ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2017 Vol. 27. No. 8. 930~936 DOI : https://doi.org/10.5352/JLS.2017.27.8.930

Flocculating Properties of Bioflocculant Biopol32 from *Pseudomonas* sp. GP32

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Received July 21, 2017 /Revised August 14, 2017 /Accepted August 15, 2017

The flocculating properties of bioflocculant Biopol32 produced by *Pseudomonas* sp. GP32 were investigated for application in industrial wastewater treatment. The major flocculating substance of bioflocculant Biopol32 was identified as polysaccharide. Many anionic flocculants need a counter ion to induce higher flocculating activity. The flocculating activity of bioflocculant Biopol32 was markedly increased by the addition of cationic ions (Ca^{2+}, Al^{3+}). The flocculating activity of bioflocculant Biopol32 was the most effective when 7.0 mM $CaCl_2 \cdot 2H_2O$ as coflocculant was added. The flocculating activity on the effect of pH and the temperature of the bioflocculant Biopol32 was compared with anionic commercial flocculant (polyacrylamide) and bioflocculant (zooglan from *Zoogloea ramigera*). In kaolin suspension, the highest flocculating activity was obtained at the bioflocculant Biopol32 concentration of 1.5 mg/l. A high flocculating activity was observed in the pH range of 5.0 to 8.0. The flocculating activity of bioflocculant Biopol32 was sustained up to 60℃, but decreased rapidly at over 70℃. In the batch culture, the charge density of bioflocculant Biopol32 was compared with flocculating activity. The larger the anionic charge density and apparent viscosity of bioflocculant Biopol32, the higher the flocculating activity. Therefore, we confirmed that the flocculating activity and apparent viscosity of bioflocculant Biopol32 was closely related to the charge density of bioflocculant Biopol32.

Key words : Bioflocculant, coflocculant, flocculating activity, flocculating property, polysaccharide

Introduction

Many different flocculants have been used in wastewater treatment, dredging and downstream processing techniques [4, 9, 14]. Among these, organic synthetic and large polymeric flocculants have been most frequently used since they are both very cost-effective and strong agents with their use recently increasing [8, 18, 21]. These flocculants have been used for various purposes, depending on their physicochemical properties and toxicity. However, most of 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 [2]. Bioflocculants produced by microorganisms are useful for the treatment of wastewater due to their flocculating activity, biodegradation, and environmental safety. In recent years, to solve these environmental problems, flocculants produced by microorganisms have been utilized due to their biodegradability and the harmlessness of their degradation intermediates to the environment [9]. Studies on flocculating substances from microorganisms have examined them from various viewpoints, such as coagulation of kaolin clay and removal of microorganisms in the fermentation industry [8, 21]. Among natural polymeric flocculants, chitosan, obtainable by deacetylation of chitin obtained from shells of lobster, crab, or shrimp, and algin extracted from brown seawood have shown high flocculating activity on suspended solids [19]. The floc-producing bacteria from activated sludge, were almost limited to the bacteria such as *Zoogloea*, *Alcaligenes*, *Flavobacterium*, and *Norcardia* [14]. Especially, *Zoogloea ramigera* produced extracellular polysaccharide in different carbon and nitrogen sources and the extracellular polysaccharide produced played an important role in decreasing COD (chemical oxygen demand) and BOD (biochemical oxygen demand) of wastewater [3, 8]. Recently, it has been reported that some microorganisms, including *Pseudomonas* sp. GP32, *Pestalotiopsis* sp. *Rhodococcus erythopolis* and *Aspergillus sojae* [7, 10, 13, 15] produce certain kinds of flocculating substances. Many researchers have smartly been working on microbial flocclulant to replace the organic synthetic floccu-

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lant. Thus, they have conducted studies on screening process to find new flocclulant-producing microorganisms, the conditions of flocclulating processes on wastewater treatment and other industrial fields.

In this paper, the flocculating properties of Biopol32 form *Pseusdomonas* GP32 are reported.

