

# Optimization of Photo-Hydrogen Production by Immobilized *Rhodopseudomonas Faecalis* RLD-53

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## ABSTRACT

*In this work, the optimization of hydrogen production by photo-fermentation bacteria immobilized on agar gel granule was systematic investigated in batch culture. Experiment focus on the effect of some important affecting factors on photo-hydrogen production. Results indicated that immobilized Rhodopseudomonas faecalis RLD-53 exhibited the highest hydrogen yield of 3.15 mol H<sub>2</sub>/mol acetate under follow optimal condition: agar granule diameter of 2.5 mm, inoculum age of 24 h, agar concentration of 2%, biomass of 4 mg/ml in agar and light intensity of 9000 lux. More importantly, immobilized photo-fermentation bacteria not only can enhance hydrogen production but can increase acids-tolerance capacity, even at pH 5.0 hydrogen also was produced, and thus hopefully immobilized photo-fermentation bacteria can be applied in the combination of dark and photo-fermentation for hydrogen production with high yield.*

**Keywords:** Hydrogen Production, Photo-Fermentation, Agar Gel, Immobilized *Rhodopseudomonas Faecalis*, Acids-Tolerance Capacity

## 1. Introduction

The shortage of fossil fuels, the pollution of global environment and emissions of greenhouse gas are attracting more and more attention of researches. But, up to now, the major source of energy is still supplied from fossil fuels. Hence, we urgent need to develop new and renewable energy to replace fossil fuels. Bio-hydrogen is a clean, environmental friendly and recycle energy carrier and recognized as a promising substitute of fossil fuels in future. At present, two main pathways, dark and photo-fermentation, were used for bio-hydrogen production [1]. Recently, bio-hydrogen production by photo-fermentation has exhibited great potential. In particular dark-fermentation process produced short chain acids, which can be utilizing by photo-fermentation process for additional hydrogen production. Thus, the combination of dark and photo fermentation for hydrogen production can achieve a high overall hydrogen yield, which hopefully close to theoretically maximum value of 12 mol H<sub>2</sub>/mol hexose. The overall hydrogen yield of 13.7 mol H<sub>2</sub>/mol-sucrose (equivalent to 6.85 mol H<sub>2</sub>/mol hexose) was obtained using sequential dark and photo-fermentation from beet molasses [2]. Previous our work showed that the two-step of dark and photo fermentation reached a nice hydrogen yield of 6.32 [3] and 5.37 [4] mol H<sub>2</sub>/mol glucose in batch experiment. Other some

reports also demonstrated that the total hydrogen yield of sequential dark and photo-fermentation was obviously higher than that of a single dark fermentation process using single substrate as sole carbon source [5-8].

The immobilization technology for dark fermentation hydrogen production has been widely studied [9,10]. Usually, bacteria main were immobilized on carrier such as polyacrylamide [11], polyvinyl alcohol [12], or agar gel [13], etc. Nearly all their work showed that immobilized dark fermentative bacteria can enhance and stabilize hydrogen production process. There are only a few reports about the immobilization of photo-fermentation bacteria for improving the bio-hydrogen process [14,15]. However, these reports have not focused on the optimization of immobilized condition.

Therefore, in this study photo-fermentation bacteria were immobilized on agar gel granule to explore their hydrogen production capacity. The some key immobilized parameters, for example, agar granule diameter, inoculum age, agar concentration, pH, biomass in agar and light intensity, were systematic optimized for improving photo-hydrogen production by batch culture.

## 2. Method

### 2.1. Bacterial Strain and Medium

Photo-fermentative bacterium *Rhodopseudomonas fae-*

*calis* strain RLD-53, the previously isolated from fresh water pond, used in this study [16]. The medium for growth and hydrogen production was same with previous description by Liu *et al.* [17]. Acetate of 50 mmol/l was used as sole carbon source in medium for hydrogen production. The strain RLD-53 was pre-cultured at 35°C for 24 h under light intensity of 2000 lux with incandescent lamps (60 W) and argon was used to maintain anaerobic condition.

## 2.2. Hydrogen Production Procedure

The experiment was carried out in 100 ml serum bottles, which were sealed by rubber plugs and filled with argon to maintain anaerobic conditions. 80 ml medium for hydrogen production was put into reaction bottles. The bottles with liquid medium were sterilized at 121°C for 15 min. Operation parameters were as follows: inoculant age of 24 h, OD<sub>660</sub> of 1.68, inoculant volume of 10% (v/v), the bottles were shaken on the constant temperature incubation oscillator at 120 rpm, culture temperature of 35°C, The light intensity of outside surface of the bottles was maintained at 4000 lux by a incandescent lamps of 60 W.

