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# Physico-Chemical and Rheological Properties of a Bioflocculant BF-56 from *Bacillus* sp. A56

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**Abstract** *Bacillus* sp. A56 was studied, because of its high flocculating activity. The flocculating substance produced by this strain was purified by ethanol precipitation, cetylpyridinium chloride (CPC) precipitation, and gel permeation chromatography (GPC). The FT-IR spectrum of the purified bioflocculant, designated as BF-56, showed typical characteristics of polysaccharides. The non-sugar substituents, and sugar components of BF-56 containing glucose, fucose, glucuronic acid, and galactose in an approximate molar ratio of 2.76: 1.10:1:0.12, suggested that it was a novel bioflocculant with an estimated molecular mass of over $7\times10^3$ kDa. Rheological analysis of BF-56 revealed that it was a pseudoplastic that had higher apparent viscosity rate at dilute concentrations than those of zooglan. The solution of bioflocculant BF-56 exhibited non-Newtonian characteristics and it was compatible to high concentrations of salts such as KCl, NaCl, $CaCl_2$, or $FeCl_3$. The present results suggested strong possibility of bioflocculant BF-56 to be fully applicable to industries such as wastewater treatment.

**Key words:** Novel bioflocculant, bacterial polysaccharide, rheology

Various flocculants are widely used in industrial fields such as tap water production, wastewater treatment, dredging, downstream techniques, fermentation processes, and food industries [12, 16]. Flocculants are generally divided into three major groups: (1) inorganic flocculants such as aluminum sulfate and polyaluminum chloride, (2) organic synthetic polymers including polyacrylic acid, polyacrylamide derivatives, and polyethylene imine, and (3) naturally occuring flocculants such as chitosan, alginate, and other microbial flocculants. These flocculants have been used for various purposes according to their physico-chemical properties and toxicity. Of these, the organic synthetic polymer flocculants have been most frequently used due to their higher flocculating activity and lower production costs. However, most of these synthetic polymers, especially polyacrylamides, have shown some limitations in their widespread use in that they are not only hardly degradable in nature but also are neurotoxic or strongly carcinogenic to humans [8]. As environmental pollution problems and transfer of toxic compounds to humans through ingestion of crops and contaminated water are becoming important issues, the use of biodegradable compounds are increasingly urgent to overcome these problems. In this regard, due to their easily biodegradable nature and safeness, numerous biopolymers produced from microorganisms have been documented and some of them are currently being marketed for their use in the field of wastewater treatment. Microbial flocculants have shown to be very efficient in coagulation of kaolin and in removal of microorganisms in various fermentation industries. Kurane *et al.* [12] reported that *Rhodococcus erythropolis* produced a flocculant which was effective for various colloidal suspensions and pigments. A polysaccharide that was obtained from *Alcaligenes latus* B-16 [17] showed flocculating and water-absorbing activities. However, researches on screening and application of novel microbial polymers possessing superior rheological properties and flocculating activity to wastewater treatment have not been extensively performed.

Previously, a soil bacterium producing a flocculant was isolated. It was effective for various suspended solids, including organic materials such as microorganisms and inorganic materials such as active carbon, and the bacterium was identified as a strain of *Bacillus* species and tentatively named *Bacillus* sp. A56. Subsequently, optimized conditions

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for the production of bioflocculant from this strain have been reported [22]. In the present paper, we report physico-chemical and rheological characteristics of the bioflocculant BF-56 produced from this strain. The results that the solution of bioflocculant BF-56 had physico-chemical and rheological properties better than or at least comparable to those of zooglan suggest its potential in developing novel bioflocculant for wastewater treatment and other industries such as fermentation processes.

## MATERIALS AND METHODS

### Microorganisms and Bioflocculant Production Conditions

The isolation medium for bioflocculant-producing bacteria was Suh's basic mineral salt medium [22] supplemented with glucose (2.0 g/l) as a sole carbon source. A medium for flocculant production by *Bacillus* sp. A56 contained 20 g of glucose, 0.5 g of $NH_4NO_3$, 1.0 g of $K_2HPO_4$, 0.8 g of $KH_2PO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.3 g of $MnSO_4{\cdot}4{\sim}6H_2O$, 0.5 g of $CaCO_3$, 0.3 g of yeast extract, and 0.3 g of tryptone in 1 l distilled water. The initial pH of the medium was adjusted to 6.5 with 1.0 N NaOH. The seed culture was derived from a single colony and grown for 20 h on a rotary shaker at 30°C. This strain was then cultured at 30°C for 30 h in a 7-l jar fermentor (KF-7, Cobiotech., Korea) containing 5 l of the above medium.