Materials and Methods

Cutivation of floccuant-producing microorganism

Pseudomonas sp. GP32 was isolated from soil samples [15] and used in this experiment. A medium composition for cultivation of *Pseudomonas* sp. GP32 contained 30 g galactose, 0.6 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.08 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 4{\sim}5H_2O$, 0.05 g NaCl in 1 l distilled water. The initial pH of the medium was adjusted to 7.5 with 1.0 N NaOH and 1.0 N HCl. The seed culture was derived from a single colony and grown for 30 hr on a rotary shaking incubator at 32℃.

Measurement of flocculating activity

The measurement of flocculating activity was based on the flocculation process of wastewater treatment, and adjusted to laboratory the procedure as reported previously [15]. Standard substance used was kaolin clay (Junsei chemical Co., Japan) and flocculating activity was investigated by flocculation of kaolin clay suspension (5,000 ppm).

Flocculating activity = 1/A - 1/B

Where A is the optical density of sample at 550 nm and B is optical density of a reference at 550 nm.

Effect of cationic ion

For comparing bioflocculant Biopol32 with other flocculants, polyacrylamide as organic synthetic flocculant and zooglan from *Zoogloea ramigera* as a well-known microbial flocculant were used in flocculation test. Generally, when polymeric flocculants were used at flocculation experiments, the cationic salts as coflocculants were used to increase flocculating efficiency [5]. The effects of various cationic ions were investigated on flocculating activity. The various cationic ions used were KCl, NaCl, $MnSO_4 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$ and $FeCl_3 \cdot 6H_2O$, at 6.5 mM, final concentrations. The effect of cationic ion of flocculant was investigated on flocculation of kaolin suspension (5,000 mg/l).

Effect of concentration

For investigating the effects of three flocculants (Biopol32, zooglan, polyacrylamide) concentration on flocculating activity, the final concentration of flocculants was adjusted to 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l, respectively. The effect of concentration of flocculant was investigated on flocculation of kaolin suspension (5,000 mg/l).

Effect of pH and temperature

According to the difference of pHs and temperatures, the flocculating activity of bioflocculant Biopol32 from *Pseudomonas* sp. GP32 was investigated. The range of pH and temperature was from pH 3.0 to pH 11.0 and from 20℃ to 100℃, respectively. The flocculation of flocculants was dependent on the pH of solution since the pH governed the ionization of the functional groups of flocculants. The pH of kaolin suspension was adjusted with 1.0 N HCl and 1.0 N NaOH. Also, as temperature governed the dispersion of flocculants in aqueous system, the effect of temperature was investigated on flocculant of kaolin suspension.

Measurement of charge density

The charge density of bioflocculant Biopol32 produced at aeration condition was measured by the following procedure. Potassium poly (vinyl) sulfate (PVSK) solution was used for the anionic charge titration and poly (diallyl) dimethylammonium chloride (PDAC) solution was used for the cationic charge titration. The indicator used was toluidine blue with cationic charge. The measurement of charge density [6] was carried out in duplicate.

The cationic charge density measurement

Five mililiters of polymer solution (0.05%) were diluted with 45 ml distilled water in 100-ml Erlenmeyer flask and thoroughly mixed for 5 min at 300-400 rpm on a multi-stirrer. After the addition of 300 μl of 0.1% toluidine blue, the mixture was titrated by biuret with 2.5 mM PVSK solution. When the blue color changed to red, addition of PVSK solution was stopped and the end point was determined.

The anionic charge density measurement

Two mililiters of polymer solution (0.05%) were diluted with 48 ml distilled water in 100-ml Erlenmeyer flask and thoroughly mixed for 5 min at 300-400 rpm on a multi-stirrer. Five mililiters of 2.5 mM PDAC solution were added and mixed 300-400 rpm for 5 min by using a multi-stirrer.

The other procedure was the same as described above. The charge density was calculated as follows :

Charge density (meq/g) = 2.5 × A × (X-Y)/1,000 × B × C

Where A is volume of Biopol32, X is volume of PVSK solution used for titration, Y is volume of PVSK solution used for basic experiment, B is weight of bioflocculant Biopol32, C is volume of Biopol32 solution.

