



# Enhanced hydrogen production of *Rhodobacter sphaeroides* promoted by extracellular H<sup>+</sup> of *Halobacterium salinarum*

Jiang-Yu Ye <sup>\*</sup>, Yue Pan, Yong Wang and Yi-Chao Wang

## Abstract

**Purpose:** This study utilized the principle that the bacteriorhodopsin (BR) produced by *Halobacterium salinarum* could increase the hydrogen production of *Rhodobacter sphaeroides*. *H. salinarum* are co-cultured with *R. sphaeroides* to determine the impact of purple membrane fragments (PM) on *R. sphaeroides* and improve its hydrogen production capacity.

**Methods:** In this study, low-salinity in 14 % NaCl domesticates *H salinarum*. Then, 0–160 nmol of different concentration gradient groups of bacteriorhodopsin (BR) and *R. sphaeroides* was co-cultivated, and the hydrogen production and pH are measured; then, *R. sphaeroides* and immobilized BR of different concentrations are used to produce hydrogen to detect the amount of hydrogen. Two-chamber microbial hydrogen production system with proton exchange membrane-assisted proton flow was established, and the system was operated. As additional electricity added under 0.3 V, the hydrogen production rate increased with voltages in the coupled system.

**Results:** *H salinarum* can still grow well after low salt in 14% NaCl domestication. When the BR concentration is 80 nmol, the highest hydrogen production reached 217 mL per hour. Both immobilized PC (packed cells) and immobilized PM (purple membrane) of *H. salinarum* could promote hydrogen production of *R. sphaeroides* to some extent. The highest production of hydrogen was obtained by the coupled system with 40 nmol BR of immobilized PC, which increased from 127 to 232 mL, and the maximum H<sub>2</sub> production rate was 18.2 mL<sup>-1</sup> h<sup>-1</sup> L culture. In the 192 h experiment time, when the potential is 0.3 V, the hydrogen production amount can reach 920 mL, which is 50.3% higher than the control group.

**Conclusions:** The stability of the system greatly improved after PC was immobilized, and the time for hydrogen production of *R. sphaeroides* significantly extended on same condition. As additional electricity added under 0.3 V, the hydrogen production rate increased with voltages in the coupled system. These results are helpful to build a hydrogen production-coupled system by nitrogenase of *R. sphaeroides* and proton pump of *H. salinarum*.

**Keywords:** Hydrogen production, Proton pump, *Halobacterium salinarum*, *Rhodobacter sphaeroides*, Bacteriorhodopsin

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## Introduction

*Rhodobacter sphaeroides* is one of the most famous non-sulfur purple bacteria for its capability of using a wide variety of substrates and its high activity in hydrogen production under anaerobic condition (Asada et al. 2006; Koku et al. 2003; Laurinavichene et al. 2018; Yetis et al. 2000). In the absence of nitrogen, the nitrogenase in *R. sphaeroides* can produce molecular hydrogen with protons under anaerobic condition (Zabut et al. 2006). In general, to enhance the rate and yield of hydrogen production, *R. sphaeroides* was often co-cultured or mixed cultured with other bacteria, e.g., its reduced-pigment mutant MTP4 (Kondo et al. 2006), *Clostridium butyricum* (Laurinavichene et al. 2018), *Enterobacter cloacae* (Nath et al. 2005), *Lactobacillus delbrueckii* (Asada et al. 2006), and *Halobacterium salinarum* (Zabut et al. 2006).

*H. salinarum* is a model organism in the halophilic branch of the archaea, which can live in saturated salt solutions (4 mmol salt or higher). It can live with light as the only energy source due to its activity of the retinal protein bacteriorhodopsin (BR), a light-driven proton pump. Proton electrochemical potential, produced by the transmembrane proton, can be utilized to drive ATP synthesis by membrane bound H<sup>+</sup>ATPase (Anandakrishnana et al. 2016). Although protons are generated by illumination in these microorganisms, protons cannot be converted to molecular hydrogen because normally they lack hydrogenase enzymes (Lata et al. 2007; Sediroglu et al. 1999). Its cells will lyse in distilled water, but its purple membrane (PM) still keeps relative stability in structure and integrated in function for a long time. Therefore, its packed cells (PC) or PM might be combined with the other system for producing hydrogen (Khan and Bhatt 1997; Sediroglu et al. 1999; Frank et al. 2016; Zabut et al. 2006).

