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Isolation, optimization, characterization and application of bioflocculant BA-CGB produced by novel *Bacillus atrophaeus* isolated from Richards Bay Harbour, South Africa

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A novel bacterium with bioflocculant-producing potential was amongst bacterial isolates isolated from soil sample at Tuzi Gazi, Richards Bay Harbour, Kwazulu-Natal, Republic of South Africa and screened for bioflocculant producing potential after cultivation using a production medium at 30 ^{o}C for 72 hours (160 rpm against 4g/L Kaolin clay suspension. The bacterium with an initial flocculating activity of 74% was further identified using 16S rRNA molecular sequencing method as *Bacillus atrophaeus* SRCM 101359 (with 98.64% similarity). Optimisation of *Bacillus atrophaeus* culture conditions were carried out resulting in a yield of 3.165 g pure BA-CGB, recovered after 96 hours of fermentation from 1 litre of production broth in the presence of 4% inoculum size, C/N ratio (40:1) glucose (carbon source), ammonium chloride (nitrogen source), at pH 9 and temperature of 35 ^{o}C as well as shaking speed of 110 rpm. Flocculating activity was stimulated with 1% Ba^{2+} solution. Characterisation of the pure bioflocculant using Fourier transform infrared spectroscopy (FT-IR) of purified BA-CGB revealed the presence of functional groups such as hydroxyl, carboxyl, amine and amide. Elements such as carbon and oxygen among others, were present in the molecular chain of bioflocculant BA-CGB. This could serve as a confirmation that the bioflocculant is with a polysaccharide backbone; accounting for its thermal stability character as it managed to attain more than 60% flocculating activity at 121 °C for 15 min, with 0.4 mg/mL dosage size and Ca^{2+} as a stimulating agent. Images of BA-CGB from scanning electron microscopy (SEM) showed that it is an amorphous and irregularly shaped biopolymer. Further analysis of the bioflocculant BA-CGB showed a chemical composition of carbohydrates (65%), uronic acid (25%) and trace proteins (10%). The flocculating activity of purified BA-CGB was greatly influenced by a wide pH range (3-12). This bioflocculant holds a promising future to replace in-use chemical flocculants in wastewater treatment.

Keywords: *Bacillus atrophaeus; BA-CGB;* Bioflocculants, kaolin clay suspension, flocculating activity, polysaccharide, thermostable

INTRODUCTION

Flocculation is one of the methods of choice for removing contaminants and organic matter from water using flocculating agents (Lee et al.,

2012; Lee, 2017). Flocculating agents such as synthetic chemical flocculants, naturally occurring bioflocculants and grafted synthetic flocculants form aggregate flocs with the suspended matters in water (Lee et al., 2013; Pathak et al., 2014). Chemical flocculants such as polyacrylamides, as a high-polymer organic flocculant have been in use over time because they are inexpensive, highly effective and require less skilled personnel (Li et al., 2013). Some of them are not easily biodegradable and have been found to be harmful to the health of humans (Buthelezi et al., 2012). The search for biodegradable, economical and sustainable extracellular biopolymers has been on the rise globally (More et al., 2012; Subudhi et al., 2015). The most recent grafted synthetic flocculants are a combination of the properties of synthetic polymers and that of natural polymers backbone to obtain a tailor-made grafted flocculant (Mishra et al. 2011). Bioflocculant can be classified as glycoprotein, polysaccharides and nucleic acids in nature (Ugbenyen et al., 2012). Marine sediments have been reported to contain a wide array of unique, diversified and novel organisms than those found in terrestrial environments (Cosa et al., 2012).

The biochemical composition of bioflocculant is influenced by various factors during the growth phases of the microorganisms such as carbon source, microbial species, the downstream extraction methods and nutrient supplementation. These factors range from inoculum size, temperature, pH of the medium, presence of metal ions, salinity, colloid types, mixing speed, aeration, cultivation time and bioflocculant concentration (Aljuboori et al., 2015; Nouha et al., 2019) which also with its physical properties usually determines the capability and mechanisms of bioflocculants. In order to optimize these culture medium conditions for better bioflocculant yields as well as improved flocculating activity, several strategies have been developed (Li et al. 2009; Abd el Salam et al. 2017) to circumvent the limited setbacks of restrictive financial cost to the industrial use of produced bioflocculants (Okaiyeto et al. 2013; Li et al. 2015). This is carried out by minimising the cost and maximising the output in the production of bioflocculants (Montgomery, 2005). One of the most important parameters in the optimisation of culture conditions for bioflocculant production is the inoculum size. The inoculum size is pivotal to the other parameter optimisation experiments as it does not only affect the growth pattern of the organism but also the final product produced by the organism (Ntozonke, 2015). Ugbenyen et al., (2014), reported that 3% inoculum size was preferred by *Bacillus* sp. Gilbert for optimum bioflocculant production. *Klebsiella pneumoniae* was reported to prefer 1% inoculum size (Luo et al., 2008) while a similar inoculum size (1%) was reported for *Bacillus licheniformis* X14 as well (Abd El Salam et al., 2017). Optimization of inoculum sizes is reported necessary and crucial, as it gives optimum bioflocculant activity and also differs with regards to different organisms (Ntozonke, 2015).

MATERIALS AND METHODS

Cultivation temperature is a crucial factor in any microbial growth as the metabolism of microorganisms is directly influenced by the cultivation temperature. Different strains of microorganisms prefer different cultivation temperatures. The attainment of maximal growth, enzymatic and microbial activity is highly influenced (directly) by the cultivation temperature (Li et al., 2008). The optimal temperature for bioflocculant production varies in the range of 25 ^{o}C and 37 ^{o}C (Salehizadeh and Shojaosadati, 2001). However, this is also dependent on the optimal temperature of the strain. *Bacillus* sp. Gilbert isolated from Algoa Bay of South Africa preferred an optimal cultivation temperature of 28 ^{o}C (Piyo et al., 2011). Different bioflocculant strains have different optimal shaking speed in aerobic fermentation which is used as a determinant for the concentration of dissolved oxygen needed for nutrient absorption and enzymatic reactions (Li et al. 2009). The shaking speed requirement is dependent on the oxygen requirement for each phase of the organisms. This accounts for the disparity between different organisms (Li et al. 2009). *Bacillus* sp. Gilbert produced optimally at an agitation speed of 160 rpm (Piyo et al. 2011).

Cations play a major role in the neutralization of surface charge of bioflocculant and in the stabilization of residual net charges formed due to the charges of the functional group on the bioflocculant (Wu et al. 2007). Bioflocculant due to the carboxyl groups of amino acids in their structure are mostly negatively charged; thus, exist an electrostatic attraction distances that needs to be reduced between bioflocculant and suspended particles (Wang et al. 2011). When cation binds to the carboxylate group sites, it increases the floc in suspension and enhanced sedimentation (Li et al. 2007). A bioflocculant obtained from *Klebsiella* sp. strain S11, a

bacterium isolated from activated sludge was reported to flocculate effectively in the presence of Ca^{2+} with a flocculating activity of 69% (Dermlim et al. 1999). The initial pH of a production medium determines on the electrification of cells creating reduced oxidation potential that enables the absorption of nutrients by cells from the environment (Salehizadeh and Shojaosadati, 2001). It is one of the major factors in the production of bioflocculant which also affects its flocculating efficiency (Zheng et al. 2008).The production fluctuates with different periods of growth such as biosynthesis, cell autolysis, complexing of metals and enzymatic action in the cell medium (Cosa, 2010; Li et al. 2009; Lu et al. 2005). Different microorganism prefers different fermentation time at which the production of the highest amount of bioflocculant is yielded. This study was focused mainly on optimisation of bioflocculant production, characterisation of produced bioflocculant from novel bacterial isolates from marine environments with high flocculating activity and high polymer yield.

RESULTS AND DISCUSSION

2.1 Isolation, screening and identification of bioflocculant producing bacterium

The isolated bioflocculant-producing bacterium labelled as S15 was obtained from soil sample of Tuzi Gazi Harbour beach line, Richards Bay Harbour in the Kwazulu Natal, South Africa. *Bacillus atrophaeus* on nutrient agar plate, appeared to be circular and possess smooth and milky white colonies, with irregular edges, mostly flat. It also possesses mucoid and ropy colony morphology which has been reported to be the basic identification properties for bioflocculant producing potential such as *Bacillus* sp. UPMB13 (Amir et al. 2003; Zulkeflee et al. 2012). The bacterium (S15) was further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacterium, the universal primers 27F and 1492R were used (Lane, 1991; Turner et al. 1999). The bacterial strain was analysed using the 16S rRNA and Nucleotide sequence analysis based on gyraseA gene using the Basic Local Alignment Search Tool (BLAST) of the 16S rRNA revealed that the S15 bacterium has 98.64% similarity to *Bacillus atrophaeus* (*B. atrophaeus*) strain SRCM101359 with accession number CP021500.1. The selected organism was simply referred to as *Bacillus atrophaeus.* It has been identified to belong to a group of useful bacteria with known production of biomolecules (Ma et al. 2018). *Bacillus atrophaeus (atrophaeus – black, phalis – brown, atrophaelis, dark brown)* is a Gram-positive rod-shaped bacterium, motile, facultative anaerobic that produces ellipsoidal endospores with characteristics virtually closer to that of *Bacillus subtilis,* however on a medium containing organic nitrogen source they produce a brown pigment after 2 to 6 days (Nakamura, 1989; Burke et al. 2004).

2.2 Optimisation of pre-culture medium conditions of *Bacillus atrophaeus*

Different parameters known to have effect on bioflocculant production were investigated including inoculum size, carbon and nitrogen (energy) sources, cations effect on flocculating activity, pH, temperature, shaking speed, fermentation time in order to improve flocculating activity and increase in yield of bioflocculant production of *B. atrophaeus*.

2.2.1 Effect of inoculum size on bioflocculant production

Inoculum size is the cell percent that is required to inoculate the bioflocculant production medium. The inoculum size is pivotal to the other parameter optimisation experiments as it does not only affect the growth pattern of the organism but also the final product produced by the organism (Liu et al. 2017). The effect of inoculum size on bioflocculant production was investigated for *Bacillus atrophaeus* ranging from 1- 5% (v/v). From Figure 1, it is evident that the flocculating activity increased as the inoculum size increases from 1% until it reached the highest flocculating activity of 91% at 4% (v/v). The flocculating activity thereafter decreased drastically to 12% at 5% (v/v) inoculum size. This decrease in the flocculating activity is an indication of less bioflocculant being produced. The inoculum size of 4% (v/v) was used in the subsequent experiments. Bacillus licheniformis and *Bacillus pumilus* were reported to optimally produce bioflocculant at an inoculum size of 4% (v/v) (Xiong et al. 2010; Makapela et al. 2016). Contrary to the findings of this study, Wang et al., (2007) reported highest flocculating activity by Klebsiella mobilis at an inoculum size of 5% while Luvuyo et al., (2013) reported the highest flocculating activity of 92% with an inoculum size of 1% obtained from a mixed culture of *Methylobacterium* sp. and *Actinobacterium* sp.

2.2.2. Effect of carbon and nitrogen sources on bioflocculant production

In a number of studies, carbon and nitrogen sources are reported as important nutrient factors, which have significant effects on the production medium of bioflocculant(Ugbenyen and Okoh, 2013; Salehizadeh and Yan, 2014).*Bacillus atrophaeus* in this study, preferably utilised glucose (91%) as the best carbon sources (Figure 2).The bacterium also utilised sucrose (85%), fructose (86%), maltose (68%), galactose (6%) and starch (1%), meanwhile, glucose was used in all subsequent tests as it was statistically significant to other sugar sources.

