Characterization of a Bioflocculant Produced by an Isolate, *Bacillus megaterium* G31

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Abstract – A bacterial strain capable of producing a novel bioflocculant was isolated from a biofilm sample and identified as *Bacillus megaterium* G31. The highest biopolymer yield was achieved when the organism was cultivated in a medium containing acetate as the sole carbon source and $(NH_4)_2HPO_4$ as the nitrogen source. In kaolin suspension, the flocculating activity was highest at 170 mg l^{-1} and decreased at the higher bioflocculant concentrations. The crude bioflocculant produced by the organism was purified by ethanol precipitation and gel permeation chromatography. The FTIR spectrum of the purified bioflocculant revealed that the bioflocculant might be a heterogeneous polysaccharide composed of hexosamines and neutral sugars. The analysis of sugar components of the bioflocculant using high performance anion-exchange chromatography showed that the sugar constituents of the bioflocculant were glucosamine, fucose, galactosamine, galactose, glucose, mannose in approximate molar ratio of 4 : 1 : 6 : 3 : 8 : 19. Its flocculating activity was stimulated by various cations. The bioflocculant was thermo-stable and retained 64% of its original activity after heating at 100°C for 50 min.

Key words : bioflocculant, biopolymer, flocculating activity, *Bacillus megaterium* G31

INTRODUCTION

A number of flocculants are widely used in a variety of industrial fields including wastewater treatment, tap water production, downstream processing, food and fermentation processes (Kurane *et al.* 1986; Salehizadeh and Vossough 2000; He *et al.* 2002). Among the flocculants, inorganic flocculants, such as aluminum sulfate and polyaluminum chloride, and organic synthetic flocculants like polyacrylamide derivatives are frequently used because they are not only cost-effective but also have strong flocculating activity. However, some reports have indicated that aluminum salts may induce health problems (e.g. Alzheimer's disease) (Masters *et al.* 1985; Kowall *et al.* 1989). Moreover, there is evidence that polyacrylamide derivatives, a representative group of the organic synthetic flocculants, are non-degradable in nature and the acrylamide monomers derived from them are neurotoxic and human carcinogens (Dearfield *et al.* 1988). Therefore, the development of harmless, more efficient and environment-friendly flocculants is essential as an alternative.

Recently microbial polymers that have flocculating activity have been the focus of great interest as promi-

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sing candidates for large-scale production of bioflocculants since they are generally biodegradable and harmless to the environment and humans. Several types of microbial polymers including polysaccharides, proteins, and glycoproteins have been previously reported, and some of them showed efficient flocculating activities comparable to those of the synthetic flocculants (Lee et al. 1995; Shih and Van 2001; Zhang et al. 2002; Deng et al. 2003). However, none of them has been practically applied in industry because of their high production cost compared with the inorganic and organic synthetic flocculants as well as their low productivity after fermentation. Therefore, screening new microorganisms capable of producing flocculants with high flocculating activity and optimizing of fermentation process to achieve a high yield and productivity of bioflocculant have become another research focus in recent years.

In this study, we report the production of a novel bioflocculant by an isolate, *Bacillus megateium* G31, using an inexpensive carbon substrate, acetic acid, together with a description of the characterization of the bioflocculant produced by the isolate.

MATERIALS AND METHODS

1. Isolation of a flocculant-producing bacterial strain

Bacterial strains were isolated by conventional methods from a wide variety of environmental samples including biofilm samples from domestic drainages. They were cultivated on a basal agar medium supplemented with 0.05 g L^{-1} aniline blue. Each liter of basal medium contained 10 g sodium acetate, 1 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.05 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3$, and 0.1 g yeast extract. After 2 days of incubation at 30°C, colonies showing blue-color on the plates were regarded as the bioflocculant producers (Kim et al. 2003), and these were tested for their flocculating activity for kaolin clay suspension (*vide infra*). Among the isolates, one strain, G31, which exhibited a relatively high flocculating activity, was selected and used for the further study.

