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Characterization of a thermostable polysaccharide bioflocculant produced by *Virgibacillus* species isolated from Algoa bay

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We evaluated the bioflocculant production by *Virgibacillus* species isolated from a marine environment in the Eastern Cape Province of South Africa. Culture conditions for optimal production of the bioflocculant included: glucose as sole carbon source (flocculating activity 91.8%); complex nitrogen source (urea + yeast extract + $(NH_4)_2SO_4$); inoculum size of 2% (v/v); initial pH 10; and calcium as mediating factor. Optimum dose of the purified bioflocculant for the clarification of 4 g/l kaolin clay suspension at neutral pH was 0.3 mg/ml. Chemical analysis of the purified bioflocculant revealed it to be mainly composed of uronic acids (87% w/w). Scanning electron microscopic observations were indicative of a porous bioflocculant structure. Fourier transform infrared (FTIR) spectrum revealed the presence of carboxyl and hydroxyl groups whilst the themogravimetric analysis (TGA) showed degradation temperature (*Td*) of 150°C. The bioflocculant appears to hold promise in vast biotechnological applications in a more environmentally friendly and economical manner.

Keywords: *Virgibacillus* sp., bioflocculant, thermostable, polysaccharide.

INTRODUCTION

Over the past three decades, microbial flocculants have attracted a number of researchers from different countries (Zhang et al., 1999) in response to the questions about the safety of synthetic flocculants. Environmental consideration dictated the development of strong, economically viable and eco-friendly substitutes of conventional synthetic flocculants. If these are ever discharged to the environment they would naturally degrade and allow least net global warming and sustainability (Sharma et al., 2006). In response, various universities and research institutes hone the development of new microbial flocculants (bioflocculants) as their research precedence pursuant to developing new microbial flocculants with better prospects for application (Liang et al., 2010).

Literature searches reveal that a number of microbes are capable of growing in diverse environments and producing secondary metabolites (viz. extracellular polysaccharides, proteins (lectins), lipids) during their growth (Subramanian et al., 2010). For example, *Bacillus* sp. I-471 (Kumar et al., 2004), *Vagococcus* sp. W31 (Gao et al., 2006) and *Halomonas* sp. OKOH (Mabinya et al., 2011) produces polysaccharide bioflocculants. *Nocardia amarae* YK-1 (Koizumi et al., 1991), *Bacillus licheniformis* (Shih et al., 2001) and *Rhodococcus erythropolis* (Tadeka et al., 1991) produce protein flocculant, whereas *Arcuadendron* sp. TS-4 (Lee et al., 1995), *Arathrobacter* sp. (Wang et al., 1995), and *Halobacillus* sp. Mvuyo (Cosa et al., 2012) produce glycoprotein bioflocculant. Extracellular substances that are typically made of polysac-

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charides and proteins are thought to mediate cell-cell interactions or adherence of cells to surfaces (Zhang et al., 2002). They are a diverse and versatile class of materials and act as new biomaterials that have potential applications in many sectors of the economy (Jamil et al., 2008). They are used in food industry as viscosfying, stabilizing and emulsifying agents (Kunmani et al., 2011). Moreover, according to Kumar et al. (2004) and Kanmani et al. (2011), these compounds have become of interest as anti-tumor, anti-viral, and anti-inflammatory agents and as inducers for interferon, platelet aggregation inhibitors and in colony stimulating factor synthesis utilized in various medical and pharmaceutical industries.

Microorganisms produce extracellular substances as flocculants (bioflocculants). Their functions in flocculation and adhesion are similar to some amphoteric polymeric flocculants. These materials virtually possess the desired properties of conventional synthetic flocculants and are biodegradable, environmentally friendly (Sharma et al., 2006), and can be uniformly and reliably produced by fermentation processes (He et al., 2010). Although a number of bioflocculants have been evaluated as alternative flocculants, however it has been hard to actualize industrialization of these bioflocculants due to culturing costs and low production yield (Gao et al., 2006). Thus, research has been conducted on bioflocculants in order to reduce bioflocculant production costs and increase flocculant yield and applicability.

According to reported literature, the production of microbial flocculants may be significantly influenced by the culture medium composition and several other physicochemical parameters. Prominent among these are the composition of the growth medium, inoculum size, pH values, temperature, carbon and nitrogen sources (Suh et al., 1998). In our previous study (Cosa et al., 2011), we reported that, a polysaccharide bioflocculant was produced by *Virgibacillus* sp. Rob. In this current study, we assessed the bioflocculant production by the bacterium using a different set of culture condition. A series of experiments was undertaken to characterize the bioflocculant in detail, study its flocculating properties and subsequently compare its efficiency with synthetic flocculants.

MATERIALS AND METHODS

Test bacterium

The test bacterium was previously isolated from marine sediments of Algoa Bay, South Africa, identified as *Virgibacillus* sp. Rob by 16S rDNA sequence (Cosa et al., 2011). The organism was preserved in 20% glycerol at -80ºC as part of the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, Alice, South Africa.

Bioflocculant production media

This present study utilized a medium described by Zhang et al. (2007) with minor modification. It incorporates additional mineral salts and made use of multiple nitrogen sources from the onset and composed of the following: 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH4)_2SO_4$, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.1 g NaCl, and 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 L seawater.

Culturing of bioflocculant-producing bacterium

The pre-culture was prepared by inoculating a colony of the strain from the stock culture into a 150-ml flask containing 50 ml bioflocculant-production medium and incubated for 72 h in a rotatory shaker at 30°C, 160 rpm. This was then used as the standard inoculum preparation for all experiments. From the pre-culture, 0.5 ml was inoculated into a 250-ml flask containing 50 ml production medium. Fermentation was carried out in flasks with a shaking speed of 160 rpm at 30°C for 72 h. After the incubation period, the obtained fermentation broth was centrifuged (8 000 *g*, 30 minto separate the cells. The cell-free supernatant was used as source of bioflocculant and assessed for flocculating activity.

Measurements of flocculating activity

The flocculating activity test was done in accordance with the method previously described by Kurane et al. (1994). The Kaolin clay was used as suspension material in 4 g/l concentration. A control was prepared by replacing the bioflocculant solution with deionized water and measured under similar conditions. Flocculating activity was then expressed in percentage using the formula below:

Flocculating activity (%) = [(A-B)/A] x 100

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

Optimization of culture parameters on bioflocculant production

To establish the optimum medium culture conditions for enhanced bioflocculant production and flocculating activity, the carbon and nitrogen sources, initial pH of culture medium and inoculum size (%, v/v) were varied.

Carbon and nitrogen source

To determine a suitable carbon source for bioflocculant production, various carbon sources including: sucrose, fructose, maltose, galactose, xylose, phthalate, sodium acetate and sodium carbonate were incorporated each in basal bioflocculant medium by replacing glucose with same concentration (20 g/l). The flasks containing sterilized production medium were then inoculated separately with the bacterial strain and cultivated as described above.

The effect of different organic nitrogen (peptone, tryptone, urea, yeast extract and casein) and inorganic nitrogen (ammonium chloride, ammonium sulphate and sodium nitrate) sources were examined by incorporating each in basal medium replacing the initial multiple nitrogen (urea (0.5 g) yeast extract (0.5 g), $(NH_4)_2SO_4$ (0.2 g) (control).