Results and Dissussion

Preparation of bioflocculant Biopol32

In order to remove the bacterial cells, the culture broth (10 l) of *Pseudomanas* sp. GP32 was diluted with ten vol. of distilled water. Most of the bacterial cells were removed by centrifugation at 10,000 x *g* for 40 min**.** The cell free culture broth was concentrated to 3.7 l. The concentrated supernatant was precipitated by the addition of two vol. of ethanol. The precipitated crude flocculant was dried with a vacuum evaporator (4.3 g), and redissolved in distilled water. Then, 10% cetylpyridinium chloride (CPC, cationic detergent) solution was added until no more precipitate was formed. The insoluble acidic flocculant-CPC complex was collected by centrifugation, and redissolved in a 10% sodium chloride solution. After dialysis against distilled water, the bioflocculant was precipitated by the addition of two vol. of ethanol and dissolved in distilled water, and the residual ethanol was removed by evaporation. To obtain the white powder of bioflocculant Biopol32, it was freeze-dried and ground to fine powder.

Bioflocculant Biopol32 solution (0.1%) was loaded onto a Sephacryl S-500 column (2.5×150 cm) and eluted with distilled water. Fractions of 5 ml eluate each were collected and polysaccharide was quantified examined by the phenol-sulfuric acid method [1]. The peak pattern of flocculating activity agreed in position with that of a polysaccharide, indicating that the main flocculating factor is a polysaccharide (Fig. 1). It has been reported that the main flocculating factor of the flocculants produced from *Pacilomyces* sp. is protein, while *Zoogloea* sp. and *Alcaligenes cupidus* produced polysaccharide as flocculant [3, 17, 18].

Effect of cationic ions on the flocculating activity

Because surface of kaolin particle in an aqueous solution was negatively charged, the repulsive force between other kaolin particles was decreased due to electrical neutralization by addition of cationic ions [5]. The binding distance

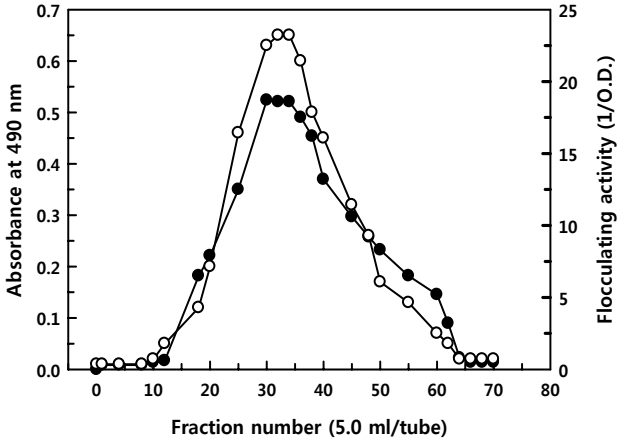

Fig. 1. Gel permeation chromatogram of bioflocculant Biopol32 with Sephacryl S-500. Biopol32 solution (0.1%) was loaded onto a Sephacryl S-500 column (2.5×150 cm). Symbols : ○ polysaccharide, ● flocculating activity.

between kaolin particles is shortened, resulting in coagulation. In this respect, the flocculating activity was measured at the indicated concentration of flocculant with kaolin suspension (5,000 mg/l) containing source of various cationic ions (K^+, Na^+, Mn^{2+}, Ca^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}). The flocculating activity of bioflocculant Biopol32 was markedly increased by the addition of Ca^{2+}, Zn^{2+}, Mn^{2+} and Al^{3+}, especially the highest at 7.0 mM at Ca^{2+} (Fig. 2). The flocculating activity of bioflocculant Biopol32 is similar to that of polysaccharide flocculant produced by *Alcaligenes cupidus* [18] and *Bacillus* sp. PY-90 [20], and the bioflocculant produced

