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Production of Biopolymer Flocculant by *Bacillus subtilis* TB11

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Abstract A microbial flocculant-producing gram-positive bacterium, strain TB11, was isolated from soil samples, and was identified as *Bacillus subtilis* by using the Midi system, the Biolog system, 16S rDNA sequence analysis, and some physiological and morphological characteristics. The maximum flocculant capsular biopolymer of TB11 strain (BCP, 4.9 mg/ml) was obtained when it was grown in GA broth medium containing 3% glutamic acid, 2% glycerol, 0.5% citric acid, 0.5% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, and 0.004% $FeCl_3 \cdot 6H_2O$, pH 7.2, at 30°C for 70 h with shaking. When glycerol was used as an additional carbon source in the GA medium, TB11 produced only flocculant BCP without any by-product. The flocculant (BCP) was found to aggregate suspended kaolin and activated charcoal powder without cations, and its flocculating activity was significantly enhanced by the addition of bivalent cations such as Ca^{2+}, Zn^{2+}, and Mn^{2+}. The flocculation activity by addition of Ca^{2+} was high in an acidic pH 4.0. In the case of Zn^{2+}, high flocculating activity remained without significant loss in the broad range of pH 4.0 to 9.0.

Key words: Flocculant, biopolymer, *Bacillus subtilis*, Midi system, Biolog, 16S rDNA

Many kinds of flocculating agents are used in a wide range of industrial fields such as tap water production, wastewater treatment, dredging, fermentation processes, and food industries. Flocculants are generally divided into three major groups: [1] inorganic flocculants: aluminum sulfate and polyaluminum chloride, [2] organic synthetic high polymers: polyacrylic acid, polyacrylamide derivatives, and polyethyleneimine, and [3] natural flocculants: chitosan, sodium alginate and microbial flocculants. But widely used inorganic flocculants and synthetic polymer flocculants give rise to environmental problems in that some of them are not readily biodegradable and intermediate products of their degradation are harmful to humans, In recent years, to solve these environmental problems, utilization of flocculants produced by microorganisms has been anticipated due to their biodegradability and the harmlessness of their degradation intermediates to the environment. Most flocculants produced by microorganisms are usually high molecular weight polymers (>10^6 Da), and some are proteins, glycoproteins, polysaccharides, DNA or others [3, 4, 10, 14]. For example, *Rhodococcus erythropolis* S-1 produces a protein flocculant [9]. Some polysaccharide flocculants are produced by *Alcaligenes* KT201 or *Alcaligenes latus* B-16 [5, 6]. It has also been reported that the flocculant produced by *Arthrobacter* sp. SB-6 is a kind of nucleoprotein [10].

This paper deals with the screening of new flocculant-producing bacteria, the taxonomic characteristics of the isolated bacteria, and the composition of broth medium for its flocculant production, and also discusses some properties of a flocculating agent produced by *Bacillus subtilis* TB11.

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MATERIALS AND METHODS

Screening for Flocculant-producing Bacterium

A flocculant-producing bacterium was screened from soil by using the flocculant test materials of kaolin clay. Soil samples collected from many places were suspended in sterile distilled water. The diluted suspensions were spread onto YE agar plates composed of 2.0% yeast extract, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 1.2% agar, pH 7.4. After incubation at 30°C for 2 days, one loop of highly mucous colony was taken from an agar plate and suspended in 1 ml water, and then the cells were removed by centrifugation. This mucous solution (100 µl) was added to 10 ml of 5000 ppm kaolin solution in a test tube, and the mixture was observed following a 5 min wait.

Taxonomic characterization of TB11 Strain

The identification of the isolated TB11 strain was based on fatty acid analysis of the cell wall by the MIDI

(Microbial Identification Inc.) system, its 16S rDNA sequences analysis, utilization of 95 sole-carbon sources by the Biolog gram-positive (GP) system, the Biolog gram-negative (GN) system, and physiological characteristics. The culture temperature was 30°C.