Microbial electrolysis cell (MEC) is a promising new approach for producing hydrogen gas from biodegradable organic matter using exoelectrogenic microbes (Merrill and Logan 2009). This research has profound significance for the exploration of hydrogen energy. In some studies, additional voltages were added to enhance the potential generated by bacteria, allowing for various products to be generated at the cathode, such as hydrogen, methane, and hydrogen peroxide (Hong et al. 2005; Logan 2010; Rozendal et al. 2009). The packed cells of *H. salinarum* coupled with an electrochemical system were also found to be very promising and facilitative, since protons came from light instead of nutrients (Khan and Bhatt 1997; Taqui Khan et al. 1992).

In this study, two methods were used for *R. sphaeroides* hydrogen production with *H. salinarum* providing protons. The first was a co-cultured system in a column photobioreactor for improving biological hydrogen

production (Wang et al. 2019). In the second way, a device, which is similar to BEAMR (bioelectrochemically assisted microbial reactor), was used to promote hydrogen production through an electrically assisted proton flow. In the cathode chamber, *R. sphaeroides* could produce hydrogen, and extra protons from anode chamber could enhance hydrogen production, while protons were provided by *H. salinarum* through a proton exchange membrane (PEM). These two methods above could promote the generation of hydrogen, and the latter has profound research significance and broad research prospects in MEC.

## Materials and methods

### Media and bacterial growth

*R. sphaeroides* and *H. salinarum* (DSM670) were purchased from CGMCC (China General Microbiological Culture Collection Center).

*R. sphaeroides* was grown in a 200-mL conical flask at 32 °C, under continuous illumination of 7000 lx. The *R. sphaeroides* growth medium (GM) was modified from Fang et al (Fang et al. 2006) and contained 4.0 g/L sodium succinate and 1.25 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The hydrogen production medium (HM) was similar to the growth medium, but contained double the amount of sodium succinate (8.1 g/L) and sodium glutamate (0.75 g/L) replaced (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the nitrogen source to prevent ammonium ion repression of the production of nitrogenase. The pH of the growth medium and the hydrogen-production medium are both 7.0.

The strain of *H. salinarum* was grown at 38 °C in a sterilized medium containing 4.27 mol/L NaCl, 20 g MgSO<sub>4</sub>·2H<sub>2</sub>O, 3.64 g/L trisodium citrate, 2.0 g/L KCL, and 10 g/L bacteriological peptone L-37 at pH 7.0 in a shaking incubator (Yücel et al. 1995). This experiment is slightly modified based on Yücel's. The organism was shake-flask cultured firstly in growth medium with 4.27 mol/L NaCl under light at 38 °C. Exponentially growing cells were harvested and resuspended in fresh medium with gradually decreased NaCl content to final 0.86 mol/L NaCl during the whole domestication period of more than 24 weeks. Growth was determined by an ultraviolet spectrophotometer at 660 nm (OD 660) optical density in the end of each acclimation period.

### Preparation of packed cells and measurement of BR of *H. salinarum*

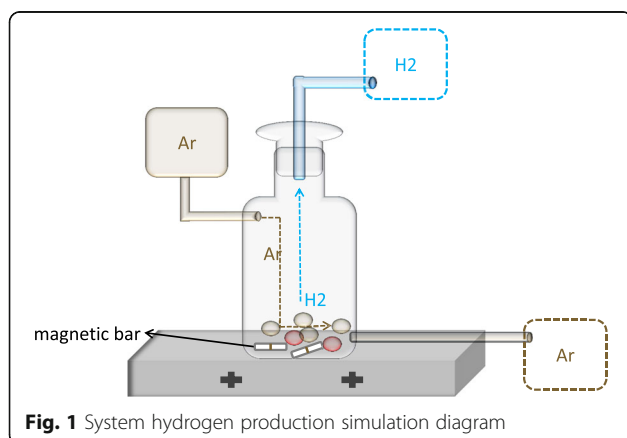
The cells of *H. salinarum* were collected after 7 days culture by centrifugating at 6000 rpm for 20 min. The precipitated cells were collected as packed cells (PC), and then, were suspended in 20 mL of basal salt solution (the growth medium without peptone), which prevents the osmotic rupture of the cells. The concentration of BR was determined by measuring the absorbance at 570 nm

and taking the extinction coefficient  $6.3 \text{ mL nm}^{-1} \text{ mol}^{-1}$ . The isolation of the purple membrane (PM) fragments was carried out according to Yücel et al. (1995), and the measurement of BR photoactivity followed Zabut et al. (2006).

