J. Microbiol. Biotechnol. (2005), **15**(3), 626–632

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Novel Cationic Microbial Polyglucosamine Biopolymer from New *Enterobacter* sp. BL-2 and Its Bioflocculation Efficacy

SON, MI-KYUNG, HYUN-DONG SHIN, TAE-LIN HUH, JIN-HO JANG[1], AND YONG-HYUN LEE*

Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea
[1]Regional Research Center for Processing and Application of Agriculture Products, Daegu University, Kyungsangbukdo 712-714, South Korea

Received: October 25, 2004
Accepted: January 11, 2005

Abstract A new bacterium BL-2 excreting a novel cationic polyglucosamine biopolymer was isolated from the spoiled leaves of Chinese cabbage and identified as *Enterobacter* sp. BL-2. The isolated *Enterobacter* sp. BL-2 was cultivated in pH-stat fed-batch culture using acetic acid as the feeding stock at pH 8.0, resulting in 17.11 g/l of cells and 1.53 g/l of an extracellular biopolymer after 72 h. The excreted biopolymer was purified by a three-step procedure, involving ethanol precipitation and deproteinizations, to a nearly homogeneous state, and its molecular weight was found to be 106 kDa. It was composed of glucosamine, rhamnose, and galactose at a molar ratio of 86.4:1.6:1.0, respectively, indicating a rarely found novel high-glucosamine-containing biopolymer. The FT-IR and ^{13}C-NMR spectra of the novel cationic polyglucosamine biopolymer PGB-1 revealed a close identity with chitosan from crab shell. It can effectively flocculate various suspended solids, including kaolin clay, $Ca(OH)_2$, Al_2O_3, active carbon, microbial cells, and acidic dyes.

Key words: Bioflocculant, *Enterobacter* sp. BL-2, pH-stat fed-batch cultivation, polyglucosamine biopolymer

Flocculants are widely utilized in water and wastewater treatments and downstream processing, and can be classified into three groups; inorganic, organic polymeric, and bio-flocculants including chitosan, alginate, and microbial polymers [17, 21]. Inorganic and organic polymeric flocculants have been mainly utilized, because of their effectiveness and cost benefit, however, their non-decomposable, carcinogenic, or neurotoxic nature make them somewhat restrictive in certain processes [2, 15].

Recently, microbial biopolymers have received considerable attention as flocculating agents; e.g., those from *Bacillus* [3, 27], *Rhodovulum* [29], *Rhodococcus* [13], *Klebsiella* [4], *Paenibacillus* [18], *Alcaligenes* [24], *Enterobacter* [26], and *Citrobacter* [5, 9]. The polysaccharide-type bioflocculants are more effective and stable than protein and glycoprotein-type bioflocculants. Also, the cationic flocculants are known to be more powerful for treatment of the negatively charged organic waste in sewage or the recovery of microbial cells. Most microbial polysaccharides are produced as a less effective anionic or neutral type; nevertheless, the microbial cationic bioflocculant, except for the natural biopolymer chitosan, has rarely been developed.

Chitosan is manufactured from crab shells through deproteinization and deacetylation using concentrated acid and alkali [22]; however, the cost and hazardous residual chemical prohibits its practical utilization. A microbial cationic polysaccharide that can replace current cationic inorganic and organic synthetic polymeric flocculants is urgently needed. Recently, Fujita *et al.* [6] reported a noticeable bioflocculant excreted by *Citrobacter* sp. TKF04, containing as high as 29.4% hexosamine. Hexosamine, containing an amine group in the C2 position, is a positively charged monomer, and its characteristics suggest the possibility of screening a cationic bioflocculant among microbial sources.

In the current study, a new microbial strain BL-2 excreting a cationic biopolymer was screened from spoiled leaves of Chinese cabbage, based on its hexosamine content and flocculating efficiency against a kaolin suspension. Phenotype characteristics and phylogenetic analysis of the 16S rDNA of the new isolate were investigated. The newly identified *Enterobacter* sp. BL-2 was cultivated in pH-stat fed-batch culture for excreting production of the biopolymer. The excreted biopolymer was purified by a three-step purification procedure to determine the molecular weight and chemical composition.

The structural features of the novel polyglucosamine biopolymer PGB-1 were compared with the natural polyglucosamine biopolymer chitosan using FT-IR and NMR spectroscopies. Finally, the flocculating efficacy of

*Corresponding author
Phone: 82-53-950-5384; Fax: 82-53-959-8314;
E-mail: leeyh@knu.ac.kr

excreted novel microbial cationic polyglucosamine biopolymer PGB-1 was evaluated against suspended solids, microbial cells, and dyes.

