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Polyphosphate metabolism by purple non-sulfur bacteria and its possible application on photo-microbial fuel cell

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A purple non-sulfur bacterium, *Rhodopseudomonas palustris* G11, was isolated from an activated sludge plant that treats domestic wastewater. This isolation resulted in the effective accumulation of polyphosphate in cells upon reaching the stationary growth phase. However, when the carbon and/or energy source were/was removed, this bacterium released intracellular polyphosphate or poly-β-hydroxybutyrate to obtain energy to grow or maintain its growth. Furthermore, a novel photo-microbial fuel cell (PMFC) design was proposed. The unique capability of purple non-sulfur bacteria to capture light energy for polyphosphate accumulation was maximized. After *R. palustris* G11 accumulated considerable polyphosphate and was transferred to a fresh medium, the PMFC system exhibited a maximum voltage of approximately 0.03 V undt illumination. The chemical oxygen demand removal efficiency, Coulomb efficiency, and power density were 95.8%, 0.62%, and 0.15 mW/m^2, respectively. The test microorganisms converted most of the light energy in growth and caused the low power production. The microorganisms grew slowly and produced less power under dark conditions than under light illumination. However, these microorganisms used the previously stored polyphosphate or poly-β-hydroxybutyrate for electricity production when they were incubated in a growth-insufficient condition. This novel concept can be improved and optimized in the future for new PMFC applications, such as rechargeable cells, to treat wastewater and restore energy simultaneously.

[**Key words:** Purple non-sulfur bacterium; *Rhodopseudomonas palustris*; Polyphosphate; Poly-β-hydroxybutyrate; Growth-insufficient condition]

The use of microbial fuel cell (MFC) to generate biological electricity has elicited considerable attention. The system performance of MFC may be affected by system architecture, materials, microbial community, substrate, and operating conditions (1,2). Photo-microbial fuel cell (PMFC) have been developed in the past decade. PMFC are devices that convert light into electricity by exploiting the photosynthetic activity of phototrophic microorganisms (3). PMFC degrade organic matter in the presence of light to simultaneously produce electricity. Therefore, light affects PMFC performance by altering the metabolism of phototrophic microorganisms. Various PMFC designs have been developed and tested by applying different photosynthetic microorganisms. Electricity generation with purple non-sulfur bacteria (PNSB) has also been studied. *Rhodoferax ferrireducens* can generate currents by oxidizing glucose to CO_2 (4). Increased power density (2720 mW/m^2) can be achieved by *Rhodopseudomonas palustris* DX-1 when acetate is utilized as the carbon source (5). However, previous research has revealed that not all PNSB strains can produce electricity. For example, *R. palustris* RE-2 produces low power of approximately 0.14 mW/m^2 (6).

PNSB are phototrophic microorganisms that can produce energy via photosynthesis. Although most PNSB strains are α-proteobacteria, several strains are β-proteobacteria (7). PNSB are isolated from lakes, lagoons, ponds, and other marine environments. The wide distribution of PNSB results from their extraordinary metabolic versatility, as manifested by photoautotrophic, photoheterotrophic, chemoheterotrophic, and chemoautotrophic bacteria. PNSB have been investigated and applied to wastewater treatment, recalcitrant organism degradation, and fermentative hydrogen production. In addition, PNSB can accumulate polyphosphate. Several PNSB strains were isolated in a previous study (8). The results showed that under anaerobic illumination, PNSB in the stationary phase can generate polyphosphate. The study also proved that light allows PNSB to accumulate polyphosphate and stabilizes phosphorus removal efficiency. The phosphorus content during illuminated anaerobic incubation ranges from 5% to 13% of the bacterial cell dry weight (8).

Polyphosphate is a linear biopolymer composed of three to hundreds of phosphate residues that are linked by high-energy phosphoanhydride bonds (9). It is also one of the most ubiquitous natural biopolymers in numerous microorganisms, such as bacteria, fungi, yeasts, plants, and animals (9–11). Microorganisms store excess energy from light as polyphosphate and release energy when starved. Polyphosphate kinase (PPK) is the key enzyme for the microbial synthesis of intracellular polyphosphate. PPK converts the terminal phosphate of adenosine triphosphate (ATP) to a general polyphosphate (12–15). Different functions of polyphosphate, such as reservoir of energy and phosphate, have been revealed in the physiological adaptation of microorganisms during growth and development (16).

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Recent MFC research has mostly focused on modifying electrode material or optimizing reactors, but not on applying or inducing the abilities of microorganisms, to enhance MFC system performance. Therefore, this study utilized PNSB, which can capture light energy and store energy as polyphosphate, as the biocatalyst for microbial fuel cell. Intracellular polyphosphate in PNSB cells is an energy-containing substance that may be used as an extra energy source to generate power in MFC systems. MFC can be regarded as a rechargeable battery that can release and store energy via polyphosphate metabolism. In this study, energy-rich intracellular polyphosphate was strategically released to enhance MFC power generation. The application of polyphosphate metabolism to enhance power generation was investigated through transferring microorganisms to growth-insufficient environments.

