Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 24, supplement 1, 2014, pp. 11-17

EXTRACTION, CHARACTERIZATION AND APPLICATION OF A BIOFLOCCULANT PRODUCED FROM A MIXED CULTURE OF TWO BACTERIA STRAINS ISOLATED FROM ALGOA BAY, SOUTH AFRICA

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ABSTRACT. Two bioflocculant-producing marine bacteria isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa identified as *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert (accession number JF799092, and HQ537128 respectively) were used in a mixed culture to produce a bioflocculant of high yield and enhanced flocculating activity. The characteristics of the bioflocculant produced by the consortium of the two strains showed an optimum flocculation (89.8 %) of kaolin suspension when the dosage concentration was 0.2 mg/ml, under neutral ph, and Ca^{2+} as a coagulant aid. The FTIR analysis of the bioflocculant indicated the presence of hydroxyl and carbonyl functional groups. Scanning electron microscopy (SEM) image of the bioflocculant revealed crystal linear spongy-like structure. The produced bioflocculant was highly efficient in removing turbidity and reducing chemical oxygen demand (COD) from brewery wastewater, diary wastewater and river water. The bioflocculant from the consortium showed a relatively higher flocculating activity compared with traditional flocculants such as alum and polyacrylamide. This boflocculant is relatively inert, eco-friendly and save for use and could serve as an important alternative to the hazardous inorganic and synthetic organic flocculants that are currently popularly used in water/ wastewater treatment and other downstream processes.

Keywords: bioflocculant, consortium, characteristic, efficient, wastewater.

INTRODUCTION

Increasing industrialization has been accepted as an enviable choice due to its contribution to economic growth. However, it has considerably raised the rate of water pollution especially from industrial sources and this has become a major environmental concern (Sarkar *et al.*, 2006). The disposal of effluents without appropriate treatment could result in long term undesirable negative impact especially on the environment and human health (Lin & Harichund, 2011).Thus, necessitating the need for adequate treatment of wastewater before discharge into the environment.

Flocculation, as a means of water treatment, is a process where colloids aggregate out of suspension in the form of floc or flake (Iupac, 1997). Bioflocculation therefore is the use of microbial flocculant to treat water. Bioflocculants are metabolite products of microorganisms produced during their growth. They are biodegradable and are a new type of high security, high efficiency, non-toxic biopolymers (Li *et al.*, 2009).

Many bioflocculant-producing microorganisms are widely distributed in soil and water, and these include bacteria, fungi, actinomycetes, yeast and algae. The composition of these biopolymers also varied, and could be polysaccharides, proteins, nucleic acids, cellulose, sugar or poly amino acids (Toeda & Kurane, 1991; Yokoi *et al*., 1997; Lee *et al.*, 1995; Liu *et al.*, 2010). Over the past decades, emphasis with regards to the bioflocculant field was mainly placed on axenic cultures. However, some few researchers have reported that the combination of strains of microorganisms in consortia could result in higher bioflocculant yield and activity than those of pure cultures (Zhu *et al.*, 2004; Zhang *et al*., 2007).

Bioflocculants are widely used in the treatment of water and wastewater, in downstream processing, food and chemicals processing and are also useful in mining and milling operations and in petroleum refineries (Salehizadeh & Shojaosadati, 2002; Deng *et al*., 2005; Ghosh *et al.*, 2009).

In this study we extracted, purified and characterised bioflocculant produced by a mixed culture of two marine bacteria previously isolated from the sediment sample of Algoa Bay in South Africa. The produced bioflocculant was highly efficient in reducing turbidity and lowering chemical oxygen demand (COD) from brewery wastewater, dairy wastewater and river water, when compared with other traditional flocculants.

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Article received: April 2014; published: May 2014

MATERIALS AND METHODS

Bacteria and Culture Conditions

The bacteria were isolated from sediment samples of Algoa Bay in the Eastern Cape Province of South Africa as part of the bioflocculant producing 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^{o}C. The culture medium consisted of 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH4)_2SO_4$, 2 g KH_2PO_4, 5 g K_2HPO_4, 0.1 g NaCl and 0.2 g $MgSO_4$.7H2O in a liter of filtered natural sea water using whatman filter paper (Zhang et al., 2007). A loopful of each bacterial colony were inoculated separately into 50 mL of the medium and incubated with shaking at 160 rpm for 72 h at 28^{o}C and were used as pre-culture for subsequent inoculations. For the bulk fermentation 10 mL of each pre-culture were inoculated into 1 L of the culture medium indicating 2 % (v/v) inoculums size, incubated with shaking at 160 rpm for 72 h at 28^{o}C.