### Preparation of BF-56 Hydrolysate

Purified BF-56 was dissolved in 20 ml of distilled, deionized water ($ddH_2O$, 0.2%, w/v) and then hydrolyzed at 121°C for 1 h after adding an equal volume of 4.0 M trifluoroacetic acid (TFA). Insoluble materials were removed by centrifugation at 32,000 ×g for 30 min and the supernatant was filtered through a 0.2-μm pore-size filter. The filtrate was dried on Speed Vac and redissolved in $ddH_2O$. To completely remove residual TFA, this procedure was repeated 3–4 times.

### Measurement of Molecular Mass and Infrared Spectrometry

A high performance liquid chromatography (HPLC) (LC 10, Shimadzu, Japan) equipped with PL-GFC 1000 Å column (8 μ, 10×325 mm) (Polymer Laboratory, U.S.A.) and Refractive Index Detector (RID-6A, Shimadzu, Japan), was employed for investigating the molecular weight (MW) distribution of BF-56. Ten microliters of the sample solution were injected and eluted with HPLC grade water at a flow rate of 1 ml/min at room temperature. The molecular weight markers used were dextrans (Sigma Chem. Co., U.S.A.) of 2,000, 580, 143, 73, and 15 kDa. The molecular mass of BF-56 was determined by comparing its retention time with those of the standards. For infrared spectrometry, a finely ground BF-56 powder was thoroughly mixed with potassium bromide (KBr, 20%, w/w) and the mixture was dried at 60°C *in vacuo*. Under pressure, the potassium bromide melts and seals the compound into a matrix. The infrared spectrum of BF-56 was recorded by IR spectrophotometer (RFX-65, Laserprecision Analytical, U.S.A.).

### Analysis of Sugar Components

Complete hydrolysis of the bioflocculant BF-56 was carried out with 4.0 M TFA at 121°C for 1 h. After hydrolysis, the solution was neutralized with 1.0 N NaOH and lyophilized. Sugar constituents in the TFA-hydrolysate were analyzed by either thin layer chromatography (TLC) or HPLC. For the TLC analysis, a portion of the acid hydrolysate was applied to silica gel TLC plate and developed in a solvent system of acetonitrile:water (85:15, v/v). The chromatogram was then heated at 100°C for 10 min and visualized by using a mixture of diphenylamine:aniline:phosphoric acid [6]. A standard sugar solution (1%, w/v) was prepared in water and 20 μl each was spotted.

Monosaccharide composition of BF-56 was also analyzed by applying the acid hydrolysate to an HPLC system equipped with Microspherogel carbohydrate column (6.5× 300 mm, Beckman, U.S.A.) and Refractive Index Detector (RID). Carbohydrates were eluted with water at a flow rate of 0.5 ml/min.

### Measurement of Charge Density

The charge density of BF-56 produced at aeration and nonaeration conditions was measured by the following procedure [15]. For measurement of cationic charge density of BF-56, 5 ml of polymer solution (0.05%) was diluted with 45 ml $dH_2O$ in 100-ml Erlenmeyer flask and thoroughly mixed for 5 min at 300–400 rpm on a multistirrer. After the addition of 300 μl of 0.1% toluidine blue, the mixture was titrated by biuret with 2.5 mM potassium poly(vinyl) sulfate (PVSK) solution. When the blue color changed to red, addition of PVSK solution was stopped and the end point was determined. In a similar manner, anionic charge density of BF-56 was also examined. Briefly, 2 ml of polymer solution (0.05%) was diluted with 48 ml of $dH_2O$ and mixed for 5 min at 300–400 rpm. The mixture was titrated with 5 ml of 2.5 mM poly(diallyl)dimethylammonium chloride (PDAC) solution.

The charge density was calculated as follows:

$$\text{Charge density (meq/g)}=2.5\times A\times(X-Y)/1000\times B\times C$$

where A is volume of BF-56 solution; B is weight of BF-56; C is volume of BF-56 solution; X is volume of PVSK solution used for titration; and Y is volume of PVSK solution used for basic experiment.