MATERIALS AND METHODS

Screening of Strain

The screening was carried out in LB agar plates (pH 7.5) supplemented with 1.0% (w/v) glucose and 0.5% sodium acetate at 30°C for 72 h. The mucous and ropy colonies were first selected, and cultivated in 10 ml of a basal liquid medium, composed of 1.0% (w/v) sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2.0% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, and 0.05% trace elements, for 48 h. The strain excreting a biopolymer with high hexosamine content and flocculation efficiency in a kaolin suspension was selected.

Phenotype Characteristics and Phylogenetic Analysis

Physiological, biochemical, and cultural characteristics of the isolate were investigated according to the procedures of *Bergey's Manual of Systematic Bacteriology* [12] and *Methods for General and Molecular Bacteriology* [7]. Partial 16S rDNA of the isolate was cloned by a PCR using the primer sets, BSF-I (5'-TAACACATGCAAGTC-3') and BSR1407 (5'-GACGGGCGGTGTGTAC-3'), and sequenced using Automatic Sequencer ABI 377 (Applied Biosystems, Foster City, CA, U.S.A.). The sequence was aligned with other *Enterobacteriaceae* families registered in the GenBank, and a phylogenetic tree was constructed according to the neighbor-joining method [20] using Clustal W [25].

pH-Stat Fed-Batch Cultivation

The isolated strain was cultivated in pH-stat fed-batch culture in a 5-l jar fermenter (KoBiotech Co., Incheon, Korea) containing 3 l of a liquid medium composed of 1.5% (w/v) sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.1% yeast extract, 2.0% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, and 0.05% trace elements, at 30°C, 500 rpm, and 0.3 vvm for 96 h. A 2 M acetic acid solution was fed intermittently to maintain the pH at 8.0.

Purification of Biopolymer

The extracellular biopolymer excreted from the isolated strain was mixed with three volumes of absolute ethanol and precipitated at 4°C for 12 h [28]. The precipitant was then treated twice with 2 M NaOH at 121°C for 10 min to remove the residual protein and then neutralized with 2 M HCl to near neutrality. The recovered biopolymer was lyophilized after dialysis against distilled water.

Determination of Molecular Weight and Electric Charge of Purified Biopolymer

The molecular weight of the purified biopolymer was measured by gel permeation chromatography using an Agilent 1100 system equipped with a PSM 3000 column and RI detector (Agilent Technologies, Palo Alto, CA, U.S.A.) using dextran-polysaccharide standards (Phenomnex Inc., Torrance, CA, U.S.A.).

The electric charge was determined using agarose gel electrophoresis [11] after minor modification. Five volumes of 1% (w/v) purified biopolymer solution were mixed with one volume of a gel-loading buffer, containing 0.25% dyes (Sigma Chemical Co., St. Louis, MO, U.S.A.), 40% sucrose, toluidine blue O for a cationic polymer, and Congo red for an anionic polymer. Five μl of the above mixed sample solution was then loaded on 0.5% agarose gel in 0.1 M Tris-borate buffer (pH 8.3) and developed by electrophoresis at 200 V for 10 min against chitosan, alginate, carboxymethylcellulose (CMC), and blue dextran (Sigma Chemical Co.).

Measurement of Flocculating Activity

The flocculating activity of the culture broth was measured by the method of Kurane *et al.* [13], by which 1 ml of the culture broth was vigorously mixed with 9 ml of 5 g/l kaolin (Kishida Chemical Co., Osaka, Japan) suspension at pH 7.0. After standing for 5 min, absorbance of the upper phase was measured at 550 nm. The flocculating efficiency was defined as $100 \times \{(B-A)/B\}$, where A was the absorbance of the sample solution and B was that of distilled water mixed with 9 ml of the kaolin suspension.

The flocculating activity of the purified biopolymer was also evaluated using other suspended solids, such as active carbon, soil, acidic and basic clay, and microbial cells, including Gram-negative bacteria *Escherichia coli*, Gram-positive bacteria *Bacillus subtilis*, and yeast *Sacharomyces cerevisiae*, at neutral pH [23] and 10 mg/l concentration.

Determination of Hexosamine Content of Biopolymer

The hexosamine content in the biopolymer was determined by the modified Elson-Morgan method [10] using glucosamine (Sigma Chemical Co.) as the standard. The purified biopolymer or crude biopolymer in the culture broth was completely hydrolyzed in 6 M HCl for 2 h and neutralized with 6 M NaOH. The hydrolysate was mixed with one volume of 5% (v/v) acetylacetone dissolved in 1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6), and then heated at 105°C for 15 min. The cooled solution was mixed with five volumes of ethanol and one volume of Ehrlich's reagent prepared with 0.8 g of ρ-dimethylaminobensaldehyde dissolved in 30 ml of ethanol and 30 ml of HCl, and incubated at 65°C for 10 min for colorization, and then the absorbance was measured at 530 nm.