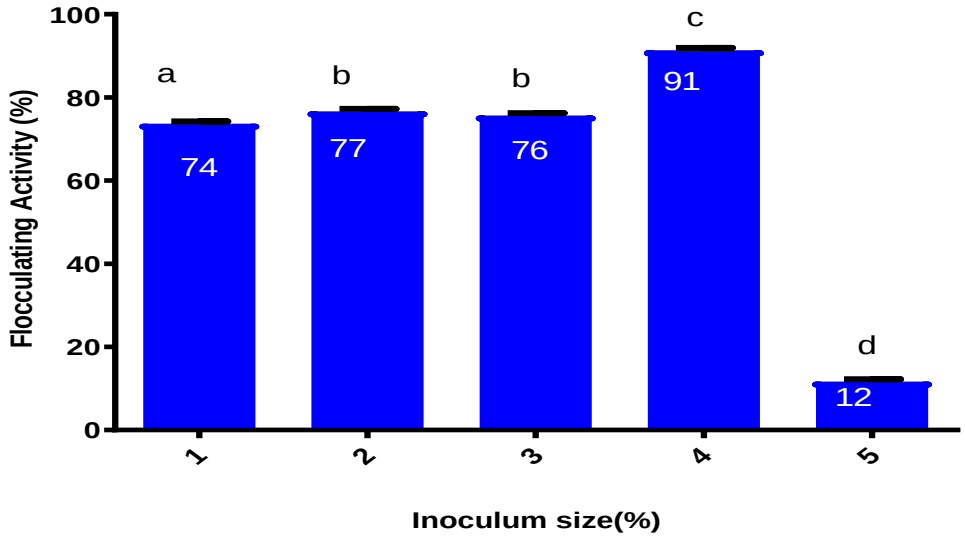

Figure 1: The effect of inoculum size on bioflocculant production. Percentage flocculating activities with different alphabets (a, b, c and d) are significantly different (p < 0.05) from each other.

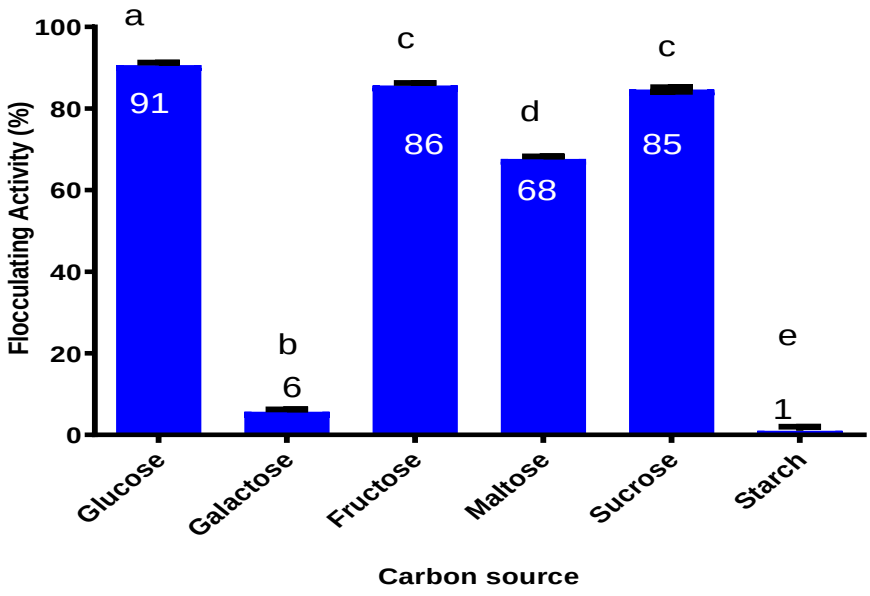

Figure 2: The effect of carbon sources on bioflocculant production. Percentage flocculating activities with different alphabets (a, b, c, d, e) are significantly different (p < 0.05) from each other.

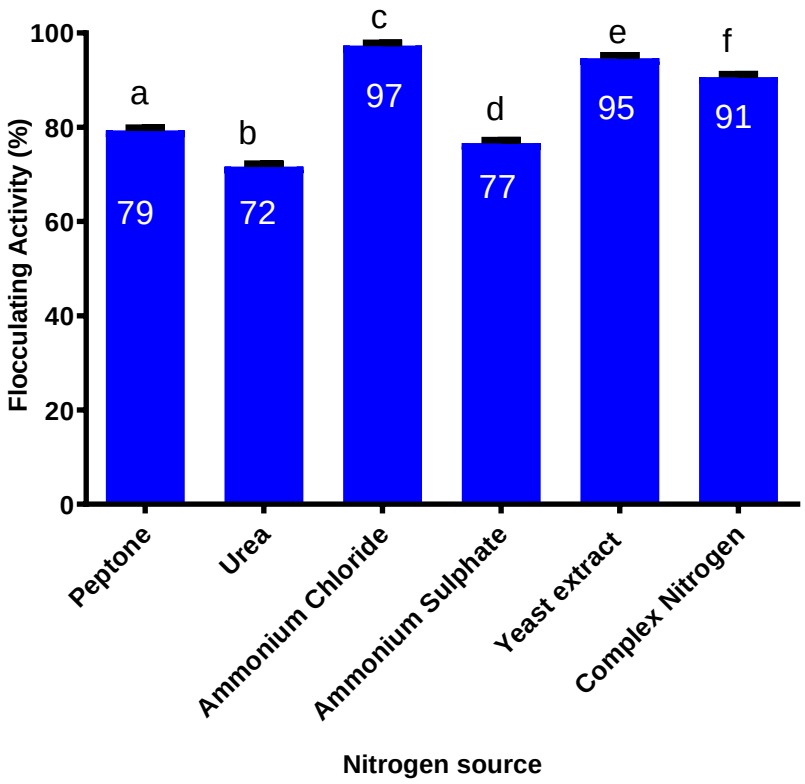

Figure 3: The effect of nitrogen sources on bioflocculant production. Percentage flocculating activities with different alphabets (a, b, c, d, e, f) are significantly different ($p < 0.05$) from each other.

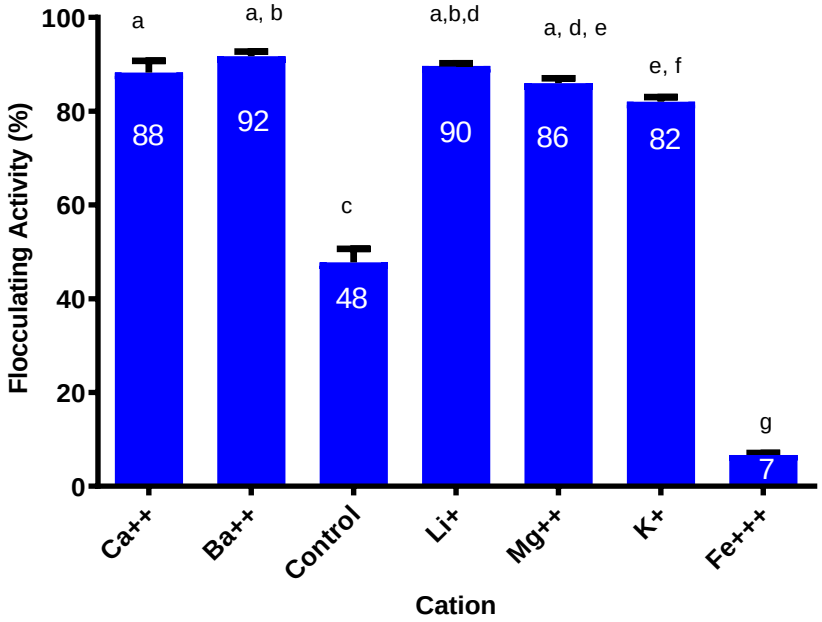

Figure 4: The effect of cations on flocculating activity. Percentage flocculating activities with different alphabets (a, b, c, d, e, f and g) are significantly different ($p < 0.05$) from each other.

Similar result to this study where the *Bacillus atrophaeus* could utilise sucrose, showed that there is an indicated feasibility that *the tested organism* could utilise the by-product of sugarcane processing industry such as sugarcane molasses or bagasse which are cheaper substances than glucose (Okaiyeto et al. 2016). It is also noted that, Liu and Cheng (2010) substituted molasses as carbon source in the production of bioflocculant by *Penicillium* sp. HHE-P7 in place of glucose.

The most preferred nitrogen for the tested bacterium was ammonium chloride (97%), yeast extract as well as complex nitrogen (91%). Flocculating activity with other array of nitrogen sources were reported as peptone (79%), urea (72%) and ammonium sulphate (77%) (Figure 3). Cosa et al., (2013a) reported that complex nitrogen supported the bioflocculant production by *Virgibacillus* sp. *Proteus mirabilis* TJ-1 was reported to have utilised peptone (Xia et al. 2008) while *Bacillus licheniformis* X14 utilised ammonium chloride as nitrogen source for bioflocculant production (Lee et al. 2001). Piyo et al., (2011) also reported *Bacillus sp*. Gilbert utilised ammonium chloride (91%) as nitrogen source.

2.2.3. Effect of cations on flocculating activity

The presence of cations in the bioflocculation process enhances the rate of flocculating activity (Li et al. 2007). In this study, the flocculating rate was highly stimulated by Ba^{2+} (92%) followed by a comparable non-significant stimulation by Li^{+} (90%) and Ca^{2+} (88%) cations (Figure 4). This implies that there is a significant difference existing between the flocculating activities obtained using Ba^{2+} (92%) and Li^{+} (90%) as cations on the activity of the bioflocculant. This is judged as such, since the p- value obtained is less than 0.05. It therefore implies that Ba^{2+} cannot be substituted for by Lithium cation. It is noteworthy that all cations tested except ferrous chloride (7%) stimulated flocculation process with flocculating activity above 80%. It has been reported that calcium chloride and aluminium chloride stimulated the flocculation rate of bioflocculant produced by marine bacteria (Cosa et al. 2013b; Okaiyeto et al. 2015). In a study on MBF-6 produced by *Klebsiella pneumoniae*, the bioflocculation process was inhibited by Al^{3+}, but stimulated by Na^{+}, K^{+}. Ca^{2+}, Mg^{2+} and Zn^{2+} (Luo et al. 2014). The flocculating activity of the bioflocculant without the addition of any cation (control) as in Fig 4 was barely 50% indicating that the bioflocculant cannot yield a better flocculation activity without the use of cations and it is therefore cation dependent.

2.2.4. Effect of initial pH on bioflocculant production

The initial pH of a cell culture medium affects the redox potential and influence electrification state of the bacterial cells. This can affect enzyme reaction as it affects the biomolecules production (Luo et al. 2014). Figure 5 shows the effect of initial pH on the flocculating activity where this activity was steadily increased from pH 3 (75%) to pH 8 (81%) and reached the optimum flocculating activity of 92% at pH 9. A remarkable decrease thereafter was observed at pH 10 (41%) to pH 12 (43%). The reason for the significant decline could be as a result of re-stabilization of the kaolin particles which further prevents agglomeration and bridging by the bioflocculant (Wang et al. 2011). The bioflocculant production from the test bacterium was favoured by acidic pH (pH 3), neutral and slightly alkaline pH (pH 9). Piyo *et al.* (Piyo et al. 2011) reported that the pH 8.42 for the habitat of *Bacillus* sp. Gilbert did not have any bearing on its bioflocculant production. However, Deng et al., (2005) reported that *Aspergillus parasiticus* preferred acidic conditions for bioflocculant production.