### Measurement of Rheological Properties

An apparent viscosity of BF-56 was measured by using a rheometer (DV-III, Brookfield, U.S.A.) equipped with a high-viscosity adaptor (SC4-34) and a low-viscosity adaptor (SC4-18). Unless otherwise stated, measurements were carried out at 25°C with the sample volume of 8 ml. The rheological properties of BF-56 were compared with those of zooglan (Sigma, U.S.A.). The consistence index or flow behavior index were calculated according to the Ostwald's Power-low equation from the measured shear rate and shear stress of biopolymer fluids [1]: $\tau=k(\gamma^n)$, where $\tau$ is shear stress (Dyne/cm$^2$), k is consistency index (cP), $\gamma$ is shear rate (sec$^{-1}$), and n is flow index, n.

### Others

Elemental analysis of BF-56 was performed with an elemental analyzer (EA1108, CarloErba Ins., Italy). The total carbohydrate content of the flocculant was determined by the phenol-sulfuric acid method [7] and expressed as the glucose equivalent. The protein moiety in the flocculant molecule was determined by the Bradford method [4] with bovine serum albumin as a standard. Sugar derivatives were investigated as follows; by the carbazole method [5] for uronic acid, the Friedmann method [9] for pyruvic acid, and the hydroxamic acid method [13] for acetic acid.

## RESULTS AND DISCUSSION

### Selection of Bioflocculant-Producing Bacteria

For this study, a biopolymer produced from a strain of *Bacillus* sp. A56, which was previously isolated and identified in our laboratory, was used [22]. From more than 200 bacterial isolates that excreted mucous material on the agar plates containing the isolation medium, this strain was finally selected because of its highest flocculating activity against kaolin clay and activated carbon [22].

### Purification of Bioflocculant BF-56

In order to remove bacterial cells from the sticky materials, the culture broth (7 l) of *Bacillus* sp. A56 was dilluted with 10 vol. of distilled water. Most of the bacterial cells were removed by centrifugation at 9,000 ×g for 30 min. The cell-free culture broth was concentrated to 2.3 l and the bioflocculant was precipitated by adding 2 vol. of ethanol. The precipitated crude flocculant was dried on a vacuum evaporator (3.6 g) and redissolved in distilled water. Also, 10% of CPC solution was added to the distilled water until no more precipitate was formed. The insoluble acidic flocculant-CPC complex was collected by centrifugation and redissolved in 10% sodium chloride solution. After dialysis against distilled water, the flocculant was precipitated by adding 2 vol. of ethanol and dissolved in distilled water. The acidic flocculant obtained was dialyzed against distilled water and lyophilized. Purified flocculant solution (0.1%) was loaded onto a Sephacryl S-500 column (2.8× 104 cm) and eluted with water. Fractions of 5 ml each were collected and examined by the phenolsulfuric acid method [7]. The flocculating activity coincided with the position of a polysaccharide, indicating that the major flocculating factor was a polysaccharide (data not shown). It has been reported that the major flocculating factors of the flocculants produced from *R. erythropolis* [24] and *Pacilomyces* sp. [23] are protein in nature, while *Zoogloea* sp. [26] and *Alcaligenes cupidus* [25] produced extracellular polysaccharides with flocculating activity. Fractions containing polysaccharide were combined, lyophilized (the final preparation was named BF-56), and then subjected to both chemical and biological analyses.

### Molecular Weight of BF-56

The bioflocculant BF-56 was investigated for its molecular weight by using HPLC. An HPLC pattern of the BF-56 is shown in Fig. 1. The molecular weight of BF-56 was estimated to be over $7\times10^3$ kDa. Generally, the molecular weight of a flocculant is related to the chain length of the polymer, which is an important factor in the flocculating reaction. It has been reported that most effective flocculants are water soluble polymers of high molecular weight, usually greater than $3\times10^3$ kDa, with linear configurations [2]. The molecular weight of BF-56 in the present study is similar to that of polysaccharide from *Drechslera spicifera*

