

Characterization of An Extracellular Bioflocculant Produced by *Cobetia* sp. OAUIFE Isolated from a Marine Sediment

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The characteristics of a bioflocculant produced by *Cobetia* sp. OAUIFE isolated from sediment samples of Algoa Bay, South Africa was investigated. The acidic extracellular polysaccharide bioflocculant optimally flocculated kaolin suspension at a dose of 0.4 mg/ml, neutral pH of 7, and Ca^{2+} as a coagulant aid. The bioflocculant is thermostable, with a high residual flocculating activity of 72 % after heating at 100 °C for 30 min. Thermogravimetric property of the bioflocculant revealed only about 50 % loss of initial weight when heated at 500 °C. FTIR analysis of the compound bioflocculant indicated the presence of hydroxyl, carboxyl and methoxyl functional groups. Scanning electron microscopy (SEM) image revealed a crystal-linear spongy-like bioflocculant structure.

Key words: *Cobetia* sp., Bioflocculant, Polysaccharide, Thermostable.

Bioflocculants are metabolite products of microorganisms produced during their growth. They are usually high molecular weight biopolymers, and are synthesized and released outside the cell¹. Many bioflocculants have been reported to be either polysaccharides, proteins, glycoproteins and nucleic acids²⁻⁵.

In recent years, bioflocculants have aroused great interest because of their biodegradability and the harmlessness of their degradative intermediates⁶ compared to synthetic flocculants such as polyacrylamide whose

degradative intermediate acrylamide has been implicated in cancer⁷. Screening new microorganisms for bioflocculant production with excellent flocculating activities has become a subject of intensive investigations globally.

Many researchers have reported studies on characterization of bioflocculants from different microorganisms. Some examples includes the study by Deng *et al.*⁸, they reported a bioflocculant MBFA9, produced by *Bacillus mucilaginosus* which composed of 47.4 % neutral sugar, 19.1 % uronic acid and 2.7 % amino sugar, while He *et al.*⁹ reported a bioflocculant REA-11, produced by *Corynebacterium glutamicum* which is mainly polygalacturonic acid.

In our previous study¹⁰ we reported a polysaccharide bioflocculant produced by *Cobetia* sp. OAUIFE composed of 93 % uronic acid, 1.2 % neutral sugar and 5 % protein. In this current study, we characterized and assessed the effect of dosage concentration, pH, metal ion and thermal stability on flocculating efficiency of the purified bioflocculant produced by *Cobetia* sp. OAUIFE.

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MATERIALS AND METHODS

Bacteria and Culture Conditions

The bacteria was isolated from sedimentary 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. The bacteria were maintained in 20% glycerol at -80 °C.

The culture medium consisted of 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g (NH₄)₂SO₄, 2 g KH₂PO₄, 5 g K₂HPO₄, 0.1 g NaCl and 0.2 g MgSO₄·7H₂O in a liter of filtered natural sea water using whatman filter paper¹¹. Two loopfuls of bacterial colonies were inoculated into 50 mL of the medium and incubated with shaking at 160 rpm for 72 h at 28°C and was used as a pre-culture for subsequent inoculation. For the bulk fermentation 20 mL of the pre-culture was inoculated into 1 L of the culture medium indicating 2 % (v/v) inoculum size, incubated with shaking at 160 rpm for 72 h at 28 °C.

Purification of Bioflocculant

Isolation and purification of the bioflocculant was done according to the method described by previous reports¹²⁻¹⁶. After 72 h of fermentation, the culture broth was centrifuged (8000 g, 30 min) to remove bacterial cells. One volume of distilled water was added to the supernatant phase and centrifuged (8000 g, 15 min) to remove insoluble substances. To the supernatant, two volumes of ethanol was added, 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.

Flocculation Test of Bioflocculant

Flocculating activity was measured as described elsewhere¹⁷, with modifications. Briefly, 3 mL of 1 % CaCl₂ and 2 mL of bioflocculant solution were added to 100 mL kaolin suspended solution (4 g/L) in 250 mL flask. The mixture was vigorously stirred and poured into a 100 mL measuring cylinder and allowed to stand for 5 min. 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, except that the bioflocculant solution was replaced with distilled water (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 at 550 nm.