Initial pH of the medium

The initial pH of the culture medium was adjusted to 3.0; 4.0; 5.0; 6.0; 7.0; 8.0; 9.0; 10.0; 11.0 and 12.0 with HCl or NaOH.

Effect of inoculum size

In 150 ml flasks containing 50 ml of bioflocculant production medium, different inoculum sizes (0.5, 1.0, 1.5 and 2.0 ml) of pre-culture were separately inoculated.

For each of the above experiments, the cultivation temperature was maintained at 30°C and the rotary shaking speed was set at 160 rpm. The samples were incubated for a period of for 72 h. For each studied experiment, all other constituents were kept constant. After the incubation period, the obtained fermentation broth was centrifuged (8 000 g, 30 min) and was assessed for flocculating activity (Yim et al., 2007; Mabinya et al., 2011; Piyo et al., 2011).

Effect of culture time on bioflocculant activity

The effect of culture time on bioflocculant activity was conducted in accordance with a previous study (Piyo et al., 2011) with minor modifications. The strains were cultured separately under optimal growth conditions. From the seed culture, 2% (v/v) was inoculated into 200 ml of bioflocculant production medium in 500 ml flasks (prepared in triplicates) and incubated on a rotatory shaker (160 rpm) at 30°C. Samples were drawn every 12 h, centrifuged at 8000 *g* for 30 min and the cell free supernatant was used to determine the flocculating activity and this was done for a period of five days. The pH of the broth samples was also measured using a pH meter (Model: EZDO PL-600 pH meter).

Bulk fermentation, extraction and purification of bioflocculant

Based on the findings of optimal growth conditions, a 1 L production medium was prepared in 2 L flasks, sterilized by autoclave, allowed to cool and inoculated with 2% (v/v) test bacterium suspension and incubated at 30°C for 72 h at 160 rpm. Purification of the polysaccharide bioflocculant was done in accordance to the methods described elsewhere (Chang et al., 1998; Chen et al., 2002; Cosa et al., 2011) with slight modification. Briefly, after 72 h of fermentation, the culture solution was centrifuged at 4,600 rpm for 30 min to remove bacterial cells. One volume of distilled water was added to the upper phase and centrifuged at 4,600 rpm for 15 min to remove insoluble substances. To the supernatant, two volumes of ethanol were added, and the mixed solution was stirred and left to stand at 4°C for 12 h. The precipitate was vacuum dried to obtain the crude biopolymer. The crude product was directly dissolved in distilled water to yield a solution, to which one volume of the mixed solution of chloroform and *n*-butyl alcohol (5:2 v/v) was added. After stirring, the mixture was set aside for 12 h at room temperature (about 20°C). The upper phase was centrifuged at 4 600 rpm for 15 min and two volumes of ethanol were added to recover the precipitate, vacuum-dried and then re-dissolved in distilled water, dialyzed against de-ionized water overnight and then vacuum dried to obtain a purified bioflocculant.

Physical and chemical analysis of purified bioflocculant

Composition analysis of purified bioflocculants

The measurement of polysaccharide content was done using the method of Dubois et al. (1956) with glucose as a standard. The total protein content was determined using the Folin-Lowry method with bovine serum albumin (BSA) as a standard (Lowry et al., 1951). The presence of uronic acid content was measured by carbazole method as described by Cesaretti et al. (2003).

Scanning electron microscopic observations on bioflocculant

Few drops of bioflocculant powder were dropped and fixed on the iron stub. The fixed specimen was gold coated and examined with a (JEOL - JSM-6390LV, Japan) Scanning electron microscope.

FTIR analysis

The polysaccharide bioflocculant was characterized by using Fourier Transform Infrared Spectrophotometer (Perkin Elmer System 2000, England). The dried bioflocculant powder was ground with potassium bromide (Kbr) and pressed into pellets for FTIR spectral measurement in the frequency range of 4000- 400 cm^{-1}.

Thermal stability of bioflocculant

Thermo-stability of purified bioflocculant was examined by measuring residual flocculating activity.The bioflocculant solutions were incubated in water bath fixed at 50, 80 and 100°C periods up to 30 min. Samples were drawn at appropriate time intervals and then analyzed for residual flocculating activity as also previously described by Gong et al. (2008).

Thermo-gravimetric analysis

The degradation temperature of the bioflocculant was studied using Thermo-gravimetric (STA 449/C Jupiter Netz, Germany; Perkin Elmer TGA7 Thermogravimetric Analyzer, USA) instrument. The measured bioflocculant was heated from 22 to 900°C at a rate constant of 10°C min^{-1} under constant flow of nitrogen gas.

Determination of bioflocculant dosage using jar test

Jar-test experiments were carried out using 1 L six place paddle stirrer (Model: FC6S VELP Scientifica, Europe) to determine the optimum dose of the purified bioflocculant for the clarification of kaolin clay suspension (4 g/l) at neutral pH. Different concentrations of the purified bioflocculant (powder) ranging from 0.1 to 1 mg/ml were prepared. The operational procedures included rapid mixing at 180 rpm for 1 min, the flocculation period at 45 rpm for 3 min and the sedimentation was 5 min as reported by Lee et al. (2001) with minor modifications. After settling, the upper phase was sampled to measure flocculating activity

Effect of pH on flocculation activity of the purified bioflocculants

To evaluate the effect of pH on flocculating activity of the purified bioflocculants, the pH of the bioflocculant solutions were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 by the addition of HCl or NaOH.

Effects of cation dependency on flocculating activity of the purified bioflocculant

To investigate this effect, (+) cation (3 ml) and bioflocculant (2 ml) were added to the kaolin suspension. $CaCl_2$ solution previously used as stimulating agent was replaced by various metal salt solutions. Potassium chloride (KCl), sodium chloride (NaCl), and lithium chloride (LiCl) acted as monovalent cation source; manganese

Table 1. Effect of carbon sources and nitrogen sources on bioflocculant activity.

Carbon source	FA (%)	nitrogen source	FA (%)
Glucose	91.8 ± 3.08 [a]	Peptone	79.9 ± 1.47[b]
Maltose	78.8 ± 1.89[b]	Tryptone	78.9 ± 4.1[b]
Galactose	74.02 ± 2.82[b]	Casein	70.8 ± 3.51[a,b]
Fructose	64.36 ± 2.18[c]	Yeast	69.8 ± 2.53[a,b]
Sucrose	58.25 ± 5.24[c]	Urea	67.9 ± 1.51[a,b]
Xylose	15.5 ± 4.36[d]	Ammonium sulphate	78.9 ± 3.04[b]
Sodium carbonate	72.15 ± 5.16[e]	Ammonium nitrate	75.3 ± 4.1[a,b]
Sodium acetate	49.56 ± 2.87[f]	Ammonium chloride	37 ± 1.01[c]
Phyltalate	42.53 ± 2.77[f]	Complex nitrogen (Urea+ Yeast extract+ $(NH_4)_2SO_2$)	91.8± 3.08[a]

The results are represented as mean values of triplicates ± Standard deviation. Percentage flocculating activity (FA) with different alphabets are significantly ($p < 0.05$) different.

phosphate ($MnPO_4$) and magnesium chloride ($MgCl_2$) acted as divalent cation source; and aluminium chloride ($AlCl_3$) and iron (III) sulfate ($FeSO_4$) acted as trivalent cation source. The flocculating activity was measured as mentioned above (Zulkeflee et al., 2012).

