Flocculating Properties of a Bioflocculant Produced by *Bacillus* sp. Isolated from a Marine Environment in South Africa

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We assessed the bioflocculant production by a bacteria isolated from a marine environment in South Africa. The 16S rDNA nucleotide sequence analyses revealed the bacteria to have a 99 % similarity to *Bacillus* sp. A-5A and the sequence was deposited in the Genbank as *Bacillus* sp. MAYA (accession number JF799093). Bioflocculant production by the bacteria was optimal when glucose (95.6 % flocculating activity) and ammonium nitrate (83.3 % flocculating activity) were used as carbon and nitrogen sources respectively; inoculum size was 2 % (v/v); initial pH 6; and Ca^{2+} as coagulant aid. Chemical analysis of the purified bioflocculant shows that it is composed of uronic acid, neutral sugar and protein. FTIR analysis reveals the presence of methoxyl, hydroxyl, carboxyl and amino-groups in the compound bioflocculant. The bioflocculant is thermostable with about 65.6 % residual flocculating activity retained after heating the bioflocculant at 100 °C for 25 minutes.

Key words:
Bioflocculant, production, bacteria, glucose, inoculums, analysis

Introduction

Flocculation is a process whereby chemicals (flocculants) stimulate aggregation of colloids and other suspended particles in a suspension to form floc. This is different from precipitation, because prior to flocculation, colloids are suspended in a liquid and not completely dissolved in a solution.[1] Flocculation finds usefulness in many fields such as wastewater treatment, downstream processing, food and fermentation processes.[2–4] Flocculating agents could be inorganic, synthetic or natural. Typical examples of natural flocculating agents are bioflocculants produced by microorganisms. Bioflocculants are the metabolite product of microorganisms produced during their growth. They are usually high molecular weight biopolymers, and are synthesised and released outside the cell.[2] Compared with the conventional flocculants, bioflocculants are safe for use because of their relative inert nature.[5] Thus, screening new microorganisms for bioflocculant with excellent flocculating activity has become a subject of intensive investigations globally.

Bacillus species are positive to Gram-staining and are rod-like, their morphological arrangement are either in pairs or chains with round or square ends having single endospore. The genus currently comprises in excess of 60 species[6] and have been reported to produce bioflocculant[2,7–13] but isolates from marine environment are scanty in literature. Marine bacteria are amongst the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about 50 % of all discovered bioactive secondary metabolites.[14] The rate of discovery of new compounds from terrestrial bacteria has decreased, whereas the rate of re-isolation of known compounds has increased, suggesting the need for exploring underexploited habitats such as the marine environment as sources of novel bioactive compounds including bioflocculants. In this report, we assessed the potential of Bacillus specie isolated from marine sediment in South Africa for the production of bioflocculant.

Materials and methods

Bacterium isolation

The bacterium was isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa and was preserved in 20 % glycerol at –80 °C.

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Identification of the bioflocculant-producing bacteria

DNA extraction

The bacterial genomic DNA was extracted using the boiling method.[15] Briefly, 2 – 3 colonies of the test bacteria were suspended in 70 µL of sterile double-distilled water and heated at 100 °C for 10 minutes to lyse the cells, allowed to cool for 5 minutes, and thereafter centrifuged at 3000 rpm for 5 minutes to pellet cell debris. The supernatant was transferred to a clean tube and used as template DNA for 16S rRNA gene amplification.

PCR amplification of 16S rRNA gene

PCR amplification of the 16S rRNA gene of the bacteria was carried out in 50 µL reaction volume containing 2 mmol L^{-1} $MgCl_2$, 2 U Supertherm Taq polymerase, 150 mmol L^{-1} of each dNTP, each primer 0.5 mmol L^{-1} (F1: 5'-AGAGTTTGATCITGGCTCAG-3'; I = inosine and primer R5: 5'-ACGGITACCTTGTTACGACTT-3') and 2 mL template DNA in line with previous descriptions.[15–17] The PCR condition included an initial denaturation (96 °C for 2 minutes), 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s), and extension (72 °C for 2 minutes), and a final extension (72 °C for 5 minutes). Gel electrophoresis was run on 1 % agarose gels to confirm that the fragment of correct size had been amplified.

