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Effects of culture conditions of *Pseudomonas aeruginosa* strain RB on the synthesis of CdSe nanoparticles

Hiroyuki Ayano, Masashi Kuroda, Satoshi Soda, and Michihiko Ike*

Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan

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Cadmium selenide (CdSe) was synthesized by *Pseudomonas aeruginosa* strain RB in a culture containing lactic acid as a carbon source, 1 mM selenite, and 1 mM cadmium under various conditions. High purity (1.02–1.16 of the atomic ratio of Se to Cd) and efficient synthesis of biogenic CdSe nanoparticles were observed at 25–30°C, 0.05–10 g L^{-1} NaCl, and neutral pH conditions compared with other tested conditions. However, the size and shape of synthesized CdSe nanoparticles were not changed by changing culture conditions. The contents of S and Se in the particles respectively increased under alkaline and weak acidic conditions. Furthermore, high temperature (>37°C), high salinity (>10 g L^{-1} NaCl), and alkaline pH affected the CdSe–synthesizing rate by strain RB. This report is the first optimizing the culture conditions for synthesizing biogenic CdSe nanoparticles in a batch processing.

[**Key words:** *Pseudomonas aeruginosa*; Cadmium selenide nanoparticles; Selenite reduction; Cadmium resistance; Optimization]

Cadmium selenide quantum dots (CdSe QDs), which are 1–10 nm semiconductor nanoparticles, attract great concern for their wide applications, such as bio-imaging and biosensing, associated with their unique properties (1,2). However, chemical synthesis of CdSe involves toxic solvents and high temperatures (3).

To overcome such shortcomings of the chemical methods, biological synthesis of CdSe at ambient temperatures and pressures without toxic solvents has been investigated as an environmentally friendly process (1,4–8). Actually, CdSe nanoparticles can be synthesized by microbial reduction of tetravalent Se (selenite or selenium chloride) to selenide followed by chemical reaction of selenide with Cd ion. Kumar et al. (1) and our group (8) achieved one-vessel processes in which microbes were synthesized CdSe in the co-presence of selenite and cadmium ion, the others (4–7) were by two-vessels processes, probably because of Cd toxicity on microbes. The one-vessel process is expected to improve economic efficiency through its simple operation of fewer reaction vessels.

Pseudomonas aeruginosa strain RB, previously isolated from a soil sample by our research group, has capacities of both selenite reduction and Cd resistance (over 5 mM of Cd ion). This bacterium can synthesize CdSe particles of <10–20 nm inside and on the cells from selenite and Cd ion by a one-vessel reaction. Particles larger than 100 nm were also observed outside the cells. Although the ideal value of the atomic Se/Cd ratio of the synthesized particles is 1.0, the experimentally observed ratio was 1.18 with sulfur (S) content of 20% (8). The synthesis conditions used were 28°C and initial pH of 7.0, which happen to be ideal conditions for the bacterial growth, although they might not necessarily be the optimum conditions for CdSe nanoparticles synthesis.

To realize the industrial production of CdSe nanoparticles by strain RB, the culturing conditions should be optimized to prepare sufficient amounts of cells and to improve the purity and synthesis rates of CdSe nanoparticles. In biological synthesis of gold or silver nanoparticles, the particle size and shape can be varied by changing the pH and temperature (9–12). However, effects of culturing conditions on microbial synthesis of binary metal compounds such as CdSe remain unknown. Compared with single-component metals, synthesis of binary metal compounds are expected be more complex and to require stricter control of synthesizing conditions to obtain pure products with high efficiency, especially in a one-vessel reaction. Therefore, this study investigated effects of culture conditions of the strain RB, such as pH, temperature, and the NaCl concentration, on the purity and the rate of CdSe synthesis.

MATERIALS AND METHODS

Bacterial strain and media Strain RB was used for this study. This strain was revealed to synthesize CdSe nanoparticles by reducing selenite to selenide and reacting it with cadmium (8). The draft genome sequence of this strain was reported later by our previous study (13).

A basal salt medium (BSM: 0.5 g K_2HPO_4, 1 g NH_4Cl, 0.05 g NaCl, 0.2 g $MgCl_2 \cdot 7H_2O$, 0.01 g $FeCl_3$, 0.01 g $CaCl_2$, 0.05 g Na_2SO_4, 0.06 mg H_3BO_3, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.12 mg $CoCl_2 \cdot 6H_2O$, 0.07 mg $ZnCl_2$, 0.025 mg $NiCl_2 \cdot 6H_2O$, 0.015 mg $CuCl_2 \cdot 2H_2O$, 0.025 mg $Na_2MoO_4 \cdot 2H_2O$, 0.02 g Bacto Yeast Extract in 1 L of ultrapure water) containing 20 mM sodium lactate as the carbon source (designated as L-BSM) was used for the strain RB cultivation. For growth tests, 20 mM of potassium hydrogen oxalate (pH 4.5–5), phosphate buffer (pH 6–6.5), 50 mM of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7–8), or CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) buffer (pH 9–10) was added to the medium to stabilize its pH value. For cadmium resistance tests and CdSe synthesis tests, nitrilotriacetic

* Corresponding author. Tel.: +81 6 6879 7672; fax: +81 6 6879 7675.
E-mail address: ike@see.eng.osaka-u.ac.jp (M. Ike).

