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# Production and Rheological Properties of Bioflocculant Produced by *Bacillus* sp. DP-152

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**Abstract** The culture conditions for *Bacillus* sp. DP-152 in the flask were investigated for the production of polysaccharide flocculant, DP-152. The optimum pH and temperature for the flocculant production were 8.0 and 30°C, respectively. The favorable substrates for flocculant production were soluble starch and ammonium nitrate. The medium composition was optimized as follows: 30 g soluble starch, 0.75 g $NH_4NO_3$, 2.0 g $K_2HPO_4$, 0.1 g $KH_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $MnSO_4 \cdot 4\sim5H_2O$ in 1 l of distilled water. Under this optimized condition, flocculating activity has been improved 4-fold compared with that of the basal medium. In the culture flask, the highest flocculating activity was obtained after 70 h of cultivation and the amount of bioflocculant DP-152 yielded was 12.4 g/l. The solution of bioflocculant DP-152 showed non-Newtonian characteristics. Bioflocculant DP-152 exhibited apparently higher viscosity at all concentrations compared to that of zooglan (from *Zoogloea ramigera*), and it was stable over a wide range of temperatures and pHs.

**Key words:** *Bacillus* species, bioflocculant production, rheology

Various flocculants have been used to aggregate colloidal substances, for example, to treat the cell and cell debris, and have also widely been used in the industrial field for many purposes, such as for tap water production, wastewater treatment, dredging fields, and downstream processing, as well as for cell removal during the fermentation processes [9]. Bioflocculants produced by microorganisms are useful for the treatment of wastewater due to their flocculating activity, biodegradation, and environmental safty. Recently, it has been reported that some microorganisms, including *Bacillus* sp. A56 [17], *Pestalotiopsis* sp. [10], *Rhodococcus erythropolis* [8], *Aspergillus sojae* [13], and *Paecilomyces* sp. [19] produce certain kinds of flocculating substances.

The production of extracellular polysaccharide as a bioflocculant is significantly affected by the culture medium composition. Generally, media containing a high ratio of carbon to a limiting amount of nutrient, often nitrogen, are favoured for polysaccharide flocculant production [2, 15]. The composition of the growth medium can also indirectly affect polysaccharide flocculant yield. For example, changes of pH may yield different medium compositions. In addition, high media concentrations resulting from high polymer concentrations may lead to oxygen limitation or heterogeneity due to an increased viscosity within the fermentor prior to the carbon source being exhausted and, thus, might indirectly affect the productivity. The cell growth and production of extracellular polysaccharide flocculants by microorganisms are determined by a wide range of environmental parameters and compositions of medium.

Rheological properties are important when bioflocculant DP-152 powder product is re-constituted into solutions for the use of viscofiers, and there are indications that post-fermention heat treatment of the powder alters these properties as well as the addition of solutes. Also, because of the mixing problems associated with bioflocculant DP-152 fermentation, re-constituted solutions have been used extensively for the understanding of them for use as an experimental model system. Therefore, it is important to know how well the re-constituted solutions mimic the properties of real broths. Because of our interest in using both bioflocculant DP-152 solution and its mixture to improve bioflocculant DP-152 fermentation processes and the general utility of the data, it was decided to study a flocculant field of bioflocculant DP-152 solutions.

In a series of screening programs searching for a novel bioflocculant, a bacterium has been found to produce a

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flocculant which is effective for various suspended solids. The bacterium was designated *Bacillus* sp. DP-152 and its charactristics of the flocculant (bioflocculant DP-152) from *Bacillus* sp. DP-152 had been reported previously [16].

In this paper, we report the investigation of the production and rheological properties for industrial applicability of bioflocculant DP-152 from *Bacillus* sp. DP-152.