MATERIALS AND METHODS

Bacterial isolation and incubation conditions Pure strains of PNSB were isolated from activated sludge tanks operating in the enhanced biological phosphorus removal mode. The isolates were cultured following the parameters described by Pfennig (17). The *Rhodospirillaceae* medium contained 1.0 g/L of sodium acetate, 0.5 g/L of KH_2PO_4, 0.5 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.4 g/L of NaCl, 0.05 g/L of $CaCl_2 \cdot 2H_2O$, 0.2 g/L of yeast extract, 0.4 g/L of glutamate, 0.005 g/L of Fe–citrate, 0.01 mg/L of cyanocobalamin (vitamin B_{12}), and 1 mL/L of trace element solution SL7. SL7 comprised 1 mL/L of 25% (v/v) HCl, 70 mg/L of $ZnCl_2$, 100 mg/L of $MnCl_2 \cdot 4H_2O$, 60 mg/L of H_3BO_3, 200 mg/L of $CoCl_2 \cdot 6H_2O$, 20 mg/L of $CuCl_2 \cdot 2H_2O$, 20 mg/L of $NiCl_2 \cdot 6H_2O$, and 40 mg/L of $Na_2MoO_4 \cdot 2H_2O$. pH was adjusted to 6.8 ± 0.01 for optimality. PNSB isolation and incubation were performed at 30°C under anaerobic illumination with 4500-5000 lux light intensity and a nitrogen gas headspace. Among all pure strains of isolated PNSB, the ratio of phosphorus content to the biomass of G11 was approximately 13%–15%, which was higher than those of other strains. Therefore, strain G11 was selected and used in this study. The polymerase chain reaction (PCR) product of G11, which was amplified with *pufM* primer, was sequenced and identified as *R. palustris* (Gene bank accession number GU080287.1).

PMFC construction and operation Single-chamber, rectangular PMFC with a 250-mL working volume were used. Carbon cloth was utilized as the anode (10 cm × 3 cm, 60 cm^2 projected area) and was autoclaved at 121°C for at least 20 min prior to use. An air cathode (projected surface area of 7 cm^2) was constructed from carbon cloth with four PTFE diffusion layers and a 0.5-mg Pt cm^{-2} catalyst layer (18). The composition of the solution was similar to that of the culture medium, but the phosphate concentration was adjusted to 100 mM to increase electrical conductivity and buffer capacity. Anaerobic conditions were generated by sparging the reactor with nitrogen gas before each batch experiment. Given that the internal resistance of the system was close to 1 kΩ, the system was operated at 1 kΩ fixed external resistance with a constant temperature of $35°C \pm 1°C$ under 1000 ± 50 lux light. Voltage was measured at 10-min-intervals with a Model 7700 multimeter/digital data acquisition system (Keithley, USA). Coulomb efficiency was calculated to estimate system performance (19).

Gene expression analysis Gene expression analysis was performed via a two-step experiment that involved RNA reverse transcription and real-time PCR. In the first step, RNA was extracted with an SV Total RNA Isolation System (Promega, USA) and reverse-transcribed to cDNA with an ImProm-II Reverse Transcription System (Promega, USA). The reaction mixture for reverse transcription contained 4.5 μL (<1 μg) of total RNA, 0.5 μL (10 mM) of the reverse primer, 1.0 μL of ImProm-II reverse transcriptase, 0.5 μL of Recombinant RNase Ribonuclease inhibitor, 1.0 μL of dNTP, 4.8 μL of $MgCl_2$ (final concentration of 6 mM), 4.0 μL ImProm-II 5× reaction buffer, and 3.7 μL of ddH_2O (total volume of 20 μL). The reverse transcription program was as follows: 25°C for 5 min, 45°C for 60 min, and 75°C for 15 min. The cDNA samples were then quantified via real-time PCR performed with a LightCycler 1.5 (Roche, Mannheim, Germany). Four primer sets *pufM*557F (5′ – GGA CAG AAA GAC CCT ATG AA – 3′)/*pufM*750R (5′ – TCA GCC TGT TAT CCC TAG AG – 3′) (20), *ppk*127F (5′ – GAC TCC GAG GTG ATT G – 3′)/*ppk*271R (5′ – GCG GAT TGA CCG ATT CTT – 3′) (21), *ppx*232F (5′ – ATG AAG GTC GGC AAG GTG T – 3′)/*ppx*553R (5′ – AGT GAT TTC TGC GAG GCG T – 3′) (21), and 27f (5′ – AGA GTT TGA TCM TGG CTC AG – 3′)/109r (5′ – ACG YGT TAC KCA CCC GT – 3′) (22), which targeted purple non-sulfur bacteria phototrophic functional gene, polyphosphate kinase gene, exopolyphosphatase gene, and bacterial 16S rRNA gene, respectively, were individually added to 20 μL LightCycler capillaries (Roche) mixed with the reagent provided with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) industrial kit. Each reaction volume contained 2.5 μL of cDNA, 1 μL of primer sets, 2 μL of 5× Master Mix (0.5 mM of $MgCl_2$, FastStart Taq polymerase, reagent buffer, SYBR Green I, and deoxynucleoside triphosphate mix), and 4.5 μL of ddH_2O. Real-time PCR was programmed as follows: initial denaturation at 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 17 s with single signal acquisition (for *pufM*); 45 cycles of 95°C for 10 s, 57°C for 5 s, and 72°C for 12 s with single signal acquisition (for *ppk*); 45 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 16 s with single signal acquisition (for *ppx*); 45 cycles of 95°C for 10 s, 55°C for 5 s, and 72°C for 12 s with single signal acquisition (for 16S rRNA gene); melting curve analysis: 95°C for 0 s, 65°C for 15 s, and 98°C for a 0.1°C/s ramp rate with continuous signal acquisition; and final cooling at 40°C for 30 s. Data were collected and analyzed with LightCycler Software 2.0 (Roche). The fold change in three functional genes, which was normalized to a reference gene (16S rRNA gene) and relative to expression at time zero, was calculated for each sample following a previous study (23). The mean and SD were determined from the triplicate samples at each time point.

