial growth as opposed to cell autolysis (Maliehe et al., 2016). Okaiyeto et al., (2015) reported similar results, whereby the microbial flocculant was produced during the lag phase of the bacterial growth. In comparison, it was reported that the bioflocculant produced by *B. cereus* showed flocculating activity of over 90% after 72 hrs, late lag phase and stationery phase of growth (Arafa et al., 2014). *Bacillus* spp KC782848.1 produced the bioflocculant which can be viewed as cost-effective since it resulted in a maximum flocculating activity (93.3%) within a short cultivation time, that is most preferable at industrial level (Scheper et al., 2003). The initial pH of the production medium dropped from 5.19 to 2.54 after 120 hrs of incubation. This was ascribed to the secreted acidic constituents of the produced bioflocculant (Okaiyeto et al., 2013).

Extraction and purification of a bioflocculant

After extraction and purification, 1.522 g of purified bioflocculant was recovered from 1L of fermentation broth. The recovered bioflocculant was white in colour and water soluble. The yield of this bioflocculant is compared better with previously obtained yields, including that reported by Ntsangani (2016) and Makapela (2015), who produced 0.78 g/l bioflocculant from *Bacillus sp.* and 0.289 g/l bioflocculant produced from *Bacillus pumilus*, respectively.

Chemical analysis of a purified bioflocculant

The chemical composition of the bioflocculant is an important factor influencing its flocculating activity and also determines its flocculation mechanisms (Nie et al., 2011). This helps in enhancing its rate of practical applicability thereby optimizing its flocculation parameters. Most bioflocculants are mainly composed of polysaccharides, proteins, fatty acids, and nucleic acids (Zheng et al., 2008). This bioflocculant is composed of total sugar (79%), uronic acid (08%), and proteins (03%) with sugar as a main component. The presence of both carbohydrate and protein contents reveals that the bioflocculant is a glycoprotein. This indicates the presence of multiple functional moieties, which suggests the bioflocculant has got many adsorption sites for colloidal particles and enhance great flocculating efficiency (Okaiyeto et al., 2015).