Extraction and Purification of Bioflocculant

Isolation and purification of the bioflocculant was done according to the method described by previous reports (Chang *et al.*, 1998; Chen *et al.*, 2002; Gao *et al.*, 2006; Cosa *et al.*, 2011; Ugbenyen *et al.*, 2012a). 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^{o}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 elsewhere (Kurane *et al.*, 1986; Ugbenyen *et al.*, 2012b), with modifications. Briefly, 3 mL of 1% $CaCl_2$ 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 the control at 550 nm. For application with real wastewater, kaolin suspension was replaced with the various wastewater types i.e. brewery wastewater, dairy wastewater and river water.

Effect of Concentration Dosage

Different concentrations of the purified bioflocculant were dissolved in distilled water yielding bioflocculant solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The flocculation assay of the different bioflocculant solutions was performed as described above.

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.*, (2010). 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^{+}, Mg^{2+}, Mn^{2+}, Al^{3+} and Fe^{3+} as their chloride salts. With regards to the effects of metal ion assays, flocculating activity assay were conducted as described above, but $CaCl_2$ solution was replaced by a solution of the above metal ion candidates.

Analysis of purified bioflocculant

Total protein content of bioflocculants was determined by the Lowry's method using Bovine Serum Albumin (BSA) as a standard (Lowry *et al.*, 1951). Total sugar content of bioflocculant was determined by the phenol-sulphuric acid method using glucose as standard solution (Dubios *et al.*, 1956) and uronic acid was quantified by the carbazole method (Jayaraman, 1981).

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}.

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

Analysis of wastewaters

River water, dairy wastewaters and brewery wastewaters were collected from the Tyume River, dairy factory and brewery factory all in the Eastern Cape Province of South Africa. COD (Chemical oxygen demand), turbidity and pH, of the river water and wastewaters were measured using a spectrophotometer (Spectroquant Pharo 100 M, EU), turbidimeter (HACH, USA) and pH meter respectively. Calibration of the pH meter was achieved using different buffer solutions of pH 4, 7 and 11. Traditional flocculants (alum and polyacrylamide) were assessed by replacing the bioflocculant with each.

The residual COD and turbidity were determined according to the method of Gong *et al*. (2008) and the removal efficiency was calculated as follows:

$$\text{Removal efficiency (\%)} = [C_0 - C/ C_0] \times 100$$

Where C_0 is the initial value and C is the value after the flocculation treatment

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 calculated from three replication.

RESULTS

Yield and composition of the bioflocculant

Purification of the fermentation product resulted in a bioflocculant yield of 0.6 g/L. The biochemical analysis of the purified bioflocculant showed that it was composed of uronic acid (62.5 %), protein (5.05 %) and neutral sugar (1.21%) (Table 1).

Table 1

Percentage composition of purified bioflocculant produced by the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert

Composite	% (w/w)
Protein	5.05
Neutral sugar	1.21
Uronic acid	62.5

Effect of concentration dose

The effect of different concentration doses on the flocculating activity of purified bioflocculant from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert is described in Fig. 1. The concentration dose at 0.2 mg/ml showed the highest flocculation (89.8 %) of the different doses used for the bioflocculant from the consortium.

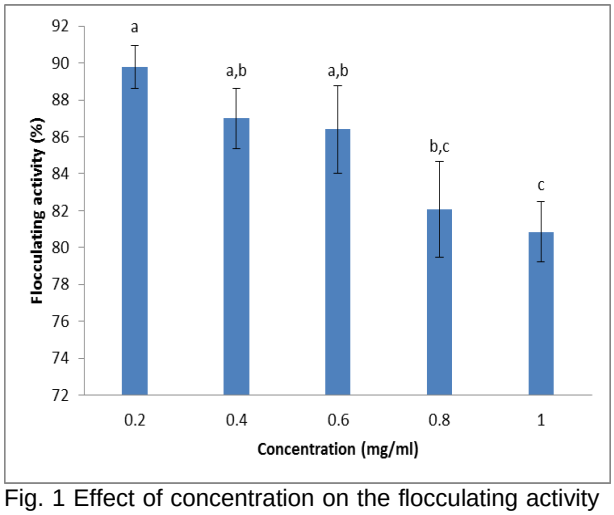

Fig. 1 Effect of concentration on the flocculating activity of the purified bioflocculants from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert. Concentrations sharing the same letter (a-c) are not significantly different ($p< 0.05$) from each other.

