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Production, characterisation and flocculation mechanism of bioflocculant TMT^{-1} from marine *Bacillus pumilus* JX860616

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Bioflocculant from marine *Bacillus pumilus* JX860616 was characterised and its flocculation mechanism determined. The bacterium was identified by 16S rRNA and the bioflocculant was obtained through solvent extraction after optimum medium composition and culture conditions were established. The physicochemical analysis of the bioflocculant were obtained by scanning electron microscopic (SEM) equipped with elemental detector, Fourier transform infrared (IR) spectrophotometry. The highest flocculating activity (93.3%) was obtained with optimum medium composition of the energy sources of glucose and $(NH_4)_2SO_4$ and culture conditions of; initial pH 6; and Ba^{2+} after 72 h, at the inoculum size of 2% (v/v). The bioflocculant (2.4 g/L) revealed to have a crystal-like porous structure and had the total carbohydrate of 83.1% w/w and proteins content of 6% w/w. The elemental analysis showed the presence of C (17.0), O (46.0), Na (4.3), Mg (6.8), P (4.1), S (7.0), Cl (5.9), K (7.4) and Ca (0.7) (% w/w). IR observations were indicative of hydroxyl, vinyl, amide and aliphatic amine groups. The bridging mechanism mediated by Ba^{2+} on colloidal Kaolin particles was proposed. The high flocculating activity of TMT^{-1} implied that it has a promise in industrial applications.

Key words: *Bacillus pumilus* JX860616, bioflocculant TMT^{-1}, flocculating activity and flocculation mechanism.

INTRODUCTION

Natural water often consists of thermodynamically unstable and kinetically non-labile colloidal particles that do not even settle out under gravity (Spellman, 2014). Colloidal particles contribute to water turbidity and often shelter some pathogens from inactivation by disinfectants (Mines, 2014). Colloids removal is a paramount goal in wastewater treatment that extensively employs chemo-physico methods such as flocculation (McCarthy, 2011; Karthiga and Natarajan, 2015). Flocculation is a purification technique whereby polymers form bridges with colloids and bind them into large and settled able agglomerates (Davis and Masten, 2014). Inorganic and synthetic organic flocculants are extensively used in vast biotechnological applications due to their cost

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effectiveness. However, they are both reported to impose health risks and environmental hazards (Salehizadeh and Shojaosadati, 2001). Inorganic and chemical flocculants are not degradable and impose carcinogenic and neurotoxic effects. Thus, novel flocculants that are eco-friendly and innocuous to humans are needed.

Bioflocculants are macro biopolymers produced by microorganisms during their exponential growth (More et al*.,* 2014). *Levure casseeuse* was the first reported biofocculant producing fungi (Pasteur, 1878). Two years later, it was observed in bacterial strains (Bordet, 1899) and *Zoogloea*-forming bacteria became first bioflocculant-producing bacterium to be discovered (Butterfield, 1835). To date, over 100 species of bioflocculant-producing microorganisms have been reported (Aljuboori et al*.,* 2015). Bioflocculants lack secondary pollution and thus are environmentally friendly and there are no health threats of any sort that have been reported regarding their usage (Cong-Liang et al*.,* 2012; Yumei et al*.,* 2014). Soil, activated sludge and fresh water bodies have been the predominant reservoirs for isolation of bioflocculant-producers (Salehizadeh and Shojaosadati, 2001; Karthiga and Natarajan, 2015). However, marine remains an untapped reservoir (Okaiyeto et al*.*, 2015). It is predicted that bioflocculants from marine microorganisms have high flocculating activities since they possess different morphology, physiology and metabolic adaptations to adverse environments in seas when compared to those found in fresh waters and terrestrial (He et al*.,* 2010). Thus, the study aimed at characterising and determining the flocculation mechanism of bioflocculant from marine *Bacillus pumilus* JX860616.

MATERIALS AND METHODS

Source and identification of bacterium

The bacterial strain was isolated from the sediment sample from Sodwana Bay in the province of Kwazulu-Natal in South Africa and was stored and maintained in 20% glycerol at -80°C at the Department of Biochemistry and Microbiology at the University of Zululand, South Africa. The growth medium for bioflocculant producing bacterium was nutrient agar (NB). The identification of bacterial strain was performed based on the 16 S rRNA gene nucleotide sequence.

Determination of bioflocculant production

The bacterial strain was inoculated into a 250 mL flask containing 50 mL of sterilized (115°C for 15 min) production medium prepared according to Zhang et al. (2007), and incubated at 30°C in a shaker at 160 rpm for 72 h. The pre-culture was centrifuged at 8000 × *g* for 30 min at 4°C to remove bacterial cells and flocculating activity (FA) determined.

Flocculating activity (FA)

The method adopted from Kurane et al. (1994) was used for determination of the flocculating activity. Hundred millilitre of Kaolin suspension (4 g/L) was measured into 250 mL flask and 3 ml of 1% w/v $CaCl_2$ and 2 mL of obtained cell free supernatant were added. The mixture was shaken for 1 min and then poured into 100 mL measuring cylinder. The sediment was allowed to stand for 5 min at room temperature as well as the control medium. The optical density (OD) of the clarifying solution was measured with ultra violet (UV) spectrophotometer at 550 nm and the flocculating activity was determined using the formula:

$$FA\ (\%) = [(A - B/A)] \times 100$$

Where, A is the optical density of control at 550 nm and B is optical density of a sample at 550 nm.

Optimisation of culture conditions for bioflocculant production

The optimum medium culture conditions for bioflocculant production were assessed. The inoculum sizes were varied (0.5 - 2.5 ml, v/v), representing (1-5%). Various carbon sources (xylose, glucose, sucrose, maltose, starch, galactose, lactose and molasses) and nitrogen sources (peptone, urea, yeast, ammonium sulphate, tryptone and casein) were utilized for determination of their effect on flocculating activity. Different cations (K^+, Na^+, Li^+, Ca^{2+}, Mn^{2+}, Ba^{2+} and Fe^{3+}) in volumes of 3 ml of 1% w/v were used in the place of $CaCl_2$ and flocculating activities determined. The rotary speeds (0- 220 rpm), initial pH of the culture medium (3 to 12), cultivation temperatures (20 to 50°C) were varied, respectively.

Time course assay

The bacterial strain was cultured under previously obtained optimal growth conditions. Samples were drawn every 12 h over a period of 96 h and of this, 2 ml was centrifuged (8000 *g*, 30 min), and the cell free supernatant was used to assess the flocculating activity. The optical density (OD 550 nm) and pH were also determined.

Extraction and partial purification of the bioflocculant

The concentration and purification of bioflocculant from the bioflocculant-rich broth was done in accordance with the methods of Chang et al, (1998). The culture broth was centrifuged at 8,000 × g at 4°C for 30 min to remove bacterial cells. Two volumes of ethanol were added to the supernatant. The solution was thoroughly agitated and left standing at 4°C for 12 h. The precipitate was freeze-dried to obtain crude bioflocculant and the crude product was dissolved in distilled water to yield a solution (1% w/v). 1 volume of a mixture of chloroform and methanol (2:1 v/v) was added, agitated and left standing at room temperature for 12 h. The supernatant was then centrifuged (8,000 × *g* for 30 min, 4°C) and freeze-dried in order to obtain a purified bioflocculant. Solubility assay of the purified bioflocculant was done by dissolving 0.05 g of bioflocculant in 2 ml of different solvents (water, acetone, chloroform, dichloromethane, benzene, hexane, ethyl acetate, ethanol, methanol and butanol).

Effect of bioflocculant concentration and cations on flocculation activity

The influence of bioflocculant concentration towards the flocculating activity was assayed by varying different concentrations of the bioflocculant (0.2 to 1 mg/Ml), as described previously (Zhang et al*.,* 2007). The effect of the following cations; KCl, NaCl, LiCl, $MnCl_2$, $BaCl_2$ and $FeCl_3$ (1% w/v) on flocculating activity of the purified bioflocculant was evaluated.