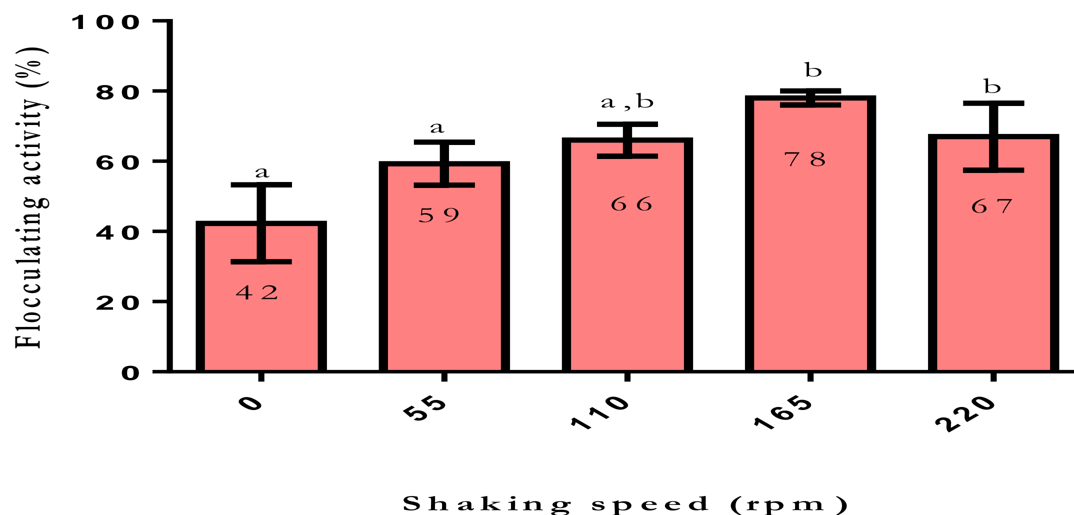

Figure 7: Effect of shaking speed on bioflocculant production

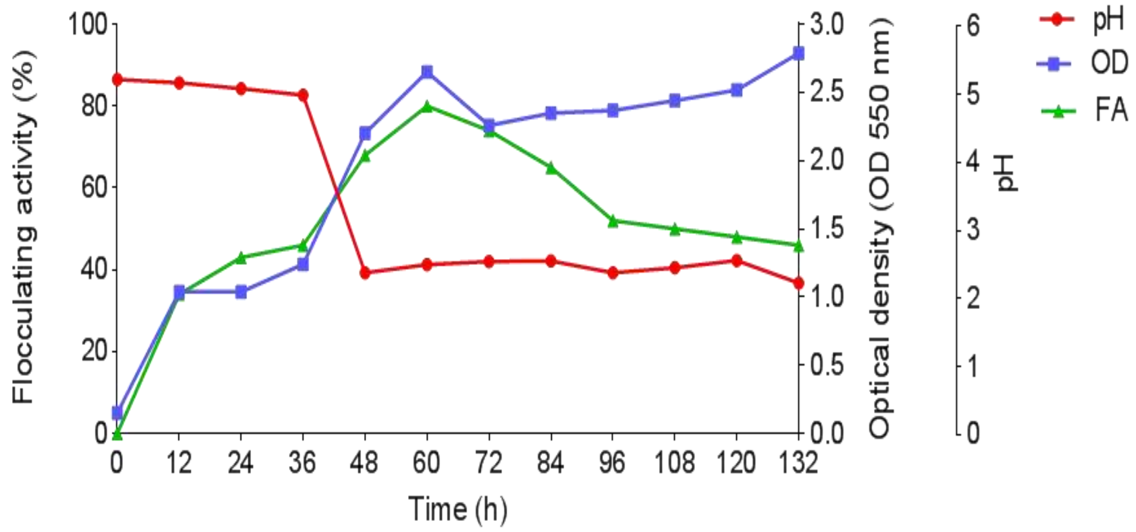

Figure 8: Effect of production time on bioflocculant production by *Bacillus spp*.