Effect of metal ions

All the metals stimulate flocculating activity of the bioflocculant from the consortium above 60 % (Fig. 2).

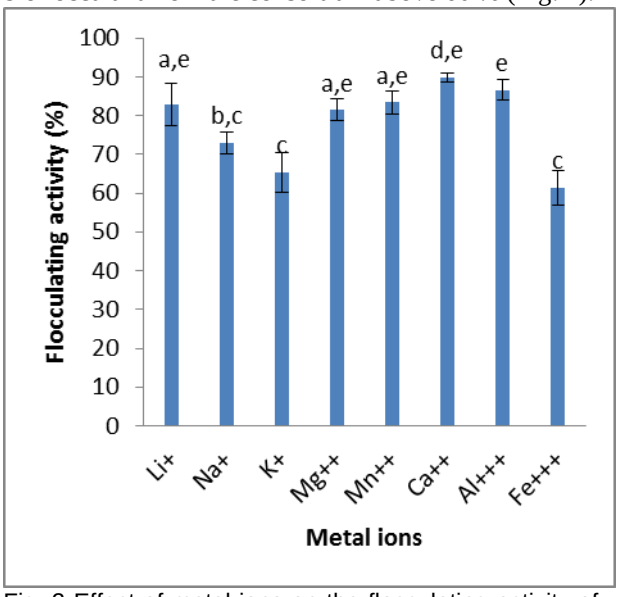

Fig. 2 Effect of metal ions on the flocculating activity of purified bioflocculants from a consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert. Metal ions sharing the same letter (a-e) are not significantly different ($p< 0.05$) from each other.

Effect of pH

The effect of pH on flocculating activity of the bioflocculant from the consortium was assessed at a concentration of 0.2 mg/ml with the pH of the solution ranging from 3- 12 and the result is shown in Fig. 3. The flocculating activity of the bioflocculant was optimum at a neutral pH of 7 and began to drop at alkaline pH.

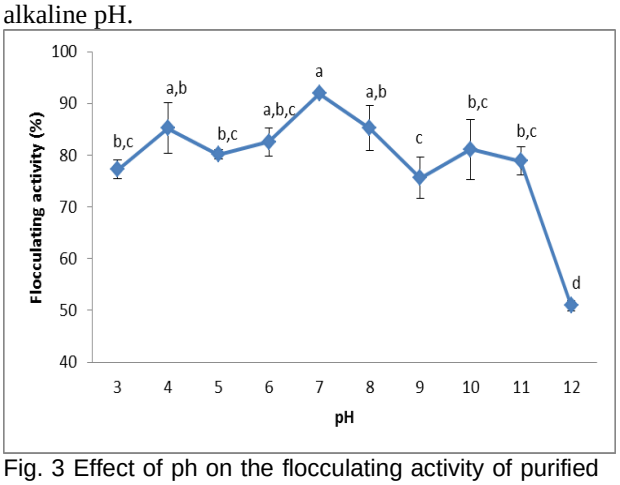

Fig. 3 Effect of ph on the flocculating activity of purified bioflocculant from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp.Gilbert. Ph values sharing the same letter (a-d) are not significantly different ($p< 0.05$) from each other.

FTIR Analysis

The FTIR spectrum analysis is shown in Fig. 4. The peak at 3414 cm^{-1} is suggestive of the presence of hydroxyl in the purified bioflocculant, while the peak at 2971 cm^{-1} is indicative of methylene group in the structure. The peaks between 1452 cm^{-1} to 1732 cm^{-1} are indicative of the presence of a carbonyl group, while the peaks between 871 cm^{-1} to 1109cm^{-1} are characteristic of all sugar derivatives.