2.2.5. Effect of cultivation temperature and shaking speed on bioflocculant production

Different strains of microorganisms prefer different cultivation temperatures as well as agitation speeds. *Bacillus atrophaeus* strain tested in this study showed low flocculating activity at 20 ^{o}C (52%) and steadily increased from 25 ^{o}C (75%) until it reached an optimum cultivation temperature at 35 ^{o}C (91%). This implies that the tested organism's (*Bacillus atrophaeus*) optimum temperature falls within the relative optimal temperature for Bacillus, which is between 25 - 35 ^{o}C (Salehizadeh and Shojaosadati, 2001). Thereafter, a slight reduction in flocculating activities were observed at 45 ^{o}C (78%) and 50 ^{o}C (75%), with a small difference that is non-significant (Figure 6). Bacillus species have been reported to have evolved various structural adaptations to higher temperature range by producing resistant spores (Nicholson et al. 2000). Similar to this findings, Luo et al., (2014) reported a high flocculating activity of 85.5% for *Klebsiella pneumoniae* YZ-6 at 30 ^{o}C.

Through continuous agitation, the sparingly soluble oxygen stimulates aerobic growth in the

aqueous production medium (55).

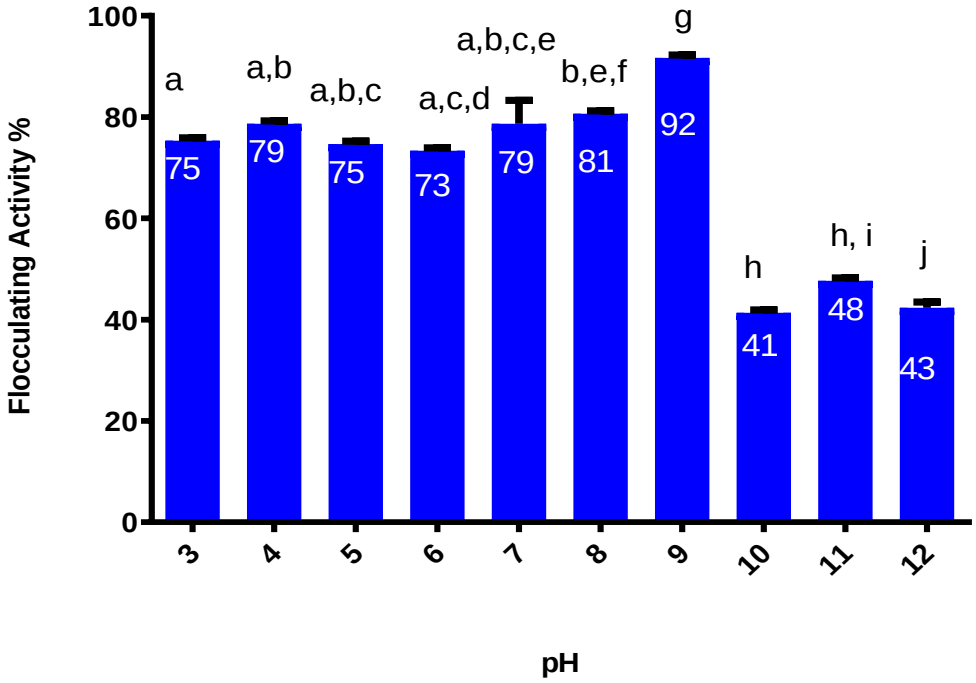

Figure 5: The effect of initial pH on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

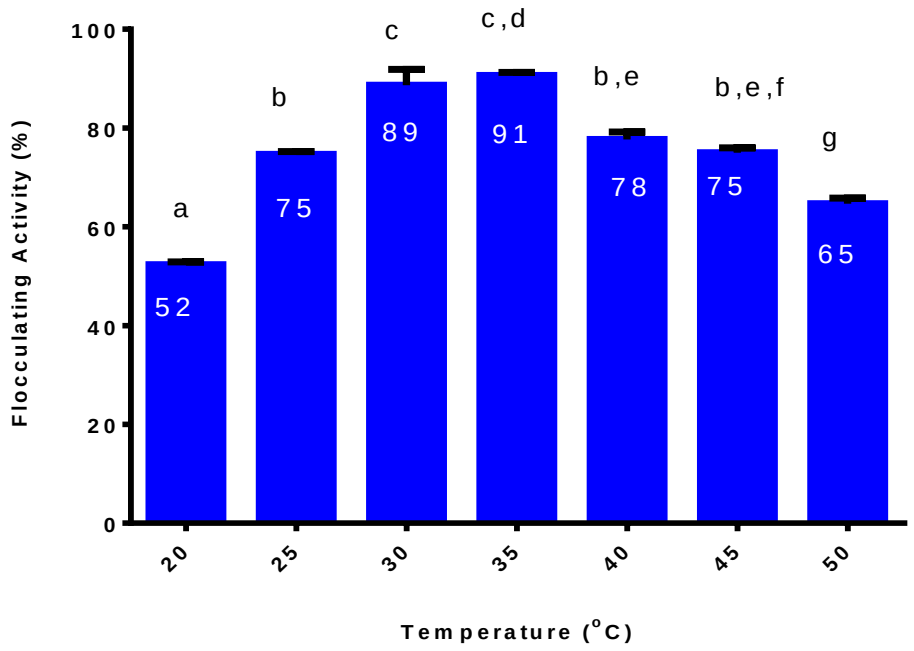

Figure 6: The effect of cultivation temperature on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

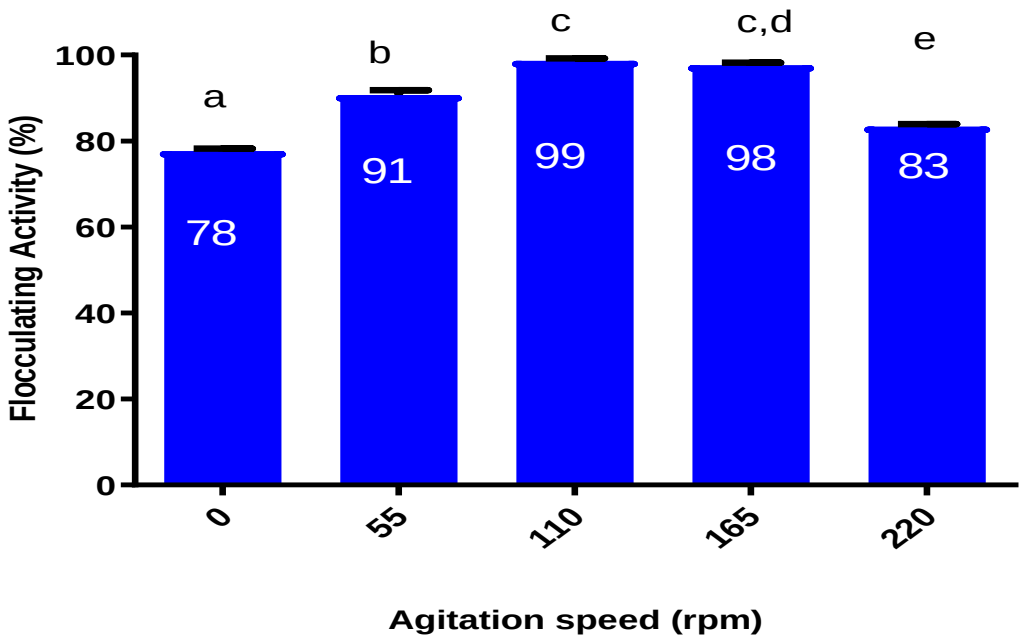

Figure 7: The effect of shaking speed on bioflocculant production. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

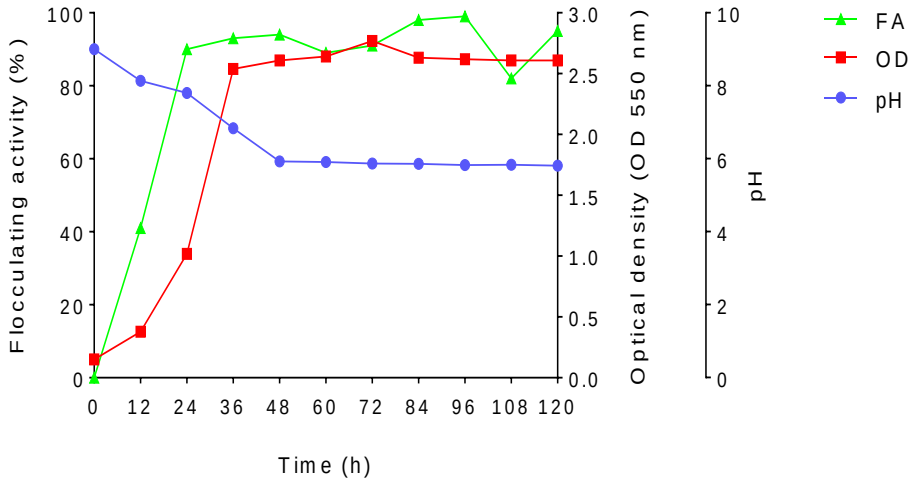

Figure 8: The time course assay on bioflocculant production for *Bacillus atrophaeus.*

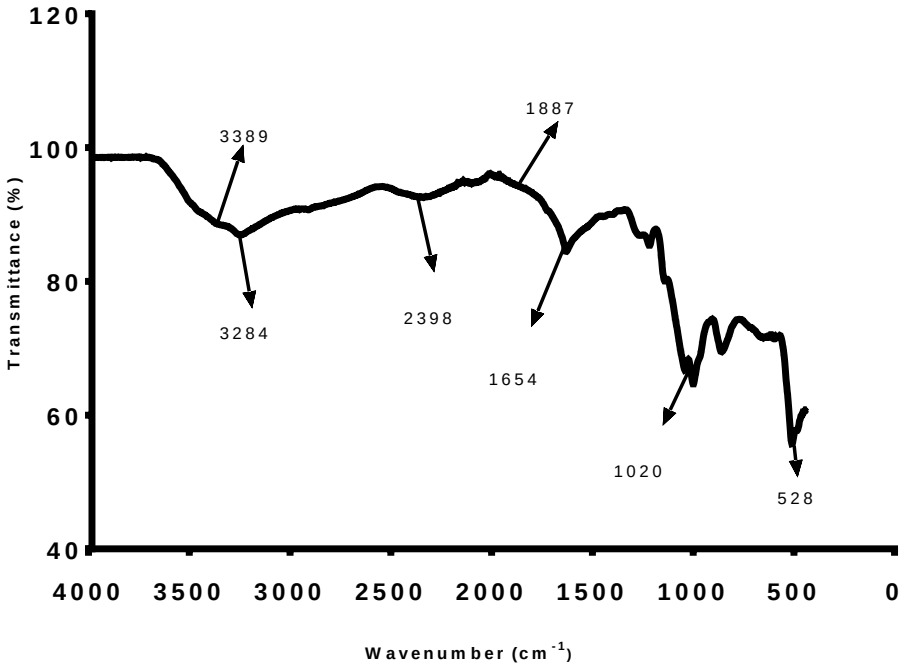

Figure 9: FT-IR spectrum of the purified bioflocculant BA-CGB.

In terms of agitation speed, the flocculating activities at 0 rpm was 78% and this steadily increased to 91% at 55 rpm until it reached its significant optimum agitation speed of 110 rpm (99%). There was a slight non-significant decrease to 98% (165 rpm) and further significant decrease to 83% (220 rpm) respectively (Figure 7). The differences in the shaking speed could be attributed to the oxygen requirement during the different growth phases of the organisms (Li et al. 2015). *Bacillus* sp. Gilbert produced optimally at an agitation speed of 160 rpm (Piyo et al. 2011).