Effect of pH and metal ions on Bioflocculant activity

The effects of pH and metal ion on flocculating activity of the bioflocculant were assessed in accordance with the description of Liu *et al.*⁵. The pH of the bioflocculant solution were varied between the range of 3 - 12 using either 0.1M HCl or NaOH, while the metal ions candidates included Na⁺, K⁺, Li⁺, Mg²⁺, Mn²⁺, Al³⁺ and Fe³⁺ as their chloride salts. With regards to the effects of metal ion assays, flocculating activity assay were conducted as described above, but CaCl₂ solution was replaced by a solution of the above metal ion candidates.

Thermal Stability of the Purified Bioflocculant

The effect of heat on the purified bioflocculant was assessed according to the method of Gong *et al.*¹⁸. Three different temperature regimes (50 °C, 80 °C and 100 °C) were used with the aid of a waterbath. A solution of the purified bioflocculant was placed in the waterbath and heated over a period of 30 min. Samples (2 mL) were drawn at 5 min intervals and assessed for residual flocculating activity.

FTIR, TGA and SEM Analysis of Purified Bioflocculant

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 370 cm⁻¹.

The degradation temperature of the purified bioflocculant was studied using Thermo-gravimetric analyser (STA 449/C Jupiter Netz, Germany, Perkin Elmer Thermo-gravimetric analyser 7, TGA analyser, USA) instrument. The purified bioflocculant was heated from 0- 500 °C at a constant rate of 10 °C /min under constant flow of nitrogen gas.

Scanning electron microscopy (SEM) image of the purified bioflocculant was taken using JEOL (JSM-6390LV, Japan).

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

Effects of Bioflocculant Dose

Fig. 1 shows the effect of bioflocculant dosage on flocculating activity within the concentration range of 0.2- 2 mg/mL. Flocculating activities of more than 80 % were obtained within the bioflocculant concentration range of 0.2- 0.6 mg/mL with the maximum observed at 0.4 mg/mL (flocculating activity of 83.5 %). A sharp decrease in flocculating activity (60.4 %) was observed at a concentration of 0.8 mg/ml after which it fluctuated between 75 % and 50 % (Fig. 1).

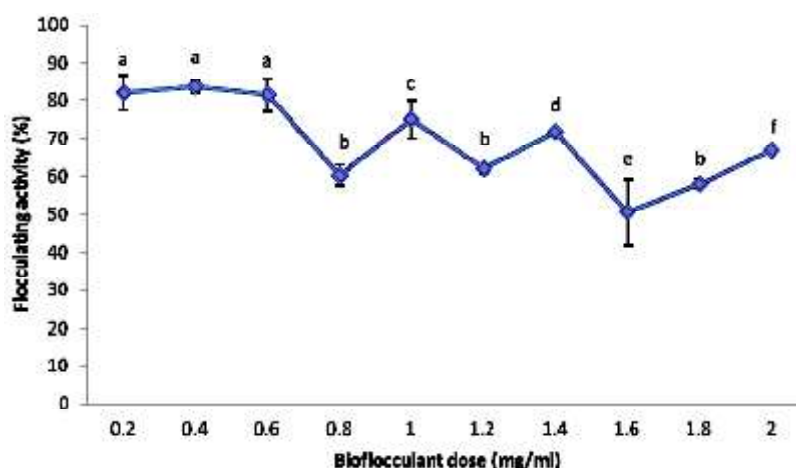


Fig. 1. Effect of bioflocculant concentration on flocculating activity of the purified bioflocculant. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other

Effects of pH

The effect of pH on flocculating activity of the purified bioflocculant was assessed using a bioflocculant concentration of 0.4 mg/ml with the pH of the solution ranging from 3- 12 (Table 1). A flocculating activity of more than 70% was maintained over the entire pH range with the maximum of 87% recorded at a neutral pH 7 (Table 1).

Effects of Metal ions

The result of the effect of various metal ions on flocculating activity is as shown in Figure 2. Single valency metal ions (K^+ , and Na^+) did not show any significant stimulating effect of flocculating activity of the bioflocculant as shown by low flocculating activities of 2.4 % and 9.1 % obtained for K^+ and Na^+ respectively with Li^+ completely inhibiting flocculating activity (Figure

Table 1. Effect of pH on flocculating activity of the purified bioflocculant

pH	Flocculating activity (%)
3	75.14 ± 0.45 ^a
4	73.71 ± 5.04 ^a
5	74.28 ± 1.20 ^a
6	79.50 ± 2.90 ^b
7	87.05 ± 1.30 ^c
8	82.65 ± 2.11 ^d
9	79.02 ± 4.35 ^b
10	83.31 ± 4.74 ^d
11	77.15 ± 1.37 ^b
12	76.23 ± 2.68 ^b

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

2). On the contrary, divalent cations stimulated flocculating activity albeit to varying degrees with Ca^{2+} supporting the maximum activity of 83.5%.

