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## Characterization of bioflocculant produced by *Bacillus* species isolated from uMlalazi estuary, Mthunzini (KZN) and its application in wastewater treatment

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The use of chemically synthesized flocculants in wastewater treatment is highly contentious as it induces Alzheimer's disease in humans and produce large amounts of sludge. Microbial flocculants therefore, have great potential in industrial water purification applications. A bioflocculant-producing bacterium was isolated from water samples collected from the uMlalazi estuary, Mthunzini, KwaZulu-Natal (KZN), South Africa. The bacterium was identified through partial 16S rRNA, using PCR with universal primers and the Genbank database revealed that bacterium is related to the genera *Bacillus*. The effect of culture conditions on the bioflocculant production reached an optimum with 1%(v/v) inoculum size, glucose, a mixed nitrogen source (Urea, Yeast and Ammonium sulphate), KCl, pH 6, shaking speed of 120 rpm at 40 °C for 84 hours of cultivation. Under these optimal conditions, maximum flocculating activity of 92% was attained. Chemical composition analysis showed that the purified bioflocculant is composed of carbohydrate (59% w/w), protein (10% w/w) and uronic acid (3% w/w). The elemental analyser revealed the presence of C (19.0), N (1.0), O (48.8), Na (0.7), Mg (2.7), P (7.2), S (0.1), Cl (0.7) and Ca (7.3) in mass proportion (% w/w). The presence of carboxyl, hydroxyl and amino group functional groups of the purified bioflocculant was revealed. The application of the bioflocculant on wastewater water indicated great potential to substitute hazardous chemical flocculants commonly used in wastewater treatment.

**Keywords:** *Bacillus* sp., Biodegradable, Bioflocculant, Flocculating activity.

### INTRODUCTION

Bioflocculation is a natural and simple process of aggregation and settling down of colloids, suspended solids and detritus by microorganisms or any substance produced by microorganisms. Microbial flocculants are by-product metabolites produced by microorganisms, including bacteria,

fungi, and actinomycetes as they're maturing, as polymers (Nakaruma et al., 1976; Kurane et al., 1986; Ugbenyen et al., 2012; Ntsaluba et al., 2013; Cosa and Okoh, 2014; Nwodo et al., 2014; Okaiyeto et al., 2014). They (polymers) can be found excreted in a solution or as an outer membrane covering the bacteria as slime

(Desouky et al., 2008). They play vital role in promoting flocculation by forming bridges between themselves and suspended particles. Generally, this results in coming together of suspended particles to form large flocs, which can be removed easily (Deng et al., 2003; Abdel-Aziz et al., 2013). Bioflocculant-producing bacteria have been screened and isolated from different environments including marine water, soil, sediments, wastewater and activated sludge (Okaiyeto et al., 2016b).

Most bioflocculant-producing microorganisms are reportedly isolated from soil and activated sludge (Desouky et al., 2008). These microbial flocculants are known to improve the settling down process of fine particles. They can also play a vital role in improving filtration, which is one of the most important steps in the treatment of water and industrial processes involving flocculation. Bioflocculants have won a monumental attention scientifically and biotechnologically because of their biodegradability and environmental friendly nature (Gao et al., 2006; Mabinya et al., 2011; Cong-Liang et al., 2012; More et al., 2014; Karthiga and Natarajan, 2015). They are pH depending and produce no sludge, which is easily degraded by microorganisms (Okaiyeto et al., 2016a).

Polysaccharides, protein, lipids, glycoproteins and glycolipids are the most important components of the bioflocculants (Gao et al., 2006; Zheng et al., 2008; Subramanian et al., 2010). These polymers are used by a microorganism for protection against harsh environmental conditions including temperature, high osmotic pressure and toxic compounds. Polymers play a vital role in the usage of metal ions, the prevention of dehydration under extreme conditions. They also prevent absorption of bacteriophages and engulfment by protozoa (Nichols et al., 2005; Buthelezi et al., 2010).

These characteristics of polymers make them useful in a number of industries including those that produce cosmetics, processing food, cleansing agents and mining industries. They may be used in water purification, oil recovery, wastewater treatment and in downstream processes (Salehizadeh and Shojaosadati, 2001; Sutherland, 2002; Nwodo and Okoh, 2013). Other studies have reported that some polymers have elements that inhibit tumour and inflammation in cell growth as well as function as an activator of enteron and factors that stimulate colonies for bacteria (Kumar et al., 2004; Lin and Zhang, 2004; Kunmani et al., 2011).

Fermentation costs and yields seem to be a major problem limiting large-scale production and industrial applicability of these microbial by-products which also lowering progress in scientific studies (Zhang et al., 1999; He et al., 2010; Zhao et al., 2012).

Chemically synthesized flocculants are conventionally implemented in wastewater treatment, in the purification of drinking water, in food industries, in downstream processing as well, as a result of their economic advantage and potency (Takagi and Kadowaki, 1985; Lin and Harichund, 2012).

These flocculants are grouped into three classes, namely: organic flocculants, inorganic flocculants and natural occurring flocculants. Inorganic flocculants include polyaluminium chloride (PAC), aluminium sulphate and ferric chloride, organic flocculants such as polyethylene amine, polyacrylic acid, and polyacrylamide (PAA) derivatives and naturally occurring flocculants such as bioflocculants, chitosan, cellulose, mucilage, gum and tannin (Kurane and Nohata, 1994; Salehizadeh et al., 2000; Xia et al., 2008; Maliehe et al., 2020)

The extensive use of chemically synthesised flocculants has fuelled major health and environmental concerns. Aluminium salts have reported to be associated with Alzheimer's disease (Arezo, 2002) and that residual acrylamide monomers contained in polyacrylamides are neurotoxic and carcinogenic to humans (Li et al., 2008). They are reported to be highly influenced by pH (Sharma et al., 2006). They do not produce at low *temperatures* and the large volumes are still a key factor for effective flocculation. This results in a higher amount of sludge being generated which is still a concern in water purification treatment (Sharma et al., 2006; Zhang et al., 2012).

Due to these characteristics and the environmental protection concern, among other factors, there is a huge requirement for a continual search of different bacterial environment for new bioflocculants with enhanced bioflocculation activity, high stability, and that are innocuous to human life (Qiang et al., 2010). Optimisation known to have an essential role it plays in bioflocculant production. The optimisation of culture conditions as well as factors effecting flocculating activity may enhance the flocculant-producing ability of microorganisms and also optimising the activity of bioflocculants. An understanding of the flocculation mechanism and of the physical and chemical structure of

bioflocculants, still need to be taken into consideration (Jang et al., 2001; Shih et al., 2001; He et al., 2004). Therefore, this study focused mainly on the screening of high flocculating bacterial strains from marine sediment from uMlalazi estuary, Mthunzini in KwaZulu-Natal, Republic of South Africa, was investigated. The study also sought to find low cost substrates and optimal fermentation conditions for treatment of wastewaters.

## MATERIALS AND METHODS

### Experimental section

#### Isolation of bioflocculant-producing bacteria

A number of bacteria were isolated from sediment and water samples of uMlalazi estuary, Mthunzini in the Province of KwaZulu-Natal in RSA. In the laboratory, the water samples were serially diluted into sterile saline water. About 100 microliters of the diluted solution were spread on Nutrient agar plates. About 1ml of the sediment samples was transferred into 9 ml of sterile saline water and agitated for 30 seconds. From these, serial dilutions were made. Similarly, 100 microliters of the serially diluted sample were spread on the surface of Nutrient agar plates, following the method of Jensen et al. (1990). The Nutrient agar plates were incubated for 3 days at 37 °C. Colonies were randomly picked and subcultured on fresh nutrient agar plates and incubated overnight at 37 °C.

#### 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 sodium chloride was prepared. About 5 ml of the activation media was measured into different test tubes and autoclaved at 121 °C for 15 min. the isolates were inoculated into the tubes and incubated in a rotary shaker for 24 hrs at 28 °C, 160 rpm.

#### Evaluation of bioflocculant production

#### Bioflocculant producing medium and cultivation

The sample was prepared following the method used by Zhang et al. (2007) and Cosa et al. (2011). A pre-culture medium made up of glucose (20 g), urea (0.5 g), yeast extract (0.5 g), (NH)<sub>2</sub>SO<sub>4</sub> (0.2 g), KH<sub>2</sub>PO<sub>4</sub> (2 g), K<sub>2</sub>HPO<sub>4</sub> (5 g), NaCl (0.1 g) and Mg SO<sub>4</sub> (0.2 g) was prepared in 1000 ml with filtered marine water. Fifty

millimeters of a medium was poured into a conical flask (100 ml) and autoclaved at 121 °C for 15 min. The sterile pre-culture broth was inoculated with a single colony of bacteria. Each flask with the mixture was placed in a shaking incubated at a speed of 160 rpm and temperature of 30 °C for a period of 72 hrs. After 3 days the broth culture was centrifuged for 30 min at 8,000 g, 4 °C to remove bacterial cells and the top solution (supernatant) was used to measure a bioflocculating activity (Kurane et al., 1994).

#### Bioflocculating activity assay

Bioflocculating activity assay was conducted following the procedure used by Ugbenyen et al. (2012). Kaolin solution was utilized to determine the bioflocculating activity. Kaolin solution, 0.4% (w/v) in distilled water, was prepared. Hundred milliliters of kaolin solution was transferred into 250 ml conical flask. Two millimeters of the centrifuged culture broth and 3 ml from 1% (w/v) CaCl<sub>2</sub> solution were mixed with kaolin solution. The mixture was vigorously shaken for 1 min and transferred to a graduated measuring cylinder (100 ml). The sediment was allowed to settle for 5 min at room temperature. Same method was used for a control, except that 2 ml of a supernatant was replaced with 2 ml of freshly prepared fermentation medium. The optical density (OD) of the supernatant was measured at 550 nm wavelength using spectrophotometer. Bioflocculating activity was determined in percentage applying the following equation:

$$\text{Bioflocculating Activity (\%)} = [(A - B)/A] \times 100$$

where A represents the absorbance of a control measured at 550 nm and B represents the absorbance of a sample at 550 nm.

#### Identification of bioflocculant –producing bacteria using molecular technique

Bioflocculant-producing strains were isolated from uMlalazi estuary in Mthunzini area, KZN, RSA. The DNA extraction kit™ (Zymo Research) (Omega Bio-Tek, Inc., USA) was employed to obtain DNA from the bacterial strain. PCR amplification was carried out utilizing universal primers, forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer (5'-CGGTTACCTTGTACGACTT-3'), and DreamTaq™ DNA polymerase (thermo Scientific™) to measure the 16S rRNA gene sequence. PCR products were gel extracted using Zymo Research, Zymoclean™ Gel DNA Recovery kit and sequenced in forward and reverse directions on the ABI PRISM™ 3500xl genetic Analyser.

Purified sequencing products (Zym Research, ZR-96 DNA sequencing Clean-up kit™) were analysed using CLC Main Workbench 7. The NCBI database was used to compare a bacterial strain after 16S rRNA gene sequencing (Altschul et al., 1997).