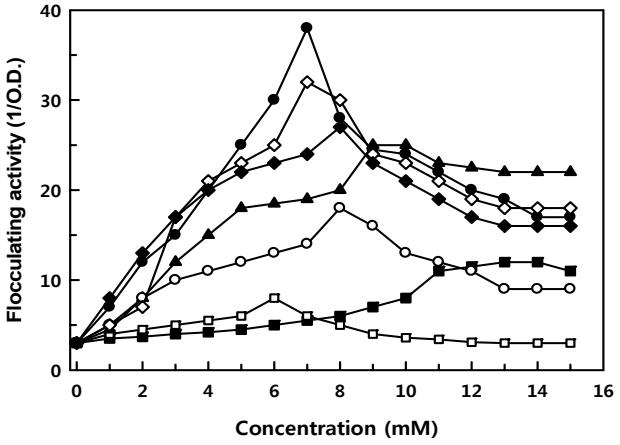

Fig. 2. Effect of several cationic ions on flocculating activity of bioflocculant Biopol32. The flocculating activity was measured in the reaction mixture consisting of 96 ml of kaolin suspension (5,000 mg/l), 2 ml of 6.5 mM cationic solution, 2 ml of bioflocculant Biopol32 (50 mg/l). Symbols: ■ K^+, □ Na^+, ▲ Mn^{2+}, ● Ca^{2+}, ○ Zn^{2+}, ◇ Al^{3+}, △ Fe^{3+}.

by *Nocardia amarae* increased upon addition of several cationic ions (Na^+, Ca^{2+}, Al^{3+} and Fe^{3+}) [14].

These results thus suggested that the bioflocculant Biopol 32 and other microbial flocculants have positive effect on flocculation by adding cationic ions as coflocculant. For investigating the correlation between bioflocculant Biopol32, Ca^{2+}(coflocculant) and kaolin particles, the flocculating activity of bioflocculant Biopol32 was measured according to the addition order of Ca^{2+}. The optimum concentration of $CaCl_2 \cdot 2H_2O$ for flocculation reaction in kaolin suspension (5,000 mg/l) was 7.0 mM.

The stability of kaolin particles in aqueous system is dependent on the balance of the electrostatic in repulsive forces and the London-van der Waals attractive forces [5]. This balance is disrupted by the addition of Ca^{2+}. Addition of Ca^{2+} coagulated the particles by reducing the electrostatic interaction, and flocculation is induced by the addition of bioflocculant Biopol32. As bioflocculant Biopol32 was precipitated by cetylpyridimium chloride (cationic detergent), it was considered to be an anionic polymer flocculant. Therefore, flocculating activity of bioflocculant Biopol32 was more effective when cationic solution (Ca^{2+}) was added.

Effect of bioflocculant Biopol32 concentration

The flocculation reactions were performed at different concentrations ranging from 0.1 to 4.0 mg/l in kaolin suspensions (5,000 mg/l) containing 7.0 mM $CaCl_2 \cdot 2H_2O$. As shown in Fig. 3, the flocculating activity of bioflocculant Biopol32 was the highest at 1.5 mg/l and decreased at higher concentration than optimized concentration of bioflocculant Biopol32. The flocculating activity of polyacrylamide (anionic commercial flocculant) and zooglan (bioflocculant from *Zoogloea ramigera*) was the highest at 1.5 mg/l and 3.5 mg/l, respectively.

The flocculating activity initially increased with increasing flocculant dosage, but then decreased as the adsorption of excess flocculant restabilized the particles. Because of incomplete dispersion of excess flocculants, only particles around flocculants participated in the flocculating reaction in a moment. Therefore, other particles did not participate in the flocculating reaction and the flocculating activity decreased. The relationship between flocculant concentration and flocculating activity of bioflocculant Biopol32 is similar to that of bioflocculant produced by *Rhodococcus erythropolis* [7]. The bioflocculant Biopol32 was especially effective in the flocculating reaction at a low flocculant concentration. These results thus suggested that the bioflocculant Biopol32 can be substituted for commercial flocculant polyacrylamide in respect to flocculation.

