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Characterization and flocculation properties of a carbohydrate bioflocculant from a newly isolated *Bacillus velezensis* 40B

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Abstract

In this study, a bioflocculant with a high flocculation activity (>98%) produced by strain 40B, which was isolated from a brackish water was investigated. By 16S rDNA sequence analysis, strain 40B was identified as *Bacillus velezensis*. Chemical analysis of the bioflocculant 40B indicated that it contained 2% protein and 98% carbohydrates. FTIR analysis showed the presence of carboxyl, hydroxyl and amino groups, which were preferred for the flocculation process. The optimal concentration for the flocculation activity was 3.5 mg l^{-1}. This polysaccharide could also flocculate kaolin suspension over a wide range of pH (1–10) and temperature (5–85 °C) in the presence of $CaCl_2$. The stability of the bioflocculant 40B under various conditions suggests its possible use in the industries and environmental applications. However, no previous report exists on the isolation and characterization of a bioflocculant from the *Bacillus velezensis*.

Key words

Bacillus velezensis, Brackish water, Bioflocculant, Stability, Bioflocculant property

Introduction

Flocculation is an essential phenomenon used in domestic and industrial wastewater treatment for separation of suspended solids from wastewater. It is achieved with the help of flocculants, which are used for the aggregation of colloidal substances and cellular materials (Nakata and Kurane, 1999). Flocculants are generally classified into three major categories; (i) inorganic flocculants such as aluminum sulfate and polyaluminum chloride, (ii) organic synthetic polymeric flocculants, such as polyacrylamide derivatives and polyethylene amine, and (iii) naturally occurring flocculants, such as chitosan and sodium alginate, and microbial flocculants (He *et al.*, 2002). Despite their efficiency, cost effectiveness and easy availability, the synthetic flocculants' status has not been favorable currently. Most of the high molecular weight flocculants are recalcitrant. Monomers of polyacrylamide are a potent carcinogen and neurotoxic to humans and other animals (Vanhoric *et al.*, 1983). Compared with conventional synthetic organic flocculants, bioflocculants are environmental friendly, since they are biodegradable, non toxic and non carcinogenic. Moreover, they have high flocculation ability; low dosage requirements, diverse applications and end of pipe technologies are not required (Salehizadeh and Shojaosadati, 2001).

Many bacteria, fungi and actinomycetes have been reported to produce extracellular biopolymers, such as polysaccharides, functional proteins and glycoproteins with considerable flocculation activity. These microorganisms include; *Bacillus subtilis* F-2-01 (Taniguchi *et al.*, 2005), *Bacillus* sp. F19 (Zheng *et al.*, 2008), *Serratia ficaria* (Gong *et al.*, 2008), *Bacillus licheniformis* X14 (Li *et al.*, 2009),

Rhodococcus erythropolis (Chang *et al.*, 2009), *Chryseobacterium daeguense* (Liu *et al.*, 2010). Bioflocculants produced by *Gyrodinium impudicum* KG03 (Yim *et al.*, 2007), *Pseudoalteromonas* sp. SM9913 (Li *et al.*, 2008), *Bacillus subtilis* DYU1 (Wu and Ye, 2007), *Vagococcus* sp. W31 (Gao *et al.*, 2006), *Enterobacter aerogenes* W-23 (Lu *et al.*, 2005), *Bacillus* sp. I-471 (Kumar *et al.*, 2004), *Halomonas* sp. V3a (He *et al.,* 2009), *Alcaligenes cupidus* KT201 (Toeda and Kurane, 1991) and *Bacillus subtilis* IFO3335 (Yokoi *et al.*, 1996) were polysaccharides.

Although, so many studies about bioflocculants have been done, flocculation activity and culture cost of bioflocculants are still the major limiting factors with regard to their application (Zhang *et al.*, 2010). Consequently, there is a need to identify new microorganisms (especially from unusual environments like salt or brackish water lakes) with a high bioflocculant-producing ability and improve upon the flocculation efficiency of the known bioflocculants. Since microorganisms in such habitats have evolved the greatest genomic and metabolic diversity, they have become an important point in the search for novel microbial products (Lam, 2006). Brackish water has more salinity than fresh water, but not as much as seawater. It contains between 0.5 and 30 g of salt l^{-1}. Thus, it covers a range of salinity regimes and is not considered as a precisely defined condition. As environmental conditions of the brackish water are different from terrestrial ones, it is surmised that microorganisms may be present in this kind of water have unusual characteristics in comparing to those of terrestrial counterparts. Therefore, they might produce novel types of bioactive compounds.