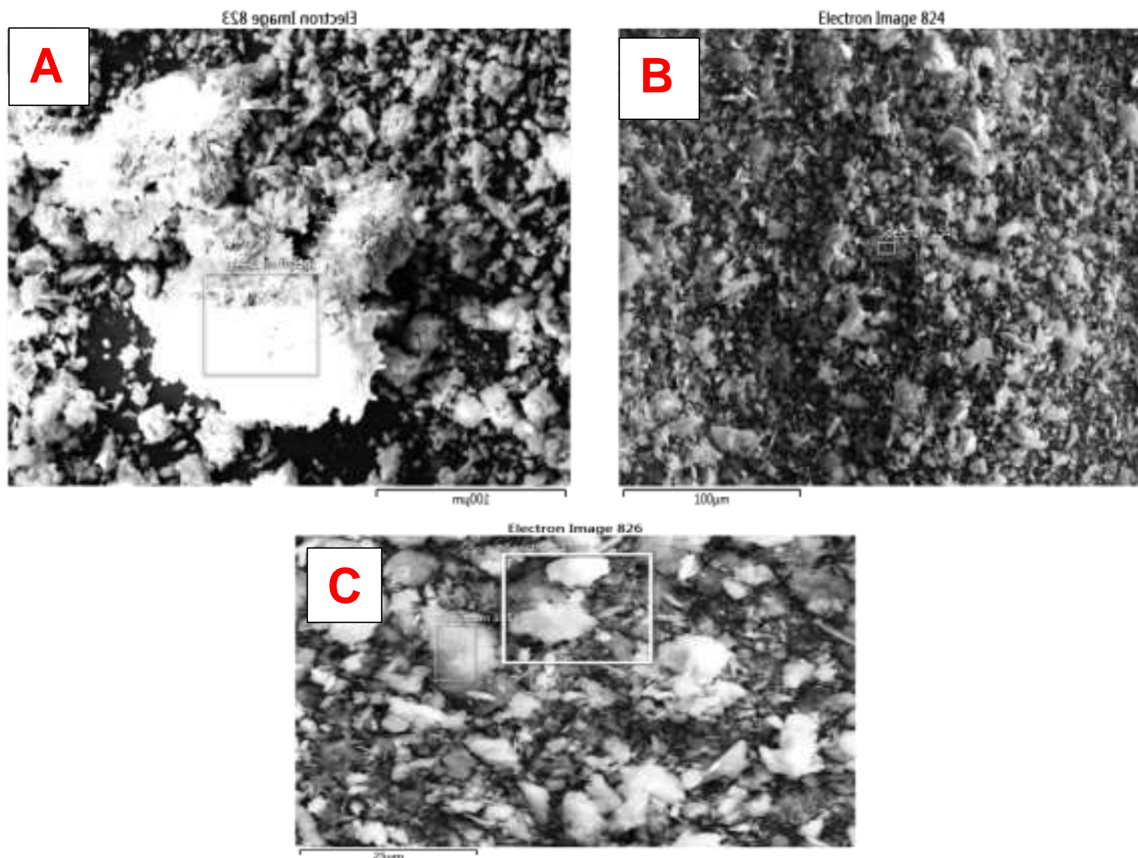

Figure 9: SEM images of kaolin clay (A), bioflocculant (B), and flocculated kaolin (C)