## MATERIALS AND METHODS

### Microorganism and Culture Conditions

*Bacillus* sp. DP-152 was isolated from soil samples [16] and used in this experiment. A basal medium for flocculant production by *Bacillus* sp. DP-152 contained 20 g glucose, 1.0 g $NH_4NO_3$, 1.0 g $K_2HPO_4$, 0.8 g $KH_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $MnSO_4 \cdot 4\sim5H_2O$ in 1 l distilled water. A solid medium was prepared by dissolving 15 g/l agar in the above medium. The initial pH of the medium was adjusted to 7.0 with 1.0 N NaOH. The seed culture was derived from a single colony and grown for 30 h on a rotary shaker at 30°C. This strain was then cultured at 30°C for 3 d in a 250-ml Erlenmeyer flask containing 50 ml of the above medium by rotary-shaking (150 rpm) with 1.0 ml of seed culture.

### Determination of Dry Cell Weight

The rate of cell growth was estimated by measuring the optical density of the cell suspension at 660 nm using a spectrophotometer (UV-160A, Shimadzu, Japan). Dry cell weight (DCW) was measured as follows; 10 ml of culture broth was first centrifuged at 10,000×g for 10 min, and packed cells were washed with saline solution followed by distilled water. Finally, the cells were dried by desiccation at 105°C to a constant weight prior to measuring the dry weight.

### Measurements of Flocculating Activity

Kaolin clay (Junsei Chemical Co.) was used as the suspension material for estimating the flocculating activity as reported previously [16]. The flocculating activity was calculated by the following equation.

$$\text{Flocculating activity (F.A.)} = 1/A - 1/B$$

where A is the optical density of control and B is that of a sample.

### Determination of Flocculant Amount

The total accumulated carbohydrate in the culture broth was measured by the phenol-sulfuric acid method [3] after cells were removed by centrifugation. Because bioflocculant DP-152 is a polysaccharide flocculant [16], the amount of flocculant was estimated using an equivalent amount of glucose as the standard.

### Measurement of Rheological Properties

The apparent viscosity of bioflocculant DP-152 and zooglan were measured by a rheometer (DV-III; Brookfield, U.S.A.) fitted spindle No. 3 at a shear rate of 30 $sec^{-1}$. Samples of 8 ml were used and measurements were carried out at 25°C, unless otherwise stated.

## RESULTS AND DISCUSSION

### Effects of pH and Temperature on Flocculant Production

Preliminary studies were performed in shake-flasks to investigate the principal processing steps for cell concentration, flocculant concentration, and flocculating activity. As shown in Figs. 1 and 2, these processes were affected by the initial pH and temperature. A high flocculating activity was observed in the pH range of 6.5 to 8.0 but at pHs lower than 6.0, flocculating activity decreased markedly. Especially at pH 8.0, the flocculating activity was maximal and the flocculant concentration was 6.1 g/l. The optimum pH for flocculant production at pH 8 observed in the present study is similar to that reported by Kurane *et al.* [8], but was one unit higher than the pH 7.0 reported by Shimofuruya *et al.* [14]. Nakamura *et al.* [13] have also reported that pH influences strongly for the flocculant production.

Because temperature is one of the most important environmental factors affecting cell concentration and flocculant production [11], flocculant production by

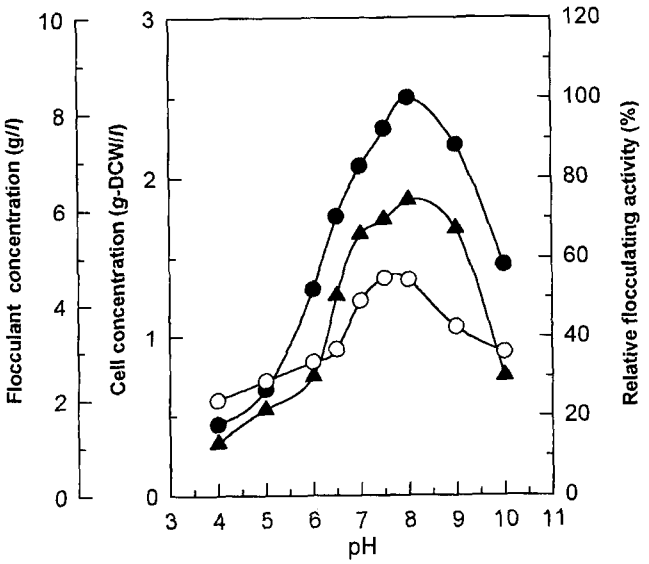


**Fig. 1.** Effect of initial pH on the flocculant production.

Cells were incubated with shaking at 30°C for 3 d in the basal medium. The basal medium was described in Materials and Methods. Symbols: (○) cell concentration, (●) flocculating activity, (▲) flocculant concentration.