Media preparation and culture conditions

The pre-culture medium consisted of 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 5 g K_2HPO_4, 0.1 g NaCl and 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 liter of filtered natural seawater using a Whatman filter paper. The initial pH of the medium was 6.[18] Two loopfuls of the bacteria colonies were inoculated into 50 mL of the medium and incubated with shaking at 160 rpm for 72 h at 28 °C. At the end of the incubation period, 2 mL of the fermentation broth was centrifuged (8000 g, 30 minutes) to separate bacterial cells and the cell free culture supernatant was analysed for flocculating activity. The pre-culture was stored at 4 °C and used for subsequent inoculations. Production medium contained the same components as the pre-culture medium.[18]

Determination of flocculating activity

Flocculating activity was determined as described elsewhere.[16,19–21] Briefly, 3 mL of 1 % $CaCl_2$ and 2 mL of cell free supernatant were added to 100 mL kaolin suspended solution (4 g L^{-1}) in 250 mL flask. The mixture was shaken vigorously and poured into a 100 mL measuring cylinder, and allowed to stand for 5 minutes. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. A control experiment was prepared using the same method, but the bioflocculant was replaced by the fresh culture medium (B). The flocculating activity was measured using the equation

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

where A is the absorbance of the sample experiment at 550 nm; B is the absorbance of control experiment at 550 nm.

Effects of cultivation conditions

Inoculum size

Inoculum size is an important parameter in the production of bioflocculant.[18,22] Hence, we assessed the effect of different inoculum sizes on bioflocculant production. Flasks (150 mL size) containing 50 mL production medium were separately inoculated with 0.5 mL, 1.0 mL, 1.5 mL, and 2.0 mL (amounting to 1 %, 2 %, 3 % and 4 % (v/v) respectively) pre-culture of the bacteria cultivated at 28 °C with agitation at 160 rpm for 72 h. At the end of the incubation period, the fermentation broths were centrifuged (8000 g, 30 minutes) to separate the cells and the cell free supernatants were analysed for flocculating activity.

Carbon and nitrogen source

The effects of organic and inorganic carbon sources on bioflocculant production were assessed. The organic carbon source candidates included glucose, sucrose, fructose, maltose, galactose and xylose; while the inorganic carbon sources include phthalate, sodium acetate and sodium carbonate. Also, the organic nitrogen sources such as peptone, tryptone, urea, yeast extract and casein, as well as inorganic nitrogen sources such as ammonium chloride, ammonium sulphate and ammonium nitrate were assessed for their effect on bioflocculant production.

Initial pH and cations

The effects of initial pH and cations on bioflocculant production were assessed in accordance with the description of Liu et al.[5] The initial pH of the production medium were varied in the range of 3–12 using 0.1 mol L^{-1} HCl and NaOH, while the cation candidates included Na^+, K^+, Li^+, Mg^{2+}, Mn^{2+}, Al^{3+} and Fe^{3+} as their chloride salts. With regards to the effects of cation assays, flocculant tests were conducted as described above, but the $CaCl_2$ solution was replaced by a solution of the above cation candidates, and the flocculating activity was measured.

Time course assay

The medium for the time course assay was composed based on the previously determined optimal growth conditions. Samples were drawn every 12 h over a period of 5 days and of this, 2 mL was centrifuged (8000 g, 30 minutes), and the cell free supernatant was used to determine the flocculating activity. The optical density (OD) of the broth was also measured at 660 nm.

Extraction and purification of bioflocculant

Extraction and purification of bioflocculant was done according to the method described by previous reports.[16,20,23–25] After 72 h of fermentation, the culture broth was centrifuged (8000 g, 30 minutes) to remove bacterial cells. One volume of distilled water was added to the supernatant phase and centrifuged (8000 g, 15 minutes) to remove insoluble substances. After adding two volumes of ethanol, the supernatant was stirred and left to stand for 12 h at 4 °C. The precipitate was vacuum dried to obtain crude bioflocculant. The crude product was dissolved in distilled water to yield a solution, to which one volume of a mixed solution of chloroform and n-butylalcohol (5:2, v/v) was added, stirred and allowed to stand for 12 h at room temperature. Two volumes of ethanol were again added to recover the precipitate, which was then lyophilized.

