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Correlation between bio-hydrogen production and polyhydroxybutyrate (PHB) synthesis by *Rhodopseudomonas palustris* WP3-5

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ABSTRACT

The aim of this study was to determine the competition between H_2 production and polyhydroxybutyrate (PHB) accumulation of *Rhodopseudomonas palustris* WP3-5 when grown on six different substrates. From the results, strain WP3-5 can utilize acetate, propionate, malate, and lactate to produce H_2 but can only synthesize PHB on acetate and propionate. The substrate conversion efficiency (SCE) on acetate and propionate increased significantly after the maximum PHB content was achieved, illustrating a competition for reducing power when PHB synthesis occurred. However, when strain WP3-5 was cultivated at suboptimal pH values on acetate, the synthesized PHB prevented strain WP3-5 from the stress of the inappropriate pH and retained H_2 producing efficiency as at optimal pH value. Consequently, although PHB synthesis does compete with H_2 production in *R. palustris* WP3-5, it is still conducive to H_2 production when strain WP3-5 is in a stressful condition.

1. Introduction

Nowadays, fossil fuel combustion is faced with several obstacles, including exhausted sources, emission of green house gases, and the release of hazardous pollutants into the environment. Hydrogen (H_2) that is biologically produced by microorganisms has been extensively developed because of its high-energy content (122 kJ/g) and clean product after combustion. Moreover, the biological manufacture of H_2 shows lower cost and fewer pollutants released into the environment when compared with the chemical manufacture. Among the various biological processes to produce H_2, the photobiological H_2 production via purple non-sulfur bacteria (PNSB) is becoming a promising technology attributed to its high-purity H_2, lower energy requirement, and flexible applicability (Turner et al., 2008).

Several species of PNSB are capable of producing H_2 photobiologically by utilizing organic acids as the substrate, such as *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sulfidophilus*, and *Rhodopseudomonas palustris*. These PNSB gain electrons and protons by degrading organic acids and generate ATP from photosystems. The harvest of the electrons, protons, and ATP can further support nitrogenase to achieve nitrogen fixation under nitrogen-limiting conditions. When both ammonium and nitrogen gas are absent, nitrogenase can act as a non-specific enzyme and converts the proton to H_2 with electrons and thus produce H_2. H_2 production by PNSB is complex and is affected by many factors with many intercellular aspects such as cell growth, energy distribution, light harvesting efficiency, and enzyme activity. For instance, the nitrogenase activity is vulnerable to the oxygen and ammonium content, as oxygen and ammonium can seriously disrupt its capability to produce H_2. It has been reported that when changing the nitrogen source from glutamate to ammonium chloride, the volume of H_2 produced by *R. palustris* P4 decreased by approximately 50% (Oh et al., 2004). In addition to the influence on the nitrogenase activity, the biochemical properties of the substrate also play an important role in the H_2 producing efficiency. Various types of substrates result in different numbers of intermediate metabolites and varying energy costs due to the use of different metabolic routes, causing a large difference in the H_2-producing behavior. Moreover, it has been indicated that the oxidation state of the substrate was another significant factor because it is related to the electron availability (Yilmaz et al., 2010). McKinlay and Harwood (2011) noted that a substrate with a negative oxidation state (e.g., butyrate or acetate) showed a higher energy flux in the biomass synthesis and metabolic energy cost than those substrates with positive oxidation states (e.g., fumarate or succinate). Additionally, the H_2 production of PNSB is also affected by several cultivation conditions, including inoculum age, light intensity, pH, temperature, and type of operation (Basak and Das, 2007).

To further develop the photobiological production of H_2 for commercial purposes, a variety of studies to enhance the H_2 production via PNSB have been conducted. The most investigated strategy was optimizing the basic parameters, including the

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operating conditions, substrate selection, immobilization of PNSB cells for a higher retention time, and an integrated system by combining different types of H_2-producing microorganisms (Eroglu and Melis, 2011). On the other hand, genetic engineering approaches, such as inactivation of the uptake hydrogenase, exclusion of energy storage compounds, and improvement for ammonia tolerance, were also employed to increase the efficiency of H_2 production. Among these genetic strategies, the elimination of poly-β-hydroxybutyrate (PHB) notably increased the amount of H_2 produced by *R. sphaeroides* KD131 (Kim et al., 2006). PHB is an energy- and carbon-storage compound found in a wide variety of microorganisms when they are faced with a suboptimal environment. It can be mobilized and used for survival when the carbon source becomes a limiting resource. However, PHB synthesis consumes a large number of metabolites and reducing equivalents that are required for the H_2 production in PNSB. Therefore, several studies indicated that PHB accumulation in PNSB should compete with H_2 production for electrons and energy distribution (Hustede et al., 1993; Vincenzini et al., 1997).

Because of the possible competition between PHB synthesis and H_2 production, the correlation between PHB formation and H_2 production of *R. palustris* WP3-5 was investigated in this study. Due to the effect of the substrate type and oxidation state on H_2 production, acetate, propionate, malate, lactate, glucose, and lactose were used as the substrates to extensively study the H_2 production and PHB formation by *R. palustris* WP3-5. By measuring the cumulative H_2 volume and PHB content, this study tried to evaluate whether a competition in the reducing power distribution between PHB synthesis and H_2 production is present.