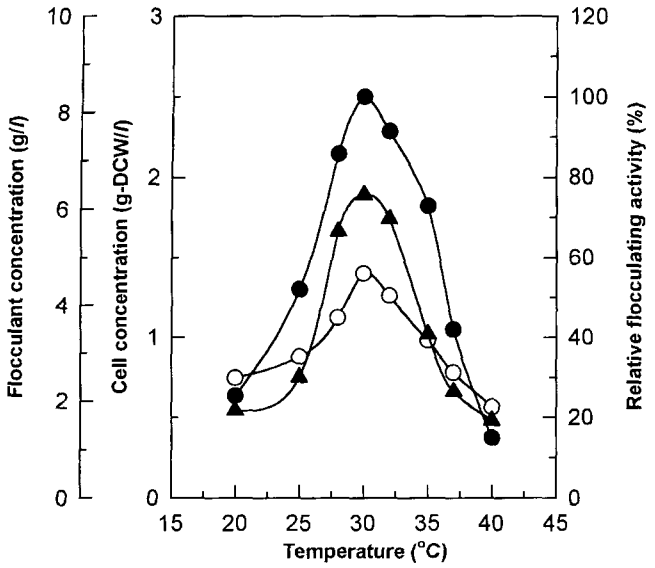


**Fig. 2.** Effect of temperature on the flocculant production.

Initial pH of the medium was adjusted to 8.0. The basal medium used was as described in Fig. 1. Symbols: (○) cell concentration, (●) flocculating activity, (▲) flocculant concentration.

*Bacillus* sp. DP-152 at various temperatures between 20 and 40°C was examined (Fig. 2). Cell concentration and flocculant production were increased with increasing temperature and reached the maximum value at 30°C. However, at higher than 32°C, flocculant production and cell concentration decreased precipitiously. Based on these results, it is concluded that 30°C is the optimum temperature for flocculant production using *Bacillus* sp. DP-152. Kurane *et al.* [9] have also reported that flocculant production by *R. erythropolis* was carried out at 30°C.

## Effects of Carbon and Nitrogen Sources on Flocculant Production

The assimilation of various carbon and nitrogen sources by *Bacillus* sp. DP-152 was examined in a batch culture. Carbon sources were added to the basal medium instead of glucose, and the flocculating activity was measured after 3 d of cultivation. Each carbon source was added to the medium at a concentration of 20 g/l. Soluble starch and galactose gave a high production of flocculant, but soluble starch showed the highest specific productivity (g-flocculant/g-cell) than that of galactose (Table 1). On the other hand, xylose, fructose, raffinose, and inulin were not much favorable for either flocculant production or cell growth. Therefore, soluble starch was used as the carbon source for flocculant production. Glucose and galactose have been reported to be good substrates for flocculant production in *Alcaligenes cupidus* KT201 [20], while fructose was reported to be a good substrate in *R. erythropolis* [8].

**Table 1.** Effect of carbon sources on the flocculant production.

| Carbon source | Cell concentration (g-DCW/l) | Flocculant concentration (g/l) | Relative flocculating activity (%) |
|---|---|---|---|
| Soluble starch | 2.33 | 8.5 | 100 |
| Galactose | 2.31 | 7.4 | 82.4 |
| Glucose | 1.63 | 5.8 | 68.3 |
| Lactose | 1.69 | 5.3 | 65.4 |
| Inositol | 1.71 | 5.1 | 62.3 |
| Sucrose | 1.38 | 4.7 | 57.4 |
| Mannitol | 1.56 | 3.8 | 43.5 |
| Raffinose | 1.10 | 3.4 | 39.8 |
| Inulin | 0.65 | 2.4 | 25.9 |
| Fructose | 1.12 | 0.4 | 14.8 |
| Xylose | 0.82 | 1.6 | 11.1 |
| Rhamnose | 0.57 | 0.3 | 6.4 |
| None | 0.05 | 0.0 | 2.5 |

Carbon sources were added to the basal culture medium at the concentration of 20 g/l without glucose. Initial pH and temperature were at 8.0 and 30°C, respectively.

