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Production and characterization of a bioflocculant produced by the consortium of *Bacillus safensis* and *Bacillus* sp. isolated from uMlalazi catchment, Mthunzini area, KwaZulu-Natal

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Abstract: Multiple microorganism consortia have been reported to improve bioflocculant yield and flocculating activity compared to single strains. Consequently, the study aimed at improving bioflocculant yield through construction of a consortium of *Bacillus safensis* KX694275 and *Bacillus* sp. KC782848. The bacteria were previously isolated from uMlalazi catchment, in the Province of KwaZulu-Natal, Republic of South Africa. The bioflocculant was produced optimally using 2% (v/v) inoculum size, starch and ammonium sulphate as energy sources, Fe^{3+} as a stimulating agent, shaking speed of 165 rpm, temperature of 30 ^{o}C and initial pH 3. Compared to single strains yields of 1.55 g (*Bacillus* sp.) and 2.10 g (*B*. *safensis*) per Litre of fermented broth, a mixture of the two cultures produced 8.5 g bioflocculant yield from 1 Litre of fermentation broth after 72 hrs incubation. 0.4 mg/mL dosage was optimally clarified 4 g of kaolin clay in 1000 mL of distilled at neutral pH. The thermostable bioflocculant was effective over a wide range of pH with the maximum flocculating activity of 95% obtained at pH 8. The microbial flocculant was composed of carbohydrate (78%), protein (05%) and uronic acid (16%). FTIR analysis revealed the presence of hydroxyl, carboxylic and amine group in its molecular chain as the main functional groups responsible for flocculation process. The high flocculation efficiency obtained with the purified bioflocculant portends its industrial applicability.

Keywords: *Bacillus safensis,* consortium, bioflocculant, flocculating activity, kaolin clay, *Bacillus* sp.

INTRODUCTION

Organic water frequently holds rigid pollutants that can be classified as suspended colloids, detritus or dissolved particles (Spellman, 2014). These pollutants have the ability to root water-borne diseases including cholera, if not effectively removed (Hemsen and Dube, 2004). They are often suspensions of nanoparticles that are thermodynamically unstable, kinetically non-labile which do not easily settle down. Effective disposal of these colloidal dispersions in water and wastewater is greatly impacted by the electrokinetic properties on the surface of the colloids (Spellman, 2014). Physico-chemical treatment such as flocculation has being employed in the removal of these colloidal pollutants (Karthiga and Natarajan, 2015).

Flocculation, as a treatment method, is a

physical purification technique whereby destabilized colloidal particles are enhanced to agglomerate and form large flocs (Salehizadeh and Shojaosadati, 2001). These flocs formed can be effectively and easily detached through sedimentation, floatation and filtration processes. Flocculants are flocculating agents which are grouped according to their chemical composition into three groups, namely inorganic, organic synthetic and biological flocculants (Yim et al., 2007). Synthetic (organic and inorganic) flocculants have been primarily actionable in different biotechnological fields due to their cost effectiveness and reliability (Yumei et al., 2014). However, they have been reported to have negative effects towards human health and the environment (Arafa et al. 2014). Reports show that they cause neurotoxin and cancer derivatives (Serdar et al. 2011) and they're non-degradable in nature (Ahmad et al. 2015).

Bioflocculants are extracellular polymeric substances often secreted by bacteria, as they grow, as a result of bacterial interaction with the environment due to substrate metabolism, bacterial maturation, cell lysis and degradation of bacteria or bacterial chemical components (Komilis et al., 2016). Bioflocculants are composed of organic components including polysaccharides, lipids, proteins, nucleic acids and other polymeric compounds (Carlos et al. 2011). They are effective at low dosage concentrations, benign in nature, innocuous to humans and animals and dearth secondary pollution. However, bioflocculant manufacturing has been restricted by insufficient production yields by microorganisms (Maliehe et al. 2016). The lack of knowledge concerning the characteristics of active components has also contributed to their restrictions (Liu et al. 2015). Nevertheless, researchers have recently focused attention on increasing their yields through the use of microbial consortia (Zhang et al. 2007). As the microorganisms naturally do not reside in segregation but they exist together and result in synergistic and symbiotic relationships. Reports show that the fusion of strains of microorganisms in consortia result in bioflocculants with better flocculation efficiency and greater bioflocculant yields as opposed to individual strains (Zhu et al. 2004). Thus the study focused mainly on the production and characterization of bioflocculant from the consortium of *B*acillus *safensis* KX694275 and *Bacillus* sp. KC782848 in the attempt to improve the yield.

MATERIALS AND METHODS

Isolation and maintenance of bacterial strains

The bacteria were isolated from the sediments and water samples collected from uMlalazi catchment, Mthunzini area in the Province of KwaZulu-Natal. Serial dilutions and spread plate techniques were performed as describe by Jensen et al. (2005). Hundred microliters (100 µL) of diluted and undiluted water samples were grown into the nutrient agar plates up to 72 hrs at 37 ^{o}C. Colonies were selected randomly based on their morphology, size as well as structure and subcultured into nutrient agar plates to obtain pure cultures. Pure cultures were maintained in 20% glycerol broth at −80 °C refrigeration in the Microbiology laboratory at the University of Zululand.

Sample preparation

The sample was prepared according to the description of Cosa et al. (2011). A production medium made up of glucose (20 g), urea (0.5 g), yeast extract (0.5 g), $(NH_4)_2SO_4$ (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 1000 mL of filtered natural sea water. About 50 mL of production medium was transferred into 100 mL conical flasks prior to autoclaving at 121 ^{0}C for 15 min. Fifty millilitres of sterile production medium was inoculated with one loop full of bacterial colony and incubated for 72 hrs at 30 °C in a shaker at 160 rpm. Two millilitres (after incubation) was centrifuged at 8000 x g for 30 min at 4 °C to remove bacterial cells. The cell-free culture supernatants were used to determine flocculating activities.

Bacterial consortium

B. safensis KX694275 and *Bacillus sp* KC782848 were used in mixture to build bacterial consortium. One loop full colony from each strain was inoculated separately into 50 mL of the fermentation broth and incubated for 72 hrs at 30 °C with the shaking speed of 160 rpm. The fermented broth was then used as the standard inoculum. For construction of the bacterial consortium, 1 mL of the standard inoculum of bacterial strains were both inoculated into the same 50 mL of the freshly prepared fermentation medium and incubated at 30 °C for 72 hrs in the shaking incubator with a speed adjusted to 160 rpm. After 72 hrs of fermentation, the fermented broth was centrifuged at 8000 x g for 30 min, 4°C and the clear supernatant was used for

determination of flocculating activity (FA) (Zhang et al. 2007).

Determination of flocculating activity

The method used by Ugbenyen et al. (2012), a modification of Kurane et al. (1986) was used for determination of the flocculating activity of the constructed bacterial consortium. Kaolin clay was used as the test material. A kaolin clay solution of 0.4% was prepared in 1 L of distilled water. 100 mL of kaolin clay solution was dispensed measured into 250 mL conical flask. 3 mL of 1% w/v $CaCl_2$ and 2 mL of obtained cell-free supernatant were added. The mixture was shaken vigorously for 1 min and transferred into 100 mL measuring cylinder. The mixture was then allowed to sediment for 5 min at room temperature. The control was prepared by replacing the cell-free supernatant with freshly prepared production medium. The optical density (OD) of the clarifying supernatant was measured using ultra-violet (UV) spectrophotometer at 550 nm. The flocculating activity was calculated using the following formula:

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

Where A is the optical density of control at 550 nm and B is optical density of a sample at 550 nm.

Optimisation of culture medium conditions for bioflocculant production

The optimum medium culture conditions for improved flocculating activity as well as bioflocculant production were established. The inoculum size (%, v/v), nutrients sources, initial pH of culture medium, shaking speed, temperature, cations, and time course were varied.

Determination of inoculum size

Different concentrations of the constructed consortium broth culture were prepared ranging from 1- 5% (v/v). Inoculations were made to 50 mL production medium and incubated for 72 hrs at 30 ^oC and 160 rpm shaking speed. After 72 hrs of incubation, their flocculating activities were evaluated using kaolin clay solution (0.4% w/v). The flocculating activity was measured as described above (Piyo et al. 2011).

Effects of energy sources on flocculating activity

To determine an appropriate energy (carbon and nitrogen) sources for bioflocculant production, flocculating bacteria in consortia were supplied with various carbon sources (glucose, fructose, sucrose, lactose, maltose, xylose and starch). Preparations were made by substituting glucose with same concentration (20 g/L) in the production medium. The effects of various organic nitrogen sources (yeast extract, peptone and urea) and inorganic nitrogen sources [$(NH_4)_2SO_4$ and $(NH_4)_2NO_3$] were assessed by incorporating each in basal medium replacing the initial nitrogen sources of 1.2 g made up of urea (0.5 g) yeast extract (0.5 g) and $(NH_4)_2SO_4$ (0.2 g) in equivalent amounts. Flocculating activities were measured thereafter (Zhang et al. 2010).

Effect of shaking speed on flocculating activity

The effect of shaking speed on the flocculating activity was investigated using the method described by Ogunsade et al. (2015). Different shaking speeds were used in the range of 0 - 220 rpm at 30 °C for 72 hrs on a rotary shaker prior to the determination of the flocculating activity of the test microorganisms.

Effect of initial pH on flocculating activity

The initial pH of the culture medium was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with 1 N HCl and 1 N NaOH prior to incubation at each of these pH values. The flocculating activities were measured (Ntozonke et al., 2017; Akapo et al. 2019).

Effect of heat on flocculating activity

To study the effect of heat on the flocculating activity of an organism, the method described by Makapela et al. (2016) was used. The flocculating bacterial consortium in optimum medium was incubated at different temperatures such as 20, 25, 30, 35, 40, 45 and 50 °C, for 72 hrs at 160 rpm. The flocculating activity was determined thereafter as described above.

Effect of metal ions on flocculating activity

To investigate the effect of metal ions, 3 mL cation and 2 mL bioflocculant were added into 100 mL kaolin solution. $CaCl_2$ solution (1% w/v) used as a stimulating agent was replaced by various metal salt solutions including 1% (w/v) sodium chloride (NaCl), potassium chloride (KCl), lithium chloride (LiCl), manganese chloride ($MnCl_2$), barium chloride ($BaCl_2$), magnesium chloride ($MgCl_2$) iron chloride ($FeCl_3$) and flocculating activities were measured (Nie et al. 2013).