2.3 Time course assay on bioflocculant production

The pH of production medium, as observed in the time course assay experiment (Figure 8) with *Bacillus atrophaeus* decreased from 9.00 to 5.84 during the 5 days of the experiment. The test organism has a minimal flocculating activity of 41% after first 12 hours of incubation and the cell growth represented by an OD_{550} was observed to be 0.382. Studies have reported that at this stage of growth (lag phase), the production of bioflocculant is not majorly associated with the cell growth (Li et al. 2007), indicating that the bioflocculant was produced extracellularly by biosynthesis during its growth as a secondary metabolite (Lu et al. 2005) to keep the cells close together. There was a remarkable increase in flocculating activity to 88% after 24 hours of incubation observed with OD_{550} of 1.020. The highest flocculating activity was observed after 96 hours (99%) of growth and the cell growth increased to an OD_{550} of 2.622. After 96 hours of growth, there was a decrease in flocculating activity to 82% at 108 hours and an observed cell growth with an OD_{550} of 2,611. There was however an increase in flocculating activity observed at 120 hours to 95% but with an observed reduced cell growth with OD_{550} of 2.609. The decrease in flocculating activity from 99% to 82% at this period (108 hours) has been reported to be due to autolysis of bacterial cell and or in combination with bioflocculant degrading enzymes (Ugbenyen et al. 2012). A similar finding was reported on a study of *Pantoea* sp., a novel organism isolated from Mtunzini Beach (28.9597° S, 31.7501° E) which reached the maximum flocculating activity after 96 hours (Ugbenyen et al. 2017). Change in pH of the medium may also be attributed to the production of organic acids in the production medium during the process of formation of the bioflocculant components or from the glucose metabolism (Ntsaluba et al. 2011). Culture time could influence the production of bioflocculant and flocculating activity. Cosa *and co-workers* (Cosa et al. 2011) reported a study

where the pH of the medium remained between (6.2 – 6.4) throughout the incubation period. This growth pattern of microorganism can be manipulated in order to minimise cost (Ntozonke et al. 2017). The highest flocculating activity of the test bacterium was reached at 96 hours with the organism in the stationary phase. bioflocculant production process is in synonymous with biosynthesis (Nwodo et al. 2012). *Bacillus licheniformis* cell growth and bioflocculant production *was* reported to reach the highest peak during the stationary phase at 96 hours simultaneously (Shih et al. 2001). This is in tandem to the test bacterium in this study. On the contrary, Liu and Cheng (2010) observed that the secretion of bioflocculant is not correlated with higher biomass as reported with *Penicilium* strain HHE-P7 cultivation on beef extract where a flocculating activity of 80% was produced with a growth biomass of below 3 g/L while a yeast extract yielded a flocculating activity of above 90% with a biomass growth of 9 g/L (Liu and Cheng, 2010).

2.4 Extraction and purification of bioflocculant

After extraction, the crude bioflocculant (BA-CGB) from *Bacillus atrophaeus* strain yielded 5.916 g/L, a milky white colour substance. The purified bioflocculant, BA-CGB was a white powdered substance with a yield of 3.165 g recovered from 1l fermentation broth. The yield of BA-CGB in this study is higher than the 1.6 g/L yield obtained from *Bacillus* sp. AEMREG7 (Okaiyeto, 2016). However, the produced bioflocculant was smaller in yield compared to a yield of 4.52 g/L reported for *Halomonas* sp. V3a by He *et al.* (2010) and a yield of 3.8 g/L reported by *Ochrobactium cicero* W2 yield of 3.8 g/L reported by Wang *et al.* (2013). A polysaccharide yield of 25.63 g/L produced from a culture of *Paenibacillus elgii* B69 using sucrose as carbon source was reported by Li et al., (Li et al. 2013), which is 8 times higher than the tested bacterium produced. The yield of bioflocculant by microorganism is an important factor when considering its use in industry (Cosa and Okoh, 2014), for the tested bacterium, the observations of yield at the different phase can be further manipulated to increase the yield or with the use of bioreactor or genetic engineering (Liu et al., 2017).

2.5 Characterization of the purified bioflocculant BA-CGB

2.5.1 FT-IR analysis of the purified bioflocculant BA-CGB

The FT-IR spectrum indicates important peaks, which correlates with the presence of various functional groups in the molecular chain of the bioflocculant (Okaiyeto, 2016). The FT-IR analysis for bioflocculant BA-CGB showed a broad stretching peak observed at 3389 cm^{-1} which indicated the presence of amine groups. The infrared spectrum revealed the presence of hydroxyl (3384 cm^{-1}), strong amide (1654 cm^{-1}) and amino (1090 cm^{-1}) groups (Figure 9). The presence of sugar derivatives was revealed at the weak peaks of 859 cm^{-1} whereas, the peak at 1460.62 cm^{-1} indicated the presence of uronic acid in the polysaccharide. The IR vibration peak at 1020 cm^{-1} revealed strong C-O stretching vibration for alcohols and this further predicts the presence of OH group and carboxylate ion in the bioflocculant (BA-CGB). The peak at 1654 cm^{-1} can be attributed to C-O stretching in NH_2 bending, $CO\text{-}NH_2$ group from the polymeric and dimeric OH stretches of phenol or tertiary alcohol bends with the presence of carboxyl and hydroxyl groups. The strong absorption peak observed at 528 cm^{-1} is for halo-alkanes C-X bending vibrations and a typical characteristic of sugar derivatives. The presence of amino groups was reported by Abu-Elreesh and Abd-El-Haleem in bioflocculants produced by tested strains QUST2, QUST6 and QUST9 (Abu-Elreesh and Abd-El-Haleem, 2014). The presence of hydroxyl, amide and amino groups may also be because of vibration of OH^- or NH_2 groups present in the sugar ring (Cosa et al. 2011). Spectrum peaks between 1304 cm^{-1} and 1654 cm^{-1} has been reported to be consistent with the presence of carboxylate (Abu-Elreesh and Abd-El-Haleem, 2014). The indicated functional groups of carboxyl, hydroxyl and amino groups revealed in the infrared spectrum of the reported bioflocculant MBF-6 (Luo et al., 2014) and in BA-CGB could serve as a confirmation that the bioflocculant is with a polysaccharide backbone; accounting for its thermal stability (Okaiyeto, 2016). This could explain the rationale behind the observed high flocculating efficiency of BA-CGB observed in this study with the various functional groups as enhancement of the flocculating process and binding capacity to the cations and the particles (Freitas et al., 2009; Aljuboori et al., 2014)

2.5.2 SEM and EDX analysis of bioflocculant BA-CGB

SEM Analysis

SEM analysis shows the surface images of sample as high-energy beam of electrons scan in a faster scan pattern. The signal containing information about the sample surface is revealed as the electrons interact with the atoms of the sample (Selepe, 2017; He et al., 2010). The surface morphology of purified BA-CGB was examined with its flocculation of kaolin clay powder by SEM (Figure 10).

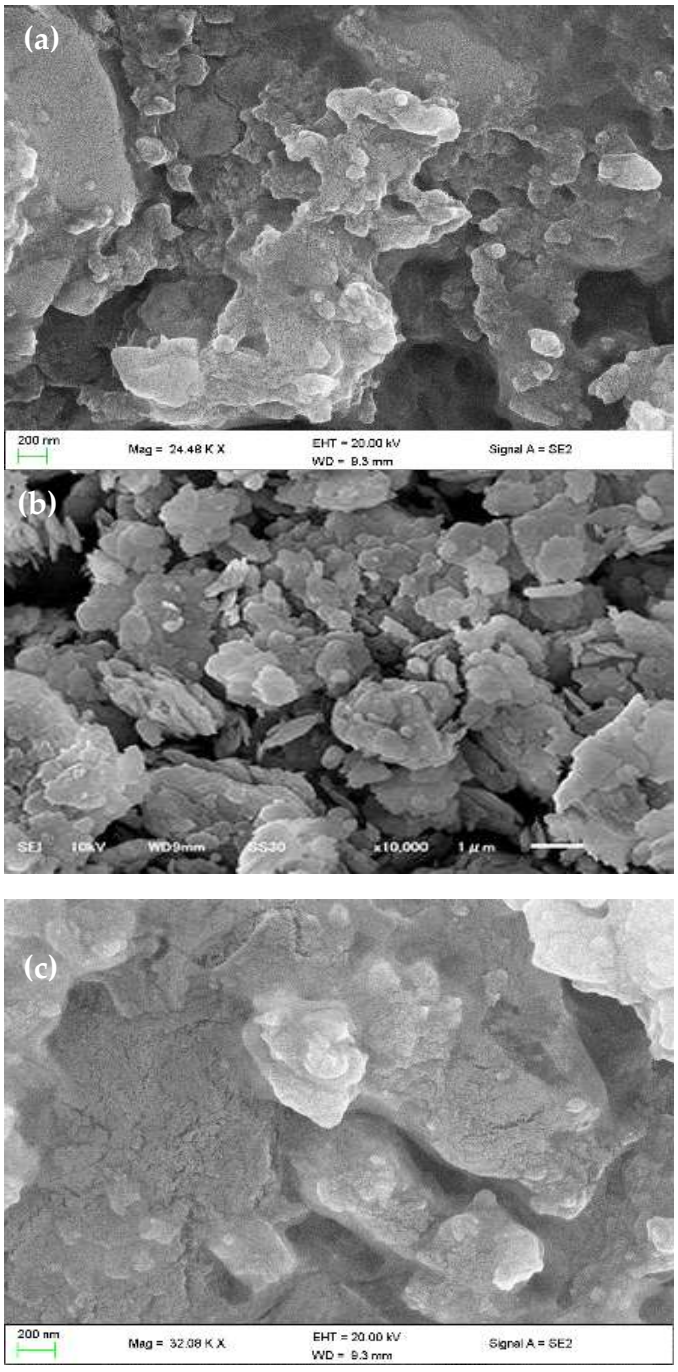

Figure 10: SEM images of the (a) bioflocculant, (b) kaolin particles and (c) flocculated kaolin particles

Figure 10 shows the images of purified bioflocculant (a) kaolin clay particles before flocculation (b) and flocculated kaolin clay particle (c).Figure 10(a) shows the coarse and amorphous structure of the bioflocculant (BA-CGB). The SEM image of the bioflocculant BA-CGB showed that it is an amorphous and irregularly shaped biopolymer. Figure 10(b) shows the fine and white scattered uniform-sized kaolin clay particles before flocculation. Figure 10(a) is comparable to Figure 10(b) and 10(c) in terms of structure and sizes. It was observed that larger flocs were easily formed and precipitated due to gravity as the kaolin clay particles connects to the large size particles of the bioflocculant (Figure 10). These observations were consistent with the findings of glycoprotein PRKF5 bioflocculant produced from *Providencia rettgeri* KF534469 (Selepe, 2017).

SEM EDX analysis for BA-CGB

The SEM-EDX analysis of the purified bioflocculant depicted in Table 1 shows its elemental composition (% wt) to comprise of C, O, Na, Mg, P, Cl, K, Ca in the percentage of 3.46%, 44.14%, 5.41%, 4.61%, 19.97%, 2.33%, 19.34%, and 0.75%, respectively. The elemental composition indicates the presence of polysaccharide as a backbone in the compound bioflocculant. FTIR analysis spectrum also revealed the presence of amino, hydroxyl and carboxyl groups in the bioflocculant. The elemental analysis of a novel bacterial bioflocculant revealed the presence of C, N, O, Na, P, and Cl in the macromolecule as 31.20, 6.12, 46.39, 5.67, 10.40, and 0.23 %, respectively (Pathak et al. 2017).

Table 1: Percentage composition of elements in the purified bioflocculant (BA-CGB).

Element	% Weight
C	3.46
O	44.14
Na	5.41
Mg	4.61
P	19.97
Cl	2.33
K	19.34
Ca	0.75
Total	100.00

Chemical composition of bioflocculant BA-CGB

The chemical composition of bioflocculant BA-CGB was found to contain approximately 65% of sugar, 25% of uronic acid and is approximately 10% of trace protein. In a reported study by Feng and Xu (Feng and Xu, 2008), bioflocculant BF3-3 was composed of 66.1% (polysaccharide) and 29.3% (protein) while Deng et al., (2005) reported *Aspergillus parasiticus* bioflocculant was composed of carbohydrate (76.3%) and protein (21.6%). However, on the contrary, the bioflocculant produced by *Halomonas* sp. V3a consist of majorly polysaccharide (29%), neutral sugar component (20.6%), uronic acid (7.6%), amino acids (1.6%) and a sulfate group (5.3%) (63). The bioflocculant BA-CGB could then be described as mainly polysaccharides, hence, its thermostability.