Statistical analysis

Treatments used for each experiment were done in minimum of three replicates with mean and standard deviation values determined. Microsoft excel office 2007 version was used to determine means and standard deviations. Statistically significant differences of the percentage bioflocculant activity among the treatment means were analysed using the analysis of variance (ANOVA) test (Minitab Student Release 12) where differences were considered significant at 0.05 confidence level.

RESULTS

Factors affecting the bioflocculant activity

Effect of carbon and nitrogen sources

The effect of various carbon and nitrogen sources on bioflocculant activity was carried out and results are depicted in Table 1. The effect of various carbon sources showed that glucose followed by maltose, galactose and sodium carbonate were best favoured carbon sources with flocculating activity of 91.8, 78.8, 74.02 and 72.15% respectively. On the other hand, phthalate was the least preferred carbon source with 42.53% flocculating activity. With respect to nitrogen source, Table 1 shows that peptone (79.9% flocculating activity) followed by tryptone (78.9% flocculating activity), casein (70.8% flocculating activity) and yeast (69.8% flocculating activity) were the best nitrogen sources while ammonium chloride (37% flocculating activity) was the least favourable nitrogen source. Although, higher flocculating activity of 91.8% was obtained for the test bacteria when a multiple nitrogen source consisting of urea, yeast extract and ammonium sulphate was used.

Effect of initial pH and inoculum size

The effect of initial pH of the medium was examined over the pH range of 3-12 and results are shown in Figure 1. From the results of this study, initial medium pH 3, 4, 5 and 6 resulted in bioflocculant production with flocculating activities of 83, 72.5, 73.8 and 73.6%, respectively; however alkaline pH 10 showed optimum flocculating activity of 85.8%.

Figure 2 shows that inoculums size of 2% (v/v) resulted in optimum production of bioflocculant (flocculating activity 91.8%). Subsequent increases in inoculum size resulted in almost constant activity without further increase in flocculating activity.

Effect of fermentation duration

With regards to the bioflocculant production kinetics (Figure 3), flocculating activity of about 82% was observed within the initial 12 h of fermentation and remained relatively constant with time but attained a peaked (87% flocculating activity) in 72 h after which flocculating activity declined.

Physical and chemical analysis of bioflocculant

Bioflocculant yield

After purification, a total bioflocculant yield of 2.43 g (Table 2) was recovered from 1 L culture broth and was slightly better when compared with other different yields of bioflocculant production as shown in Table 2.

Chemical composition analysis of the bioflocculant

The chemical properties of the purified bioflocculant showed the proportions of the neutral sugar content as

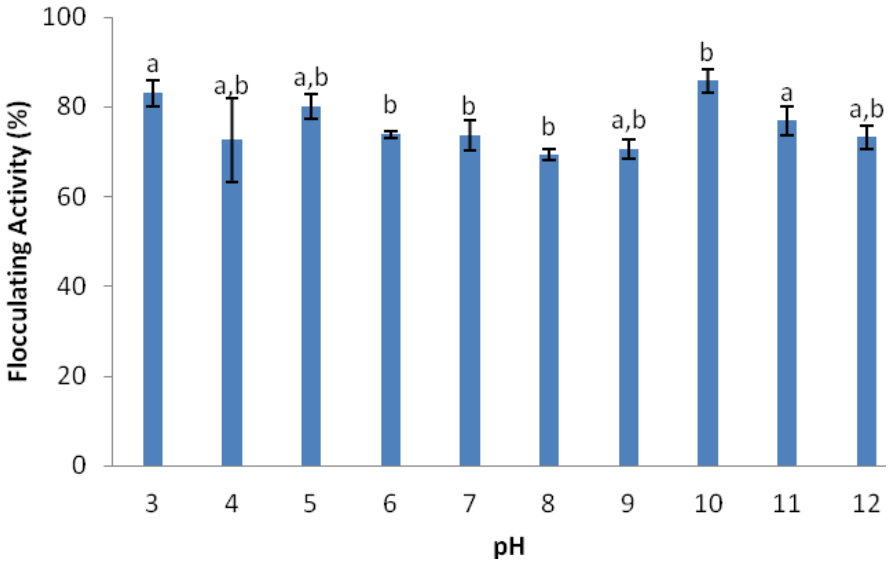

Figure 1. Effect of initial pH on bioflocculant activity of *Virgibacillus* sp. Rob. Percentage flocculating activity with different letters (a, b) are significantly ($p < 0.05$) different.

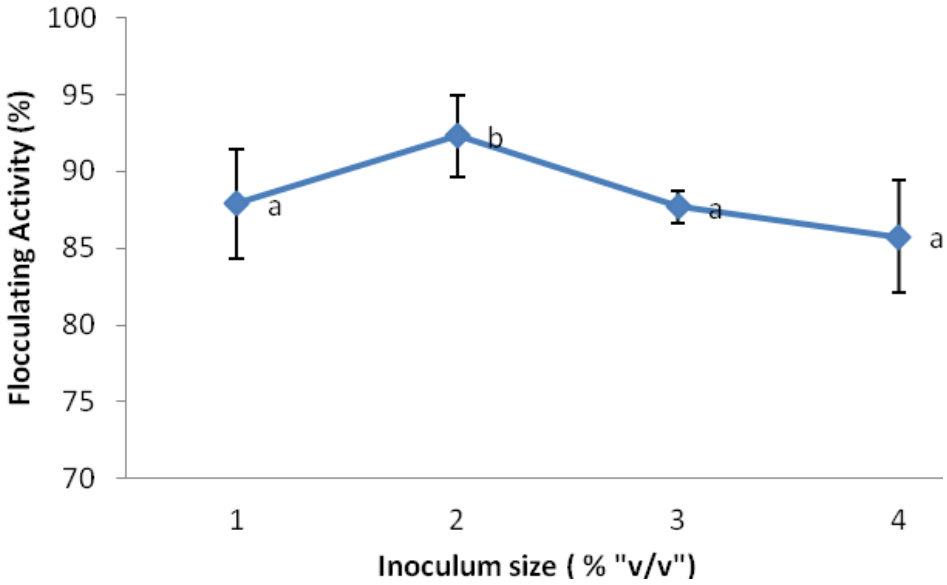

Figure 2. Effect of inoculum size on bioflocculant activity of *Virgibacillus* sp. Rob. Percentage flocculating activity with different letters (a, b) are significantly ($p < 0.05$) different.

6.6 % while uronic acids were 87.7% with no protein detected.

Further details of the elemental composition revealed the bioflocculant to contain C, N, O, P major elements, while S was present as minor element on the surface of the bioflocculants with 3.82, 7.43, 40.7, 1.69, 11.69 weight percentages respectively.

