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Photo-biological hydrogen production by an acid tolerant mutant of *Rhodovulum sulfidophilum* P5 generated by transposon mutagenesis

Jinling Cai [a,*], Guangce Wang [a,b,*]

[a] Tianjin Key Laboratory of Marine Resources and Chemistry, College of Marine Science and Engineering, Tianjin University of Science and Technology, Tianjin 300457, PR China
[b] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, Shandong, PR China

HIGHLIGHTS

- Transposon mutagenesis was used to enhance H_2 yield of a photosynthetic bacterium.
- A transposon mutagenesis library of *Rhodovulum sulfidophilum* P5 was constructed.
- Mutant TH-102 had higher aciduric and temperature resistant ability.
- TH-102 can produce H_2 at dark fermentation effluent environment (pH 5.5 and 35 °C).
- In continuous culture, H_2 yield and rate were 17 and 15-fold higher than the WT.

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ABSTRACT

Most of the photosynthetic bacterial strains exhibit optimum hydrogen production at neutral initial pH, and lower initial pH resulted in a sharp decrease in hydrogen yield. Thus, screening of acid-tolerant hydrogen-producing photosynthetic bacteria is very important. To obtain acid tolerant mutants, a Tn7-based transposon was randomly inserted into the genomic DNA of *Rhodovulum sulfidophilum* P5. An acid tolerant mutant strain TH-102 exhibited increased hydrogen production in acidic environment (pH 4.5–6.5) and at higher temperatures (35 and 37 °C) than the wild-type strain. At pH 5.5 and 35 °C, the mutant strain TH-102 continuously produced hydrogen. The hydrogen yield and average rate were 2.16 ± 0.10 mol/mol acetate and 10.06 ± 0.47 mL/L h, which was about 17.32 and 15.37-fold higher than that of the wild-type strain, respectively. This acid- and temperature-tolerant mutant strain TH-102 could be used in a cost-effective hydrogen production process employing both dark fermentative and photosynthetic bacteria.

1. Introduction

Due to the limited availability of traditional energy from a non-renewable reservoir and as a result of ever-growing energy demand, there is an increasing interest in the search for renewable energy sources to meet the current and future energy requirements. Numerous alternative and renewable energy resources have been explored. Among them, hydrogen is an attractive potential alternative energy source due to its non-polluting and environmental-friendly nature, and currently, sustainable biological hydrogen production is under active investigation.

The low yield and production rate are still major barriers for commercial biological production of hydrogen. It has been estimated that a hydrogen yield of 8 mol H_2/mol glucose would be sufficient for practical production (Keskin et al., 2011). However, the hydrogen yield of all the principal biological hydrogen production methods such as direct and indirect biophotolysis, photo fermentation, and dark fermentation, is relatively below this benchmark. In some studies, integration of dark and photo fermentations has been reported to produce yields of up to 7.1 mol H_2/mol hexose (Asada et al., 2006; Chen et al., 2008) (even up to 8.3 mol H_2/mol hexose (Kim et al., 2006)). Thus, further investigations are required for the development of these systems for practical purposes.

The combined use of dark-fermentative bacteria and photosynthetic bacteria, which is a promising hydrogen production approach, can markedly enhance hydrogen yield. Most of the dark-fermentative bacteria can produce hydrogen at high rate, but cannot degrade organic compounds completely due to thermodynamic limitations. The maximum theoretical hydrogen yield of dark fermentation is relatively low, ranging from 2 to 4 mol/mol hexose according to the composition of the organic acids produced.

* Corresponding authors at: Tianjin Key Laboratory of Marine Resources and Chemistry, College of Marine Science and Engineering, Tianjin University of Science and Technology, Tianjin 300457, PR China. Tel./fax: +86 022 60601305 (G. Wang).
E-mail addresses: jinlingcai@tust.edu.cn (J. Cai), gcwang@ms.qdio.ac.cn (G. Wang).

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As a result, the dark-fermentative hydrogen-producing effluent primarily contains organic acids (mainly acetate and butyrate) and alcohols (Lee et al., 2010), mostly in their undissociated forms, which can cross the cell membrane at a low pH and can adversely affect hydrogen production (Wang et al., 2008). Furthermore, the presence of organic acids and alcohols in the residual medium poses a disposal issue. However, the organic acids could be further metabolized to produce additional hydrogen by photosynthetic bacteria under photoheterotrophic conditions, which increases the theoretical hydrogen yield from 4 to 12 mol H_2/mol glucose and reduces the organic content of the final residual waste. Several studies have reported that the combined use of both dark-fermentative and photosynthetic bacteria could achieve higher hydrogen yields from various substrates, when compared with the hydrogen yields obtained using either of these bacteria (Lee et al., 2010; Liu et al., 2009; Patel et al., 2012). Thus, the combined use of dark-fermentative and photosynthetic bacteria may overcome individual limitations and exhibit greater advantages.

Low pH of the dark-fermentative effluent inhibits hydrogen production and even growth of the photosynthetic bacteria, which is one of the major barriers for the stable operation of the integrated process. The pH for dark fermentative effluent and optimum pH for hydrogen production are in the acidic range of 4.5–5.5 (Karadag and Pahuakka, 2010; Luo et al., 2010; Masset et al., 2012; Sigurbjornsdottir and Orlygsson, 2012). However, the optimum pH for hydrogen production by most of the photosynthetic bacteria is neutral (Nath and Das, 2009; Wu et al., 2012; Yang et al., 2012; Zhu et al., 2010) to weak alkaline (Cai et al., 2012; Cai and Wang, 2012; Pandey et al., 2012).