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acid of 0.216 g L^{-1} per 1 mM of cadmium was added to the medium as the cadmium ion chelator: L-BSM containing × mM of selenite is designated as LSe(x)-BSM; L-BSM medium containing y mM of $CdCl_2 \cdot 2.5H_2O$ with a corresponding concentration of nitrilotriacetic acid is designated as LCd(y)-BSM. LCd(1)-BSM containing 1 mM sodium selenite is designated as LCdSe-BSM.

Carbon source utilization test Commercially available Biolog GN2 plates (Biolog Inc., USA) were used to determine the carbon source utilization profiles of strain RB. Strain RB cells in the stationary phase were washed twice with saline (0.85 g L^{-1} NaCl). Then the optical density at 600 nm (OD_{600}) of the suspension was adjusted to 0.01. Then a 150-μL aliquot of the cell suspension was inoculated into each well of the plate. The plate was incubated at 30°C for 48 h under dark conditions. Absorbance at 595 nm was measured using a microplate reader (Vient XS; DS Pharma Biomedical Co. Ltd., Osaka, Japan).

Growth and resistance tests Strain RB was cultivated aerobically in 20 mL L-BSM in a 50-mL serum bottle for 24 h at 28°C on a rotary shaker at 120 rpm (radius of gyration, 50 mm). Subsequently, 1 mL of the culture was transferred to 20 mL of fresh L-BSM and cultivated under the same conditions for 12 h (prior cultivation). The culture was inoculated into 100 mL of fresh L-BSM in a 300-mL Erlenmeyer flask at 0.02 of initial OD_{600} and was cultivated at various temperatures (10–50°C), pH (4.5–10), salinity (0.05–50 g L^{-1}), and the concentrations of cadmium ion (0–20 mM) and selenite (0–20 mM). The specific growth rate was calculated from the time course of the OD_{600} value during the log-phase.

CdSe synthesis tests Strain RB was inoculated into 100 mL of LCdSe-BSM in a 300-mL Erlenmeyer flask in the same way as growth tests and was cultivated under various temperatures (25°C, 30°C, 37°C, and 45°C), pH (6.5, 7, 7.5, 8, and 8.5), and salinity (0.05, 1, 5, 10, and 20 g L^{-1} NaCl).

The cell density of strain RB was estimated from the protein concentration. Aliquot cultures were diluted and the cells were disrupted by ultrasonication (130 W, 1 min) on ice. The protein concentration was measured by Coomassie Brilliant Blue staining (CBB, bundled in Protein Quantification Kit-Rapid; Dojindo Molecular Technologies Inc., Kumamoto, Japan).

Characterization of the cells and synthesized nanoparticles Transmission electron microscopy (TEM, H-7650; Hitachi Ltd., Tokyo, Japan) was used to observe the cells and synthesized particles. The cells were observed as described previously (8). For observation of the cell-free particles, cells containing the particles were collected using centrifugation (15,000 ×g, 4°C, 10 min) from 50 mL of the culture, and washed three times with ultrapure water. Subsequently, the pellet was resuspended in 5 mL of wash buffer described by Park et al. (6) and followed by cell-disruption with an ultrasonicator (130 W, 10 min, Vibracell VCX-130; Sonics and Materials Inc., CT, USA). The suspensions were filtered with 0.45 μm and 0.2 μm pore size cellulose acetate filters (Advantec, Tokyo, Japan). Then the filtrates were collected using centrifugation (21,600 ×g, 4°C, 10 min) and suspended in 5 mL of ultrapure water. Subsequently, 1 mL of them was transferred to 1.5 mL microtubes for washing three times with ultrapure water. Finally, 10 μL of the suspensions were dried on carbon-supported copper grids (ELS-C10; Okenshoji Co. Ltd., Tokyo, Japan) for observation of the extracted particles.

Elemental analysis of the synthesized particles was conducted using an energy-dispersive X-ray spectra system (EDS, EX-24063 JGT; JEOL Ltd., Tokyo, Japan). Samples were prepared in the same manner as those used for TEM observations.