2. Identification of the isolate

Identification of the isolate was based on the fatty acid profile and 16S rDNA sequence. Fatty acids as their methyl esters were extracted from cells grown on trypticase soy broth agar, and separated using Hewlett Packard 6890 gas chromatography. Fatty acids were identified by the Microbial Identification System (Microbial ID Inc, USA). For analyzing the 16S rDNA sequence, a partial 16S rDNA of the isolate was enzymatically amplified by using universal primers, 8F and 1522R (Lane 1991). The resultant PCR product was purified using a DNA PrepMate kit (Bioneer Co., Korea). The purified PCR amplicon was directly sequenced using a Silver Sequence DNA Sequencing System (Promega Co., USA) as described previously (Kang et al. 2001). The 16S rDNA sequence of the isolate was aligned with those from strains of the genus *Bacillus* on the basis of similarities in the primary and secondary RNA structures using the PHYDIT program (Chun 1995).

3. Cultivation

Shake flask cultures were carried out aerobically in 500 ml Erlenmeyer flasks containing 100 ml of basal medium. Batch fermentations were conducted in a 5 l jar fermentor with a working volume of 3 l. The medium was inoculated with a 5% (v/v) inoculum of an overnight culture in the same basal medium. The temperature and pH were automatically controlled at optimal values, 30°C and 7.0, respectively. The air flow rate was 0.33 vvm and agitation speed was 170 rpm. Cell growth was monitored spectrophotometrically at 660 nm. All experiments were repeated at least three times.

4. Measurement of flocculating activity

The flocculating activity was evaluated by measurement of the turbidity of a kaoline suspension (Kurane and Matsuyama 1994). Kaoline clay (Junsei Chemical Co.) was suspended in deionized water at 5 g L^{-1}. Five ml of kaoline suspension was mixed with 0.5 ml of sample (supernatant of the culture broth or the crude bioflocculant) in a test tube. The reaction mixture was stirred for 20 seconds and then allowed to stand for 20 min. The turbidity of upper phase was measured with a

spectrophotometer at 550 nm. A control was prepared using the same method but the sample was replaced by distilled water. The flocculating activity was calculated according to the equation:

Flocculating activity (1/OD) = 1/A - 1/B

where A is the turbidity obtained by addition of the sample and B is turbidity in the control case.

5. Purification of the bioflocculant

In order to purify the bioflocculant, the culture broth was centrifuged at 7000 rpm for 20 min to remove cells. The culture supernatant was mixed with 5 volumes of ethanol and left overnight at $-10°C$. The crude bioflocculant was collected by centrifugation and then dissolved in distilled water. For further purification, the bioflocculant solution was loaded onto a Sephacryl S-400HR column (2.4 × 95 cm) and eluted with distilled water at a flow rate of 48 ml per hour. Active fractions showing flocculating activity were collected and concentrated, which was used in this study as the purified bioflocculant.

6. Analytical methods

After hydrolysis of the purified bioflocculant with 2 M trifluoroacetic acid and 6 N HCl at 100°C for 4 h, the sugar components were analyzed by using high performance anion-exchange chromatography (Dionex Co., USA) with CarboPak PA1 column. The FTIR-ATR spectra was obtained using a Digilab FTS 80 (Bio-Rad) spectrophotometer equipped with a KRS 5 internal reflection element (incidence angle, 45°). Differential scanning calorimetry (DSC) analysis was performed as previously described (Kim et al. 1999).

RESULTS AND DISCUSSION

1. Identification of the isolate

The isolate G31 was a Gram-positive, aerobic, motile, catalase-positive, and spore-forming rod. The major fatty acids were i-15 : 0 (38.0%), ai-15 : 0 (35.5%), and i-14 : 0 (6.3%). The fatty acid profile of the isolate G31 showed 66% similarlity with that of Bacillus mega-

terium. A total of 425 nucleotides of the 16S rDNA of strain G31 were determined and aligned with those of reference strains from GenBank. Phylogenetic analysis of the 16S rDNA sequences of G31 showed that the isolate was closely linked with B. megaterium IAM 13418, with a highest sequence similarity of 99.5%. Based on these results, the isolated strain G31 could be identified as B. megaterium.

2. Production of bioflocculant

B. megaterium G31 was tested for production of bioflocculant in a basal medium containing various carbon sources. The flocculating activity of the culture suspension was almost the same as that of its cell-free supernatant, indicating that the bioflocculant is extracellular. Table 1 shows the results of shake flask experiments for G31 grown with various carbon substrates including saccharides, sugar-alcohols, and fatty acids. Most of saccharides and sugar-alcohols were better substrates for cell growth than fatty acids. However, the organism produced biopolymer at higher levels (317-430 mg L^{-1}) when fatty acids, such as acetate, propionate and butyrate, were used as the sole carbon substrate. In particular, the bioflocculant concentration produced from acetate was the highest among those tested, although acetate was not favorable for the cell growth. These results indicate that the bioflocculant production by G31 strain is not closely linked to the cell mass.