To prepare samples for MIDI (Microbial Identification Inc.) analysis, fatty acid methyl esters were obtained from the wet cell biomass (40 mg) by saponification, methylation and extraction according to the instructions given by MIDI, and were separated by gas chromatography (HP 5890A). Identity and composition of fatty acids were determined by the Microbial Identification System Library Software (Midi, Newark, Del, USA).

For PCR amplification of the 16S rDNA genes, crude cell lysates were prepared and used as sources of PCR templates. 16S rDNA fragments that corresponded to 8 bp to 1,510 bp of the *Escherichia coli* 16S rDNA were amplified by PCR using universal primers fD1 and rP2 [11] and purified by a combination of agarose gel electrophoresis and glass powder binding. Detailed information on these procedures has been given previously [2]. The gel-purified 16S rDNA fragments were subcloned with a PCR cloning ligation kit (Pharmacia, Upsala, Sweden). Transformation of *E. coli* DH5α was performed according to the manual of standard molecular cloning [8]. Sequencing was performed with subcloned 16S rDNA as the template according to Sanger's method [8]. The 16S rDNA sequence determined in this study was analyzed in the GenBank nucleotide sequence databases.

Procedures for Biolog analysis were performed as follows. The inoculum for TB11 strain was prepared from Biolog Universal Growth Medium (BUGM). The bacterium were grown for 4 to 18 h. A swab was gently rolled over the colonies to prevent carryover of nutrients from the agar medium into the saline (0.85%) suspension of bacteria. The inoculumn should be used within 10 min. The plates were inoculated with 150 µl per well and incubated at 30°C. The plates were read at 4 h for the ability of the bacterium to utilize 95 carbon sources on a computer-controlled microplate reader. Its metabolic profiles were analyzed on the database software provided by Biolog System (Biolog, Inc., Hayward, U.S.A.).

TB11 Cultures

Spore suspensions for inocula were prepared by growing TB11 strain on nutrient agar with 0.5% yeast extract for 5 days at 30°C. The spores were harvested in water, washed twice, heated at 65°C for 30 min to kill vegetative cells, and then stored at -20°C. Usually, an inoculum of 3×10^8 spores per 200 ml of broth media in a 1-l Erlemmeyer flask was used by diluting the stock suspension with water for shaking cultures.

Both solid media (YE) and broth media (GA) of TB11 strain for flocculant polymer production were prepared as follows: YE agar media; 2.0% yeast extract, 0.1% KH_2PO_4, 0.1% $MgSO_4\cdot7H_2O$, GA broth media; 2% glycerol, 0.5% ammonium chloride, 3% L-glutamic acid, 0.05% K_2HPO_4, 0.05% $MgSO_4\cdot7H_2O$, 0.004% $FeCl_3\cdot6H_2O$, at pH 7.2. The media were used after autoclaving at 121°C for 15 min. Cultivation was done aerobically with shaking (150 rpm) at 30°C for 3 days.

Isolation of Bacterial Capsular Polymer (BCP)

The isolation methods of flocculant capsular polymer of TB11 strain grown on YE agar media, designated as BCP, were performed as follows. Spore suspension (100 µl of 10^8 cells/ml) was spread evenly over the YE agar plates as a lawn. After 48 h of growth at 30°C, the cells were harvested from the growth medium by scraping with a glass micro slide into a beaker containing 0.01 M Tris (pH 7.1)-0.03 M NaCl, and blended for 2 min at room temperature to shear the capsule from the cells. On blending, the cells were then removed by centrifugation at 16,000×g, and the supernatant were combined with 4 volumes of cold ethanol and left overnight at -20°C. The precipitated capsule was concentrated by centrifugation at 48,000×g for 20 min at -20°C, resuspended in a minimal volume of H_2O, and dialyzed against distilled water (DW) for 24 to 48 h at 4°C. The dialysate product obtained was lyophilized.