#### **Optimisation of bioflocculation conditions**

In order to obtain an improved bioflocculating activity together with the improved bioflocculant production, broth culture optimum conditions were established. The following parameters were evaluated, such as carbon sources, the inoculum size (% v/v), nitrogen sources, broth culture pH, shaking speed, temperature together with cations

#### **Determination of inoculum size**

Different inoculum sizes ranging from 1 to 4% (v/v) of the seed culture were used to inoculate the production medium. Culture inoculum volumes of 0.5 ml, 1 ml, 1.5 ml and 2 ml in proportion to the fermentation volume (50 ml) were respectively evaluated for bioflocculant production. To standardize, fifty millimeter of sterile saline solution was inoculated with a loop of pure colonies of the isolate. The cultures were incubated at a temperature of 30 °C for 72 h at 160 rpm. After 72 hrs the flocculating activities were evaluated. Kaolin clay [0.4% (w/v)] was prepared in distilled water. The kaolin solution, free-cell culture broth and CaCl<sub>2</sub> were used to determine the flocculating activity at 550 nm, while other conditions were kept constant (Piyo et al., 2011).

#### **Effect of carbon and nitrogen sources on bioflocculant production**

For the assessment of suitable nutrients sources, different carbon and nitrogen sources were used for the production of a bioflocculant. To assess carbon sources, the bacterium was cultivated in molasses, sucrose, lactose, maltose, xylose, starch and glucose. These carbohydrates, each, were added in a production medium substituting glucose with the similar amount of 20 g/l. Flasks with production media (50 ml) of different carbohydrates and bacterium were incubated at 30 °C for 72 hrs. After 72 hrs, the flocculating activity in each flask was determined. A carbohydrate with highest flocculating activity will be used for all tests followed.

The effect of different nitrogen sources was evaluated using yeast extract, peptone, urea, casein, ammonium molybdate, ammonium sulphate and ammonium nitrate each to substitute

urea (0.5 g), yeast extract (0.5 g) and ammonium sulphate (0.2 g) in a production medium with equivalent amounts (1.2 g) (Cosa et al., 2013). Flasks with production medium (50 ml) of different nitrogen sources and bacterium were incubated at 30 °C for 72 hrs. After 72 hrs, the flocculating activity in each flask was determined. A nitrogen source with highest flocculating activity will be used for all tests followed.

#### **Effect of shaking speed on bioflocculant production**

To measure the shaking speed effect on the bioflocculant production, the method used by Zhang et al. (2007) was followed. Various shaking speeds (60-220 rpm) were used. Different flasks with 50 ml production medium and 0.5 ml inoculum were incubated in a shaking incubators at different speeds (60, 80, 100, 120, 140, 160, 180, 200 and 220 rpm) at the temperature of 30 °C for the period of 72 hrs. After 72 hrs the bioflocculating activities were measured.

#### **Effect of initial pH on bioflocculating activity**

To determine the effect of initial pH on bioflocculating activity, pH of a cultivation medium was adjusted using 1 N NaOH and 1 N HCl prior to sterilisation and inoculation. The pH used were 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The bioflocculating activity was measured at each pH (Cosa et al., 2013).

#### **Effect of cultivation temperature on bioflocculating activity**

A method used by Zhang et al. (2007) was followed for the investigation of cultivation temperature effect on the bioflocculating activity. A test bacterium was inoculated in the production media and incubated at different temperatures of 20, 25, 30, 35, 40, 45, 50, 55 and 60 °C for 72 hrs. After 72 hrs the flocculating activity was measured.

#### **Effect of metal ions on bioflocculating activity**

To investigate metal ions effect on bioflocculating activity, a method used by Nie et al., (2011) was followed. Three milliliters of cation solution (1% w/v) and 2 ml of a bioflocculant solution were added into 100 ml kaolin solution. 1% (w/v) CaCl<sub>2</sub> solution used as a cation was replaced by various metal salt solutions (NaCl, KCl, LiCl, MnCl<sub>2</sub>, BaCl<sub>2</sub> and FeCl<sub>3</sub>) and experimental control was prepared by putting free-cell supernatant in a kaolin solution, no cation was

added. The bioflocculating activity was determined (Okaiyeto et al., 2015b).

### Time course assay

The effect of culture time on bioflocculating activity of the bacterium was conducted in accordance with a previous study by Piyo et al. (2011). A production medium made up of glucose (20 g), urea (0.5 g), yeast extract (0.5 g),  $(\text{NH}_2\text{SO}_4$  (0.2 g),  $\text{KH}_2\text{PO}_4$  (2 g),  $\text{K}_2\text{HPO}_4$  (5 g),  $\text{NaCl}$  (0.1 g) and  $\text{MgSO}_4$  (0.2 g) was prepared in 1000 ml of filtered marine water. The pH was adjusted to 6 with either 1 N NaOH or 1 N HCl. The mixture was autoclaved at 121 °C for 15 min. The strain was cultivated separately under optimal growth conditions. To standardize, 50 ml of sterile saline solution was inoculated with a loop of pure colonies of the isolate, vortexed and the suspension standardized to  $\text{OD}_{660\text{nm}}$  0.1. Briefly, the standardized saline solution was used as seed culture for inoculum preparation. From the seed culture, the optimum inoculum size of 1% (v/v) was inoculated into 100 ml of a sterile culture broth in 250 ml flasks (prepared in triplicate) and incubated on a rotary shaker (120 rpm) at 40 °C. Samples were drawn every 12 hrs. Two millimeter of the sample was centrifuged at 8,000 g for 30 min. the supernatant was used to determine the flocculating activity. The optical density at 660 nm ( $\text{OD}_{660\text{ nm}}$ ) was measured every 12 hrs together with the pH of the medium for a period of 5 days.

### Extraction and purification of the bioflocculant

For extraction and purification of a microbial flocculant, a method used by Chen et al. (2003) and Makapela et al. (2016) was used, with minor modifications. After 84 hrs of fermentation, the culture broth was centrifuged at 8,000 g at 4 °C for 15 min. One thousand millimetres of distilled water was added to the supernatant phase and then centrifuged at 8,000 g for 15 min, at 4 °C. Two thousand millimetres of ice-cold ethanol were added to the supernatant, and the mixture was shaken and then left at 4 °C for 12 hrs. The precipitate was vacuum-dried to obtain the crude bioflocculant and dissolved in distilled water to form a solution (w/v). One hundred millimetres of a mixture of chloroform and butanol (5:2 v/v) was added. After shaking, the mixture was left at room temperature for 12 hrs. The supernatant was then centrifuged at 8,000 g for 15 min at 4 °C and vacuum-dried to obtain a purified bioflocculant.

### Chemical analysis of a purified bioflocculant

#### Composition analysis of the purified bioflocculant

The total sugar content was determined by the phenol-sulphuric acid method as described by Dubois et al. (1956). D-glucose was used to prepare a standard curve. Total protein concentration was measured using Folin-Lowry method (Lowry et al., 1951), standardized with bovine serum albumin (BSA). Carbozole method was used to estimate the amount of uronic acid present in a purified bioflocculant, standardized with D-glucuronic acid (Cesaretti et al., 2003).

#### Elemental analysis

To analyse the elements present in a purified bioflocculant, a scanning electron microscope (SEM) (SEM-Sipma-VP-03-67, Zeiss, P-Sigma, Germany) equipped with an elemental analyser was used. Prior to SEM analysis, 5 mg of bioflocculant was added on slides coated with silicon and fixed by a spin coater at 1000 rpm, 60 s.

#### FTIR analysis

Functional groups analysis for a purified bioflocculant was carried out using Fourier transform infrared spectrophotometer (FTIR) (Perkin Elmer System 2000, Cambridge, England). The powdered bioflocculant was mixed with potassium bromide powder and pressed into pellets for FTIR spectral measurement in the frequency range of 4000-400  $\text{cm}^{-1}$  (Zhang et al., 2015).

#### Thermogravimetric analysis of the bioflocculant

To analyse the pyrolysis of a bioflocculant, the thermo-gravimetric analyzer (STA 449/C Jupiter, Netzsch, Wittelsbacherstraße, Germany) was used. The instrument was adjusted to the temperature range between 30 °C and 800 °C under a nitrogen gas constantly flowing at 20.0 ml/min. The rate was kept constant at 10 °C  $\text{min}^{-1}$  (Okaiyeto et al., 2013).

#### Optimisation of flocculating activity of a purified bioflocculant

##### Effect of bioflocculant dosage on flocculating activity (Jar test)

To investigate the dosage of a purified microbial flocculant by *Bacillus* sp., the method described by Wan et al. (2013) was followed. To

determine a dosage, various concentrations of bioflocculant solution were prepared ranging from the concentration of 0.2 to 1.0 mg/ml (w/v). From each solution, 2 ml was mixed with 100 ml of kaolin solution (4 g kaolin powder in 100 ml distilled water) and 1% (w/v) of CaCl<sub>2</sub> solution in 250 ml conical flasks. The mixture was agitated quickly, poured into a measuring cylinder (100 ml) and allowed to sediment for 5 min at room temperature. Two millimeters was drawn from upper part of the solution and used for bioflocculating activity measurements.

#### Effect of heat on the bioflocculating activity

The thermal stability of a purified bioflocculant was assessed using the description by Makapela et al. (2016). 0.4 mg/ml solution of a bioflocculant was used in all tests. Tubes containing 2 ml of a bioflocculant solution were transferred into various cultivation temperatures range (50, 60, 70, 80, 90 and 100 °C) for a period of 30 min. Another tube was heated in an autoclave (works with steam under pressure) at 121 °C for 15 min. All tubes were used for bioflocculating activity measurements.

#### Effect of pH on the bioflocculating activity

A method described by Zhang et al. (2013) was used to investigate the effect of pH on the bioflocculating activity. A pH of kaolin solution (100 ml) in different flasks (250 ml) was adjusted with either 1 N NaOH or 1 N of HCl in a pH range of 3-12. pH 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were used to assess flocculating activity of a bioflocculant. To each flask 2 ml of 0.4 mg/ml bioflocculant solution was mixed with kaolin solution and used for bioflocculating activity assessment.

#### Effect of cations on the purified bioflocculant

A method used by You et al. (2006) was followed to investigate the metal ions effect towards bioflocculating activity of the produced bioflocculant. Different salt solutions (1% w/v) such as NaCl, LiCl, KCl, BaCl<sub>2</sub>, MnCl<sub>2</sub> and FeCl<sub>3</sub> were used to substitute 1% (w/v) CaCl<sub>2</sub> solution. Experimental control was prepared by putting free-cell supernatant in a kaolin solution, no cation was added.

#### Flocculation of wastewaters

Wastewater samples (2 liters) were freshly collected from Thendele coal mine at KwaSomkhele area in Mtubatuba, KwaZulu-Natal, South Africa and domestic wastewater from

KwaDlangezwa, Empangeni, KZN. Parameters such BOD, pH, sulphide content and COD were determined by a pH meter and spectra-quant (Pharos 100, Merck KGaA, Germany) before flocculation (Li et al., 2013).