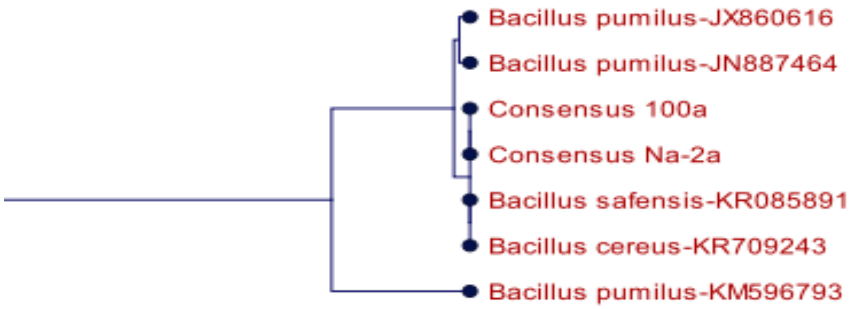

Figure 1. A phylogenetic tree showing the relationships among the selected strains and other related sequences collected from the Gene Bank.

Physicochemical composition of bioflocculant

The morphological surface of structures of the bioflocculant, kaolin particles and floc were examined under a scanning electron microscope (SEM-Sipma-VP-03-67) (Karthiga and Natarajan, 2015). Characterization of the electric charge of the bioflocculant was evaluated using Malvern Zetasizer Nano. The total sugar content was determined by phenol-sulphuric acid method described by Chaplin and Kennedy (1994). The qualitative analysis of proteins, nucleic acids and amino acids were determined by UV-vis spectrophotometry (Harrington and Raper, 1968) and Ninhydrin method (Kay et al., 1956), respectively. The quantitative analysis of the total protein content of the bioflocculant was determined by Bradford (1976). The elemental analysis was carried out with SEM (SEM-Sipma-VP-03-67) equipped with Oxford Instruments-X-Max^N. Prior to SEM analysis, 5 mg of bioflocculant, was added on slide coated with silicon and fixed by spin coater (1000 rpm, 60 s). The functional groups of the bioflocculant were determined by Fourier transform infrared (IR) spectroscopy.

Thermal, pH and salinity stability of the bioflocculant

The pH was varied in the range of 3.0 - 10 for assessment of pH stability of the bioflocculant and the bioflocculant was kept for 60 min in various temperatures (50-100°C) for determination of its thermal stability. The degradation temperature of the purified bioflocculant (10 mg) was studied using thermo-gravimetric instrument. The bioflocculant was heated from 22 to 900°C at a rate constant of 10°C under constant flow of nitrogen gas. The effect of salinity on bioflocculant on flocculating activity was determined by varying different concentrations of NaCl (5-35 g/L) in Kaolin solution (4 g/L).

Proposed flocculation mechanism

The zeta potential of the bioflocculant TMT^{-1}, Kaolin solution, Kaolin solution with $BaCl_2$, Kaolin solution with both $CaCl_2$ and bioflocculant TMT^{-1} were measured by a Zetasizer Nano so to determine flocculation mechanism of the bioflocculant (Aljuboori et al., 2015).

Statistical analysis

The mean and standard deviation mean of three experiments were determined. Data were subjected to one-way analysis of variance (ANOVA). P values ≤0.05 were regarded as significant and P values ≤ 0.01 as very significant.

RESULTS

Molecular identification of bacterium strain

A BLAST search against GenBank indicated that the 16S rRNA gene nucleotide sequence of species was similar to that of the *B. pumilus* JX860616. A phylogenetic tree (Figure 1) was constructed between it and similar sequences found in GenBank.

Optimization of cultivation conditions

Optimization of the cultivation process improves the production of bioproducts. Thus, the varying of different constituents of the culture medium and fermentation conditions resulted in improved flocculating action when compared to the flocculation activity prior to optimisation. *B. pumilus* JX860616 did demonstrate the potential flocculating activity of 64% in the original pre-culture medium and conditions.

Inoculum size

The results of the effect of inoculum size of *B. pumilus* JX860616 on flocculating activity are illustrated in Table 1. The observations showed that the inoculum size of 2% (v/v) was the best as it revealed the highest flocculating activity of 76.8%. An increase or decrease in the inoculum size did result in the slight decrease in flocculating activities.

Carbon and nitrogen sources

B. pumilus JX860616 utilized various carbon and nitrogen sources, 20 and 1.2 g/L, respectively, resulting in bioflocculant production. Glucose, lactose and sucrose were the most preferred carbon sources showing insignificantly different flocculating activities (Table 1). Lactose was gave the highest flocculating activity of 84.1±4.35%. Maltose and starch were poor carbon

Table 1. Effect of inoculum size of *B. pumilus* JX860616 on flocculating activity.

Inoculum size (% v/v)	FA (%)±SD	Carbon source	FA (%)±SD	Nitrogen source	FA (%)±SD	Cation	FA (%)±SD
1	56.5±5.84^{a}	Maltose	47.8±0.20^{a}	Casein	59.1±2.06^{a}	Control	58.2±9.47^{a}
2	76.8±4.03^{b}	Molasses	53.7±4.38^{a,b}	Urea	71.4±1.19^{a,c}	K^{+}	60.8±0.15^{a}
3	70.6±0.76^{b}	Fructose	61.8±0.21^{b,c}	Peptone	73.3±7.72^{a,b}	Na^{+}	60.6±0.32^{a}
4	70.6±6.41^{b}	Galactose	64.1±1.40^{c}	Yeast extract	74.4±3.40^{a,b}	Li^{+}	67.1±2.52^{a}
5	55.9±6.16^{a}	Starch	68.5±5.38^{c}	Tryptone	79.5±10.7^{b,c}	Mn^{2+}	69.9±4.43^{a,b}
		Glucose	83.7±3.12^{d}	$(NH_4)_2SO_4$	90.1±7.89^{b}	Ca^{2+}	79.2±2.54^{b}
		Sucrose	84.0±1.71^{d}			Ba^{2+}	79.6±3.47^{b}
		Lactose	84.1±4.35^{d}			Fe^{3+}	58.2±2.62^{a}

Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p<0.05$) different.

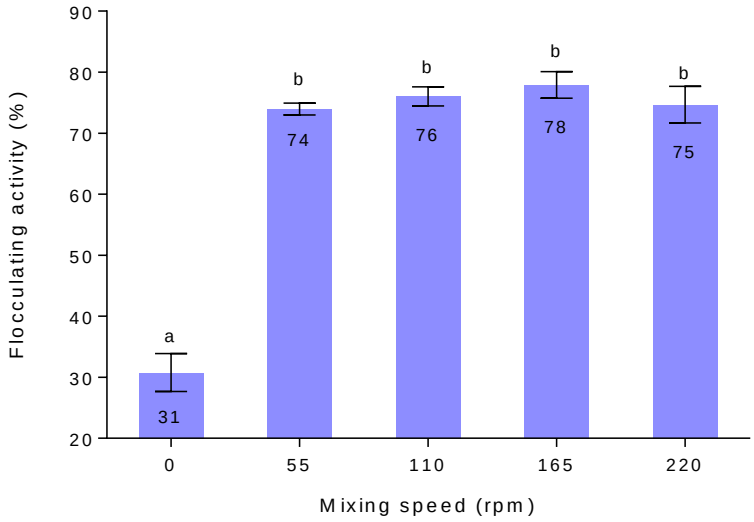

Figure 2. Effect of mixing speed on flocculating activity.

sources and gave 47.8±0.20 and 53.7±4.38% of flocculating activities, respectively. The effect of different nitrogen sources was assessed and the results were illustrated in Table 1. Ammonium sulphate was the best nitrogen source, with an outstanding flocculating activity of 90.1±7.89%. All other nitrogen sources used, (with the exception of casein, which showed the lowest flocculating activity (59.1±2.06%) showed the potential of been good nitrogen sources for bioflocculant production by *B. pumilus* JX860616, giving the flocculating activities above 70% (Table 1).

Cations effect on flocculation activity

Table 1 shows the effect of different metal cations on flocculating activity. It was clearly observed that Ba^{2+} greatly stimulated flocculating activity, resulting with the highest activity (79.6±3.47%). The monovalent (K^{+}, Na^{+} and Li^{+}) cations did also enhance flocculating activity, giving flocculating activities above 60%. However, the trivalent metal cation (Fe^{3+}) demonstrated the lowest flocculating activity of 58.2±2.62% (Table 1).

Effect of mixing speed

Figure 2 illustrates the effect of shaking speed on the bioflocculant production. The shaking speed of 165 rpm was the most conducive, giving the highest flocculating activity of 78%. The decrease in flocculating activity was observed when the shaking speed was below or above 165 rpm.