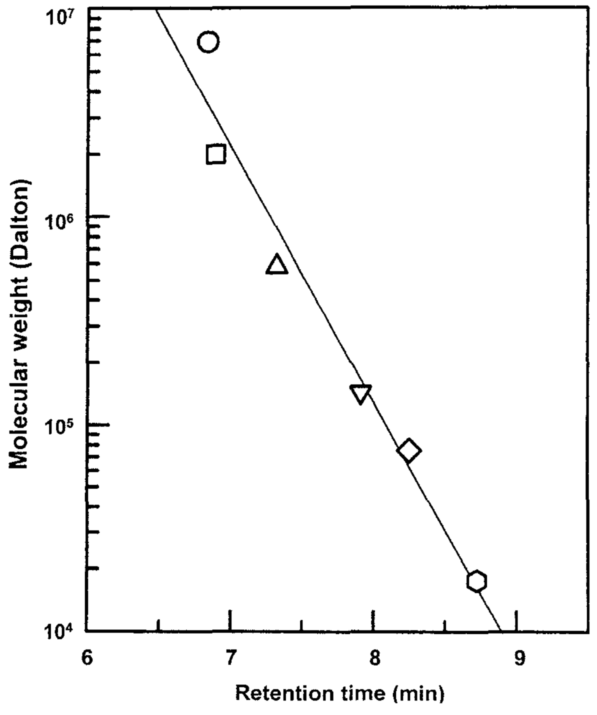

**Fig. 1.** Estimation of molecular weight of BF-56 using gel permeation chromatography with HPLC.
Standard marker: (○) BF-56, (□) 200 kDa dextran, (△) 580 kDa dextran, (▽) 143 kDa dextran, (◇) 73 kDa dextran, (○) 15 kDa dextran.

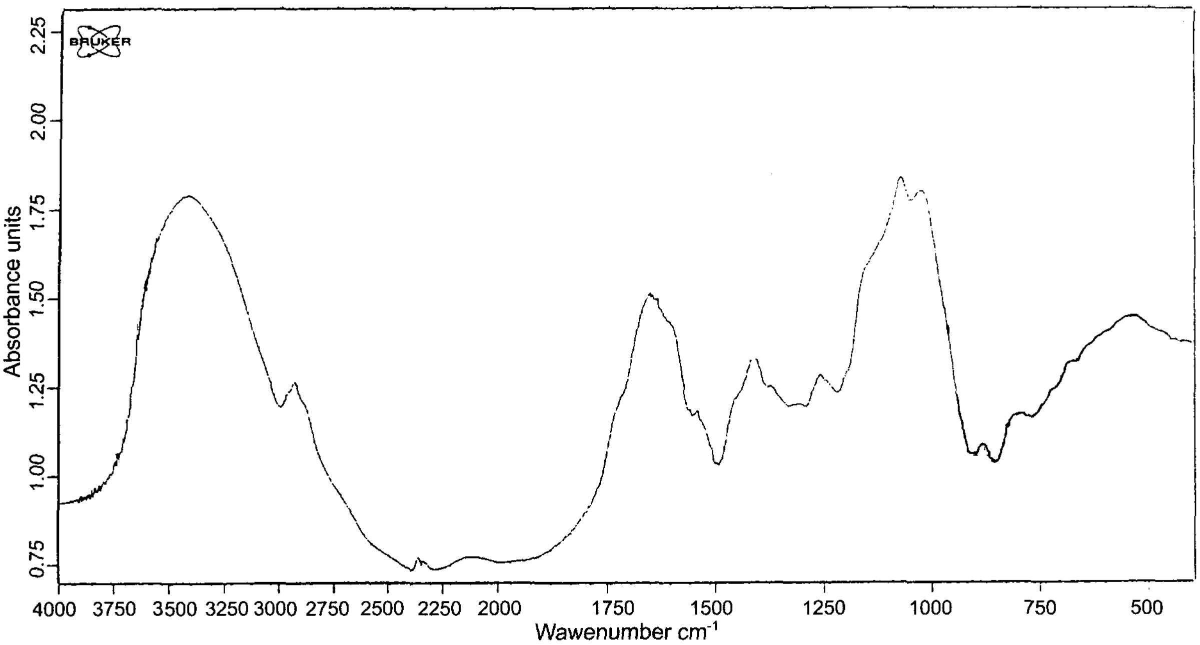

**Fig. 2.** Infrared absorption spectrum of BF-56 in KBr.

[3]. Seo *et al.* [21] reported that the extracellular polysaccharide produced by *Penibacillus* sp. has the molecular weight of about 31.5 MDa. A larger flocculant molecular weight results in a larger floc size in the flocculation reaction [10]. Since large-molecular-weight flocculant has more functional groups, it forms stronger flocs, which coagulate many suspended particles in the solution [10]. The molecular weights of available flocculants range from a few thousand to several million daltons [14]. Michaels [14] reported that an anionic polyacrylamide with a higher molecular weight was more effective in removing suspended solids such as clay particles.