Each wastewater sample (100 ml) was poured in a 250 ml beaker together with 2 ml of 0.4 mg/ml bioflocculant solution and 3 ml of 1% (w/v) BaCl<sub>2</sub> solution. The mixture was vigorously agitated at 200 rpm for 3 min. The speed was then reduced to 45 rpm for 5 min. The flasks were left standing at room temperature for 10 min. The residual COD, BOD, and sulphide content and flocculation efficiency were determined using supernatant. The conventionally-used chemical flocculants such as ferric chloride and alum were used for comparison, replacing the bioflocculant. Pollutant removal rate (efficiency) (RE) was determined using the formula below:

$$\text{Removal Efficiency (RE) (\%)} = \left[ \frac{C_0 - C}{C_0} \right] \times 100$$

where: C<sub>0</sub> is the value before flocculation and C is the final value after the flocculation process.

#### Statistical analysis

Tests were conducted in triplicates. The means and standard deviations were determined using Microsoft Excel Office version.

## RESULTS AND DISCUSSION

### Screening, isolation and identification of bioflocculant-producing bacteria

More than 40 isolates were obtained from sediment and water samples from uMlalazi estuary, Mthunzini and screened for bioflocculant production. Among the screened isolates, the isolate with the highest flocculating activity (70%) against kaolin suspension was used for the production of bioflocculant. An isolate appeared on the nutrient agar plate as white, circular and moist colonies. The cells of a selected isolate were observed to be Gram positive and rod shaped. Its identity was confirmed by the 16S rDNA sequencing method. A comparative analysis of the 16S rDNA sequence of the isolate in the GenBank database showed 98% similarity with *Bacillus safensis* and its repository accession number was KX694275.1. The isolate was named a *Bacillus* species. *Bacillus safensis* has not been reported as a bioflocculant-producing strain in previous studies. A number of bacteria known to produce extracellular polymeric substances come from *Bacillus* sp. (Okaiyeto et al., 2016b). *Bacillus amyloliquefaciens* (Ogunsade et al., 2015), *Bacillus pumilus* (Makapela et al., 2016; Maliehe

et al., 2016).

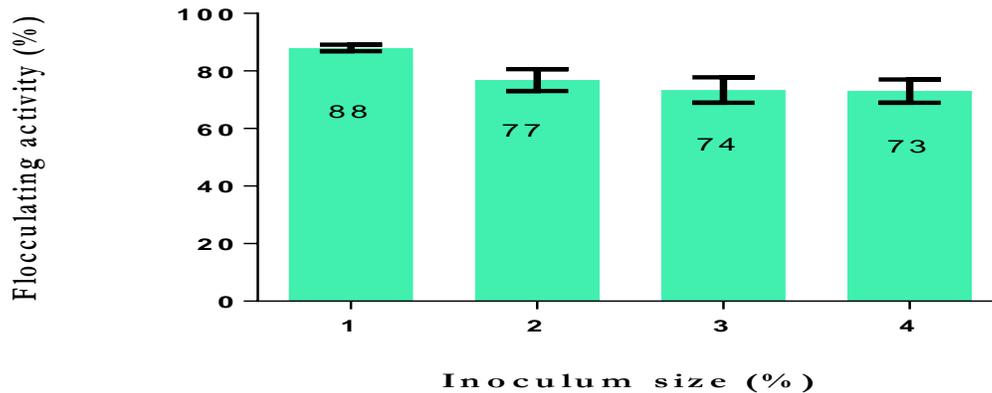
### Optimisation of culture conditions

In order to create the optimum broth culture conditions for bioflocculant production, the inoculum size (% v/v), nutrient sources, pH of culture medium, shaking speed, temperature,

and time course were examined.

### Effect of inoculum size on bioflocculant production

The inoculum size effect on the production of a bioflocculant by *Bacillus* species was evaluated in Figure 1.



**Figure 1: Effect of inoculum size on bioflocculant production by *Bacillus* sp.**

Inoculum size plays a major role in microbial growth and in bioflocculant production (Wang et al., 2011). A low inoculum size delay the bacterial growth, while a high inoculum size cause niches of the overlap excessively and consequently inhibit the bioflocculant production (Salehizadeh and Shojaosadati, 2001). As observed in Figure 1, all inoculum sizes used were favourable for bioflocculant production as they produced more than 70% bioflocculating activity. A 1% (v/v) inoculum size was more effective than others with a bioflocculating activity of 88%.

An increase in the inoculum size resulted in a slightly decrease in flocculating activity. An inoculum size of 0.5 ml (1%) was enough to allow the bacterium to grow and multiply in the broth culture resulting in the stimulation of an extracellular polymeric substance manufacturing (Li *et al.*, 2009). A 0.5 ml (1%) inoculum size was used to the entire tests followed.

Gong et al. (2008) and Luo et al. (2014) recorded the similar results whereby an optimum bioflocculant production obtained with 1% inoculum size. Contrary to the study findings, the inoculum sizes of 2% (Jang et al., 2001; Aljuboori et al., 2013), 4% (Jang et al., 2001) and 8% (Zhao et al., 2012) were reported to produce the highest bioflocculating activities.

### Effect of carbon sources on bioflocculant production

Literature studies have revealed that carbon sources also play an important part as they uplift the production of bioflocculant by bacteria (Nakumura et al., 1976; Xia et al., 2008; Goo et al., 2013; Nwodo and Okoh, 2013; Pathak et al., 2014)

The conditions of bioflocculant production were optimised by using different carbon sources, as shown in Figures 2. It was observed that among all the carbon sources assessed glucose had the highest flocculating activity of 88%. This greatly improved bioflocculant production. Glucose was followed by sucrose, maltose and starch with flocculating activities of 85%, 79%, 59% respectively. Flocculating activities below 50% were observed for lactose and xylose, that of 46% and 45% respectively. Among other carbon sources, molasses showed a very little effect on bioflocculating activity with 38% being recorded. Therefore, glucose was used in all tests followed. This is because glucose is a monosaccharide or a simplest carbohydrate ( $C_6H_{12}O_6$ ), which is easily utilized by microorganisms. Unlike other carbon sources which are made up of a combination of two monosaccharides (disaccharide), with formula  $2(C_6H_{12}O_6)$  or more monosaccharides (polysaccharides), with formula  $3(C_6H_{12}O_6)$ , linked with glycosidic bonds which are not easy for

microorganisms to utilize as they require to first simplify them.

Several studies revealed glucose to be more effective for bioflocculant production which is similar to these research findings. Cosa *et al.* (2013), reported that a number of bacteria used in the production of bioflocculants favoured glucose. For example, Liu *et al.* (2015) reported glucose to be effective in the production of bioflocculants. Contrary, Patil *et al.* (2011); Mabinya *et al.* (2012) reported sucrose and lactose as favoured carbon sources for optimum bioflocculant production, respectively.

### Effect of nitrogen sources on bioflocculant production

One of the most important nutrient sources reported to have an impact on the production of bioflocculant is nitrogen (Okaiyeto *et al.*, 2016a).

Figure 3: Effects of nitrogen sources on bioflocculant production by *Bacillus* sp.

Various nitrogen sources were tested for bioflocculant production by *Bacillus* sp. The complex nitrogen sources (made up of urea, ammonium sulphate and yeast extract) produced the highest flocculating activity (88%) followed by yeast extract (82%), ammonium sulphate (79%) ammonium nitrate (78%), ammonium molybdate (76%), casein (74%), peptone (53%) and urea (46%) respectively (Figure 3). Therefore, complex nitrogen sources were used in all tests followed.

Gong *et al.* (2008), Zhao *et al.* (2013) and Ntsaluba *et al.* (2013) recorded the similar results, in which multiple nitrogen sources resulted in the highest flocculating activity.

Contrary to this findings, other researchers reported individual nitrogen sources to be more effective in bioflocculant production than multiple nitrogen sources. For example, ammonium sulfate (Cosa *et al.*, 2011), ammonium chloride (Mabinya *et al.*, 2012; Piyo *et al.*, 2011), ammonium nitrate (Zheng *et al.*, 2008) and urea (Mabinya *et al.*, 2011) were reported to produce the highest flocculating activity.

### Effect of cations on the flocculating activity

Literature studies revealed that both bioflocculant and suspended particles carried negative charges which could be neutralised by cations addition and eventually enhance the absorption of the microbial flocculant onto the suspended particles (Okaiyeto *et al.*, 2013). In this study, metal ions (monovalent, divalent and trivalent) effect was also evaluated (Figure 4). It was observed that flocculation was stimulated by

both monovalent and divalent cations, with  $K^+$  and  $Ba^{2+}$  produced the highest flocculating activity of 95% and 94% respectively. This was followed by  $Na^+$ ,  $Ca^{2+}$ ,  $Li^+$ , and  $Mn^{2+}$  with flocculating activity of 90%, 88%, 86%, and 82% respectively. Blank (no cation used) and trivalent cation  $Fe^{3+}$  and were less effective as they resulted in the lowest (33% and 10% respectively) flocculating activities.  $K^+$  was therefore selected as the cation for the subsequent experiments.

Similar, Piyo *et al.* (2011) and Okaiyeto *et al.*, (2016b) in their studies reported  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $K^+$  and  $Fe^{2+}$  to be more effective in stimulating the production of bioflocculants.

### Effect of pH on bioflocculant production

The flocculating activity of a bioflocculant is highly influenced by the pH of the environment (Tang *et al.*, 2014). The effect of initial pH of a growth medium ranging from pH 3 to 12 on the production of bioflocculant by *Bacillus* sp. was examined. In Figure 5, the increased in bioflocculating activity from pH 3 to pH 6 was noticed. The optimum bioflocculating activity of 93% was recorded at pH 6.

An increase in pH above 6 led to the decrease in the bioflocculating activity. Increasing pH to above 6 and decreasing the pH to below 6 of a cultivating broth resulted in the decrease of the bioflocculating activity. A drastically decrease in bioflocculating activity was observed in pHs above 7 which may be due to alkaline degradation of the polysaccharide (Zhong *et al.*, 2014). This degradation could cause the fragmentation of the polysaccharide chain or molecular rearrangement of its surface changes (Zhong *et al.*, 2014). Ugbenyen *et al.* (2014) reported that different microorganisms prefer different pH range for bioflocculants production.

Similarly, Wang *et al.* (2007) and Ogunsade *et al.* (2015) reported pH 6 to be more effective in the bioflocculation productions.

Contrary to the findings of this study, Pathak *et al.* (2014) and Luo *et al.* (2014) reported pHs other than pH 6 to highly improve the bioflocculant production.

### Effect of temperature on bioflocculant production

A number of researchers clearly indicated that the cultivation temperature is among the most important factors that influence a bioflocculant production and a flocculation process by microorganisms (Nakata and Kurane, 1999; Ntsaluba *et al.*, 2011). Literature studies have

shown that the optimum range for bioflocculant production varies between 25 °C and 37 °C (Okaiyeto et al., 2016a). The effect of temperature on growth ranging from 20 °C to 60 °C in the

production of bioflocculant by *Bacillus* sp. was examined.