However, until this manuscript there have been no publications dealing with the isolation and characterization of bioflocculant-producing microorganisms from the Egyptian lakes. As well as, to our best knowledge, no previous work describing a bioflocculant produced by *Bacillus velezensis* has been reported till date. A series of experiments were performed to characterize and study the flocculation properties of the bioflocculant (designated as 40B) for further application.

Materials and Methods

Sampling : Water samples were collected from the borders of the main five Egyptian lakes; Burullus, Manzala, Qarun, Bardweel and Marriott. The samples were placed in sterilized air-tight bottles that were labeled and transported to the laboratory within 5 hrs in a cool container and maintained in a refrigerator at 4 °C until preparation. Lake Burullus is a salt water lake in North shore of River Nile Delta, Western corner in Kafr El-Sheikh Governorate. Lake Manzala is a brackish lake, in northeastern Egypt on the Nile Delta near Port Said. It is the largest of the northern deltaic lakes of Egypt (47 km long and 30 km wide). Qarun Lake is in the northwest of the Fayoum Oasis, 80 km southwest of Cairo. It was fresh water in prehistory, but today it is a brackish water lake. Its area is estimated to vary between 1,270 km² and 1,700 km². Lake Bardweel is a large, saline lake in Egypt on the north coast of the Sinai Peninsula. The lagoon is shallow (reaching a depth of about 3 m) and is separated from the Mediterranean Sea by a narrow sandbar. It is about 90 km long and 22 km wide. Lake Marriott is a salt lake, or rather brackish, with an area of about 250 km^2 in northern Egypt. It is separated from the Mediterranean Sea by the narrow isthmus on which the city of Alexandria was built.

Media and cultivation conditions : The prescreening medium which was used to screen for bioflocculant-producing microorganisms contained (per liter) 1% glucose, 0.35% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% NaCl and 1.5 % agar. The broth of this medium was used as a seeding medium. The fermentation medium contained (per liter) 20 g L-glutamic acid, 7 g NH_4Cl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 40 mg $FeCl_3$, 150 mg $CaCl_2$ and 140 mg $MnSO_4$. The initial pH of all media was adjusted to 7.2 to 7.5 with 1 M NaOH and 0.5 M HCl. All the media were prepared with distilled water and sterilized at 121°C for 20 min. All cultivations were done at 30°C.

Isolation and time course determination : The water samples were diluted and then plated on the selective medium described above. Strains with different colony morphologies were selected and inoculated into 250-ml flasks containing 50 ml of the fermentation medium. The strains were incubated for 48 h at 30 °C with shaking at 200 rpm. The flocculation activities of the free-cell culture supernatants were determined as described below. The strain with the highest flocculation activity (40B) was selected and stored on slant medium at 4 °C for further research. To determine the time course and growth profile, strain 40B was pre-cultured for 24 hrs in the selective medium and inoculated into the fermentation medium with the addition of 2% starter culture on a rotary shaker (200 rpm) at 30 ºC for five days. Samples were taken every 12 hr to measure cell growth and flocculation activity.

Comparative sequence analysis of 16S rDNA gene : Molecular identification of the selected isolate was performed by the amplification of 16S rDNA with eubacterial universal primers 27F and 1492R (Lane, 1991). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, Calif.). The sequence was analyzed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database Project (Altschul *et al.*, 1991) also analyzed using the BLAST program (National Centre

for Biotechnology Information) to determine the closest available database sequences. Selected rDNA sequences were aligned using the ClustalW program (Shingler, 1996). Published sequences were obtained from the GenBank. A phylogenetic tree was constructed using ClustalW by distance matrix analysis and the neighbor-joining method (Saitou and Nei, 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