One hundred milliliters sterile water and 4 g sodium alginate (SA) were taken into a 250-mL sterile beaker and stirred until all sodium alginate completely dissolved. The crosslinking solution was prepared by adding an additional 0.05 mol  $\text{CaCl}_2$  to the growth media and agitated by a magnetic stirrer. The alginate solution mixture was dripped from 20-cm height into 1000 mL crosslinking solution. The immobilized balls were washed with sterile distilled water and stored at  $-20^\circ\text{C}$  until use.

### Hydrogen production and mixed hydrogen production

Hydrogen production experiments were carried out in a 200-mL volume jar. Three outlets were in the reactor as shown in Fig. 1: one was for  $\text{H}_2$  gas outlet connected to the  $\text{H}_2$  collection system, the second one for removal of argon, the third one for argon inlet and the sample collection. The reaction temperature was regulated by a water jacket and a magnetic stirrer. The reactor had a flattened bottom, which allowed the use of a 1.5-cm magnetic bar. The stirring speed was 300 rpm. After the setup was sterilized by steam-heated, it was filled with fresh sterilized culture medium. Then, 5% v/v of *H. sphaeroides* (48 h grown culture) and the calculated appropriate amount of *H. salinarum* were inoculated into culture medium. Argon gas was added into the reactor and sealed with paraffin to prevent any leakage for anaerobic condition. The reactor was illuminated by a ring light with 7000 lx intensity, and its temperature was kept at  $32^\circ\text{C}$ . The samples were collected and measured at 12 h intervals. The evolved gas was collected by reversible displacement of water, in which a low concentration of NaOH was readied for  $\text{CO}_2$  removal. All experiments were operated in parallel in three to four reactors.



**Fig. 1** System hydrogen production simulation diagram

Hydrogen concentration was analyzed by a Shimadzu GCRI1A gas-chromatograph with molecular sieve 5A column ( $3.0 \text{ mm} \times 150 \text{ mm}$ ). The pH levels of solutions were monitored by pH meter. The temperature of the detector is  $100^\circ\text{C}$ , and column oven temperature is  $40^\circ\text{C}$ .

### Electrochemically assisted microbial production of hydrogen

The setup was a two-chamber reactor with the anode and cathode in different chambers separated by a proton exchange membrane (PEM, ULTREX CMI-7000, Membrane International Incorp). The volume of each chamber was 500 mL, and the separated PEM between two chambers was  $9.4 \text{ cm}^2$ . The anode and the cathode were externally connected to the power supply through an electrical circuit. The anode electrode was plain carbon cloth, and the cathode electrode was made of carbon paper containing  $0.5 \text{ mg Pt/cm}^2$ . Each electrode had a surface area of  $60 \text{ cm}^2$ , and the distance between the two electrodes was 15 cm. The anode chamber contained nutrient medium for domesticated *H. salinarum* as a proton donor, while the cathode chamber contained hydrogen medium for *R. sphaeroides*. Then, the two strains in exponential phase were inoculated into chambers, respectively, and the inoculation amounts were 5%. Experiments were performed at  $32^\circ\text{C}$  under anaerobic conditions by argon gas added. Illumination was achieved by a ring light with about 7000 lx light intensity. Voltage in the range of 0~500 mV was applied to the circuit by connecting the positive pole of a programmable power supply to the anode, and the negative pole to the cathode. The evolved hydrogen was collected from cathode chamber.

### Statistical methods

There are 3 parallel trials, and their averaged values with statistical deviations were used for the data analysis. Statistical analysis, which includes the error bar, standard deviation, and averaged values, was conducted in this study by using the Microsoft Excel program.