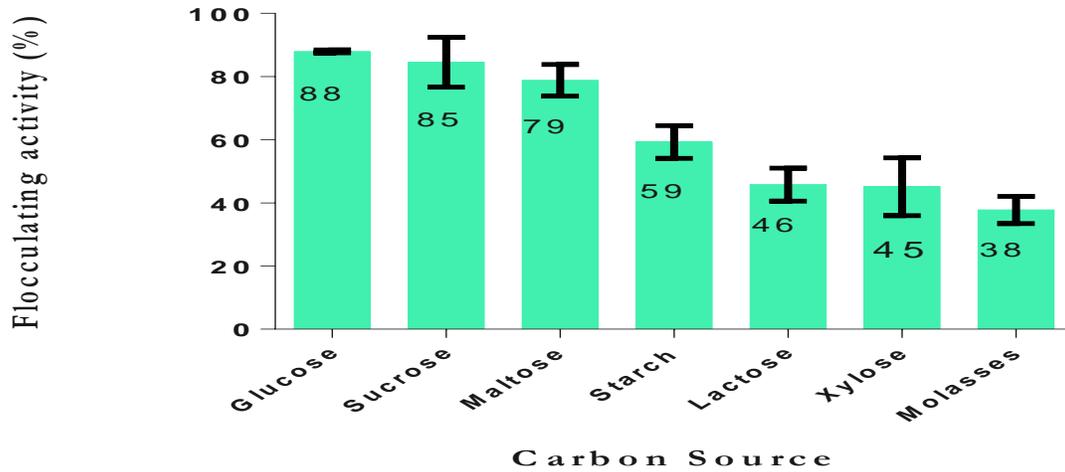


Figure 2: Effect of carbon sources on production of a bioflocculant by *Bacillus* sp.

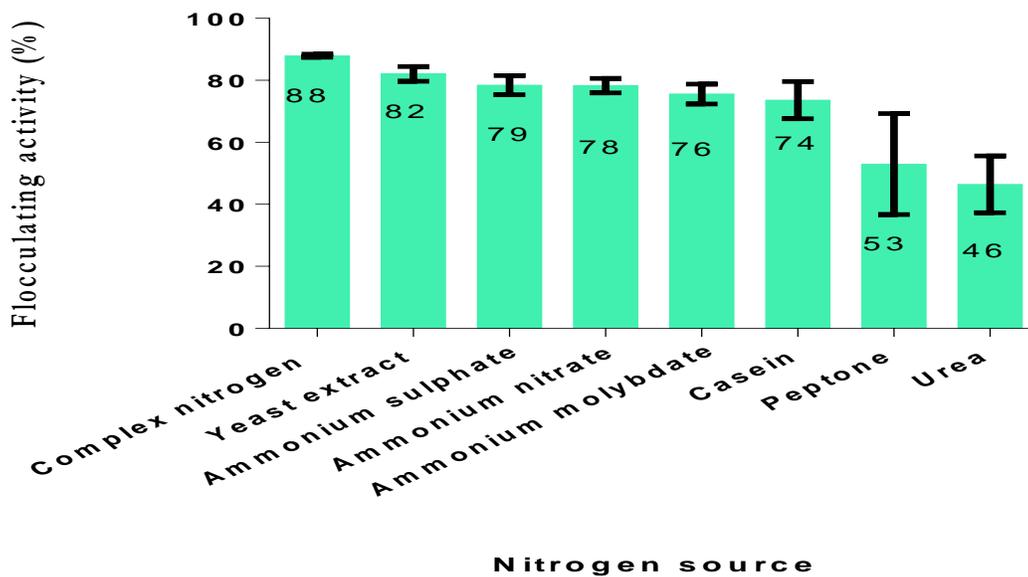


Figure 3: Effect of nitrogen sources on bioflocculant production by *Bacillus* sp.

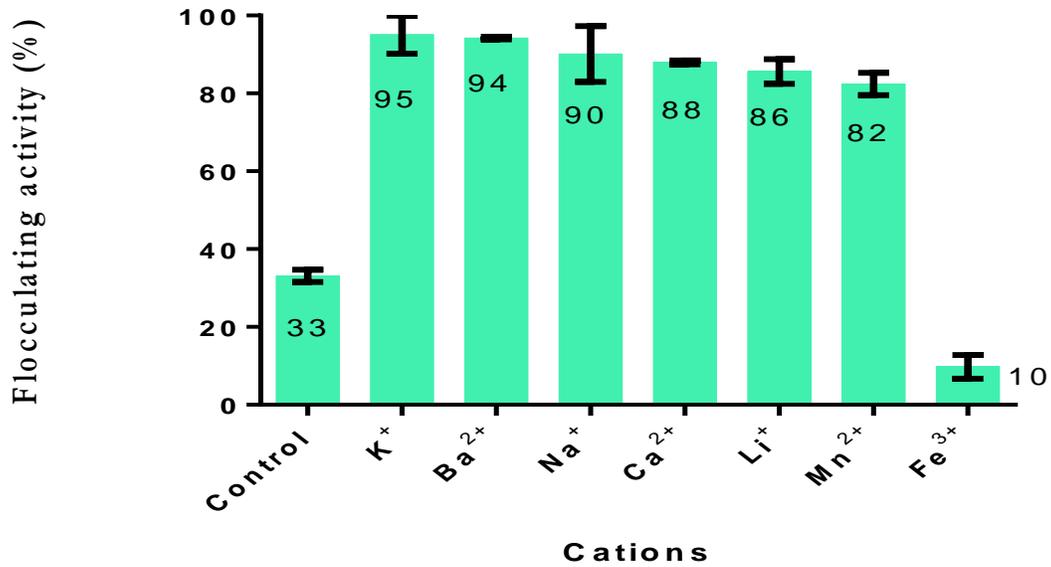


Figure 4: Effect of cations on bioflocculant production by *Bacillus* sp.

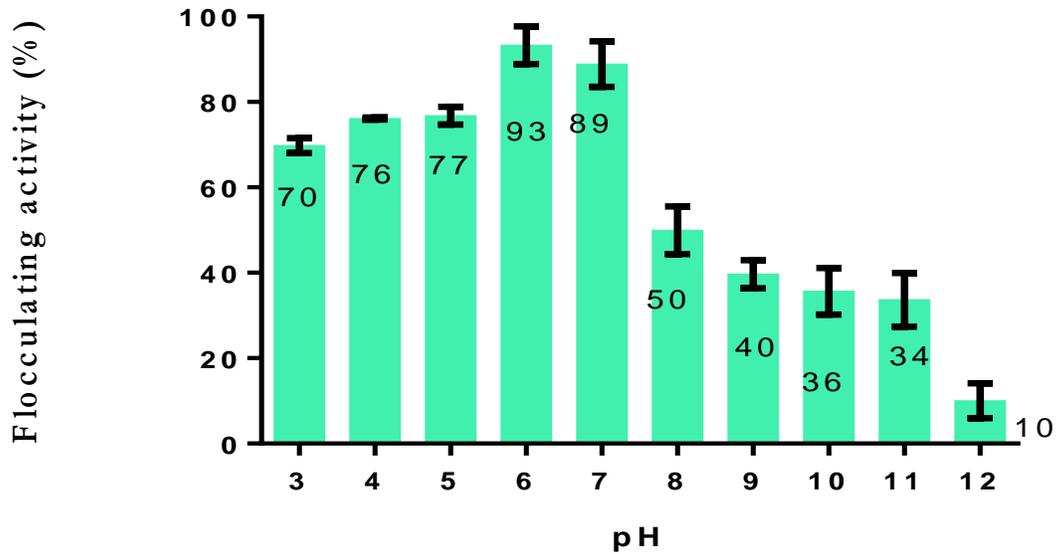
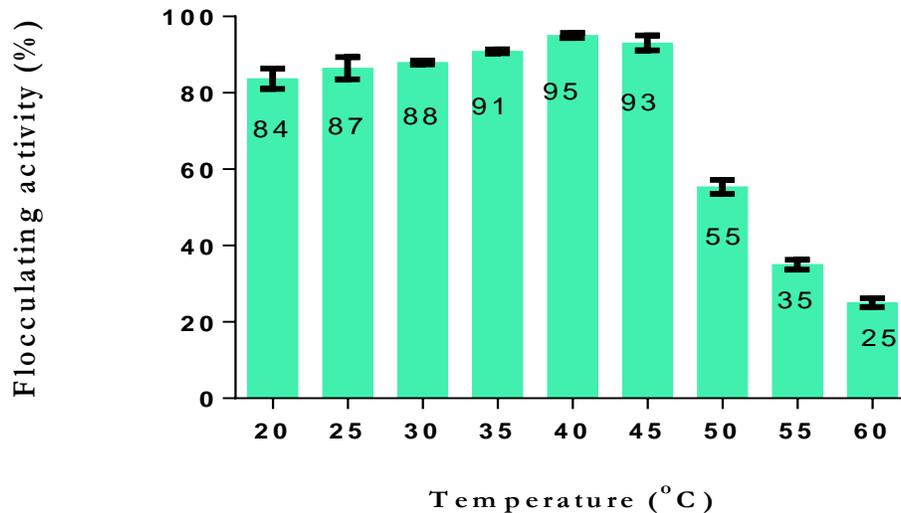


Figure 5: Effect of pH on bioflocculant production by *Bacillus* sp.



**Figure 6: Effect of temperature on bioflocculant production by *Bacillus* sp**

In Figure 6 the flocculating activity of *Bacillus* sp. increased from a temperature of 20 °C to 40 °C. The optimum bioflocculating activity (95%) was recorded at 40 °C. Furthermore, the increase and decrease in cultivation temperature above 40 °C have led to the decline of the bioflocculating activity. This is due to the enzyme responsible for the production of bioflocculant being activated at an optimum temperature (Zhang et al., 2007). Similarly, *Staphylococcus cohnii* was reported to produce a bioflocculant from Palm Oil Mill Effluent (POME) at 40 °C (Wong et al., 2012). Xiong et al. (2010) obtained the maximum flocculating activity of 700 U/ml after cultivation of *Bacillus licheniformis* at 37 °C for 48 h.

Contrary to this study, Makapela et al. (2016) reported an optimum flocculating activity at 28 °C when assessment of *Bacillus poilus* isolated from freshwater milieu for bioflocculant production. *Virgibacillus* species isolated from Algoa bay was reported to produce a thermostable polysaccharide bioflocculant at 30 °C (Cosa et al., 2013). Liu et al. (2010) reported the bioflocculant produced by *K. pneumoniae* to flocculate well in the range of 4-50 °C with flocculating activity of above 88%, and the highest flocculating activity of 97.5% was achieved at 30 °C.