## 2.3. Preparation of Immobilized Cell

The cells of photo-fermentation bacteria were harvested by centrifugation at 6000 rpm for 10 min. Agar was dissolved in 20 ml of sterile water at 0.4% (w/v) and incubated at 100°C, and then it was used for the immobilization of bacterial cells. Agar solution was cooled to 40-50 °C and mixed with 20 ml prepared suspension of centrifuged *R. faecalis* RLD-53 in a beaker. The above mixture was immediately inhaled into a syringe of 50 ml by hand and until agar gel was formed, and then agar gel of containing bacterial cells was injected into the medium through a syringe. The dry weight of the bacterial cells in the each agar gel granule with the average diameter of about 2.5 mm was approximately 0.113 mg.

## 2.4. Analytical Method

Hydrogen analysis in evolved gas was performed using a gas chromatograph (GC) (Model SC-II, Shanghai Analysis Instrument Factory) equipped with a thermal conductivity detector and a 2-m stainless column packed with 5 Å molecular sieves. The operational temperatures at the injection port, the column oven and detector were 100, 60 and 105 °C, respectively. Argon was used as carrier gas at a flow rate of 70 ml/min. The volatile fatty acids in supernatant of culture broth were determined using a second GC (Model GC122, Shanghai Analysis Instrument Factory) equipped with a flame ionization detector and a 30 m×0.25 mm×0.25 mm fused-silica capillary column. The liquor samples were first centrifuged at

12,000 rpm for 5 min, and then acidified with hydrochloric acid and filtered through a 0.2-µm membrane before free acids were analyzed. Nitrogen was used as carrier gas.

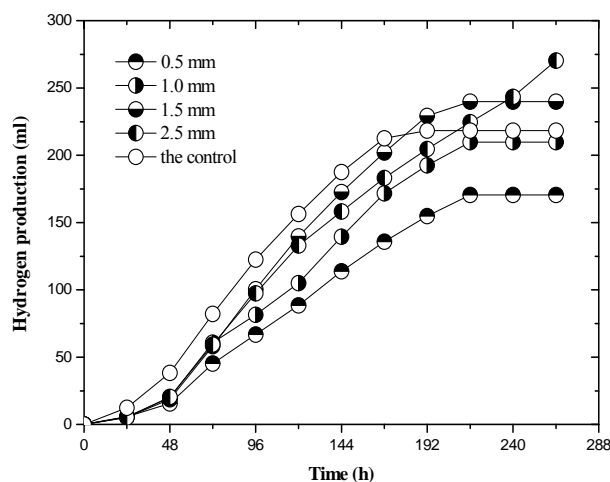
The light intensity (lux) was measured by using a digital luxmeter (TES1330A, Junkai Co.). Cell concentration was determined by an Amersham Pharmacia Biotech ultraspec 34300 UV/Vis spectrophotometer.

## 3. Results and Discussion

### 3.1. The Effect of Agar Gel Granule Size

Agar granule size was an important factor affecting hydrogen production by photo-fermentation. Agar granule size of 0.5 mm, 1 mm, 1.5 mm, 2.5 mm made by different sizes of syringe. Acetate of 50 mmol/l, glutamate of 10 mmol/l, reaction system of 80 ml, incubation temperature of 35°C and light intensity of 4 000 lux were used.

Hydrogen production increased gradually with the increase of agar granule size from 0.5 - 2.5 mm (**Figure 1**). Agar granule size at 0.5, 1.0, 1.5 and 2.5 mm, cumulative volume of hydrogen was 170, 210, 240 and 270 ml/reactor, respectively, and the control was 218 ml/reactor. This result indicated that hydrogen yield was higher compared to other size when agar granule size was at 1.5 and 2.5 mm. Agar granule size at 2.5 mm, hydrogen yield reached maximum value of 3.15 mol H<sub>2</sub>/mol acetate, conversion efficiency of substrate was 78.75% and hydrogen content was between 75% - 85%. In addition, immobilized bacterial cells can lengthen the time of hydrogen production. Hydrogen production of non-immobilized cell stopped at 192 h, but hydrogen production of immobilized cell stopped at 264 h. The utilization efficiency of substrate of immobilized cell



