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Effect of AI crude extract on PHB accumulation and hydrogen photoproduction in *Rhodobacter sphaeroides*

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ABSTRACT

Poly-β-hydroxy butyric acid (PHB) accumulation and gaseous H_2 release are regarded as alternatives for expending reducing power. Some researchers suggested that quorum-sensing system affects PHB accumulation in *Rhodobacter sphaeroides*, but whether the system plays regulation role between hydrogen producing and PHB synthesis is still unknown. By adding autoinducer of *R. sphaeroides* into its culture solutions, measuring its total hydrogen production, PHB content and PHB synthase activity, the function of quorum-sensing on PHB accumulation and hydrogen production was preliminarily investigated. Compared with the control, the total gas productions in experimental groups increased accompanying slight decrease of PHB contents, which was partially caused by the reduction of PHB synthase activities. Biolog tests indicated the carbon source utilization profiles, especially those involving fatty acids and butanoate metabolism, had partly changed after exogenous signal molecules added. These results suggest that quorum-sensing is involved in signal regulation between PHB accumulation and hydrogen production in *R. sphaeroides*.

1. Introduction

Microbial communities communicating via quorum-sensing employ various chemical signals to dominate their surrounding environment, alter genetic expression and gain advantage over their competitors [1]. Quorum-sensing bacteria produce and release chemical signal molecules termed autoinducers (AI) whose external concentration increases as a function of increasing cell-population density [2–5]. Bacteria detect the accumulation of a minimal threshold stimulatory concentration of these autoinducers and alter their gene expression, and therefore their behavior in response to the variation of the concentration of autoinducers. Signal molecules implicated in cell-to-cell communication are now known as autoinducers or quorum-sensing molecules, and their function is to regulate gene expression in other cells of the community, which, in turn, control a number of bacterial responses [6–10]. Using these signal-response systems, bacteria synchronize particular behaviors on a wide scale of population and thus function as multicellular organisms [2,11]. In *Rhodobacter sphaeroides*, the *cerR/cerI* quorum-sensing codes for LuxR-type regulatory protein and acylhomoserine

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lactone synthase, and its signaling molecule was *N*-(7-cis-tetradecenoyl)-L-homoserine lactone (tdeDHL) [12,13].

Hydrogen production and PHB accumulation are important physiological behaviors of *R. sphaeroides*. In the absence of nitrogen, nitrogenase can produce molecular hydrogen from protons under anaerobic conditions, a reaction also associated with consumption of ATP [14]. PHB is a polymer of the polyhydroxyalcanoates (PHAs) family of polyesters, which are synthesized by a wide range of bacterial and archeal species to form carbon and energy reserve materials [15–19]. For similar physiological role to hydrogen production, PHB is particularly critical, and that is why it is regarded as a carbon and/or energy storage compound or a sink for reducing equivalents and as a solid fermentation product in *R. sphaeroides* [20–22]. Thus the release of hydrogen and the accumulation of PHB represent two alternatives for expending reducing power in *R. sphaeroides* [23,24]. Under aerobic growth conditions, deletion of chromosomal DNA including *cerR* and *cerI* or insertional interruption of *cerI* resulted in two-fold increase in the cellular poly-β-hydrobutyrate (PHB) content in comparison with the wild-type [25]. However, up to now it is still unclear whether QS system plays a key role between hydrogen producing and PHB synthesis.

In this study, we measured hydrogen production, PHB accumulation, PHB synthase activity, and the sole carbon source utilization in *R. sphaeroides* after exogenous signal molecules added, attempted to explore whether QS system is involved in regulation of PHB accumulation and photoproducing hydrogen.