Initial pH

The effect of the initial pH of the growth medium on flocculating activity was assessed and the result presented in Figure 3. The flocculating activity was

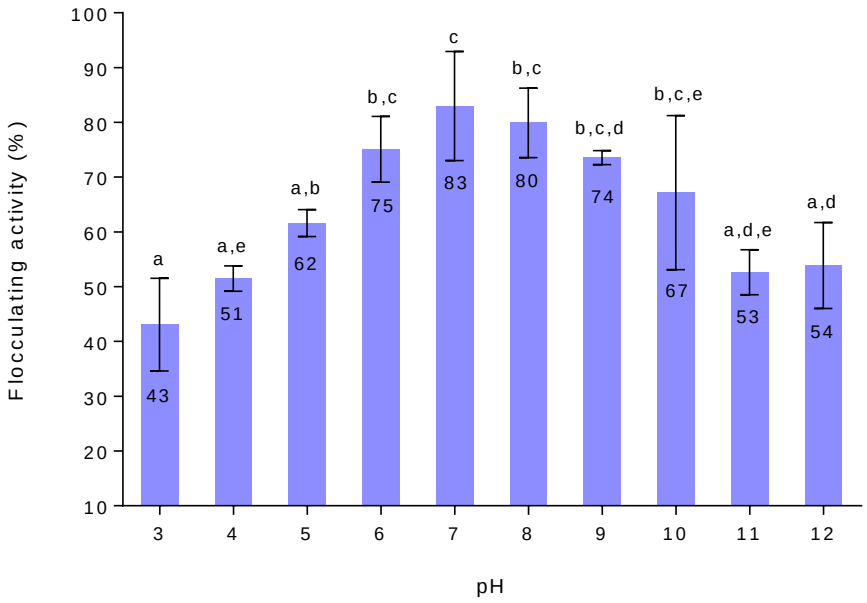

Figure 3. Effect of initial pH on bioflocculant production.

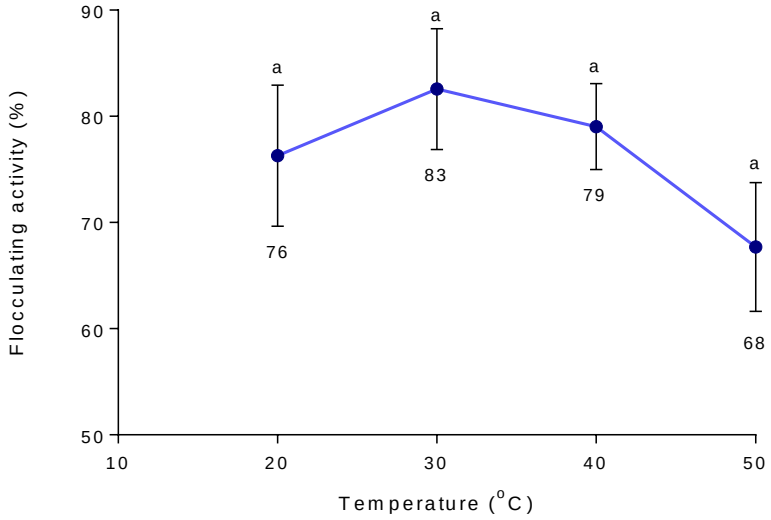

Figure 4. Effect of different temperature regime on flocculating activity.

highest (83%) when the initial pH was 7. The observations also showed that *B. pumilus* JX860616 has a potential to survive and produce bioflocculant in the range of the weak acid and alkaline (pH 6-9), as it gave the flocculating activities above 70%. pH 3 demostrated the lowest flocculating activity of 43%.

Temperature effect on flocculation activity

Temperature is an environmental factor that greatly affects bioflocculant production. 30ºC was the optimum temperature for bioflocculant production and yielded 83% of flocculating activity as illustrated in Figure 4. The decline in flocculating activity was observed when the temperature was altered above or below 30ºC.

Time course

The effect of time course on flocculating activity (FA), bacterium growth and pH are shown in Figure 5. The flocculating activity increased relatively to the *B. pumilus* JX860616 growth until 60 h, where the maximum of

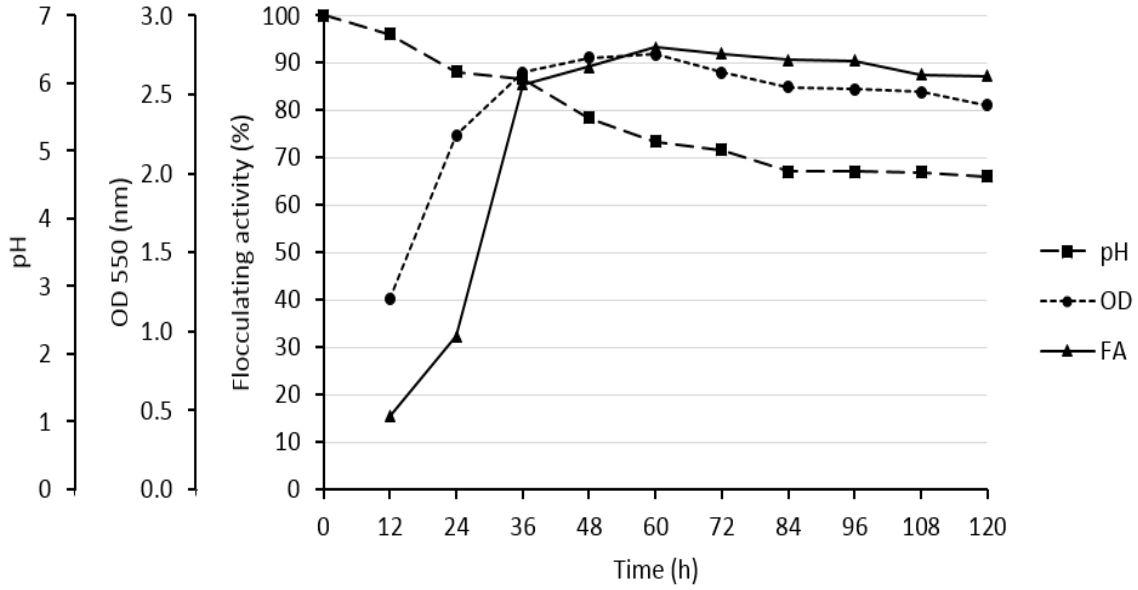

Figure 5. The graph of time course includes growth curve, pH and flocculating activity.

Table 2. Effect of different concentrations of bioflocculant TMT^{-1}.

Dosage (ml)	FA (%)±SD	Cation	FA (%)±SD
0.2	$81.73±1.88^{a,b}$	Control	$66.87±2.34^{a}$
0.4	$82.0±0.74^{a,b}$	Li^{+}	$51.57±1.72^{b}$
0.6	$82.77±1.0^{a}$	Na^{+}	$95.47±0.42^{c}$
0.8	$78.90±0.53^{b}$	K^{+}	$96.07±0.35^{c}$
1	$79.20±1.92^{a,b}$	Mn^{2+}	$83.27±1.19^{d}$
		Ba^{2+}	$95.23±1.54^{c}$
		Fe^{3+}	$80.63±1.39^{d}$

Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p<0.05$) different.

flocculation activity (93.3%) was obtained. After 60 h interval, the bacterium got into stationary phase and the flocculation activity decreased slightly. On the other hand, the pH of the medium dropped uniformly from the initial pH of 7 to the final pH of 4.7.

Bioflocculant yield

One thousand millilitres of fermented broth culture yielded 2.4 g of the purified bioflocculant TMT^{-1}. The bioflocculant was insoluble in all solvents (acetone, methanol, butanol, ethanol, hexane, benzene, chloroform, ethyl-acetate and dichloromethane), with the exception of water, of which it dissolved (Table 2).

Bioflocculant concentration and cations effects on flocculation activity

Table 2 illustrates the effect of different concentrations of bioflocculant TMT^{-1}. TMT^{-1} had the highest flocculation activity (82.77%) at the concentration (0.6 mg/ml). However, 0.2 mg/ml was the most preferred dosage size since it showed high flocculating activity (81.73±1.88). Moreover, there was no statistical difference shown between the high concentration of 0.6 and 0.2 mg/ml. An increase or decrease in the TMT^{-1} concentrations resulted in the slight decrease in flocculating activities. The effect of various metal cations on the purified bioflocculant TMT^{-1} was determined. All monovalent cations (with the exception of Li^{+}) and divalent cations significantly enhanced flocculation activities, giving flocculation activities above 80% (Table 2). Ba^{2+} was the most preferred metal cation with the highest flocculation activity of 95.23±1.54%, while Li^{+} showed the lowest flocculating activity of 51.57±1.72%.

