Isolation of a Flocculent Photosynthetic Bacterium
Rhodovulum sp. and Characterization of Nucleic Acids
Dependent Flocculation

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Summary: A marine photosynthetic bacterium (PS88) identified as Rhodovulum sp.
with flocculating ability was isolated from the sea sediment mud of a shrimp
cultivation farm in Thailand. This bacterium flocculated in glutamate/malate
medium during aerobic/dark or anaerobic/light cultivation. The flocculating ability
was enhanced with the increase of NaCl concentration to 6% (w/v). Flocculation and
growth were promoted by Mg$^{2+}$ up to 10 mM in culture medium. Kaolin, anionic
colloidal particle was flocculated only in the simultaneous presence of extracellular
polymeric substance (EPS) derived from bacterial cells, and metal cations. With the
advantage of self-flocculation, high density cell cultivation was accomplished by
continuous cultivation to 43 g dry matter l$^{-1}$ with acetate as a substrate consumption
at 22.5 g l$^{-1}$d$^{-1}$.

Introduction Photosynthetic bacteria such as Rhodospirillaceae have been used for
industrial organic wastewater (soybean curd and sugar cane manufacturing) treatment
process because they can use various organic materials as substrates. However, the
separation of photosynthetic bacteria after wastewater treatment is a problem for the
practical applications due to their poor solid/liquid separation. Generally flocculation
provides some advantages for industrial process as the simple separation of cell mass
from wastewater. In practice, microorganism with flocculent ability have been
employed for industrial application such as activated sludge treatment, continuous
ethanol fermentation by flocculent yeasts, and methane fermentation with upflow
anaerobic sludge blanket reactors. In the present study, a excellent flocculent
photosynthetic bacterium was isolated, and identified as Rhodovulum sp. To
understand the flocculation mechanism for the further application of cell surface
engineering, higher valency of metal cations and extracellular polymeric substances
(EPS) were found to provide kaolin aggregation. EPS was then shown to contain nucleic
acids, especilly RNA as the essential component for kaolin aggregation. Utilizing
bacterial flocculent characteristic, high cell density culture was accomplished as model
continuous wastewater treatment process as acetate for the substrate.

Materials and Methods
Strain isolation For the isolation, samples of sea sediment mud of a shrimp culti-
vation pond were stabbed into modified glutamate/malate (GM) agar medium (pH 8.0)
in screw-cap tubes and overlaid with sterile liquid paraffin to provide anaerobic light
(photoheterotrophic) conditions. The tubes were then incubated at 35 °C under 38.1-
57.1 µmol photons m$^{-2}$s$^{-1}$ incandescent illumination. After several times enrichment
cultures, the colonies with different colors and sizes were isolated by crossstreaking for
pure cultures. PS88 strain was thus isolated as a rapidly growing photosynthetic bacte-
rion with rare flocculent ability. PS88 strain was stored on GM plus 3% NaCl agar
slants after cultivation for 2–3 d under anaerobic light conditions (95.2 µmol photons
m⁻²s⁻¹) at 30°C. Upon inoculum from agar slants, PS 88 was grown aerobically in 100 ml GM medium (pH 8.0) with 3% NaCl at 20°C in a 300 ml conical flask in the dark for 1–7 d. Aerobic conditions were provided by rotary shaking at 100 rpm.

**Extraction of extracellular polymeric substance (EPS)** PS 88 strain cells grown at 20°C and pH 8.0 for 5 d were harvested at 10,000 g, 15 min, and washed once with 5 mM phosphate buffer (pH 8.0) at 4°C. Cell pellet (approx. 50 mg wet weight) was transferred to 10 ml of the buffer in a 20-ml screw-cap tube. To deflocculate cells in the absence of NaCl, the tube was incubated for 30 min at 40°C with reciprocal shaking at 100 rpm and 10-cm amplitude. After 5,000 g for 15 min, supernatant was used as EPS fraction. PS 88 cells were cultivated aerobically in 100 ml GM medium (pH 8.0) with 3% NaCl at 20°C in a 300 ml conical flask in the dark for 1–7 d. Aerobic conditions were provided by rotary shaking at 100 rpm.

**Flocculation assay** According to Kamekura and Onishi (1978) with slight modification for the estimation of cell mass, flocculation was determined. Total protein concentration of each portion in their method as cell mass because it was linearly correlated with dry cell weight (R = 0.966). Thus, flocculation (F) was defined as follows:

\[ F(%) = (1-2C_{UP}/C_{TP}) \times 100 = \left(\frac{C_{LP}-C_{UP}}{C_{LP}+C_{UP}}\right) \times 100 \]

where C_{TP} is the total protein in 100 ml culture broth (mg/100 ml), C_{UP} is the upper (50 ml) protein fraction (mg/50 ml), and C_{LP} is the lower (50 ml) protein fraction (mg/50 ml) after standing in a 100 ml cylinder for 1 min. To evaluate cell distribution between upper and lower halves in the cylinder, the fractions were separated quickly and the cells were solubilized in 2 M NaOH before protein assay.

**Measurement of kaolin flocculation** Flocculating activity of EPS in a kaolin suspension was determined based on the method of Toeda and Kurane (1991).

**Single-tower fermentor** A single-tower fermentor with working volume of 660 ml consisted of a reaction zone (lower part, φ 4.1 cm x 29.5 cm height, 390 ml) and a settling zone (upper part, φ 8.0 cm, 270 ml) designed for effective gas/liquid separation. During continuous operation, temperature was maintained at 20°C by a water jacket. Pure N₂ gas or O₂ gas, and air was supplied from the bottom of the reactor with a sparger (φ 40 mm) at 300-500 ml min⁻¹ as flow rates.