Chemical analysis Liquid samples were filtered through a 0.22-μm pore filter prior to chemical analyses. To determine chemical oxygen demand (COD), the closed reflux titrimetric method was performed in accordance with a standard method (24) The residual phosphorus concentration in each sample was determined colorimetrically with Test N Tube Reagent Vials (HACH, USA) following the manufacturer's instructions. pH variation was measured with a pH meter (HACH sensION 156 Meter). All experiments were performed in duplicate.

4′,6-Diamidino-2-phenylindole and poly-β-hydroxybutyrate staining 4′,6-Diamidino-2-phenylindole (DAPI) is a fluorescent DNA stain that strongly binds to AT-rich DNA regions. The stain exhibits maximum absorption at a wavelength of 360 nm. When bound to double-stranded DNA, its maximum emission wavelength is at 461 nm. DAPI has also been utilized to detect intracellular polyphosphate. The emission wavelength of the DAPI-polyphosphate complex is 525 nm when excited at 360 nm (25,26). In this study, target cells were fixed on a slide and stained with 1 μg/mL of DAPI at normal atmospheric temperature for 15 min. The slide was washed twice with 1 mL of cold ddH_2O to remove excess DAPI stain and then air-dried overnight. Sudan Black B staining was applied to visualize PHA (27), which contains poly-β-hydroxybutyrate (PHB), polyhydroxyvalerate, polyhydroxyhexanoate, polyhydroxyoctanoate, and their copolymers. All of them could be visualized by Sudan Black B staining. In this study, Sudan Black B staining was mainly applied to observe PHB, which could be synthesized by *R. palustris*. Stained bacterial samples were visualized with an optical Axioskop 2 Plus epifluorescent microscope (Carl Zeiss, Germany) equipped with a HBO 100 W Hg vapor lamp. All images were obtained under similar conditions with an exposure time of 100 ms.

RESULTS AND DISCUSSION

Polyphosphate accumulation capacity of G11 and practical strategy for releasing intracellular polyphosphate A previous study has shown that light illumination is important for phosphate accumulation by PNSB. Under anaerobic illumination after 3 days of incubation and in the growth stationary phase, the amount of intracellular phosphorus that *R. palustris* G11 accumulated amounted to 13%–15% of its biomass (8). In this study, the practical operation strategy for releasing the intracellular polyphosphate was tested. The phosphorus removal capacity of the isolated *R. palustris* G11 under illuminated anaerobic conditions was evaluated as baseline data. Fig. 1 shows the bacterial growth curve and nutrient consumption in the medium, including organic carbon and phosphorus consumption. At 79 h of incubation under illuminated anaerobic conditions, *R. palustris* G11 simultaneously reduced phosphorus concentration from 231 mg/L to 208 mg/L and COD from 1331 mg/L to 720 mg/L in the liquid medium. During this period, moreover, *R. palustris* G11 utilized COD as its carbon and energy sources. Between 0 h and 45 h of incubation (exponential growth phase), the phosphorus concentration in the medium decreased from 231 mg/L to 220 mg/L. After an additional significant decrease in the phosphorus medium, the concentration from 220 mg/L to 208 mg/L from 48 h to 63 h of incubation corresponded to the growth stationary phase (Fig. 1). Intracellular polyphosphate accumulation in the isolated *R. palustris* G11 is presented in Fig. 2. The amount of intracellular polyphosphate, which emits yellow fluorescence signals, increased with incubation time. Only a few cells accumulated polyphosphate during exponential growth. By contrast, *R. palustris* G11 accumulated polyphosphate grains after entering the stationary growth phase. Prokaryotes can directly convert pyruvate to phosphoenolpyruvate (PEP) by using PEP synthetase (PPS) or pyruvate–phosphate dikinase (PPDK). These

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