2.6 Characteristics of a purified bioflocculant BA-CGB

2.6.1 Effect of dosage size on flocculating activity of BA-CGB

Appropriate dosage size helps in minimising cost and to obtain better flocculating performance in industrial processes and treatments (Okaiyeto et al. 2016). A bioflocculant solution of 0.2 mg/mL showed flocculating activity of 72%, 0.4 mg/mL (85%), 0.6 mg/mL (83%), 0.6 mg/mL (82%) and 1.0 mg/mL (87%), respectively (Figure 11) with an addition of 1 mL (3%) calcium chloride as Ca^{2+} cation. No significant difference was observed between the dosage concentrations values from 0.4 mg/mL and 1.0 mg/mL; therefore, further experiments were carried out using 0.4 mg/mL dosage concentration as the preferable dosage size, since low dosage is preferable and beneficial in industrial scale application in order to reduce cost. Similar to the study, 0.4 mg/mL dosage concentration was reported to produce optimum flocculating activity (Maliehe et al. 2016). Contrary to this study, the maximum dosage size of 1.2 mg/mL for *C. daeguense* resulted to a flocculating activity of 96.9%, was reported by Liu and Cheng (2010). Agunbiade et al*., (2017*) reported an optimum dosage size of 0.8 mg/mL for a consortium of *Cobetia* spp and *Bacillus* sp. However, a dosage concentration size of 2.0 mg/mL was reported for a consortium of *Methylobacterium* sp. Obi and *Actinobacterium* sp. Mayor (Ntsaluba et al. 2011). Wang et al., (2011) reported a maximum flocculating activity (96.21%) of a bioflocculant at 12 mg/mL produced

by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6 (Wang et al. 2011).

2.6.2. Effect of cation on bioflocculant activity

Divalent cations (Figure 12) largely supported the flocculating activity of BA-CGB with Ca^{2+} at 85%, Ba^{2+} (73%), Mn^{2+} (74%), Mg^{2+} (81%) while the effect of monovalent cations were moderate with Li^{+} (50%), K^{+} (58%) and trivalent cations Fe^{3+} sitting at 37%. Therefore, Ca^{2+} was used for all subsequent experiments.Similar results were reported on *Achromobacter* sp. TERI-IASST N which produced a bioflocculant that preferred Ca^{2+} as cation, and urea as nitrogen source. These findings were also similar to the results obtained using a bioflocculants from *Enterococcus hirae* (Subudhi et al. 2014). The bioflocculant BA-CGB is a cation-dependent as less than 5% flocculating activity was observed during the experiment.

2.6.3 Effect of heat on flocculating activity of bioflocculant BA-CGB

The bioflocculant BA-CGB showed a strong thermal stability within the range of 50 -90 ^{o}C, and the flocculating efficiency was 76% at 50 ^{o}C, increased slightly to 77% at 60 ^{o}C (Figure 13). A slight decrease in flocculating activity from 77% at 90 °C to 69% at 100 ^{o}C was observed. The bioflocculant is thermally stable as it retained above 60% flocculating activity at 100 ^{o}C. Although the bioflocculants have a strong thermal stability, increase in heat above 90 ^{o}C had a significant effect on the flocculating activity. Okaiyeto (2016) reported similar findings where all the three thermal-stable bioflocculants (REG-6, MBF-W7 and MBF-UFH) retained above 80% flocculating activity against kaolin clay suspension in the presence of divalent cations at 100 ^{o}C (Okaiyeto, 2016). The bioflocculant BA-CGB solution was also autoclaved at a temperature of 121 ^{o}C and pressure of 103.4 kPa for 15 minutes and retained over 60% flocculating activity. Autoclaving is one of the methods of sterilisation using steam under high-pressure method to sterilize objects used in various fields. Sterilisation utilises many different methods including steam or dry heat, chemicals and radiation (Tao, 2012). This bioflocculant's (BA-CGB) high flocculating activity can be due to its polysaccharide backbone, which has been reported to become extended on exposure to high temperature; thereby exposing more sites for binding to form flocs and increase flocculating activity (Gir et al., 2015). Bioflocculants that are rich in polysaccharide has been reported to have better thermal resistance than those of proteins and nucleic acids; owing to possessing of heat resistance properties (Zhang et al., 2012). Bioflocculants containing sugars as main flocculation component have been reported to be heat stable as they can retain up to 50% of their flocculating activity when heated in water (Lu et al., 2005).

2.6.4 Effect of pH on the flocculating activity of pure BA-CGB

One of the factors that contributes to the flocculation efficiency is the increased surface area that is initiated by different pH regimes during optimisation (Ntozonke et al., 2017). The flocculating activity of purified BA-CGB was greatly influenced by all pH tested, ranging from 3 - 12. Figure 14 shows the maximum flocculating activity that was observed at the extreme alkaline pH of 11 (94%). The flocculating activity for BA-CGB was above 70% for all pH range from pH 3 to pH 12 except pH 5 (65%). This kind of behaviour is very important for industrial application since the bioflocculant can be used without adjusting the pH of the sample. The decrease in flocculating activity around pH 5 (65%), may be due to adsorption of the hydroxyl ions (OH^{-}) close to neutral pH 7. There was a similar result where a bioflocculant MBF-UFH showed a good flocculating activity from pH 3 to pH 8, but had a sharp reduction in flocculating activity at pH 9. The reason for this sharp reduction is that MBF-UFH exhibited different spatial arrangement at different pH as a result of different electric states (Okaiyeto et al. 2016). This may interfere with the cation-mediated neutralisation of charges between Ca^{2+} and kaolin clay suspension (Cosa et al., 2011). Aljuboori et al., (2014) reported the pH stability of the purified bioflocculant promises good industrial applicability (Aljuboori et al., 2014). Ugbenyen et al., (2014) reported similar results to this study, where a bioflocculant produced by a consortium of *Cobetia* and *Bacillus* species demonstrated a flocculating activity of over 70% across a wide pH range of 3 - 11 but at variance to this study, the highest flocculating activity was attained at pH 8 (Ugbenyen et al., 2014).

2.6.5 Thermogravimetric analysis of the purified bioflocculant

Thermogravimetric analysis (TGA) was used to study the pyrolysis property of the bioflocculant BA-CGB. The results are depicted in Figure 15. An initial weight loss of about 19% was observed

between 0 - 200 ^{o}C. This downward trend continued with corresponding increase in temperature resulting in 40% weight loss being recorded at 792 ^{o}C with a final residual weight of 60%. This first weight loss under 200 ^{o}C has been reported to be due to moisture loss (Kumar and Anand,1998). It has also been reported to be due to protein part associated with protein content molecules (Selepe, 2017).

The decomposition of BA-CGB started between 30 ^{o}C and 67 ^{o}C. The thermogravimetric results for BA-CGB exhibited about four decomposition steps. These steps were at 67 °C, 121 °C, 208 °C, and 258 °C. The four decomposition steps of BA-CGB showed corresponding weight loss percentages of 7.51, 5.57, 8.40 and 6.88 %, respectively.

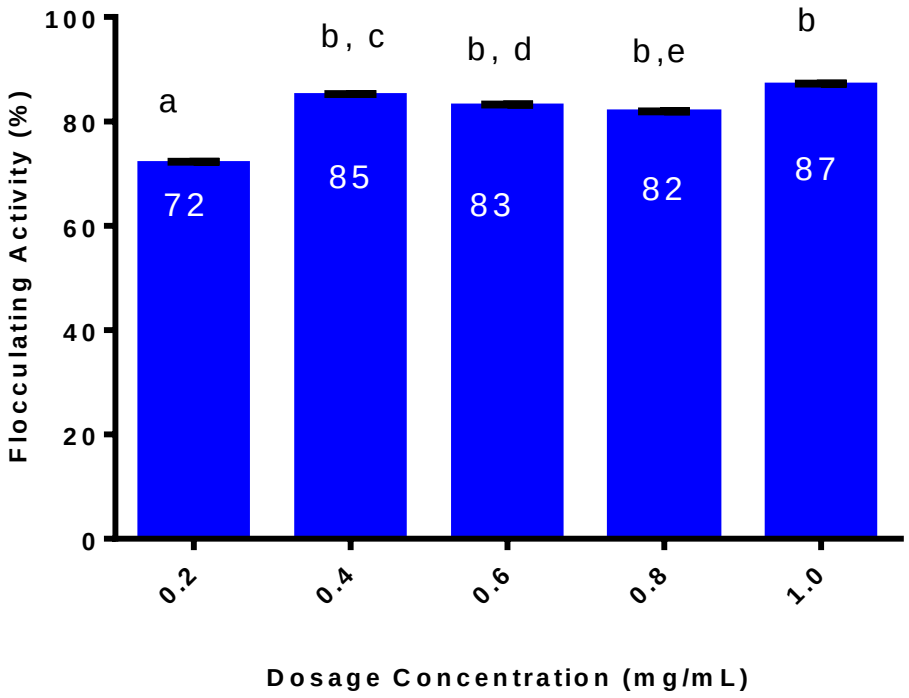

Figure 11: The effect of dosage size on flocculating activity of (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

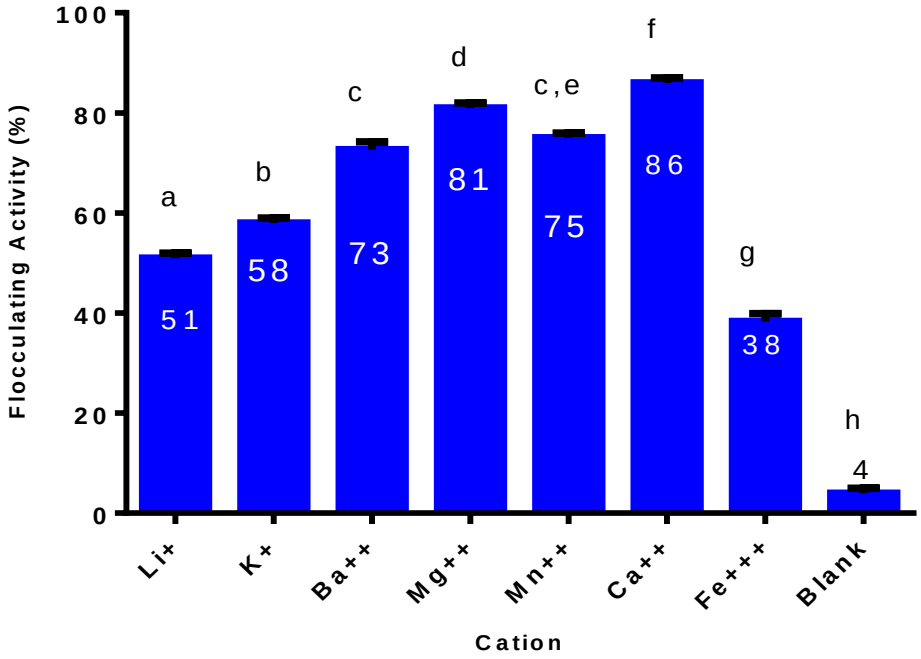

Figure 12: The effect of cations on the flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different (p < 0.05) from each other.