Physicochemical composition of bioflocculant

Figure 6 shows the SEM surface images of the purified

bioflocculant, flocculated Kaolin particles and Kaolin particles. TMT^{-1} had a crystal-like structure (Figure 6a). The clump like structure was observed in the flocculated Kaolin particles (Figure 6b) and Kaolin particles revealed a fine and smooth structure (Figure 6c). The surface charge of TMT^{-1} was determined and the results illustrated in Figure 7. The zeta potential of TMT^{-1} (-11.6 mV) revealed the bioflocculant as an anionic biomolecule.

Chemical composition of bioflocculant TMT^{-1}

TMT^{-1} was Ninhydrin positive. The UV-vis spectrum of TMT^{-1} revealed a sharp peak at 290 nm that characterised protein content (Figure 8). The quantitative chemical analysis of the bioflocculant TMT^{-1} was carried out and the main chemical constituents revealed were poly-saccharide (93.1%) and trace protein content (6.0%) (Table 3).

Elemental and infra-red (IR) analysis

The elemental analysis of the bioflocculant revealed its elemental composition in mass proportion (% w/t): C (17.0), N (0.0), O (46.0), Na (4.3), Mg (6.8), P (4.1), S (7.0), Cl (5.9), K (7.4) and Ca (0.7) (Figure 9). The functional groups of TMT^{-1} were determined and the results illustrated in Figure 10. The IR spectrophotometric analysis of TMT^{-1} showed the occurrence of hydroxyl, vinyl, aliphatic amine and amide groups.

Thermal, pH and salinity stability of TMT^{-1}

Figure 11 shows the effect of temperature on flocculating activity of the purified bioflocculant TMT^{-1}. TMT^{-1} was heat stable showing flocculating activity of 79% at 100°C after an hour of heat exposure. The pyrolysis property of TMT^{-1} was also studied and the results illustrated in Figure 12. The degradation temperature (*T*d) of 100°C was observed and it validated the thermal stability of TMT^{-1}.

The effect of pH on flocculating activity of the purified bioflocculant TMT^{-1} was assessed and the results are presented in Figure 13. TMT^{-1} was more stable in a wide range of pH (pH 3.0-10), giving flocculation activities above 70%. TMT^{-1} effectively flocculated Kaolin particles and gave the maximum flocculation activity (81%) at pH 6.0.

Figure 14 represented the results obtained during the determination of the effect on Na^+ concentration on the flocculating activity of bioflocculant TMT^{-1}. The flocculating activity of the bioflocculant decreased proportionally with the increase in Na^+ concentration. However, TMT^{-1} did maintain high flocculation activity (72%) even at high salinity (35 g/L).

Proposed flocculation mechanism of TMT^{-1}

Table 4 presents the zeta potential of the bioflocculant, Kaolin suspension, Kaolin plus cation and Kaolin suspension flocculated with $BaCl_2$ and TMT^{-1}. The electrical charges of the bioflocculant and Kaolin particles were both negative (-11.6 and -9.24, respectively) and the addition of Ba^{++} to Kaolin suspension and Kaolin suspension plus bioflocculant TMT^{-1} resulted in reduction on zeta-potential (-6.96 and -2.09 mV, respectively).

DISCUSSION

Marine *Bacillus* spp. has been good bioflocculant producers (Okaiyeto et al*.,* 2015). However, rarely have these bacterial strains isolated from nature produce bioflocculants at sufficiently high concentrations enough for commercialisation (Martinko and Madigan, 2006). It was observed that the identified marine *B. pumilus* JX860616 was capable of producing bioflocculant but at low flocculant activity (64%) at natural conditions. This was due to the fact that the bioflocculant is often not essential for the bacterial growth and reproductive metabolism. Thus, optimisation of cultivation process was done so to improve bioflocculant production and flocculation activity.

The quality and quantity of seed culture affects the growth kinetics of cultured bacteria and bioflocculant synthesis (Scheper et al*.,* 2003). Young and fresh cultures have active viable cells that easily grow and produce bioflocculants, while small inoculum sizes prolong the lag phase time of bioflocculant production and a large inoculum sizes decrease the amount of bioflocculant produced. Thus, the smaller the inoculum size, the more appreciated. Inoculum size of 2% (v/v) (Table 1) was optimal for bioflocculant production by *B. pumilus* JX860616. The inoculum size of 2% (v/v) was better in comparison to the optimum inoculum size of 4% (v/v) reported for *Bacillus licheniformis* (He et al*.,* 2010).

Bacterial strains utilize nutrients in fermentation medium to synthesise bioflocculants and for energy used during metabolic processes for cell growth and reproduction. The bulk of net energy–yielding or energy consuming metabolic reactions in bacteria involves change in the oxidation state of carbon and nitrogen elements in substrates (Manahan, 2005). Lactose, glucose and sucrose were evidently the most effective carbon sources resulting in flocculating activity above 80%. The cost of carbon source strongly accounts for its selection (Scheper et al*.,* 2003). The flocculation activity activated by glucose had insignificant statistical difference with that of lactose and sucrose (Table 1) and thus, glucose was used during the experiment due to its availability at low market prices. Although molasses is an industrial by-product of economic insignificant (Smith, 2009), it demonstrated a very low flocculating activity

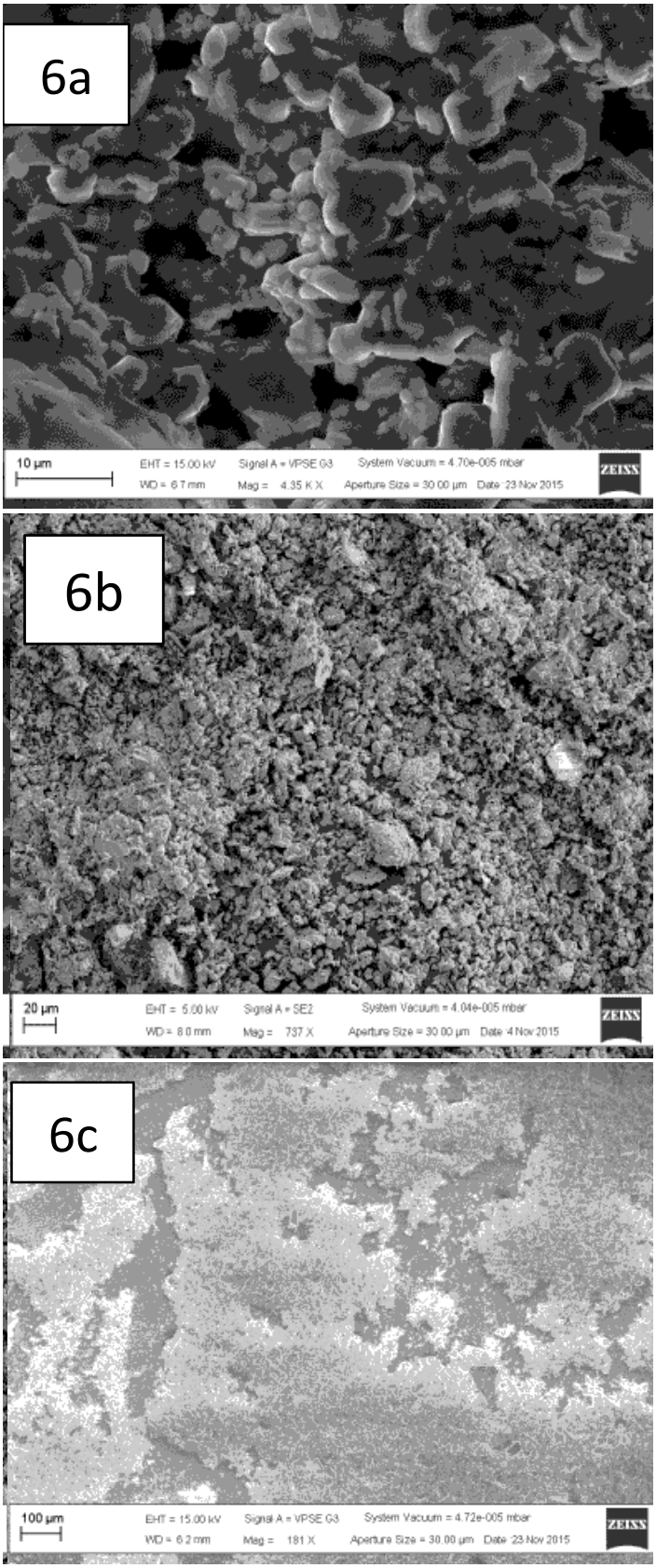

Figure 6. SEM surface images of the (a) bioflocculant (b) flocculated Kaolin particles and (c) Kaolin particles.