Time course assay

The effect of fermentation time on flocculating activity by the test organisms was conducted in accordance with a method by Gao et al. (2006). Bacterial culture in consortium was grown under obtained optimal growth conditions. Seed culture was prepared by inoculating 50 mL enriched medium with 2% inoculum of the consortium and incubated at 30 ^{o}C in a rotary shaker (165 rpm). For optical density ($OD_{600\ nm}$) test, freshly prepared saline solution was used to dilute fermented broth to 0.1. From the seed culture, 2% inoculum was inoculated to 50 mL of sterile production medium in 100 mL conical flask and incubated for 7 days at 30 ^{o}C and shaken at 165 rpm. About 10 mL aliquot were withdrawn periodically at timed intervals of 12 hrs for 5 days to measure pH and cell growth ($OD_{600\ nm}$). 2 mL was centrifuged at 8,000 x g for 30 min and the cell- free supernatant was used to determine the flocculating activity as described by Kurane et al. (1986).

Extraction and purification of the bioflocculant

The concentration and purification of bioflocculant from the bioflocculant-rich broth was done in accordance with the methods of Chang et al. (1998) and Chen et al. (2003), with minor alterations. After 72 hrs of fermentation, the culture broth was centrifuged at 8,000 x g, 4 °C for 15 min. To eliminate the undissolved materials, one volume of deionized water was mixed with the supernatant phase and re-centrifuged at 8,000 x g for 15 min, 4 °C. Two volumes of ice-cold ethanol were added to the supernatant and the mixture was incubated at 4 °C for 12 hrs. After 12 hrs of incubation, the precipitate was collected and vacuum-dried to obtain a crude bioflocculant. The obtained crude product was dissolved in 100 mL distilled water to yield a solution (w/v). One volume of a mixture of chloroform and n-butyl alcohol or methanol (5:2 v/v) (2:1) was added into the bioflocculant solution. After agitating, the mixture was left to stand at room temperature for 12 hrs to remove impurities. The top layer of the mixture was collected and vacuum dried to obtain a purified bioflocculant.

Characterization of a purified bioflocculant

Chemical composition analysis of the purified bioflocculant

The total sugar content was determined using the phenol-sulfuric method as described by Chaplin and Kennedy (1994) with glucose used as a standard. Bradford method as described by Bradford et al. (1976) with Bovine serum albumin (BSA) as a standard was used to estimate the total protein content of the purified bioflocculant. Uronic acid content of a bioflocculant was measured using carbozole method (Cesaretti et al., 2003) with glucoronic acid as a standard.

Scanning electron microscope (SEM) analysis of a bioflocculant

The surface morphological structure of the purified bioflocculant as well as the elements present in the bioflocculant was determined using scanning electron microscope equipped with an energy-dispersive X-ray analyser (EDX) (JEOL JSM-6100). A Tungsten (W) filament and acceleration voltage of 12 kV was used to take images with an emission current of 100 Å. Bioflocculant powder was placed on copper stubs with double-sided carbon tape and carbon coated using the JEOL vacuum evaporator. The samples were then analysed and EDX was performed using JEOL JSM 6100 SEM with Bruker Quantax Esprit software. The elemental composition of a purified bioflocculant in percentage weight was recorded.

Fourier transform infrared spectrophotometer (FT-IR) analysis

The presence of functional groups in the purified bioflocculant was investigated using the Bruker Tensor 27 Fourier transform infrared spectrophotometer (Bruker, Gauteng, South Africa) with standard ATR cell. Prior to every analysis, the surface of the cell was cleaned with ethanol. For proper contact between the surfaces, the pressure was adjusted to 90 gauge.

Thermogravimetric analysis (TGA)

The pyrolysis analysis of the purified bioflocculant was assessed using thermogravimetric analyser, Perkin-Elmer Thermal Analysis Pyris 6 TGA in a closed perforated aluminium pan under nitrogen gas with a flow rate of 40 cc/min. To analyse, 10 mg of a bioflocculant was used over the temperature range of 30 – 800 ^{o}C at a heating rate of 10.00 ^{o}C/min.

Flocculation characteristics of a purified bioflocculant

Effect of dosage concentration on flocculating activity (Jar test)

To determine the optimum bioflocculant dosage, the method used by Ogunsade et al. (2015) was followed with minor changes. Various concentrations of aqueous bioflocculant solutions were prepared and used for the determination of the optimum bioflocculant dosage. Concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were used. Two millimetres of the bioflocculant solution was mixed with 100 mL of kaolin suspension (0.4% w/v) containing 1% (w/v) $CaCl_2$ in 250 mL conical flask. The solution was agitated rapidly and transferred into 100 mL graduated cylinder and left to settle for 5 min at room temperature. About 1 mL of the clear upper phase of the supernatant was removed for determination of flocculating activity. The flocculating activity was measured at 550 nm as previously described.

Effect of cations on flocculating activity of a purified bioflocculant

To investigate the influence of different metal ions on flocculating activity of a purified bioflocculant, the method by Zulkeflee et al. (2012) was utilized. The analysis was done by replacing the 1% (w/v) $CaCl_2$ solution with various salt solutions including KCl, NaCl, LiCl, $BaCl_2$, $MgCl_2$, and $FeCl_3$, at the same concentration. Flocculating activity was measured as previously described using the optimum bioflocculant concentration.

The effect of heat on flocculating activity of a bioflocculant

To investigate the thermal stability of the purified bioflocculant, the method by Ahmad et al. (2015) was utilized. The optimum bioflocculant was dissolved in distilled water to prepare a bioflocculant solution (w/v). 2 mL aliquots of the bioflocculant solutions were heated for 30 min at different temperatures ranging from 50 – 100 ^oC. The residual flocculating activities were measured using kaolin solution (0.4% w/v) as described previously at room temperature.

The pH stability of a bioflocculant

The pH stability of a purified bioflocculant was determined according the method used by Okaiyeto et al. (2013). The pH of kaolin solutions in separate flasks were adjusted to different pH values ranging from pH 3-12 using 1N NaOH and 1N HCl prior to the assessment of flocculating activity. Flocculating activity was measured as previously described using the optimum bioflocculant concentration.

Statistical analysis

All experiments were conducted in triplicate, with the mean and standard deviation for each experiment determined using Graph Pad Prism ™ 6.1. Statistical differences in the percentage bioflocculant activity amongst the treatment mean was analysed using the analysis of variance (ANOVA) test, where differences greater than 0.05 are considered significant.

RESULTS AND DISCUSSION

The secretion of biological active microbial flocculants is naturally very low. This is due to the fact they are less important for the microbial growth or only the small amount they need for satisfaction of their primary needs. Normally, the less synthesis of active compounds is often in line with unfriendliness economically, particularly at industrial level (Barredo, 2005). Therefore, optimization of culture condition for cultivation is done not only to increase microbial flocculants production but also to improve flocculating activity. Naturally, microbes coincide in bionomical niches and also possess correlations that have number of practical biotic effect adequacy for the entire interacting species. According to Maliehe et al. (2016), microorganisms biologically interact with each other as well as their nearby parameters (physical and chemical) in different ways resulting in a complete complicated relationship, including synergism and symbiosis. Reports, however, show that the use of combination of strains in consortium has proven to yield a better bioflocculant with an improved flocculating activity as opposed to utilization of single strains of bacteria (Cosa and Okoh, 2013). Thus, this study was focussed mainly on the production and characterization of a bioflocculant from the combination of two microorganisms in consortium.

Optimization of culture conditions for bioflocculant production

The effect of inoculum size on bioflocculant production

Inoculum size is a fundamental factor in the cell growth as well as in the bioflocculant production as the small inoculum size extends the lag phase of growth which can hinder the bioflocculant production and outsized inoculum

size makes the niche of the strains overlap extremely and inhibiting the growth and production due to a lack of oxygen and not enough supplier of the nutrients (Tawila et al., 2018). In this study, the effect of inoculum size range of 0.5 mL (1% v/v) - 2.5 mL (5% v/v) in 50 mL of sterile production medium was investigated and the results obtained are depicted in Figure 1.

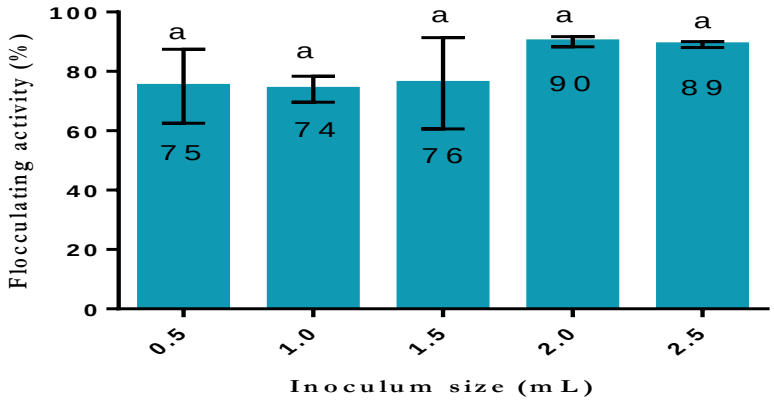


Figure 1: Effect of inoculum size on flocculating activity of the mixed culture

The flocculating activity greater than 70% was observed in all inoculum sizes tested with the highest flocculating activity of 90% obtained with 2 mL [4% (v/v)] inoculum size. Above and below 4% (2 mL) inoculum size, the flocculating activity decreased. Therefore, the 4% (v/v) inoculum size endorsed the adaptation of strains to the production medium thus promoting the production of bioflocculant (Li et al., 2009). An inoculum size of 4% (v/v) was used in all subsequent tests. Similar optimum inoculum size (4% v/v) was reported by Ntsangani et al. (2017) for bioflocculant production by *Bacillus* sp. AEMREG4. 4% (v/v) inoculum size was also documented by Okaiyeto *et al*. (2014) whereby *Micrococcus sp*. Leo produced a bioflocculant with an optimum flocculating activity above 80%. Contrary to the study finding, Gong et al. (2008) reported 2% optimum inoculum size for the bioflocculants produced by *Serratia ficaria*.