## Results and discussion

### Acclimation and growth of *H. salinarum*

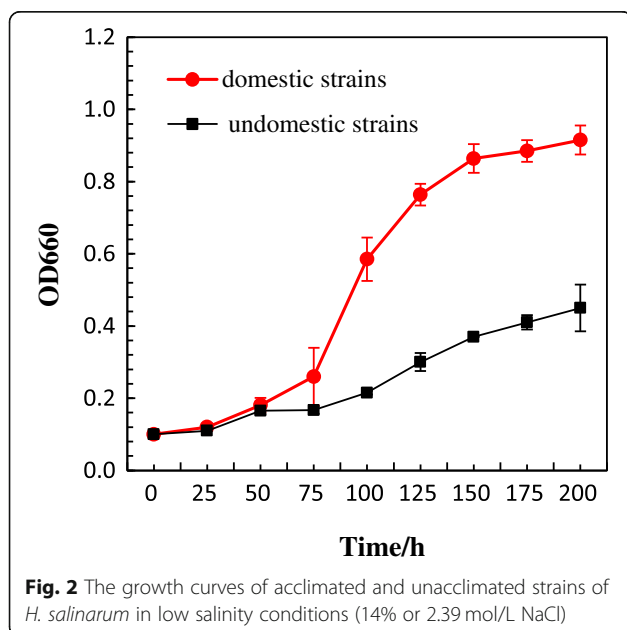
*H. salinarum* generally lives in salt evaporation ponds, natural salt lakes, and other hyperhaline environments, where the salt concentration comes close to saturation. The experimental results indicated that the cells of *H. salinarum* were well-grown in salinity of 17~25% (2.91–4.27 mol/L), and its vitality would drop quickly at salinities below 17%. In normal cell membranes of *H. salinarum*, the photoactivity of BR is promoted by a high ionic strength, so the other microbes in combined system are required to be tolerant strains (Zabut et al. 2006).

The alternative idea is to acclimatize *H. salinarum* to grow in a low-salt environment until it could adapt to a degressive ionic strength. Through a series of culture and inoculation in the media of salinity reduced gradually more than 24 weeks, *H. salinarum* strain had adapted to grow in a medium with a relative low salinity.

The growth curves of *H. salinarum* in a medium with 14% NaCl (2.39 mol/L NaCl) are shown in Fig. 2. Although domesticated organisms have normal morphological character and color, it could grow well in a medium with relatively low salinity. Compared with the wild strain, an obvious logarithmic phase was observed in domesticated bacterium between 48 and 130 h culture (Fig. 2). Domestication is effective and slow in the desired direction of selection process, which induces the cells of *H. salinarum* physiological adaptation to low-salinity conditions.

#### Hydrogen production by *R. sphaeroides* combined with PC of *H. salinarum*

The results of hydrogen production by *R. sphaeroides* combined with pack cells of *H. salinarum* are shown in Fig. 3. In experimental groups, the concentration of *R. sphaeroides* were kept at constant, but the packed cells of *H. salinarum* containing BR concentrations were different, from 0 to 160 nmol, respectively. PC was not vital, but BR in the native membrane in its original environment was still active (continue to pump protons upon illumination) (Zabut et al. 2006). Comparing 80 with 160 nmol BR concentration of *H. salinarum* added, the latter did not increase the hydrogen production rate in *R. sphaeroides*, so 80 nmol BR concentration was



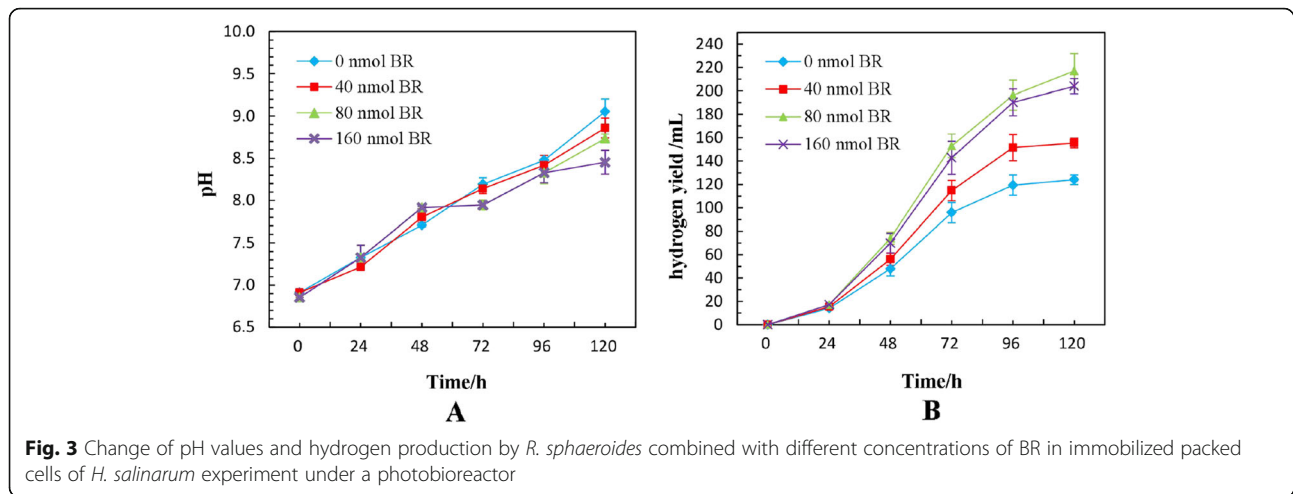
chosen to be the highest amount of concentration for further experiments.