#### **Effect of shaking speed on bioflocculant production**

To study the influence of shaking speed on the production of a bioflocculant by *Bacillus* sp., a shaking speed range of 60-220 rpm was used. Figure 7 shows the effect of agitation on *Bacillus* sp. and its flocculation activity in the different shaking speeds used. From Figure 7, it can be seen that the flocculating activity increased with an increase in shaking speed until reaching a speed of 120 rpm (95% flocculating activity), followed by a decrease thereafter. A decrease in flocculating activity is caused by shaking speed that is either higher or lower than 120 rpm. The optimum shaking speed determines the concentration of dissolved oxygen that influences nutrient adsorption and enzymatic reaction (Lopez et al., 2003). The shaking speed of 120 rpm was used in subsequent experiments.

Similarly, Suryani et al., (2011) reported a shaking speed of 120 rpm to be more effective for the bioflocculant production.

Li et al. (2009) and Tang et al. (2014) reported a shaking speed of 140–160 rpm and 180 rpm, respectively, to be optimal for bioflocculants production.

#### **Time course of the bioflocculant production by *Bacillus* sp.**

The time course of bioflocculant produced by

*Bacillus* sp. was determined, where the interrelationship between bioflocculating activity, pH and cell multiplication was investigated over a cultivation period of 120 hrs (Figure 8).

The pH of the production medium decreased from 7.14 to 5.29 during the 5 days of the experiment. The decrease in pH was as a result of acids being produced either from a sugar (in the medium) degradation by microorganism or the availability of the acidic component on the bioflocculant polymer being produced (Ntsaluba et al., 2011). As stated in literature, the initial pH of the culture medium measures the cell charges and the oxidation-reduction rate which can influence both nutrient absorption and enzymatic reaction.

An increase in bioflocculant production starting from 12hrs time continued until it reached a peak after 84 hrs was observed. It is also observed from Figure 8 that the cell growth (OD<sub>660 nm</sub>) is parallel to the flocculating activity. A maximal production of bioflocculant of 92% was observed on the 84 hrs of fermentation. The flocculating activity decreased thereafter. This drop in flocculating activity may be assigned to either cell autolysis or the action of bioflocculant-degrading enzymes produced by microorganisms (Kim et al., 2011; Okaiyeto et al., 2016b). At this time (84 hrs) the exponential growth of cells was also observed. This suggests that the bioflocculant was produced by biosynthesis. Furthermore, the absorbance of the growth medium increases for the rest of the fermentation process, which may be resulted from the metabolic wastes and dead cells contributing to the increase in turbidity of the fermented broth. After 96 hrs of incubation flocculating activity decreases while cell growth (OD<sub>660 nm</sub>), the decreased in flocculation has to do with the shortage of nutrients in the production medium and the presence of bioflocculant-degrading enzymes being produced. Optical density increases was due to the accumulation of dead cells and metabolic wastes which increases the turbidity of the fermentation broth.

Piyo et al. (2011) reported a similar phenomenon whereby a bioflocculant by *Bacillus* sp. Gilbert was produced where the increase in growth period was parallel to the bioflocculant activity. This phenomenon seems to be essential as it implies that the bioflocculant production can be done using the growth pattern of the producing organisms as it is produced through biosynthesis. This could be more useful in terms of the cost reduction.

Cosa et al. (2011) observed the same where *Virgibacillus* sp. Rob showed the highest flocculating activity at day 4 (96 hrs). In addition, *Cobetia* sp. and *Halomonas* sp. OKOH produced a bioflocculant during the exponential phase of cultivation (Ugbenyan et al., 2012).

#### **Extraction and purification of a bioflocculant**

After extraction and purification, the used optimum culture conditions resulted in a yield of about 2.082 g/l of the purified bioflocculant. This figure seems to be a better bioflocculant yield when compared to the yields reported for several microorganisms. For an example, Yang et al. (2012) reported a bioflocculant yield of 1.8 g/l produced by *Klebsiella* sp. while Okaiyeto et al. (2014) reported a maximum yield of 0.738 g/l of the bioflocculant produced by *Micrococcus* sp. The obtained yield is low when compared to 15 g/l reported by Zhang et al. (2007) from multiple-microorganisms' consortium.

#### **Chemical analyses of the purified bioflocculant**

According to Li et al. (2014), it is important to determine the components of the bioflocculant to clarify their flocculation mechanisms. This would help in the optimisation of the flocculating parameters, which would result in an improvement of their efficiency in practical application. In this study, a chemical analysis of the purified bioflocculant from *Bacillus* sp. was revealed to be composed of sugar, protein and uronic acid with sugar being dominant. The chemical analysis revealed that the proportions of the sugar content, protein content and uronic acid were 59%(w/w), 10%(w/w) and 3%(w/w), respectively. The bioflocculant was dominated by carbohydrates which make it heat stable and confirm the hypothesis that the predominant components of bioflocculants are carbohydrates (More et al., 2014). This leads to the assumption that carbohydrates are the most active components in flocculation processes.

Similarly, a number of studies reported bioflocculants to have been dominated by a polysaccharide component (Maliehe et al., 2016). Contrary, Cosa and Okoh, (2014) reported an acidic polysaccharide bioflocculant composed of uronic acid (69%), protein (26%) and sugar (05%).

#### **Fourier transform infrared spectroscopy (FTIR) analysis**

The Fourier transform infrared spectroscopy was used to investigate the functional groups

present in molecular chain of a bioflocculant produced by *Bacillus* sp. The functional groups in the molecular chain of purified bioflocculant by *Bacillus* sp. were assessed using FTIR. The functional moieties of a bioflocculant revealed by IR spectrum (Figure 9) displayed the presence of strong broad O-H bond and weak N-H bond stretching peak at  $3303\text{ cm}^{-1}$ .

These bonds indicated the presence of both alcohol and secondary amine. The presence of hydroxyl group within the polymer favoured the possibility of hydrogen bonding with one or more molecules of water, so all bioflocculants exhibited high solubility in an aqueous solution (Desouky et al., 2008). A sharp absorption peak at  $1666\text{ cm}^{-1}$  indicated the presence of carbonyl group. Carbonyl groups allow the chain to spread out as a result of electrostatic repulsion. A stretched molecular chain provides the adsorption site for particle attachment (Pathak et al., 2014).

A small absorption peak at  $1247\text{ cm}^{-1}$  revealed a C-O bond which indicates the presence of sugar. The spectrum showed strong peaks at  $1063\text{ cm}^{-1}$  and  $1028\text{ cm}^{-1}$  which represent aliphatic amine. The sharp peak at  $893\text{ cm}^{-1}$  indicates the presence of furan sugar (saccharides) and the strong peak observed at  $554\text{ cm}^{-1}$  represents halo compound. Peng et al. (2014) reported a similar spectrum for bioflocculant produced by *Rhodococcus erythropolis*. The availability of the functional groups (carboxyl and hydroxyl) in a molecular chain of a bioflocculant and the strong peak in a range of  $1000\text{ to }1200\text{ cm}^{-1}$  (C-O) are known to represent the normal features the entire saccharides moieties (Luo et al., 2014). Thus, the bioflocculant has a polysaccharide as its main component. The showed functional groups are the major adsorptive forces of a bioflocculant and were perceived to have been involved in bioflocculating activity of the bioflocculant (Cosa and Okoh, 2014). The carboxyl group observed may act as the binding sites for cations, permitting an enhanced flocculation process (Zheng et al., 2008). The infrared spectrum obtained in this study is in line with other spectra generated for a various bioflocculants produced by different microorganisms (Peng et al., 2014; Cosa and Okoh, 2014).

### Thermogravimetric analysis of the purified bioflocculant

To study the pyrolysis property of the purified bioflocculant produced by *Bacillus* sp., the thermogravimetric analyzer was used and the results are shown in Figure 11. The initial weight

of the purified bioflocculant was 99.992% (w/w) at a temperature of  $29.9\text{ }^{\circ}\text{C}$ . A weight loss of about 6.79% was observed when the temperature was increased from  $29.9\text{ }^{\circ}\text{C}$  to  $100.90\text{ }^{\circ}\text{C}$  with the resultant residual weight of 93.207% (w/w). A further increase in temperature to  $150\text{ }^{\circ}\text{C}$  resulted in about 15.54% reduction weight (residual weight of 84.46% (w/w)). The initial weight loss might be due to a loss of moisture content (Okaiyeto et al., 2016b). This moisture content resulted from the availability of both carboxyl and hydroxyl groups in the molecular chain of the purified bioflocculant (Ugbenyen et al., 2014). The higher the carboxyl content of a bioflocculant, the greater the affinity of the polysaccharides for water molecules. The observed decrease in weight from  $150\text{ }^{\circ}\text{C}$  to  $400.9\text{ }^{\circ}\text{C}$  indicates that the decomposition of the main molecular chain of the purified bioflocculant resulted in about 27.90% weight loss. A further increase in temperature to  $794.9\text{ }^{\circ}\text{C}$  resulted in about 33.20% (w/w) weight loss of the initial weight of the purified bioflocculant. More than 60% of the bioflocculant produced by *Bacillus* sp. that appears to be not decomposed. From the obtained results, it can be deduced that the bioflocculant produced by *Bacillus* sp. was thermo-stable and of high molecular weight.

### SEM analyses

SEM analyses were carried out to elucidate the surface morphological structure of the bioflocculant before and after flocculation to kaolin clay. The scanning electron microscope pictures (Figure 11 (a)) showed a crystal-like bioflocculant which is white in colour. This structure reveals an excellent flocculating ability that the bioflocculant might have when in contact with other molecules, such as kaolin clay molecules. The kaolin clay particles in Figure 11(b) appeared to be fine and scattered before flocculation. In Figure 11(c), SEM imaging revealed the very big flocs being formed as the bioflocculant and the kaolin clay coming together, which results in the simple precipitation of flocs due to gravity. The observations seemed to be similar to the findings of Zhang et al. (2007) and Ugbenyen and Okoh (2014) whereby aggregation of flocculated kaolin clay was witnessed.

### Elemental analysis

The elemental analysis of the purified bioflocculant revealed its elemental composition in mass proportion (% w/t): C (19.0), N (1.0), O (48.8), Na (0.7), Mg (2.7), P (7.2), S (0.1), Cl (0.7), K (1.0) and Ca (7.3) (Figure 12). The presence of

a number of elements may have an impact on the flexibility and stability of the bioflocculant. Nearly the same results were reported by Okaiyeto et al. (2016b) and Maliehe et al. (2016) whereby the bioflocculant MBF-W7 possessed differences in mass proportion (% w/w): C (5.25), N (5.55), O

(23.73) and P (10.23) and the bioflocculant TPT<sup>1</sup> composed of N (1.3), C (15.0), O (44.8), P (0.8), Ca (9.0), Cl (2.8), Mg (0.4), S (12.1), K (11.4) and Na (1.9), in mass proportion (% w/t), respectively.