Monosaccharide and Elemental Composition Analysis

The monosaccharide composition was determined by gas chromatography (GC) using an Autosystem XL Gas Chromatograph (Perkin-Elmer Ins., Norwalk, CA, U.S.A.)

with a flame ionization detector and SP-2380 column (Supelco, Bellefonte, PV). For GC analysis, the purified biopolymer was modified to methanolysis, followed by subsequent trimethysilylation [1, 14]. An element analysis was carried out using an Elemental Analyzer (Elementar Vario EL, Elementar Analysensysteme, Germany).

FT-IR and ^{13}C-NMR Analyses

The FT-IR spectrum was recorded using an FT-IR spectrometer (Galaxy 7020A, Mattson Instruments, Inc., Fremont, CA, U.S.A.), after preparation of KBr pellets [8]. For ^{13}C-NMR spectroscopy, the biopolymer was dissolved with 2% (v/v) DCl in D_2O at a concentration of 30 mg/ml and reacted at 80°C [19, 30]. The ^{13}C-NMR spectrum was obtained from FT-NMR spectroscopy (Varian Unity Inova, VSEA, Gloucester, MA, U.S.A.) at 500 MHz with an acquisition time of 1.30 s.

Measurement of Bleaching Efficacy

A mixture of 1 ml of the biopolymer (50 mg/l) and 9 ml of a dye solution (100 mg/l) was left to stand for 1 h, and the absorbance of the upper layer was then measured at different wavelengths: 500 nm for acid red 4, 600 nm for dispersed blue 1, and 530 nm for basic red 2 (Aldrich Chemical Co., Milwaukee, WI, U.S.A.).

Results and Discussion

Isolation of Strain Excreting Hexosamine Biopolymer and Its Phenotypic Characteristics

The strains forming mucous and ropy colonies on the screening agar plate were picked up, and then re-cultivated in a basal liquid medium. Based on the hexosamine content and flocculating efficiency of the excreted biopolymer, a strain BL-2 was isolated from the spoiled leaves of Chinese cabbage. The isolated strain BL-2 was Gram-negative, facultative anaerobic, catalase positive, and negative on oxidase and urease. The strain also exhibited an acid-forming capability from glucose and other sugars, KCN restriction, positive on methyl red and Voges-Proskauer tests, motility, and yet was indole production negative (data not shown). It was a straight rod-shaped bacterium, as shown in Fig. 1. According to *Bergeys' Manual of Systematic Bacteriology* [12], the isolated strain BL-2 seemed to belong to the family *Enterobacteriaceae.*

Phylogenetic Analysis of Isolated Strain BL-2

The 16S rDNA sequence of the isolated bacterium BL-2 was determined and it was registered in the GenBank (accession number, AY488028). It showed as high as 99% homology with the genus *Enterobacter*, including *Pantoea agglomerans* SP1 (AP199029), *Enterobacter agglomerans* (AF157688), and *Enterobacter colacea* (AF157695) and belonged to *Enterobacteriaceae* families by BLAST [16].

A phylogenetic tree was also constructed with ten other species in the *Enterobacteriaceae* family in the GenBank/EMBL public databases [31]. As shown in Fig. 2, the closest relatives appeared to be *Pantoea agglomerans* SP1, *Enterobacter agglomerans*, and *Enterobacter colacea*; however, the evolutional distance was distinctly different from other *Enterobacter* species. The isolated strain BL-2, which excretes the hexosamine biopolymer and showed flocculating activity, seems to be a new strain. It was deposited as *Enterobacter* sp. BL-2 in the Korean Agricultural Culture Collection (accession number, KACC 91086).

pH-Stat Fed-Batch Cultivation of New *Enterobacter* sp. BL-2

The new strain *Enterobacter* sp. BL-2 was facultative anaerobic, and also preferably assimilated acetic acid as a carbon source. *Enterobacter* sp. BL-2 was cultivated in pH-stat fed-batch culture containing a basal medium, using 2 M acetic acid as the fed-batch feeding stock to maintain

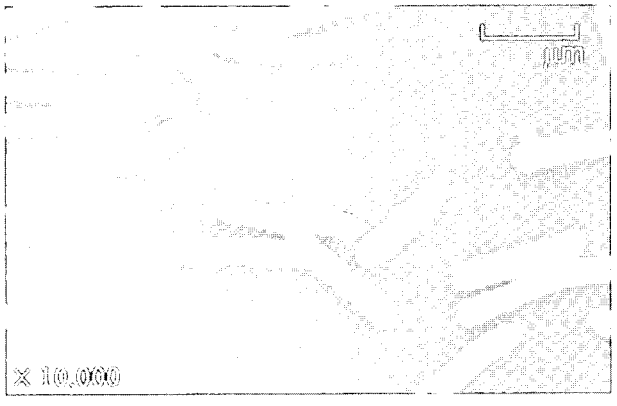

Fig. 1. Scanning electron microphotography (SEM) of new strain BL-2 which produces bioflocculant containing high hexosamine.