Table 1. Effects of carbon substrates on the cell growth and bioflocculant production from B. megaterium G31.

Carbon source	Cell growth (OD 660 nm)	Bioflocculant (mg L⁻¹)
Glucose	3.18	57
Fructose	5.52	200
Galactose	5.58	93
Lactose	5.77	107
Sucrose	3.19	310
Mannitol	8.09	180
Sorbitol	0.57	243
Glycerol	2.32	20
Acetate	1.44	430
Propionate	0.29	317
Butyrate	0.83	350
Lactate	0.03	27

Cells were grown aerobically in 500 ml Erlenmeyer flasks containing 100 ml basal medium at 30°C for 48 hr.

Acetate is an inexpensive carbon substrate which can be easily produced from various organic wastes, and thus the ability of producing bioflocculant from acetate is potentially useful for the commercial production of bioflocculant since price reductions can be achieved by reduced substrate cost (Fujita *et al.* 2000). To examine the effect of acetate concentration on cell growth and bioflocculant production, batch cultures were carried out with varying amounts of acetate from 5 to 30 g L^{-1} (Fig. 1). The maximum biomass yield was relatively high when the initial acetate concentration was between 15 and 25 g L^{-1}, and was significantly decreased when acetate concentration exceeded 25 g L^{-1}. However, the optimum concentration of acetate for the bioflocculant production was 20 g L^{-1}. The effects of inorganic nitrogen
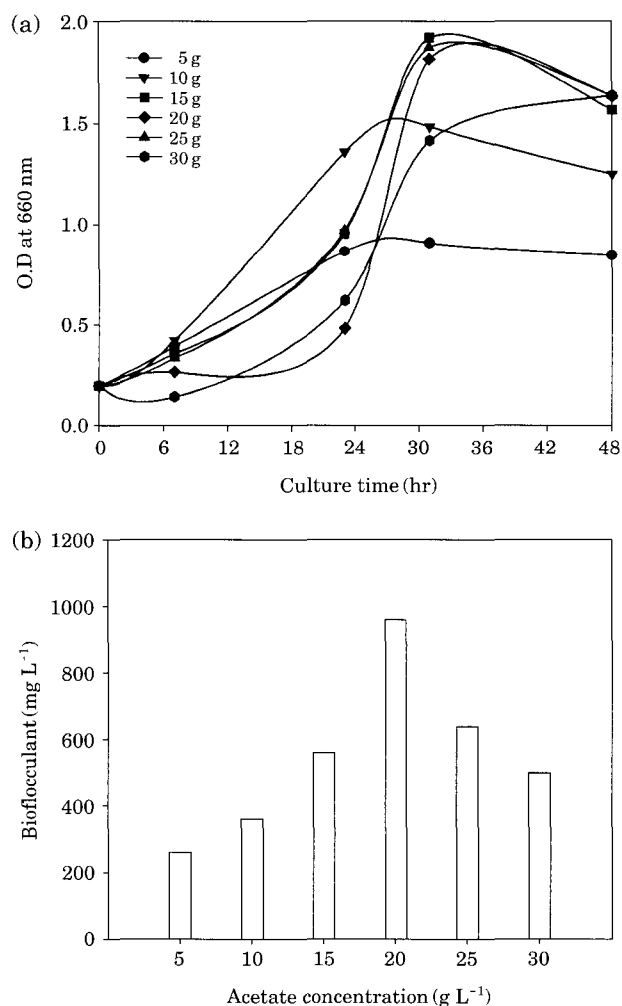
source were also investigated when acetate was used as a carbon source. Among inorganic nitrogen sources tested, $(NH_4)_2HPO_4$ was most effective for the bioflocculant production. The concentration of bioflocculant produced from $(NH_4)_2HPO_4$ exceeded 870 mg L^{-1}, which was approximately five times higher than that achieved from other inorganic nitrogen sources, such as $(NH_4)_2SO_4$, NH_4NO_3, and NH_4Cl, at a same concentration (data not shown). Meanwhile, the initial pH of the medium showed no significant differences in the bioflocculant production within the pH range of 7.0-8.5. The optimum temperature for both cell growth and bioflocculant production was found to be 30°C.