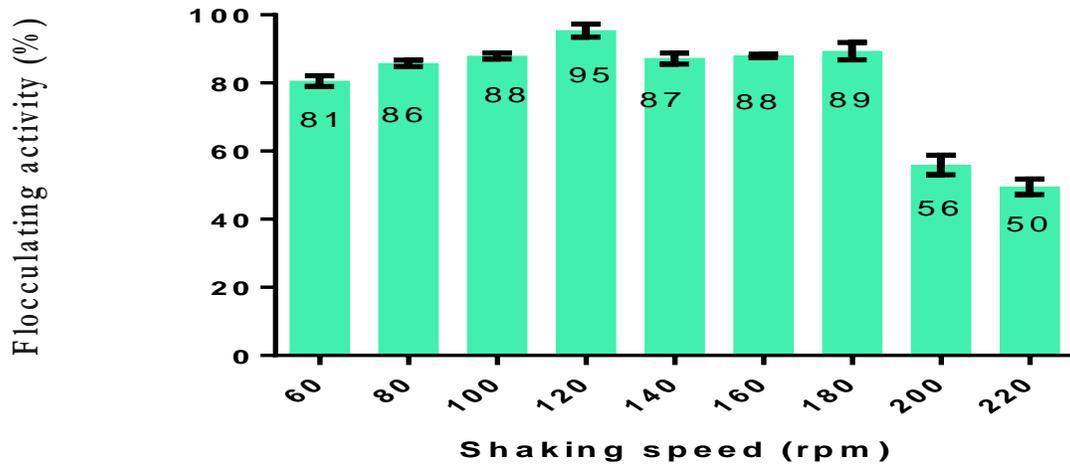


Figure 7: Effect of shaking speed/ agitation on bioflocculant production by *Bacillus* sp.

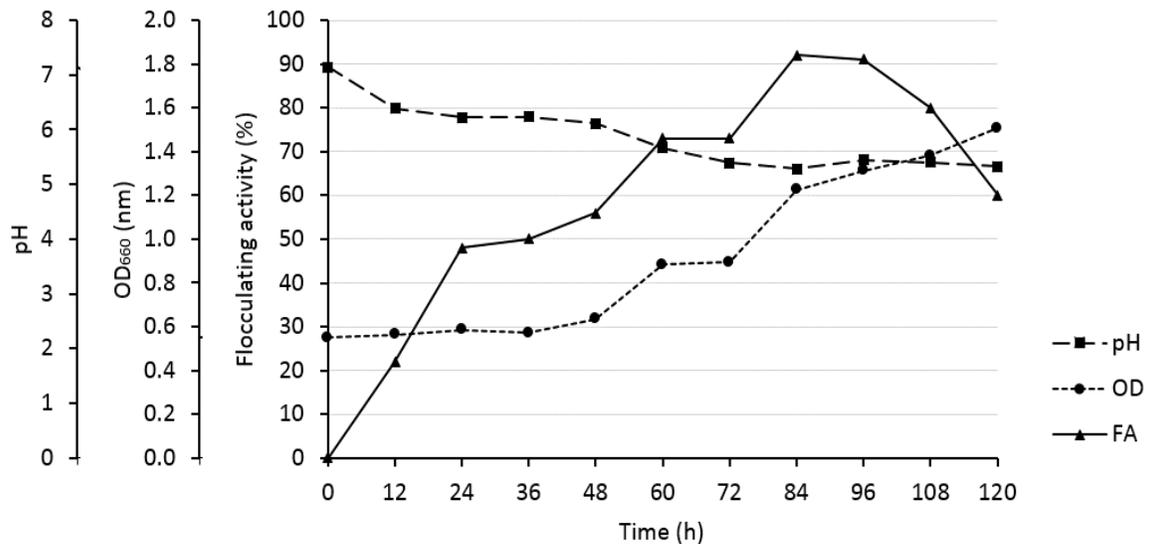


Figure 8: Effect of cultivation time on bioflocculant production

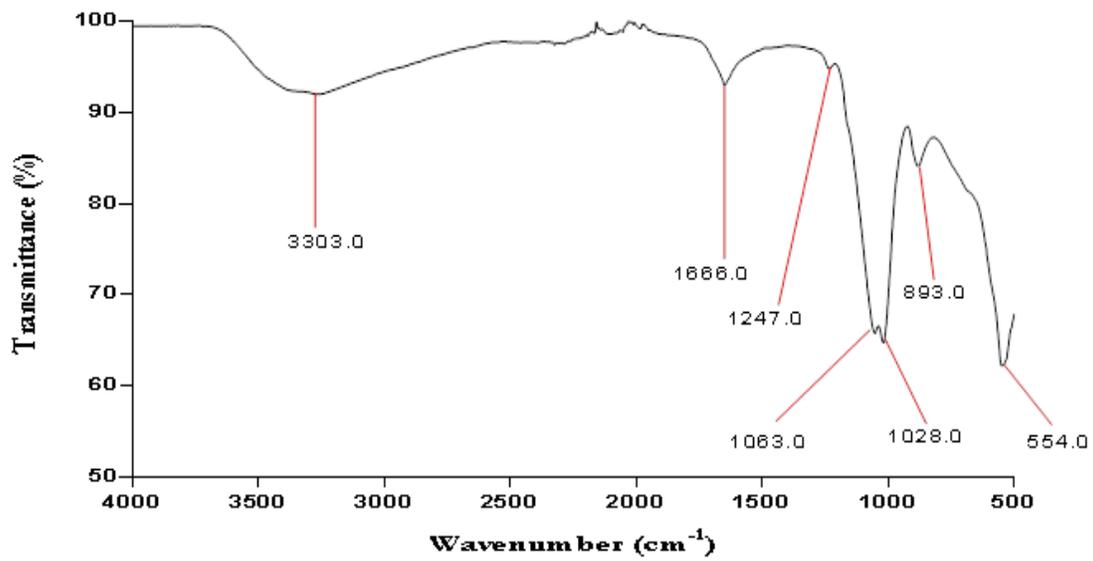


Figure 9: IR spectrophotometric analysis of the purified bioflocculant

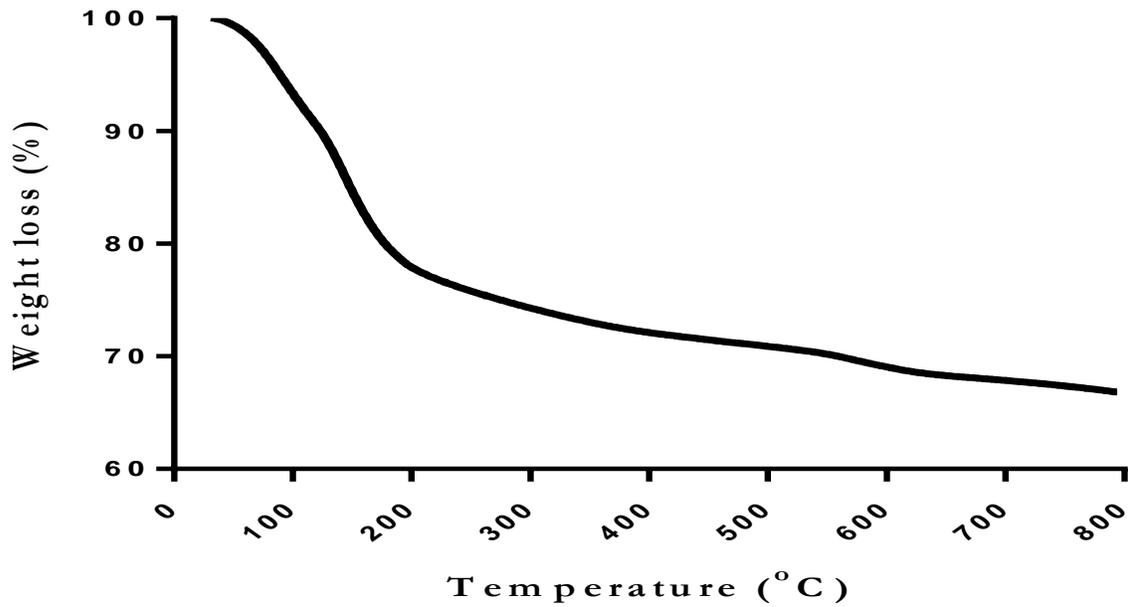
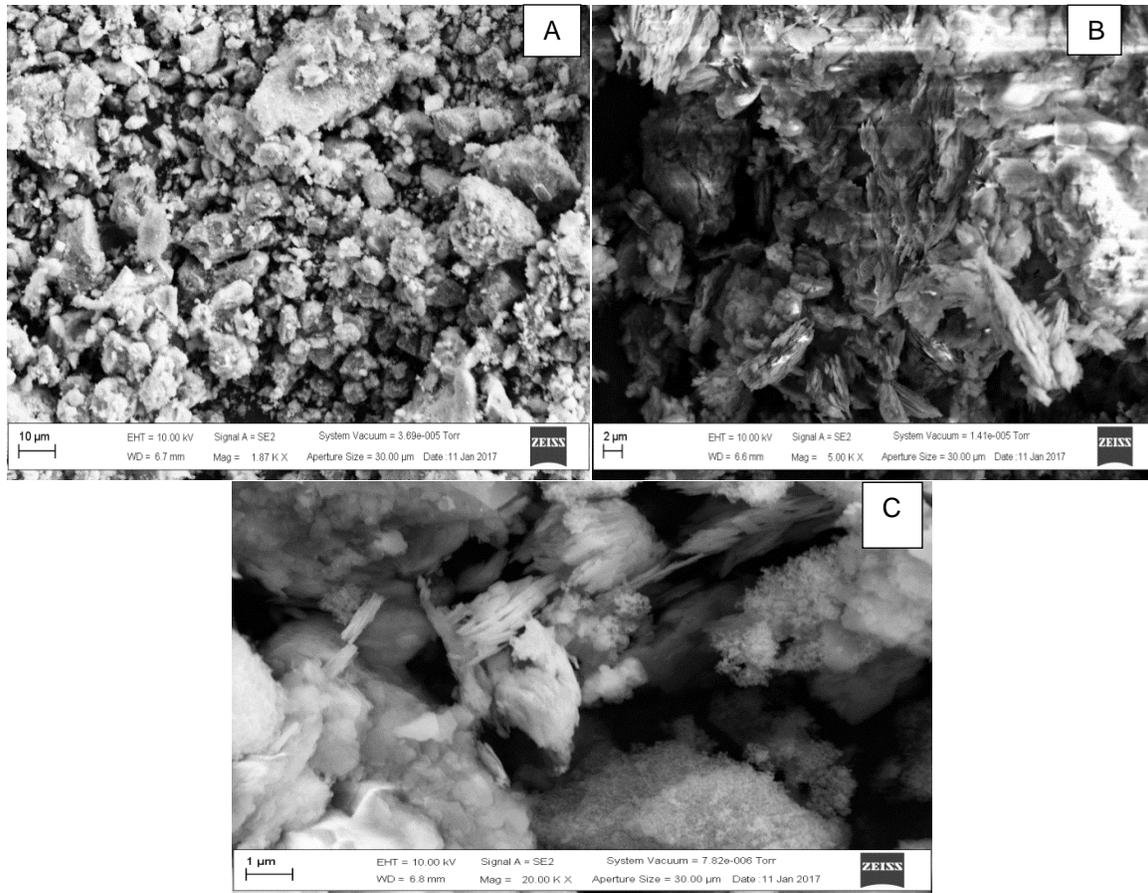