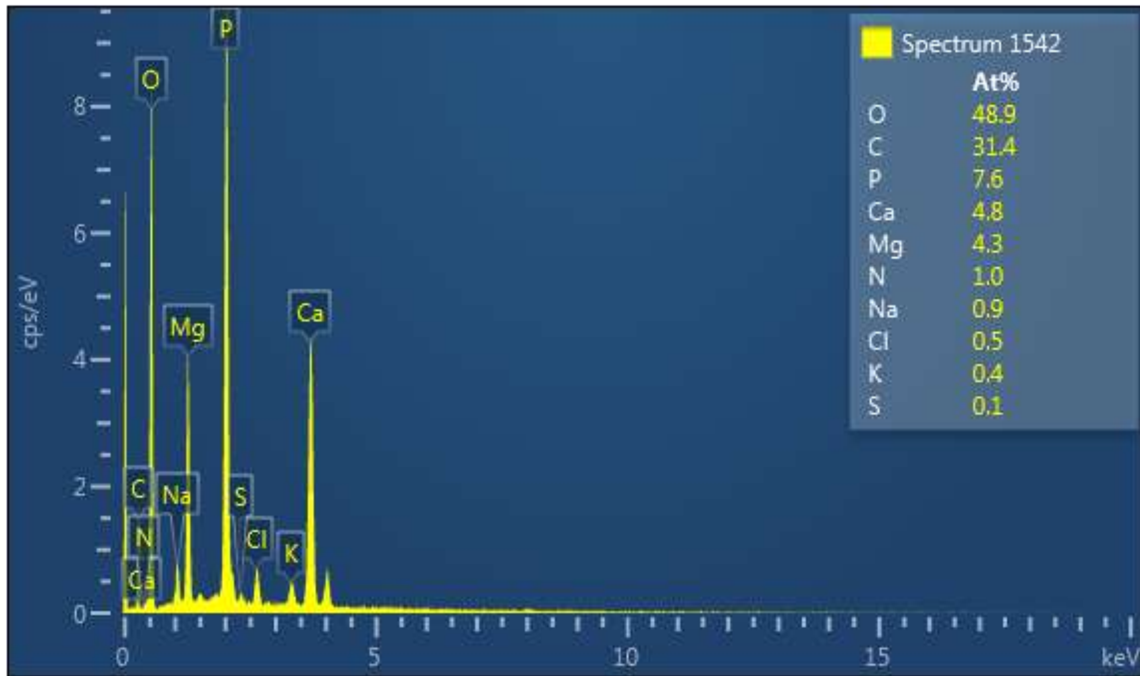

Figure 10: SEM –EDX analysis of the bioflocculant

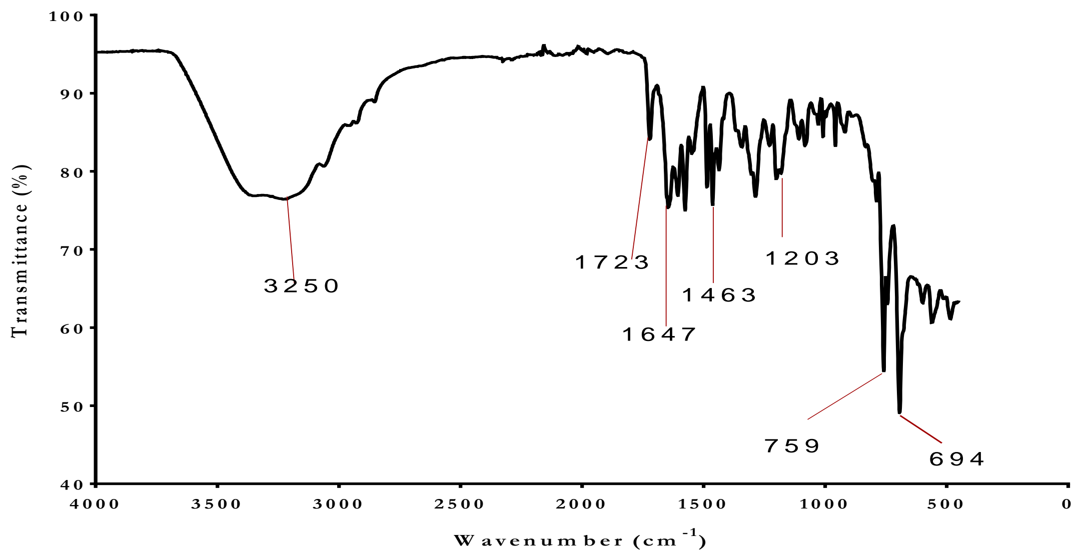

Figure 11: FT-IR spectrum of bioflocculant

The carbohydrate derivatives of the bioflocculant were known to be responsible for the stability features presented by the bioflocculant and the high flocculating efficiency (More et al., 2014). Nie et al., (2011) and Ntsangani et al., (2017) also reported the bioflocculants with carbohydrates as their main components.

Scanning electron microscopy analysis

Scanning electron microscopy analyses were carried out to elucidate the surface morphological structure of the bioflocculant before and after flocculation to kaolin clay suspension. The scanning electron microscope pictures, Figure 9(a) showed an amorphous, white coloured 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 9(b). In Figure 9(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 bioflocculants.

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 11 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 derivatives. The sharp peak at 759 cm^{-1} marks the existence of furan sugar (saccharides) and the peak observed at 694 cm^{-1} signifies halo compound (Desouky et al., 2008). The produced bioflocculant showed the FTIR spectrum which was consistent with the findings of other microbial flocculants produced by various microorganisms (Feng *et al*., 2008). The presence of hydroxyl and carboxyl groups in the bioflocculant's functional groups indicates that the bioflocculant is mostly a polysaccharide (Zhao et al., 2013).

Thermogravimetric analysis

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 lost observed between 30 and 150 ^{o}C (Figure 12) 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 ^{o}C 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; this marks the thermal stability of the bioflocculant due to high carbohydrate content present (Ugbenyen et al., 2014)

Optimisation 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 mg/ml (Figure 13). 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 floc 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 the 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*., 2016a). 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%), Fe^{3+} (70%). The flocculating activity of cation-free bioflocculant was 36%.