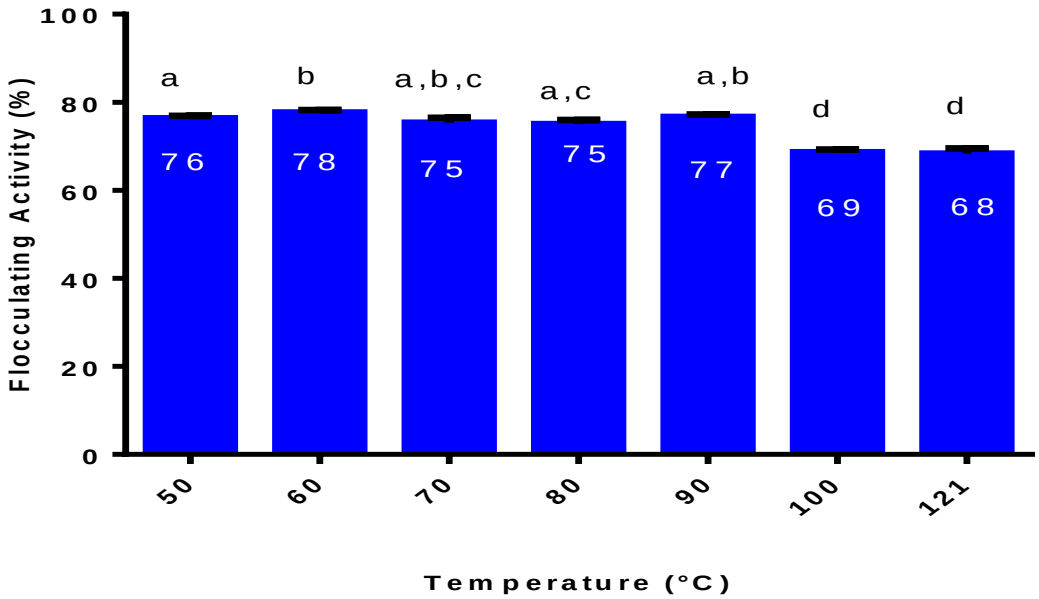

Figure 13: The effect of temperature on flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different (p < 0.05) from each other.

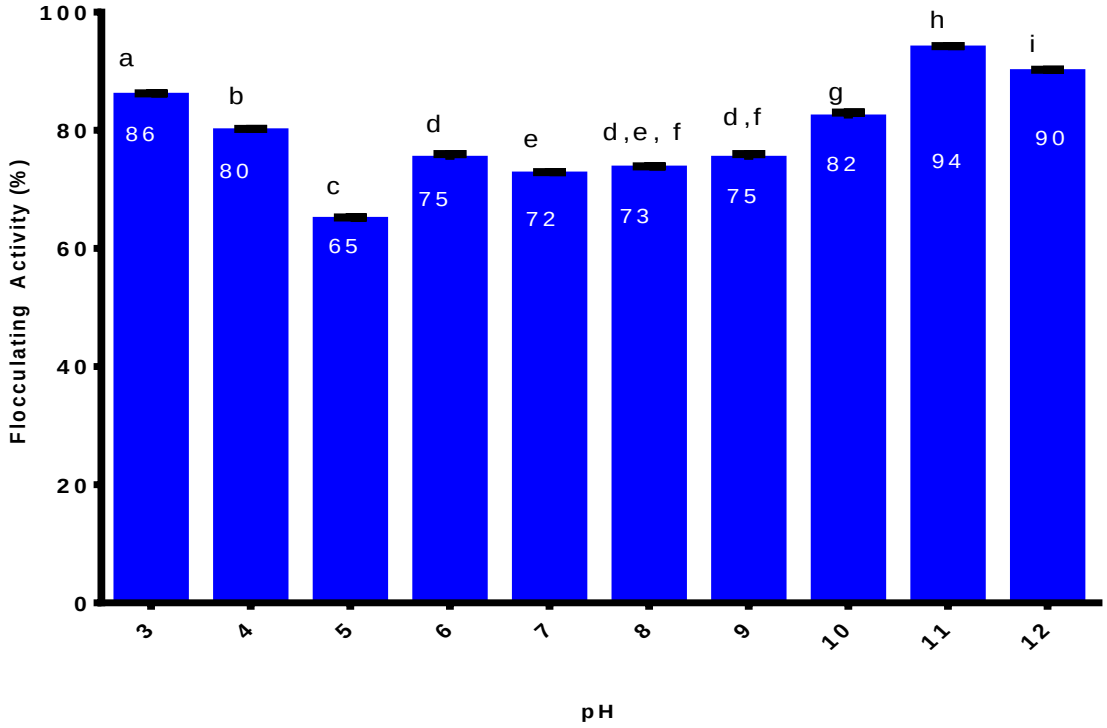

Figure 14: The effect of pH stability on flocculating activity of purified (BA-CGB) bioflocculant. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

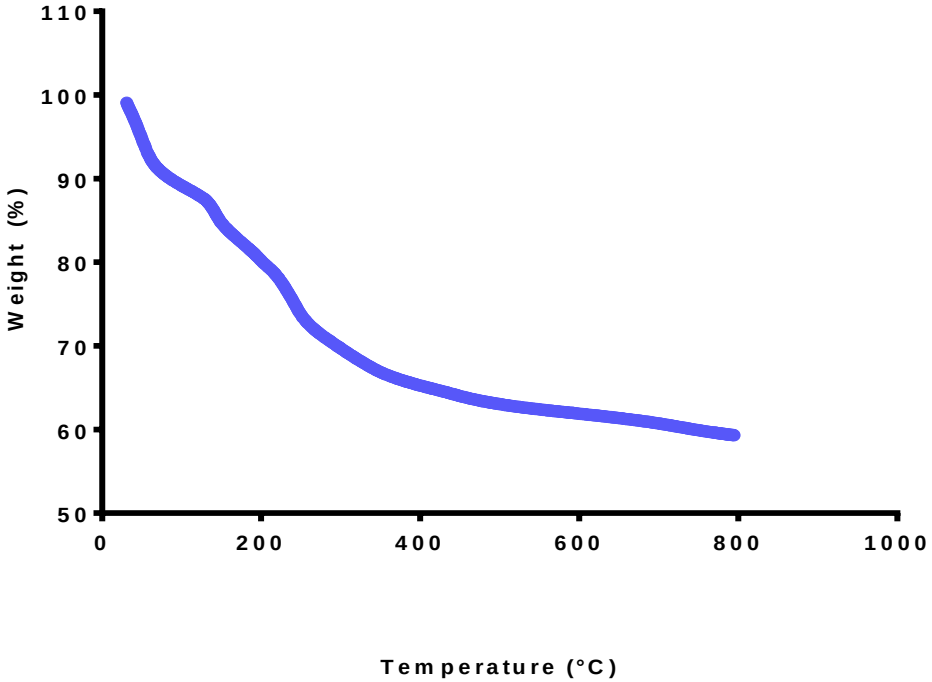

Figure 15: TGA of purified bioflocculant BA-CGB from *Bacillus atrophaeus.*

The corresponding weight loss of the bioflocculant as observed in 30-100 ^{o}C (10%), 100-200 ^{o}C (19%), 200-500 ^{o}C (36%) and 500-791 ^{o}C (40%) (Figure 15). The thermal analysis patterns for BA-CGB exhibited distinct decomposition steps which represented mass loss at every stage. The various decomposition steps reflect the number of distinctive compounds present in the bioflocculant BA-CGB which further affirms the results obtained with FT-IR and EDX elemental analysis. There was a 40% decomposition of bioflocculant BA-CGB at a temperature of 791 ^{o}C, this indicates a good thermal stability as more than 60 % weight was retained. Nwodo and Okoh (2013) reported similar findings with four decomposition steps and complete decomposition above 600 ^{o}C. The complete thermal decomposition of the bioflocculant was achieved at a temperature above 600 ^{o}C, thus implying a good thermal stability. Wang et al., (2011) reported a slightly different initial loss of 10% between 20 ^{o}C and 150 ^{o}C for a bioflocculant produced using a consortium of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6, then a further decrease at 400 ^{o}C by 40% and total weight loss at 1000 ^{o}C.

2.7 Application of bioflocculant on treatment of wastewater

2.7.1 Comparison of flocculating efficiencies of BA-CGB and conventional flocculants

The flocculating efficiency of BA-CGB was compared with other conventionally used chemical flocculants in wastewater treatment using the method described by Ugbenyen et al., (2014) with modifications as described by Okaiyeto et al., (2016). The optimum dosage (0.4 mg/mL) for BA-CGB was also compared with same dosage concentration for the other flocculants using kaolin clay suspension (Table 2).

Table 2: Comparison of flocculating activity of BA-CGB with conventional flocculants.

Flocculants	Conc. (mg/mL)	Flocculant activity (%)
Polyacrylamide	0.4	93
BA-CGB	0.4	91
Alum	0.4	83
Ferric Chloride	0.4	91

Note: All values obtained in triplicate

The results showed that polyacrylamide has the highest flocculating activity of 93%, followed by BA-CGB and ferric chloride at 91% respectively. All the three values obtained above are statistically insignificant from each other. However, Alum, a locally used flocculant has a flocculating activity of 83% (Figure 16) without the addition of cation. The results show that BA-CGB was comparably significant to both polyacrylamide as well as ferric chloride and was more efficient than alum. A similar result was reported by Okaiyeto et al., (2015), where the bioflocculant MBF-UFH was comparable to polyacrylamide. However, in that study, the reported flocculating activity of aluminium chloride (67.99%) and $FeCl_3$ (42.78%) was much lower than the results obtained in comparison with BA-CGB in this study. The results suggest that BA-CGB could possess great potential in the treatment of wastewater or river water.

2.7.2 Application of bioflocculant BA-CGB in treatment of Vulindlela wastewater and Mzingazi river water

Bad odours and anaerobic conditions are results of high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in stagnant waters that do not support aquatic life (Mhelcic and Zimmerman, 2010). Ma et al., (2018) have reported efficient removal of suspended solids, microorganisms, COD, BOD and heavy metals using bioflocculants. Waste water from Vulindlela domestic and Mzingazi river were treated with bioflocculant BA-CGB, thereafter compared with the conventional flocculant (polyacrylamide) and the removal efficiency were tabulated in Table 3 and Table 4.

Table 3 showed the physical properties of BA-CGB application in wastewater and river water treatment. Removal efficiency of COD for polyacrylamide (84%) was comparable to that of BA-CGB (80%). The removal efficiency for BOD on Vulindlela wastewater was 87% for BA-CGB and 89% for polyacrylamide. The removal efficiency of sulphate 60% compared to that of polyacrylamide (82%). Bioflocculant BA-CGB showed lower efficacy in the removal of phosphate (52%) and nitrate (88%) when compared with polyacrylamide (phosphate 96%, nitrate 93%), respectively.

Table 4 also showed that both bioflocculant BA-CGB and polyacrylamide had removal efficiency above 75% for all the parameters assessed in this study on Mzingazi river water. Li et al., (2013) reported a bioflocculant produced by *P. elgii* B69 with a 68% COD removal, 83% turbidity reduction with a dye colour removal efficiency of 88%. Contrary to the findings in this study, bioflocculant MBFA9 showed a COD

removal of 68.5% (82), while the bioflocculant produced from *Bacillus mucilaginous* had 74.6% COD and 42.3% BOD removal, respectively. Selepe (2017) conducted a comparative study on bioflocculant ORY84 with other conventional flocculants on Tendele coal mine and Erwat wastewater treatment plants. The findings showed that the COD removal efficiency value of 94% showed no significant difference in comparison with ferric chloride (94%) and alum (93%), respectively. The removal of COD, BOD and other constituents of wastewater plays an important factor in the re-use of water effluents.