The effect of carbon sources on bioflocculant production

Bioflocculant production is influenced by numerous factors such the constituents of the cultivation medium as well as culture conditions. The effect of different carbon sources for bioflocculant production by the bacteria was evaluated and the results are shown in Figure 2. Among the tested carbon sources starch was the best carbon source with a flocculating activity of 93% (Figure 2). Glucose (83%), xylose (64%) and fructose (59%) also improved the flocculating activity for bioflocculant production with sucrose showed less flocculating activity (42%). Starch was then chosen as the sole carbon source for all subsequent experiments. Reports show that a number of bioflocculant- producing bacteria prefer organic carbon sources for them to produce optimum microbial flocculant yields, thus supporting the study observations (Cosa et al., 2011). For example, bioflocculant produced by *Bacillus licheniformis* X14, in which the starch and sucrose among other tested carbon source were the most preferred carbon sources was reported by Li et al. (2009). Starch as a sole carbon sources was also reported to produce bioflocculant optimally (Ntsangani et al. 2017). This finding is similar to the study finding with the exception that sucrose was a least preferred carbon source. Contrary, glucose has been well documented as fine carbon source for microbial flocculant production (Suh et al., 1998). For example, different carbohydrates were tested for bioflocculant production by *Paenibacillus polymyxa* BY-28 with glucose as the best (Gong et al. 2003).

The effect of nitrogen sources on bioflocculant production

The effect of nitrogen sources on bioflocculant production was assessed and the results are shown in Figure 3. Both organic (peptone) and inorganic nitrogen sources (ammonium sulphate) favoured the production of bioflocculant by the consortium in this study with ammonium sulphate

being more effectively used and resulted to the flocculating activity of 74%, while peptone has produced 71% flocculating activity. But all tested nitrogen sources producing more than 50% flocculating activity with yeast extract as a least preferred nitrogen source. Therefore, the inorganic nitrogen source ammonium sulphate was used for subsequent tests. Similar to the study, Gouveia et al. (2019) reported ammonium sulphate as the most utilized inorganic nitrogen sources for the production of bioflocculant by *Bacillus megaterium* from sugarcane crop soil or from sludge of agro-industrial effluent. Ammonium sulphate as a nitrogen source was also reported to optimally promote the production of bioflocculant by *Bacillus sp.* from the sediment of Algoa Bay of the Eastern Cape Province (South Africa) (Okaiyeto et al. 2016). Peptone as the best nitrogen source was reported in the production of a bioflocculant by *Paenibacillus elgii* B69 (Li et al. 2013). On the other hand, yeast extract was a choice of nitrogen source to produce a bioflocculant by *Rhizobium radiobacter* SZ4S7S14 was also reported (Rasulov et al. 2017).

The effect of initial pH on bioflocculant production

The initial pH of the medium plays an important role in cell charges determination and oxidation-reduction potential which interns affect the nutrients absorption and enzymatic reactions of microbial cells (Zhang et al. 2013). This implies that different microorganisms adapt on different ranges of pH, thus, varying the pH may have the negative effect in the fermentation medium composition resulting in altered bioflocculant activities (Salehizadeh and Shojaosadati, 2001). In this study, bacteria adapted and produced an optimum bioflocculant when they were exposed to more acidic environment (pH 3, 4 & 5) with pH 3 has the highest flocculation rate of 83% (Figure 4). The slightly decrease in flocculating activity is observed from pH 4 (81%) and drastically at pH 12 (42%) which may be due to the microorganisms producing the acid as they use the nutrients available (Okaiyeto et al. 2013). Just like the bioflocculant produced in this study, Gouveia et al. (2019) also reported pH 3 for optimum production of a bioflocculant by *Bacillus thuringiensis*. The decrease in flocculating activity as the medium pH increases is attributed to the interference of the hydroxyl ion (OH^-) during the formation process of the flocs through the complex of bioflocculant and clay in the presence of cations, resulting to its suspension. Contrary to the study findings, Cosa et al. (2013) reported that a basic pH is more effective in the production of thermostable polysaccharide bioflocculant from *Virgibacillus* species isolated from Algoa bay with pH 10 as the most preferable one. Agunbiade et al. (2018) reported neutral pH 7 for an optimum bioflocculant production by *Streptomyces platensis*. The effect of initial pH of the medium for production on microbial synthesis varies with different bacterial strains, conditions being used and medium composition (Shu and Lung, 2004).

The effect of cations on flocculating activity

The production of microbial flocculants is influenced by the availability of metal ions in the production medium (Shen et al., 2009). The importance of cations during flocculation process involves the initiation of the bridging formation between particles. Different cations (monovalent, divalent and trivalent) were assessed for their effect on flocculating activity and their results are depicted in Figure 5. Iron chloride (trivalent cation) was the best cation among other with the highest flocculating activity of 84%, followed by divalent cations with 71% (manganese chloride) and 65% (barium chloride) flocculating activities with monovalent cations having least flocculating activities of 51% (potassium chloride), 48% (lithium chloride) and 47% (sodium chloride). The bioflocculant produced in this study is cation-dependent as less than 15% flocculating activity was achieved where no cation was added. The effectiveness for trivalent cation used in this study is may be due to more ions (charges) available in iron (Fe^{3+}) which are capable of neutralising negatively charged functional groups of both the bioflocculant molecules and the suspended particles thereby weakening the electrostatic repulsive force resulting in the enhancement of the flocculation rate (Piyo et al. 2011). Sheng et al. (2006) also reported trivalent cations Al^{3+} and Fe^{3+} to highly enhance the flocculating activity of *Klebsiella* sp. A9, while Zufarzaana et al. (2012) reported trivalent cation (Al^{3+}) to have an inhibitory effect on the flocculating activity of the bioflocculant produced by *Bacillus* species, in contrast. Tang et al. (2014) reported the cation-independent, pH tolerant and thermostable bioflocculant produced by *Enterobacter* sp. ETH-2 as opposed to the cation-dependent bioflocculant produced in this study.

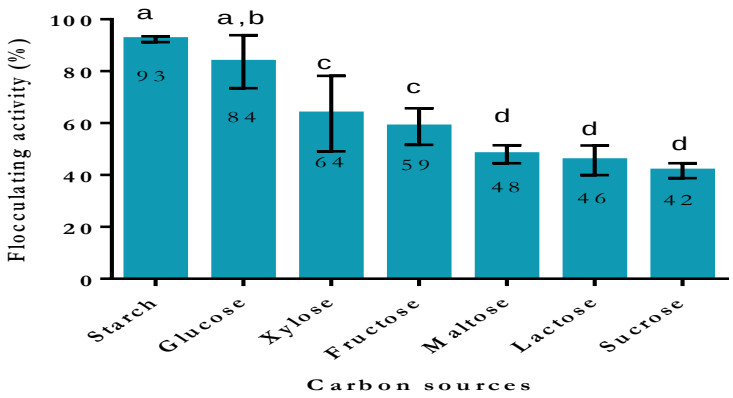


Figure 2: The effect of carbon source on bioflocculant production

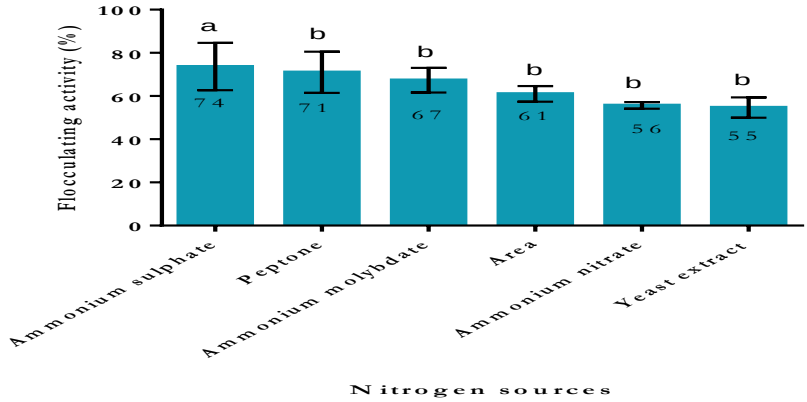


Figure 3: The effect of nitrogen sources on bioflocculant production

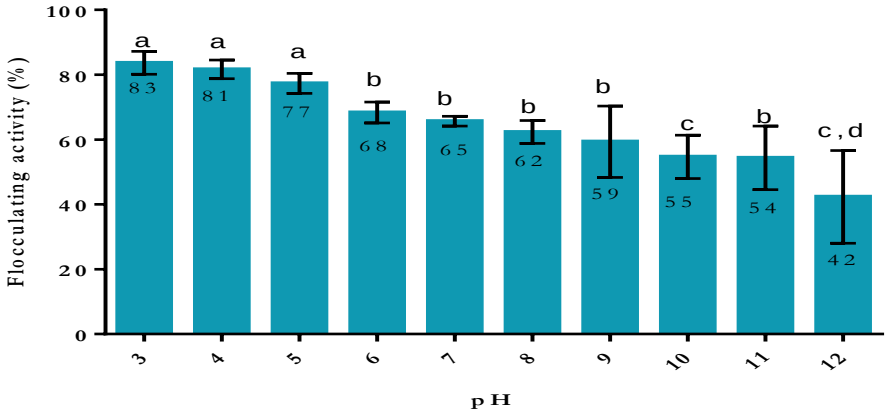


Figure 4: The effect of initial pH on flocculating activity of the bacterial consortium