The effect of the nitrogen source on flocculant production was tested after 3 d of cultivation (data not shown). The nitrogen sources were divided into two groups; inorganic nitrogens such as $(NH_4)_2SO_4$, $NaNO_3$, and $NH_4NO_3$, and organic nitrogens such as yeast extract, peptone, and casamino acid. Of the inorganic nitrogens, the most suitable source was $NH_4NO_3$, which was also reported to be a favorable nitrogen source for flocculant production in *Zoogloea* MP6 [21].

When $NH_4NO_3$ at the concentration of 1.0 g/l was present, an additional inclusion of the organic nitrogen source, such as casamino acid, malt extract, and beef extract to the culture medium did not further increase the flocculant production. Although yeast extract has been used in general as the organic nirtrogen source for the flocculant production processes [9, 14], the presently investigating strain showed the higher flocculating activity only in the inorganic nitrogen sources. Therefore, no organic nitrogen source was added throughout the present experiment.

## Effects of Mineral Ions on Flocculant Production

Mineral ions are required for cellular metabolic activity and enzyme regulation [6]. As shown in Table 2, $MgSO_4$ and $MnSO_4$ significantly increased cell growth and flocculant production. $CuSO_4$ and $CoCl_2$ were not much effective for either flocculant production nor cell growth. From these results, we concluded that $MgSO_4$ and $MnSO_4$ played an important role in cell growth and flocculant production. $MgSO_4$ is an important cofactor for several enzymes catalyzing the transfer of phosphates and phosphorous groups, and also acts as a stabilizer for ribosome and nucleic acid structure [5]. $MnSO_4$ is used as a cofactor for superoxide dismutase, *etc.* [5, 6]. In order to determine the optimum concentration for $MgSO_4$

**Table 2.** Effect of mineral ions on the flocculant production.

| Mineral ion | Cell concentration (g-DCW/l) | Flocculant concentration (g/l) | Relative flocculating activity (%) |
|---|---|---|---|
| $MgSO_4$ | 2.58 | 8.9 | 100 |
| $MnSO_4$ | 2.54 | 8.7 | 96.3 |
| $SnCl_4$ | 2.68 | 7.7 | 82.5 |
| $ZnSO_4$ | 3.17 | 7.5 | 78.3 |
| $CaCl_2$ | 2.26 | 6.5 | 67.7 |
| $BaCl_2$ | 3.06 | 5.3 | 62.8 |
| $CaCO_3$ | 3.12 | 4.4 | 57.3 |
| None | 1.62 | 3.6 | 37.4 |
| $FeSO_4$ | 2.60 | 2.9 | 30.4 |
| $CoCl_2$ | 0.42 | 0 | 4.87 |
| $CuSO_4$ | 0.17 | 0 | 2.4 |

*Mineral ions were added to the culture medium at the concentration of* 0.1 g/l. Culture medium contained 20 g/l soluble starch, 1.0 g/l $NH_4NO_3$, 1.0 g/l $K_2HPO_4$, and 0.8 g/l $KH_2PO_4$.

and $MnSO_4$ requirement, varying amounts of each element, from 0 to 1.0 g/l, were added. The $MgSO_4$ concentration higher than 0.5 g/l resulted in a very sharp decline in flocculant production as well as for cell growth (Fig. 3). In the case of $MnSO_4$, a broad spectrum of cell growth and flocculant production were obtained at concentrations from 0.1 to 1.0 g/l (data not shown). The optimal concentrations of $MgSO_4$ and $MnSO_4$ for flocculant production were achieved at 0.2 g/l. A similiar pattern of the effective concentration of $MgSO_4$ and $MnSO_4$ for both cell growth and flocculant production was observed.

