Characterization of an extracellular polysaccharide produced by *Bacillus* sp.RL-2

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**Abstract:** A strain secreting a strongly acidic polysaccharide flocculating agent was isolated from activated sludge, and identified as *Bacillus brevis*. The bioflocculant was produced by RL-2 during the late logarithmic growth in the batch culture and was recovered from supernatant by ethanol precipitation. The bioflocculant is thermo-stable as its activity remains stable after heated at 100 °C for 45 min. Its flocculating activity with kaolin suspensions was stimulated by the addition of Ca<sup>2+</sup>, Al<sup>3+</sup>, and Cu<sup>2+</sup>. The flocculant consists of glucose, mannose, and galacturonic acid. Its average molecular mass was estimated to be approximately 2.86×10<sup>5</sup> by the method of viscosity. The flocculant aggregates various inorganic and organic compounds in solution.

**Keywords:** bioflocculant; *Bacillus* sp.RL-2; extracellular polymer; bioflocculation

1 Introduction

Bioflocculation is a dynamic process resulting from synthesis of extracellular polymer by living cells. Flocculant are widely used in industrial processes including wastewater treatment, downstream processing, and food and fermentation processes [1-6]. The flocculants used can be classified into three groups: 1) inorganic flocculants such as aluminum sulfate and poly-aluminum chloride; 2) synthetic organic high-polymer flocculants such as polyacrylamide derivatives and polyethylene imine; and 3) naturally occurring flocculants such as chitosan, sodium alginate, and the microbial flocculants. Although chemical flocculants have been used widely due to their effective flocculating activity and low cost, their use give rise to environmental problems in that some of them are not readily biodegradable and the intermediate products of their degradation are harmful to humans. To solve these environmental problems, the use of biodegradable biopolymer flocculants produced by microorganisms has been investigated [7-9]. Microbial flocculants are biodegradable and their degradation products are harmless to the ecosystem.

In this study, the isolation of a microorganism that produces a new biopolymer flocculant is reported together with descriptions of flocculating and chemical properties of the flocculant.

2 Materials and methods

2.1 Screening and identification of a biopolymer flocculant-producing bacterium

Flocculant-producing strains were isolated from soil, drain and activated sludge samples by an agar plate culture using a basal medium consisting of 0.3% beef extract, 1% polypepton and 0.5% NaCl. A kaolin suspension was used as a flocculation test material. RL-2 was identified based on its morphological and physiological characteristics [10].

2.2 Preparation of biopolymer flocculant

One loop, full of flocculant producing bacteria isolated from activated sludge and grown on slant agar at 28 °C, was inoculated to a 50 mL flask containing 25 mL medium. The composition of screening medium (PT-1) was as follows: 20 g glucose, 2 g KH<sub>2</sub>PO<sub>4</sub>, 5 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g NaCl, 5 g yeast extract and 0.5 g urea, in 1 L distilled water. The pH of the medium was adjusted to 8.0. Then the culture flask was transferred to a rotary shaker at 30 °C, 150 r/min for 3 days. The broth culture was used for testing the flocculation activity.
2.3 Bioflocculant purification

In order to purify the bioflocculant, the culture broth was diluted with a double volume of distilled water and centrifuged at 4,000 g for 30 min to remove cells. Thereafter the diluted culture broth was concentrated. A double volume of cold ethanol at 4 °C were added to the supernatant and the crude bioflocculant precipitate was vacuum-dried in a desiccator overnight. In distilled water, the dry crude bioflocculant was dissolved, and cetylpyridinium chloride (CPC) with a mass concentration of 20 g/L was added until no more insoluble CPC-bioflocculant complex was formed. The precipitate was redissolved in a saline solution, in which a supernatant was separated and concentrated by dialysis against water for 24 h. Cold ethanol at 4 °C doubling the volume of the dialyzed supernatant was added to obtain precipitate which was then washed twice with cold ethanol and finally vacuum-dried to get partially purified RL-2 product [4,5].

2.4 Chemical and physical characterizations analyses

The total sugar content of the bioflocculant was determined by the phenol−sulfuric acid method; the total protein content by the Coomassie brilliant blue G-250 method; the amino sugars by the Elson–Morgan method; the uronic acid by the carbazol method; and the Neutral sugar by Anthrone reaction. The purified bioflocculant was hydrolyzed at 100 °C for 5 h using 2 mol/L HCl; its sugar components were analyzed by thin layer chromatography and its relative molecular mass was determined by Viscosity method [9,10].