A typical time course of growth and bioflocculant production of strain G31 in basal medium containing 10 g L^{-1} sodium acetate is shown in Fig. 2. The cell mass was highest (0.9 g L^{-1}) after 48 h cultivation and decreased slightly thereafter. The bioflocculant was released into the culture broth in parallel with cell growth and continued to increase rapidly after growth had ceased; the amount of crude bioflocculant, 1.2 g L^{-1}, was obtained after 110 h cultivation. In contrast, the flocculating activity reached its maximum after 80 h cultivation and decreased thereafter.

In order to understand the relationship between the bioflocculant concentration and the flocculating activity, flocculation reactions were performed at different concentrations ranging from 5 to 700 mg L^{-1} in kaoline suspension (Fig. 3). The flocculating activity was highest at 170 mg L^{-1} and decreased at the higher flocculant concentrations. More than 80% of the flocculating activity was observed at a concentration ranging from 120 to 230 mg L^{-1}. These results suggest that the excess dosage of the bioflocculant cause the resuspension of kaoline particles and leads to the decrease of flocculating activity. This phenomenon may be ascribed to the oversaturation of many binding sites in the surface of kaoline particles, thus the attractive forces of other particles are reduced and flocculating activity decreases (Yokoi *et al.* 1995; Kwon *et al.* 1996).

3. Characteristics of the bioflocculant

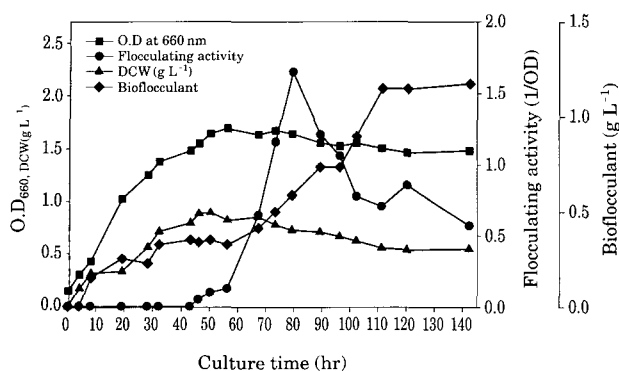The freeze-dried purified bioflocculant was whitish and soluble in water. It contained sugars as determined

Fig. 1. Effect of acetate concentration on the cell growth (a) and bioflocculant production (b).

Fig. 2. Fermentation kinetics of bioflocculant production during the batch fermentation of *B. megaterium* G31 in a basal medium containing acetate (10 g L^{-1}) as the carbon source.
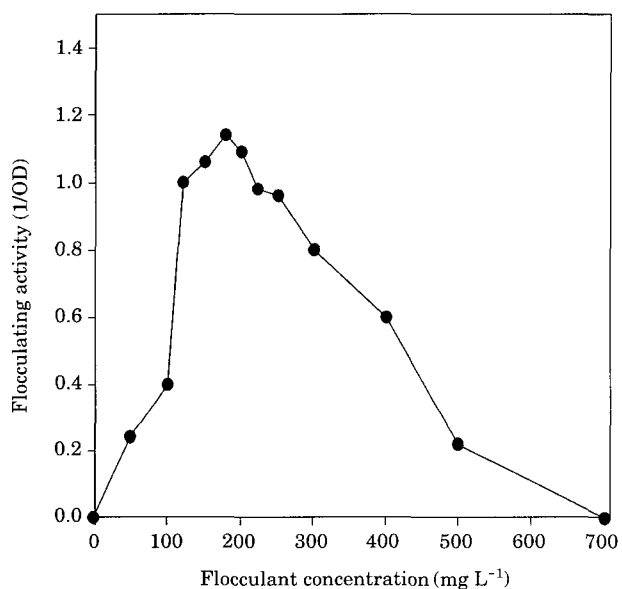

Fig. 4. Analysis of sugar constituents of the purified bioflocculant from *B. megaterium* G31 using high performance anion-exchange chromatography.

Fig. 3. Effect of bioflocculant concentration on the flocculating activity.