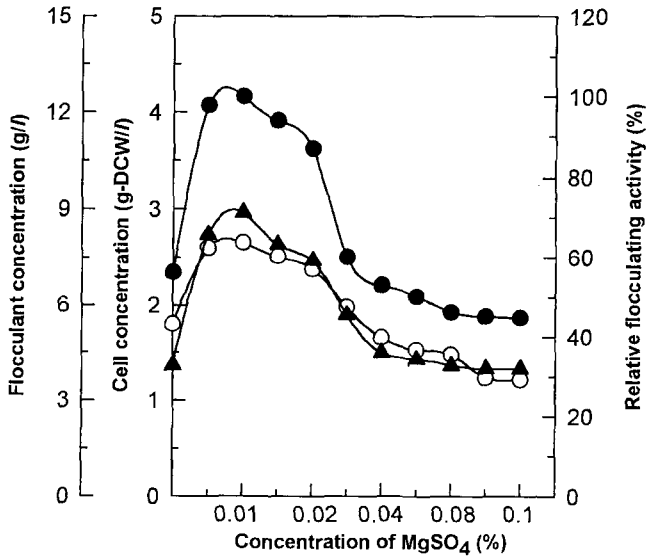


**Fig. 3.** Effect of $MgSO_4$ concentration on the flocculant production.

Cells were incubated with shaking at 30°C for 3 d in the culture medium containing $MgSO_4$ of various concentrations. A culture medium contained 20 g/l soluble starch, 1.0 g/l $NH_4NO_3$, 1.0 g/l $K_2HPO_4$, 0.8 g/l $KH_2PO_4$. Initial pH of the medium was adjusted to 8.0. Symbols: (○) cell concentration, (●) flocculating activity, (▲) flocculant concentration.

Suh *et al.* [17] reported that addition of $MgSO_4$ and $MnSO_4$ to the culture medium resulted in a large increase in flocculant production by *Bacillus* sp. A56.

The effects of phosphate ions on flocculant production and cell growth were investigated (data not shown). Phosphate content is an important factor in the regulation of polymer production [15]. $K_2HPO_4$ and $KH_2PO_4$ were supplied as phosphate sources. The maximum cell concentration was obtained when 1.6 g/l $K_2HPO_4$ and 0.1 g/l $KH_2PO_4$ were added. The highest bioflocculant concentration was attained at a concentration of 2.0 g/l $K_2HPO_4$ and 0.1 g/l $KH_2PO_4$. The buffering action of phosphate ions in preventing a decrease in pH during culture had great influence on bioflocculant production and cell growth. Friedman and Dugan [4] reported that bioflocculant production by *Zoogloea* sp. was effective in a culture medium containing 2.0 g/l $K_2HPO_4$ and 1.0 g/l $KH_2PO_4$.

## Effect of C/N Ratios on the Flocculant Production

In general, polysaccharide flocculant production is favoured by a high ratio of carbon to nitrogen (C/N ratio) in many bacterial species and also in some fungi [21]. The effective concentration of soluble starch on the flocculant production was investigated with culture medium containing 10, 20, 30, 40, and 50 g/l. The concentration of $NH_4NO_3$ was fixed to 1.0 g/l. The highest flocculating activity was reached at 30 g/l soluble starch and the cell concentration was 2.87 g/l. The addition of more than 40 g/l soluble starch depressed the production of flocculants (data not shown).

In order to determine the optimum nitrogen ratio for the production of polysaccharide flocculant by *Bacillus* sp. DP-152, several media containing different C/N ratios with $NH_4NO_3$ as a nitrogen source were investigated. The constituents of the culture medium were the same as previously described, except that various combinations of the carbon and nitrogen sources were empolyed to give different carbon-to-nitrogen ratios. Initial C/N ratios were adjusted to 10, 20, 40, 70, and 100. The concentration of soluble starch was fixed to 30 g/l. The effects of C/N ratio on cell growth and flocculant production of *Bacillus* sp. DP-152 are summarized in Fig. 4: The optimum C/N ratio for flocculant production was obtained at 40. Production of polysaccharide flocculant was greatly influenced and stimulated by the limitation of nitrogen sources. Consequently, it appears that the polysaccharide flocculant may have been produced under the condition of nitrogen limitation. This result suggests that the ratio of flocculant to cell is maintained high under the condition of relatively low nitrogen. Similar patterns have been also observed with other polysaccharides, *e.g.*, xanthan and pullulan [11, 15]. The optimum medium for the production of bioflocculant DP-152 was as follows: 30 g soluble