BCP isolation from the GA culture broth was performed as follows. The GA culture broth of TB11 cultivated for 3 days was diluted with an equal volume of DW, and centrifuged at 16,000×g for 30 min. The supernatant was poured into 3 volumes of ethanol. After standing for overnight at 4°C, the resulting precipitate was collected by centrifugation (12,000×g, 20 min) and washed with ethanol. The precipitate was dissolved in water, followed by dialysis against deionized water. The dialyzed solution was centrifuged (16,000×g, 30 min) and the supernatant was lyophilized to give BCP.

Assay of Flocculating Activity

Assay of flocculating activity was carried out as follows. Five µl of culture broth or biopolymer flocculant solution, 0.05 ml of 100 mM $CaCl_2$, and 10 ml of 5 g/l kaolin suspension adjusted to pH 7.0 were mixed in a test tube (I.D.: 18 mm ×Height: 200 mm). The mixture was stirred with a Vortex mixer and left standing for 5 min. Two ml of supernatant was carefully removed from the upper layer in the test tube and its optical density at 550 nm $(OD_{550})_T$ was measured with a spectrophotometer (Baush & Lomb, Spectronic 20). A control experiment without a culture broth or biopolymer flocculant solution was carried out in the same manner and the optical density $(OD_{550})_C$ was measured. Flocculating activity was calculated using the following equation.

Table 1. Cellular fatty acid (CFA) composition of TB11 and *B. subtilis* (ATCC 37015).

Strain	Profiles of Fatty Acids (%)									
	14:0 iso	14:0	15:0 iso	15:0 ante	16:0 iso	16:1 w11c	16:0 iso	17:1 w10c	17:0 iso	17:0 ante
TB11	0.69	1.68	**22.93**	**38.93**	1.21	3.58	10.96	1.70	**8.58**	**7.77**
ATCC 37015	1.14	0.62	**32.75**	**37.37**	2.02	2.03	3.02	2.43	**8.45**	**7.82**

$$\text{Flocculating activity} = 1/(OD_{550})_T - 1/(OD_{550})_c$$

The effects of various cations on the flocculating activity of BCP in a kaolin suspension (5,000 ppm) were examined using NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $MnSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, and $ZnCl_2$ as the cation sources.

RESULTS AND DISCUSSION

Screening for Flocculant Producing Bacteria

Seventeen strains having the ability to flocculate kaolin clay, activated charcoal, and muddy soils were isolated from 52 different regional soil samples. The TB11 strain was found to have the highest flocculating activity for kaolin clay, activated charcoal powder, and muddy soil (Fig. 1). It was isolated from Byung-Jeom in Kyung-Gi province.

Identification of TB11

The TB11 strain was a gram-positive rod, motile with peritrichous flagella, and had aerobic growth and spore-forming ability. The bacterium was grown in nutrient broth containing 10% NaCl, not grown at 55°C.

The fatty acid profiles and composition of TB11 strain were investigated by the MIDI system. Table 1 shows that strain TB11 is characterized by a relatively simple profile made up largely of anti-iso $C_{15:0}$ (39%), iso $C_{15:0}$ (23%), $C_{16:0}$ (11%), iso $C_{17:0}$ (8.6%), and anti-iso $C_{17:0}$ (7.8%). The fatty acid profiles of TB11 were attributed to the *Bacillus* fatty acid group according to analysis of the data generated with numbers of *Bacillus* strains. The phenotypic expression of fatty acids within bacterial cells depends on various factors including medium composition, growth temperature, and growth rate. The variability of the fatty acid profile analysis was assessed by repeated testing of the strain.