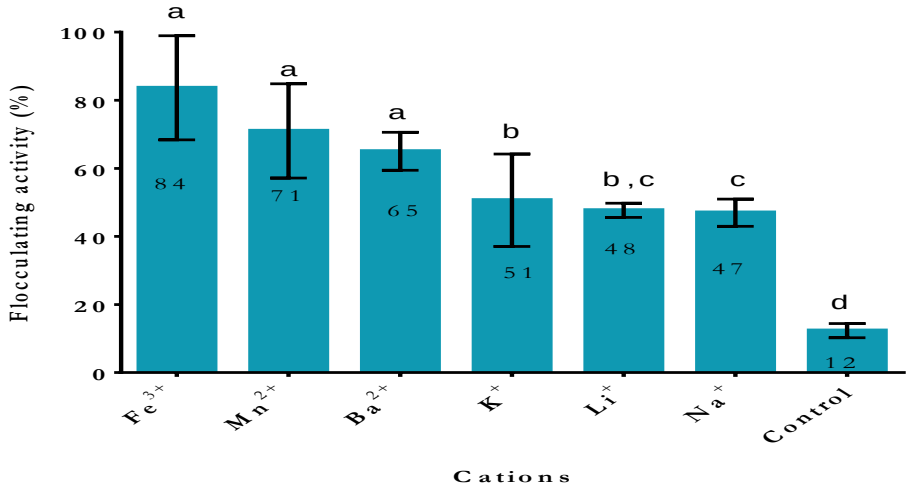


Figure 5: Effect of cations on flocculating activity of bacterial strains

The effect of shaking speed on flocculating activity

Agitation speed regulates the dissolved oxygen concentration, which in turn can also affect the nutrient absorption and enzymatic reaction (Li et al. 2017). Microorganisms vary in their oxygen demand. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activity. The speed also suspends the microbial cells and nutrients evenly throughout the medium to ensure that nutrients are available to all microbial cells. Different strains of bacteria require different shaking speeds for their optimum production of bioflocculants (Dlamini et al. 2019). Figure 6 shows the effect of mixing speed on flocculating activity. The flocculating activity observed to be parallel to the increase in mixing speed with all tested agitation speeds resulted in flocculating activity of 70% to above with the highest flocculating activity of 90% achieved at 165 rpm. The optimum speed (165 rpm) stimulated the optimum concentration of oxygen to settle in and promoted the thoroughly distribution of nutrients as well as well agitation of the multiplying cells of the microorganisms used for the production of bioflocculant (Lopez et al. 2003). The slightly decreased in flocculating activity (85%) was observed when the shaking speed was increased above 165 rpm. Excessive mixing speed may normally produce high shear pressure and resulted in negative impact towards cell growth as well as bioflocculant production (Maliehe et al. 2019). Similar results were presented by Maliehe et al. (2019), whereby the shaking speed of 165 rpm produced an optimum flocculating activity (77.9%) of the produced bioflocculant by *A. faecalis* HBC2. A shaking speed of 150 rpm was reported by Diao et al. (2018) to produce the highest flocculating activity for bioflocculant production by *Klebsiella* sp M1.

The effect of temperature on bioflocculant production

Heat has a significant role it plays in providing suitable environmental conditions for microbial growth and bioflocculant production. Reports show that the enzymes responsible for bioflocculant production are normal active in a temperate range between 25 – 37 ^{o}C (Xia et al., 2008). In the present study, the microorganisms (in consortium) have the highest cell growth as well as bioflocculant production at 30 ^{o}C. Below and above 30 ^{o}C temperatures there was a decrease in flocculating activity of the tested bacteria. Similarly, a bacterium *Enterobactor* sp. ETH-2 was reported to produce a bioflocculant optimally at 30 ^{o}C (Tang et al. 2014). Contrary, Zhong et al. (2009) reported 37 ^{o}C as an optimum for *Bacillus licheniformis* X14 growth as well as bioflocculant production. Temperature of 30 ^{o}C was used in all subsequent tests even though there was no significant difference in terms of statistical analysis since 30 ^{O}C is the standard temperature for bioflocculant production.

Time course of the bioflocculant

The effect of time course on flocculating activity (FA), cell growth in terms of optical density (OD) at 600 nm and pH are shown in Figure 8. The flocculating activity increases parallel to the cell growth (optical density) until it reached the peak at 72 hrs of incubation. The decrease in flocculating activity was observed thereafter. The observed decrease in flocculating activity might be due to enzymatic degradation of a polymer

bioflocculant (Okaiyeto et al. 2016). The increase in optical density with increase in time evident that the bioflocculant was produced by biosynthesis process through bacterial growth as opposed to cell autolysis (Okaiyeto et al., 2013). The pH decrease from the initial pH 6.0 to the final pH 4 after 72 hrs was observed which has to do with the carbohydrate (starch) or polymer flocculant metabolism resulting in the production of organic acids (Mabinya et al. 2012). After 72 hrs of incubation, there was a gradually pH increases to around 9.4 which is may be due to hydroxide ions freed related to the metal ions being drained out (Lors et al. 2009). This phenomenon shows that the bacteria are capable of changing their kinetic features in order to adapt towards environmental changes (Mabinya et al. 2012). Bacteria tend to behave differently in terms of cultivation time for the bioflocculant productions. For example, *Aspergillus flavus* produced its bioflocculant after 60 hrs of incubation as the cells were still multiplying in the presence of sucrose, peptone magnesium sulphate, potassium chloride iron sulphate, K_2HPO_4 and the medium was adjusted to initial pH of 6 (Aljuboori et al. 2013). Simirlar to this study, Ugbenyen et al. (2018) reported that *Alcalegenes faecalis* obtained from Sodwana Bay Estuary produced a bioflocculant after 7 hrs of incubation through biosynthesis process at pH 9.

Extraction and purification of a bioflocculant

A bacterial consortium reported to improve both the bioflocculant yield and flocculation rate (Zhu et al. 2004). Thus, about 8.5 g of a purified bioflocculant was recovered from 1 L of the fermented production medium by the consortium of *B. safensis* KX694275 and *Bacillus* sp. KC782848 after extraction and purification. The combined strains seem to improve the production rate of a bioflocculant, as expected, compared to the pure strains for the individual microorganisms which produced the yield of 1.522 g/L by *Bacillus* sp. (Ntombela et al. 2019) and 2.100 g/L by *B. safensis* (Ntombela et al. 2020). The yield is much higher than the bioflocculant (0.256 g/L) produced by the consortium of two bacteria (*Cobetia* and *Bacillus* species) reported by Ugbenyen and Okoh (2014). Maliehe et al. (2016) reported 3 g/L of a bioflocculant (TPT^{-1}) from the consortium of two bacteria (*B. pumilus* JX 860616 and *A. faecalis* HCB2) which is two times lower than the yield produced in this study.

Chemical analysis of a purified bioflocculant

Chemical composition analysis of the purified bioflocculant

Assessment of the active ingredients of microbial flocculants is essential to elucidate their flocculation mechanisms, which would be suitable for the optimization of flocculating parameters and consequently enhance their rate in practical implementations (Li et al. 2014). In this study, the chemical analyses of the purified bioflocculant revealed it to be made up of carbohydrates (78%) and protein (05%), suggesting that the produced bioflocculant is mainly polysaccharide. Further assessment showed that the microbial flocculant consisted of uronic acid (16%), suggesting that the bioflocculant is an acidic polysaccharide. According to Okaiyeto et al. (2016), bioflocculants with mainly protein component have lower molecular weights with lesser functional groups resulting in lower flocculation rate compared to bioflocculant with polysaccharide as their main component. A number of bioflocculants with polysaccharide as their main component have been documented (Zhao et al. 2013; Nwodo et al. 2014).

SEM analysis

The surface morphological structure has a significant impact on the bioflocculation process as it is liable for flocculating efficiency of the bioflocculants (Zhang et al. 2007). Morphological structure surfaces of the produced bioflocculant, kaolin powder and flocculated kaolin powder were revealed using Scanning Electron Microscope (SEM) (Figure 9). SEM analysis showed that the bioflocculant was whitish in colour and quadrilateral-like in shape, which may be responsible for its high flocculating efficiency, Figure 9(A). On the other hand, kaolin powder particles appeared fine and scattered before flocculation as shown in Figure 9(B). Flocculated kaolin particles appeared to have formed flocs in clusters. These appeared flocs in clusters are due to the absorption of kaolin particles towards the binding sites of the bioflocculant resulting in the formation of large flocs through their interaction, Figure 9(C). The formation of large flocs is in line with rapid sedimentation due to weight (Xiong et al. 2010).

Elemental analysis of a purified bioflocculant

According to Cosa et al. (2011), elemental composition of the microbial flocculant has a significant role it plays in the bioflocculant

structure as well as flocculating rate. Different elements give rise to the stability and flexibility of the bioflocculants. Perceptible analysis of the elemental make-up of the produced bioflocculant from consortium was investigated and the results are depicted in Figure 11. The presence of elements such as C: O: Na: Mg: Al: P: S: Cl: K and Ca accountable for 50.22: 38.07: 1.26: 0.12: 0.11: 0.86: 3.06: 0.97: 2.11 and 3.22 (% Wt), respectively, shown in the elemental spectrum using EDX analysis. The presence of C and O elements in the molecular chain of the bioflocculant is an indication of the glycoprotein-like polymer (Pathak *et al*., 2015). A similar finding of the bioflocculant with carbon and oxygen as its main elements was reported by Sun et al. (2015).

FT-IR analysis

The functional groups of a purified bioflocculant from the consortium of two marine bacteria were assessed and the results are shown in Figure 12. The FT-IR spectrum (Figure 12) exhibited a broad stretching observed at around 3456 cm^{-1} as an indication of the presence of -OH group (Lian et al. 2008). The vibration band observed at 2929 cm^{-1} may be attributed by the presence of C-H groups towards binding site of the bioflocculant. The peak at 1856 cm^{-1} corresponds to the bond stretching due to the availability of alkenes group. The strong stretching peaks between 1000 and 1110 cm^{-1} are for alkoxy group in the molecular chain of a bioflocculant. The weak bands observed from 829.9 to 543.2 cm^{-1} represent or are characteristics of all sugar derivatives. The results indicate clearly the presence of hydroxyl, alkoxy, and carboxylic groups in the molecular chain of a bioflocculant which are essential as they promote flocculation process. Moreover, the functional groups, carboxylic group in particular, has an advantage of working as functional moieties to generate novel and modified carbohydrate variants using various methods such as polymer engineering or new emergence designing, thereby linking the carbohydrate with other produced polymers (Yim et al. 2007). The said functional groups have the advantage of participating in the linkage of hydrogen ions and hydroxyl ions present on the surface of the particles resulting in the formation of the hydrogen bonds when bioflocculant molecular chains approach surface of the particles (Cosa et al. 2013). Similar findings for functional groups present in the bioflocculant were reported by Feng and Xu (2008).