Determination of flocculation activity : Bioflocculation activity was determined with jar test equipment (Jar Tester Model CZ150) comprising six paddle rotors (24.5 mm 63.5 mm), equipped with six beakers of 1 l each. The mixture contain 190 ml kaolin clay suspension (5 g l^{-1}, pH 7.0), 0.5 ml free-cell supernatant and 10 ml $CaCl_2$ solution (3%, pH 7.0). Subsequently, the mixture was stirred with rapid mixing at 200 rpm for 2 min, followed by slow mixing at 80 rpm for 3 min and left standing for 5 min. The supernatant was measured for absorbance at 550 nm. A control was prepared by using the same method, but the sample was replaced by distilled water. The flocculation activity was calculated according to the equation:

$$\text{Flocculation activity}(\%) = \left[\frac{A-B}{A0}\right] x\,100$$

where A and B are the supernatant optical densities (OD) of the control (clay suspension without any bioflocculant addition) and sample respectively, at 550 nm.

Purification of the bioflocculant : To purify bioflocculant, the method described previously by Li *et al.*, (2009) was used. The cell-free supernatant of strain 40B was concentrated to 0.2 volumes with a rotary evaporator and dialyzed overnight at 4 ºC in de-ionized water. Thereafter, three volumes of cold anhydrous ethanol (4 ºC) were added to the dialyzed broth. The precipitate obtained was re-dissolved in de-ionized water followed by the addition of 10% cetylpyridinium chloride (CPC) with stirring. After several hours, the resultant precipitate was collected by centrifugation (5000 rpm, 15 min) and dissolved in 0.5 M NaCl. Subsequently, three volumes of cold anhydrous ethanol (4 ºC) were added to obtain the precipitate, which was then washed with 75% ethanol three times and lyophilized to obtain purified bioflocculant.

Physical and chemical analyses of the bioflocculant : The protein content of the bioflocculant 40B was determined according to Bradford's method (Bradford, 1976). Total sugars were determined by the phenol-sulfuric acid reaction using the procedure of Chaplin and Kennedy (Chaplin *et al.*, 1994). Its composition from sugars and amino acids was determined by using amino acid analyzer (Abd-El-Haleem *et al.*, 2006) and HPLC analysis (Stroop *et al.*, 2002), respectively. Furthermore, it was analyzed with a Fourier transform infrared (FTIR) spectrophotometer (Shimadzu FTIR-8400 S) using disc technique with KBr as a matrix over a wave number range of 4,000 to 500 cm^{-1}. Moreover, its chemical stability was determined against different organic solvents, alkaline and acidic media.

Flocculation properties of the bioflocculant : The effect of pH and temperature on the flocculation activity of the purified bioflocculant 40B was studied. Its pH and temperature stabilities were determined by measuring the residual activity after 24 hr and 30 min of pre-incubation at various pHs (pH 1–11) and temperatures (5–100 °C) compared with that of the normal bioflocculant solution at pH 7 and 30 °C, respectively. Furthermore, effects of bioflocculant concentrations (0 –5 mg l^{-1}) and cation types (NaCl, KCl, $CaCl_2$, $MgSO_4$, $FeSO_4$ and $FeCl_3$) were investigated. The effect of the concentration of the best cation on the flocculation activity was also studied. The obtained result was the mean of the results of three independent experiments.

Results and Discussion

A total of 56 colonies were isolated from the brackish water samples collected from the main five Egyptian lakes (Marriott, Burullus, Manzala, Qarun and Bardweel), and 22 pure culture strains with slimy or mucoid appearance were screened on the basis of kaolin suspension flocculation activity over 85% (data not shown). Among them, one strain having kaolin at flocculation activity exceeding 98% was selected for further studies (Gong *et al*., 2008).

The batch culture of strain 40B was performed and the flocculation activity of its fermentation medium was measured simultaneously (Fig. 2). Its flocculation activity was in parallel with cell growth and reached its maximum value in later logarithmic growth phase (at 24 hr) with crude biopolymer yield of 3.54 g l^{-1}. This shows that strain 40B had a higher productivity in the bioflocculant production in a shorter time compared with other strains. During cultivation beyond 24 hrs the flocculation activity decreased gradually from 99.9% to 64.2% at 108 hrs, indicating that strain 40B did use the bioflocculant as a carbon source. Only the supernatant rather than the cells has flocculation activity throughout the fermentation process. This suggested that the bioflocculant was not produced by cell autolysis but instead by biosynthesis. To obtain the bioflocculant with high flocculation activity, we chose 24 hr as culture time in the following studies. Previously, many reported bioflocculants were collected in the later logarithmic growth phase and the early stationary phase because the flocculants production would not increase after those stages and at the worst case it would decrease due to the production of deflocculation enzymes (Lu *et al.*, 2005, Xia *et al.*, 2008).