Chemical analysis of purified bioflocculant

Qualitative detection of protein, sugars and hexouronic acid was done using the Folin-Lowry,[26] phenol-sulphuric acid[27] and carbazole[28] methods, respectively. FTIR analysis of the purified bioflocculant was done using a Fourier-transform infrared spectrophotometer (Perkin Elmer System 2000, England) over a wave number range of 4000 to 500 cm^{-1}. The thermal stability of the purified bioflocculant was also assessed by incubating solutions of the bioflocculant at 80 °C and 100 °C for 25 minutes[29] after which residual flocculating activity was determined. Scanning electron microscopy (SEM) image of the purified bioflocculant was taken using JEOL (JSM-6390LV, Japan) and the Energy Dispersive X- ray analysis (EDX) was measured by a Thermo Super Dry II X- ray Detector using a Noran System Six Software package.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using MINITAB Student Release 12 statistical package. A significance level of $p < 0.05$ was used. The mean values are of three replications.

Results and discussion

PCR amplification of the 16s rRNA gene of the test bacteria

BLAST (Basic Local Alignment Search Tool) analyses of the nucleotide sequence of the amplified (Fig. 1) 16S rDNA revealed the bacteria to have 99 % similarity to that of *Bacillus* sp. A-5A and the nucleotide sequence was deposited in GenBank as *Bacillus* sp. MAYA with accession number JF799093.

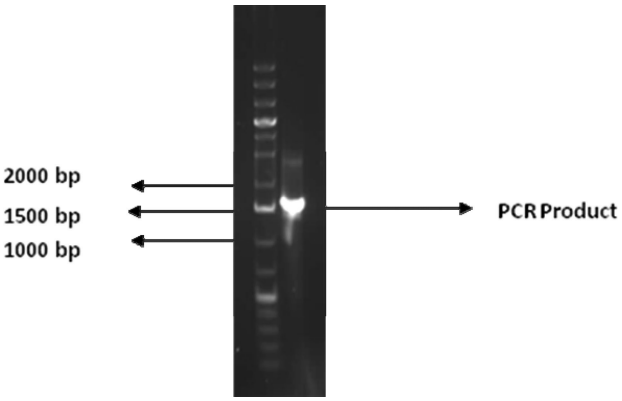

Fig. 1 – *Polymerase Chain Reaction (PCR) product of the 16S rDNA of the test bacteria in 1 % agarose gel*

Effects of culture conditions

The result of the effect of inoculum size on bioflocculant production is as shown in Fig. 2. Inoculum size of 2 % (v/v) resulted in the highest ($p < 0.05$) flocculating activity compared to the other three sizes. As a result, an inoculum size of 2 % was used for all subsequent experiments.

Findings have revealed that inoculum size impacts the growth kinetics of cultured organism greatly, as well as the product formation of such organism.[30–32] From previous report, bioflocculant production by different microorganisms was affected by inoculum size. An inoculum size of 1 % fa-

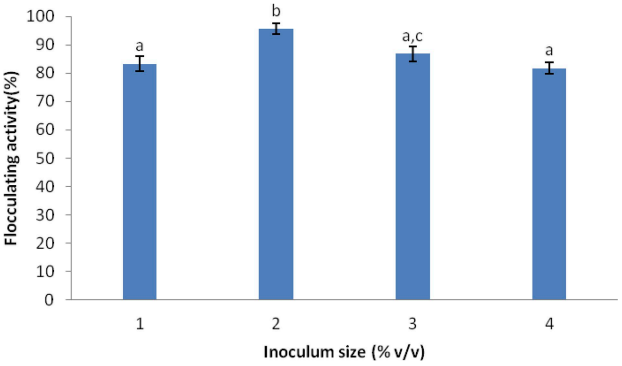

Fig. 2 – *Effect of inoculums size on bioflocculant production by the bacteria. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other*

voured flocculant production by *Serratia ficaria*,[29] 2 % inoculum size favoured *Proteus mirabilis*,[33] 4 % was best for the production of bioflocculant for *Bacillus licheniformis* X14[12] and 5 % for the flocculant produced by *Citrobacter* sp.[34]

The impact of various carbon sources on bioflocculant production is represented in Fig. 3. From the organic and inorganic carbon sources used, the bacteria showed preference to glucose for bioflocculant production, which resulted in flocculating activity of 95.6 %, followed by sodium carbonate an inorganic carbon source with a flocculating activity of 67.2 %. Fructose, xylose and sodium acetate were poorly utilised by the bacteria for bioflocculant production as suggested by the weak flocculating activities of 36.7 %, 44.4 % and 35.7 % respectively. As a result, glucose was used for all subsequent cultures.