3. Experimental Section (Materials and methods)

3.1 Sample collection, bacterial isolation and identification

Soil, water and sediment samples were collected from Richards Bay harbour (28.8000° S, 32.0833° E), KwaZulu Natal, Republic of South Africa. Samples were collected aseptically in sterile plastic bottles of 250 mL capacity and McCartney bottles (25 mL), transported for analysis in a cooler box with ice to the Microbiology Laboratory of the University of Zululand, KwaDlangezwa, South Africa, and analysed within six hours after collection.

3.2.2 Cultivation media

Nutrient agar, yeast extract agar (YEA) and M1 medium (YEA + starch) were used for the cultivation of microorganisms. In the cultivation of microorganism, non-selective media such as Nutrient agar medium is used for general cultivation and maintenance. However, there are also fastidious organism that grows on enriched media such as yeast extract agar and M1 medium (YEA + starch). M1 agar and YEA agar were used for the isolation of actinomycetes with modifications (83). The basal ingredient in nutrient agar per litre consists of meat extract (1.0 g), peptone (5.0 g), yeast extract (2.0 g), sodium chloride (8.0 g), and bacteriological agar (15 g) as solidifying agent. All ingredients were dissolved in 1 litre of filtered seawater. M1 consist of 10 g starch, 4 g yeast extract, 2 g peptone and 18 g bacteriological agar were dissolved in 1 litre of filtered seawater as described by Ogunmwonyi *et al.* (2010) and Mincer et al., (2002). Yeast extract agar composed of yeast extract (3 g), peptone (5 g) and bacteriological agar (15 g) supplemented with 10% starch (2.3 g) in 1 litre of filtered marine water.

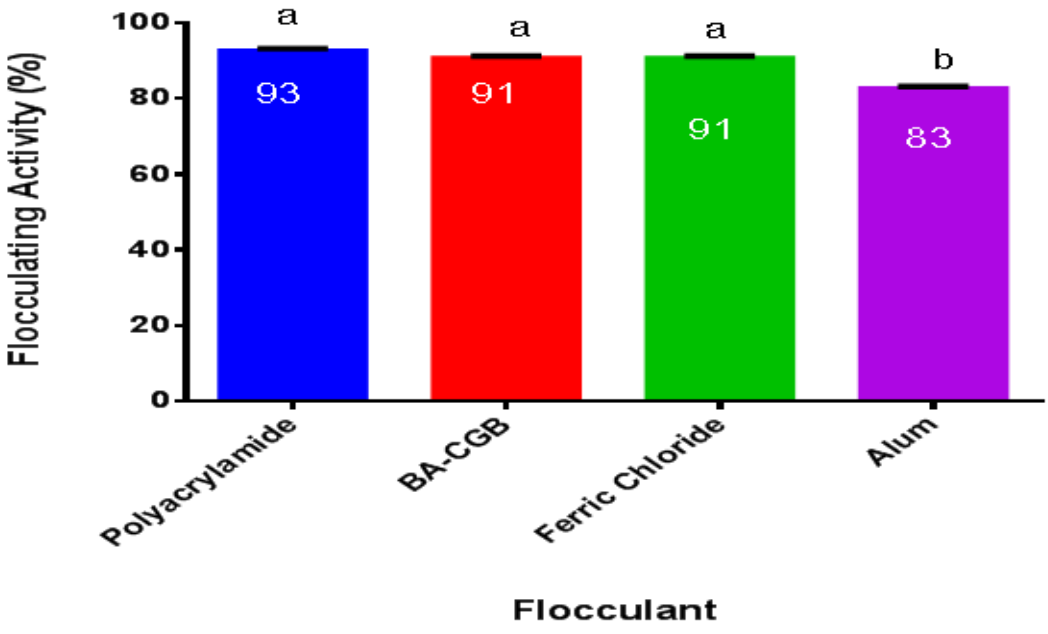

Figure 16: Comparison of flocculating activity of purified bioflocculants BA-CGB with conventional flocculants. Percentage flocculating activities with different alphabets are significantly different ($p < 0.05$) from each other.

Table 3: Removal efficiency of bioflocculant BA-CGB in the treatment of Vulindlela wastewater.

Flocculants		COD (mg/L)	BOD (mg/L)	Sulphate (mg/L)	Phosphate (mg/L)	Nitrate (mg/L)
Polyacrylamide	Before	422±0.0	335±0.0	245±0.0	6,7±0.0	0,8±0.0
	After	67± 0.58	38±0.58	45±0.58	0,25± 0.06	<0,1±0.00
	Removal efficiency (%)	84	89	82	96	93
BA-CGB	Before	422±0.0	335±0.0	245±0.0	6,7±0.0	0,8±0.00
	After	85±1.00	43±0.58	99±0.58	3,2±0.06	0,1±1.00
	Removal efficiency (%)	80	87	60	52	88

*All values obtained in triplicate

Table 4: Removal efficiency of bioflocculant BA-CGB in the treatment of Mzingazi river.

Flocculants		COD (mg/L)	BOD (mg/L)	Sulphate (mg/L)	Phosphate (mg/L)	Nitrate (mg/L)
Polyacrylamide	Before	50±0.0	32±0.0	210±0.0	1,5±0.0	0,5±0.0
	After	3,5±0.03	5±0.06	32± 0.58	<0.1±0.01	<0.1± 0.01
	Removal efficiency (%)	93	85	85	95	90
BA-CGB	Before	50±0.0	32±0.0	210±0.0	1,5±0.0	0,5±0.0
	After	6.8±0.01	8.0±0.01	44±0.58	0,3±0.01	<0.1±0.01
	Removal efficiency (%)	86	76	78	80	85

*All values obtained in triplicate

3.2.3 Isolation of microorganisms

Tenfold serial dilution of the water samples was prepared with sterile saline solution. One hundred microliter (100 µL) of the diluted water samples was inoculated to nutrient agar, yeast extract agar and M1 agar plates using spread plate techniques. To isolate microorganisms from the sediment samples, the dry/dilute and dry/stamp method slightly modified were used (Jensen et al. 2005). All plates were incubated at 37 ^{o}C for 24-72 hours after which the plates were observed for growth. Single colonies were selected randomly based on their colour, size, structure, morphology, sub cultured onto nutrient agar plates and incubated at 37 ^{o}C for 24-72 hours to obtain pure cultures. Pure colonies obtained after the incubation (or sub culturing) for 24-72 hours at 37 ^{o}C were used to screen for bioflocculant-producing bacteria and identification. Identification was done based on their morphology, cultural characteristics using Bergey's manual of systematic bacteriology (Christen, 2008).

3.3 Screening for bioflocculant production

3.3.1 Production medium and bacterial growth

The production medium composed of ingredients listed in Table 5 was prepared as described by (Zhang et al. 2007) in filtered marine water.

Table 5: Production medium for screening microorganism for production of bioflocculant.

Bioflocculant pre-culture composition	Ingredients
Ingredients	**Amount**
Glucose	20.0 g
Yeast Extract powder	0.5 g
Urea	0.5 g
$(NH_4)_2SO4$	0.2 g
K_2HPO_4	5.0 g
KH_2PO4	2.0 g
NaCl	0.1 g
$MgSO_4.7H_20$	0.2 g
Marine water (Filtered)	1 litre

The production medium was prepared by dissolving the ingredients (Table 2) in filtered marine water and autoclaved for 15 minutes at 121 ^{o}C. One loopful of bacterial colony was inoculated into 50 mL of sterile production medium in a 100 mL conical flask. This standard method was used for the production medium and was carried out according to Zhang et al., (2007). A standardised inoculum was used before the inoculum size for the selected bacterium was

obtained. The inoculated medium was incubated at 30 ^{o}C for 72 hours in a shaking incubator with a rotating speed of 160 rpm. After the incubation period, 2 mL of the fermentation broth was then centrifuged (8000 xg, for 30 minutes at 4 ^{o}C) to remove the cells, while the cell-free supernatant was used as crude bioflocculant to determine the flocculating activity (Xia et al. 2008).

3.3.2 Determination of flocculating activity

The flocculating activity tests were carried out according to the method previously described by Kurane and Matsuyama (1994) with slight modifications as described by Ugbenyen and Okoh (2013). Four gram per litre of kaolin clay suspension was used as a test material. Three millilitres (3 mL) of 1% $CaCl_2$ and 2 mL of cell-free supernatant were added to 100 mL of kaolin clay suspension (4.0 g/L) contained in a 250 mL conical flask. The mixture was agitated vigorously, poured into a 100 mL measuring cylinder and allowed to stand for 5 minutes at room temperature for sedimentation. A control experiment was prepared using the same method but the bioflocculant was replaced by sterile culture medium. The top clear solution (1 cm) was used to determine the optical density at 550 nm with a spectrophotometer (Pharo 100, Merck KGaA, Germany) (Xia et al. 2008).

Flocculating activity was calculated using the following formula:

Flocculating Activity = {(A - B)/A} x 100%

Where *A* = optical density at 550 nm (OD_{550}) of control and *B* = optical density at 550 nm (OD_{550}) of a sample.

3.4 Molecular identification of the organism using 16S rRNA gene

The bacterial isolate was cultured in 50 mL fresh Luria Broth (LB) and incubated at 37 ^{o}C on rotatory shaker speed of 200 rpm for 16 hours. The bacterium was further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacterium, the universal primers 27F and 1492R were used to amplify the 16S target region of this bacterium (Lane, 1991; Turner et al. 1999). The bacterial strain was analysed using the 16S rRNA and the phylogenetic tree was constructed with similar sequences found in Gen Bank. Nucleotide sequence analysis based on gyraseA gene using the Basic Local Alignment Search Tool (BLAST) of the 16S rRNA. The amplified and purified PCR products were used to determine the sequence of the bacteria. The findings were compared with the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree was thereafter constructed as described by Okaiyeto et al., (2013). These isolates were stored in the 20% glycerol broth at -80 ^{o}C freezer in the Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, KwaZulu-Natal, South Africa.

3.5 Optimisation of culture conditions for bioflocculant production

To enhance and increase the yield and flocculating efficiency of a bioflocculant, the conditions in the culture broth must be optimised by varying factors such as carbon and nitrogen sources, metal ions, aeration ratio, initial pH effect, inoculum size, culture incubation time and temperature effect (Salehizadeh and Yan, 2014).

3.5.1 Effect of inoculum size on bioflocculant production

To determine the optimum inoculum size, the stock culture ranging from 1% (0.5 mL), 2% (1.0 mL), 3% (1.5 mL), 4% (2.0 mL) to 5% (2.5 mL) was prepared and inoculated into 50 mL of production medium. Flasks were incubated at 30 ^{o}C for 72 hours at 160 rpm. To measure the flocculating activity, three millilitres of 1% $CaCl_2$ (w/v) and 2 mL of supernatant from the centrifuged production medium were added to 250 mL conical flasks with 100 mL kaolin clay suspension (4 g/L). The solution was agitated and poured into a graduated 100 mL measuring cylinder and allowed to stand for 5 minutes at room temperature for sedimentation. One millilitre of clear supernatant was withdrawn and the flocculating activity was determined at 550 nm using spectrophotometer (Ntozonke et al., 2017).

3.5.2 Effect of different carbon, nitrogen sources on bioflocculating production

Different carbon sources such as glucose, lactose, fructose, sucrose, carbonate, starch (20 g/L) were used and flocculating activities were measured. The impact of various organic and inorganic nitrogen sources using yeast extract powder, peptone, urea, $(NH4)_2SO_4$ and NH_4Cl were also monitored. The multiple nitrogen sources in the original pre culture medium was replaced with equivalent amount (1.2 g/L) contained in the basal production medium as described by Lachhwani (2005).

3.5.3 Effect of initial pH of the production medium

The effect of initial pH of the production medium was assessed by adjusting the pH of the production medium with 0.1 N NaOH and 0.1 N HCl to pH values ranging from 3 -12 and the flocculating activity was determined as previously described (Ntozonke et al. 2017).