SEM observations

Surface morphology structure of the bioflocculant as revealed by Scanning Electron Microscopy (SEM) showed that the bioflocculant was whitish in color and porous (Figure 4i). On the other hand, the kaolin particles before flocculation are fine and scattered as shown in

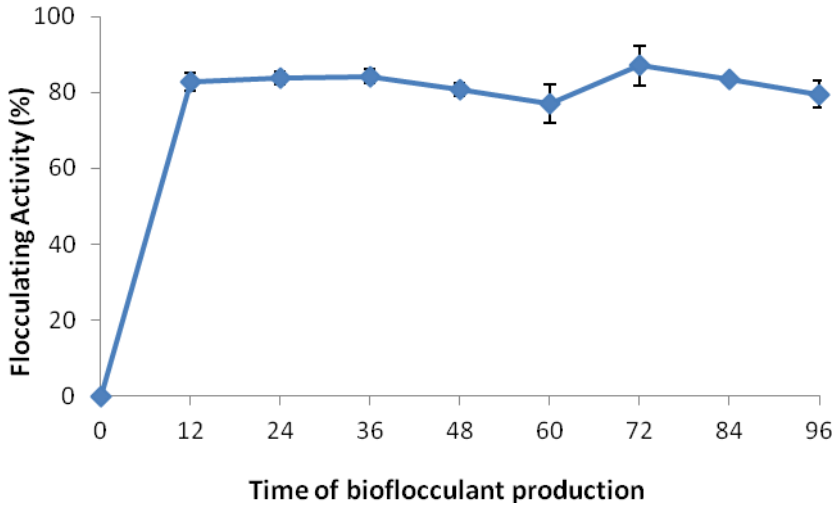

Figure 3. Time course assay of bioflocculant production by *Virgibacillus* sp. Rob

Table 2. Comparison of the purified bioflocculant produced by *Virgibacillus* sp. Rob with other bioflocculants.

Strain	Bioflocculant yield (g/L)	Carbon/Nitrogen sources in fermentation	Components	Reference
Enterobacter cloacae WD7	2.27	Glucose/Sucrose, $(NH_4)_2SO_4$	acidic hetero-polysaccharide	Prasertsan et al., 2006
Proteus mirabilis TJ-1	1.33	Glucose, peptone	Acid polysaccharide and protein	Xia et al., 2008
Bacillus firmus	1.36	Glucose, peptone, yeast extract	Acidic polysaccharide	Chen et al., 2002
Bacillus sp. Strain F19	1.47	Sucrose, yeast extract	Mainly polysaccharide	Zheng et al., 2008
Aeromonas sp.	2.25	Sucrose, yeast extract, ammonium sulphate, urea	Not specified	Li et al., 2007
Vagococcus sp. strain W31	2.3	Glucose, ammonium sulphate	Polysaccharide	Gao et al., 2006
Virgibacillus sp. Rob	2.43	Glucose, peptone	Polysaccharide	Our study

Figure 4(ii) while after flocculation, the bioflocculant and its kaolin interaction were coupled as represented in Figure 4(iii).

Functional groups of the purified bioflocculants

An analysis of the functional groups of purified bioflocculant from *Virgibacillus* sp. Rob showed a broad stretching peak at 3414.51 cm^{-1} which is a common characteristic of hydroxyl and amino groups. Two peaks at 2374.75 and 2063.74 cm^{-1}, weak peaks observed at 1638.43 and 1619.13 cm^{-1} suggestive of the presence of carboxyl groups with C =O in an amide group and 1400.88 cm^{-1} and small absorption peaks were exhibited around 1000 to 1137 cm^{-1} attributed to the asymmetrical stretching vibration of a C-O-C ester linkage.

Analysis of thermal stability of bioflocculant

The effect of heat on the bioflocculant was investigated at three different temperatures (50, 80 and 100°C) for 30 min each. The bioflocculant retained about 84, 80 and 79% flocculating activity at 50, 80 and 100°C respectively although these variations were not significantly different

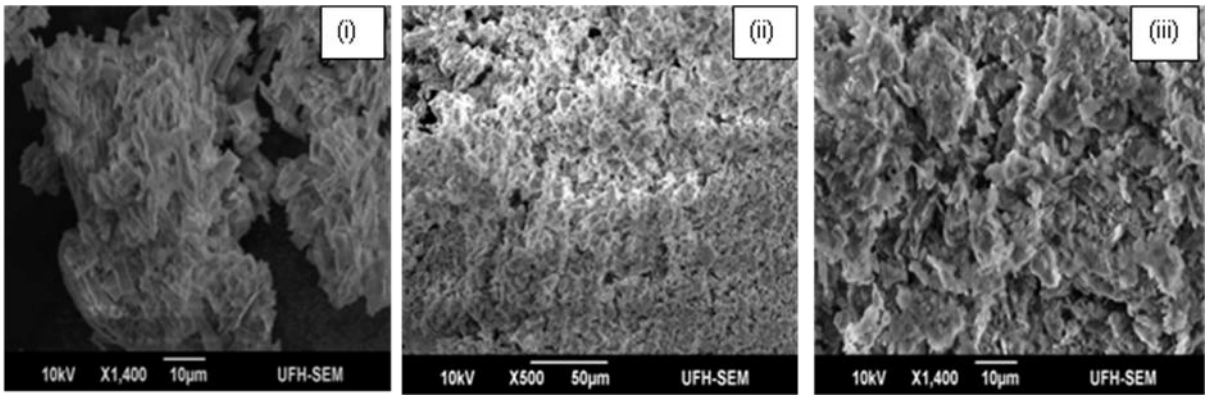

Figure 4. SEM images of (i) kaolin clay (ii) bioflocculant by *Virgibacillus* sp. Rob (iii) kaolin clay flocculated by the extracted bioflocculant.

($p < 0.05$).

Thermo-gravimetric analysis of purified bioflocculant

The pyrolysis property of the purified bioflocculant was studied using thermo-gravimetric analysis (TGA). A degradation temperature (T_d) of 150°C was observed (Figure 7). An initial weight loss of about 2% in the bioflocculant was found between 110 and 140°C. Further increase in temperature to 580°C resulted in about 20% reduction in mass fraction thereafter a gradual decrease in mass weight was observed.

Flocculating properties of purified bioflocculant

Effect of bioflocculant concentrations

Jar-test experiment revealed that flocculating activity increased with the increasing concentration of bioflocculant and a peak of 88.7% was attained at a dose of 0.3 mg/ml. Thereafter, as bioflocculant concentration increased beyond this optimum, flocculating activity decreased (Figure 8).

Effect of pH on flocculation by the purified bioflocculant

The effect of pH on flocculating activity of the purified bioflocculant was examined using 0.3 mg/ml concentration of the purified bioflocculant and Ca^{2+} as the mediating cation. At acidic conditions, the flocculating activity was maintained between 71.2 to 82.9% while at alkaline conditions, the flocculation activity was between 33.6 to 79.8%. However, the highest flocculation activity was obtained at neutral pH (84%).

Effect of various cations on flocculation

Experiments on effect of various cations demonstrated that, flocculating activity was altered in presence of some cations (Figure 10). Flocculation was enhanced in the presence of Ca^{2+}, Al^{3+}, Mn^{2+} and Mg^{2+} with 91.7, 83.2, 68.2 and 62.9% flocculating activity, respectively. On the other hand, Fe^{3+}, Li^{+}, K^{+} and Na^{+}, showed least and/or no flocculating activity (Figure 10).