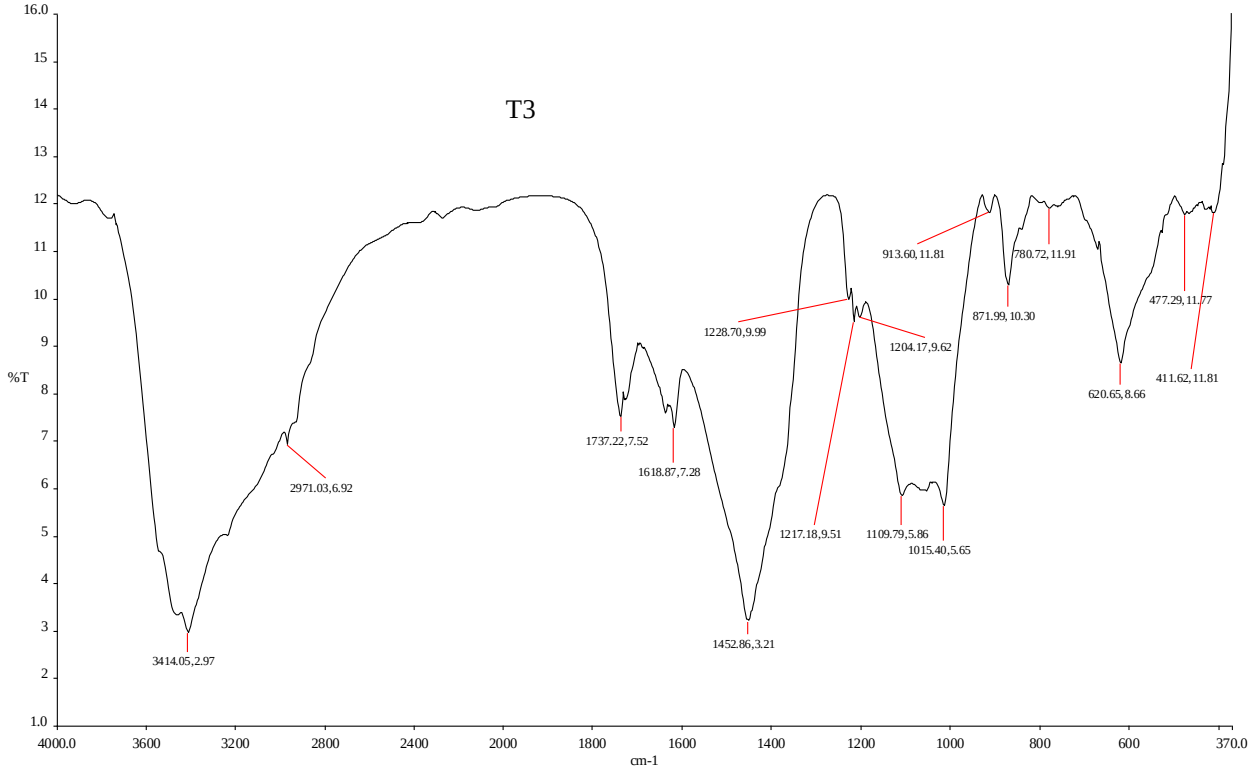

Fig. 4 FTIR Spectrum of the purified bioflocculant from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp Gilbert.

SEM Analysis

The morphology of the purified bioflocculant shown in Fig. 5a is crystal linear spongy-like structure, while Fig. 5b shows the formation of large floc as a result of the interaction between the bioflocculant and suspended kaolin particles.

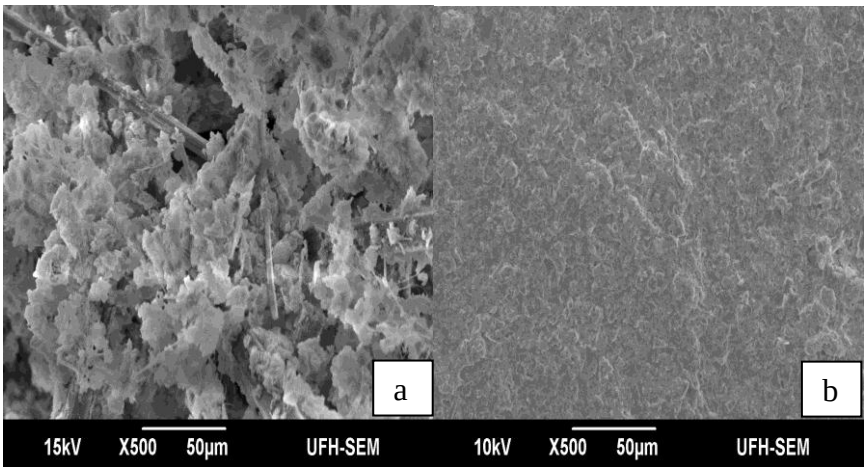

Fig. 5 (a) SEM analysis of the purified bioflocculant from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp Gilbert (b) SEM analysis of the purified bioflocculant from the consortium flocculating kaolin suspension.