Fig. 5. FTIR-ATR spectra of the purified bioflocculant from *B. megaterium* G31.

by the phenol-sulfuric acid method, while protein was not detected as determined by the Bradford method. The higher amount of total sugar compared to other components and the absence of protein indicated that the bioflocculant was a polysaccharide. The analysis of sugar components of the bioflocculant using high performance anion-exchange chromatography is depicted in Fig. 4. The sugar constituents of the bioflocculant were glucosamine, fucose, galactosamine, galactose, glucose, mannose in approximate molar ratio of 4 : 1 : 6 : 3 : 8 : 19.

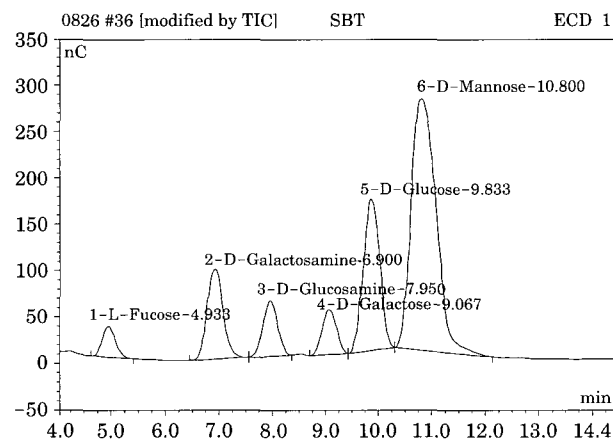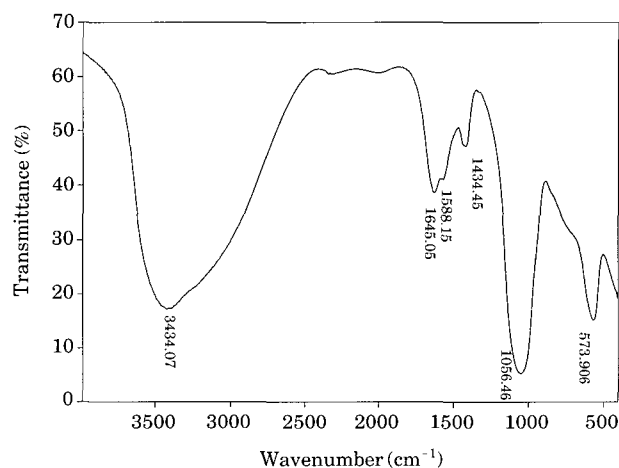The FTIR-ATR spectrum of the purified bioflocculant

exhibited absorption peaks of a hydroxyl band at 3434 cm^{-1}, an amine band at 1645 cm^{-1}, and a C-N stretching at 1056 cm^{-1} as characteristic bands (Fig. 5). The bands pattern of the bioflocculant thus shows the presence of hydroxyl and amine groups. These functional groups are well known to contribute to the flocculation of clay particles (Divakaran and Pillai 2001). Based on these results, it was presumed that the bioflocculant might be a heterogeneous polysaccharide composed of hexosamines and neutral sugars. For the complete identification of the chemical structure of the bioflocculant, further detailed studies are required.

In addition to protein (or poly-glutamic acid) biofloc-

culants (Yokoi *et al.* 1995; Shih *et al.* 2001), polysaccharide bioflocculants are reported to be produced by many *Bacillus* spp. (Suh *et al.* 1997; Salehizadeh and Shojaosadati 2002; Deng *et al.* 2003). However, the components and composition of the bioflocculant produced by *B. megaterium* G31 differs from those of other polysaccharides produced from *Bacillus* spp. and other bacteria in that it contains high amount of hexosamines (approximately 25 mol%). The extracellular production of polysaccharide bioflocculant containing high amount of hexosamine by bacteria has not been reported within the range of our reference search. Exceptionally, only a bacterial strain *Citrobacter* sp. has been recently reported to produce a bioflocculant containing glucosamine as a major component (Fujita *et al.* 2000). The molecular weight of the bioflocculant was higher than 1×10^5 Da as determined by MALDI-TOF. The thermal property of this polysaccharide investigated by DSC exhibited a crystalline melting point at 275°C.