## Infrared Spectrometry of BF-56

From the infrared spectrum of the BF-56 pelleted with KBr, the characteristic chemical groups were analyzed (Fig. 2). The absorption peak at 3,420 cm$^{-1}$ was a characteristic of OH stretching from the bound hydroxyl group, and adsorbed water molecules. The peaks in the range of 2,900 and 2,800 cm$^{-1}$ indicated aliphatic C-H stretching. The absorption peaks around 1,654 cm$^{-1}$ and 1,071 cm$^{-1}$ were characteristics of C=O and C-O groups. N-H bending occurred around 1,640–1,550 cm$^{-1}$ for primary and secondary amides [19]. The strong absorption peaks observed in the range of 1,000 and 1,200 cm$^{-1}$ are generally known to be typical characteristics of all sugar derivatives. Therefore, the infrared spectrum of this flocculant showed the presence of carboxyl, hydroxyl, and amide groups. Zajic and Knettig reported that functional groups known to contribute to the flocculation of clays were carboxyl, hydroxyl, and amino groups [27]. The BF-56 reacted with the cationic colloid reagent, poly(diallyldimethylammonium) chloride, suggesting that it is an anionic flocculant having a charge density of −1.71 meq/g. This result was in good

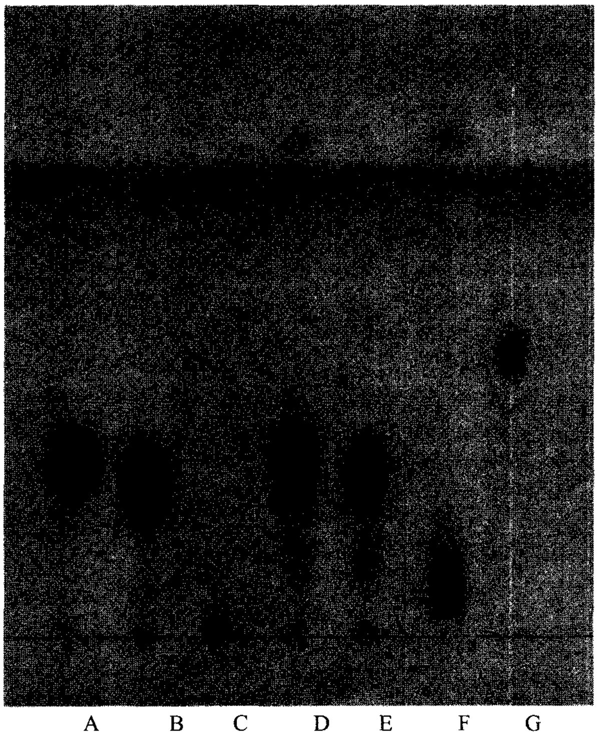

**Fig. 3.** Thin layer chromatogram of BF-56 hydrolysate.
(A) Glucose, (B) Galactose, (C) Glucosamine, (D) BF-56 hydrolysate, (E) Mixture of standard carbohydrate, (F) Glucuronic acid, (G) Fucose.

**Table 1.** Chemical components of bioflocculant BF-56.

| Chemical components | Contents (w/w, %) | Elemental analysis | Contents (w/w, %) |
|---|---|---|---|
| Total sugar | 81.7 | Carbon | 40.20 |
| Protein | 5.9 | Hydrogen | 6.70 |
| Acetic acid | 3.5 | Oxygen | 52.10 |
| Pyruvic acid | 5.2 | Nitrogen | 0.67 |
| Uronic acid | 27.4 | Sulfur | 0.30 |

agreement with the charge density (−1.69 meq/g) of Al-201 from *Alcaligenes cupidus* KT201 [25].

### Monosaccharide Compositions of BF-56

Component sugars of BF-56 were determined after complete hydrolysis of BF-56 with dilute trifluoroacetic acid (TFA). The acid hydrolysate was subjected to the TLC analysis by using a mixture of acetonitrile:water (85:15, v/v) as a developing solvent. A chromatogram that was developed showed sugar spots equivalent to those of glucose, fucose, glucuronic acid, and galactose (Fig. 3). HPLC chromatogram of acid hydrolysate also showed the same monosaccharide compositions (data not shown), indicating that BF-56 is a heteropolysaccharide. The molar ratios of glucose, fucose, glucuronic acid, and galactose were approximately 2.76:1.10:1:0.12.