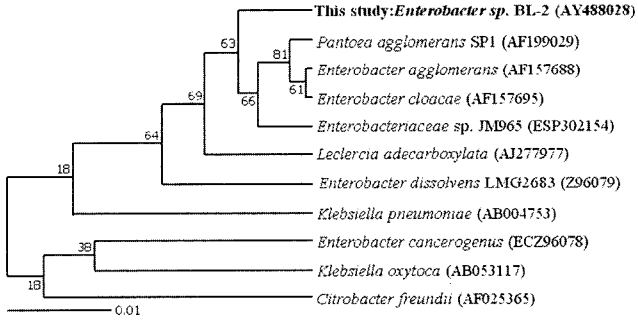

Fig. 2. Phylogenetic tree of 16S rDNA for new strain, BL-2, and other strains from the *Enterobacteriaceae* family.
The phylogenetic analysis was performed using the neighbor-joining method adapted from www.genebee.msu.su. The scale bar indicates 0.01 substitutions per site, while the numbers at the nodes are the percentage of bootstrap confidence values based on 1,000 replicates.

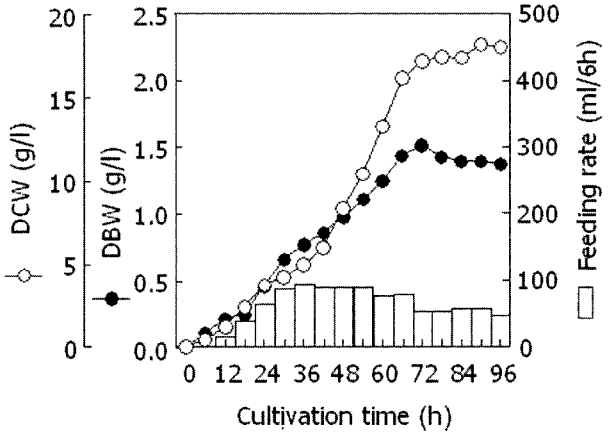

Fig. 3. pH-stat fed-batch cultivation of *Enterobacter* sp. BL-2 for excreting production of bioflocculant containing high hexosamine.

The cells were cultivated in a basal medium, containing 1.0% (w/v) sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2.0% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, and 0.05% trace elements, at 500 rpm and 0.3 vvm for 96 h using 2 M acetic acid as the feeding stock to maintain pH at 8.0. Abbreviations: DCW, dried cell weight; DBW, dried biopolymer weight.

pH at 8.0 for the overproduction of the hexosamine biopolymer. As shown in Fig. 3, the biopolymer excretion was closely associated with cell growth, and 1.53 g/l of the biopolymer was excreted in 72 h.

Purification and Chemical Composition of Biopolymer Excreted from *Enterobacter* sp. BL-2

The excreted crude biopolymer was purified by a three-step procedure: ethanol precipitation, followed by two-step 2 M NaOH treatments for deproteinization. The composition of the initial biopolymer preparation was of 63.4% hexosamine, 21.9% protein, and 14.7% miscellaneous compounds. As shown in Table 1, the recovery yields of the biopolymer were 98.6%, 86.9%, and 78.4% after the ethanol precipitation, and the first and second NaOH treatments, respectively. The ethanol precipitant was treated by protease, but the flocculating activity was not influenced by the above treatment, implying that the flocculating activity originated from the hexosamine fraction rather than the protein fraction (data not shown). The protein in the ethanol precipitated biopolymer can successfully be excluded through the two-step NaOH treatments rather than through expensive protease treatment.

Table 2. Monosaccharide and elemental compositions of purified biopolymer.

Monosaccharide composition (molar ratio)	
Glucosamine	86.4
Rhamnose	1.6
Galactose	1.0
Elemental composition (%, w/w)	
Carbon	34.5
Hydrogen	7.1
Oxygen	52.4
Nitrogen	5.9
Sulfur	trace

The elemental composition of the purified biopolymer was 34.5% carbon, 7.1% hydrogen, 52.4% oxygen, 5.9% nitrogen, and a trace amount of sulfur. As shown in Table 2, it was also composed of glucosamine, rhamnose, and galactose at the molar ratio of 86.4:1.6:1.0, respectively, implying that the excreted biopolymer is almost a homo-biopolymer, mainly consisting of glucosamine, yet containing a small amount of rhamnose and galactose.