2. Methods

2.1. Microorganism and cell preservation

The purple non-sulfur bacterial species used in this study, *R. palustris* WP3-5, was isolated from an activated sludge of a hoggery wastewater treatment plant. The medium used for the cell preservation was prepared according to previous study (Lee et al., 2002). To preserve the cell in a solid culture, a single colony was streaked onto a fresh plate and incubated in an anaerobic jar with a gas generating kit and anaerobic indicator (Oxoid). The cultivated temperature was 32 °C, and the luminous intensity was controlled between 6000 and 7000 lx using a tungsten lamp. For the liquid culture, the inoculum can be obtained from a solid culture or directly from the last liquid culture. After inoculation, the headspace of the liquid culture was stuffed with medium to avoid the influence of O_2.

2.2. Batch experiments for substrates utilization and pH value influence

To prepare sufficient biomass for the batch experiments, *R. palustris* WP3-5 was precultured in a large volume for 3 days under the same cultivation conditions as mentioned earlier. After the preculture, cells were harvested by centrifugation at 4 °C and 6000 rpm and washed twice by a sterile buffer solution (0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.4 g/L NaCl, and 1 g/L $CaCl_2{\cdot}2H_2O$ at pH 6.8) to eliminate the spent medium. Substrates including acetate, propionate, malate, lactate, glucose, and lactose were individually investigated as a sole carbon source in the medium and the substrate initial concentration was fixed at 1 g/L. The culture medium was then mixed with the washed cell to a cell density of optical density (O.D.) 0.3 at 660 nm. The mixture was then divided into serum bottles, sealed with a rubber seal, sparged with argon gas for 5 min to maintain an anaerobic environment, and cultivated as above. The procedures for the pH value batch experiments were identical to those of the substrate utilization experiments. However, only the acetate and malate substrates were investigated for the pH value experiments. The pH value was controlled at 6.0, 6.8, and 8.0 for both substrates. The buffer system used for the pH 6.8 experiment was a phosphate buffer in the same concentration as the medium, whereas a 10 mM tris buffer was used for the pH 8.0 experiment and a 10 mM bis–tris buffer was used for pH 6.0 experiment. To maintain the pH value, 1 N NaOH or HCl was used to adjust the pH value after every sampling. The serum bottle for the next sampling was sterilely opened for the pH adjustment, then resealed and re-sparged for further cultivation.

The gas volume was measured by the water replacement method and its composition was determined by gas chromatography (GC, HP6890 series) equipped with a thermal conductivity detector (TCD). The cell density and pH value were measured beforehand, and the substrate concentration was analyzed after the liquid sample was filtered through a 0.22-μm filter. Then, 85 mL of the residual cells was collected by centrifugation at 4 °C and 6000 rpm and the pellet was stored at −20 °C to determine its PHB content.

2.3. Analytical methods

After measuring the gas volume in the serum bottle by the water replacement method, the gas composition can be correctly sampled due to the atmospheric pressure equilibration. The gas composition was analyzed by GC/TCD. The column was a Supelco 60/80 Carboxen-1000 stainless steel column and the carrier gas was argon, which operated at a flow rate of 19.2 mL/min. The temperatures of the inlet and the detector were 100 and 190 °C, respectively. The temperature program of the oven was held at 65 °C for 2 min and increased at 20 °C per min to 180 °C.

The cell dried weight was determined based on a correlation equation between the cell density and dried weight. The cell density was measured by a UV/VIS spectrophotometer (HITACHI, U-2800) at the wavelength of 660 nm and the cell dried weight was analyzed according to Standard Methods (APHA, 1996). The pH was measured by a pH meter (WTW inoLab pH/ION LEVEL2) and was judged by pH indicator strips (Neutralit®, Merck, Germany) when adjusting the pH value during batch experiments. To determine the substrate concentration, acetate and propionate were analyzed by a GC equipped with a flame ionization detector (FID). Then, 20 μL of 1 N H_2SO_4 was added to the liquid samples for acidification. The column used for GC/FID was a DB-WAXetr column (30 m × 0.35 mm with 0.1 μm film thickness) and the carrier gas was N_2, which operated at 3.0 mL/min. The sample volume for the injection was 1 μL. The temperature of the injector and the detector was controlled at 250 °C, and the temperature program of the oven was held at 60 °C for 5 min and rose by 15 °C per min to 180 °C. For malate and lactate analysis, the sample was treated according to the study of Gasslmaier et al. (2000) before being injecting into the high performance liquid chromatography (L7100, Hitachi, Tokyo, Japan). The column used to separate malate and lactate was the Mightysil RP-18 column and the mobile phase was phosphoric acid, which operated at 0.8 mL/min. The elution was monitored at 220 nm by a UV detector (L7400, Hitachi, Tokyo, Japan). Additionally, the glucose and lactose concentration was determined by the phenol–sulfur method (Dubois et al., 1956). The calculation of the substrate conversion efficiency (SCE) for all batch experiments was according to the study from Vincenzini et al. (1982).

The PHB content was analyzed according to the study by Satoh et al. (1996) but was modified as described below. To extract the intercellular PHB, the cell pellet was freeze-dried and then mixed with 2 mL of an acidic methanol solution (20% H_2SO_4) and 2 mL chloroform and was then heated to 100 °C for 3.5 h. After the