As shown in Table 1, the results of elemental analysis revealed that the constituent elements of BF-56 were of 40.2% carbon, 6.7% hydrogen, 52.1% oxygen, and 0.67% nitrogen. Measurement of the total sugar by the phenol-sulfuric acid method [7] showed that 81.7% of the polysaccharide was made up of sugar and also of acetic acid, pyruvic acid, and uronic acid according to other analyses. It is generally accepted that the negatively charged groups of bioflocculant are involved in its coagulation with positively charged suspended particles. The results suggested that the BF-56 acted as a polyelectrolyte, which aggregated suspended solids into a floc network through electrostatic interaction. Because the components and composition of BF-56 differ from those of other bacterial polysaccharides known, it appears to be a new polysaccharide flocculant.

### Rheological Properties

Most commercial applications of microbial polysaccharides depend on their rheological properties, which can be influenced by both their sugar composition and spatial structure of their basic units [1]. Among the rheological properties, apparent viscosity is an important factor which can be used to approximate rheological characteristics of the polymer solution. To evaluate their applicability to the treatment of wastewater, an effective viscosity of polymeric bioflocculant needs to be examined under various conditions such as varying concentrations of polymer, and changing pH and temperatures. Therefore, the characteristic flow behavior of BF-56 solution was studied and compared with the solution of zooglan that was produced by *Zoogloea ramigera*.

The combined relationships of apparent viscosity and shear stress versus shear rate at various concentrations of BF-56 and zooglan were investigated by using spindle SC4-34 and SC4-18 at 25°C. Figure 4 shows a plot of an apparent viscosity ($\eta$) against the shear rate ($\gamma$) with varying concentrations of flocculant. Apparent viscosity of BF-56 solution decreased rapidly with increasing shear rate at all concentrations tested. A decrease in apparent viscosity with an increase in shear rate may indicate a change in the degree of orientation of the molecule, a change in the shape of flexible molecules, and the effect of flow on intermolecular interactions. These phenomena indicated that BF-56 and zooglan had the highest degree of pseudoplasticity, identified as shear thinning, among the characters of non-Newtonian fluids. At each shear rate, the apparent viscosity of the BF-56 fluid was found to be approximately 2.3 times higher than the zooglan fluid at each concentration. The fact that the effect of the shear rate on apparent viscosity was greater than on that of zooglan indicated that the flocculant BF-56 is highly charged and asymmetric.

To determine the yield stress (that is, the shear stress required to initiate flow) of BF-56, shear rate versus shear stress was plotted at 0.1% concentration. The shear stress of BF-56 increased nonlinearly to the higher level than that of zooglan with increasing shear rate (Fig. 5). A similar phenomenon was

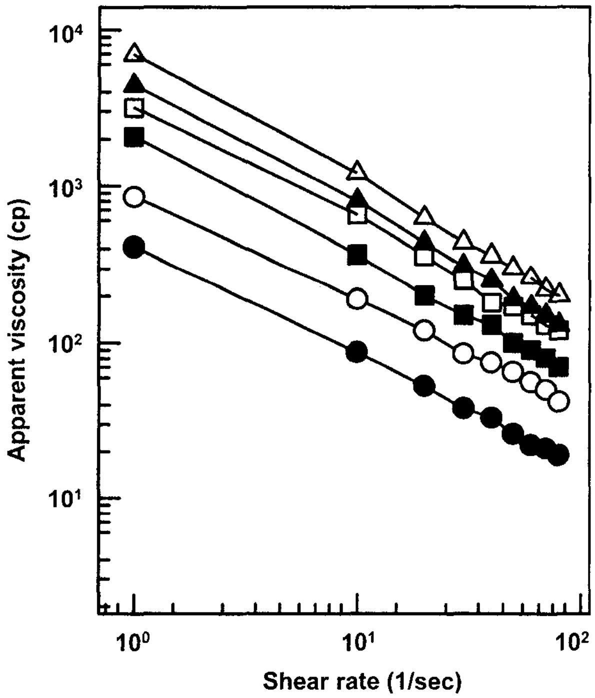

**Fig. 4.** Relationship between shear rate and apparent viscosity at different concentrations of BF-56 and zooglan.

Symbols: (○) 0.1 BF-56, (●) 0.1% zooglan, (□) 0.3% BF-56, (■) 0.3% zooglan, (△) 0.5% BF-56, (▲) 0.5% zooglan.