The extracellular microbial biopolymer containing as high as 95.1% glucosamine has never been reported in the literature, except in a recent work of Fujita *et al.* [6], who reported a microbial bioflocculant containing 29.4% of hexosamine. The microbial polyglucosamine biopolymer from new *Enterobacter* sp. BL-2 seems to be quite a distinct novel biopolymer, and was named as microbial polyglucosamine biopolymer PGB-1.

Molecular Weight and Electric Charge of Polyglucosamine Biopolymer PGB-1

As shown in Fig. 4A, the homogeneity of the purified polyglucosamine biopolymer PGB-1 was confirmed by a compact peak in a gel permeation chromatogram. The molecular weight of the homo-polyglucosamine biopolymer PGB-1 was measured to be about 106 kDa (Fig. 4B), showing a relatively small molecular weight, compared with other known microbial carbohydrate bioflocculants, such

Table 1. Purification of biopolymer excreted from *Enterobacter* sp. BL-2.

Purification	DPW[1] (g/l)	Yield[2] (%)	Composition (%)		
			Hexosamine	Protein	Others
Crude biopolymer	1.53	100.0	63.4	21.9	14.7
Ethanol treatment for precipitation	1.51	98.6	77.3	17.2	5.0
NaOH treatment (1) for deproteinization	1.33	86.9	87.5	6.7	5.8
NaOH treatment (2) for deproteinization	1.20	78.4	95.1	N.D.	4.9

[1] DPW: Dried polymer weight.
[2] Yield: recovery yield.

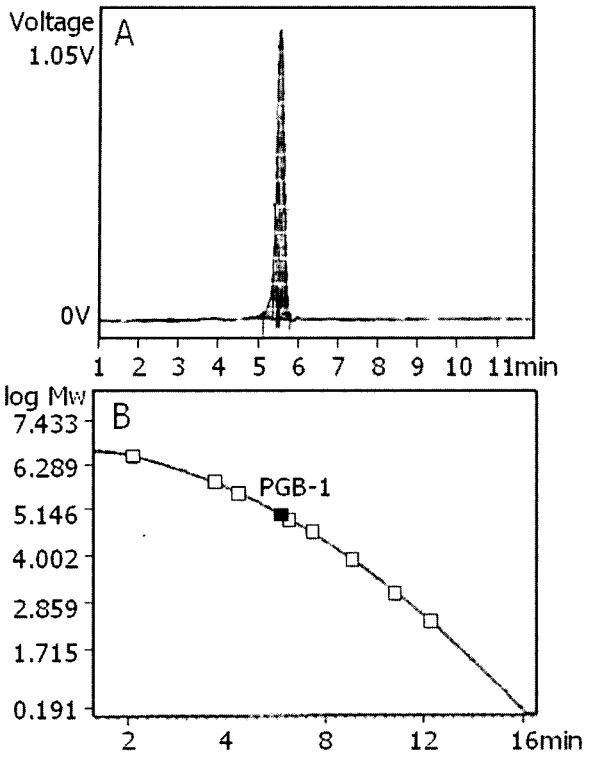

Fig. 4. Gel permeation chromatogram (A) and molecular weight calibration curve (B) of polyglucosamine biopolymer PGB-1.

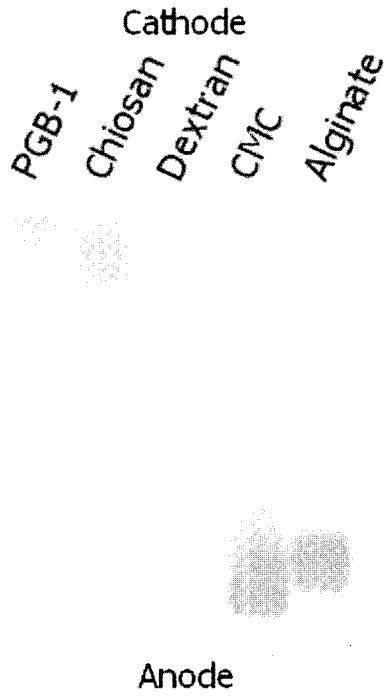

Fig. 5. Agarose gel electrophoresis of polyglucosamine biopolymer PGB-1 with chitosan, dextran, CMC, and alginate.

as 320 kDa for *Citrobacter* sp. [6], 2,600 kDa for *Baccillus mucilaginosus* and *Klebsiella* sp. [3, 4], and 2,500 kDa for *Enterobacter* sp. [26]. The average molecular weight was measured to be 105 kDa, corresponding to the polydiversity (M_w/M_n) of 1.01. This implies that the purified polyglucosamine biopolymer PGB-1 is a near homogeneous polymer.