The results of experiments carried out with free or immobilized packed cells of *H. salinarum* were compared with the control experiment. As is seen from Fig. 3b, the  $H_2$  production was enhanced by increasing the amount of BR from PC of *H. salinarum*. The highest hydrogen production was obtained through the mixed culture system with 80 nmol BR. Total gas production increased from 124 to 217 mL, and  $H_2$  production rate increased from 5.160 to 9.042 mL<sup>-1</sup> h<sup>-1</sup> L culture, which could be ascribed to the additional protons coming from the light-induced proton pumping of BR. When BR concentration was more than 80 nmol, for instance, 160 nmol, the total hydrogen production did not increase further, which could be possibly explained that there was viscosity increase and transmittance decrease in bacterial liquid caused by the overfull suspended PC. Another phenomenon was observed in Fig. 3, pH values rose more quickly in the group of *R. sphaeroides* alone and in the groups containing lower BR concentrations. Comparatively, the pH values rose more slowly and maintained at around 7.8 in the two groups with higher BR concentrations after 48~72 h of inoculation, during which the most efficient hydrogen production took place. Obviously, there is an extreme relation between the stable pH value and higher hydrogen production rate. Upon illumination, BR pumps out protons and most probably this process is accompanied by deprotonation of BR (Sediroglu et al. 1999; Yücel et al. 1995). Under a limiting condition of nitrogen, these protons were readily used for hydrogen production by nitrogenase of *R. sphaeroides* (Akroum-Amrouche et al. 2019). Otherwise, the buffering effect of BR in PC was also helpful in enhancing hydrogen production. Overall, the combining of *R. sphaeroides* with PC of *H. salinarum* was more efficient for photobiological  $H_2$  production. The positive effect of BR on hydrogen production has been confirmed by different authors on different systems.

The immobilization might have increased the effective utilization of light energy, and BR does not lose its photoactivity on sodium alginate for at least 2 months (Elkholy et al. 2020). Figure 4 shows *R. sphaeroides* produced hydrogen by the coupled system with immobilized PC or PM of *H. salinarum*.

From Fig. 4, immobilized PC or immobilized PM of *H. salinarum*, no matter what style, could promote hydrogen production of *R. sphaeroides* to some extent. The highest production of hydrogen was obtained by the coupled system with 40 nmol BR of immobilized PC, which total gas production increased from 127 (the control group with 0 nmol BR) to 232 mL, and the maximum  $H_2$  production rate was 18.2 mL<sup>-1</sup> h<sup>-1</sup> L.