In recent years, the nucleotide sequence comparison of 16S rDNA sequence has also been used as a powerful tool for the identification of various bacterial species and the determination of the exact phylogenetic and taxonomic positions of similar genera and species because 16S rDNA is highly conserved in evolutionary aspects. We determined the primary structure of the 16S rDNA (1431 bp) by sequencing a subcloned 16S rDNA of TB11. As a result of a homology search with the GenBank databases, TB11 strain was found to be most similar to members of the *Bacillus* species, with *B. subtilis* as the nearest phylogenetic neighbour. The levels of binary sequence similarity between our TB11 strain and the *B. subtilis* is 99%.

The Biolog system was introduced in 1989 for the identification of aerobic gram-negative bacteria (enteric bacilli, nonfermenters, and fastidious species) by determination of carbon source utilization profiles. Recently, Biolog has added the capability to identify a broad range (cocci, bacilli, and spore-forming bacilli) of aerobic gram-positive

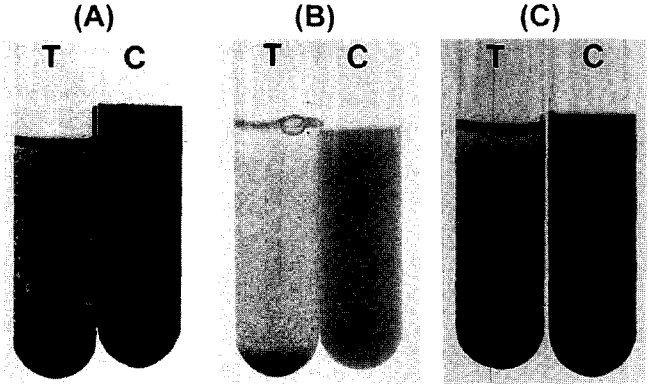

Fig. 1. Early flocculation of activated charcoal powder (A), kaolin clay (B), and muddy clay (C) suspensions with flocculant biopolymer (BCP) produced by TB11 strain.
*T: BCP addition, C: DW addition.

Table 2. Catabolic profiles of TB11 by Biolog GP microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A			++	+++	++							
B				++	++				++	+	+++	
C			++			+++					+++	
D			++	++	++					+	++	+++
E		+	+++									
F						+++			++			
G			+									
H	++		+	++	++							

*The Biolog GP Microplate are 96-well dehydrated panels containing tetrazolium violet, a buffered nutrient medium, and a different carbon source for each well, except the control, which does not contain a carbon source.
*Percent change in optical density versus A1 control well +: ≤99, ++: ≤199, +++: ≥200.

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TB11  AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC GGACAGATGG GAGCTTGCTC CCTGATGTTA GCGGCGGACG  100
B.sub ********** ********** ********** ********** ********** ********** ******G*** ********** ********** **********

TB11  GGTGAGTAAC ACGTGGGTAA CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT GGTTGTCTGA ACCGCATGGT TCAAACATAA  200
B.sub ********** ********** ********** ********** ********** ********** ********** ******T*** ********** **********

TB11  AAGGTGGCTT CGGCTACCAC TTACAGATGG ACCCGCGGCG CATTAGCTAG TTGGTGAGGT AACGGCTCAC CAAGGCGACG ATGCGTATCC GACCTGAGAG  300
B.sub ********** ********** ********** ********** ********** ********** ********** ******A*** *******G** **********

TB11  GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCG  400
B.sub ********** ********** ********** ********** ********** ********** ********** ********** ********** *********C

TB11  GCGTGAGTGA TGAAGGTTTT CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTGCCG TTCAAATAGG GCGGCTCCTT GACGGTACCT AACCAGAAAG  500
B.sub ********** ********** ********** ********** ********** ******A*** ***G**C*** ****TA**** ********** **********

TB11  CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC GCAGGCGGTT TCTTAAGTCA  600
B.sub ********** ********** ********** ********** ********** **T******* ********** ********** ********** *********T

TB11  GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA AACTGGGGAA CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ACGTGTAGCG GTGAAATGCG  700
B.sub ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