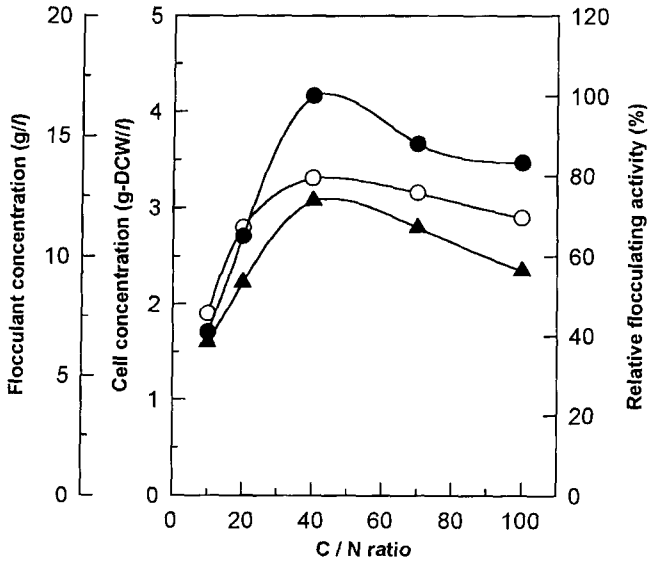


**Fig. 4.** Effect of C/N ratio on the flocculant production.

Cells were incubated with shaking at 30°C for 3 d in the culture medium containing $NH_4NO_3$ of various concentrations. A culture medium contained 30 g/l soluble starch, 2.0 g/l $K_2HPO_4$, 0.1 g/l $KH_2PO_4$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.2 g/l $MnSO_4 \cdot 4\sim5H_2O$. Initial pH of the medium was adjusted to 8.0. Symbols: (○) cell concentration, (●) flocculating activity, (▲) flocculant concentration.

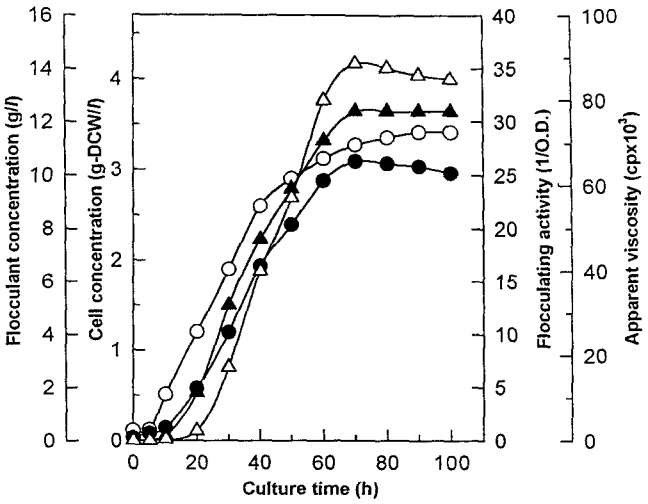


**Fig. 5.** Time course of the flocculant production.

Cells were incubated at 30°C for 120 h in the optimized medium. An optimized medium contained 30 g/l soluble starch, 0.75 g/l $NH_4NO_3$, 2.0 g/l $K_2HPO_4$, 0.1 g/l $KH_2PO_4$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.2 g/l $MnSO_4 \cdot 4\sim5H_2O$. The initial pH was maintained at 8.0. Symbols: (○) cell concentration, (●) flocculating activity, (▲) flocculant concentration, (△) viscosity.

starch, 0.75 g $NH_4NO_3$, 2.0 g $K_2HPO_4$, 0.1 g $KH_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $MnSO_4 \cdot 4\sim5H_2O$ in 1 l of distilled water. By using this medium, the flocculating activity observed was about 4 times higher than that with the basal medium.