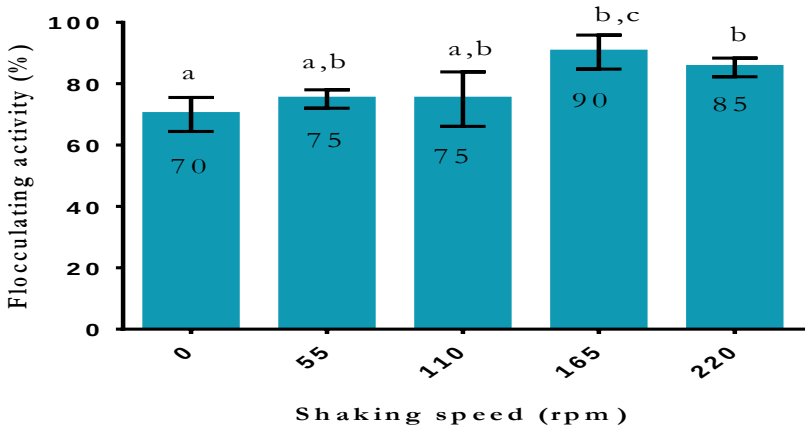


Figure 6: Effect of shaking speed on bioflocculant production

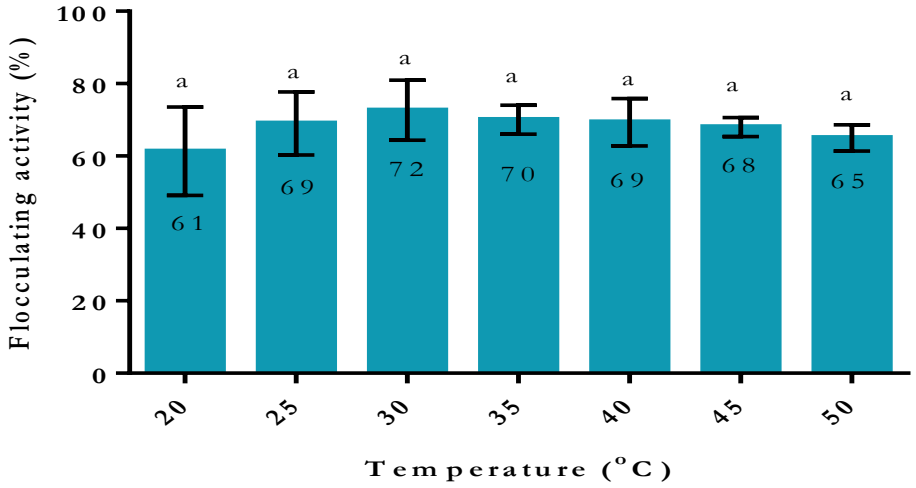


Figure 7: Effect of temperature on bioflocculant production

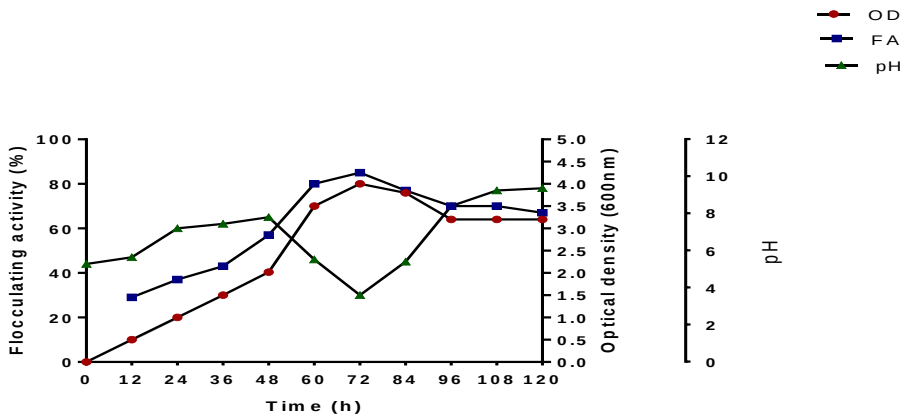


Figure 8: Effect of fermentation time on bioflocculant production

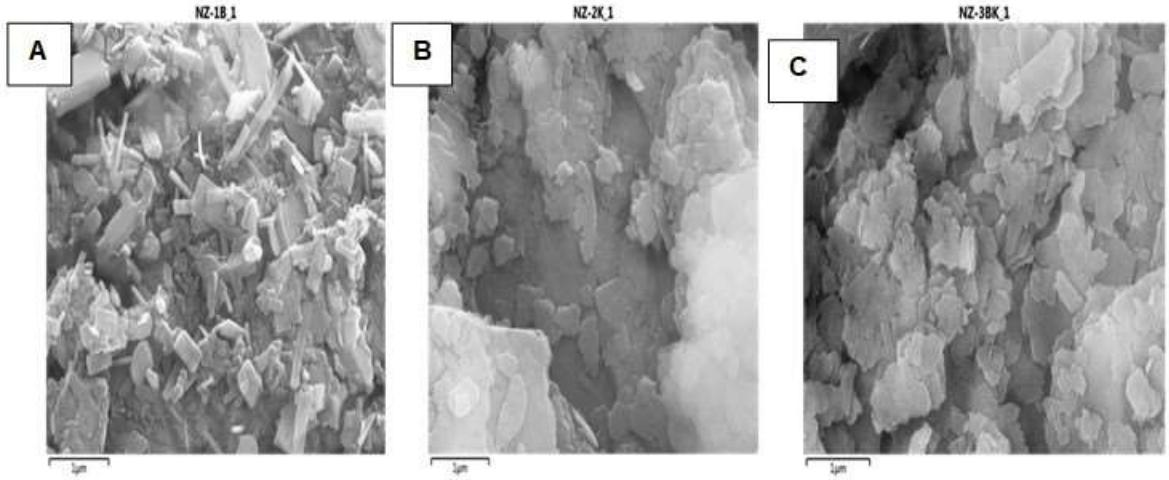
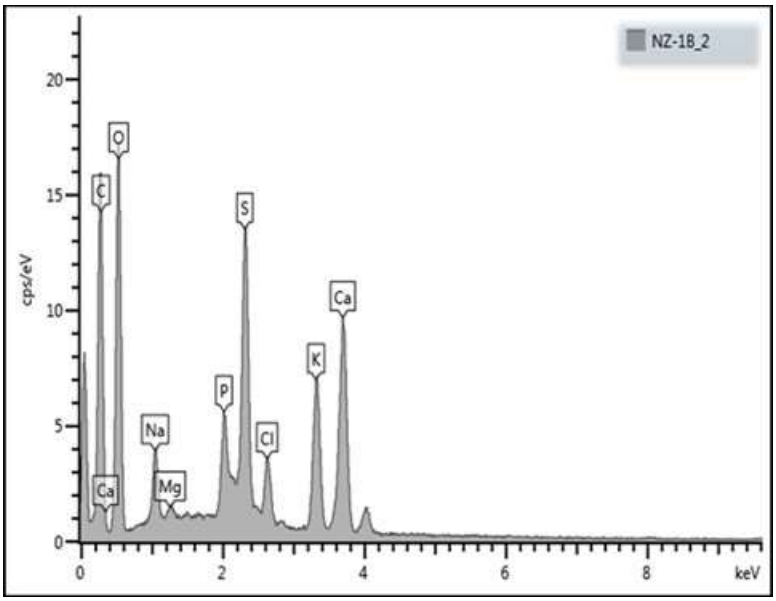


Figure 9: SEM analysis of a bioflocculant (A), kaolin powder (B) and flocculated kaolin clay (C).



Element	Wt%
C	50.22
O	38.07
Na	1.26
Mg	0.12
Al	0.11
P	0.86
S	3.06
Cl	0.97
K	2.11
Ca	3.22
Total	100.00