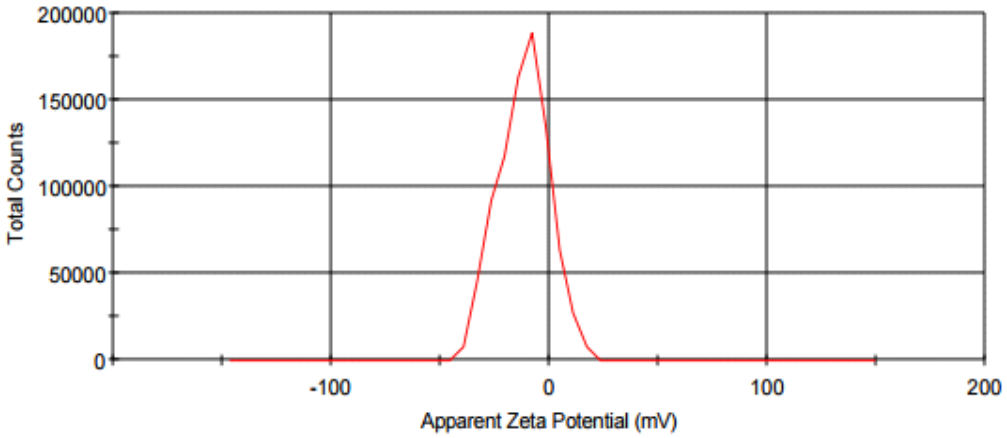

Figure 7. Zeta potential of the purified TMT^{-1}.

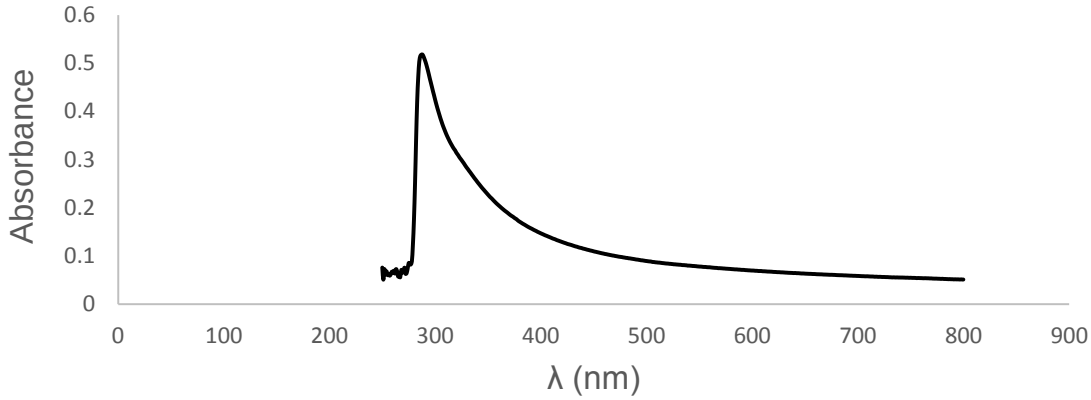

Figure 8. UV-vis spectrum of TMT^{-1}.

Table 3. Chemical components of the purified bioflocculant TMT^{-1}.

Chemical component	Concentration (% w/w)
Protein	6.0
Carbohydrate	93.1

(53.7±4.38%) and thus, was neglected to be used as a carbon source. Maximum flocculating activity of 90.1±7.89% was also obtained when $(NH_4)_2SO_4$ was used as a nitrogen source (Table 1). The observations were in agreement with those of Okaiyeto et al. (2016), whereby ammonium sulphate promoted the growth of marine *Bacillus* sp. and bioflocculant production, resulting with the flocculating activity of 79.89±6.65%.

Flocculating activity of the bioflocculant was stimulated by different cations (Table 1). Ba^{2+} greatly promoted flocculating activity, resulting in the highest flocculating activity of 79.6±3.47%. Generally, divalent cations (Mn^{2+}, Ca^{2+} and Ba^{2+}) were the most favourable cations showing flocculating activities above 70%. Monovalent and trivalent cations slightly inhibited flocculation activity of the bioflocculant and the Fe^{3+} showed the least flocculating activity (58.2±2.62%). Arafa et al. (2014), also obtained similar observations, whereby the flocculanting activities of different bacterial strains were greatly enhanced by divalent (Mn^{2+} and Ca^{2+}) and trivalent (Fe^{3+}) cations.

The effect of shaking speed on the bioflocculant production revealed that the shaking speed of 165 rpm was the most conducive, giving the highest flocculating activity (78%) (Figure 2). Any change in mixing speed led to the decrease in flocculating activity. There was a decrease in flocculating activity when the shaking speed was above or below 165 rpm. This was perhaps due to the denaturation of the bioflocculant by the mechanical damage as some cells are highly fragile (Smith, 2009). Shaking speed determines the concentration of the dissolved oxygen, which can influence the biochemical absorption of the nutrients and enzymatic activity of

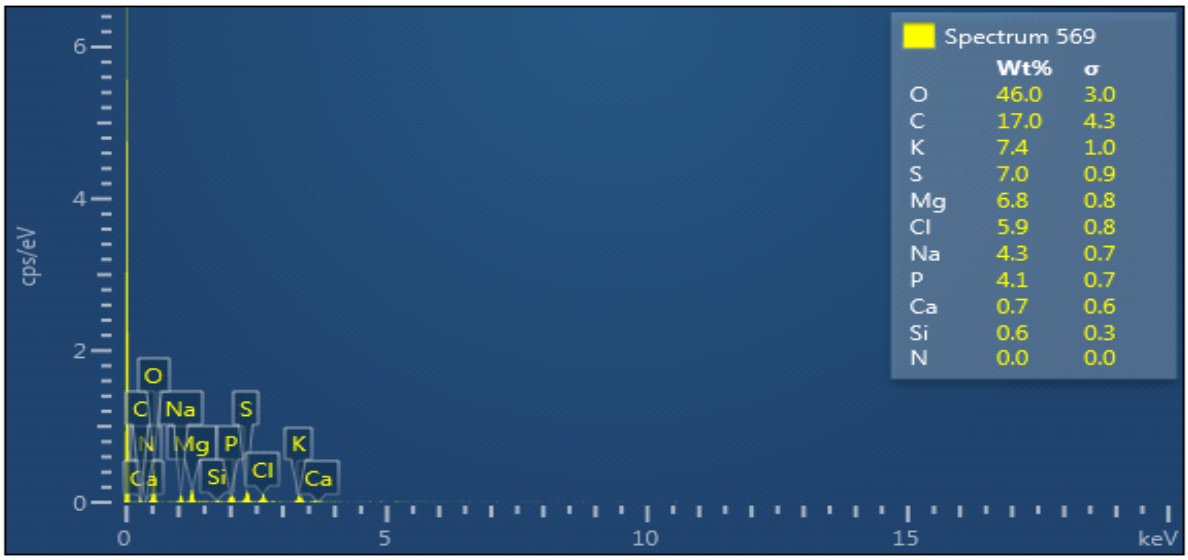

Figure 9. Elemental analysis of TMT^{-1}.

bacterium (Adebayo-Tayo and Adebami, 2014). Moreover, optimum shaking speed allows evenly distribution of nutrients and proper mixing of growing cells (Garg et al*.,* 2011). Presumably at 165 rpm, there was an enhanced supply of dissolved oxygen, uptake of nutrients to the bacterium cells and bioflocculant production (Smith, 2009). The results were in close conformity with those reported by Piyo et al. (2011), whereby the best flocculation was obtained at 160 rpm for *Bacillus* sp. Gilbert.

The catalytic activity of enzymes relies on the specific state of ionisation imposed on its functional groups by pH alterations (Walker and Wilson, 2005). Thus, pH determines the electric charge of the bacterial cells and the oxidation-reduction potential which in turn can directly affect adsorption of nutrients and enzymatic reactions (Xia et al., 2008). According to Wong et al*.* (2012), pH alterations can result in denaturation of enzymes and proteins, disturb the pumping of ions at the bacterial cell membrane and affect biosynthesis of bioflocculant. The optimal initial pH of the culture medium of *B. pumilus* JX860616 oscillates in a range of pH 6 to 9, with pH 7 giving the highest flocculating activity (83%) (Figure 3). Sathiyanarayanan et al. (2013) reported similar results that the maximum flocculating activity was highest when the pH was adjusted to 7.