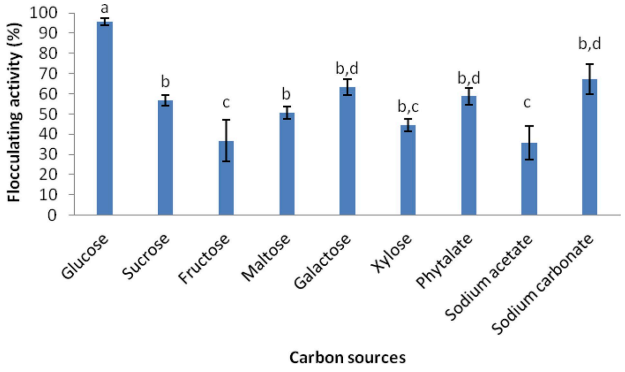

Fig. 3 – *Effect of carbon sources on bioflocculant production by the bacteria. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other*

Fig. 4 shows the result of the effect of various nitrogen sources on bioflocculant production. All the nitrogen sources appeared to be useful for bioflocculant production as they resulted in flocculating activity of at least 60 %. Ammonium nitrate yielded the highest flocculating activity (83.3 %), although this seems no different statistically from those of the other nitrogen sources except peptone.

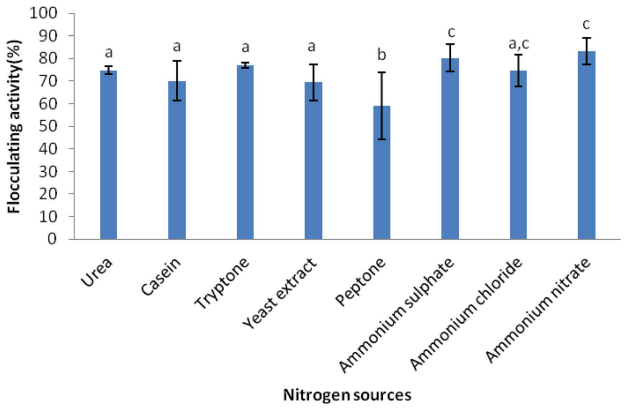

Fig. 4 – *Effect of nitrogen sources on bioflocculant production by the bacteria. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other*

Carbon and nitrogen sources are important nutrient factors for the production of bioflocculant.[5,10,11,35–37] Hence, in this study we investigated the effect of carbon and nitrogen sources in bioflocculant production. Amongst the carbon sources, glucose was the most preferred by the bacteria for bioflocculant production with a flocculating activity of 95.6 %, others were poorly utilised by the bacteria. Similar findings show that *Penicillium* sp, *Halomonas* sp V3a', *Virgibacillus* sp. Rob, *Proteus mirabilis* all favoured the use of glucose for bioflocculant production.[16,33,35,37]

All the nitrogen sources were well utilised by the bacteria with a flocculating activity of above 70 % except for peptone, which has a flocculating activity of 58.9 %. Optimal bioflocculant production was observed with ammonium nitrate with a flocculating activity of 83.3 %. Previous report on *Penicilium* sp shows similarity with our finding, with the bacteria utilising all investigated nitrogen sources effectively for bioflocculant production.[35]

The effect of initial pH of the medium on bioflocculant production was investigated and the results are presented in Fig. 5. The bacteria produced bioflocculant well in a wide range of pH 3–9, i.e. under acidic conditions and weak alkaline pH. There was a slight drop in flocculating activity at pH 5 and 7. It could be observed that highly alkaline pH 10–12 had not favoured bioflocculant production by this organism. Bioflocculant was produced optimally at pH 6 (75.4 %).