## Time Course of the Flocculant Production

Figure 5 shows the time courses of cell concentration and flocculant production of *Bacillus* sp. DP-152 in optimized medium at the flask level. In the flask, the culture broth became progressively viscous with the production of bioflocculant DP-152. The cell concentration increased gradually with the extension of cultivation time and reached the maxium value of 3.41 g-DCW/l after 90 h of cultivation. The concentration of bioflocculant DP-152 increased in proportion to the increasing cell concentration and reached the maxium level of 12.4 g/l after 70 h of cultivation. This result indicates that the concentration of polysaccharide (bioflocculant DP-152) from *Bacillus* sp. DP-152 is much higher than that of other polysaccharide-producing microorganisms [1, 18, 19]. The viscosity of the culture broth at that time was 88,700 centipoise, and then, decreased gradually with prolonged cultivation. Because the concentration of flocculant remained constant, the decrease of the viscosity in later stages of cultivation might suggest that structural changes occurred during this stage. The flocculating activity of *Bacillus* sp. DP-152 was parallel to cell concentration, and a large amount of flocculating substance (DP-152) was released at the end of the exponential phase to give the maximum flocculating activity to aggregate suspended kaolin particles.

In the case of *R. erythropolis* [9] and *A. sojae* [13], a flocculating substance appears to be released into the culture broth in proportion to cell growth and reached to the maximum flocculating activity in the stationary phase. In contrast, in the case of *Streptomyces griseus* [14], a flocculating substance was released in the death phase. These results suggested that the production mechanism of flocculating substances of those microorganisms might be different.

Our results showed that *Bacillus* sp. DP-152 highly produced flocculants in the medium (containing soluble starch and $NH_4NO_3$) without expensive organic nitrogen sources such as peptone and yeast extract. It has not been previously reported that strains produce the flocculants in high concentrations by using only inorganic nitrogen sources. This finding shows the possibility that *Bacillus* sp. DP-152 can be regarded as a useful strain in the commercial production of flocculants.

## Rheological Properties of the Bioflocculant DP-152

One of the most important functions of biopolymer flocculants for industrial application, namely, the effective viscosity of biopolymeric flocculant which is influenced by the changes of polymer concentration, pH and temperature, is dependent on the method to test for the industrial wastewaters under various situation. Then, the rheological property of polymeric bioflocculant is an important factor to evaluate for the possible use on the treatment of wastewater. Most commercial applications

of microbial polysaccharides depend on their rheological properties, which can be influenced by both their sugar composition and the spatial structure of their basic units [11]. Among the rheological properties, apparent viscosity is an important factor which can measure rheological characteristics of polymer solutions. The characteristic flow behavior of the bioflocculant DP-152 solution has been studied in comparison with the zooglan solution (from *Z. ramigera*).

The shear rate varied between 80 and 1 $sec^{-1}$. The resulting changes in the apparent viscosity of the flocculant solutions were measured. The bioflocculant DP-152 and the zooglan solution were pseudoplastic non-Newtonian fluids at concentrations above 0.05 g/l. At each shear rate, the apparent viscosity of the bioflocculant DP-152 solution was found to be about 4 times higher than the zooglan solution at each concentration (0.05, 0.1, 0.2, 0.3, and 0.5%) (Fig. 6). In particular, the most important property of the bioflocculant DP-152 is that it has a higher viscosity than the zooglan solution at a low concentration. The values of apparent viscosity of different types of polysaccharides are remarkably different at the same concentration [12]. It was proposed to be determined according to structural differences such as molecular weight, distribution of side chains, charge density, and shape of the linkage [7].

As shown in Fig. 6, apparent viscosities decreased with increasing shear rate in both flocculants showing pseudoplastic behavior. A decrease in apparent viscosity with an increase in shear rate may indicate the degree of orientation of the molecule, the change in the shape of flexible molecules, and the effect of flow on intermolecular interactions. The thixotropic effect is more dominant at higher concentrations of flocculants. The effect of the shear rate on apparent viscosity is greater than on that of zooglan, indicating that the flocculant molecule is highly charged and asymmetric. The relationship between apparent viscosity and shear rate in the case of the bioflocculant DP-152 was similar to that in the case of zooglan.