The two trivalent cations, Al^{3+} and Fe^{3+} showed little stimulating effect with lower than 40% flocculating activity recorded (Fig. 2).

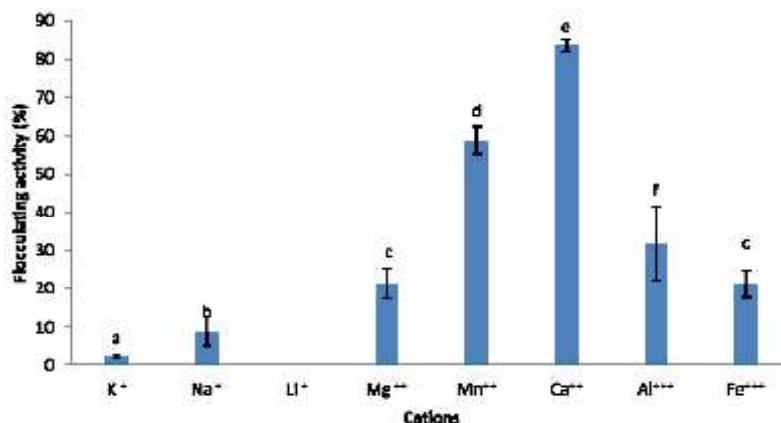


Fig. 2. Effect of metal ions on purified bioflocculant from *Cobetia* sp OAUIFE. Percentage flocculating activities with different alphabet are significantly different ($p < 0.05$) from each other

Thermal Stability

With regards to the effect of heat on flocculating activity, fairly constant residual flocculating activities ranging between 72% and 80% were exhibited at 100 °C over a period of 30 min. Heating at both 50 °C and 80 °C over the same period showed a decline in flocculating activity (Fig. 3).

Functional group analysis

The result of the FTIR spectrum analysis

is shown in Fig. 4. The spectrum shows peaks indicating the presence of some functional groups. Peak at 3486 cm^{-1} , suggests the presences of hydroxyl group in the purified bioflocculant. The bands at 1649 cm^{-1} and 1384 cm^{-1} are indicative of the presence of a carbonyl group, while the peaks at 1231 cm^{-1} and 1137 cm^{-1} are suggestive of the presence of methoxyl group. The absorption peaks at 988 cm^{-1} – 1063 cm^{-1} are characteristic of all sugar derivatives.