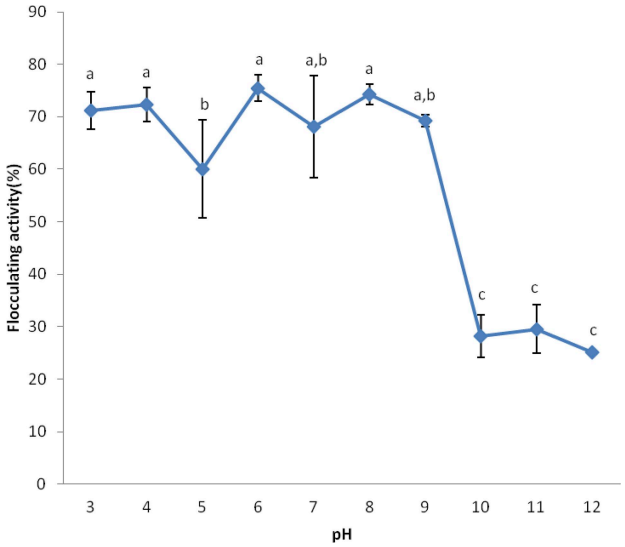

Fig. 5 – *Effect of pH variation on bioflocculant production by the bacteria. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other*

The effect of initial pH of the culture on bioflocculant production has been investigated by many researchers.[10,11,33,35,36] It has been observed that the initial pH for optimum production of bioflocculant differs with respect to the organism. The electrical charge on cells, as well as the oxido-reduction potential which affects absorption of nutrient and other enzymatic reactions of cells is dependent on the initial pH of the culture medium.[2,33,38]

From this study, the test bacteria produced bioflocculant well in a wide range of pH 3–9, it was observed that highly alkaline pH 10–12 had not favoured bioflocculant production as observed by the flocculating activities (25.1 % to 29.5 %). The optimal initial pH was 6 with a flocculating activity of 75.4 %. This is similar to the report by Deng et al.,[39] where bioflocculant production by *Aspergillus parasiticus* was well produced in the range of pH 3–9 and optimal flocculating activity observed at pH 6.

The result of the effect of metal ions on bioflocculant production revealed that all the metal ions could significantly stimulate flocculating activity, except for Fe^{3+}, which resulted in a poor flocculating activity of 25.8 % (Table 1). Ca^{2+} and Mn^{2+} ions are the most preferred cations having resulted in flocculating activity of 95.6 % and 92.4 %, respectively. As a result, Ca^{2+} was used for all subsequent experiment.

Table 1 – *Effect of metal ions on bioflocculant production by Bacillus sp. MAYA*

Metal ions	Flocculating activity (%)
Na^{+}	82.51 ± 1.36^{a}
K^{+}	88.94 ± 1.08^{b}
Li^{+}	87.50 ± 2.66^{b}
Ca^{2+}	95.60 ± 1.88^{c}
Mn^{2+}	92.45 ± 1.50^{c}
Mg^{2+}	$86.55 + 4.69^{b}$
Al^{3+}	88.92 ± 1.67^{b}
Fe^{3+}	25.82 ± 3.58^{d}

Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other.

Metal ions serve as coagulant aid of many microbial bioflocculants.[40] This is due to their charges and ionic radii which enable them to neutralise and stabilise opposite charge on the functional group of the bioflocculant,[2] and also reduce the particle surface charge density of the suspended particle, so that the bioflocculant molecule and the suspended particle can come together.[41,42] In this work, all the metal ions investigated stimulate flocculating activity well above 80 %, except Fe^{3+} that shows a flocculating activity of 25.8 %. Optimal flocculating activity of 95.6 % was observed with calcium ion. This is in congruent with the report of Li et al. [43] and Mabinya et al.,[21] both showed that Ca^{2+} mediated the highest flocculating activity amongst the metal ions examined.

Time course assay

The result of the time course assay of bioflocculant production is presented in Fig. 6. The flocculating activity rose to 82.7 % after 12 h of incubation from an initial flocculation activity of about 15 % and remained almost constant thereafter.