**Results and Discussion**

**Deflocculation of flocculent cells** Flocculent cells were treated either physico-chemically or enzymatically to obtain deflocculated cells and to understand flocculation mechanism. Chemical treatment with 1 M NaOH had a remarkable effect on deflocculation, whereas 1 M HCl did not (Table 1). In addition, cells were completely deflocculated by incubation at 40°C in the absence of NaCl even for 30 min, but not in the presence of 1% NaCl, suggesting that flocculation of the strain PS88 can be destabilized in NaCl-free condition and at relatively high temperature. Enzyme treatments also supported that DNA and/or RNA may be involved in flocculation of strain PS88 (Table 2).

**Table 1** Physico-chemical deflocculation of flocculated cells of PS88. The control contained 5 mM phosphate buffer (pH 8.0, without NaCl) without agent. Each agent was dissolved in 5 mM phosphate buffer (pH 8.0, without NaCl) except for the treatments with 1 M NaCl and 1 M HCl. ++ Complete deflocculation; + considerable deflocculation; – no deflocculation. Estimations of deflocculation were performed according to the method of Kamada and Murata (1983). Flocculated cells obtained after 120 h cultivation at 20°C under 3% NaCl in glutamate/malate medium were used.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reaction temperature (°C)</th>
<th>Deflocculation</th>
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<tbody>
<tr>
<td>1 M HCl</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>1 M NaOH</td>
<td>20</td>
<td>+ +</td>
</tr>
<tr>
<td>0.1 M EDTA 4Na</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>2% Tween 80</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>2% Triton X-100</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>40</td>
<td>+ +</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>–</td>
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**EPS formation** Flocculent cells cultured at 3.5% NaCl (Fig. 1A) were observed by scanning electron microscopy (Fig. 1B), where coccus-, ovoid- and rod-type cells were observed to be covered with and connected by strand-like sticky materials. However, deflocculated cells obtained by Na-free incubation at 40°C for 30 min had almost the same appearance in SEM (data not shown). This may be due to the incomplete removal of the exopolymers even though cells were completely deflocculated.

**Taxonomic characteristics** Strain PS88 was examined in terms of carbon assimilation, morphology, membrane ultrastructure. The existence of intracytoplasmic membrane of the vesicular type (data not shown) indicated that PS88 can be defined as *Rhodovulum* sp. (Hiraiishi and Ueda 1995).

**Effect of divalent cations on growth and flocculation** As shown in Fig. 2, 10 mM Mg\(^{2+}\) showed the optimal growth (ca. 1.0 g DCW l\(^{-1}\)) and flocculation (F(%) ≥ 90). This concentration was almost the same as that in coastal sea water with lower Mg\(^{2+}\) concentration than ocean sea water (ca. 50 mM Mg\(^{2+}\)), because it is diluted by influx of river water. In case of Ca\(^{2+}\) (Fig. 2c), only slight flocculation was observed for various Ca\(^{2+}\) concentrations in spite of good growth. However, when Ca\(^{2+}\) was added together with 10 mM MgSO\(_4\), good growth and flocculation were constantly observed with a variety of Ca\(^{2+}\) concentrations (Fig. 2c). This result suggested that PS88 strongly requires Mg\(^{2+}\) both for flocculation and growth. On the other hand, trivalent cations such as Fe\(^{3+}\) and Al\(^{3+}\) could not support growth and flocculation in place of Ca\(^{2+}\) and Mg\(^{2+}\) (data not shown).
Effect of cations on kaolin flocculating activity Since flocculation of strain PS88 (intact cell system) was enhanced by the addition of cations, and EPS produced was seemed to be a weak-anionic polymer from effect of pH on kaolin flocculation (data not shown), effect of various cations on the flocculating activity was examined for kaolin suspension (Fig. 3). Monovalent cations such as $K^+$ and $Na^+$ had a slight promotive effect on kaolin flocculation, and more than 500 mM NaCl or 300 mM KCl was required for the flocculation. Addition of divalent cations such as $Ca^{2+}$, $Mg^{2+}$, $Al^{3+}$, and $Fe^{3+}$ also promoted kaolin flocculation to one to two-order higher degrees than monovalent cations (Fig. 3b, 3c). These results suggest that promotive effect on kaolin flocculation with higher valency cations is dependent on both concentrations and valence of the ions.

Continuous culture with flocculent cells in single-tower fermenter system Continuous culture was operated to investigate whether a high density of the flocculated cells of Rhodovulum sp. was attainable in the single-tower fermenter. As shown in Fig.4, high density cell cultivation was accomplished to 42.7 g MLSS l$^{-1}$ at 750 h culture with high consumption rate of acetate (22.5 g l$^{-1}$ d$^{-1}$). This value of 42.7 g MLSS l$^{-1}$ was the highest level of photosynthetic bacterial cultivation reported (Sakato et al. 1992). Flocculated cells exhibited good settling characteristics in later growth phase (600 h ~) i.e. SVI values was lower than 30 ml g$^{-1}$ MLSS even if the SV$30$ increased due to the increase of MLSS. Turbidity of settling zone (OD$_{660}$) was kept at relatively low value, indicating the
good flocculation of cells. These characteristics indicating good settling property, which would be useful for cell harvesting/separation.

Moreover, DO was also closely related with settling characteristics of flocculent cells. When DO level decreased to 3 mg l⁻¹ after 350 h, increase of SVI value and decrease of cell mass were observed. This result may also supported a hypothesis that mass transport was limited in flocculated cells and deflocculation would occur by self-digestion.

References


Publication List