Comparative analysis of flocculation efficiencies of different chemical flocculants and the biofloculants

The purified bioflocculant was more efficient than the conventional inorganic ferric chloride and alum, as well as polyethylimine flocculants as revealed by the optimum dose of 0.3, 0.1, 0.2 and 0.2 mg/ml compared to 0.3, 0.8 and 1 mg/ml for polyethylimine, ferric chloride and alum respectively. The bioflocculants were however less efficient than polyacrylamide which had an optimum dosage of 0.1 mg/ml (Table 3).

DISCUSSION

In our previously study (Cosa et al., 2011), *Virgibacillus* sp. Rob was established as a bioflocculant producing bacteria. Some preliminary culture conditions were examined. Conversely, in this present study, additional sources (carbon and nitrogen sources), other parameters (pH, inoculum size and thermal stability) and cultivation time that are known to indirectly affect the bioflocculant yield and activity were investigated, the fundamental rationale been to improve upon the bioflocculant production efficiency of the bacteria.

Bioflocculant production is affected by numerous factors, such as the constituents of the culture medium

Table 3. Comparison of bioflocculant efficiency with some chemical flocculants.

Flocculant	Dosage (mg/ml)	Turbidity reduction (%)
Polyacrylamide	0.3	95.2
Bioflocculant	0.3	89.0
Polyethylimine	0.1	82.6
Ferric chloride	0.8	74.5
Alum	1	70.9

and the culture conditions. Since the carbon source plays a very significant role in growth, particularly in extracellular polysaccharide production, several sugars were investigated and results are articulated in Table 1. Flocculating activity was significantly higher ($p < 0.05$) when glucose (flocculating activity 91.8%) was used as sole source of carbon compared to the other carbon sources. This confirms our previously reported study (Cosa et al., 2011) although in this case the percentage flocculating activity increased. The increase may be due to the supplemented nitrogen sources or fermentation period. Glucose had been vastly documented as fine substrate for bioflocculant production (Suh et al., 1998). Furthermore, a literature search shows that numerous bioflocculant producing bacteria prefer organic carbon sources for bioflocculant production, thus supporting the observation for the *Virgibacillus* sp. Rob. For instance, Gong et al. (2003) concluded that sucrose, maltose, xylitol, lactose and glucose are suitable substrates for flocculant production by *Paenibacillus polymyxa* BY-28, with glucose being the best.

Furthermore, we investigated the influence of nitrogen source on bioflocculant production by the bacteria with glucose as carbon source. In place of the multiple-nitrogen sources (yeast, urea and ammonium sulphate) used, individual nitrogen source was incorporated, however the concentration used amounted to the sum of that multiple-nitrogen source used in the previous report. Results revealed that flocculating activity was highest when peptone was used, though not significantly different in comparison to tryptone, ammonium nitrate and ammonium sulphate (Table 1). Organic nitrogen sources are reported to be more suitable for bioflocculant production but a complex nitrogen substrate consisting of urea, yeast extract and $(NH_4)_2SO_4$ enhanced cell activity more significantly and was better suitable for the bioflocculant production than the other nitrogen sources tested. In line with our results, Gong et al. (2008) also found that a complex nitrogen source consisting of beef extract and urea is better than a single inorganic or organic nitrogen sources. Kurane and Matsuyama (1994), reported a bioflocculant production by a mixed culture in which the production medium incorporated a combination of ammonium sulphate and yeast extract as the nitrogen sources. On the contrary, the literature (Gandhi et al., 1998; Hwang et al., 2003; Wang et al., 2011) reports that organic nitrogen sources are more favourable for bioflocculant production and in addition are more easily absorbed by the cells than the inorganic nitrogen sources.

Based on literature (Zhang et al., 2007), the initial pH of the production medium determines the electric charge of the cells and the oxidation-reduction potential, which can affect absorption of nutrients and enzymatic reaction of test bacteria. Hence, altering the pH may affect the medium composition thus resulting in varied bioflocculant activities. As illustrated in the results, it is evident that the acidic, neutral to alkaline conditions supported bioflocculant production (Figure 1). Alkaline pH 10 appeared to be optimum in contrast to pH 11 reported in the previous study (Cosa et al., 2011). Although the initial pH of the production medium determines the electric charge of the cells and the oxidation-reduction potential which consecutively can influence nutrient absorption and enzymatic reaction, this influence differs with each studied organism (Salehizadeh and Shojaosadati, 2001). Mabinya et al. (2011) reported that pH 7 was optimal for bioflocculant production by *Halomonas* sp. OKOH. Kurane et al. (1994) documented that for bioflocculant production by *Rhodococcus erythropolis*, alkaline pH (8.0-9.5) was most favourable. In the case of the present study, the bioflocculants from *Virgibacillus* sp. was produced maximally under high alkaline conditions

Amongst a number of bacteria's physiological properties, inoculum size may play a significant role in biological development (Gancel et al., 1994). According to documented literature (Chen et al., 2008; Wang et al., 2011) the inoculum size and liquid volume may play a vital role in cell reproduction and bioflocculant production. Experimental results revealed that inoculum size had an effect on bioflocculant production and activity. When the inoculum size was increased a decrease in flocculating activity was observed, while at 2% inoculum size the maximum flocculating activity was achieved, reaching a plateau thereafter (Figure 2). The obtained flocculating activity at 2% inoculum size was significantly higher ($p < 0.05$) than other inoculum sizes tested. The optimal inoculum size allowed the adaptation of strains to the production medium thereby promoting the production of bioflocculants (Li et al., 2009). In addition, the plateauing in flocculating activity may be attributed to excess inoculum size, which causes an excessive niche overlap, resulting in inhibition of further bioflocculant production due to the limit of nutrient distribution (Salehizadeh and Shojaosadati, 2001; Zhang et al., 2012). Similar phenomenon was reported for bioflocculant production by *Serratia ficaria* (Gong et al., 2008).

When the bioflocculant production kinetics was investigated, flocculating activity increased with culture time, subsequently flocculating activity declined (Figure

3). The decline on flocculating activity may be due to the enzymatic degradation and cessation of bioflocculant production (Zhu et al., 2011). The bioflocculant was deduced an extracellular product since it was extracted from culture medium free of cells (Zhu et al., 2011). Similar results were reported by Li et al. (2007) whereby *Aeromonas* sp. bioflocculant reached its flocculating activity maxima after 3 days of fermentation. Prasertsan et al. (2006) reported *Enterobacter cloacae* WD7 exhibiting the highest flocculating activity after 72 h of cultivation.

As noted in Piyo et al. (2011), cultivation time for bioflocculant release into the medium varies with diverse microorganisms. *Bacillus licheniformis* CGMCC 2876 showed relatively short fermentation period of 40 h (Xiong et al., 2010). The observed corresponding pH to the profile of flocculating activity, a slight increase in pH levels from 10.2 to 10.5 in the first 12 h; thereafter the pH was nearly stable the fermentation period (data not shown). This increase did not vary significantly and may be as a result of excretion of extracellular materials resulting from the partial degradation of the bioflocculant (Mabinya et al., 2011).

After the extraction and purification, our recovered bioflocculant yield was about 90% more than obtained in our previous report (Cosa et al., 2011). Different yields in bioflocculant production have been well documented as shown in Table 2 and our bioflocculant yield differed from the other microorganisms from those reported in literature (Table 2).