Chemical analysis of the bioflocculant 40B revealed that the proportions of its total carbohydrate and protein contents were 98% and 2% (w/w), respectively, indicating that the bioflocculant was mainly polysaccharide. It is known that high polysaccharide content is one of the noteworthy characteristics of a flocculant because they are usually more heat resistant in comparison with those with high protein content (Lu *et al.*, 2005). Bioflocculants that are not made of sugars as the main flocculant components show more sensitivity to temperature (He *et al.*, 2004). Bioflocculants with higher protein content are usually less heat stable as protein can be destroyed upon heating; hence, they may be of less importance in industrial waste treatment (Patil *et al.*, 2011). As stated in Table 1, the bioflocculant 40B was rich in the amino acids; glutamic (40%), aspartic (7.9%) and glycine (6.6%), respectively. However, HPLC analysis revealed that monosugar glucose was the major sugar components of the bioflocculant exhibiting 90.7% of the analyzed sample.

The infrared spectrum of the purified bioflocculant 40B (Fig. 3) displayed a broad stretching intense peak at 3,440 cm^{-1} characteristic of hydroxyl and amino groups. Two weak C–H stretching bands at 2,950 cm^{-1} and 2,177 cm^{-1} indicated aliphatic C–H bands and the bands at 1,620 cm^{-1} and 1,415 cm^{-1} could be assigned to the C=O antisymmetrical and symmetrical stretchings in the carboxylate, respectively (Deng *et al.*, 2003). The adsorption peak at 1,253 cm^{-1} and 1,074 cm^{-1} indicated the C–O stretching vibration and the presence of methoxyl groups. In addition, the peak at 1074 cm^{-1} was generally known to be typical characteristics of all sugar derivatives. The two peaks at 663 cm^{-1} and 474 cm^{-1} indicated C=O twisting vibration (Deng *et al.*, 2003). Consequently, the infrared spectrum of the bioflocculant 40B showed the presence of carboxyl, hydroxyl, amino and methoxyl groups, which are the preferred groups for the flocculation, similar to those observed in polyelectrolytes (Yim *et al.*, 2007). Presence of carboxyl and hydroxyl groups has more adsorptive forces, which result in aggregation; hence, they may be the preferred groups for flocculation process. This is a significant characteristic because (–COOH) groups might be used as functional groups to link this polysaccharide to starch like natural and synthetic polymers to form new polysaccharides having unique properties (Daolun and Shihong, 2008)

The protein and the carbohydrate contents of the purified biopolymer may explain its stability toward all studied organic solvents (acetone, methanol, ethanol, chloroform, dimethylsulphoxide). However, it has a tendency to be soluble in water as well as both acidic and alkaline media. The insolubility of the polysaccharide in organic solvents may be due to the increased number of hydroxyl groups present in the bioflocculant which favor the building up of strong forces of attraction between polysaccharide molecules resulting in the crystalline polymer solids and these forces hard to be broken by organic solvents (Kumar *et al.*, 2004).

The pH of reaction mixtures is a key factor influencing the flocculation activity. As presented in Table