Figure 11: EDX analysis of a purified bioflocculant

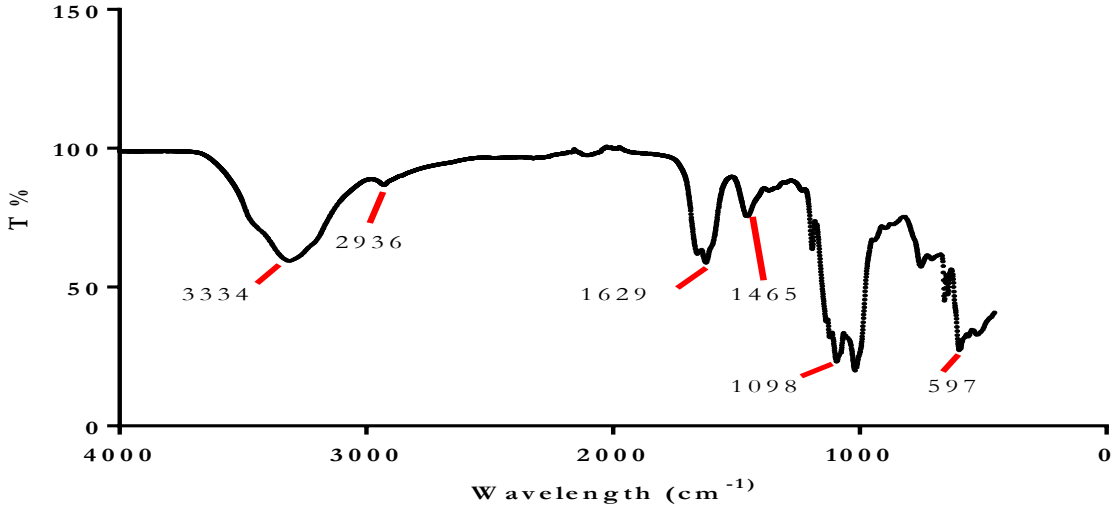


Figure 12: FT-IR analysis of a purified bioflocculant

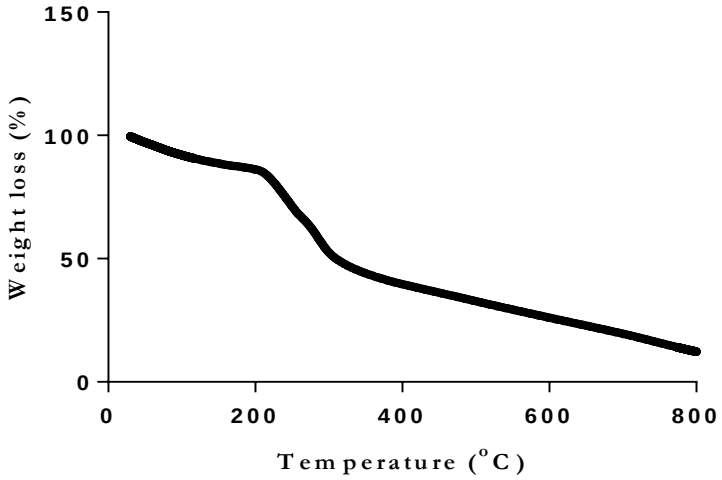


Figure 13: Thermogravimetric analysis of a purified bioflocculant

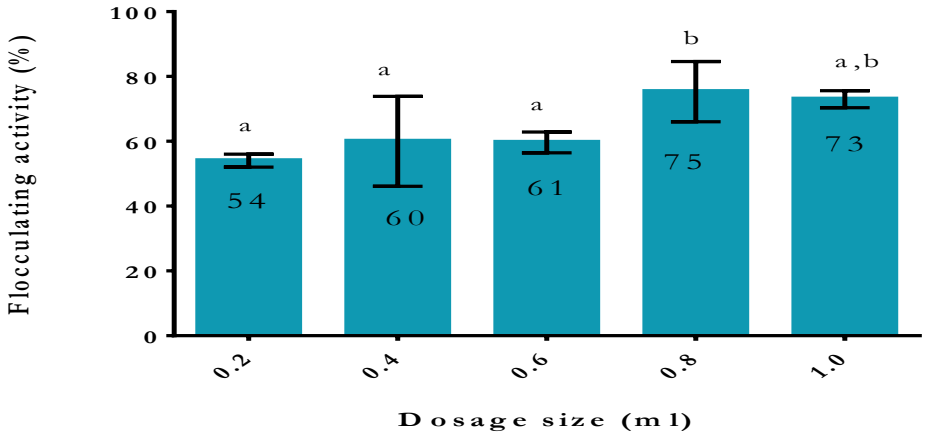


Figure 14: Effect of dosage size on flocculating activity

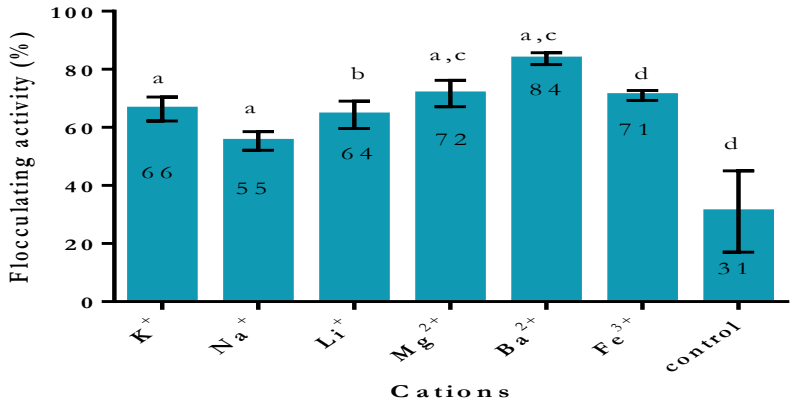


Figure 15: Effect of cations on flocculating activity

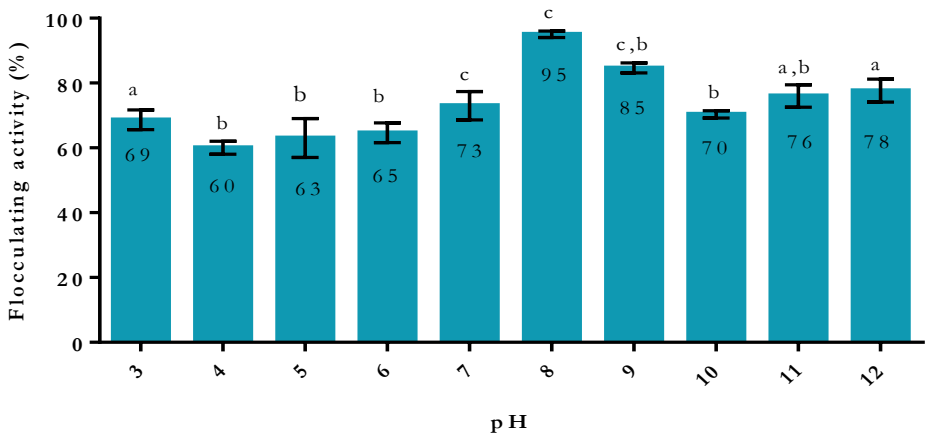


Figure 16: Effect of pH stability on flocculating activity

Thermogravimetric analysis of a purified bioflocculant

The bioflocculant from the consortium of bacteria was assessed to elucidate its thermogravimetric behaviour at varied temperatures starting from 30 - 800 ^{o}C, and the results are depicted in Figure 13. The thermogravimetric analysis is done to assist in recognizing the bioflocculant's pyrolysis character after exposed to significantly elevated heat (Yim et al. 2007). A weight loss of bioflocculant, expressed in percentage, under the effect of temperatures is shown in Figure 13. The highest weight loss (60.35%) of a bioflocculant was reported at 400 ^{o}C and the bioflocculant was almost completely pyrolyzed at temperatures above 400 ^{o}C, about approximately 11% weight loss at 150 ^{o}C and 29% at 250 ^{o}C. This bioflocculant seems to exhibit thermo-labile and thermo-stable molecular contents, as the mixture of protein and carbohydrates materials are presented as shown by examination. The first degradation of the bioflocculant or weight loss is may be due to moisture content loss or just the presence of C-O and OH functional groups in the molecular chain of the bioflocculant linked with the glycoprotein-like particles (Tawila et al. 2019). Both Wang et al. (2011) and Tawila et al. (2019) respectively reported similar observations with the bioflocculant produced by consortium of multiple bacteria and a novel bioflocculant QZ-7 from *Bacillus salmalaya* 139SI.

Flocculation properties of the purified bioflocculant

Effect of bioflocculant dosage on flocculating activity

Dosage concentration is still one of the important parameters to take into consideration when assessing the excellent conditions for the high performance of flocculants in the flocculation process. This is vital since inadequate dosage of bioflocculant leads to a poor bridging phenomenon, thus, resulting in low flocculating activity while excessive input might induce re-stabilization of kaolin particles (Hassan et al. 2009). Moreover, determination of the dosage concentration becomes more important in terms of establishing the excellent microbial flocculant dosage concentration. This could play a vital role in minimizing costs and resulting in the improved flocculation rate for its application industrially. The optimum dosage size was determined using a jar test experiment and the highest flocculating activity was obtained at 0.8 mg/mL (75%) of a bioflocculant (Figure 14). All tested dosage concentration resulted in the flocculating activity above 50% with 0.2 mg/mL the lowest resulted in the low flocculating activity compared to other dosage concentrations. Below and above 0.8 mg/mL dosage concentrations the flocculating activity decreased. This is may be due the fact that low dosage concentrations cannot neutralize the functional groups optimally and high dosage concentrations lead to poor colloids binding due to high viscosity. Therefore, for all subsequent tests 0.8 mg/mL was used. Ongubiade et al. (2017)

also reported 0.8 mg/mL as an optimum dosage for the bioflocculant produced by *Anterobacter humicola,* while Liu and Cheng (2010) reported that the bioflocculant produced by *C*. *daeguence* where more than 90% flocculating activity obtained using 1.2 mg/mL (optimum dosage concentration).

Effect of metal ions on flocculating activity of the bioflocculant

Metal ions increase flocculating activity by neutralizing and stabilizing the residual negative surface charge of both functional groups of kaolin clay particles and the bioflocculant (Wu and Ye, 2007). The results depicted in Figure 15 show that all tested metal ions stimulated the flocculating activity of a bioflocculant produced against kaolin clay suspension with great stimulation by Mg^{2+}, Ba^{2+} and Fe^{3+}. Ba^{2+} resulted in the optimum flocculating activity of 84% and was used in all subsequent tests. The bioflocculant produced in this study seems to be cation-dependent as little flocculating activity (31%) was recorded when there was no cation added. These findings are comparable to those of Tawila et al. (2019), Zheng et al. (2008), where they reported various bivalents and trivalents to optimally stimulate the flocculating activities of the produced bioflocculants. Contrary to the study findings, a cation-independent bioflocculant ETH-2 produced by *Enterobacter* sp. ETH-2 was reported by Tang et al. (2014), where the bioflocculant produced an optimum flocculating activity without being stimulated by metal ions.

The pH stability of a bioflocculant

The pH of the solution is a key parameter that controls the flocculation/coagulation process (Maliehe et al. 2019). pH may cause some modifications in microbial flocculant charge status and the structural characteristics of the colloid particles in a solution and as a result affects flocculating efficiency (Li et al. 2007). The effect of pH on flocculating activity of the purified bioflocculant was determined ranging from 3 - 12 and the results are depicted in Figure 16. The produced bioflocculant was effective within a wide range of pH (3 -12) with flocculating activity equal to/ greater than 60%. The optimum flocculating activity of 95% was obtained at pH 8.0 and lowest flocculating activity of 60% observed at pH 4.0. The slight decrease in pH is observed at acidic condition (3 - 6), this decrease might be due to the protein content present in the bioflocculant denaturation. According to Nwodo et al. (2014), this behaviour of the bioflocculant might be due to the absorption of hydrogen ions at low pH tends to weaken bioflocculant-kaolin clay complex formation process leading to poor flocculation and similar effect observed at high pH due to hydroxyl ions. Therefore, pH 8.0 was used in all subsequent tests. Similar results whereby the bioflocculant produced optimum flocculating activity at pH 8.0 was reported for bioflocculant produced by *Bacillus tequilensis* MMFG37 (Awady et al. 2018). Cosa et al. (2013) also reported a bioflocculant from the consortium of two bacterial species to be stable to wide ranges of pH, but the neutral pH had the highest flocculating activity.