2.5 Determination of flocculation activity

Unless otherwise stated, the flocculating activity was measured according to the method of Kurane et al. [1] using kaolin clay suspension as test material. Kaolin clay was suspended in distilled water at a concentration of 1 g/L (kaolin suspension). In a 100 mL beaker, 80 mL kaolin suspension was added and mixed with 2.0 mL CaCl₂ solution (ω(CaCl₂)=1%) and 2.0 mL sample (broth culture). The test beaker was vigorously stirred for 1 min, then gently shaken for 3 min, and left to stand for 5 min at room temperature. From the upper layer in the beaker, 2.0 mL of supernatant was carefully taken out, and its absorbance at 550 nm (A) was measured. A control test without the polymer solution was carried out in the same way and the absorbance at 550 nm (B) was measured. The flocculating activity was expressed in the form of flocculation efficiency given by the following equation [3].

\[ \text{Flocculating efficiency} \% = (1 - A/B) \times 100 \]

2.6 Effect of various condition on flocculating activity

The effects of various cations on the flocculating activity of the bioflocculant from strain RL-2 in kaolin suspension were carried out. Solutions of KCl, CaCl₂, MgCl₂, MnSO₄, AlCl₃, FeCl₃ and CuSO₄ were used as the sources of cation. To examine the effects of pH value of a reaction mixture on flocculating activity, the pH value of a reaction mixture containing kaolin suspension was adjusted with 1 mol/L HCl and 1 mol/L NaOH, then the flocculating activity was measured. The pH values of the test suspensions ranged from 2.0 to 12.0.

3 Results and discussion

3.1 Screening and identification of bioflocculant-producing bacteria

Among 107 pure culture strains with mucoid appearance isolated from activated sludge, soil, drain, etc., the strain RL-2, producing flocculating substances, is obtained from activated sludge, exhibiting the highest flocculating activity against kaolin clay suspension after 3 days of cultivation. Growing on basal medium agar was a slimy, cream-colored colony with a smooth edge and a rounded shape. The morphological tests showed a short rod, Gram-positive bacteria, a sporogenous, aerobic, motile strain. Only on the basis of morphological tests, the bacterium is classified into the genus “Bacillus”. According to physiological characteristics and compared with Bergey’s manual [11], RL-2 is identified as Bacillus brevis. The results are presented in Table 1.

3.2 Time course of bioflocculant production in the production medium

Fig.1 shows the time course of cell growth and flocculating activity of RL-2 strain in the batch cultivation using PT-1 medium. The course of flocculating activity is parallel to that of cell growth. The flocculating activity reaches its climax at 96.3% after 3 days of incubation and decreased thereafter, and a large amount of the bioflocculant is secreted into the culture broth during the late logarithmic growth phase. This suggests that the bioflocculant is not produced by cell autolysis but by biosynthesis.
Table 1  The morphological and physiological characteristics of Bacillus sp. RL-2

<table>
<thead>
<tr>
<th>Morphological characteristics</th>
<th>Physiological characteristics</th>
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<tbody>
<tr>
<td>Shape</td>
<td>Short rod</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Capsule</td>
<td>+</td>
</tr>
<tr>
<td>Spore</td>
<td>+</td>
</tr>
<tr>
<td>Gram-stain</td>
<td>+</td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>+</td>
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<td></td>
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Fig. 1  The curves of flocculating activity and the cell growth in terms of OD₅₅₀

Fig. 2 is the pH variation curve with the culture time. As can be seen, the decline of pH from 8.0 to 7.2 correlates with the rapid cell growth during the first 24 h, but it is followed by a slight rise that may be due to the production of organic acids from the metabolism of glucose or to the presence of organic acid components of the polymer produced.

The flocculating activity of the bioflocculant solution heated at 100 °C for 45 min under atmospheric pressure hardly changes, which demonstrates the thermo-stability of RL-2 and implies that the polymer may be an acidic polysaccharide.

The results of the qualitative and quantitative analyses for the biopolymer are shown in Table 2. Chemical and physical analyses show that neutral sugar and uronic acid are the major and minor components, respectively. The total mass fraction of all sugar is 52.2% and there is no protein, indicating that the biopolymer is mainly a polysaccharide. The hydrolysate of the purified polymer shows three spots on silica gel plates, and the \( R_f \) values of these three spots corresponded with that of the standard sugars galacturonic acid, mannose, and glucose. The average relative molecular mass of the polymer is estimated to be approximately 2.86×10⁵ Da by the method of viscosity. The precipitate occurring after the addition of CPC to the polysaccharide solution indicates the formation of the polysaccharide–CPC complex resulting from the interaction with the quaternary ammonium ion of the CPC, which manifests the existence of acidic groups in the structure of the polysaccharide [12,13]. This further concluded that the polymer is an acidic polysaccharide. The polysaccharide from Bacillus sp. RL-2 is soluble in all acidic (HCl) or basic (NaOH) solutions tested, but insoluble in organic solvents (methanol, ethanol, acetone and acetic acid).

