Note

A Protein Bioflocculant Produced by *Rhodococcus erythropolis*[†]

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Rhodococcus erythropolis S-1, a phthalate ester degrading microorganism,^{1,2)} produces a bioflocculant (NOC-1) that has the ability to flocculate many kinds of suspended solids, including kaolin.³⁾ Culture conditions for optimum flocculant production have been reported.⁴⁾ This paper describes purification protocols and chemical composition analysis of NOC-1.

The microorganism was inoculated in 100 ml of the following culture medium, and then incubated for 4–5 days at 30°C. The culture medium contained 10 g of glucose, 0.5 g of yeast extract, 0.5 g of urea, 5 g of K₂HPO₄, 2 g of KH₂PO₄, 0.1 g of NaCl, and 0.2 g of MgSO₄·7H₂O in 1 liter of distilled water. After cultivation, cells were removed by centrifugation (10,000 × *g* for 5 min) and cell-free culture broth was obtained.

Kaolin clay suspension was used to test for flocculating activity. Since multivalent cations were indispensable for flocculation of kaolin by NOC-1,^{3,4)} kaolin was suspended in 1.2 mM Al₂(SO₄)₃ solution with a concentration of 5 g/liter. The sample was added to 1 ml of this suspension, and then the assay mixture was vortexed. The aggregate that formed was sedimented by a weak centrifugation at 40 × *g* for 15 sec, and the optical density (OD) of the aqueous phase was measured at 660 nm (OD_{sample}). The OD of the sample-free kaolin suspension was also measured (OD_{blank}). Flocculating activity was calculated by the following equation,

$$\text{Flocculating activity} = \frac{\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{blank}}}$$

Cell-free culture broth and its filtrates were dialyzed and then flocculating activities were estimated (Fig. 1). The

culture broth retained its original activity after the filtration through a filter paper (Toyo No. 2, pore size; 5 μm). However, when filtered through membrane filters (pore sizes; 0.45 and 0.8 μm), the activity was reduced to less than half of the original. The activity was almost lost by filtration through a membrane filter of which the pore size was 0.22 μm. The membrane filters were products of Millipore (MF type, mixed esters of cellulose). These results suggested that the flocculant formed micelles of 0.22–5 μm in diameter.

Forty g of (NH₄)₂SO₄ and 100 ml of water-saturated *n*-butanol were added to 100 ml of the culture broth, followed by centrifugation. Three phases were appeared after centrifugation, i.e., a water lower phase, a *n*-butanol upper phase, and an insoluble layer boundary phase. The dry weight of the insoluble layer was 20–50 mg, while the amount of both butanol and water phases were less than

Fig. 1. Flocculating Activity of Culture Broth and Its Filtrates.

Fifteen μl of culture broth dialysate (A) and its filtrates passed through filters, with mean pore sizes of 0.22 (B), 0.45 (C), 0.8 (D), and 5 μm (E), were added to 1 ml of the test kaolin suspension.

Fig. 2. Effects of Flocculant Concentration on Flocculating Activity.

The standard kaolin suspensions containing 1.2 mM of Al³⁺ were tested with different flocculant preparation concentrations.

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Fig. 3. Filter Paper Electrophoresis of the Flocculant. Electrophoresis was done at a constant voltage of 500 V. A mixture of Tris-HCl buffer (pH 8.4) and pyridine (9:1) was used as an electrolyte. The filter paper used was Toyo No. 6. After the electrophoresis, the filter paper was stained with CBB G-250.

2 mg. It must be emphasized here that only the insoluble layer had flocculating activity. The active layer was further washed with acetone, suspended in distilled water, and then dialyzed. The dialysate was designated the flocculant preparation.

The flocculant preparation thus obtained lost its activity upon filtration through a membrane filter (0.22 μ m), showing that it formed micelles. When a flocculation test was done at different concentrations of the flocculant preparation, a sufficient activity was observed at the range of 10–20 mg/liter (Fig. 2). The activity decreased when an excess of the preparation (more than 20 mg/liter) was supplied (Fig. 2).

Elemental analysis showed that the content of nitrogen was 11.0%. In an infrared spectrum, two significant absorption bands (1500–1550 and 1650–1700 cm^{-1}) that were considered to correspond to mono-substituted amide

can be observed. Three mg of the flocculant preparation was suspended in 10 ml of 0.041 M potassium phosphate buffer (pH 7.5) containing 6 μ g/ml of Pronase E (protease, type XXV, Sigma) and in 10 ml of 0.01 N HCl containing 6 μ g/ml of pepsin. After incubation at 37°C for 18 hr, the mixtures were dialyzed and a flocculation test was done. Flocculating activity was decreased to less than 10% of the initial activity, suggesting that the protein portion is indispensable for flocculation.

When the flocculant preparation was analyzed by filter paper electrophoresis, a single protein band was detected (Fig. 3). However, many bands were detected on sodium dodecyl sulfate polyacrylamide electrophoresis (data not shown). It could be assumed that a complicated association of many peptides might lead to micelle formation, which is beyond the scope of this report.

Several polymer that were produced by microorganisms have been reported as flocculants.^{5–8)} Two of them had protein portions, but their activities were not lost by Pronase treatment.^{7,8)} In contrast to that, the activity of NOC-1 was lost by enzymatic digestion, suggesting that the protein portion is important.

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