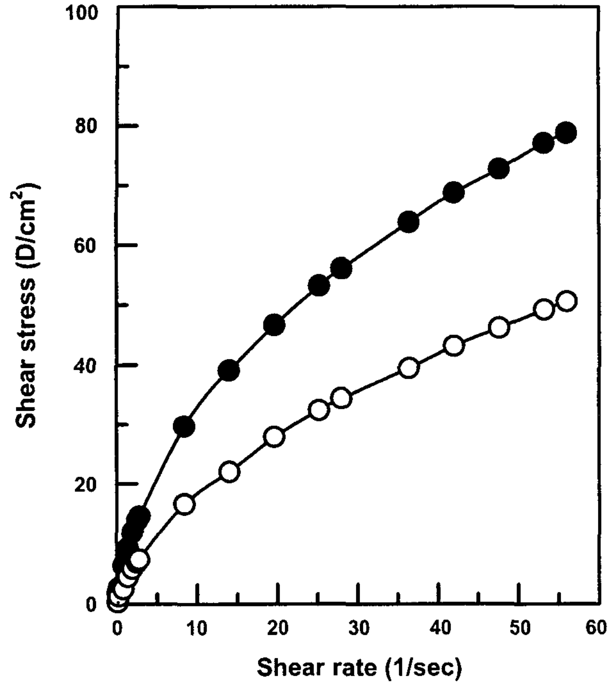

**Fig. 5.** Comparison of shear stress vs shear rate on the concentrations of BF-56 and zooglan.
Symbols: (●) 0.1% BF-56, (○) 0.1% zooglan.

observed at all concentrations (data not shown) and it was indicated with increasing concentration levels that BF-56 consisted of a higher degree of pseudoplasticity than zooglan.

Figure 6 shows the consistency level and flow index of BF-56 at each concentration. Consistency index (k) values of BF-56 fluid were higher than those of zooglan fluid. Futhermore, the flow index (n) values of BF-56 were lower than those of zooglan. Consistency index of the two fluids increased and the flow index decreased with increasing concentrations. Accordingly, pseudoplasticity of the two fluids was obvious at high concentrations due to decreased flow index with increasing concentrations. Consistency and flow index of BF-56 was higher and lower, respectively, when compared with those of zooglan at all concentrations tested. The flow behavior index of BF-56 at a concentration of 5 g/l decreased to 0.13 and that for zooglan was 0.26 under a similar condition. In general, a lower flow index indicates a higher pseudoplastisity, which is defined by a rapid decrease in viscosity as the shear rate increases.

BF-56 and zooglan fluids (0.2% each) were prepared in buffers of pH 2.0 to 13.0. The change of apparent viscosity of BF-56 was similar to that of zooglan fluid. In particular, in a strong acidic region (pHs 2–3), apparent viscosity of BF-56 fluid was less stable than that of zooglan fluid, whereas in an acidic to alkali region (pHs 4–13), apparent viscosity of BF-56 fluid was more stable than that of zooglan fluid. In fact, BF-56 fluid was found to have the highest apparent viscosity at pH 5.0 (data not shown). In contrast to the most microbial polysaccharides which are unstable in acidic conditions, BF-56 showed a unique rheological behavior of high viscosity under acidic conditions.

Apparent viscosities of BF-56 and zooglan fluids were measured at constant shear rates at varying temperatures (20, 30, 40, 50, 60, 70, and 80°C). The apparent viscosity of BF-56 was found to be higher than zooglan at every temperature tested (data not shown); in particular, that of BF-56 decreased by about 30% at temperatures over 60°C, whereas that of zooglan decreased by about 50% at over 50°C. The apparent viscosity of succinoglycan solutions has also been reported to be temperature dependent, showing its viscosity approaching that of water at 60°C [11]. The relatively higher viscosity of BF-56 at higher temperatures suggests that BF-56 can be a better choice than others as gelling agents or flocculants in industries such as food processing or wastewater treatment where higher temperature is required.