The surface morphology structure of the studied bioflocculant and its interaction with kaolin clay suspension by Scanning Electron Microscopy (SEM) showed that the bioflocculant (Figure 4) was conversely different from the crystal-linear structure of bioflocculant TJ-F1 proposed by Xia et al. (2008). However, our findings were similar to that reported by Kumar et al. (2004). Prior to flocculation, the kaolin particles were dispersed, during the process of coagulation-flocculation the scattered kaolin particles were probably knit and adsorbed onto the binding sites of the bioflocculants which thus aggregated, forming larger flocs and leading to rapid sedimentation due to gravity (Xiong et al., 2010; Wang et al., 2011).

When a chemical composition analysis of the purified bioflocculant was carried out to identify the main chemical constituents, the results showed that it was mainly acidic polysaccharide with no amount of protein present. The results revealed it to be predominantly made up of uronic acids. A number of bacterial strains that produce (acidic) polysaccharide bioflocculants have been well documented. As example, *Bacillus firmus* amongst others was reported to produce an acidic polysaccharide bioflocculant (Chen et al., 2002). Consequently, the polysaccharide in the purified bioflocculant may explicate the observed high flocculating activities.

When further analysis was conducted for elements of the purified bioflocculant the findings revealed the presence of C, N, O, S, and P on the surface of the bioflocculants. The presence of the non-sugar components is typically smaller, though it may give flexibility and stabilize the bioflocculant. In agreement with our findings, Gosh et al. (2009) reported the presence of C and N in quantities of 14.3%, 0.64% respectively while S and P were reported as trace elements on the surface of the bioflocculant produced by *Klebsiella terrigena.*

FTIR spectrophotometry revealed the presence of some functional groups in the purified bioflocculant. As shown in Figure 5, a broad stretching peak was observed at 3414.51 cm^{-1} which was indicative of hydroxyl groups and amine groups. This may also be as a result of vibration of -OH or -NH present in the sugar ring (Xiong et al., 2010). Two peaks at 2374.75 and 2063.74 cm^{-1} is indicative of C-H alphatic bonds (He et al., 2010). The weak peaks observed at 1638.43, 1619.13 cm^{-1} and 1400.88 cm^{-1} are indicative of carboxyl groups. The peaks exhibited around 1000 to 1137 cm^{-1} are suggestive of uronic acids (Kumar et al., 2004), and the weak peaks (873.58 cm^{-1}) identified are indicative of the presence of sugar derivatives. Also according to Xiong et al. (2010), the small absorption peaks may be suggestive of β-glycosidic linkages amid the sugar monomers. Hence, the FTIR spectrophotometric analysis suggests the presence of carboxyl, hydroxyl, and amine groups which are major adsorptive forces of the bioflocculant and crucial for the process of flocculation (Wang et al., 2011). The observation also suggests the carboxyl groups as the binding sites for cation allowing improved flocculation. For example, the OH, COOH, and COO^- groups may link with the H^+ and OH^- present on the surface of the particles forming hydrogen bonds when the bioflocculant chains approach the particles' surface (Zheng et al., 2008). Our findings also revealed the bioflocculant to contain functional groups similar to those reported in other studies (Wang et al., 2007; Yim et al., 2007; Feng et al., 2008). Moreover, these functional groups are preferred for the flocculation process similar to that observed in polyelectrolytes such as anionic polyacrylamides (Bolto and Gregory, 2007). Furthermore, the functional groups particularly carboxyl could also work as functional moieties to generate new and/or modified polysaccharide variants using different approaches, including polymer engineering or novel formulation designing, by linking the polysaccharide with other synthesized polymers (Yim et al., 2007).

When the effect of heat on the bioflocculant was investigated at three different temperatures (50, 80 and 100°C) the results suggested that heat had little impact on the physical and chemical properties of the bioflocculant up to 100°C (Figure 6). The thermal stability of the bioflocculant could be as a result of the

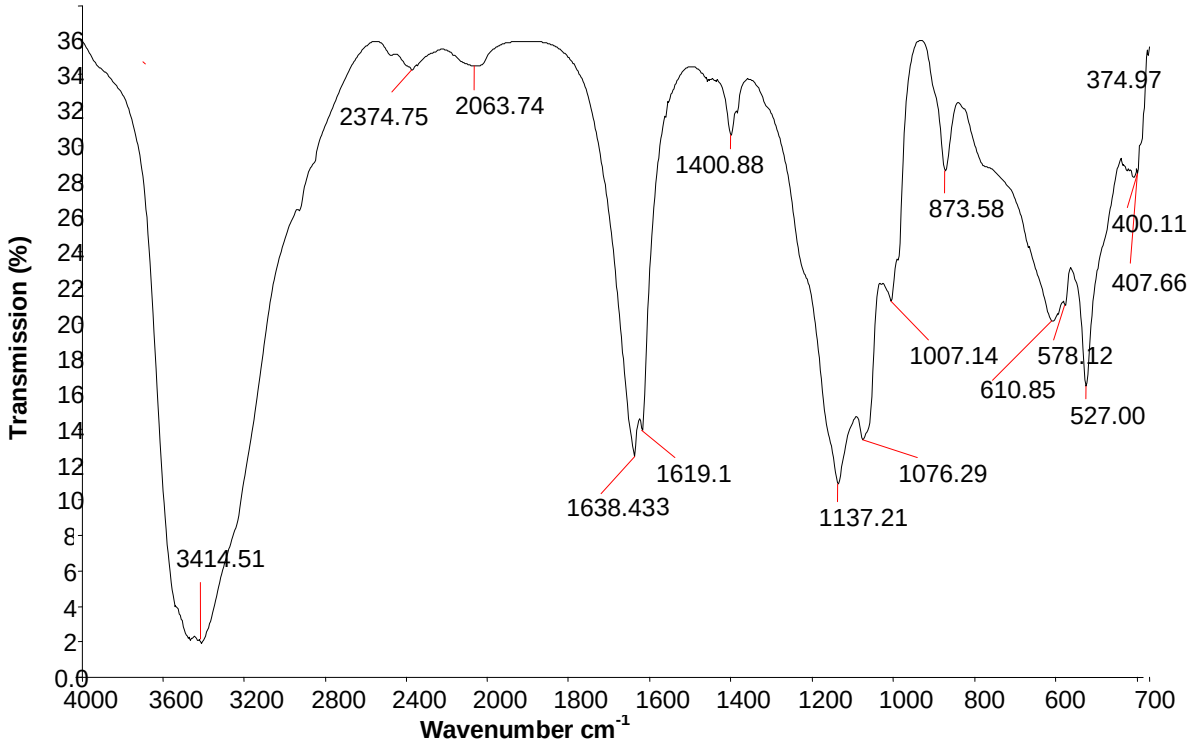

Figure 5. Infrared spectra of purified polysaccharide bioflocculant produced by *Virgibacillus* sp. Rob.