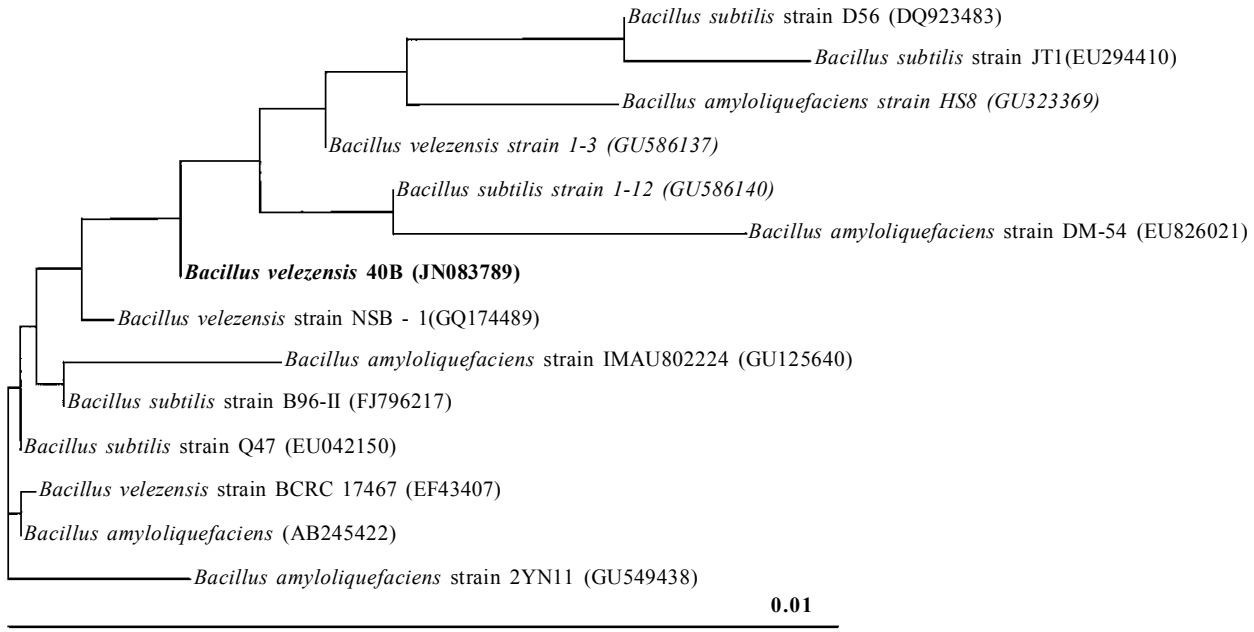

Fig. 1 : Phylogenetic tree showing the relationships among *Bacillus velezensis* 40B (in boldface) and published 16S rDNA sequences (their GenBank accession numbers are present in the brackets). The microorganism *Bacillus amyloliquefaciens* strain 2YN11 (GU549438) was used as an out-group

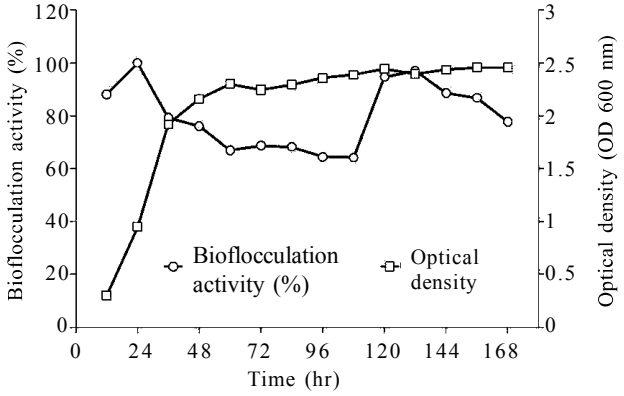

Fig. 2 : Growth profile for *Bacillus velezensis* 40B (clay concentration =5g/l, agitation speed = 2 min at 200 rpm then 3 min at 80 rpm, dosage of free- supernatant = 2.5 ml/l, dosage of $CaCl_2$ (3%) = 50 ml/l clay suspension pH = 7)

2, the purified bioflocculant 40B had the optimal pH for flocculation activity at acidic conditions, the flocculation activity increased gradually when the pH value went up and reached its maximum at pH 7.0 (Table 2). Bouchtroch *et al.* (2001) and Gao *et al.* (2006) reported similar optimal pH values (pHs 7.2 and 7.0) for the activity of the bioflocculants ERSS-31 produced by *Halomonas maura* sp. nov. and MBFW31 produced by *Vagococcus* sp. W31, respectively. Bioflocculant HBF-3, produced by a mutant *Halomonas* sp. V3a' also attained the highest flocculation activity at pH 7 (He *et al.*, 2010). This may be due to the fact of the negative natural surface of the clay particles. At low pH, the absorption of H^+ ions tend to weaken the bioflocculant-kaolin complex formation process and a similar effect is also observed at high pH values (He *et al.*, 2010). However, the preferable acidic conditions for flocculation obtained with the bioflocculant 40B was similar to that of flocculant