The electric charge was determined by the location of the PGB-1 band in agarose gel electrophoresis, as shown in Fig. 5, and the band moved toward the cathode to almost the same position as chitosan, which is known as a unique natural cationic polymer. Meanwhile, the nonionic dextran did not move, whereas the anionic CMC and alginate moved toward the anode. This implied that the polyglucosamine biopolymer PGB-1 is a cationic biopolymer having an electric charge similar to chitosan.

FT-IR and NMR Spectra of Cationic Polyglucosamine Biopolymer PGB-1

The FT-IR spectrum of PGB-1 was composed of an OH band at 3,360 cm^{-1}, CH band at 2,880 cm^{-1}, CH_2 band at 1,420 cm^{-1}, CH_3 band at 1,380 cm^{-1}, primary amine band at 1,665 cm^{-1}, and secondary amine at 1,550 cm^{-1}. It was also compared with the well-known natural polyglucosamine polymer chitosan from crab shell. As shown in Figs. 6A and 6B, the transmission pattern of PGB-1 was very similar to that of chitosan. However, the secondary amine band at 1,550 cm^{-1} was clearer than chitosan, and a small unknown peak also appeared near 3,700 cm^{-1}, indicating the existence of another functional group in the structure of PGB-1.

In the ^{13}C-NMR spectrum, the main carbon peaks appeared as follows: C_1 at 98.25, C_4 at 77.70, C_5 at 75.44, C_3 at 70.76, C_6 at 61.04, and C_2 at 56.65 ppm, as shown in Figs. 7A and 7B. The carbon peaks of polyglucosamine biopolymer PGB-1 were also almost identical to those of the natural glucosamine polymer chitosan, indicating that the biopolymer PGB-1 is composed of a glucosamine unit and (1,4)-glycosidic bond [8, 30]. No microbial polyglucosamine biopolymer with structural features similar to natural chitosan has been observed, although the accurate linkage and coupling scheme of the monomers or functional group need to be investigated.

Flocculation and Bleaching Efficacy of Cationic Polyglucosamine Biopolymer PGB-1

As shown in Table 3, the novel cationic polyglucosamine biopolymer PGB-1 excreted from *Enterobacter* sp. BL-2 can effectively flocculate most of the low-molecular weight suspended solids even more effectively than the natural chitosan. It flocculated the Gram-negative bacteria *E. coli* and *S. cerevisiae* more effectively than the chitosan, but was less effective on the Gram-positive *B. subtilis*. In contrast to the chitosan, the microbial polyglucosamine biopolymer PGB-1 can effectively flocculate and bleach

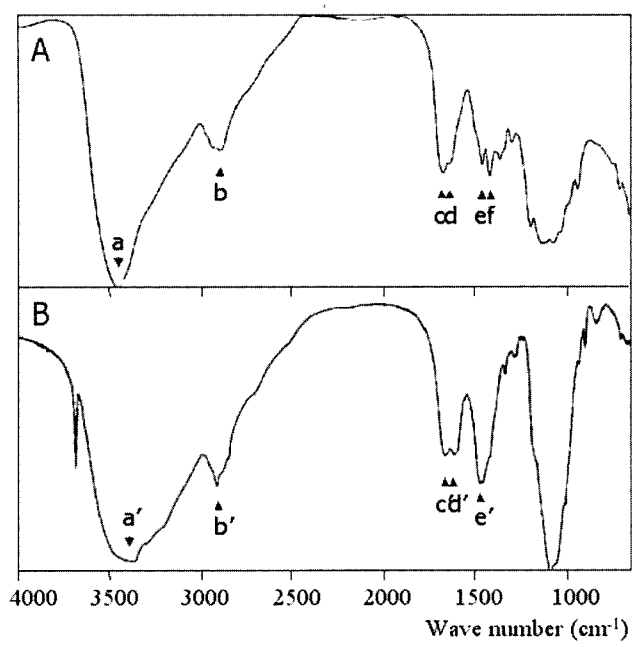

Fig. 6. FT-IR spectrum of chitosan (A) and purified polyglucosamine biopolymer PGB-1 (B).
The a-f in (A) are characteristic bands of chitosan (a, OH band; b, CH band; c, primary amine band; d, secondary amine band; e, CH_2 band; and f, CH_3 band), while the a'-e' in (B) are the characteristic bands of polyglucosamine biopolymer PGB-1.

most of the dissolved dyes, especially acidic and dispersed dyes, compared to basic dyes.

This may be due to the different functional group in both polyglucosamine biopolymers or the much lower molecular weight of the microbial polyglucosamine biopolymer PGB-1, compared to natural chitosan and other previously reported microbial bioflocculants. The present microbial cationic polyglucosamine biopolymer PGB-1 can also be potentially applied to other biotechnology fields where the natural chitosan from crab shell has been utilized.