TB11  TAGAGATGTG GAGGAACACC AGTGGCGAAG GCGACTCTCT GGTTTGTAAC TGACGCTGAG GAGCGAAAGC GTGGGGAGCG AACAGGATTA GATACCCTGG  800
B.sub ********** ********** ********** ********** ***C****** ********** ********** ********** ********** **********

TB11  TAGTCCACGC CGTAAACGAT GTGTGCTAAG TGTTAGGGGG TTTCCGCCCC TTAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGGTAGC  900
B.sub ********** ********** *A******** ********** ********** ********** ********** **G******* ********** *******C**

TB11  AAGACTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC 1000
B.sub ********** ********** ********** ********** ********** ********** ********** ********** *****T**** **********

TB11  TCTGACAATC CTAGAGATAG GACGTCCCCT TCGGGGGCAG AGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG 1100
B.sub ********** ********** ********** ******-*** ********** ********** ********** ********** ********** **********

TB11  CAACGAGCGC AACCCTTGAT CTTAGTTGCC AGCATTCAGT TGGGCACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG ACGTCAAATC 1200
B.sub ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

TB11  ATCATGCCCC TTATGACCTG GGCTACACAC GTGCTACAAT GGACAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA TCCCACAAAT CTGTTCTCAG 1300
B.sub ********** *******T** ********** ********** ********** ********** **-******* ********** ********** **********

TB11  TTCGGATCGC AGTCTGCAAC TCGACTGCGT GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT TCCCGGGCCT TGTACACACC 1400
B.sub ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

TB11  GCCCGTCACA CCACGAGAGT TTGTAACACC C 1431
B.sub ********** ********** ********** *
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Fig. 2. Comparison of homology in 16S rDNA sequences between strain TB11 and *B. subtilis*.

bacteria. Ninety-five substrates contained in the Biolog GN Microplate and the Biolog GP Microplate include carbohydrates, carboxylic acids, amides, esters, amino acids, peptides, amines, alcohols, and aromatic chemicals. The Biolog system database includes information for identification of 566 species or groups of aerobic gram-negative bacteria and 250 species or groups of gram-positive bacteria and encompasses almost all known human pathogens and most important environmental species. TB11 used 27 substrates among 95 substrates of gram-positive microplate as carbon sources. The result is shown in Table 2. Strain TB11 had a metabolic profile that closely matched *B. subtilis* in the Biolog database, indicating that the profiles obtained were specific for

Bacillus species. From the above results, we inferred flocculant-producing strain TB11 as a species of *Bacillus subtilis*. We designated it *Bacillus subtilis* TB11.

BCP Production

When *B. subtilis* TB11 was grown at 30°C for 2 days on YE agar media, colonies were surrounded by mucous materials. Viscous materials extracted from these colonies were very soluble in water and have very high flocculating activities in kaolin clay suspension. They seem to be capsular polymer produced by TB11. We designated the flocculant capsular polymer of TB11 as BCP. Generally, capsules of bacteria facilitate adhesion to surfaces, promote biofilm formation, and offer protection against a wide variety of harmful substances such as antibiotics, biocides, and adverse conditions such as predation and the immune response. When *B. subtilis* TB11 was grown at 30°C for a few days on GA broth media, it was observed that the medium became highly viscous due to the production of BCP along with the bacterial growth. In the case of shaken broth culture, GA media was adequate for the production of BCP. BCP contained only a small amount of sugar as determined by the phenol-sulfuric acid method [1], indicating that BCP of TB11 was not a polysaccharide. The growth of TB11, the production of BCP, and the relative viscosity of the medium reached a maximum after incubation for 72 hr. When the culture supernatant after 72 h of cultivation was incubated at 37°C, its flocculating activity decreased rapidly (Fig. 3). These results suggest that *B. subtilis* TB11 excreted a BCP degradation enzyme with the progress of cultivation and BCP was degraded by this enzyme.