Table 2
Characteristics of effluent waters

	Brewery wastewater	Diary wastewater	River Water
COD (mg/ l)	821	4813	92
Turbidity (NTU)	750	1382	174
pH	5.6	7.6	7.2

Table 3
Comparison of the flocculating activity, COD reduction, and turbidity removal of the bioflocculants from consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert with those of tradition flocculants.

Flocculant used	Wastewater	Turbidity removal (%)	COD reduction (%)	Flocculating activity (%)
Bioflocculant	Brewery	93.1	92.4	90.9
	Dairy	76.9	99.1	89.0
	River	98.2	67.3	94.9
Alum	Brewery	87	98.0	75.1
	Dairy	71.4	98.6	67.0
	River	33.1	70.0	57.4
Polyacrylamide	Brewery	85.4	99.5	66.1
	Dairy	72.5	98.0	67.3
	River	48.8	68.8	70.8

DISCUSSION

Factors such as the concentration of bioflocculants, the metal ion concentration and pH, affect the flocculation activity of purified bioflocculants. Many studies have reported the optimum flocculating concentration of bioflocculants isolated from individual microorganisms (Yokoi *et al.*, 1997; Yim *et al.*, 2007; Lu *et al.*, 2005; Suh *et al.*, 1997; Salehizadeh *et al.*, 2000; Nakata & Kurane, 1999; Fujita *et al*,. 2000) however, information on the effect by a consortia of microorganisms is still very scarce in literature. From this study we observed the optimum flocculating activity (89.8%), when the bioflocculant concentration of the purified bioflocculant was 0.2 mg/ml (Fig 1). A bioflocculant CBF-F26 produced from a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 also showed an optimum flocculating activity of 96% when the concentration of the bioflocculant was 12 mg/L (Wang *et al.*, 2011).

Cations stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups of bioflocculants and also by forming bridges between particles. According to Levy *et al*. (1992), the role of bivalent and trivalent cations is to increase the initial adsorption of biopolymers on suspended particles by decreasing the negative charge on both the polymer and the particle. As an example, the flocculating activity of *Enterobacter* sp. BY-29 increases in the presence of Al^{3+}, Fe^{3+}, Fe^{2+}, and Ca^{2+} (Yokoi *et al.*, 1997). From Fig 2, we also observed high flocculating activities with Ca^{2+}, Mg^{2+}, Mn^{2+} and Al^{3+}, when used as a coagulant aid for the bioflocculant produced from the mixed culture of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert, with Ca^{2+} showing the optimum flocculation (89.8%). It was also reported that the optimum pH for stimulating the flocculation by Ca^{2+} was in the range of pH 4–5 (Yokoi *et al.*, 1996). Whereas for Mg^{2+}, Fe^{2+}, and Fe^{3+}, the optimum pH was 6–7 (Yokoi *et al.*, 1996). Like many other bioflocculants reported in literature, the bioflocculant from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert flocculated kaolin suspension over a wide range of pH (Fig 3). The highest flocculating activity (90%) was achieved when the pH of the bioflocculant solution was neutral, implying that the adjustment of pH would not be necessary for achieving high flocculation with this bioflocculant. Liu *et al.* (2010) reported a similar finding for which the bioflocculant MBF-W6 showed flocculating activity over a wide range of pH, with the highest flocculating activity of 96.8% at pH 5.6.