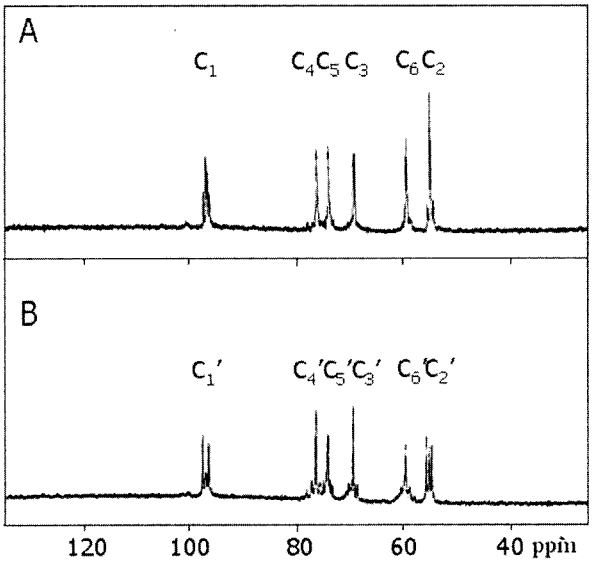

Fig. 7. ^{13}C-NMR spectrum of chitosan (A) and polyglucosamine biopolymer PGB-1 (B).
The C_1-C_6 in (A) are the six carbons of glucosamine in chitosan, while the C_1'-C_6' in (B) are those in polyglucosamine biopolymer PGB-1.

Table 3. Flocculating efficacies of cationic polyglucosamine biopolymer PGB-1 and chitosan on various suspended solids, microbial cells, and dyes.

Test materials	F. E.[1] (%)	
	PGB-1	Chitosan
Suspended solid		
Active carbon	84	65
Soil solid	91	82
Kaolin clay	98	98
Al_2O_3	90	85
$Ca(OH)_2$	83	79
Microbial cell		
E. coli	90	93
B. subtilis	21	72
S. cerevisiae	52	11
Dye		
Acid red 4	71	0
Dispersed blue 1	86	0
Basic red 2	0	0

[1] Flocculating efficiency.

Acknowledgment

This work was supported by the Brain Korea 21 project in 2004.

REFERENCES

1. Chaplin, M. F. and J. F. Kennedy. 1986. *Carbohydrate Analysis: A Practical Approach*, pp. 2–13. IRL Press, Oxford, Washington DC.
2. Dearfield, K. L., C. O. Abernathy, M. S. Ottley, J. H. Branter, and P. F. Hayes. 1988. Acrylamide: Its metabolism, developmental and reproductive effects, genotoxicity and carcinogenicity. *Mutat. Res.* **195:** 45–77.
3. Deng, S. B., R. B. Bai, X. M. Hu, and Q. Luo. 2003. Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. *Appl. Microbiol. Biotechnol.* **60:** 588–593.
4. Dermlim, W., P. Prasertsan, and H. Doelle. 1999. Screening and characterization of bioflocculant produced by isolated *Klebsiella* sp. *Appl. Microbiol. Biotechnol.* **52:** 698–703.
5. Fujita, M., M. Ike, J. H. Jang, S. M. Kim, and T. Hirao. 2001. Bioflocculation production from lower-molecular fatty acids as a novel strategy for utilization of sludge digestion liquor. *Water Sci. Technol.* **144:** 237–243.