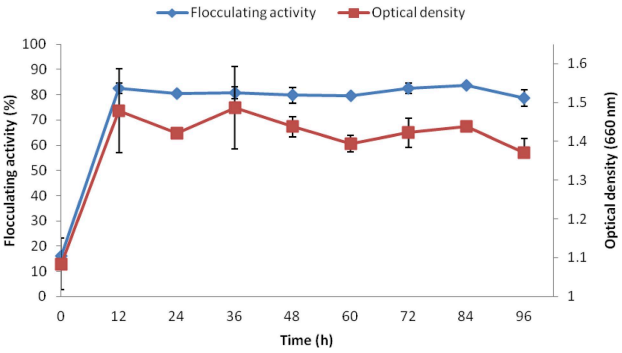

Fig. 6 – *Kinetics of bioflocculant production by the bacteria*

The cultivation time for bioflocculant production shows that flocculating activity rises rapidly after 12 h to 82.7 %, and remains almost constant thereafter. During cultivation beyond 12 h, the flocculating activity remained high, indicating that the bioflocculant had not been used up after production and/or the organisms had not produced bioflocculant degrading enzyme as in the case of bioflocculant reported by Gao et al.[25]

This bioflocculant is one of the earliest optimally produced in literature. For example, in *Bacillus firmus*, bioflocculant production peaked after 33 h.[44] In *Agrobacterium* sp. M-503 production peaked after 48 h;[45] while for *Vagococcus* sp. W31 production peaked after 60 h.[25] Also, in *Phormidium* strain J-1 production peaked after 96 h;[46] while for *Halomonas* sp. OKOH [21] and *Bacillus* sp. Gilbert [20] maximum flocculating activities were attained in 135 h and 240 h, respectively. The optical density curve was parallel to the flocculating activity curve (Fig. 6) indicating that the bioflocculant is produced by biosynthesis during the growth phase of the bacteria.

Chemical and thermal stability analysis

Qualitative chemical analysis of the purified bioflocculant revealed the presence of protein, sugars and uronic acid. Also, Fig. 7 shows the thermal stability of the purified bioflocculant. Heating the bioflocculant at 100 °C for 25 minutes, resulted in residual flocculating activity of 65.6 %, thus suggesting the bioflocculant to be thermally stable.

Bioflocculant REA-11, produced by *Corynebacterium glutamicum* when heated at the same temperature, lost flocculating activity completely.[47] The purified bioflocculant seems to be more stable when compared with As-101, a bioflocculant produced by *Bacillus* sp. As-101.[48] Heating As-101 at 100 °C for 15 minutes resulted in about 50 % loss of flocculating activity. The thermal stability of this bioflocculant may be due to the presence of uronic acid in the structure of the molecule, providing carboxyl and hydroxyl functional group that can create considerable hydrogen bonds within the molecule. FTIR analysis of the purified bioflocculant further confirmed the presence of these functional groups.

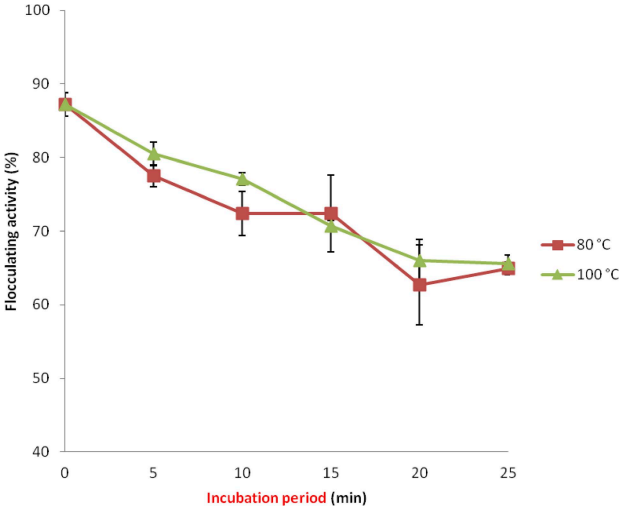

Fig. 7 – *Thermal stability of the purified bioflocculant produced by the bacteria*

FTIR, SEM and EDX analysis

The result of the FTIR spectrum analysis is shown in Fig. 8. The spectrum shows important peaks indicating the presence of some functional