Several types of bioflocculants have been reported including polysaccharides, proteins, lipids, glycolipids and glycoproteins (Kurane & Matsuyama, 1994). The flocculant biopolymers from *Bacillus* sp. DP-152 contain glucose, mannose, galactose, and fucose in an approximate molar ratio of 8:4:2:1 (Suh *et al.*, 1997). The bioflocculant APR-3 produced by R-3 mixed culture was an acidic polysaccharide made of glucose, galactose, succinic acid, and pyruvic acid in the molar ratio 5.6:1:0.6:2.5 (Kurane & Matsuyama, 1994). From Table 1, the bioflocculant produced from the consortium was mainly acidic polysaccharide containing a high level of uronic acid (62.5%). High levels of uronic acid may also be adduce for the presence of large number of carboxylate groups on the bioflocculant, which can serve as bindings sites for divalent cations (Prasertsan *et al.*, 2006).

The FTIR analysis (Fig. 4) reveals important functional groups such as hydroxyl and carbonyl group present in the bioflocculant that allows for effective flocculation of suspended particles in solution. The peak at 3414 cm^{-1} is suggestive of the presence of hydroxyl in the purified bioflocculant, while the peak at 2971 cm^{-1} is indicative of methylene group in the structure. The peaks between 1452 cm^{-1} to 1732 cm^{-1} are indicative of the presence of a carbonyl group. The latter was characteristic of C=O in an amide group (Shriner *et al.*, 1998, Wang *et al.*, 2011) while the former is assigned to the asymmetric C=O stretching in carboxylate. The peaks between 871 cm^{-1} to 1109cm^{-1} are characteristic of all sugar derivatives.

Analysis of the surface morphology of the purified bioflocculant from the mixed culture of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert as revealed by the SEM image in Fig. 5a, showed a mixture of amorphous and crystal linear structures. This is similar to the bioflocculant TJ-1 produced by *Proteus mirabilis* (Xia *et al.*, 2008) which had a crystal linear structure morphology. In Fig 5b, 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.

Until recently, many studies on microbial production of bioflocculants have been reported, however many of them focused mainly on the flocculating activity of bioflocculants for kaolin suspension. Due to the effectiveness of certain bioflocculants, researchers have tested such bioflocculants in a wide range of processes including treatment of water and wastewater (Gong *et al.*, 2008; Gao *et al.*, 2009; Guo *et al.*, 2013). From our study we observed that the bioflocculant produced from the consortium of the two bacteria (*Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert) were very effective in flocculating kaolin suspension, as well as some real wastewater streams including river water, dairy wastewater and brewery wastewater. The characteristics of these real wastewaters are shown in Table 2. River water is one of typical surface waters with low COD and turbidity. The chemical oxygen demand (COD) of the dairy wastewater was about three times that of the brewery wastewater. The pH of the dairy wastewater and the river water were almost neutral, while that of the brewery wastewater was slightly acidic.

The results of the treatment of the riverwater, brewery wastewater and dairy wastewater using bioflocculant produced from the mixed culture and traditional flocculants such as alum and polyacrylamide were shown in Table 3. The turbidity removal efficiency (%) of the bioflocculant for brewery wastewater was above 90%. The results when compared with inorganic flocculant (alum) revealed that the bioflocculant had better turbidity removal efficiencies than alum and polyacrylamide which gave 87% and 85% removal rate respectively. The bioflocculant was also very effective with dairy wastewater and river water with the turbidity removal rate being 98.2% for river water, compared to that of a traditional flocculant (alum) (33.1%). Gong *et al.* (2008) also reported that the turbidity removal rate of the bioflocculant SF-1 was 91.8–93.7% for brewery wastewater, meat-processing wastewater and soy sauce brewing wastewater which was better than polyaluminium chloride and polyacrylamide. The results of COD removal efficiency (%) from our study (Table 3) revealed above 90% COD removal rate for bioflocculant produced from the consortium for brewery and dairy wastewater.

CONCLUSION

The bioflocculant produced from the consortium of *Cobetia* sp. OAUIFE and *Bacillus* sp. Gilbert possessed functional groups that favours flocculation processes, such as hydroxyl and carboxyl group. The bioflocculant showed high efficiency in reducing turbidity and lowering chemical oxygen demand (COD) from brewery wastewater, dairy wastewater and river water when compared to other traditional flocculants such as alum and polyacrylamide. We propose that the bioflocculant could be an attractive candidate for use in water/wastewater treatment and other relevant biotechnology applications.

ACKNOWLEDGEMENTS

We are grateful to the University of Fort Hare and Govan Mbeki Research and Development Centre (GMRDC) for financial support.

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