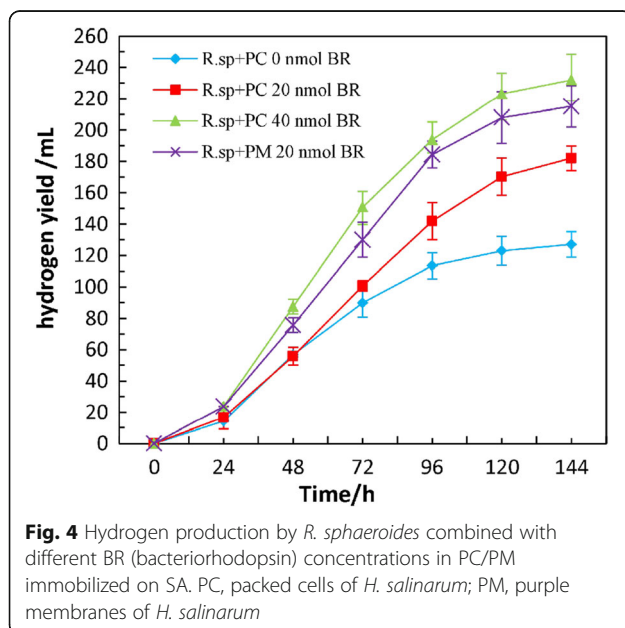
Furthermore, as seen in Figs. 3 and 4, hydrogen production ratio (HPR) obtained by using free *H. salinarum* packed cells was lower than the immobilized systems containing the same amount of BR (photoactive proton pump). When BR concentration was 20 nmol, the average  $H_2$  production rate of the coupled system with immobilized PM was  $10.7 \text{ mL}^{-1} \text{ h}^{-1} \text{ L}$ , which was higher than the maximum rate from immobilized PC with *R. sphaeroides* ( $8.6 \text{ mL}^{-1} \text{ h}^{-1} \text{ L}$ ). Especially, after inoculation for 12~36 h, the hydrogen production rate increased quickly. This may be due to an excessive bacterial growth, which made a significantly shorter lag phase of production of hydrogen (Wang et al. 2016). These results were a little different from some other similar experiments, in which BR was more effective on hydrogen production when *R. sphaeroides* was combined with PC

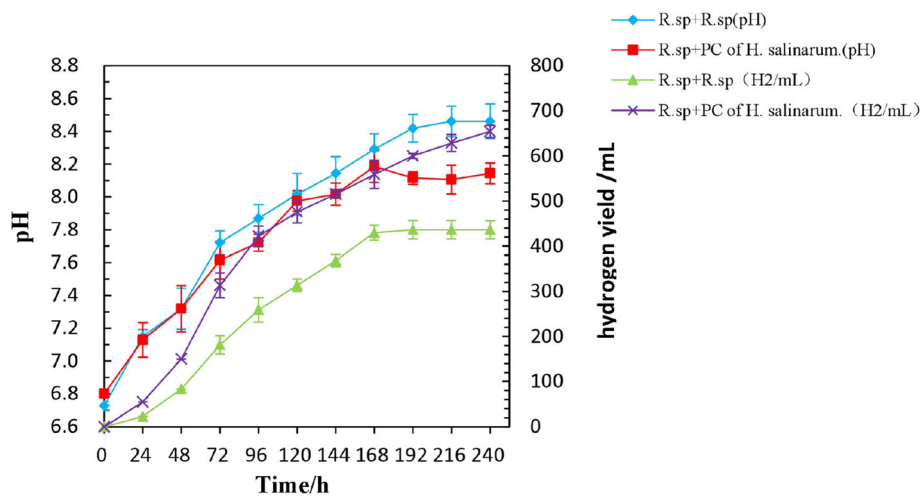
rather than *R. sphaeroides* combined with PM fragments (Zabut et al. 2006). Due to PM containing only lipids and BR instead of all cellular components, it was possible that light utilization of PM fragments might thus be better and less affected by low salt concentration. In addition, the self-orientation of PM on sodium alginate might be much better than the packed cells. However, since PM fragments require sophisticated purification techniques that might be quite expensive and time consuming, packed cells for use are cheaper than PM fragments (Sediroglu et al. 1999).

#### Hydrogen production in a two-chamber reactor

In order to further demonstrate proton-assisted hydrogen production in combined condition, a two-chamber system, connected by a proton exchange membrane to assist proton flow, was set up and operated for microbial hydrogen production. The experimental results are shown in Fig. 5.

From Fig. 5, the two-chamber combined system could continually produce hydrogen for about 168 h in control experiment, in which both of the two chambers were inoculated with *R. sphaeroides*, and then, hydrogen production appeared stagnant (Fig. 5). In experimental groups, *R. sphaeroides* was inoculated into the cathode chamber; the packed cells of *H. salinarum* for supported bacteria were poured into the anode chamber. As might be expected, the hydrogen production rate in experimental group increased more quickly, and the time of hydrogen production extended to 240 h, and total amount of hydrogen gas in comparison with the control was 1.49 times during experimental period. In the later stages of the experiments, pH value of the couple system descended more slowly. It was clear that a transmembrane proton transfer existed between the two chambers under anaerobic and illumination. The parts of entered protons were used to produce hydrogen gas by