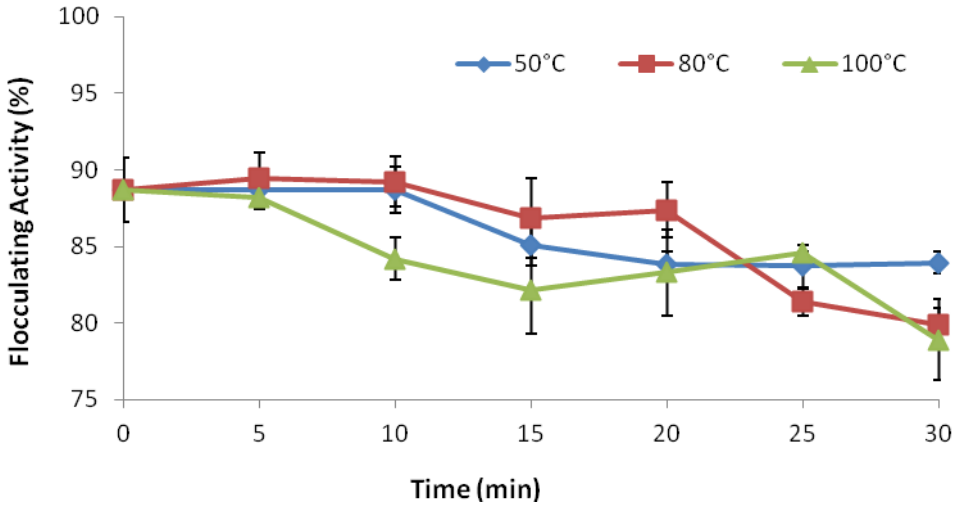

Figure 6. Thermal stability of the purified polysaccharide bioflocculant.

polysaccharide backbone of the bioflocculant (Gong et al., 2008; Lu et al., 2005). Corroborating our findings, Gong et al. (2008) and Lu et al. (2005) reported bioflocculants produced by *Serratia ficaria* and *Enterobacter aerogenes* as thermally stable. However in a study reported by Lian et al. (2008), the bioflocculant produced by *Bacillus mucilaginosus* was thermally stable but beyond 70°C noticeable reduction in the flocculation ratio that falls to about 70% was observed.

When the pyrolysis property of the purified bioflocculant was studied, a degradation temperature (T_d) of 150°C was observed which further corroborated the thermal

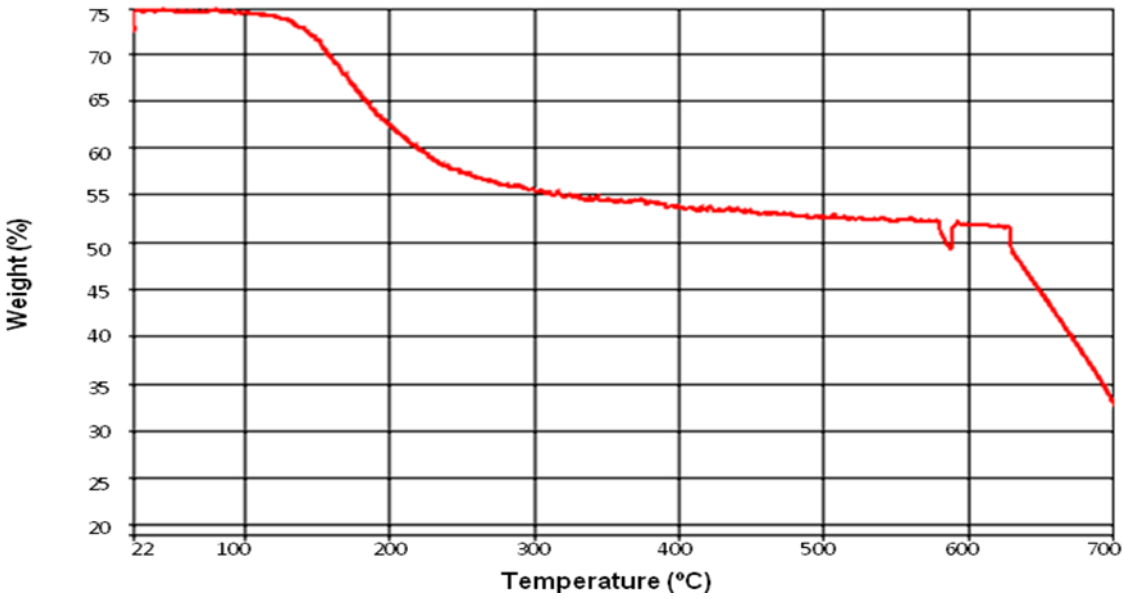

Figure 7. Thermogravimetric analysis of purified polysaccharide bioflocculant from *Virgibacillus* sp. Rob

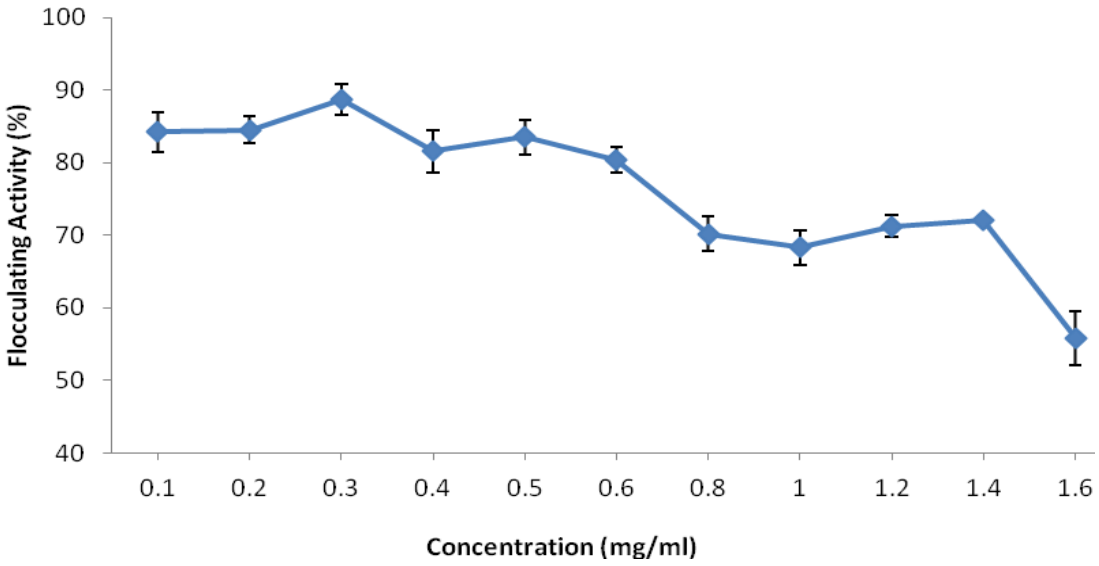

Figure 8. Effect of bioflocculant concentration on flocculation. Error bars represent the standard deviation of mean value of

stability of the bioflocculant as reported overleaf (Figure 7). The observed initial weight loss was due to moisture content as a result of the levels of carboxyl groups present in the bioflocculant. Yim et al. (2007) reported degradation temperature of 230°C for p-KG03 bioflocculant while Kumar et al. (2004) reported *Bacillus* sp. I-471 bioflocculant with Td of 307°C.