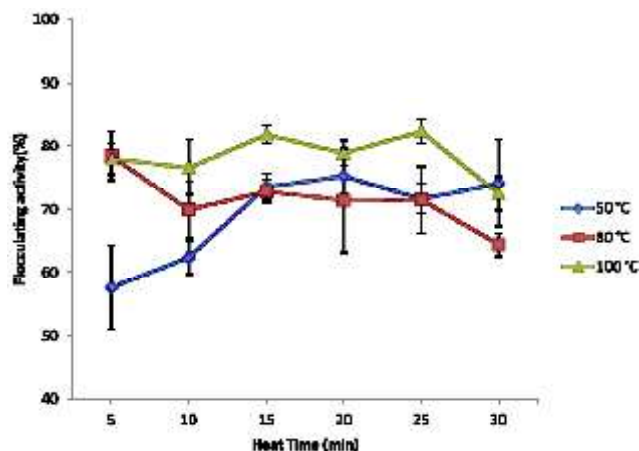


Fig. 3. Thermal stability of purified bioflocculant from *Cobetia* sp.OAUIFE

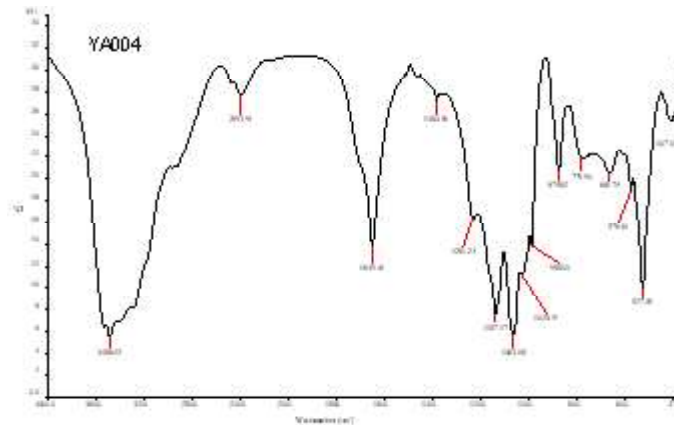


Fig. 4. FTIR Spectrum of purified bioflocculant from *Cobetia* sp. OAUIFE

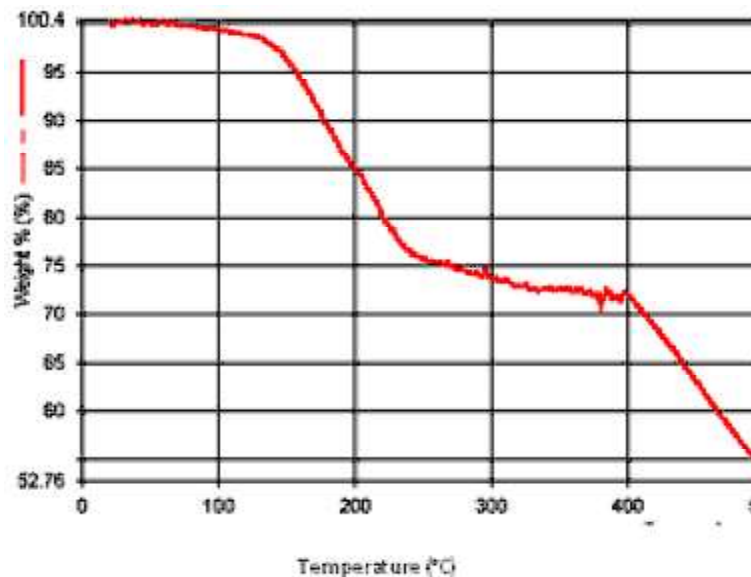


Fig. 5. Thermogravimetric analysis of purified bioflocculant from *Cobetia* sp. OAUIFE

Thermogravimetric property of the purified bioflocculant

The result of the pyrolysis property of the bioflocculant is shown in Figure 5. At 100 °C no weight loss was observed. An initial weight loss of about 15 % was only observed at 200 °C and this downward trend continued with corresponding increase in temperature resulting in 50 % weight loss being recorded at 500 °C (Fig. 5).

SEM Analysis

Figs 6a, 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. 6a is a crystal linear spongy-like structure, while Figure 6c shows the formation of large floc as a result of the interaction between the bioflocculant and suspended kaolin particles.

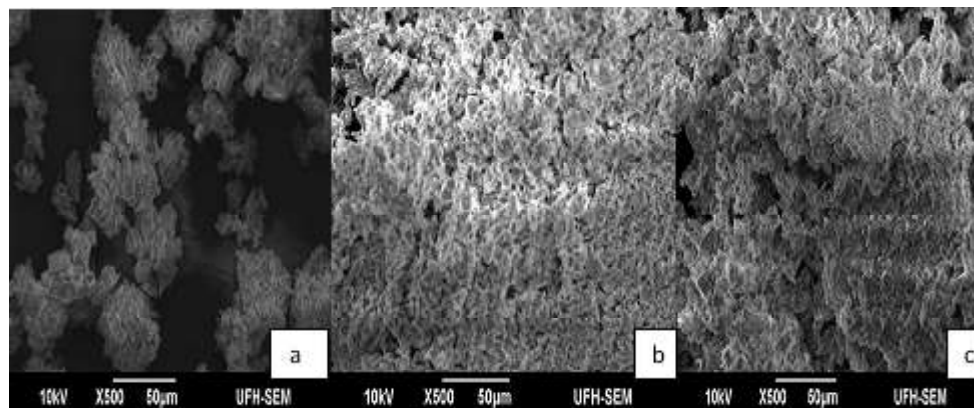


Fig. 6. SEM analysis of purified bioflocculant from *Cobetia* sp. OAUIFE

DISCUSSIONS

From recent reports, the dosage of purified bioflocculants used for kaolin flocculation was within the range 1 to 700 mg/L^{5,8,14,18-22}. From this study we observed higher flocculating activities within the concentration range of 0.2- 0.6 mg/mL (Fig. 1), with 0.4 mg/ mL being the optimum dose concentration with a resultant flocculating activity of 83.5 %.