The apparent viscosity of BF-56 was influenced by both species and concentrations of salt (Fig. 7). The change in apparent viscosity was likely due to ionic interaction, resulting from a bridge formation mediated by counterions ($Na^+$, $K^+$, $Ca^{2+}$, and $Fe^{3+}$), between two charged chains within a polysaccharide molecule. The nature of interaction of the counterions with charged groups on the macromolecular backbone is important in defining the polyelectrolyte solution properties [18]. The salt compatibility means that the apparent viscosity (rheological properties) of the polysaccharide does not show any change at high

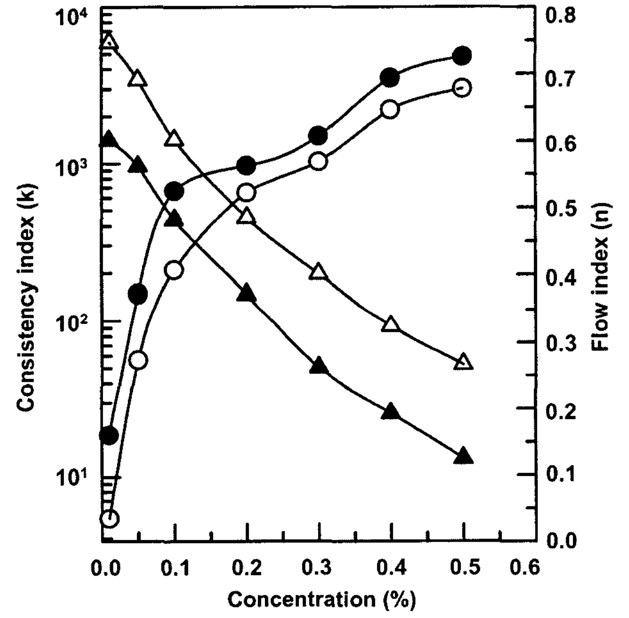

**Fig. 6.** Consistency factor and Power-law index of BF-56 and zooglan solution.
Symbols: (●) Consistency index of BF-56, (○) Consistency index of zooglan, (▲) Flow index of BF-56, (△) Flow index of zooglan.

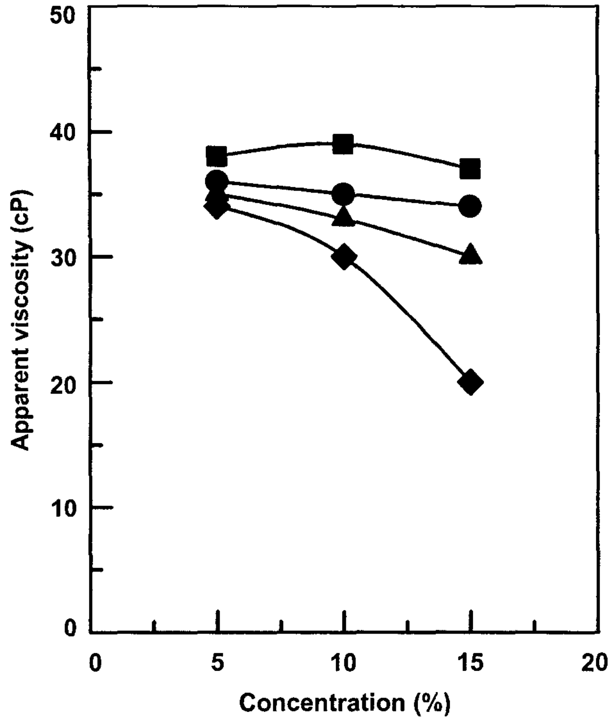

**Fig. 7.** Variation of apparent viscosity on the 0.1% BF-56 solution with various concentrations of several salts.

Apparent viscosity was measured at shear rate 14.0 sec$^{-1}$ by using spindle SC4-34. Symbols: (●) KCl, (■) NaCl, (▲) $CaCl_2$, (◆) $FeCl_3$.

concentrations of salt. This property plays an important role in applicability to industry. As shown in Fig. 7, the apparent viscosity of BF-56 decreased with an increase in salt concentrations. The decrease in apparent viscosity in the presence of salt confirmed the presence of charged groups in BF-56. In fact, BF-56 was compatible to high concentrations of salts such as KCl, NaCl, $CaCl_2$, and $FeCl_3$. The apparent viscosity of BF-56 was constantly maintained when counterions were added. These rheological characteristics of BF-56 suggested that BF-56 could replace zooglan in industrial uses.

Based on its chemical identities and rheological properties, BF-56 appears to be a new anionic polysaccharide with high viscosity at low shear rate, significant pseudoplastic property, an excellent salt compatibility, and a remarkable stability over a wide range of heat and pHs. The results in the present study strongly suggest that the BF-56 polysaccharide flocculant produced by *Bacillus* sp. A56 is highly applicable to treating various kinds of wastewater as an environmentally safe flocculant.

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