6. Fujita, M., M. Ike, S. Tachibana, G. Kitada, S. M. Kim, and S. Inoue. 2000. Characterization of a bioflocculant produced by *Citrobacter* sp. TKF04 from acetic and propionic acids. *J. Biosci. Bioeng.* **89:** 40–46.
7. Gerhardt, P., G. E. Murray, W. A. Wood, and N. R. Kreg. 1994. *Methods for General and Molecular Bacteriology*, pp. 8–292. American Society for Microbiology, Washington DC.
8. Harish Prashanth, K. V., F. S. Kittur, and R. N. Tharanathan. 2002. Solid state structure of chitosan prepared under different N-deacetylating conditions. *Carbohydr. Polym.* **50:** 27–33.
9. Jang, J. H., M. Ike, S. M. Kim, and M. Fujita. 2001. Production of a novel bioflocculant by fed-batch culture of *Citrobacter* sp. *Biotechnol. Lett.* **23:** 593–597.
10. Jang, J. H., H. C. Hia, M. Ike, T. Hirao, T. Yoshida, and M. Fujita. 2002. Acid hydrolysis and determination of total hexosamine in biopolymer produced by *Citrobacter* sp. TKF04 using modified Elson-Morgan method, p. 187. *In: Proceedings of the 80th Annual Meeting of the Society for Biotechnology of Japan*, Osaka, Japan.
11. Jegasothy, H., M. Wootton, and R. J. Fairclought. 2000. Agarose gel electrophoresis of wheat starch. *J. Cereal Sci.* **31:** 75–78.
12. Kreg, N. R. and J. G. Holt. 1984. *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Maryland.
13. Kurane, R., K. Toeda, and T. Suzuki. 1986. Culture conditions for production of microbial flocculant by *Rhodococcus erythropolis*. *Agric. Biol. Chem.* **50:** 2309–2313.
14. Lee, J. W., F. Dong, W. G. Yeomans, A. L. Allen, R. A. Gross, and D. L. Kaplan. 2001. Direct incorporation of glucosamine and *N*-acetylglucosamine into exopolymers by *Gluconacetobacter xylinus* (=*Acetobacter xylium*) ATCC 10245: Production of chitosan-cellulose and chitin-cellulose exopolymers. *Appl. Environ. Microbiol.* **67:** 3970–3975.
15. Masters, C. L., G. Multhaup, G. Simms, J. Pottgisser, R. N. Martins, and K. Beyreuther. 1985. Neuronal origin of a cerebral amyloid: Neurofibrillary tangles or Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO J.* **4:** 2757–2763.
16. Moon, G. S., C. H. Kang, Y. R. Pyun, and W. J. Kim. 2004. Isolation, identification, and characterization of a bacteriocin-producing *Enterococcus* sp. from kimchi and its application to kimchi fermentation. *J. Microbiol. Biotechnol.* **14:** 924–931.
17. Nakamura, J., S. Miyashiro, and Y. Hirose. 1976. Screening, isolation and some properties of microbial cell flocculants. *Agric. Biol. Chem.* **40:** 377–383.
18. Oh, H. M., S. J. Lee, M. H. Park, H. S. Kim, H. C. Kim, J. H. Yoon, G. S. Kwon, and B. D. Yoon. 2001. Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49. *Biotechnol. Lett.* **23:** 1229–1234.
19. Ramos, V. M., N. M. Rodríuez, M. S. Rodríuez, A. Heras, and E. Agull. 2003. Modified chitosan carrying phosphoric and alkyl groups. *Carbohydr. Polym.* **51:** 425–429.
20. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
21. Salehizadeh, H. and S. A. Shojaosadati. 2001. Extracellular biopolymer flocculants: Recent trends and biotechnological importance. *J. Biotechnol. Adv.* **19:** 371–385.
22. Sandford, P. A. and G. P. Hutchings. 1987. Chitosan - a natural, cationic biopolymer: Commercial applications, pp. 363–375. *In* M. Yalpani (ed.), *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications.* Elsevier Science Publishers, Amsterdam.
23. Shih, I. L., Y. T. Van, L. C. Yeh, H. G. Lin, and Y. N. Chang. 2001. Production of a biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties. *Biores. Technol.* **78:** 267–272.
24. Toeda, K. and R. Kurane. 1991. Microbial flocculant from *Alcaligenes cupidus* KT 201. *Agric. Biol. Chem.* **55:** 2663–2664.
25. Yeo, S. H., O. S. Lee, I. S. Lee, H. S. Kim, T. S. Yu, and Y. J. Jeong. 2004. *Gluconacetobacter persimmonnonis* sp. nov., isolated from Korean traditional persimmon vinegar. *J. Microbiol. Biotechnol.* **14:** 276–283.
26. Yokoi, H., T. Aratake, J. Hirose, S. Hayashi, and Y. Takasaki. 2001. Simultaneous production of hydrogen and bioflocculant by *Enterobacter* sp. BY-29. *World J. Microbiol. Biotechnol.* **17:** 609–631.
27. Yoon, S. H., J. K. Song, S. J. Go, and J. C. Ryu. 1998. Production of biopolymer flocculant by *Bacillus subtilis* TB-11. *J. Microbiol. Biotechnol.* **8:** 606–612.
28. Yu, K. W., K. S. Shin, Y. M. Choi, and H. J. Suh. 2004. Macrophage stimulating activity of exo-biopolymer from submerged culture of *Lentinus edodes* with rice bran. *J. Microbiol. Biotechnol.* **14:** 658–664.
29. Watanebe, M., Y. Suzuki, K. Sasaki, Y. Nakashimada, and N. Nishio. 1999. Flocculating property of extracellular polymeric substance derived from a marine photosynthetic bacterium, *Rhodovulum* sp. *J. Biosci. Bioeng.* **87:** 625–629.
30. Wishart, D. S., C. G. Bigam, J. Yao, F. Abildgaard, H. J. Dyson, E. Oldfield, J. L. Markley, and B. D. Sykes. 1995. ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **6:** 135–140.
31. Zeng, X., X. Xiao, P. Wang, and F. Wang. 2004. Screening and characterization of psychrotropic, lipolytic bacteria from deep-sea sediments. *J. Microbiol. Biotechnol.* **14:** 952–958.