One of the key factors influencing flocculating activity of bioflocculants is the pH of the reaction mixture³. In this study the flocculating activity was high over a wide range of pH from 3-12, but the highest flocculating activity of 87% was observed at a neutral pH of 7. This is similar to the study carried out by Liu *et al.*⁵, which reported enhanced flocculating activities over a wide range of pH for bioflocculant MBF-W6.

It has been well documented that achieving high flocculating activity, metal ions are usually added^{18,22,23} to aid bioflocculants. This is because metal ions can neutralize negative charges on most polysaccharide bioflocculants and the suspended kaolin particle with which they react, thereby increasing the absorption of the bioflocculants onto the kaolin particle²¹.

The stimulating effects of metal ions on bioflocculant in flocculating kaolin suspension are dependent on both the concentration and valence of the metal ion²⁴. From Fig. 2, Ca²⁺ and Mn²⁺ ions which are divalent metal ions stimulate most strongly flocculating activity of the bioflocculant, this may be adduce to the presence of large number

of carboxyl groups on the bioflocculant, that can serve as bindings sites for divalent cations²⁵. This is similar to the findings of Salehizadeh and Shojaosadati,²³ where Ca²⁺ and Mg²⁺ stimulate the flocculating activity of bioflocculant MS-102. However, the monovalent cations like K⁺ and Na⁺ could not stimulate effectively the flocculating activity of the bioflocculant, likely due to their weaker static force of pulling between cations and the bioflocculant²⁶. Salehizadeh and Shojaosadati,²³ and Elkady *et al.*²² reported similar findings where monovalent cations showed weak stimulation of flocculating activity of their respective bioflocculants.

From this study we observed that addition of trivalent metal ions (Al³⁺ and Fe³⁺) only resulted in a marginal improvement of the flocculating activity of the bioflocculant (Fig. 2), which is similar to the findings of Wu and Ye²¹.

As seen in Fig. 3, the residual flocculating activity of the bioflocculant after heating at 100°C for 30 min was 72%. A similar result was observed when the crude bioflocculant was heated at the same temperature for 25 min¹⁰, thus suggesting that the high content of uronic acid in the molecule could be responsible for the thermal strength. Gong *et al.*¹⁸ also reported a similar finding when the bioflocculant from the culture of *Serratia ficaria* was heated at same temperature. This bioflocculant appear to be more stable when compared with others in literature^{9,27,28}.

Presences of hydroxyl and carboxyl groups within the bioflocculant molecule as indicated by the FTIR spectrum (Fig. 4), enhances

the formation of hydrogen bonds which might be responsible for thermal stability of the bioflocculant. These groups allow for better interaction with water molecules, and may be responsible for the observed high solubility of the bioflocculant in water since “like dissolve like” according to James²⁹ and may also serve as a binding site for divalent cations, in bridging the bioflocculant and suspended kaolin particles during flocculation.

The thermogravimetric analysis of the bioflocculant in Figure 5 shows that at 100 °C, the loss in weight of the bioflocculant is insignificant. Weight lost at this point might be due to loss of water molecules trapped by the hydroxyl and carboxyl functional groups in the molecule. A significant decomposition was first observed at about 200 °C with a 15 % loss in initial weight, which increases to 25 % after heating to 300 °C. The bioflocculant only retain about 50 % of its initial weight at 500 °C. The result is similar to the trend showed by the bioflocculants EPS471 and CBF-F26 as reported by Kumar *et al.*³⁰ and Wang *et al.*³¹.

The surface morphology of the purified bioflocculant and its flocculation of kaolin clay were observed using scanning electron microscope as shown in Fig. 6. The bioflocculant showed a crystal-linear structure, similar to the structure of bioflocculant TJ-F1³². In Figure 6c, there is the formation of 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.

CONCLUSIONS

This study have shown that the purified acidic polysaccharide bioflocculant from *Cobetia* sp. OAUIFE is thermally stable and consist of hydroxyl and carboxyl groups that are very important in conferring the excellent flocculating properties exhibited by this bioflocculant. The bioflocculant is also cation- dependent, and could be used as an alternative to harmful inorganic and synthetic flocculants in water treatment and other relevant biotechnology applications.

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