Temperature affects microbial growth and bioflocculant production in two ways. Firstly, as temperatures rise, chemical and enzymatic reactions in cells doubles up for every 10°C increase due to the increase in velocity and kinetic energy (Atlas, 1995). The bacterial growth and bioflocculant production become faster up to a point where denaturalisation is established and the enzymes are inactivated as they lose their functional structures. Secondly, low temperatures restrict the rate of chemical and enzymatic reactions in cells. Bacterial cells tend to be metabolically inactive at lower culture temperatures and this often causes bacterial strains to hibernate and cease activation of bioflocculant production (Walker and Wilson, 2005). The optimal cultivation temperature for the production of the bioflocculant was 30°C, with the highest flocculating activity of 83% (Figure 4). The temperature below or above 30°C led to reduction in the flocculating activities. Temperature above 30°C may have had an adverse effect on the nucleic acid and enzyme system of *B. pumilus* JX860616*,* consequently leading to low bioflocculant production while temperatures below 30°C propelled the strain to hibernate and cease to produce the bioflocculant. The results were in agreement with those of Elkady et al. (2011), whereby *Bacillus mojavensis* exhibited approximately 96.1% of flocculating activity when its temperature was adjusted to 30°C during bioflocculant production.

The bacterial growth and flocculating activity were in harmony up for the first 60 h of fermentation (Figure 6). The OD curve representing the cell growth showed a sharp increase in the first 60 h and slight decline over the remaining fermentation period. The flocculating activity also increased continuously until it reached the maximum (93.3% after 60 h), indicating that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis (Lu et al*.,* 2005). The results were similar to those of Okaiyeto et al*.* (2015), in which the bioflocculant synthesis was synchronous with the bacterial growth and reached maximum flocculating activity in the late growth phase and early stationary phase. In comparison, Arafa et al*.* (2014) revealed that the bioflocculant from *B. cereus* exhibited flocculating activity of over 90% after 72 h. The selection of bioflocculant producers consider the time interval for bioflocculant production by bacteria and

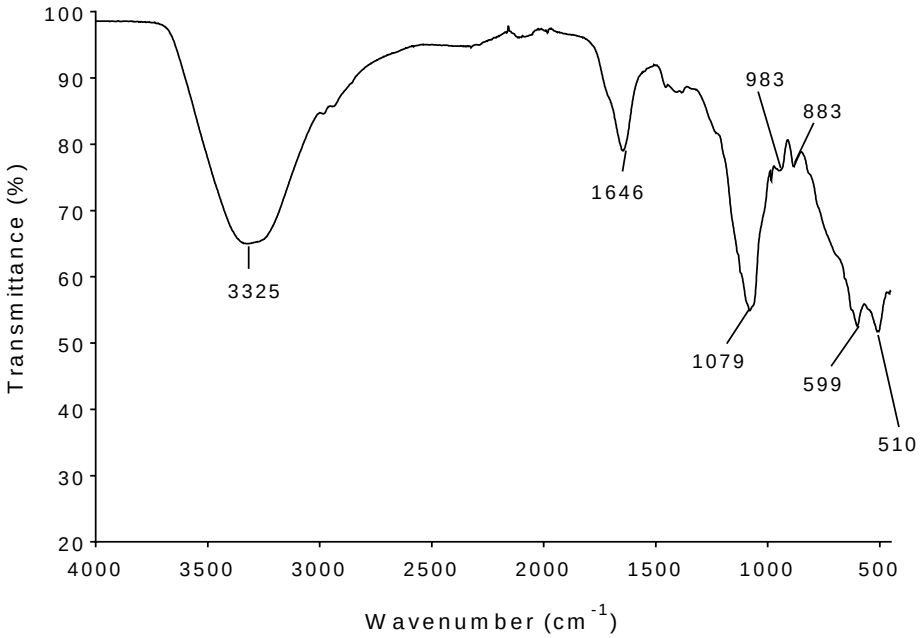

Figure 10. IR spectrum of TMT^{-1}.

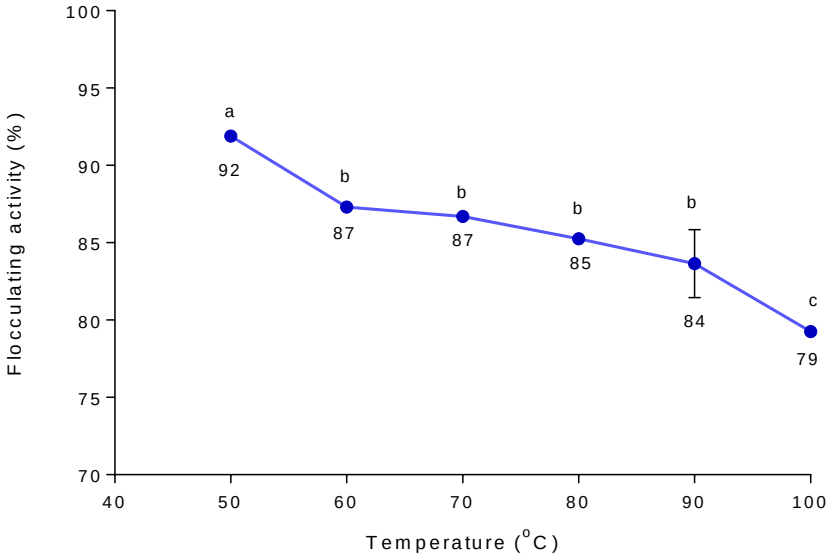

Figure 11. Effect of temperature on TMT^{-1} flocculating activity.

on short time intervals are the most preferred industrially, as they are cost effective and less time consuming (Scheper et al., 2003). Thus, the bioflocculant production by *B. pumilus* JX860616 can be viewed as an economically friendly since it gave high flocculating activity (93.3%) within a short fermentation period. The pH of the medium generally dropped from the initial pH of 7 to pH 4.99. The drop in pH might have been due to the organic acidic components of the bioflocculant polymer (Okaiyeto et al., 2013).

Yield (amount of product) and productivity (rate of product formation) are the good quantitative analysis that indicates how bacterial cells convert components in culture medium into bioflocculants (Smith, 2009). *B. pumilus* JX860616 produced about 2.4 g of the purified bioflocculnat from 1 L of fermentation broth within 60 h.

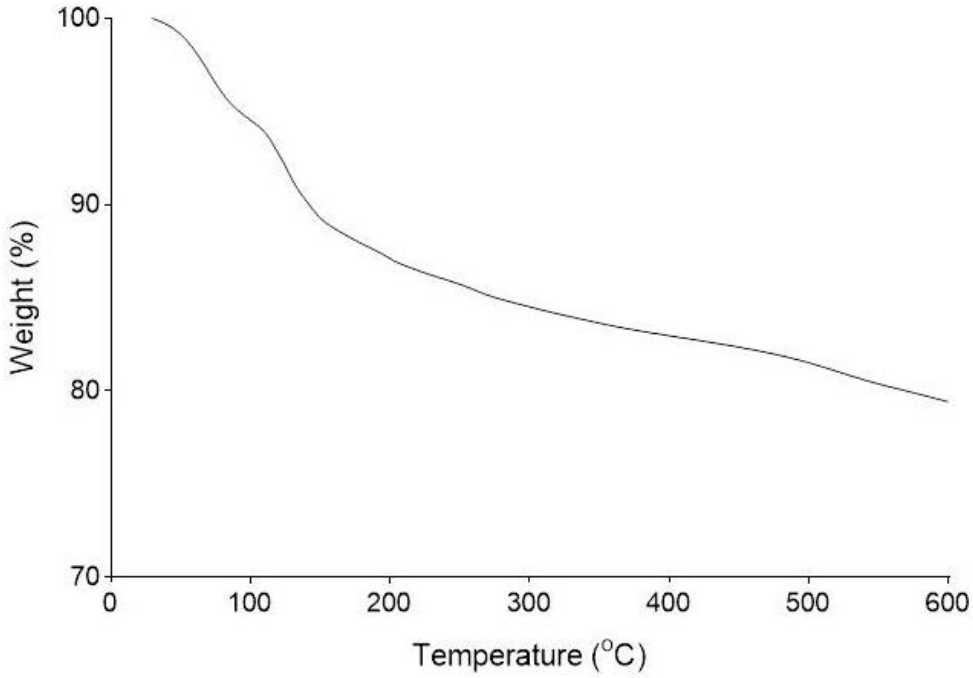

Figure 12. Thermal gravity stability of TMT^{-1}.