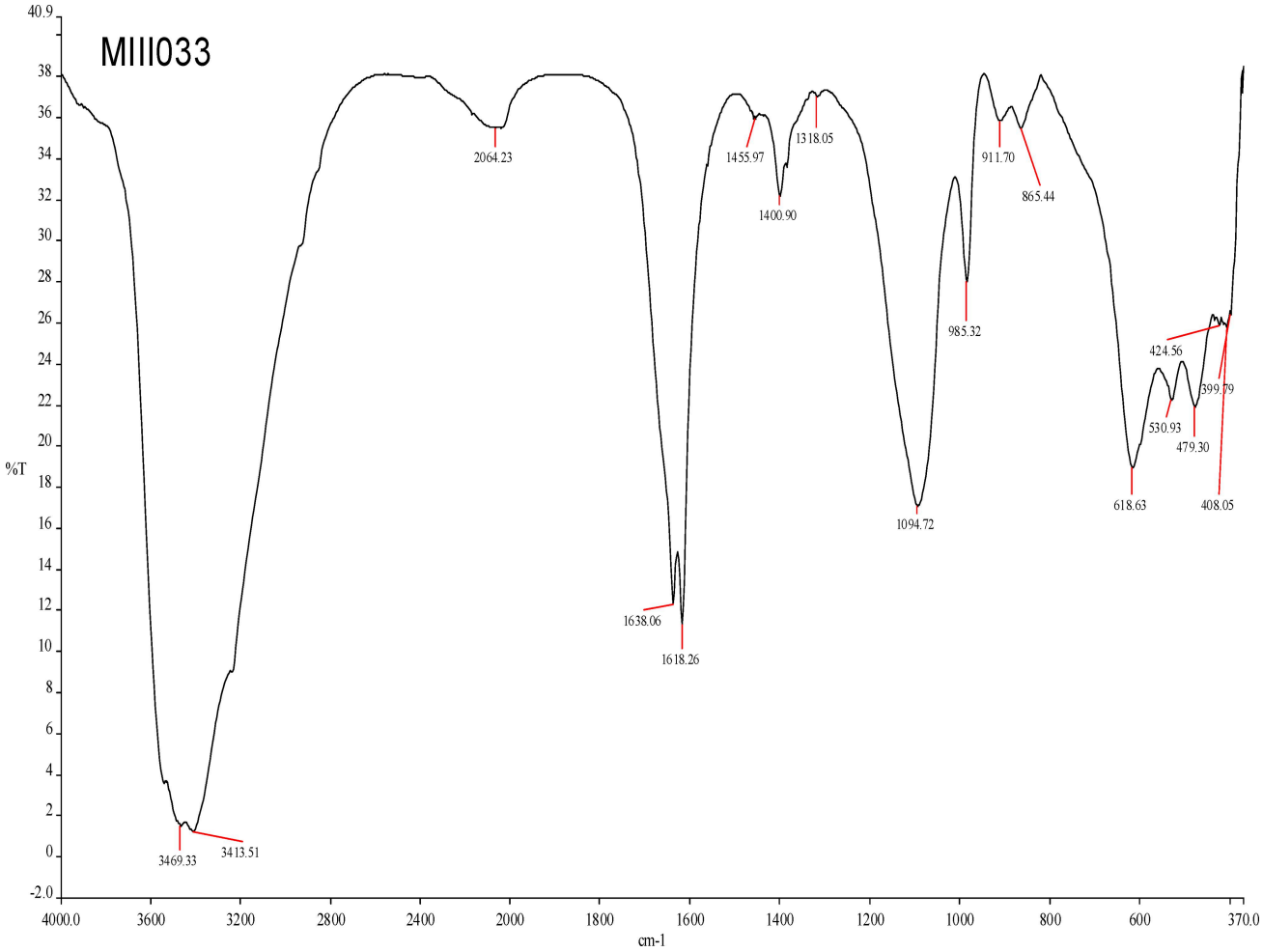

Fig. 8 – *FTIR Spectrum of the purified bioflocculant produced by the bacteria*

groups. Peaks at 3469 cm^{-1} and 3413 cm^{-1}, suggest the presence of hydroxyl and amino group in the purified bioflocculant. The peaks from 1400 cm^{-1} to 1638 cm^{-1} are indicative of the presence of a carbonyl group, while the peaks at 1091 cm^{-1} and 1215 cm^{-1} are characteristic of all sugar derivatives.