With regard to temperature effects (data not shown), the apparent viscosity of bioflocculant DP-152 was found to be higher than zooglan at every point in the temperature range (20~80°C). The changes in apparent viscosity of bioflocculant DP-152 were similiar to those of zooglan as the temperature increased. However, the apparent viscosity of bioflocculant DP-152 decreased by about 1/2 at temperatures over 50°C and bioflocculant DP-152 was comparatively unstable at various temperatures.

The buffer solutions ranging from pH 3.0 to 13.0 were prepared, and 0.2% of bioflocculant DP-152 and zooglan were dissolved in these buffer solutions. The change of apparent viscosity of bioflocculant DP-152 was similar to that of the zooglan solution. In particular, in the acidic range (pH 3~6), apparent viscosity of the

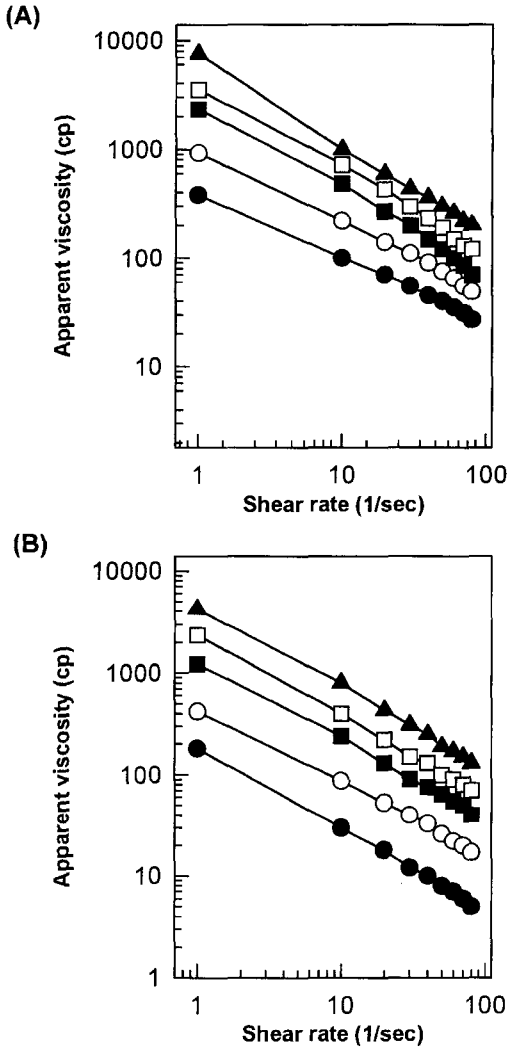


**Fig. 6.** Relationship between shear rate and apparent viscosity at the different concentration of bioflocculant DP-152 (A) and zooglan (B).
Symbols: (●) 0.05%, (○) 0.1%, ( ■ ) 0.2%, (□ ) 0.3%, (▲) 0.5%.

bioflocculant DP-152 solution was lower than that of the zooglan solution. In the alkali range of pH 7~13, the apparent viscosity of bioflocculant DP-152 solution was higher than that of the zooglan solution and was the highest at pH 8. (data not shown). Polymer-solvent interaction, steric interaction, and van der Waal's interactions of chain segments, as well as the electrostatic interaction showed be the important or dominant factors influencing the configuration, size, and shape of the macromolecules and the viscosity of macromolecular solution. These factors are important for the binding force between solvent and dissolvable groups (-COOH, $-SO_3H$, $-NH_2$, *etc.*) of macromolecules [10]. Therefore, from these results we can predict that bioflocculant DP-152 contains more ionic groups than zooglan. At various pH ranges, the ionization degree of the side residues of bioflocculant

DP-152 will be various and can be expected to contain many different ionic groups.

From the rheological properties of bioflocculant DP-152 from *Bacillus* sp. DP-152, bioflocculant DP-152 can be considered to be a new polysaccharide which shows a high apparent viscosity. It has several important properties such as a high viscosity at low concentrations, low viscosity at high shear rates, high viscosity at low shear rates, and good viscosity stability in strong acids and akalides. It is believed that bioflocculant DP-152 will be an environmentally safe flocculant for treatment of many kinds of industrial wastewater. The flocculating properties of bioflocculant DP-152 will be reported elsewhere.

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