The high yield might be owed to the ability of *B. pumilus* JX860616 to yield bioflocculant under stress conditions and the polarity of the solvents used. The high bioflocculant yield recovered is of economic importance. Though it was higher than most of bioflocculant by pure cultures (Lin and Harrichurd, 2011), it was much lower than the highest ever reported bioflocculant production (4 g/L) from pure bacterial species (Zhang et al*.,* 2007).

Bioflocculant biomolecules differ in the balance of charged, polar and hydrophobic components they possess on their surface. The bioflocculant was found to be soluble in water but insoluble in all tested organic solvents (acetone, methanol, butanol, ethanol, hexane, benzene, chloroform ethyl-acetate and dichloromethane). Jorand et al. (1998) reported that hydrophobic bioflocculants are mainly comprised of proteins, whereas the hydrophilic fraction mainly consisted of carbohydrates. TMT^{-1} is predominately a carbohydrate bioflocculant (Table 3). Thus, the charged and polar groups were solvated by aqueous molecules, thus making bioflocculant soluble and hydrophilic (Walker and Wilson, 2005). The abundance of hydroxyl groups (Figure 10) builds up strong forces of attraction between bioflocculant molecules, and may result in relatively hard crystalline solids – where hydrogen bonding can occur. Since these forces are not dissociable by organic solvents, the TMT^{-1} was insoluble in all organic solvents used. The solvation of TMT^{-1} in aqueous solution was attributed to the presence of hydroxyl group creating a hydrogen bond with a water molecule (Okaiyeto et al*.*, 2015).

Table 2 illustrates the effect of different concentrations of bioflocculant TMT^{-1}. TMT^{-1} had the highest flocculation activity (82.77%) at the concentration (0.6 mg/ml). However, 0.2 mg/ml was the most preferred dosage size since it showed high flocculating activity (81.73±1.88). Moreover, there was no statistical difference shown between the high concentration of 0.6 mg/ml and 0.2 mg/ml. Thus, the increase in dosage size from 0.2 to 0.6 mg/ml did not affect the flocculation activity of the bioflocculant. Flocculation often cease once the dosage concentrations exceed the absorption of excess negatively charged bioflocculant and destabilise Kaolin particles, thus the attractive forces of other Kaolin particles are reduced as strong repulsive forces set in and consequently, flocculation activity decreases (Okaiyeto et al*.*, 2013). Thus, the flocculation activity slightly decreased when the dosage concentration was increased further. The results are consistent with those reported by Okaiyeto et al*.* (2015). On the other hand, at low dosages concentrations (<0.6 mg/ml), bridging flocculation mechanism of the bioflocculant was not effectively enhanced as most Kaolin particles did not have enough bioflocculant molecules to bind. A bioflocculant from *Azotobacter indicus* had an optimum dosage concentration of 500 mg/l (Patil et al*.,* 2011), and this implied that TMT^{-1} is active and economical.

Cations neutralize and stabilize the negative charge of the functional groups of colloidal Kaolin particles in solution and the bioflocculant (He et al*.,* 2010). Table 2 shows that all the monovalent (with exception of Li^{+}), divalent and trivalent cations neutralized the negative

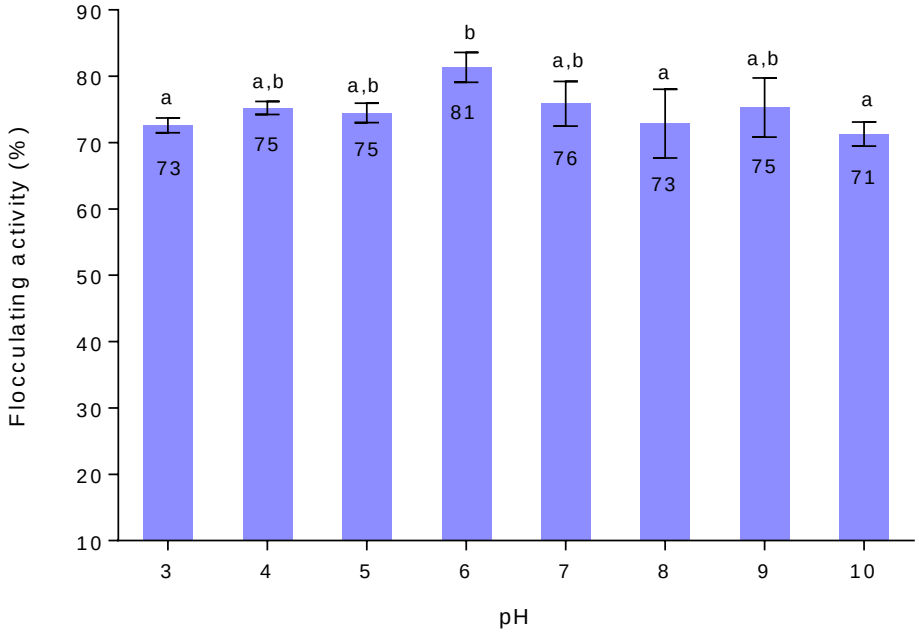

Figure 13. pH stability of bioflocculant TMT^{-1}.

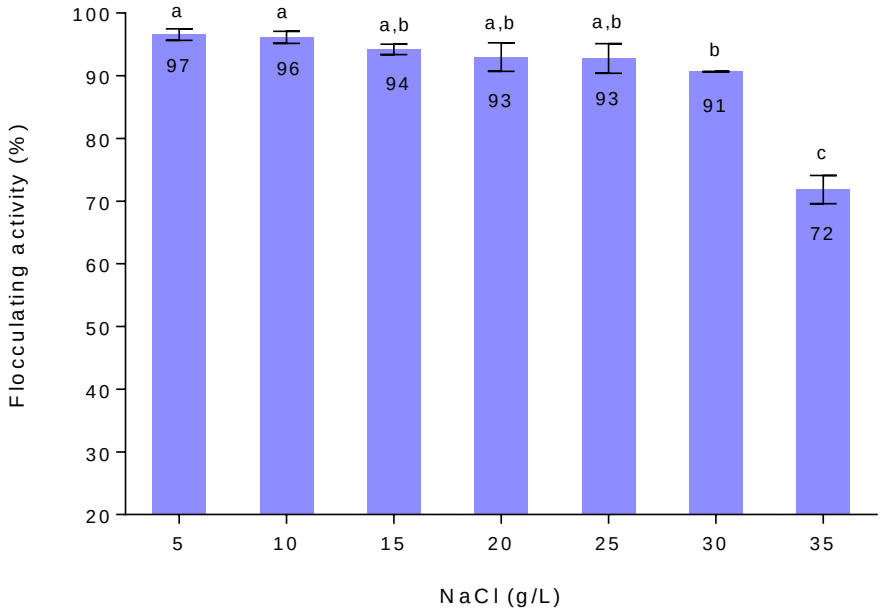

Figure 14. Effect on salinity on flocculating activity of TMT^{-1}.

Table 4. Zeta potential of different samples.

Sample	Zeta potential (mV)
Bioflocculant TMT^{-1}	-11.6
Kaolin particles	-9.24
Kaolin particles with Ba^{++}	-6.96
Kaolin particles flocculated with $BaCl_2$ and TMT^{-1}	-2.09

charges on TMT^{-1} and the colloidal Kaolin particles in solution, reducing the distance between them, increasing the initial adsorption of TMT^{-1} onto the colloidal Kaolin particles and thus leading to settleable flocs and high flocculating activities above 80%. Ba^{2+} showed an outstanding flocculating activity of 95.23±1.54% and thus, was the most preferred cation. The results were similar to the findings by Ma et al. (2015) and Okaiyeto et al. (2015), whereby the flocculation activities of CBF and MBF-UFH were greatly enhanced by addition of divalent (Ca^{2+}, Ma^{2+} and Fe^{2+}), trivalent (Al^{3+} and Fe^{3+}) and the monovalent (Na^{+}, K^{+} and Li^{+}) cations.