Thermal stability of a purified bioflocculant

The correlation between heat and flocculating efficiency of the purified bioflocculant from consortium of two bacterial strains was examined at a temperature range of 50 -100 ^{o}C for 30 min and the results are depicted in Figure 17. This is done to assess its stability when exposed to higher temperatures. The microbial flocculant retained over 70% flocculating activity when exposed to heat at 100 ^{o}C for 30 min suggesting that the bioflocculant was stable. The flocculating activity of 80% was observed at 60 ^{o}C, when the heat was increased above 60 ^{o}C there is a slight decreased in flocculating rate but interestingly, there is no significant difference in terms of statistical analysis was shown. Therefore, it was deduced that the bioflocculant was thermo stable and its flocculating efficiency was not affected when the temperature was elevated. In general, the availability of protein or peptide within the structure of a bioflocculant is highly linked with its sensitivity towards heat and those with high sugar content are more resistant to heat (Sun et al., 2012); hence it can be deduced that the bioflocculant produced by the consortium of *Bacillus safensis* and *Bacillus* sp. consisted of polysaccharide as its main component. The heat-resistance of this product suggests the presence of a hydroxyl group in its chain responsible for the emergence of hydrogen bonds in its structure (Ntombela et al. 2019). Cosa and Okoh (2014) reported a flocculating activity of more than 80% for a purified bioflocculant produced by the bacterial consortium of *Halobacillus* sp. Mvuyo and *Oceanobacillus* sp. Pinky retained after heating at 100 ^{o}C for 1 hr, thus indicating its thermo stability character.

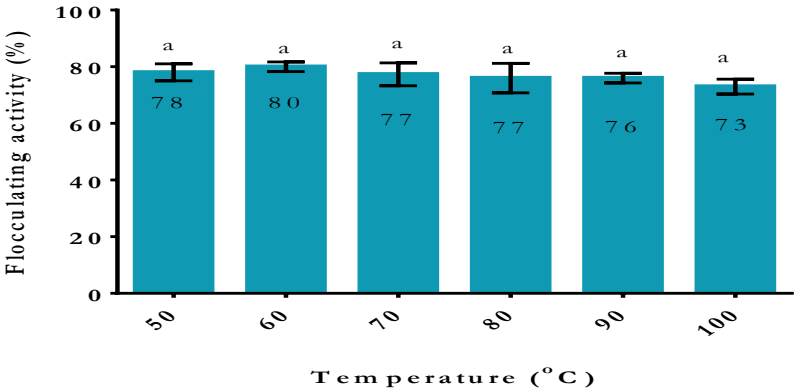


Figure 17: Effect of thermal stability on flocculating activity

CONCLUSION

Bioflocculant from a consortium of two bacteria *Bacillus safensis* and *Bacillus* sp. was produced and investigated for its flocculating efficiency as well as physicochemical properties. An anionic, heat stable glycoprotein molecules which is best produced when starch and ammonium sulphate are used as energy sources in optimum fermentation conditions (30 °C, 165 rpm, initial pH 3 and 72 hrs) in the presence of iron chloride as a mediating factor for flocculation process by neutralizing and stabilizing the bioflocculant residual functional groups. The produced bioflocculant with production yield of 8.5 g/L had a great flocculating activity at 0.8 mg/mL dosage, thermo-stable with more than 70% flocculating activity retained at 100 °C and maintained a wide pH range flocculating activity with a maximum flocculating activity of 95% at pH 8. The acidic polysaccharide bioflocculant possessed hydroxyl, carboxylic and amino groups in its molecular chain as the main functional groups which were responsible for flocculation mechanism. The revealed features of the bioflocculant suggested its potential towards industrial application. For further studies, the product will be tested for antimicrobial activity, used in biosynthesis of nano-size materials and applied on 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: BAK, ME and PVSR; Formal analysis; NZG, TT, KXN, PVSR and BAK; Investigation NZG, TT and KXN; Supervision: BAK, ME and PVSR; Writing (original draft): NZG; Writing (review and editing): NZG, BAK, PVSR and ME.

REFERENCES

Agunbiade OM, Van Heerden E, Pohl CH and Ashafa AT. (2017). Flocculating performance of a bioflocculant produced by *Arthrobacter humicola* in sewage waste water treatment. BMC Biotechnol. 17: Article number: 51 (2017).

Agunbiade M, Pohl C and Ashafa O. (2018). Bioflocculant production from Streptomyces platensis and its potential for river and waste water treatment. Brazil. J. Microbiol. Environ. Microbiol. 49(4): 73-741.

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

Akapo CSO, Ntombela ZG, Pullabhotla VSR and Basson AK. (2019). Isolation, optimization, characterization and application of bioflocculant BA-CGB produced by novel *Bacillus atrophaeus* isolated from Richards Bay Harbour, South Africa. Biosci. Res. 16(4): 3873-3902.

Aljuboori A, Idris A, Abdullah N and Mohamad R. (2013). Production and characterization of a bioflocculant produced by *Aspergillus flavus*. Biores. Technol. 127: 489-493.

Arafa RA, El-Rouby MN, Abass HA and El-Khier ZA. (2014). Bioflocculants produced by bacterial isolates from Egyptian soil 1-characterization and application of extracellular bioflocculants and nanoparticles for treatment of River Nile water. J. Pharm. Biol. Sc. 9(5): 103-114.

Awady E EL, Ghada Ibrahim S, Ayaz AO, Mouafi FE and Mahmou MG. (2018). Production and characterization of bioflocculant from isolated *Bacillus tequilensis* MMFG37. Biosci. Res. 15(4): 3581-3592.

Barredo JL. (2005). Microbial process and products-methods in Biotechnology 18. Hum. Pres. 1-18.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analy. Biochem. 72: 248-254.

Carlos DMF, Nancy GL, Glen TD and Lerslie GCP. (2011). Biological wastewater treatment. 3rd Ed. CRC Press. N. Yk. 655: 60-62.

Cesaretti M, Luppi E, Maccari F and Volpi N. (2003). A 96-well assay for uronic acid carbazole reaction. Carbohydr. Polym. 54: 59-61.

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

Chaplin MF and Kennedy JF. (1994). Carbohydrate analysis, seconded. Oxf. Univers. Press. New York. 138-141.

Chen X, Oh SW, Zheng Z, Chen HW, Shin H and Hou SX. (2003). Cyclin d-cdk4 and cyclin e-cdk2 regulate the Jak/STAT signal transduction pathway in Drosophila. Dev. Cell. 4(2): 179-190.

Cosa S, Mabinya LV, Olaniran AO, Okoh OO and Okoh IA. (2011). Bioflocculation production by *Virgibacillus* sp. Rob isolated from the bottom sediment of Algoa Bay in the Eastern Cape. South Africa. Molec. 16: 2431-2442.

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

Cosa S and Okoh A. (2013). Bioflocculant production by a consortium of two bacterial species and its potential application in industrial wastewater and river water treatment. Appl. Environ. Microbiol. Stud. 23(3): 689-696.

Cosa S and Okoh AI. (2014). Bioflocculant production by a consortium of two bacterial species and its potential application in industrial wastewater and river water treatment. Pol. J. Environ. Stud. 23: 689-696.

Diao H, Li L, Liang J and Ding X. (2018). Screening of high- performance flocculant-producing bacteria and optimization of conditions for flocculation of wheat distillery wastewater. Biores. 13(4): 7738-7757.

Dlamini NG, Basson AK and Pullabhotla VSR. (2019). Optimization and application of bioflocculant passivated copper nanoparticles in the wastewater treatment. Int. J. Environ. Res. Pub. Healt. 16: 2185.

Gao J, Bao HY, Xin MX, Liu YX, Li Q and Zhang YF. (2006). Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31. J. Zhej. Univ. Scie. B. 7(3): 186-192.

Gong X, Wang S, Luan Z and Jia Z. (2003). Screening and optimization of a bioflocculant-producing bacterium and its application in water treatment. Chin. J. Appl. Environ. Bio. 9: 196-199.

Gong W, Wang S, Sun X, Liu X, Yue Q and Gao B. (2008). Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. Biores. Technol. 99: 4668-4674.

Gouveia JG, dos S Sildal AL, dos Santos ELC, Martins ES and Lopez AMQ. (2019). Optimization of bioflocculant production by *Bacillus spp*. from sugarcane crop soil or from sludge of the agroindustrial effluent. Braz. J. Chem. Eng. 36(2).

Hassan MAA, Li TP and Noor ZZ. (2009).

Coagulation and flocculation treatment of wastewater in textile industry using chitosan. J. Chem. Nat. Res. Eng. 4: 43-53.

Hemson D and Dube B. (2004). Water services and public health: the 2000-01 cholera outbreak in KwaZulu-Natal, South Africa. 8th World Congress on Environmental Health (22-27 February). Durb. RSA.

Jensen PR, Gontang E, Mafnas C, Mincer TJ and Fenical W. (2005). Culturable marine *Actinomycetes* diversity from tropical Pacific Ocean sediments. Environ. Microbiol. Soc. Appl. Microbiol. 7(7): 1039-1048.

Karthiga K Devi and Natarajan KA. (2015). Isolation and characterization of a bioflocculant from *Bacillus megaterium* for turbidity and arsenic removal. Min. Metall. 32: 222-229.

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

Kurane R, Takeda K and Suzuki T. (1986). Screening and characteristics of microbial flocculants. J. Agric. Biol. Chem. 50: 2301-2307.

Li XM, Yang Q, Huang K, Zheng GM, Xiao DX, Liu JJ and Long WF. (2007). Screening and characterization of a bioflocculant produced by *Aeromonas* sp. Biomed. Environ. Sci. 20: 274-278.

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

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

Li Y, Li Q, Hao D, Hu Z, Song D and Yang M. (2014). Characterization and flocculation mechanism of an alkali-activated polysaccharide flocculant from *Arthrobacter* sp. B4. Bioresour. Technol. 170: 574-577.

Li L, Ma F and Zuo H. (2017). Production of a novel bioflocculant and its flocculation performance in aluminium removal. Bioengine. 7(2): 98-105.

Lian B, Ye C, Jin Z, Teng HH, Zhu L and Yua S. (2008). Microbial flocculation by *Bacillus mucilaginosus*: applications and mechanisms. Bioresour. Technol. 99: 4825-4831.

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

Liu W, Cong L, Yuan H and Yang J. (2015). The mechanism of kaolin clay flocculation by a cation-independent bioflocculant produced by *Chryseobacterium daeguense* W6. AIMS Environ. Sci. 23: 213-218.

López E, Ramos I and Sanromán MA. (2003). Extracellular polysaccharides production by *Arthrobacter viscosus*. J. Fd. Eng. 60: 463-467.