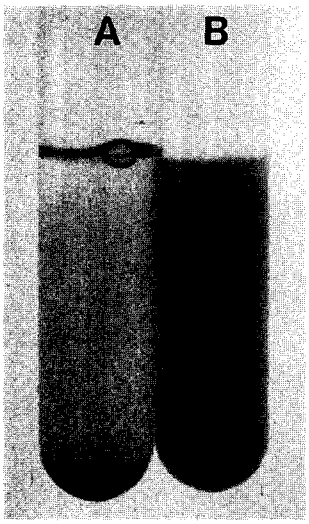

Fig. 3. Flocculation of kaolin clays with culture supernatant (A) and reaction supernatant (B), cultivated by *B. subtilis* TB11 for 3 days.

A: non-reaction culture supernatant, B: culture supernatant reacted at 37°C for 2 h.

Table 3. Production of bacterial capsular polymer (BCP) by various carbon sources.

Carbon source[1]	Growth[2]	Purified polymer (g/100 ml)	By-product[3]
None	nongrowth	-	
Glucose	++	0.72	++
Galactose	++	-	
Fructose	++	0.46	++
Glycerol	++	0.35	-
Citric acid	nongrowth	-	
Maltose	+++	0.82	++
Sucrose	+++	1.67	+++

Cells were cultivated for 70 h at pH 7.2 and 28°C.

[1]Carbon source (2 g) in the media (100 ml) with 3 g L-glutamic acid, 0.5 g NH_4Cl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g K_2HPO_4, 0.004 g $FeCl_3 \cdot 6H_2O$.

[2]Growth (cell weight/100 ml); +: <100 mg/100 ml, ++: 300~600 mg/100 ml, +++: >700 mg/100 ml.

[3]OD_{585} measured by visible spectrophotometer after phenol-sulfuric acid reaction (1) of 0.1% polymer solution. -: <0.1, ++: 0.3~0.5, +++: >0.5.

Investigation of Various Carbon Sources for BCP Production

The productivity of BCP was investigated by using 7 compounds as carbon sources in the basal media (200 ml, pH 7.2) containing 6 g of L-glutamic acid, 1 g of ammonium chloride, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of K_2HPO_4, and 0.008 g of $FeCl_3 \cdot 6H_2O$. The bacterial cells were cultivated aerobically at 30°C for 72 h. The results are shown in Table 3. Galactose was not favorable for BCP production, but favorable for growth. In contrast, sucrose, fructose, maltose, and glucose were favorable for both growth and BCP production. When the above carbon sources were used, BCP was produced together with a by-product, which seemed to be a polysaccharide. But when glycerol was used as an additional carbon source in the media, BCP was produced without any by-product. On the other hand, use of citric acid instead of glycerol as a carbon source was not adequate for the growth of TB11.

Investigation of L-Glutamic Acid, Citric Acid, or Ammonium Chloride Concentration in the Basal GA Medium for BCP Production

The BCP productivity was investigated with various concentrations of each component in the basal media (100 ml, pH 7.2) containing, 3 g of L-glutamic acid, 1 g of citric acid, 0.5 g of ammonium chloride, 1.8 ml of glycerol, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of K_2HPO_4, and 0.004 g of $FeCl_3 \cdot 6H_2O$ (Table 4). The cell dry weight, in the range of 0.3~0.6 g/100 ml of L-glutamic acid, was independent of L-glutamic acid concentration. When 3 g of L-glutamic acid was added to the medium, the highest yield of purified BCP, 4.9 mg/ml, was produced. When L-glutamic acid was not added to the medium, BCP was produced, although in small amounts.

Table 4. Effects of various concentrations of each component in GA medium on flocculant BCP production in shaken flasks.