3.5.4 Effect of shaking speed on flocculating activity for bioflocculant production

The effect of different ranges of shaking speeds on the bioflocculating activity was assessed according to the method described by Zhang et al., (2007). Different conical flasks containing 50 mL of production medium was inoculated with the 4% (v/v) inoculum size of the bioflocculant producing isolate and incubated at 30 ^{o}C for 72 hours in different speeds, ranging from 0-220 rpm. The flocculating activity was measured at 550 nm wavelength using a spectrophotometer.

3.5.5 Effect of metal ions on flocculating activity for bioflocculant production

The effect of metal ions on the flocculating activity was assessed using a method described by Nie et al., (2011). To obtain the flocculating activity, three millilitres of $CaCl_2$ (1% w/v) and 2 mL of bioflocculant solution was added to 100 mL kaolin suspension (4 g/L) in 250 mL conical flasks. From the standard method, the 1% $CaCl_2$ was replaced by various metal salt solutions (1%) (LiCl, $BaCl_2$, $FeCl_3$, $MnCl_2$, $MgCl_2$ and KCl). A control experiment was also prepared without cation added on 100 mL Kaolin solution and 2 mL supernatant. The flocculating activity was determined as described by Ugbenyen and Okoh (2013).

3.5.6 Time course Assay

For the fermentation time, the method described by Gao et al., (2006) was used with minor modifications. The optimum medium composition obtained was used. The pH of the medium was adjusted with either 1.0 N NaOH or 1.0 N HCl to pH 9.0. The production medium made up of glucose (20 g/L), K_2HPO_4 (5 g/L), KH_2PO_4 (2 g/L), NH_4Cl (1.2 g/L) and $MgSO_4$-$7H_2O$ (0.2 g/L) was dissolved in 1 litre of filtered marine water and autoclaved at 121 ^{o}C for 15 minutes. Saline solution (50 mL) was used to prepare the suspension of the bacterial isolate as the standard inoculum. The optical density of the suspension (100 µL) in distilled water (1 mL) was first measured at OD_{660} and then gradually adjusted to 0.1. The optimum volume of the bacterial suspension obtained was inoculated into 50 mL production medium in a 250 mL flask and incubated in a rotary shaking speed at 110 rpm, 35 ^{o}C for 120 hours. At every 12 hours up to 120 hours, 2 millilitres of the broth were drawn and assessed for flocculating activity in accordance with the method of Kurane and Matsuyama (1994). The bacterial growth was measured at OD_{660} in a spectrophotometer and the pH of the culture broth was also monitored for 120 hours.

3.6 Extraction and purification of bioflocculants

The method of extraction and purification of the bioflocculant was carried out as described by Cosa et al., (2011) and Chang et al., (1998) with minor modifications. After 96 hours of fermentation, the bioflocculant from the production medium was harvested by centrifugation for 15 minutes at 5000 g at 4 ^{o}C. For isolation of EPS, one volume of distilled water was added to the supernatant phase and further centrifuged for 15 minutes at 5000 g at 4 ^{o}C to remove insoluble substances. Two (2) volumes of ice-cold ethanol was added to the culture supernatant, agitated properly and stored for 12 hours at 4 ^{o}C. The precipitate was vacuum-dried to obtain the crude bioflocculant. The crude bioflocculant obtained was re-dissolved in 100 mL of distilled water and one volume of a mixture of chloroform and n-butyl alcohol (5:2 v/v) was added. The mixture was shaken vigorously and left to stand at room temperature for 12 hours. The supernatant was centrifuged at 5000 g for 15 minutes at 4 ^{o}C and vacuum dried to obtain a pure bioflocculant. The weight of the dried bioflocculant was expressed in g/L culture (Lie et al. 2016; Gupta et al. 2016).

3. 7. Physicochemical composition of the purified bioflocculants

3. 7. 1. Chemical compositional analysis of the purified bioflocculants

The total sugar content analysis was done using the phenol-sulfuric acid method with glucose being used as a standard (Chaplin and Kennedy, 1994). Bradford assay with Bovine serum Albumin (BSA) as standard was used to measure the total protein content of the bioflocculant produced (Bradford, 1976). The carbazole–sulfuric acid method as described by Bitter and Muir (1962) was used to measure the content of uronic acid.

3. 8 Chemical Analysis of a purified bioflocculant

3. 8. 1 SEM Analysis of bioflocculant

The morphological structure of the bioflocculant was investigated using the scanning electron microscope (SEM) equipped with elemental analyser (Oxford Instruments X-MaxN). Five milligram (5 mg) of purified bioflocculant was added to a silicon-coated slide. The silicon-coated slide was fixed with a spin coater at 1000 rpm for 60 seconds. The SEM image of the purified bioflocculant, kaolin clay particles and flocculated kaolin clay particles were obtained (Xia et al. 2008). The elements present in the purified bioflocculants were also determined.

3. 8. 2 Fourier transform infrared spectrophotometer (FT-IR) analysis

The functional groups analysis of the purified bioflocculant was done using the Fourier transform infrared spectrophotometer to obtain the infrared spectrum of the dried purified bioflocculant sample. This was recorded at room temperature (25 ^{o}C) at wavenumber ranges of 4000 - 400 cm $^{-1}$ using a FT-IR spectrophotometer (Cosa et al. 2013).

3.8.3 Thermo-gravimetric analysis (TGA)

Ten milligrams of the bioflocculant was analysed using a TG analyser (Perkin Elmen Pyris 6 TGA) to obtain the pyrolysis of the purified bioflocculant at a temperature range of 30 – 900 ^{o}C and at a heating rate of 10 ^{o}C per minute. This rate was kept constant under flow of nitrogen gas (Yim et al. 2007).

3.9 Flocculation characteristics of a purified bioflocculants

3.9.1 Effect of dosage concentration on flocculating activity (Jar test)

To assess the effect of dosage concentration of the purified bioflocculant, the method described by Makapela *et al.* (2016) was followed. A range of concentration of the purified bioflocculant between 0.2 and 1.0 mg/mL (w/v) were prepared. Two millilitres from each solution was mixed with 100 mL of kaolin clay suspension together with 3 mL of 1% (w/v) $CaCl_2$ in a 250 mL conical flask. The solution was vigorously agitated, transferred into 100 mL measuring cylinder and allowed to stand for sedimentation for 5 minutes. One millilitre of the supernatant was drawn and used to determine flocculating activity in a spectrophotometer at 550 nm. The obtained optimum concentration dosage was used for subsequent experiments.

3.9.2 Effect of temperature on bioflocculating activity

The purified bioflocculant was dissolved in distilled water to give an optimum concentration of 0.4 mg/ml. Two millilitres of the bioflocculant solution was heated at various cultivation temperatures such as: 50, 60, 70, 80, 90, and 100 ^{o}C for a period of 30 minutes. Another tube (2 mL) was autoclaved with steam under pressure at 121 ^{o}C for 15 minutes. The residual flocculating activity of the purified bioflocculant for kaolin suspension (4 g/L) at room temperature was determined in order to obtain the temperature dependence of the purified bioflocculant (Aljuboori et al. 2015).

3.9.3 Effect of pH on the flocculating activity of purified bioflocculant

Optimum dosage concentration (0.4 mg/ mL) of purified bioflocculant was prepared and used to measured pH stability of a bioflocculant. The pH of individual 100 mL of kaolin solutions (4 g/L) were adjusted in separate flasks, ranging from 3 – 12 prior to determination of flocculating activity. The flocculating activity values for each experiment were obtained at each pH values (Ntozonke, 2015).

3.9.4 Effect of cations on flocculating activity

The effect of cations on the flocculating activity was assessed with the method described by Zulkeflee *et al.* (2012). Different metal ions such as Fe^{2+}, Ba^{2+}, Li^{+}, K+, Mg^{2+}, Fe^{3+} and Mn^{2+} were used to replace 1% $CaCl_2$ (w/v) in the experiment. A solution of bioflocculant optimum concentration was prepared. The effects of these metal ions on the flocculating activity of the purified bioflocculant were determined.

3.10 Treatment of waste water using a purified bioflocculant

Two litres of wastewater samples were freshly collected from Vulindlela domestic waste water treatment outlet and Mzingazi River from Richards Bay. Different parameters such as Biochemical Oxygen Demand (BOD), ammonia, nitrate, phosphate, pH, sulphate content, and Chemical Oxygen Demand (COD) were determined prior to and after flocculation with a bioflocculant. This was determined using a pH meter and spectrophotometer (Pharo 100, Merck KGaA,

Germany) (Okaiyeto et al. 2016; Li et al. 2013). One hundred millilitres (100 mL) of wastewater sample was adjusted to the optimum pH of 11 in a 250 mL beaker together with 2 mL of 0.4 mg/ mL bioflocculant solution and 3 mL of 1% (w/v) calcium chloride (Ca^{2+}) ions. The mixture was vigorously agitated at 200 rpm for 3 minutes and then the speed was reduced to 45 rpm for 5 minutes. After 5 minutes of agitation, the flask was left to stand for 5 minutes at room temperature for sedimentation. The clear supernatant just 3 cm below the surface was used to determine the residual parameters of phosphate, sulphate content, nitrate, BOD and COD of the treated sample. The flocculating activity was also measured using a spectrophotometer at OD_{550}. In comparison, the bioflocculant was replaced with the chemical flocculants known as polyacrylamide in the same concentration of 0,4 mg/mL. To calculate the removal efficiency of flocculant, the following formula was used;

Removal Efficiency (RE) (%) = $[C_0 – C/C_0]$ x 100 %

Where C_0 and C are the removal efficiency values before and after the flocculation process.

3.11. Statistical analysis

All data were conducted and collected in triplicates with results expressed as mean and standard deviation values. These data were subjected to one-way analysis of variance (ANOVA) using Graph Pad Prism ™ 6.1. Differences were considered at a significant level of $p<0.05$.

CONCLUSION

Bioflocculants have shown great potential to improve quality of waste water treatment, productivities and are safe for both human and the environment compared to the in-use chemical flocculants, which have been implicated in undesirable health conditions. Bioflocculant producing strain, *Bacillus atrophaeus* strain SRCM 101359 isolate was identified through 16S rRNA nucleotide sequence and BLAST analyses. After optimization of physiochemical properties of culture medium, conditions were adjusted to pH 9, incubated at 35 ^oC with a shaking speed of 110 rpm and with Ba^{2+} cation as a stimulating agent. The optimal growth condition produced a bioflocculant yield of 3.165 g after 96 hours of fermentation obtained from one litre of production medium using 4% inoculum size, glucose and ammonium chloride as energy sources. This was produced parallel to the cells multiplication of *Bacillus atrophaeus*. The purified bioflocculant BA-CGB was characterized with the SEM showing bioflocculant BA-CGB of an amorphous structure consisting of elements in major proportion such as carbon, oxygen, phosphorus and potassium. BA-CGB was revealed to be composed of carbohydrates (mainly), proteins and uronic acid. FT-IR analysis revealed that the molecular chain of BA-CGB possess hydroxyl, amine, carboxyl and amide functional groups, characteristics favouring the flocculation mechanism. The bioflocculant BA-CGB is thermal stable within the range of 50 - 100 ^oC and retained more than 60% flocculating activity at 121 ^oC after 15 minutes of exposure. An effective performance of BA-CGB was observed at a wide range of pH 3-12 with an optimum dosage concentration of 0.4 mg/mL and Ca^{2+} cation stimulating the process. This is a great benefit as it is contemplated for the use in industrial processes. The microbial flocculant showed the potential in reducing COD and BOD and at the same time aid in the removal of efficiencies of certain parameters such as sulphate, nitrate as well as phosphorous in both Mzingazi river water and Vulindlela domestic wastewater. The bioflocculant BA-CGB could be a great substitute to in-use chemical flocculants.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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AUTHOR CONTRIBUTIONS

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