The flocculating activity of the 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^+, Na^+) were least effective, though they did enhance the flocculating activity of the bioflocculant (Figure 14). 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 range of 50- 100 °C for 30 min and 121 ^{0}C (autoclaved) for 15 min, as depicted in Figure 15. The purified bioflocculant is observed to be thermally stable as it retained about 76% flocculating activity at 100 °C. Further exposure to high autoclave temperature (121 °C, 15 min) and pressure resulted in more than 70% flocculating activity attained, but the difference in all tested heat was not significant in term of statistical analysis. Therefore, it was concluded that the bioflocculant produced by *Bacillus* species was thermo-stable 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 predominantly 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).

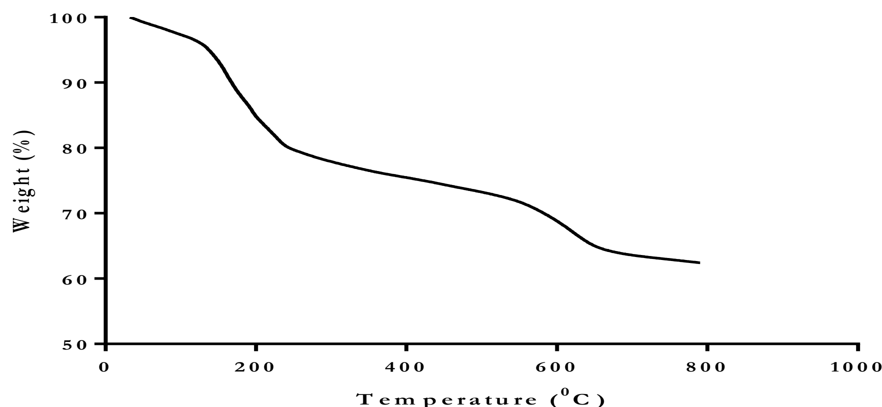

Figure 12: Thermogravimetric analysis on bioflocculant

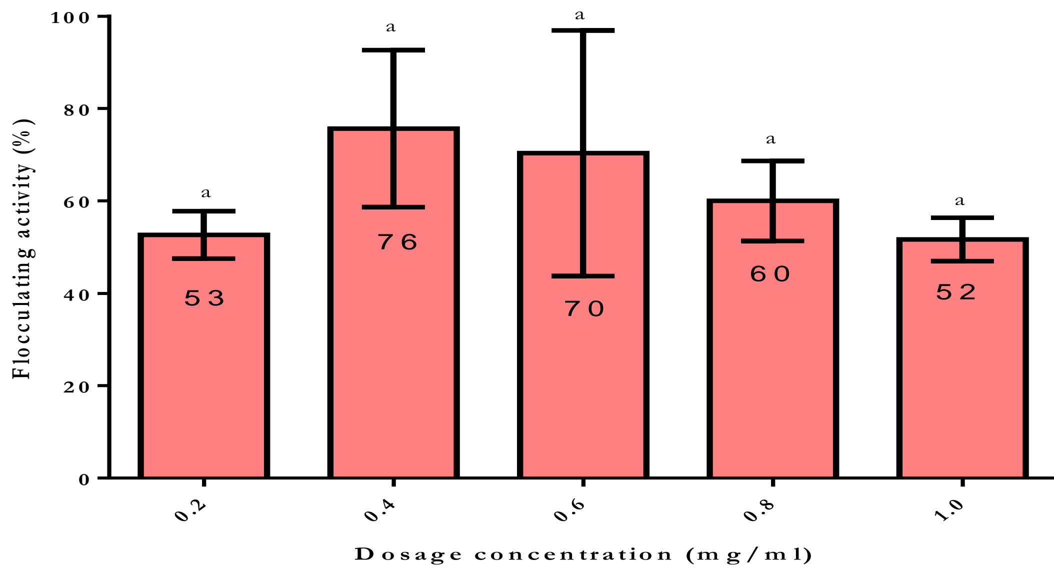