Component in GA medium	Component conc. (%)	BCP (mg/ml)
L-glutamic acid	0	1.1
	0.5	1.6
	1.0	2.2
	2.0	3.4
	3.0	4.9
	5.0	1.8
Glycerol	0	no growth
	1	2.8
	2	4.2
	4	2.6
	8	1.5
	12	1.2
Citiric acid	0	3.1
	0.5	3.8
	1.0	4.6
	2.0	1.9
	3.0	2.1
	5.0	no growth
Ammonium chloride	0	-
	0.2	4.8
	0.5	4.1
	1.0	1.2
	2.0	no growth
$MgSO_4 \cdot 7H_2O$	0	no growth
K_2HPO_4	0	no growth
$FeCl_3 \cdot 6H_2O$	0	4.6

*Cell were grown on GA medium containing concentrations of each component to investigate effects of BCP yield for 3 days at 28°C in shaken culture.

*GA medium was composed of 3 g glutamic acid, 1.8 ml glycerol, 0.5 g NH_4Cl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g K_2HPO_4, 0.004 g $FeCl_3 \cdot 6H_2O$ (per 100 ml, pH 7.2).

The productivity of BCP was strictly dependent on the concentration of L-glutamic acid up to 3 g/100 ml.

The BCP productivity was also investigated with various concentrations of citric acid in the media containing 3 g of L-glutamic acid and 0.5 g ammonium chloride. When 1.0 g of citric acid was added to the medium, the yield of BCP was 4.6 mg/ml. Even though citric acid was not added to the medium, BCP was produced, similar to the case of L-glutamic acid. The amount of BCP produced increased with the increase in citric acid concentration up to 1 g per 100 ml. When 2 g or more of citric acid was added, the amount of BCP decreased. From these results, it is clear that citric acid or L-glutamic acid was nonessential for BCP production.

When ammonium chloride was omitted from the GA medium, the cell grew well, but BCP was hardly produced. The cell dry weight decreased with the increase in ammonium chloride concentration. Thus, it was found that ammonium chloride was an essential component of the medium for BCP production and the most suitable amount was about 0.2~0.5 g/100 ml. When glutamic acid and ammonium chloride were added to the medium in small amounts (below 0.6 g of glutamic acid and 0.2 g of ammonium chloride), BCP was hardly produced. From these results, it can be presumed that BCP production is dependent on the total amount of ammonium ions supplied to GA medium.

When $MgSO_4$ was omitted, the organism did not grow well. Varying the concentration of K_2HPO_4 and $MgSO_4$ from 0.05 to 0.3 g per 100 ml had no effect on BCP yield. K_2HPO_4 is the only source of phosphorus in the medium, and the organism did not grow when it was omitted. The presence of ferric ions was not important in BCP production. The growth of *B. subtilis* TB11 was delayed in the early growth phase when iron was omitted, but was restored after 40 h.

The Effects of Various Cations on the Flocculating Activity of BCP

The effect of BCP concentration on flocculating activity is shown in Fig. 4. Flocculating activity was highest at 10 mg/l and decreased at higher concentrations of the flocculant in the reaction mixture of kaolin suspension containing 5 mM of most cations (K^+, Ca^{2+}, Mn^{2+}) except Zn^{2+}. The relationship between flocculant concentration and flocculating activity of BCP is similiar to that of a polypeptide flocculant produced by *Bacillus* sp. PY-90 [12]. The effects of various cations on the flocculating activity of BCP in a kaolin clay suspension were examined using $AlCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, KCl, $FeCl_3 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $MnSO_4$, NaCl, and $ZnCl_2$ as the cation sources (Fig. 5). Among these cations, flocculating activity was stimulated markedly by the addition of Ca^{2+}, Mn^{2+}, and Zn^{2+} , and decreased by the addition of Al^{3+}, Fe^{3+}, and Na^+. It has been known that addition of cations