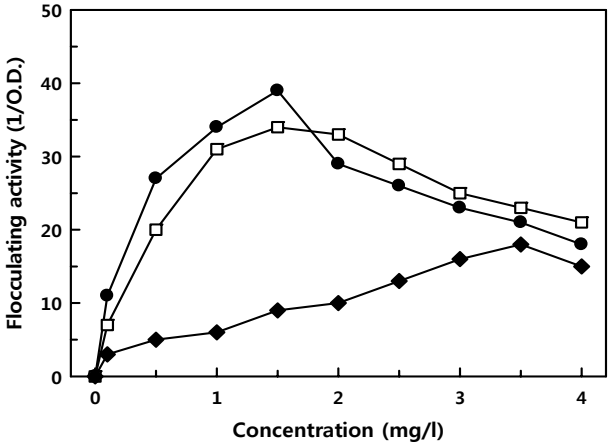

Fig. 3. Effect of flocculant concentration on the flocculating activity. The flocculating activity was measured in the reaction mixture consisting of 96 ml of kaolin suspension (5,000 mg/l), 2 ml of 7.0 mM $CaCl_2 \cdot 2H_2O$ solution, 2 ml of flocculant (5~200 mg/l). Symbols : ● bioflocculant Biopol32, □ polyacrylamide (anionic commercial flocculant), ◆ zooglan (from *Zooglea ramigera*).

Effect of pH and temperature on the flocculating activity

During flocculation of polymeric flocculants, pH and temperature of aqueous system played important roles. The pH was related to ionization of the functional group of flocculant [12]. The effect of pH and temperature of the bioflocculant Biopol32 on the flocculating activity was compared with anionic commercial flocculant (polyacrylamide), and bioflocculant (zooglan from *Zoogloea ramigera*).

In case of the pH changes, all flocculants showed the high flocculating activity generally in the range 5.0 to 8.0. Some typical flocculation curves of these flocculants in kaolin suspension are shown in Fig. 4. The flocculating activity of bioflocculant Biopol32 was the highest at pH 6.0, but at pHs higher than pH 9.0, flocculating activity was decreased markedly. At all ranges of pH, the flocculating activity of bioflocculant Biopol32 was higher than that of other two flocculants. This result shows the possibility that bioflocculant Biopol32 can be regarded as a useful flocculant in the industrial wastewater treatment [16].

The temperature was related to dispersion of flocculant molecules. Because temperature is one of the most important environmental factors affecting flocculating activity [12],

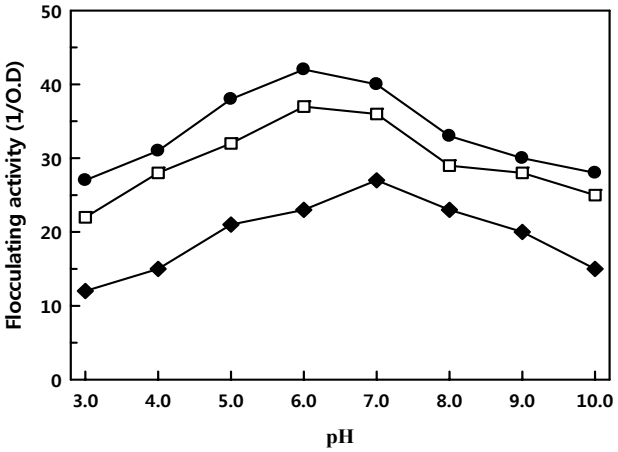

Fig. 4. Effect of pH on the flocculating activity. Each pH was adjusted with 1.0 N NaOH and 1.0 N HCl. The flocculating activity measured in the reaction mixture consisting of 96 ml of kaolin suspension (5,000 mg/l), 2 ml of 7.0 mM $CaCl_2 \cdot 2H_2O$ solution, 2 ml of flocculant (75 mg/l of bioflocculant Biopol32, 75 mg/l polyacrylamide, 100 mg/l of zooglan). Symbols : ● bioflocculant Biopol32, □ polyacrylamide (anionic commercial flocculant), ◆ zooglan (from *Zooglea ramigera*).