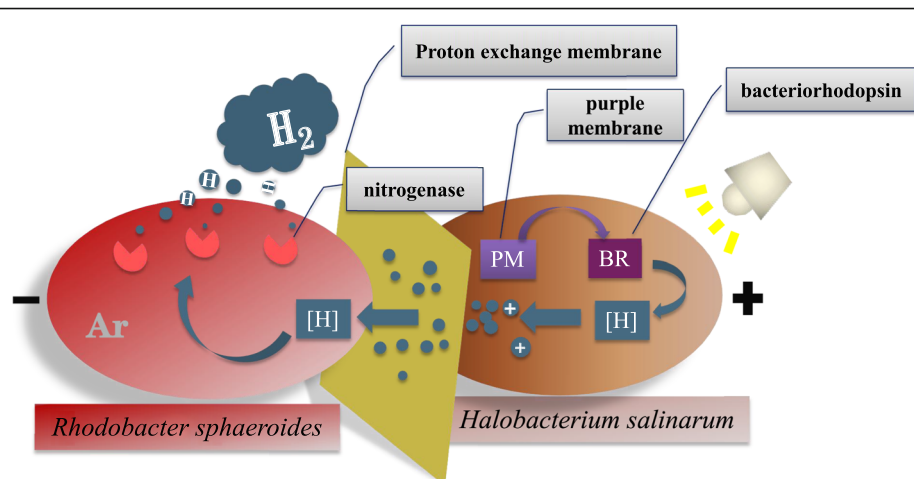


**Fig. 5** Hydrogen production by *R. sphaeroides* semi-combined with PC-SA

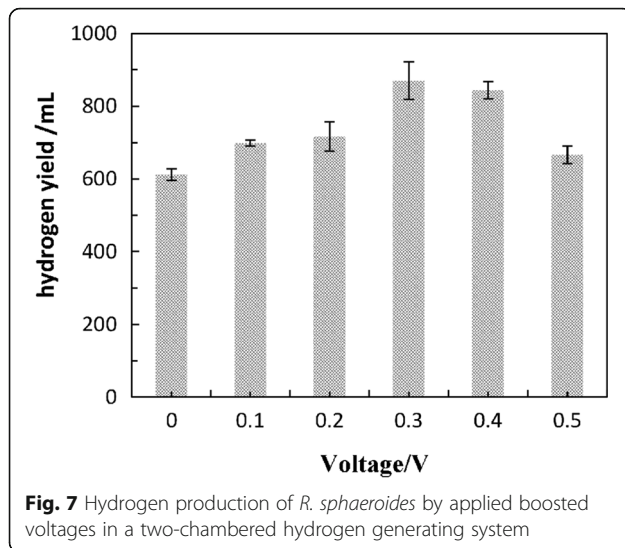
nitrogenase system of *R. sphaeroides*, and the other participated in regulating pH value.

A few years ago, microbial electrolysis cell (MEC) has been demonstrated to be a promising technique for biological hydrogen production from wastes (Hong et al. 2005; Logan et al. 2008; Rozendal et al. 2006; Rozendal et al. 2007; Sun et al. 2009). As an additional current was added, MEC could be changed to a bioelectrochemically assisted microbial reactor (BEAMR), which requires addition of small voltage to complement energy obtained from light or biomass (Hong et al. 2005). This helps to reduce the amount of electrical energy required for hydrogen production. In anode chamber, some electricity-generating microbes can directly transfer electrons to the anode surface, and then, electrons pass through a resistor or other type of electrical device to the cathode (Lovley 2006). In our experiments, a two-chamber

system, in which two separated electrodes were connected by an electrical (DC) power source, was used to boost proton flow from anode chamber to cathode chamber. As shown in Fig. 6, protons are produced by light stimulating BR on *H. salinarum*. Protons move to the interface of *R. sphaeroides* through the cation exchange membrane and synthesize hydrogen with the help of nitrogenase. The two-chamber system is similar to BEAMR hydrogen experiment, but the principle in producing hydrogen is not entirely the same. In BEAMR, degrading bacteria are added only in anode chamber, most of them being a mix of exoelectrogens; in our experiments, two different bacteria strains are respectively added into anode and cathode chambers. Secondly, hydrogen is produced from the electrode of cathode chamber in BEAMR; in our experiments, hydrogen is mainly carried out from biosynthesis of *R. sphaeroides*,



**Fig. 6** Analytical diagram of dual-chamber hydrogen production principle



partly from transmembrane protons switched by nitrogenase system. Figure 7 shows the change in the total hydrogen production of the system in 192 h with different additional voltages (from 0.1 to 0.5 V) in the cathode chamber and 0 V for the control experiment.