Chemical analysis All samples were centrifuged (15,000 ×g, 10 min, 4°C) and filtrated by 0.45 μm filter units to separate the supernatant and precipitates before analyses. The selenite concentration in the supernatant was determined using ion-exchange chromatography (IC), as described in a previous report (14). For soluble selenium and cadmium, nitric acid was added to samples at 10% of the final concentration before determination by inductively coupled plasma atomic emission spectrometry (ICP–AES, SPS7800; SII NanoTechnology Inc., Chiba, Japan).

RESULTS

Physiological characterization of strain RB A carbon source utilization profile of strain RB is shown in Table S1. Strain RB showed a wide availability of carbon sources, especially amino acids and carbonic acids, which was similar to that of typical strain of *P. aeruginosa* (15). Lactate used as a carbon source in our previous study is an excellent substrate for strain RB growth.

The specific growth rates of strain RB under various temperatures, pH, salinities, and selenite or cadmium concentrations are shown in Fig. 1. Strain RB grew under widely various conditions of 10–45°C, pH 5–9.5, and 0.05–40 g L^{-1} of NaCl (Fig. 1A–C). Moreover, they showed a maximum specific growth rate (0.88 h^{-1}) at 40°C, pH 7.0, and 0.05 g L^{-1} NaCl. The optimum temperature for growth that is most practical is expected to be 37°C because the specific growth rate dropped at temperatures higher than 40°C (Fig. 1A). Although the specific growth rate at 1 mM selenite was as high as that without selenite, it dropped over 2.5 mM selenite (Fig. 1D). On the other hand, the specific growth rate decreased by about 25% with 1–20 mM cadmium.

Effects of culture conditions on CdSe nanoparticles synthesis The time courses of the removals of selenite and cadmium ion under various conditions are shown in Fig. 2. The time courses of OD_{600} as the cell concentration are also shown in Fig. 2A–C. The OD_{600} could not be determined under the condition of 45°C, pH 6.5 or alkaline pH, and high NaCl concentrations because of the formation of firm flocs. Under most conditions, the following tendencies were found consistently. First, after a short lag-phase, selenite and cadmium ions were removed concomitantly, implying CdSe synthesis (8). This short lag-phase for the Cd/Se synthesis is attributable to the fact that selenite reduction in LSe(1)-BSM started in the late log-phase (Fig. S1). Second, as their removals proceeded, the removal rate

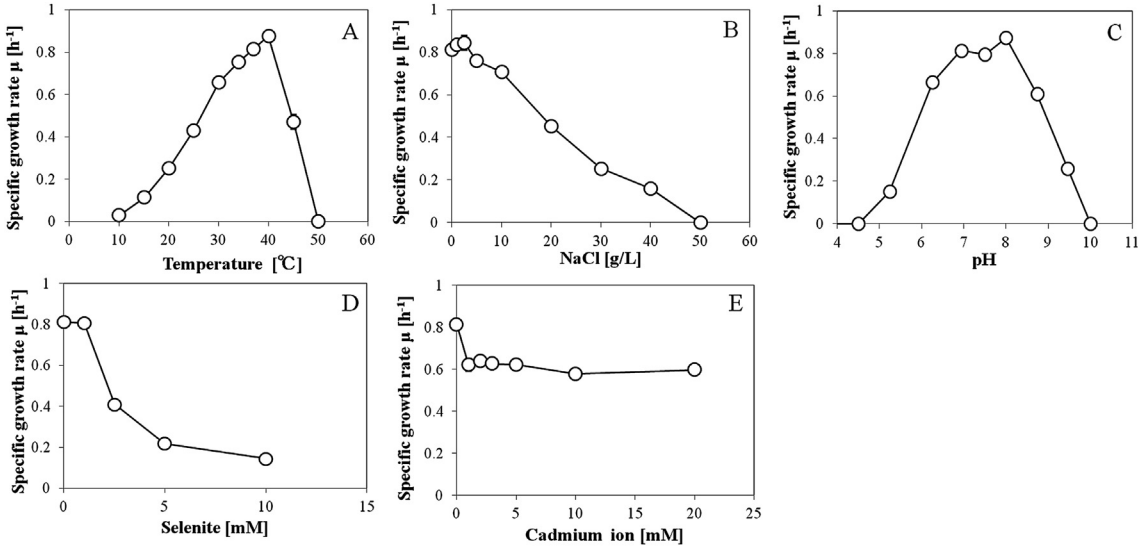

FIG. 1. Specific growth rates of strain RB under various temperatures, pH values, salinities, and selenite or cadmium ion concentrations: (A) effect of temperature, (B) effect of salinity, (C) effect of pH, (D) effect of selenite concentration, and (E) effect of cadmium ion concentrations. Vertical bars represent the standard deviation of three independent experiments.