Fig. 6 shows the effects of cations added to the reaction mixture on flocculating activity. Solutions of KCl, CaCl$_2$, NaCl, MgCl$_2$ or FeSO$_4$ · 7H$_2$O were used as the source of cations. Flocculating activity of the bioflocculant for kaoline suspension increased by the addition of these cations at a low concentration of 0.5 mM regardless of their valence states, indicating that the bioflocculant from G31 is a cation-dependent flocculant. It seems that the cations stimulate the initial adsorption of the bioflocculant on kaoline particles by decreasing the negative electrical charge of kaoline particles and the bioflocculant. Furthermore, the bioflocculant could efficiently flocculate other inorganic and organic suspended particles. It was particularly effective for activated carbon but relatively ineffective for *Candida parapsilosis* cells (data not shown). The flocculating activities for activated carbon and *C. parapsilosis* cells were approximately 4 times higher and 6 times lower, respectively, than that for kaoline.

As the major component of the bioflocculant is polysaccharide, it showed relatively high stability at high temperature (Fig. 7). The flocculating activity of the bioflocculant in kaoline suspension decreased by only 18% after heating at 100°C for 20 min and it decreased by 36% after 50 min. By contrast, protein bioflocculants are usually not heat-stable as protein can be easily
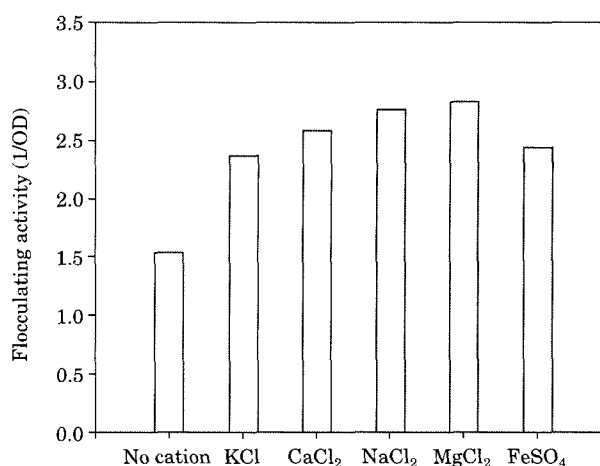

Fig. 6. Effects of cations on the flocculating activity of bioflocculant in kaoline suspension. Cation concentration in the kaoline suspension was 5 mM.
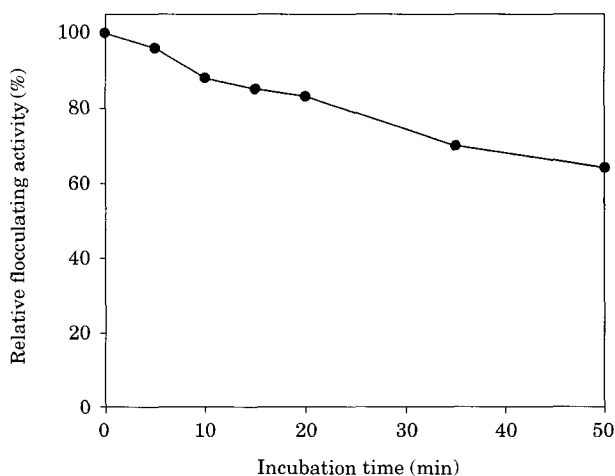

Fig. 7. Thermal stability of bioflocculant. The bioflocculant in kaoline suspension was preincubated at 100°C and the residual flocculating activity was measured under the standard conditions.

destroyed upon heating; for example, the protein bioflocculant produced by *Bacillus* sp. PY-90 lost completely its original flocculating activity after 40 min heating at 100°C (Yokoi *et al.* 1995).

4. Conclusion

This study describes the characteristics of a bioflocculant produced by a newly isolated strain, *B. megaterium* G31, using acetate as the sole carbon source. The chemical analysis revealed that the major component of

the bioflocculant produced by *B. megaterium* G31 is polysaccharide composed of hexosamines and neutral sugars. To date the production of polysaccharide bioflocculant containing high amount of hexosamine by bacteria has not been reported, and thus it is believed to be a novel polysaccharide bioflocculant. Since the bioflocculant can not only flocculate various inorganic and organic suspensions but also has strong flocculating activity in a wide range of pH and temperature, it is expected to be widely used in various industrial wastewater treatments and many other fields in environmental biotechnology. The optimal culture conditions and the development of an efficient *fermentation* process to achieve a high yield of bioflocculant are currently being conducted in our laboratory.

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