Figure 10: Thermogravimetric analysis of purified bioflocculant



**Figure 11: SEM images of a purified bioflocculant (A), kaolin clay particles (B) and kaolin clay suspension flocculated with a bioflocculant (C).**

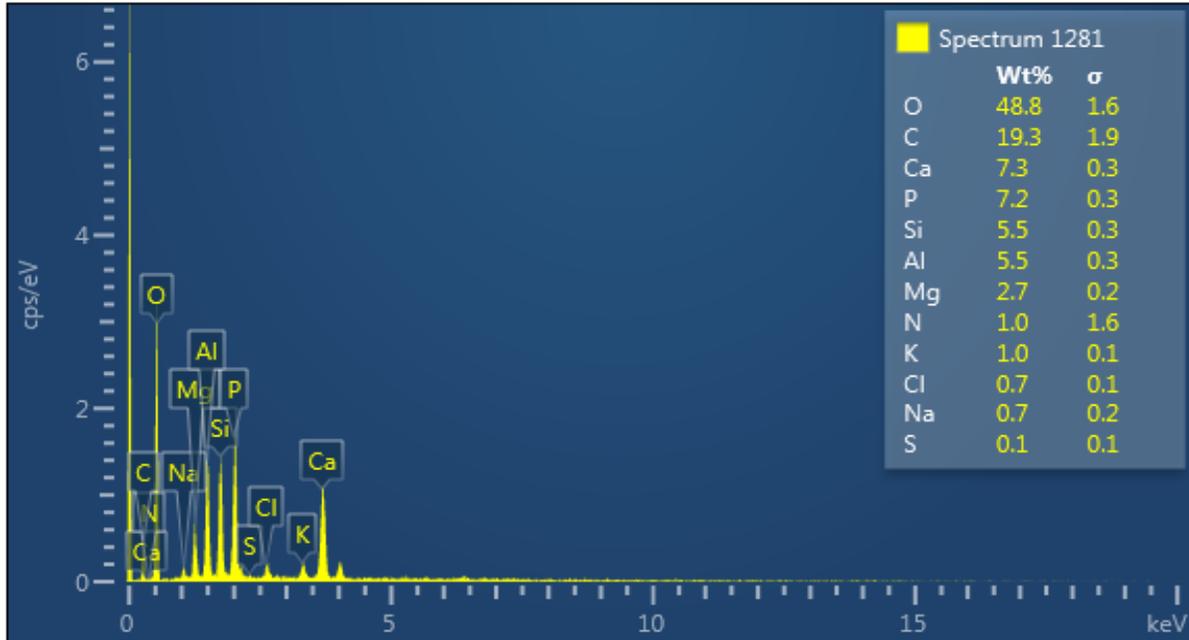


Figure 12: Elementary analysis of a produced bioflocculant.

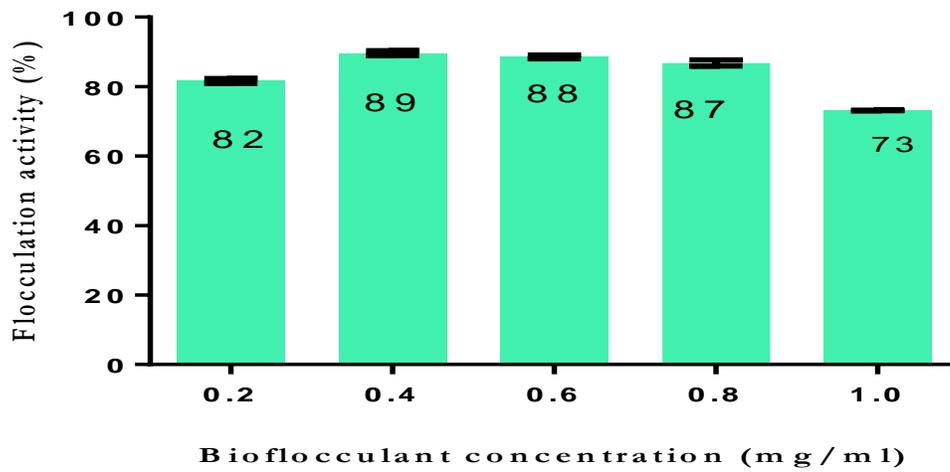


Figure 13: Effect of concentration ([ ]) on flocculating activity

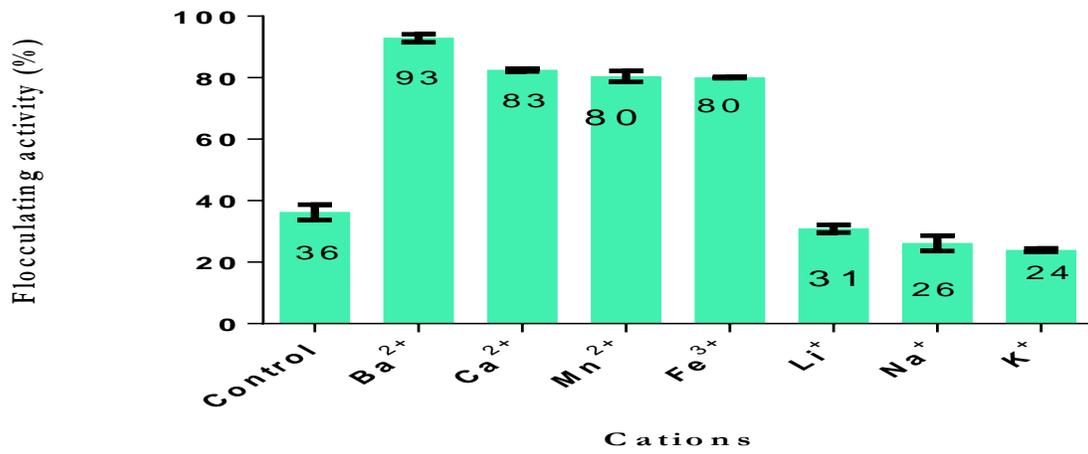


Figure 14: Effect of cations on flocculating activity

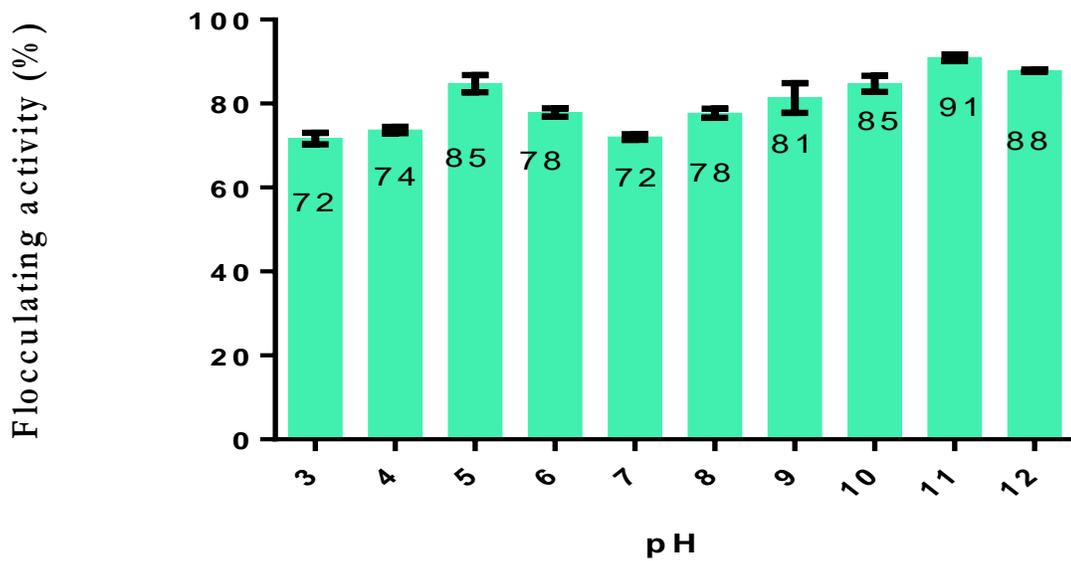
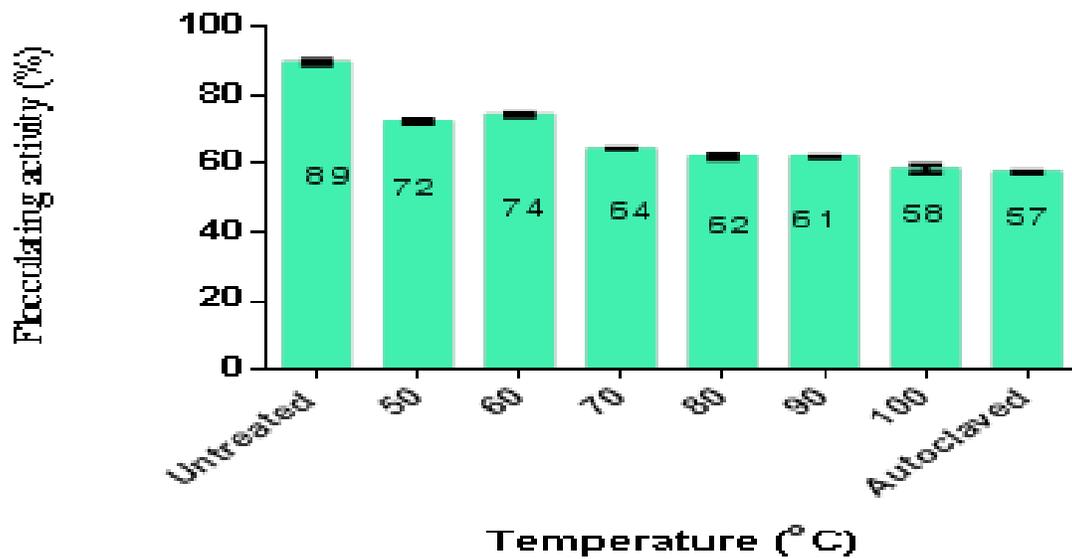


Figure 15: Effect of pH on bioflocculating activity



**Figure 16: The effect of heat on the bioflocculating activity of the bioflocculant**

#### Optimisation of conditions for flocculating activity of the purified bioflocculant

##### Effect of a concentration on flocculating activity

A concentration of a microbial flocculant plays an important role in flocculating activity (Hassan et al., 2009). The effect of different concentrations on the flocculating activity of the purified bioflocculant was investigated. A bioflocculant concentration used in subsequent experiments was determined by testing different bioflocculants concentrations within a range of 0.2 -1.0 mg/ml.

It was observed that as bioflocculants' concentration increases, so does the flocculation to the rate of 0.4 mg/ml after which flocculation began to decrease steadily (Figure 13). According to Okaiyeto et al. (2015a), lower or higher dosages induced the bioflocculant's efficiencies. Less concentration of a bioflocculant results in less absorption and no bridge formation between a bioflocculant and suspended kaolin clay particles. Higher concentration generates high viscosity and blocks the absorption sites, thereby reducing flocculation processing and flocs' formation (Zufarzaana et al., 2012). In this study, an optimum flocculating activity of 89% was recorded when the bioflocculant concentration was 0.4 mg/ml. A decrease in flocculating activity occurred at concentrations below or higher than

0.4 mg/ml. This reduction may be due to the sedimentation of flocs being inhibited by high/low bioflocculant concentrations and resulted in viscosity.