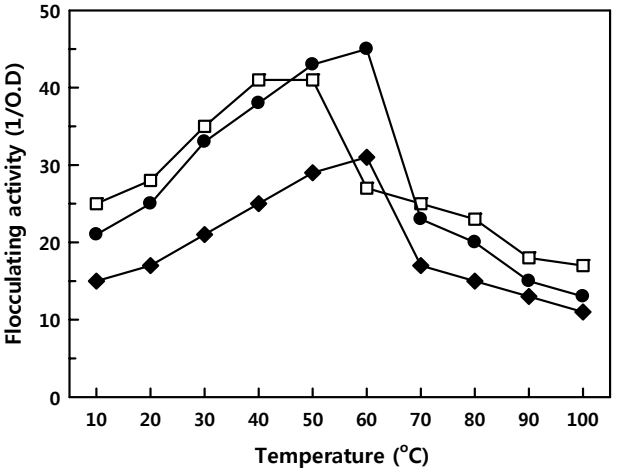

Fig. 5. Effect of temperature on the flocculating activity. The flocculating activity measured in the reaction mixture consisting of 96 ml of kaolin suspension (5,000 mg/l), 2 ml of 7.0 mM $CaCl_2 \cdot 2H_2O$ solution, 2 ml of flocculant (75 mg/l of bioflocculant Biopol32, 75 mg/l polyacrylamie, 100 mg/l of zooglan). Symbols : ● bioflocculant Biopol32, □ polyacrylamide (anionic commercialflocculant), ◆ zooglan (from *Zooglea ramigera*).

flocculating activity of bioflocculant Biopol32 at various temperatures between 10 and 100℃ was examined. As shown in Fig 5. flocculating activity of bioflocculant Biopol32 was retained up to 60℃, but decreased rapidly at over 70℃. On the other hand, the flocculating activity of polyacrylamide

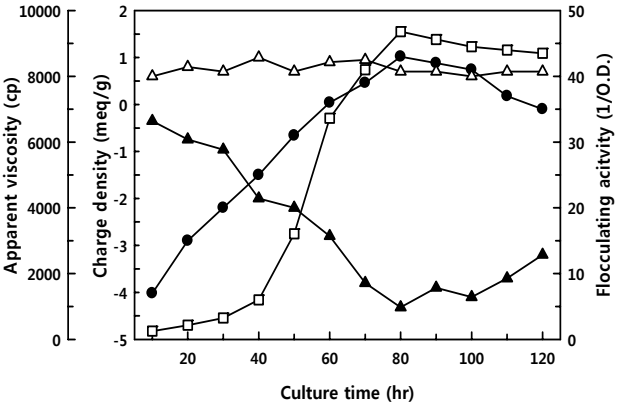

Fig. 6. Change in charge density, apparent viscosity and flocculating activity of bioflocculant Biopol32 for 120-hr culture. A medium composition for batch culture of *Pseudomonas* sp. GP32 contained 30 g galactose, 0.6 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.08 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 4{\sim}5H_2O$, 0.05 g NaCl in 1 l distilled water. The culture temperature and initial pH were 30℃ and 7.5. Symbols : ● flocculating activity, □ apparent viscosity, ▲ anionic charge density, △ cationic charge density.

decreased rapidly at temperature of over 60℃. Similar patterns have been also observed with bioflocculant from *Aspergillus sojae* [13]. Flocculating activity of zooglan from *Zoogloea ramigera* was stable at various temperature. Generally, the microbial flocculants were more stable than organic synthetic flocculant at high temperature.