Lors C, Chehade MH and Damidot D. (2009). pH variations during growth of *Acidithiobacillus thiooxidans* in buffer media designed for an assay to evaluate concrete biodegrading. Int. Biodeterior. Biodegrad. 63: 880-883.

Mabinya LV, Cosa S, Nwodo U and Okoh AI. (2012). Studies on bioflocculant production by *Arthrobacter* sp. Raats, freshwater bacteria isolated from Thyme River, South Africa. Int. J. Mol. Sci. 13: 1054-1065.

Makapela B, Okaiyeto K, Ncedo N, Nwodo UU, Green E, Mabinya LV and Okoh AI. (2016). Assessment of *Bacillus pumilus* isolated from freshwater Milieu for bioflocculant production. Appl.Scie.6(211): 1-20.

Maliehe TS, Selepe NT, Ntombela ZG, Simonis JJ, Basson AK, Ngema S, Xaba PS and Mpanza F. (2016). Production and characteristics of bioflocculant TPT^{-1} from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2. Afric. J. Microbiol. Resear. 10(37): 1561-1575.

Maliehe TS, Basson AK and Dlamini NG. (2019). Removal of pollutants in mine wastewater by a non-cytotoxic polymeric bioflocculant from *Alcaligenes faecalis* HCB2. Int. J. Environ. Res. Publ. Healt. 16(20): 4001.

Nie M, Yin X, Jia J, Liu S, Shen Q, Li P and Wang Z. (2011). Production of a novel bioflocculant MNXY1 by *Klebsiella pneumonia* strain NY1 and application in precipitation of *cyanobacteria* and municipal wastewater treatment. Applied Microbiology. 111: 547-558.

Ntombela ZG, Mthembu NS, Gasa NL, Basson AK, Simonis JJ, Madoroba E and Pullabhotla VSR. (2019). Isolation, identification and characterization of a bioflocculant producing strain, *Bacillus* sp. KC782848.1, from uMlalazi catchment, Mthunzini, KwaZulu-Natal. J.

Innov. Scientif. Inform. Serv. Netw. Bioscie. Res. 16(4): 3664-3685.

Ntombela ZG, Kubhayi TM, Basson AK, Simonis JJ, Madoroba E and Pullabhotla VSR. (2020). Characterisation of bioflocculant produced by *Bacillus* species isolated from uMlalazi estuary, Mthunzini and its application in wastewater treatment. Biosci. Res. 17(3): 1944-1970.

Ntozonke N, Okaiyeto K and Okoh AI. (2017). A Marine bacterium, *Bacillus* sp. isolated from the sediment samples of Algoa Bay in South Africa produces a polysaccharide-bioflocculant. Int. J. Environ. Res. Publ. Healt. 14(10): 1149.

Ntsaluba L, Nwodo UV, Mabinya LV and Okoh AI. (2013). Studies of bioflocculants production by a mixed culture of *Methylobactacterium* sp. Obi and *Actinobacterium sp.* BMC. Biotechnol. 1472-6750/13/62.

Ntsangani N, Okaiyeto K, Nwodo UU, Olaniran AO, Mabinya LV and Okoh AI. (2017). Bioflocculation potentials of a uronic acid-containing glycoprotein produced by *Bacillus* sp. AEMREG4 isolated from Thyme River, South Africa. 3 Biotech. Spring. Lin.7:78.

Nwodo UU, Green E, Mabinya LV, Okaiyeto K, Rumbold K, Obi LC and Okoh AL. (2014). Bioflocculant production by consortium of *Streptomycetes* and *Cellulomonas* species and media optimization via surface response model. Colloi. Surf. B: Biointerf. 116: 257-264.

Ogunsade OO, Bakare MK and Adewale IO. (2015). Purification and characterization of bioflocculant produced by *Bacillus amyloliquefaciens* ABL 19 isolated from Adeti Stream, Ilesa, Osum State, Nigeria. Nat. Scie. 13(2): 54-64.

Okaiyeto K, Nwodo UU, Mabinya LV and Okoh AI. (2013). Characterization of a bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. Inter. J. Environ. Resear. Publ. Heal. 10(10): 5097-5110.

Okaiyeto K, Nwodo UU, Mabinya LV and Okoh AI. (2014). Evaluation of the flocculation potential and characterization of bioflocculant produced by *Micrococcus* sp. Leo. Appl. Biochem. Microbiol. 50: 601-608.

Okaiyeto K, Nwodo UU, Mabinya L, Okoli A and Okoh A. (2016). Evaluation of flocculating performance of a thermostable bioflocculant produced by marine *Bacillus sp*. Environ. Technol. 37: 1829-1842.

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

Piyo N, Cosa S, Mabinya VL and Okoh IA. (2011). Assessment of bioflocculation production by *Bacillus* sp. Gillbert, a marine bacterium isolated from the bottom sediment of Angola Bay. Mar. Drug. 9: 1232-1242.

Rasulov BA, Li Li, Liu Y-H, Mohamad OA, Xiao M, Ma B-J and Li W-J. (2017). Production, characterization and structural modification of exopolysaccharide-based bioflocculant by *Rhizobium radiobacter* SZ4S7S14 and media optimization. 3Biotech. 7(3): 179; doi: 10.1007/s13205-017-0811-9.

Salehizadeh H and Shojaosadati SA. (2001). Extracellular biopolymeric flocculants: Recent trends and biotechnological importance. Biotechnol. Adv. 19: 371-385.

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

Shen M, Zeng Y-G and Shen Y-C. (2009). Isolation and characterization of a novel *Arthrobacter nitroguajacolicus* ZJUTB06-99, capable of converting acrylonitrile to acrylic acid. Proc. Biochem. 44: 781-785.

Sheng Y, Zhang Q, Sheng Y Cheng C and Wanga LH. (2006). Screening and flocculating properties of bioflocculant-producing microorganisms. J. Univ. Scie. Technol. Beij. Min. Metall. Mater. 13(4): 289-292.

Shu CH and Lung MY. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorate* in batch cultures. Process. Biochem. 39: 931-937.

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

Suh H, Kwon H, Lee CH, Kim HS, Oh HM and Yoon BD. (1998). Characterization of bioflocculant produced by *Bacillus* sp. DP-152. J. Ferment. Bioengineer. 84: 108-112.

Sun J, Zhang X, Miao X and Zhou J. (2012). Preparation and characteristics of bioflocculants from excess biological sludge. Bioresour. Technol. 126: 362-366.

Sun P, Hui C, Bai N, Yang S, Wan L, Zhang Q

and Zhao Y-H. (2015). Revealing the characteristics of a novel bioflocculant and its flocculation performance in *Microcystis aeruginosa* removal. Scientif. Rep. 5: 17465.

Tang W, Song L, Li D, Qiao J, Zhao T and Zhao H. (2014). Production, characterization, and flocculation mechanism of cation independent, pH tolerant, and thermally stable bioflocculant from *Enterobacter* sp. ETH-2. PLoS One. 8: 9(12):e114591. doi: 10.1371/J.pone.0114591.

Tawila ZMMA, Ismail S, Arezoo D and Mohammed MU. (2018). Production and characterization of a bioflocculant produced by *Bacillus salmalaya* 139SI-7 and its applications in wastewater treatment. Molecules. 23(10): 2689.

Tawila ZMMA, Ismail S, Amrc SSA and Elkhair EKA. (2019). A novel efficient bioflocculant QZ-7 for the removal of heavy metals from industrial wastewater. RSC. Adv. 9: 27825-27834.

Ugbenyen A, Cosa S, Mabinya LV, Babalola OO, Aghdasi F and Okoh AI. (2012). Thermostable bacterial bioflocculant produced by *Cobetia* sp. isolated from Algoa Bay (South Africa). Int. J. Environ. Res. Publ. Healt. 9: 2108-2120.

Ugbenyen AM, Madonsela TA, Simonis JJ and Basson AK. (2018). Assessment of the flocculating potentials of *Alcaligenes faecalis* isolated from the estuary of Sodwana Bay. Ann. Scie. Technol. 3 (2): 1-7.

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

Wu J and Ye HF. (2007). Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtillis* DYU1 isolate. Proc. Biochem. 42: 1114-1123.

Xia SQ, Zhang ZQ, Wang XJ, Yang A, Chen L, Zhao JF, Leonard D and Jaffrezic-Renault N. (2008). Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. Bioresour. Technol. 99: 6520-6527.

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

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

Yumei L, Qiang L, Dukui H, Zhiheng H, Dongxue S and Min Y. (2014). Characterization and flocculation mechanism of an alkali-activated polysaccharide flocculant from *Arthrobacter* sp. B4. Biores. Technol. 170: 574-577.

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

Zhang Z, Xia S, Zhao J and Zhang J. (2010). Characterization and flocculation mechanism of high efficiency microbial flocculant TJ-F1 from *Proteus mirabilis*. Colloids and Surfaces B. Biointerf. 75(1): 247-251.

Zhang CL, Cui YN and Wang Y. (2013). Bioflocculants produced by Gram-positive *Bacillus* xn12 and *Streptomyces* xn17 for swine wastewater application. Chem. Biochem. Eng. Q. 27: 245-250.

Zhao H, Liu H and Zhou J. (2013). Characterization of a bioflocculant MBF-5 by *Klebsiella pneumonia* and its application in *Acanthamoeba cysts* removal. Bioresour. Technol. 137: 226-232.

Zheng Y, Ye Z-L, Fang Y-H and Cai W-M. (2008). Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. Bioresour. Technol. 99: 7686-7691.

Zhong C, Lau MW, Balan V, Dale BE and Yuan YJ (2009). Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl. Microbiol. Biotechnol. 84(4): 667-76.

Zhu YB, Feng M and Yanga JX. (2004). Screening of complex bioflocculant producing bacterium and their flocculating mechanism. J. Harbin Inst. Technol. 36(6):759-762.

Zufarzaana Z, Ahmad ZA, Zulkifli HS and Mohd KY. (2012). Cation dependence, pH tolerance, and dosage requirement of a bioflocculant produced by *Bacillus* spp. UPMB13: Flocculation performance optimization through kaolin assays. The Scient. Wor. J. dx. doi. Org /10.1100/ 2012/49565910.1100/2012/495659.