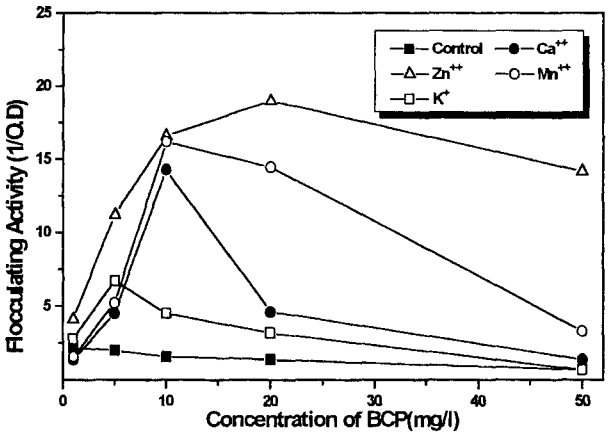

Fig. 4. Effect of BCP concentrations on flocculating activities in kaolin suspensions containing 5 mM Ca^{++} and various concentrations of BCP.

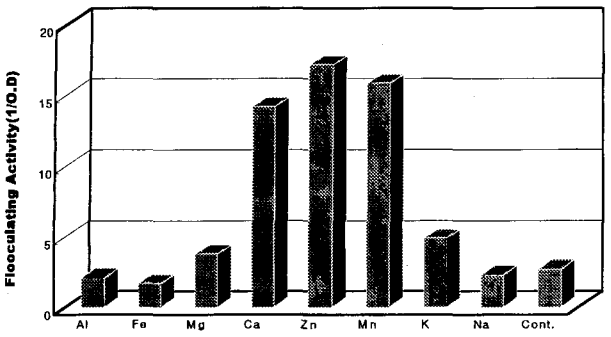

Fig. 5. Effects of cations on flocculating activity of flocculant BCP.

Flocculating activities in kaolin suspensions containing BCP 10 mg/l and 5 mM of various cations were measured.

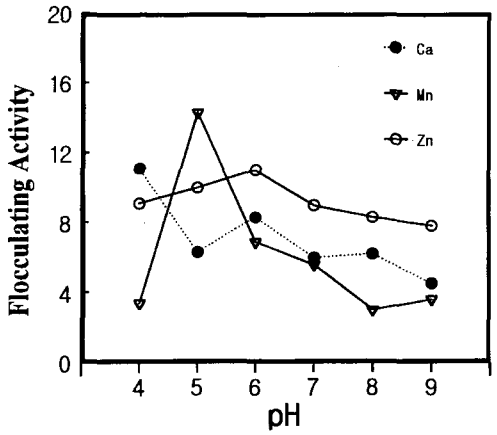

Fig. 6. Effect of pH of kaolin suspensions containing various cations on flocculating activity of BCP.

Concentrations of BCP in reaction mixture were 10 mg/l. Concentrations of Ca^{2+}, Mn^{2+}, and Zn^{2+} in reaction mixtures were 1 mM.

to suspensions is necessary to induce the effective flocculation of microbial flocculants [4, 5, 6, 9]. The microbial flocculants produced by *Rhodococcus erythropolis* and *Alcaligenes cupidus* required Ca^{2+} and Al^{3+} for their high flocculating activity. The flocculating activity of the PGA (polyglutamic acid) produced by *Bacillus* sp. PY-90 was stimulated by Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} [12, 13]. Compared with these flocculants of different sources, the effects of cations on the flocculating activity of BCP were different and the value of the flocculating activity of BCP in a kaolin suspension was equal or high. Highly synergistic effects with cations on kaolin flocculation indicate that cation effects result from neutralization of zeta potential.

Flocculation activity of BCP was affected by the pH of reaction mixtures, including a kaolin suspension and various cations, that have been adjusted to predetermined pH values using HCl and NaOH. The optimum pH for Ca^{2+} was in the weakly acidic range of pH 4.0, and that for Zn^{2+} was in broad ranges of pH 5.0 to 9.0 for better flocculating activity of BCP to kaolin suspensions (Fig. 6).

These characteristics of flocculant BCP will be useful for the application to wastewater treatment in dredging, metal processing factories, and mines.

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