2. Material and methods

2.1. Bacterial strain and the media

R. sphaeroides CICC 10287 (CICC: China Center of Industrial Culture Collection) was used in this study. The pure culture grew in the simpler culture medium, which contained (per liter of distilled water): KH_2PO_4, 0.5 g; ammonium acetate, 0.5 g; $MgSO_4.7H_2O$, 9.4 g; NaC1, 0.4 g; $CaC1_2.2H_2O$, 0.1 g; yeast extract 1 g; ferrous citrate, 0.005 g; and additional inorganic salts solution 10 mL as specified by Cohen-Bazire et al. [26]. The pH was adjusted to 6.8–7.0 before inoculation. Organisms for hydrogen production were grown in a succinic acid minimal medium (initial pH, 7.0) containing, per liter: succinic acid, 2.0 g; $(NH_4)_2SO_4$, 0.8 g; sodium glutamate, 0.10 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 1.0 g; $K_2HPO_4 \cdot 2H_2O$, 1.572 g; yeast extract, 0.5 g; EDTA, 0.02 g; nicotinic acid, 0.001 g; thiamin/HCl, 0.001 g; and additional inorganic salts as specified by Cohen-Bazire et al. [26]. Growth was estimated from measurements of the optical density at 650 nm in 1-cm cuvettes by using a spectrophotometer.

2.2. Isolation and purification of PHB granules

After anaerobic cultivation, cells in different phases were harvested by centrifugation. Cells were disruptured by using a French press, and resuspended in 50 mM Tris–HCl, pH 7.5, containing 25% (w/v) NaCl, 0.2% (w/v) KCl, 0.5% (w/v) $MgSO_4 \cdot 7H_2O$ [27]. The disrupted cells were then subjected to centrifugation (10,000 g, 30 min) to sediment intact cells and cell debris. PHB granules were then sedimented by ultracentrifugation (100,000 g, 60 min). To enhance purity, PHB granules were washed in the same buffer and centrifuged again (100,000 g, 30 min). The granules were suspended in a suitable volume of the same buffer for further use.

2.3. Spectrometric assay of PHB and PHB synthase activity

Quantitative determination of PHB was performed by spectrophotometric assay [28]. For the spectrophotometric assay of polymer, a sample containing 5–50 μg polymer in chloroform was transferred to a clean test tube. The chloroform was evaporated and 10 mL of concentrated H_2SO_4 are added, the tube was capped with a glass marble and heated for 10 min at 100 C in a water bath. The solution was cooled, and, after thorough mixing, a sample was transferred to a silica cuvette and the absorbance at 235 nm was measured against a sulfuric acid blank. The amount of crotonic acid was calculated from the molar extinction coefficient.

PHB synthase activity was determined spectrophotometrically by monitoring the release of CoA as reference [27]. The enzyme reaction was started by adding 20 μL crude extract containing 5 mg protein/mL or 0.05 mg protein/mL for assay with 3-hydroxybutyryl-CoA. In addition, also a spectroscopic assay was done. The enzyme solutions were incubated in 25 mM TRIS/HC1 buffer containing 1 mM DTNB plus 30 mM D(−)-3-hydroxybutyryl-CoA, and changes in the absorbance at 412 nm were recorded. Assays were done with crude cell extracts or the soluble protein fractions. All assays were done at 30 °C, and 1 U of enzyme activity is defined as the amount required to catalyze the transformation of 1 μmol substrate per min.

2.4. Preparation of AI crude extract

Anaerobically grown cells were harvested at the end of logarithmic phase and centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was extracted thrice with equal volume of ethyl acetate and dried as the AI crude extract of *R. sphaeroides*. AI crude extract was collected according to the method described by Pearson et al. [29]. If the extract from 10 L nutrient fluid at last was added into 1 L culture, we called it was 10× AI extract in the experiments; likewise, 1× AI means that the extract from 1 L nutrient fluid at last was added into 1 L culture [25].

2.5. Detection of AI crude extract

Two detection methods were adopted in experiments. The first one was the one described by Yang, Y. H. et al. in 2006 [30]. Sample (40 μL) was prepared and 50 μL of a 1:1 mixture of hydroxyl amine (2 M): NaOH (3.5 M) was aliquoted and mixed with the sample. Subsequently, the same amount of 1:1 mixture of ferric chloride (10% in 4 M HCl): 95% ethanol was added. Then absorption changes were measured at 520 nm. The second method was biosensor detection put forward by Ravn, L. et al. in 2001 [31]. The agar plates were prepared as follows: a pre-culture was grown in AT-medium for 24 h at 28 °C with aeration and 1 mL of the pre-culture was used to inoculate 50 mL AT-medium for JZA1. The culture was grown for 24 h at 28 °C with aeration and was poured into 100 mL AT-agar maintained at