Table 1 : Amino acids and sugars compositions of the purified biopolymer

Amino acids	Content (%)	Sugars	Content (%)
Aspartic acid	7.91	Xylose	4.21
Thereonine	2.91	Glucose	90.72
Serine	1.69	Sucrose	2.74
Glutamic acid	40.34	Lactose	2.34
Proline	3.42		
Glycine	6.62		
Alanine	5.46		
Valine	5.46		
Methionein	2.86		
Isoleucine	3.52		
Leucine	4.51		
Tyrosine	0.04		
Phenylalanine	2.71		
Histidine	5.67		
Lysine	4.39		
Ammonia	0.45		
Arginine	1.97		

produced by *B. subtilis* DYU 1 (Wu and Ye, 2007), *Bacillus* sp. PY-90 (Yokoi *et al.*, 1995), *Streptomyces griseus* (Shimofuruya *et al.*, 1996), and *Enterobacter* sp. BY-29 (Yokoi *et al.*, 1996), but different from that for bioflocculant produced by *Nannocystis* sp. NU-2 that was active in alkaline conditions ranging from pH 12–14 (Zhang *et al.*, 2002).

The effect of the temperature on a kaolin clay mixture was tested. The highest flocculation activity was achieved at 75 ºC (98.14%), and 83.45 % still remained when heated at 85 ºC (Table 2). Bioflocculants containing sugars as the main flocculation components are heat stable, and their flocculation activity could retain more than 50% when heated in boiling water (Lu *et al.*, 2005). However, this high temperature was lower than the flocculation temperature (100 °C) against cell suspension of *Escherichia coli* by cationic polysaccharide from *Paecilomyces* sp. I-1 (Takagi and Kadowaki, 1985) and higher than the optimum temperature (approximately 70 °C) for flocculation of polyglutamate from *B. subtilis* IFO 3335.

The pH stability experiments illustrated that the purified bioflocculant 40B was relatively stable over a wide pH range of 1–11 compared to that of the control at pH 7. On the other hand, the pH stability of the biopolymer solution was observed at the pH range of 3-9 and reached its maximum stability at pH 7 (Table 2). However, its flocculation activity slightly decreased with a very little limitation that ranged between 0.21-2.5% at the remaining studied pH range. It is demonstrated that the bioflocculant solution is suitable to be applied in neutral, weakly acid and weakly alkaline circumstances. However, its flocculation efficiency slightly reduces at highly acidic (pH<5) and highly alkaline (pH>9) circumstances. This may be returned to the bioflocculant which shows different electric states at different pH, in turn affects the bridging efficiency of the bioflocculant for clay powder (Yong *et al.*, 2009).

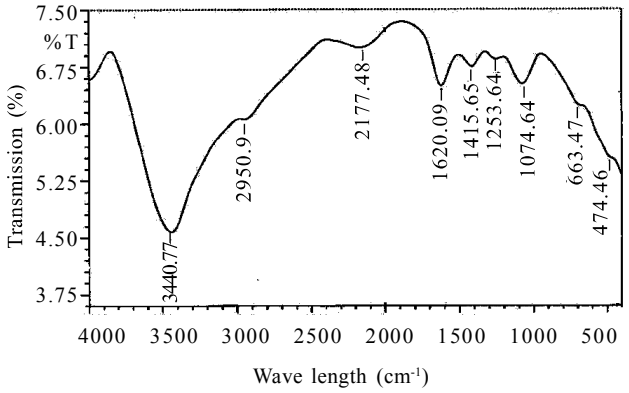

Fig 3 : FTIR analysis of the purified bioflocculant 40B

Table 2: Effects of cationic/metal salt content, flocculant dosage, pH and temperature on flocculating activity of the bioflocculant 40B