Infrared spectrum analysis of the functional groups of the bioflocculant is seen in Fig. 8. There is a broad stretching peak at 3469 cm^{-1} and 3413 m^{-1}, which is characteristic of a hydroxyl and amino group, respectively. A weak C-H stretching peak is displayed at 2061 cm^{-1}. The peaks at 1638 cm^{-1}, 1615 cm^{-1}, 1455 cm^{-1} and 1400 cm^{-1} are indicative of the presence of carbonyl group. The two latter peaks show asymmetric C = O stretching in the carboxylate (COO-) characteristic of the presence of urinate.[42] The peaks displayed at 1091 cm^{-1} and 1215 cm^{-1} are characteristic for all sugar derivatives. The FTIR spectrum was similar to the result of most bioflocculants reviewed in literature.[5,22,25]

With respect to SEM analysis, Fig. 9a, b and c shows the scanning electron micrograph of the purified bioflocculant, kaolin powder and flocculation of kaolin suspension by the purified bioflocculant, respectively. The morphology of the purified bioflocculant shown in Fig. 9a is crystal-linear in structure, while Fig. 9c shows the formation of a large floc as a result of the interaction between bioflocculant and suspended kaolin particles.

The surface morphology of the purified bioflocculant and its flocculation of kaolin clay were observed using scanning electron microscope as shown in Fig. 9. The bioflocculant shows a crystal-linear structure, similar to the structure of bioflocculant TJ-F1.[33] In Fig. 9c, there is the formation of a large floc as a result of the interaction of the bioflocculant with kaolin clay, which makes for easy settling of the floc due to gravity. This result shows that this bioflocculant flocculates kaolin clay well.

The EDX analysis of the purified bioflocculant in Table 2 shows its elemental composition (% w/w) to comprise of C, N, O, S, P in the ratio 4.77: 5.49: 32.3: 0.35: 18.8. The elemental compositions are indicative of the presence of polysaccharide and protein in the compound bioflocculant.

Table 2 – *Percentage composition of element in the purified bioflocculant produced by the bacteria*

Element	% (w/w)
Carbon	4.77
Nitrogen	5.49
Oxygen	32.3
Sulphur	0.35
Phosphorus	18.8

Conclusions

This study has shown that the bioflocculant produced by *Bacillus* sp. Maya has excellent flocculating activity of kaolin clay, and was optimally produced with glucose and ammonium nitrate as carbon and nitrogen source, respectively, in the presence of Ca^{2+} under weakly acidic pH 6. We propose that the thermostable bioflocculant could be an attractive candidate for use in water treatment and other relevant biotechnology applications.

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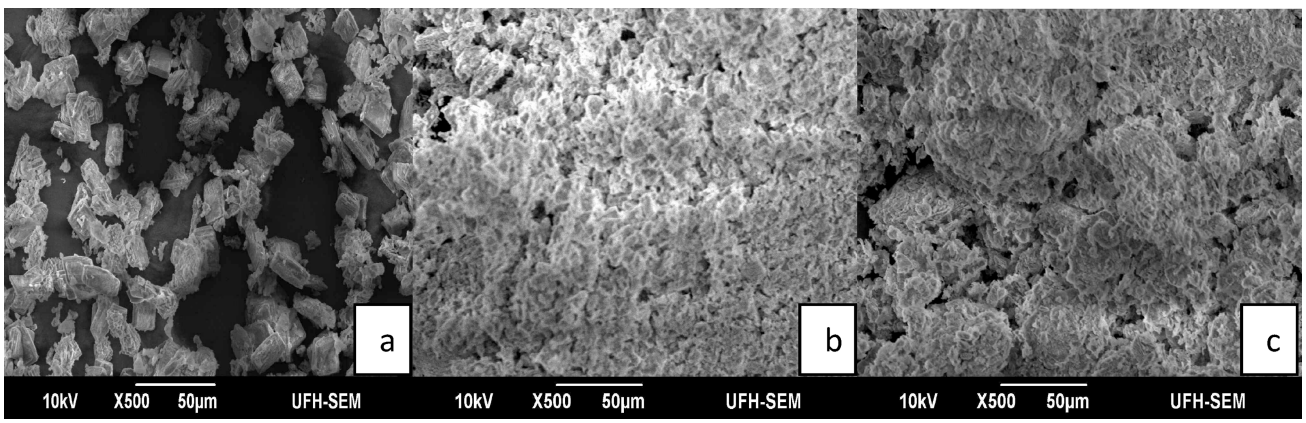

Fig. 9a,b,c – *SEM analysis of the purified bioflocculant, SEM analysis of Kaolin Powder, SEM analysis of purified bioflocculant flocculating kaolin suspension*

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