It has been observed to be necessary to control the pH of the fermentation effluent for further production of hydrogen by photosynthetic bacteria (Ljunggren et al., 2011). However, controlling the pH of the dark fermentation process requires large amounts of alkaline solution, which not only presents an economic burden, but also increases the concentration of salts in the treated effluent, requiring adoption of an efficient way to reduce the amount of alkaline solution. Hence, it is necessary to achieve acid tolerant remediation of fermentation effluent, which could reduce alkali consumption, as well as increase the hydrogen yield.

Screening of acid-tolerant hydrogen-producing photosynthetic bacteria is very important. These bacteria are not only acid tolerant, adapting to acid stress and alleviating organic acid inhibition, but are also beneficial for hydrogen production as well as for the integration of dark and photo fermentations. Although the traditional isolation strategy for screening acid-tolerant photosynthetic bacteria for hydrogen production is effective, it is time-consuming. Some powerful genetic engineering approaches could be employed to improve the hydrogen yield (Hallenbeck et al., 2012; Kim et al., 2006). Among them, transposon technology, carrying antibiotic-resistance genes, is an excellent indispensable tool in bacterial genetics, especially for those bacteria whose genetic system has not yet been developed (Ma et al., 2012; Cai and Wang, 2013).

Transposon mutagenesis is an important genetic tool for the creation and characterization of insertion mutants, and has been used to carry out insertional mutagenesis screening of mutants of photosynthetic bacteria which could produce high hydrogen yield, such as *Rhodovulum gelatinosus* (Vanzin et al., 2010), *Rhodovulum capsulatus* (Ma et al., 2012), and *R. sulfidophilum* P5 (Cai and Wang, 2013). Furthermore, the use of transposon mutagenesis technology to create a mutant bank of *R. sulfidophilum* P5 could significantly contribute to enhancing its hydrogen production as well as our understanding of the mechanism of hydrogen production.

To date, biological hydrogen production by photosynthetic bacteria in fresh conditions has been well developed. Hydrogen production by marine photosynthetic bacteria from marine wastewater has been attracting increasing attention. Some studies have reported hydrogen production by marine photosynthetic bacteria, such as *Rhodobium marinum* (Anam et al., 2012), *R. sulfidophilum* P5 (Cai and Wang, 2012), and *Rhodovulum* spp. (Matsunaga et al., 2000), as well as marine mixed phototrophic bacterial consort (Cai et al., 2012).

However, most of the investigations have been mainly focused on optimizing the basic parameters, including the operating conditions, substrate selection, immobilization of photosynthetic bacteria cells for a higher retention time, etc. Only a few studies have attempted to improve the hydrogen yield of photosynthetic bacteria through molecular biology methods (Ma et al., 2012). Thus, the main purpose of the present study was to screen acid-tolerant photosynthetic bacteria producing high hydrogen yield, which could be used in the integrated dark–photo fermentation process for hydrogen production.

2. Methods

2.1. Bacterial strain and culture medium

The transposon library of *R. sulfidophilum* P5 was created according the forward research (Cai and Wang, 2013). Transposition was performed using the GPS mutagenesis system (New England Biolabs Catalog NO E7101S), according to the manufacturer's instructions. The transprimer donor used was pGPS3, which carries kanamycin resistance. The acquired mutants were stored at −80 °C in 15% glycerol.

R. sulfidophilum strains were grown on RCVBH medium (Cai and Wang, 2012). The RCVBH medium contained the following: 20 mmol/L acetate, 10 mmol/L glutamate, 20 g/L NaCl, 75 mg/L $CaCl_2$ $2H_2O$, 120 mg/L $MgSO_4$ $7H_2O$, 10 mmol/L KPO_4 buffer, 1 mg/L thiamine hydrochloride, 20 mg/L sodium ethylenediaminetetraacetic acid, and 1 mL/L trace element solution (per 100 mL of deionized water (dH_2O): 280 mg H_3BO_3, 75.2 mg $NaMoO_4$ $2H_2O$, 159.2 mg $MnSO_4$ H_2O, $3H_2O$, 4 mg $Cu(NO_3)_2$, and 24 mg $ZnSO_4$ $7H_2O$). For basic growth, the pH was adjusted to 8.0.

2.2. Screen and characterization of acid tolerant mutant

To verify the hydrogen production ability of the mutants, hydrogen production profiles of different mutant strains were grown under acidic condition (pH 4.5) using 50 mL anaerobic tube (20 mL working volume). Among all the mutants, only 9 mutants could produce hydrogen under acidic condition (pH 4.5). These nine acid tolerant mutant strains could evolve hydrogen were further analyzed the nature of hydrogen production. A total of 50 mL of pre-cultured cells (OD660 = 0.8–1.0) were harvested by centrifugation and inoculated into 500 mL of RCVBH medium. Argon gas was purged into the bioreactors to create anaerobic conditions. The bioreactors were stirred at 150 rpm at 30 °C. The light intensity was 100 μmol photons/m^2 s.

For further analysis of the hydrogen production nature by the acid tolerant mutant strain TH-102, two series of test batches (of 500-mL working volume) were cultured under anaerobic conditions in RCVBH medium, with the WT strain used as the control. For the initial pH tests, the pH was varied from 4.0 to 9.0. For the culture temperature test, the temperatures ranged from 30 to 40 °C.

For semi-batch hydrogen production, the mutant strain TH-102 and the WT strain in exponential growth phase were harvested and inoculated into 500-mL culture medium. Initially, the reactors were operated in batch mode for 5 days to accumulate biomass for continuous operation. Thereafter, 125 mL of the exhausted medium was withdrawn and replaced with fresh medium. The reactor was operated under the same conditions as described