Figure 13: Effect of dosage concentration on flocculating activity

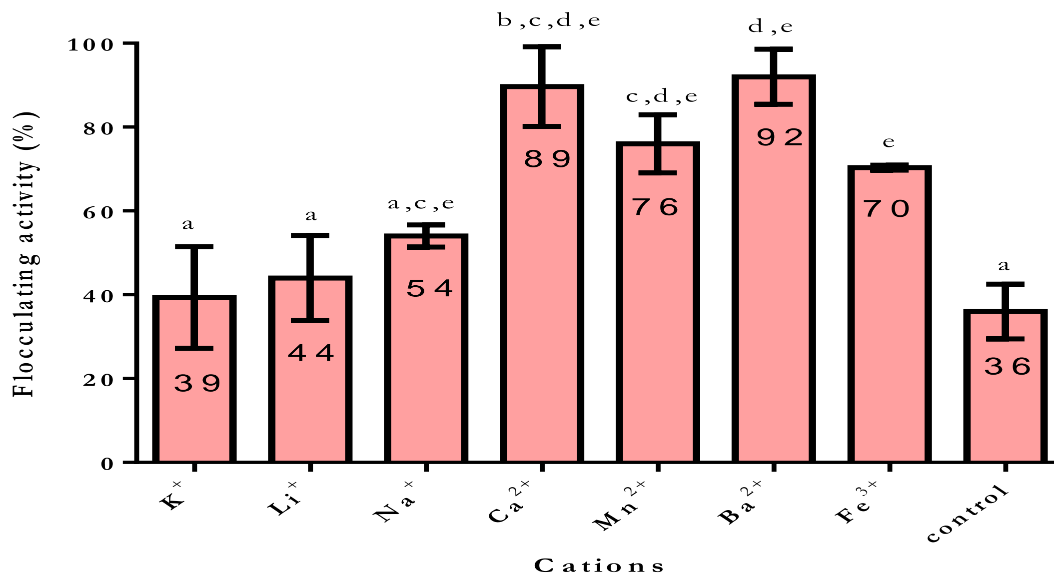

Figure 14: Effect of cations on flocculating activity

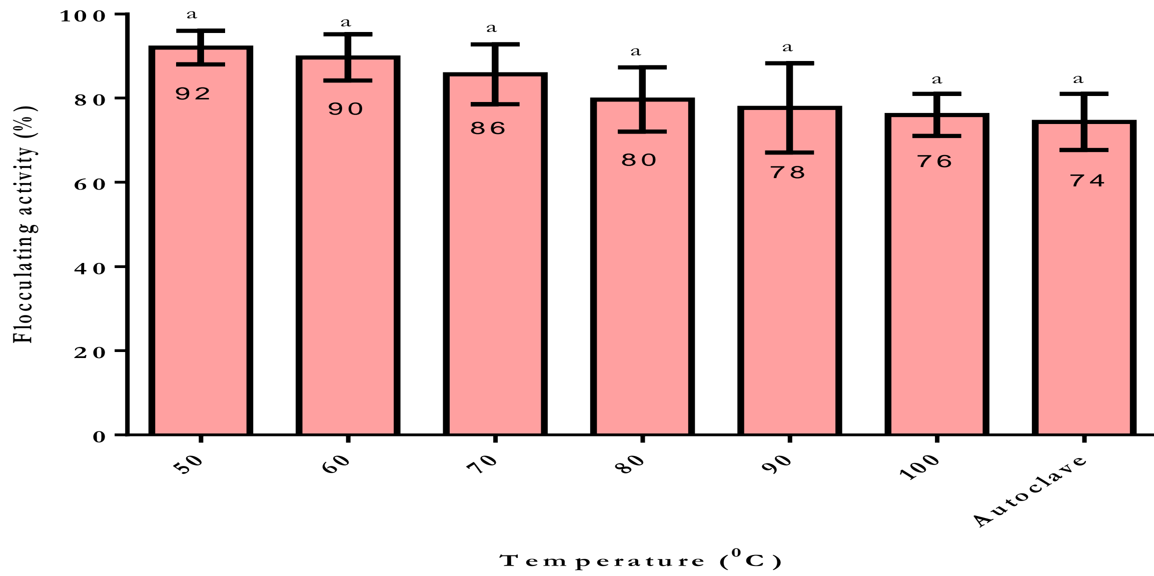

Figure 15: Effect of temperarute on flocculating activity of a bioflocculant

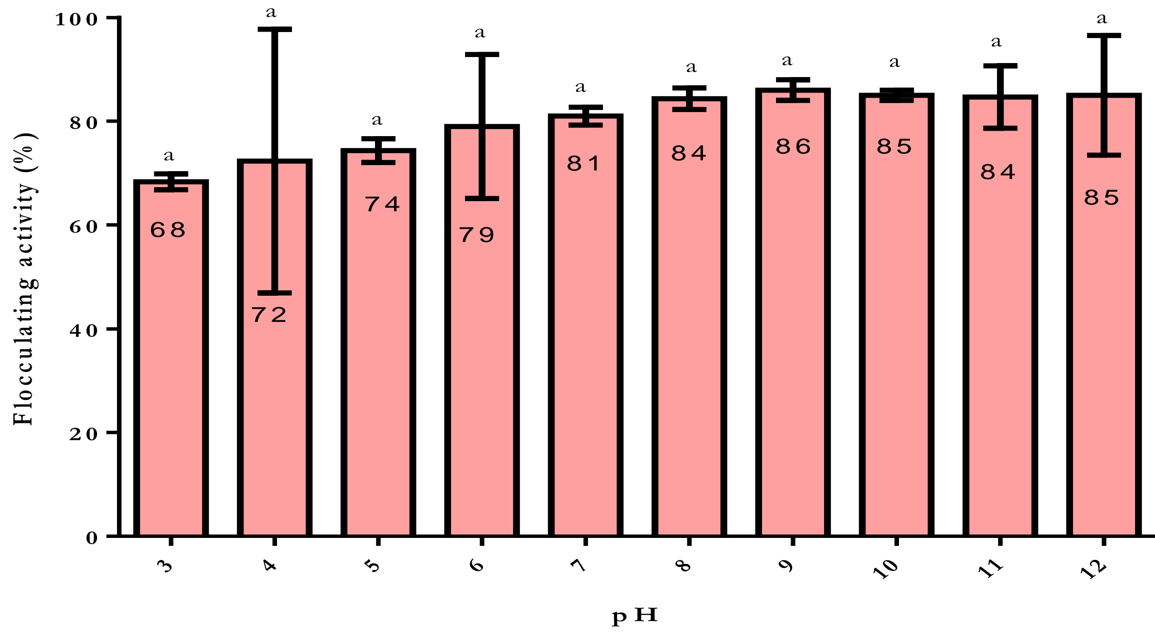

Figure 16: pH stability of a purified bioflocculant

Various studies reported bioflocculants exhibited thermal stability, for example, Li *et al.* (2007), and Tawila et al., (2018) reported a bioflocculant retained high flocculating activity 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 et al., 1999). The flocculating activity of the purified bioflocculant was tested within the pH range of 3-12, as shown in Figure 16. The tested bioflocculant was highly effective at a wide pH range of 3-12 with flocculating activities above 70%, with 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 at 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 radoibacter* F2 produced a compound biopolymer CBF-F26 with 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 range of 2-11.

CONCLUSION

The bioflocculant yield is 1.522 g/l, which is remarkably higher than some yielded by single strains in previous studies. It is a glycoprotein, and may be applied to any environment without the adjustment of pH and temperature, as it possess both pH and thermal stability features. The bioflocculant highly favours divalent cations and necessitates Ba^{2+} cation for optimal flocculating activity. Features of this bioflocculant are significantly making it attractive as an alternative to replace chemically synthesized flocculants. Application of the product in the treatment of different types of wastewater will be followed, as the bioflocculation team is extensively assessing it.

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, SJJ and PVSR; formal analysis: NZG, MNS, GNL and BAK; investigation: NZG, MNS and GNL; supervision: BKA, SJJ, ME and PVSR; writing (original draft): NZG; writing (review and editing): all authors.

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