Figure 6 demonstrates the SEM image of the bioflocculant, flocculated Kaolin particles and Kaolin particles. Bioflocculant TMT^{-1} was whitish in colour and had a crystal-like shape (Figure 6a). The configuration of TMT^{-1} might be accountable for its flocculation activity. The floc appeared clustered (Figure 6b). This was perceived to be due to the bonds between colloidal particles of Kaolin and the functional groups of TMT^{-1}. The Kaolin clay particles appeared to be fine and evenly scattered (Figure 6c). Figure 7 shows the zeta potential TMT^{-1} as a negatively charged biomolecule with the value of −11.6 mV, implying that the nature of bioflocculant TMT^{-1} is anionic. The net negative charge of the TMT^{-1} was concluded to be from the carbohydrates and proteins contents, which normally have the negatively charged functional groups (Liu et al., 2015). Ninhydrin test is the distinguishing test for all amino acids except proline (Chakravarty, 2013). The intense purple colour results due to reaction between the alpha amino group and Ninhydrin molecules. The bioflocculant was Ninhydrin positive, implying to possess free amino acids. The UV-vis spectrum revealed a sharp absorption peak at 290 nm (Figure 8). This confirmed the presence of the protein content in the bioflocculant. On the other hand, the absence of the absorption peak at 154 nm implied that the bioflocculant had no nucleic acid content. TMT^{-1} revealed high content of carbohydrate (93.1%) and negligible protein content (6.0%) (Table 3). The composition of both carbohydrates and proteins implied that the bioflocculant might have multiple functional moieties. Multiple functional moieties are indicative for many adsorption sites for the colloidal particles and this can lead to high flocculating efficiency as observed with TMT^{-1} on Kaolin particles (Verma et al., 2012). The carbohydrate derivatives in TMT^{-1} were presumed to be the most responsible components for the experientially high flocculating activity as TMT^{-1} is predominately carbohydrate and not protein. The elemental analysis of the bioflocculant revealed its elemental composition in mass proportion (% w/t) (Figure 9). The presence of various elements may have brought about the flexibility and stability of the TMT^{-1}. Okaiyeto et al. (2015), obtained almost similar result with bioflocculant MBF-UFH which possessed different in mass proportion (% w/w): C (17.21), O (40.04), Na (5.21), Mg (5.02), P (7.90), S (0.60), Cl (6.11), K (1.63) and Ca (9.63). Moreover, the absence of the detectable nitrogen element and abundance of carbon and oxygen elements affirmed the bioflocculant to be mostly of carbohydrate nature.

The flocculating activity of bioflocculants, stability and flexibility depend on their functional groups (Rao et al., 2013). The IR spectrum (Figure 10), displayed a broad stretching band with a peak at 3325 cm^{-1}; the broad peak was indicative of hydroxyl (OH) group. The sharp absorption peaks at 1646 and 1079 cm^{-1} advocated for the presence of amide and aliphatic amine groups (C=O and C-N, respectively). There was strong C-H at 983.05 and 883.39 cm^{-1} which represented the existence of vinyl group; while the peaks at 599.14 and 509.5 cm^{-1} revealed the presence of halo compounds (and C-Cl and C-Br, respectively). In overall, TMT^{-1} possess hydroxyl, amide, vinyl and aliphatic amino groups. These functional groups served as binding sites for cations and colloidal particles in solution, resulting in formation of diverse chemical bonds (Okaiyeto et al., 2015). The COOH, COO^{-} and OH groups on TMT^{-1} and groups (H^{+} and OH^{-}) on the surface of colloidal particles may interact with the bioflocculant chains to form hydrogen bonds, which permit the build-up of big flocs.

Bioflocculants have different stability as they are sensitive to heat. The bioflocculant with high protein content are generally thermally labile as compared to those rich in carbohydrate content, which are heat stable. When the major component of bioflocculant is glycoprotein, the stability of the bioflocculant depends on the relative contents of protein and carbohydrates (Walker and Wilson, 2005).

Figure 11 illustrates that heat had little impact on the physico-chemical properties of TMT^{-1}. Apparently, there was a slight decline in flocculating activity with time of heat exposure and this may be accredited to the denaturation of the protein component in TMT^{-1} (Okoh and Cosa, 2014). However, it was deduced that TMT^{-1} is a thermostable bioflocculant, as it retained high flocculating activity (81.0%) even at high temperature (100°C). The thermal-stability might be owed to the high content of carbohydrates. Figure 12 illustrated the pyrolysis property of TMT^{-1} after its exposure to high temperatures using thermogravimetric equipment. There was a decrease in weight between 35 and 157°C. The weight loss of 12% could be due to the loss of moisture content in the bioflocculant. The moisture content in TMT^{-1} is owed to the presence of carboxyl (in amide) and hydroxyl groups. High carboxyl content leads to greater affinity of the carbohydrates derivatives for water molecules (Kumar and Anand, 1998). The decline in weights above 157°C is attributed to the degradation of the bioflocculant. The TGA results validated the thermal stability of TMT^{-1}.

pH influences flocculating activity by affecting the stability of suspended particles and floc formation (Okoh et al., 2014). Figure 13 illustrates the effect of pH on the

flocculation activity of TMT^{-1}. The results vividly show that TMT^{-1}, within a wide pH range of 3-12, resulted in flocculation activity above 70%. This implied that TMT^{-1} can be effectively applied in acidic, neutral and alkaline environments (Adebayo-Tayo and Adebami, 2014). The highest activity (81%) was obtained at pH 6. These observations were similar to those of Zhang et al. (2007), whereby the flocculating activity of the obtained purified bioflocculant was highest at pH 6.

High salt concentrations have tendency to denature bioflocculants, thus affect their flocculation activities (Atlas and Bartha, 1987). High concentrations of salt interfere with charges on bioflocculants and promote loss of functional structures. Figure 14 illustrates that there was a constant decrease in flocculation activities with an increase in Na^+ concentrations. At higher concentration (35 g/L), the flocculating activity dropped drastically, implying that there was denaturation of some components of TMT^{-1}. Nevertheless, it was concluded that TMT^{-1} has salinity stability as it did maintain high flocculation activity (72%) even at the highest concentration of Na^+ (35 g/L). The saline stability of TMT^{-1} maybe due to the fact that the bioflocculant was produced by marine bacterial isolate, which survived in marine environment which is characterised by high salinity (3.5%, 35 g/L) (Abraham and Marteel-Parrish, 2014).

The flocculation process by bioflocculants mostly involves charge neutralization and bridging mechanisms (Lian et al., 2008). Charge neutralization occurs when the both particles in solution are charged oppositely while bridging mechanism occurs when the bioflocculants extend from the particle's surface into the solution for a distance greater than the distance over which the interparticle repulsion acts. The latter mostly happens where both the bioflocculant and the colloidal particles carry the same charge. The zeta potential of the bioflocculant and Kaolin particles were both negative (-11.6 and -9.24 mV, respectively) (Table 4). Addition of Ba^{2+} to Kaolin suspension and Kaolin suspension plus bioflocculant TMT^{-1} resulted in reduction of zeta-potential (-6.96 and -2.09 mV, respectively). When the negative charge is reduced or totally abolished, the repulsion force become terminated and particles easily agglomerate (Hadgson et al., 2004). Ba^{2+} increased the adsorption of bioflocculant TMT^{-1} on the surface of colloidal Kaolin particles by decreasing the negative charge on TMT^{-1} and Kaolin particles. Thus, the attractive forces were capable of weakening and overcoming the electrostatic repulsion force, reducing the distance between them by compressing the double layer of Kaolin particles while increasing the adsorption of TMT^{-1} on the colloidal Kaolin particles. Flocculation is speedy when the zeta potential is below 20 mV (Mines, 2014). It was concluded that Ba^{2+} stimulated rapid flocculation by neutralization and stabilization of residual negative charge of TMT^{-1}, forming a bridge that binds Kaolin particles to each other as the big flocs were initiated.

Conclusion

B. pumilus JX860616 produced bioflocculant TMT^{-1} (2.4 g/L) when glucose and inorganic ammonium sulphate were used as energy sources in the optimum fermentation conditions (initial pH 6, 30°C, 165 rpm and 60 h). The characterization results of bioflocculant TMT^{-1} proved that the bioflocculant was anionic glycoprotein that was porous in structure and soluble only in water. TMT^{-1} demonstrated salinity, pH and thermal stability. Ba^{2+} stimulated rapid flocculation by neutralization and stabilization of residual negative charge of TMT^{-1}, forming a bridge that binds Kaolin particles to each. The revealed properties of TMT^{-1} suggested its potential in industrial applicability. For further studies, the bioflocculant TMT^{-1} will be applied on wastewater treatment and the molecular methods will also be employed on the bacterium strain so to increase the TMT^{-1} yield.

Conflicts of Interests

The authors have not declared any conflict of interests.

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