From Fig. 7, when the additional voltages of 0.1–0.5 V were applied to the two-chamber system, the total gas produced in cathode chamber was 699 mL, 717 mL, 870 mL, 844 mL, and 667 mL, respectively. Compared with the control group of 612 mL hydrogen production, the total hydrogen production in experimental groups had increased by certain degree. In experimental time of 192 h, the peak hydrogen production of 920 mL was obtained when 0.3 V electric potential was added, which had 50.3% increase compared to the control group. In fact, the pH value of cathode chamber in experimental groups significantly increased slowly in the late process, which was similar to the results studied by other researchers (Sediroglu et al. 1999). These results suggested that power-promoting protons across the membrane did exist in the experimental groups. Obviously, the additional voltages gave bacterial cells electricity-driven that facilitated protons into cathode chamber more quickly.

Hydrogen can also be achieved by electrolysis of water. However, the reaction of the water electrolysis cannot occur without necessary energy, usually supplied by an external electrical power source. The standard potential of the water electrolysis cell is  $-1.23$  V at  $25$  °C in pH 0 ( $H^+ = 1.0$  M), but the actual potential is  $-1.8$ – $2.0$  V. This means a lower voltage cannot drive the electrolysis of water. Thus, the increased hydrogen yield could not come from water electrolysis. This also could be confirmed by another experimental phenomenon. From Fig. 7, although the lower voltages added as a whole were helpful to promote proton flow and hydrogen

production, the total hydrogen gas under 0.5 V added did not increase as anticipated but a little decreased on the contrary when compared with that under 0.3 V. It was possible that an additional higher voltage, namely 0.5 V, an inadequate electric potential for water electrolysis, destroyed the electric balance of bacterial cell and caused cell or cell membrane physiological damage to some degree. Therefore, it did not show the effect that the higher the voltage was, the more hydrogen production could be. Besides the electricity-driven accelerates proton transfer into cathode chamber, it may be another reason to explain hydrogen increase that appropriate voltages lower the activation energy in bacterial hydrogen production.

## Conclusions

A series of environmental factors are involved in photosynthetic microbial hydrogen production, e.g., dissolved oxygen, pH, and hydrogen ions. In the range of 14 to 25% salt concentration, it has been proven that salt marshes can adapt to lower salt concentrations through gradual domestication. In a mixed bacterial hydrogen production system, no matter whether PC or PM of domesticated *H. salinarum* was used, *R. sphaeroides* could increase hydrogen production significantly. When the BR is 80 nmol, the hydrogen production can reach 217 mL. The increased hydrogen was considered to come from *H. salinarum*'s protons transforming into gaseous hydrogen by nitrogenase enzyme system of *R. sphaeroides*. The view was further verified by the two-chamber combined system test and electricity-driven test, in which the separated microbes by the ion exchange membrane could also effectively increase hydrogen yield; when the voltage reaches 0.3 V, the hydrogen production is 920 mL.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13213-021-01621-z>.

**Additional file 1.** Data record sheet.

## Acknowledgements

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## Authors' contributions

Corresponding author is the first author: guide the completion of this experiment and revise their papers. The second author: do experiments, data analysis completed the first draft paper. The third author: experiment, record data. The fourth author: participating in the experiment, finished draft paper together with the second author. The authors read and approved the final manuscript.

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**Availability of data and materials**

Jiang-yu Ye and Yue Pan (2020). "Enhanced hydrogen production of *Rhodobacter sphaeroides* promoted by extracellular H<sup>+</sup> of *Halobacterium salinarum*," its original data is recorded in an Excel named "Data record sheet" and has been attached to this article.

**Ethics approval and consent to participate**

The study did not violate ethics, and all participants agreed to publish the paper.

**Consent for publication**

Not applicable.

**Competing interests**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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