Dosage is still one of the vital parameters considered when determining the optimum conditions for the performance of flocculant in the process of coagulation-flocculation since an insufficient dosage or over-dosage may lead to reduced performance in flocculation (Hassan et al., 2009). Therefore, it becomes indispensable to establish the optimum bioflocculant dose, as this could help minimize costs and attain better performance in the treatment processes. Studying the jar-test experiment results (Figure 8), the flocculating activity increased with increase of the biofloculant concentration. The variations

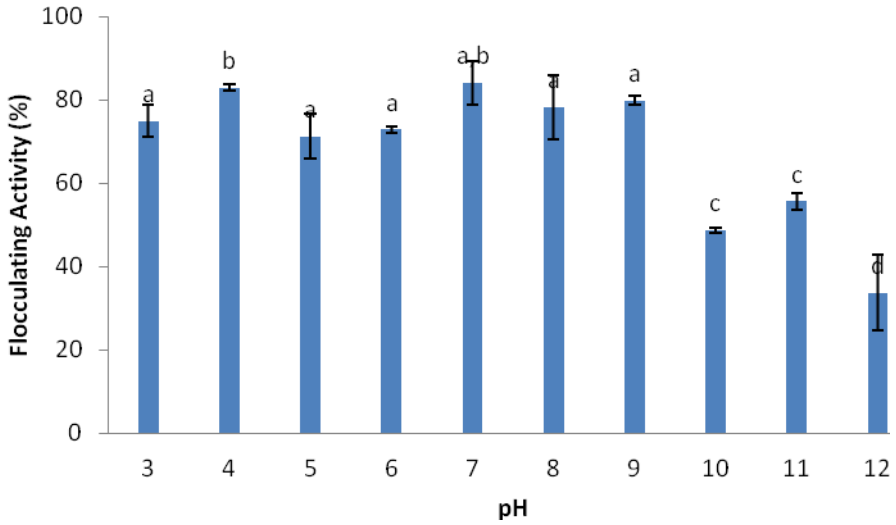

Figure 9. Effect of pH on flocculation. Percentage flocculating activity with different letters (a, b, c, d) are significantly ($p < 0.05$) different.

in flocculating activities were significantly ($p < 0.05$) different. The decrease of flocculation activity that occurred may be attributed to "flocculation deterioration" phenomenon as suggested by Liang et al. (2010) whereby some colloidal particles were enclosed up by the concentrated flocculant and a "colloid protection function" occurred, leading to reduced flocculating activity. The binding sites of the dispersive kaolin particles were blocked up by some bioflocculant molecules at high bioflocculant concentration instead of the formation of stronger bridging among the bioflocculant molecules and disperse particles in a proper flocculant concentration (He et al., 2010).

Literature stipulates that the alteration of pH may ultimately alter the bioflocculant charge status and surface characteristics of suspended materials consequently changing the flocculating ability (Zhang et al., 1999). In the present study, the flocculating activity was retained all through the pH range (3-12). However, the less flocculating activity at very alkaline conditions (pH 10-12)) (Figure 9) suggest that the alkaline degradation of the polysaccharide bioflocculant which could have resulted in a number of changes i.e. molecular rearrangements of its residue or polysaccharide chain fragmentation (Bathe and Patil, 2010). Also the mechanisms of pH influence on bioflocculation are still not quite apparent (Ma et al., 2008). However, it has been demonstrated that at very high pH the OH^- ions may impede the formed complex of the bioflocculant and kaolin particles in the mixture whilst at lower pH the bioflocculant and the kaolin may probably adsorb the H^+ leading to reduced flocculation activity. On the other hand, at neutral pH this mediating effect may be stronger (Li et al., 2007). A similar case was reported by He et al. (2010) whereby the flocculating activity of a bioflocculant produced by deep sea bacteria mutant *Halomonas* sp. V3a' peaked at neutral pH.

Lu et al. (2005) reported that cations have significant effect on flocculation. Mostly, the cation is used as coagulant aid in achieving high flocculation activity by neutralizing the negatively charged functional groups on the bioflocculant and suspended particles thereby increasing the adsorption of bioflocculant to the suspended particles (He et al., 2010; Mabinya et al., 2011). Monovalent cations (Na^+, Li^+ and K^+), trivalent Fe^{3+} had very little or no effect on flocculation activity (Figure 10). On the other hand, divalent (Ca^{2+}, Mn^{2+} and Mg^{2+}) and trivalent (Al^{3+}) enhanced flocculation. However Ca^{2+} and Al^{3+} greatly enhanced flocculation. The Ca^{2+} ion are assumed to have enhanced the process of flocculation by neutralization and stabilization of residual negative charges of carboxyl groups of uronic acid present in the acidic polysaccharide, thereby bridges between kaolin particles and to each other were formed (Suryani et al., 2011). A similar results was observed with Gong et al. (2008) and also Ca^{2+} was found to significantly improve flocculation. He et al. (2010) also reported Ca^{2+} as cation source enhancing flocculation by bioflocculant HBF-3 produced by *Halomonas* sp. V3a'.

The effectiveness of our test bioflocculant was compared with those of some conventional synthetic flocculants (polyacrilamide (PAM), polyethylimine, alum, and ferric chloride) at a concentration range of 0.1-1 mg/ml and neutral pH for flocculating kaolin suspension under similar conditions. The results are shown in Table 3. The bioflocculant was more efficient than the inorganic ferric chloride and alum, as well as polyethylimine, though less efficient than polyacrylamide. These findings

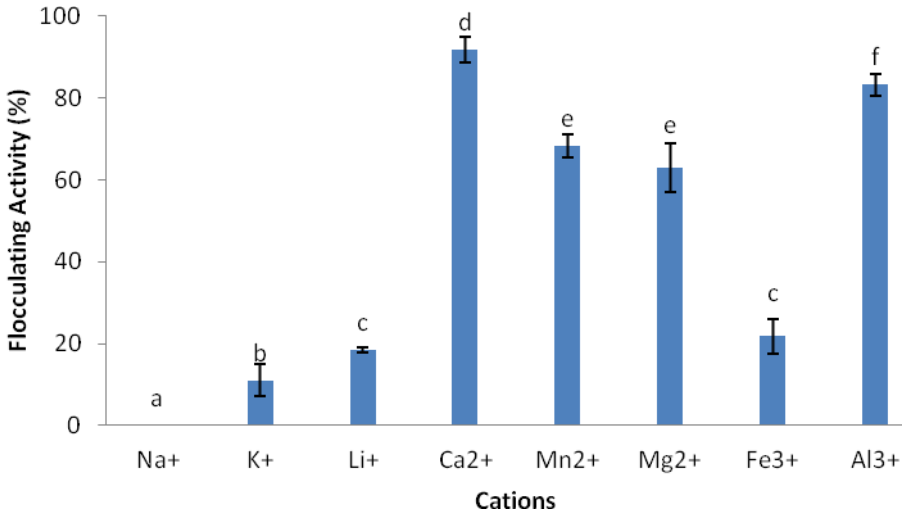

Figure 10. Effect of cations on flocculation activity. Percentage flocculating activity with different letters (a, b, c, d, e, f) are significantly ($p < 0.05$) different.

suggested that the bioflocculant could conveniently stand as alternatives to many inorganic and synthetic flocculants and facilitate their possible application in water and wastewater treatment.

Conclusions

This study corroborates our previous report and confirms that *Virgibacillus* sp. Rob produces a polysaccharide bioflocculant composed mainly of uronic acids. The low optimum dose of 0.3 mg/ml of the purified bioflocculant for the flocculation of kaolin clay; the neutral pH required for optimal activity; and the thermal stability of the bioflocculant are significant and makes it attractive as alternative to synthetic flocculants. Its applicability in the treatment of various types of wastewater is a subject on intensive investigation in our group.

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