Similar results were obtained by Feng and Xu (2008) and Ntozonke (2015) where the optimum flocculating activities were achieved using 0.4 mg/ml of bioflocculant concentration. Abu-Elreesh et al. (2011) reported the highest flocculating activity with a 0.013 mg/ml bioflocculant concentration while Okaiyeto et al. (2013) reported the highest flocculation activity with a bioflocculant concentration of 0.2 mg/ml.

##### Effect of cations on the purified bioflocculant

According to Okaiyeto *et al.* (2016a), the addition of cations during flocculating activity tests improves the neutralisation and stabilisation ability of the negative charge of both functional groups of kaolin particles in the solutions and the bioflocculants. In this study, different cations were used to examine the effect of cations on the purified bioflocculant by *Bacillus* sp.

In Figure 14, divalent cations were the most effective with  $Ba^{2+}$  leading the race by a flocculating activity of 93% followed by  $Ca^{2+}$  and  $Mn^{2+}$  with flocculating activity of 83% and 80%, respectively. Trivalent cation  $Fe^{3+}$  was also effective with 80%, while all monovalent cations had a flocculating activity of less than 35% and the blank (without cation) was 36%. The role of bivalent 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 (Ugbenyen et al., 2014). The assistance of flocculation by addition of divalent and trivalent cation is of two possible situations, which are (i) that the addition of the metal ion to kaolin suspension decreases the negative charge of the particles and (ii) cation bridging whereby the bioflocculant absorbs onto the kaolin clay particles, thus flocculating them (Sheng et al., 2006; Li et al., 2008). Because divalent and trivalent cations have more surface area for adsorption, they are usually the ones that aid in flocculation. Monovalent cations here showed little effect owing to reduction in the strength of the bonds that inevitably root a loose structure of flocs. This resulting in a decrease in floc density, size and floc resistance to shear (Wu and Ye, 2007). These observations might be due to the large capacities which are more favourable to divalent and trivalent cations than to monovalent cations (Wang et al., 2011). The same results were reported by Nwodo et al. (2012), whereby a combination of bioflocculants and divalent cations greatly enhanced flocculation.

#### **Effect of pH on flocculating activity of purified bioflocculant**

According to Wang et al. (2011), Zaki et al. (2013) and Salehizadeh and Yan (2014), the pH of the reaction mixtures has a major effect on the bioflocculating activity of bioflocculants

The effect of pH of the reaction mixture on the flocculating activity was measured (Figure 15). The effect of pH on flocculating activity of the purified bioflocculant was determined by adjusting the pH of kaolin suspension over a range of 3-12 with 1 N NaOH and 1 N HCl prior to an addition of 0.4 mg/ml bioflocculant solution. The *Bacillus* sp. produced a bioflocculant that had the highest flocculating activity at the extreme pH ranges (acidic and alkaline pH), (Figure 15). A flocculating activity of 91% was observed at pH 11 (alkaline), while at pH 5 (acidic) a flocculation activity of 85% was also observed. The bioflocculant produced in this study seems to be favoured by all pH tested as the flocculating activities of all pH used were greater than 70%. Similar results were observed by a number of researchers, including He et al. (2010), Zhao et al. (2013) and Ntozonke (2015) whereby the bioflocculants showed high flocculation rate at a wide pH range (3–11 pH). This behaviour of a bioflocculant, to be so dualistic at an acidic and

alkaline pH, is still not clear. Literature studies suggest that the pH tolerance of the bioflocculant at a wide range could indicate that it can be applied in a number of industrial fields, to treat various waters or wastewaters without having to adjust the pH of the water thus providing the bioflocculant cost-effectiveness (Okaiyeto et al., 2015b).

#### **Effect of heat on flocculating activity of the purified bioflocculant**

To investigate the effect that heat had on the flocculation of the bioflocculants, various cultivation temperatures ranging from 50 to 100 °C were applied. Another bioflocculant solution was heated with an autoclave (steam under pressure) at 121 °C for 15 min.

It was observed that untreated (unheated) sample shows the highest flocculating activity of 89% (Figure 16). The flocculating efficiency was over 60% at temperatures between 50 and 90 °C and above 50% between 100 °C and 121 °C (autoclaved). The decrease in flocculating activity might be due to the denaturation of the protein content of a bioflocculant. A number of researchers suggested that a bioflocculant's resistance to heat is in line with the general understanding that flocculants made up of high polysaccharides content have a better thermal tolerance than those made up of proteins and nucleic acids (Zhang et al., 2012). This bioflocculant seems to have a strong thermal stability, although the increase in heat had an effect on the flocculating activity. A bioflocculant seems to tolerate high temperature (121 °C) and steam under pressure from the autoclave (as it works with steam under pressure to destroy contaminants). These results could be more useful in term of its applicability as this condition provides a sterile bioflocculant. Since the steam under pressure is more useful in destroying contaminants. These observations suggested that the thermal stability of a bioflocculant is due to the carbonyl and hydroxyl groups present (Figure 9) that might have allowed a hydrogen bonds formation (Ugbenyen and Okoh, 2014; Maliehe et al., 2016).

Literature studies revealed a number of bioflocculants reported to exhibit thermal stability. For example, Li et al. (2007) and Makapela et al. (2016) reported a bioflocculant that is able to maintain and to retain high flocculating activity at 100 °C. *Virgibacillus* species isolated from Algoa bay was reported to produce a thermostable polysaccharide bioflocculant retained about 84, 80

and 79% flocculating activity at 50, 80 and 100°C respectively (Cosa et al., 2013).

#### Application of purified bioflocculant in the treatment of wastewater

The ability of the bioflocculant produced by *Bacillus* sp. to flocculate various suspended

particles in domestic wastewater as well as in coal mine wastewater (Thendele coal mine, Somkhele area, Mtubatuba, KZN, RSA) was investigated and the results shown in Table 1 and Table 2, respectively.

**Table 1: Application of the bioflocculant in the treatment of domestic wastewater.**

Flocculants		BOD <sub>5</sub> (mg/L)	COD (mg/L)	Sulfide (mg/L)	Flocculating activity at OD <sub>550 nm</sub>
Alum	Before	38	404	0.85	0.395
	After	25	231	0.35	0.083
	Removal rate/Flocculation efficiency (%)	34	43	59	79
FeCl <sub>3</sub>	Before	38	404	0.85	0.395
	After	24	251	0.27	0.089
	Removal rate/Flocculation efficiency (%)	37	38	68	78
Microbial	Before	38	404	0.85	0.395
	After	12	210	0.25	0.035
	Removal rate/Flocculation efficiency (%)	68	48	71	91

**Note:** Values are means of triplicates data:

**Table 2: Application of the bioflocculant in the treatment of coal mine wastewater.**

Flocculants		BOD <sub>5</sub> (mg/L)	COD (mg/L)	Sulfide (mg/L)	Flocculating activity at OD <sub>550 nm</sub>
Alum	Before	58	1557	0.90	1.936
	After	21	276	0.27	0.278
	Removal rate/Flocculation efficiency (%)	64	82	70	85
FeCl <sub>3</sub>	Before	58	1557	0.90	1.936
	After	27	150	0.19	0.139
	Removal rate/Flocculation efficiency (%)	53	90	79	93
Microbial	Before	58	1557	0.90	1.936
	After	0.74	116	0.15	0.098
	Removal rate/Flocculation efficiency (%)	99	93	83	95

**Note:** Values are means of triplicates data:

Two conventional flocculants (i.e. alum and iron chloride (FeCl<sub>3</sub>)) were also used for comparison. The removal efficiencies of BOD, COD and sulphide as well as the flocculation efficiency of the bioflocculant in domestic wastewater (Table 1) were 68%, 48%, 71% and 91%, respectively. The removal efficiency of the bioflocculant was better when compared to both conventional flocculants (alum and FeCl<sub>3</sub>), during

which 34% (BOD), 43% COD and 59% (sulphide) and 37% (BOD), 38% (COD) and 68% (sulphide), respectively, were recorded. Their flocculation efficiencies (79% and 78%, respectively) were much better but lower than that of a bioflocculant.

It was also found that in the treatment of coal mine wastewater, a bioflocculant has shown better removal efficiencies as well as flocculation efficiency when compared to both Alum and FeCl<sub>3</sub>

flocculants (Table 2). The removal efficiencies of BOD, COD and sulphide as well as the flocculation efficiency of a bioflocculant were 99%, 93%, 83% and 95% respectively. The removal efficiency of the bioflocculant was high compared to both conventional flocculants (alum and  $\text{FeCl}_3$ ) at 64%, (BOD), 82% (COD) and 70% (sulphide) and 53% (BOD), 90% (COD) and 79% (sulphide), respectively. The flocculation efficiency of the bioflocculant (95%) was higher than that of alum and  $\text{FeCl}_3$  flocculants, at (85% and 93%, respectively). This bioflocculant can be used in the treatment of coal mine wastewater and domestic wastewater.

The results obtained from this study were in line with the findings recorded by other researchers (Lian et al., 2008; Ugbenyen and Okoh, 2014).

Based on these findings, one can deduce that the purified microbial flocculant attained an enhancement in biochemical oxygen demand, chemical oxygen demand as well as sulphide removal and enhanced efficiencies for both domestic and coal mine wastewaters compared to both chemical synthesized flocculants

## CONCLUSION

A bacterium, *Bacillus* sp., that produced a bioflocculant was obtained from uMlalazi estuary, Mthunzini area, KZN, RSA. The production of a microbial flocculant was parallel to the cell multiplication of an organism with an optimum production achieved with an inoculum size of 1% (v/v), glucose and combined nitrogen (made up of yeast extract, ammonium sulphate and urea) were preferred as sole carbon and nitrogen sources to produce a bioflocculant, temperature of 40 °C, agitating speed of 120 rpm, initial pH 6 for 84 hrs,  $\text{K}^+$  and produced 2.082 g/l yield. The produced bioflocculant was revealed to be composed of carbohydrate (59%), protein (10%) and uronic acid (3%). The molecular chain of a bioflocculant possessed functional groups such as amino, carboxyl and hydroxyl groups. The purified microbial flocculant had high bioflocculating efficiency at 0.4 mg/ml and had an effective performance in pH range between 3 and 12. The bioflocculant can assist in reducing BOD and COD and aid in sulphide removal efficiencies in domestic and coal mine wastewater. This bioflocculant could be the better alternative to health and environmental problems associated with conventional flocculants in wastewater treatment.

## 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: AKB, VSRP, JJS and ME; Formal analysis: ZGN, KT, AKB and JJS; Investigation: ZGN and KT; Supervision: AKB, JJS, VSRP and ME; Writing (original draft): ZGN; Writing (review and editing): AKB, VSRP, JJS and ME.

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