Metal ions		Ca^{2+}		Flocculant dosage		Reaction mixture pH and temperature				Bioflocculant stability			
Metal types	*FA (%)	Con. (ml 200 ml^{-1})	FA (%)	Con. (mg l^{-1})	FA (%)	pH	FA (%)	Temp (°C)	FA (%)	pH	FA (%)	Temp (°C)	FA (%)
K	59.29	0	60.78	0.1	14.31	1	94.23	5	85.31	1	85.13	5	86.62
Na	89.18	0.5	87.54	0.2	35.68	3	92.19	25	93.54	3	87.17	25	87.55
Mg	10.00	1	97.89	0.4	67.84	5	90.52	35	92.19	5	87.35	40	86.80
Fe	15.00	2	91.41	0.5	86.05	7	97.54	50	92.56	7	87.56	60	83.46
Al	16.00	4	86.02	1.0	87.73	9	83.08	65	96.28	9	86.43	80	85.69
Zn	79.73	6	74.34	1.5	94.42	10	81.41	75	98.14	10	85.50	100	84.76
Ca	97.84	8	71.74	2.0	96.09	11	66.35	85	83.45	11	85.13		
Blank	60.78	10	67.84	2.5	96.84			100	44.98				
		15	55.39	3.0	97.76								
		20	47.95	3.5	99.90								
		25	44.23	4.0	99.90								
		30	40.52	4.5	99.90								
		35	36.80	5.0	99.90								

*FA % = Flocculation activity percent

Thermal stability studies revealed that the purified bioflocculant 40B could be classified a thermal-stable bioflocculant since its flocculation activity did not decrease in the range of 5–100 °C (Table 2). The stability of this bioflocculant was same as that of the glycoprotein produced by *Arcuadendron* sp., which was stable up to 100 °C (Lee *et al.*, 1995). This thermal stability was presumably because the main backbone of 40B was a polysaccharide (Lu *et al.*, 2005). Higher temperatures may be causing degradation of this polysaccharide chain of the bioflocculant that reduces its flocculation efficiency. This thermal behavior of the bioflocculant is supported by other studies (Gong *et al.*, 2008).

The effect of bioflocculant dosage showed flocculation activity over 90% in the range of 1.5–5.0 mg l^{-1} (Table 2). The maximum flocculation activity of 99.9% was observed at an optimum bioflocculant dosage of 3.5 mg l^{-1}, comparable to those of some chemically synthesized flocculants, such as $Al_2(SO_4)_3$ (5 mg l^{-1}) and PAM (4.5 mg l^{-1}). The dosage of PAC was 7 mg l^{-1}, and the total concentration of PAM plus $Al_2(SO_4)_3$ mixed in equal weights was 4.4 mg l^{-1}. To achieve high flocculation activity, metal cations are often added. Table 2 showed the effect of cations on flocculation. Apparently, the divalent cation (Ca^{2+}) was more effective than others. Ca^{2+} could destabilize the negatively charged kaolin particle by neutralizing and bridging (Yim *et al.*, 2007). However, trivalent cations could change the surface charge of kaolin particle and cover the adsorb sites. The competition of the positively charged particles and less adsorb sites induce the low flocculation activity (Gong *et al.*, 2008). Nevertheless, the highest effect of Ca^{2+} is one of the significant characteristics because trivalent cations are difficult to remove and can cause environmental problems. Fe^{3+} forms the gelatinous precipitate, which was more difficult to remove. Similarly, Al^{3+} gives rise to an environmental problem; therefore, $CaCl_2$ has own coagulant aid (Patil *et al.,* 2011). Hence, the bioflocculant 40B significantly improved the separation of kaolin particle in the presence of Ca^{2+}. The optimization of dosage of Ca^{2+} showed that the optimum Ca^{2+} concentration was 1 ml 200 ml^{-1} suspension. However, the higher or lower salt dosage was distinctly reducing the flocculation efficiency of the bioflocculant 40B. This result is in agreement with the expected result because as previously declared that Ca^{2+} plays a significant role in the charge neutralization for clay suspension flocculation. Therefore, the increase in the positive charge density over the clay particle surfaces may be giving the same influence of the trivalent cations presence that inhibits the biopolymer efficiency (Gong *et al.*, 2008).

In conclusion, to our knowledge this is the first report on the production of a bioflocculant from the *Bacillus velezensis* 40B isolated from a brackish water sample taken from the Qarun Lake. The bioflocculant produced could be used in industrial application and indicates the possibility of solving some environmental problems. Further study on its production mechanism and application are in progress.

Acknowledgments

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