Change of flocculating activity according to charge density

In the batch culture, charge density of bioflocculant Biopol32 was compared with flocculating activity. The medium composition for cultivation of bioflocculant Biopol32-prducing *Pseudomonas* sp. GP32 was as follows : 30 g/l galactose, 0.6 g/l $(NH_4)_2SO_4$, 1.5g/l k_2HPO_4, 0.08 g/l KH_2PO_4, 0.15 g/l $MgSO_4 \cdot 4{\sim}5H_2O$, 0.05 g/l NaCl. As shown in Fig 6. the flocculating activity of bioflocculant Biopol32 was dependent on the charge density and apparent viscosity of bioflocculant Biopol32.

For 120-hr cultivation of *Pseudomonas* sp. GP32, the anionic charge density of bioflocculant Biopol32 and apparent viscosity of bioflocculant Biopol32 were increased gradually, but the cationic charge density was constant. The larger the anionic charge density and apparent viscosity of bioflocculant Biopol32 were, the higher the flocculating activity was. Therefore, the flocculating activity was in proportion to the anionic charge density of bioflocculant Biopol32. This result was related to the polymerization of bioflocculant Biopol32

molecule. The molecular weight of bioflocculant Biopol32 was measured by gel permeation chromatography (GPC) with a Polymer PL-Gel column (8 um, 300 Å, 300×7.5 mm ; Polymer Lab., U.K), using a conventional HPLC system with a differential refractometer detector (Waters 410, U.S.A.) [15].

Generally the viscosity change in biopolymer solution is affected by changes in molecular weight. The types of molecular structure such as linear or branched chain also affect its rheological properties [11]. Therefore, we confirmed whether that the flocculating activity and apparent viscosity of bioflocculant Biopol32 was closely related to charge density of bioflocculant Biopol32. Based on these results, bioflocculant Biopol32 from *Pseudomonas* sp. GP32 is expected to be widely applied to various industrial wastewater treatments as a novel flocculating agent because of its safety to harmless toward humans and the environment.

Acknowledgement

This work was supported by a Research Grant of Gyeongnam National University of Science and Technology (2015 year).

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초록 : *Pseudomonas* sp. GP32가 생산하는 생물고분자응집제 Biopol32의 응집특성

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Pseudomonas sp. GP32가 생산하는 생물고분자응집제 Biopol32의 실제 산업폐수에서의 적용을 위하여 생물고분자 Biopol32의 응집특성을 조사하였다. Biopol32의 응집물질은 polysaccharide로 확인되었다. 음이온성 응집제들은 응집효율을 높이기 위하여 보조응집제로 counter ion을 사용하고 있다. Biopol32의 응집활성은 보조응집제로 Ca^{2+}, Al^{3+}와 같은 양이온을 첨가 하였을 때 크게 증가하였으며, 보조응집제로서 7.0 mM $CaCl_2 \cdot 2H_2O$을 첨가하였을 때 Biopol32의 응집활성이 가장 높게 나타났다. Kaolin 현탁액에 Biopol32를 1.5 mg/l의 농도로 첨가하였을 때 가장 높은 응집활성을 보였다. Biopol32의 pH와 온도에 따른 응집활성은 현재 폐수처리 현장에서 상업적으로 이용되고 있는 음이온성 유기합성고분자응집제 polyacrylamide와 *Zoogloea ramigera*로부터 생산된 생물고분자응집제 zooglan과 응집활성을 비교하였다. Biopol32의 응집활성은 pH 5.0에서 8.0의 넓은 범위의 pH에서 높은 응집활성을 보였으며, 또한 온도의 영향에서는 60℃에서 가장 높은 응집활성을 나타내었으나, 70℃ 이상의 온도에서는 응집활성이 급격히 감소하는 것으로 나타났다. Jar fermentor를 이용한 batch culture를 통하여 Biopol32의 응집활성과 전하밀도와의 관계를 조사하였다. 음이온성 전하밀도와 겉보기 점도가 높을수록 Biopol32의 응집활성이 높아져, Biopol32의 응집활성과 겉보기점도는 Biopol32의 전하밀도와 밀접한 관계가 있는 것으로 나타났다.