3.4 Characteristics of the bioflocculant from strain RL-2

The effects of various cations on the flocculation activity from strain RL-2 are shown in Fig. 3. The divalent and trivalent cations, such as Ca²⁺, Al³⁺ and
Cu\(^{2+}\), are found to be more effective than the monovalent cation. The flocculating activity of the bioflocculant is markedly increased by the addition of 8 mmol/L Ca\(^{2+}\). These results suggest that the flocculation is due to the change in the charge density: cation synergizes flocculation by neutralization of residual negative charges of the bioflocculant and kaolin particles, so increases the initial adsorption of the biopolymer on kaolin particles, forming bridges which bind kaolin particles to each other.

Table 2 Chemical characteristics of partially purified bioflocculant RL-2

<table>
<thead>
<tr>
<th>Method</th>
<th>Analyzed item</th>
<th>Mass fraction/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Ninhydrin reaction</td>
<td>α-Amino acids</td>
<td>+</td>
</tr>
<tr>
<td>2. Xanthoproteic reaction</td>
<td>Aromatic amino acids</td>
<td>−</td>
</tr>
<tr>
<td>3. Molish reaction</td>
<td>polysaccharide</td>
<td></td>
</tr>
<tr>
<td>Quantitative test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Phenol-sulfuric acid</td>
<td>Total sugar</td>
<td>52.2</td>
</tr>
<tr>
<td>2. Kaomwth lightblue method</td>
<td>protein</td>
<td>Not detected</td>
</tr>
<tr>
<td>3. Anthrone reaction</td>
<td>Neutral sugar</td>
<td>10.3</td>
</tr>
<tr>
<td>4. Carbazole-sulfate reaction</td>
<td>Uronic acids</td>
<td>16.8</td>
</tr>
<tr>
<td>5. Elson-Morgan reaction</td>
<td>Amino sugar</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Fig. 3 Effect of various cations on flocculating activity

The effects of pH value on flocculating activity are shown in Fig. 4. The flocculation efficiency initially increases with increasing pH, remains stable within a range of 3 to 9, then rapidly decrease. As the pH value of the reaction mixture changes, the biopolymer and particle surface charge density change such that the polymer adsorbs onto another particle, which causes flocculation via bridging mechanism and hence increases the flocculation efficiency.

Fig. 5 illustrates the flocculation activity of the crude bioflocculant in comparison with that of two typical synthetic flocculants, polyaluminum chloride (PAC) and polyacrylamide (PAM). For the bioflocculant, the highest flocculating activity is obtained at the concentration of 1 mg/L in the presence of 8 mmol/L CaCl\(_2\) in the kaolin suspension. The activity initially increases with increasing flocculant dosage then remains stable. It is comparable to that of PAM and much higher than that of PAC. The bioflocculant RL-2 exhibits an excellent flocculating activity compared with typical synthetic flocculants.

Fig. 4 Effect of pH on flocculating activity

Fig. 5 Effect of flocculant concentration on flocculating activity

The flocculating activity was assayed against a variety of organic and inorganic suspended particles. The materials tested were diatomite, activated carbon, bentonite, aluminum oxide, soil, cellulose powder and agar powder. The flocculant from Bacillus sp. RL-2 could effective flocculated all materials in aqueous solution. Therefore, the bioflocculant seem to have a fairly broad range of substrate specificity. On the whole, the bioflocculant of RL-2 possesses superior flocculating activity.

4 Conclusions

Bacillus sp. RL-2 isolated from activated sludge has the highest flocculating activity within 3 days of cultivation. Its flocculation of kaolin is greatly
synergized by bivalent/trivalent cations attributed to the neutralization of the zeta potential. The majority of the extracellular biopolymer secreted by Bacillus sp. RL-2 is soluble in aqueous solution but insoluble in tested organic solvents. The biopolymer is an acidic polysaccharide with a relative molecular mass of about $2.86 \times 10^5$ and possibly composed of galacturonic acid, mannose, and glucose. It has a wide flocculating spectrum, a high flocculating activity and a sound thermal stability, which reveal potential applications of the biopolymer to the treatment of a variety of wastewater.

Acknowledgments

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References