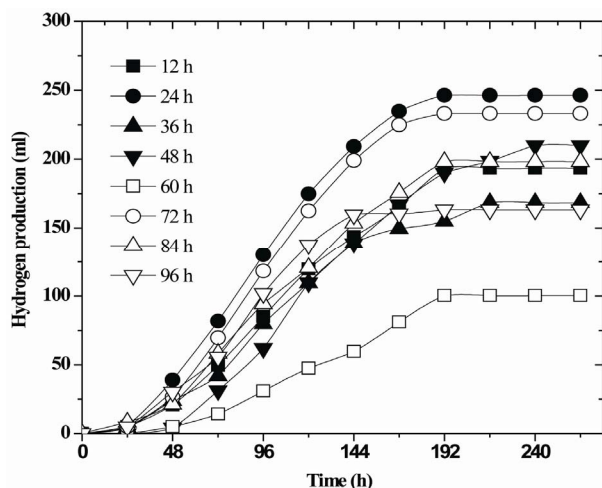
**Figure 1.** The effect of gel granule size on photo-H<sub>2</sub> Production.

was higher compared to non-immobilized cell. Within 48 h of culture, a great quantity of substrate was consumed for cell growth and the utilization efficiency of substrate was 0.04 g acetate/l/h. However, the utilization efficiency of substrate of immobilized cell was 0.018 g acetate/l/h, more acetate was for hydrogen production (**Figure 2**). These seem to imply that the bacteria were immobilized in agar gel can limit substrate for itself growth and increase hydrogen production, and lengthen the time of hydrogen production. So, agar granule size of 2.5 mm was used follow experiment for further research.

### 3.2. The Effect of Inoculant Age

In this test, the inoculant age of 12, 24, 36, 48, 60, 72, 84 and 96 h was employed to explore their hydrogen production capacity.

The difference in hydrogen production under various inoculant ages is very obviously (**Figure 3**). Higher yield and production rate of hydrogen was obtained when inoculant age was 24 h and 72 h, and cumulative volume of hydrogen was 246 ml/reactor and 233 ml/reactor, respectively. Inoculant age was at 12, 48 and 84 h, the yield and production rate of hydrogen was similarly and it was about 200 ml/reactor. Inoculant age at 36 and 96 h, cumulative volume of hydrogen was about 160 ml/reactor. However, inoculant age at 60 h, hydrogen yield only was about 100 ml/reactor. The hydrogen production by the bacteria cause the results of differences may be related to physiological state and enzymes activity of bacteria. We think that a long time of bacteria in the agar, nitrogenase activity was restored or enhanced leading to the hydrogen yield in high level. Inoculant age has a direct impact on the cell's physiological state and the chemical components of culture. Our result was consistent with Felten *et al.*'s report, which showed that the



**Figure 2.** The consumption of acetate under different agar granule size in immobilized photo-hydrogen production.

inoculant age of the immobilized bacteria is the key factor affecting hydrogen production, *R. rubrum* of inoculant age of 70 h were immobilized with the highest hydrogen production activity [18]. Therefore, inoculant age of about 24 h and 72 h for photo-hydrogen production is appropriate.

### 3.3. The Effect of Agar Concentration

Photo-hydrogen production significantly influenced by agar concentration, which directly determined the absorbance of photo-fermentative bacteria, utilization efficiency and transfer rates of substrate.

Agar concentration was in the range of 1% - 4%, agar gel granule size of 2.5 mm and inoculant age of 24 h were used in this test. When agar concentration was 1.5% and 2%, cumulative volume of hydrogen was 254.98 ml/reactor and 249.67 ml/reactor, hydrogen production capacity was higher than that of other agar concentration (**Figure 4**). The performance of hydrogen production was similar to the control under agar concentration of 1%, 3% and 4%. Agar concentration over 3%, penetration of light into inside bacterial cell decreased and the growth rate will be decreased with increasing the agar concentration, thereby affecting the utilization of its substrate and the internal mass transfer resistance increased. This result was similar to Seol. *et al.*'s research, which indicated that the substrate and products are easily transferred through the bead when agar concentration in proper experimental ranges [19]. In addition, the accumulation of cell metabolite in the agar granule caused cell toxicity and repressed infiltration capacity of substrate, will also affect the growth and hydrogen production of photo-fermentation bacteria.

### 3.4. The Effect of Bacterial Concentration in Agar Gel

The bacterial concentration in agar influenced utilization and mass transfer efficiency of substrate. Biomass in agar was 2, 4, 6, 8 and 10 mg/ml, respectively. Agar gel granule size, inoculant age, agar concentration, acetate concentration and reaction system were 3 mm, 24 h, 2%, 50 mmol/l and 80 ml, respectively.

Result indicated the biomass range of 2 - 4 mg/ml in agar may help to improve performance of hydrogen production and hydrogen yield decreased when biomass was over 6 mg/ml; high biomass in gel granule for enhancing the hydrogen production is not obvious (**Figure 5**). Reason may be due to the substrate into the gel granules was constant and excessive photo-fermentation bacteria were employed in hydrogen production process leading to the consumption of a lot of substrate to maintain the energy require for their growth, thus reducing the use of substrate for hydrogen production and immobilized cells

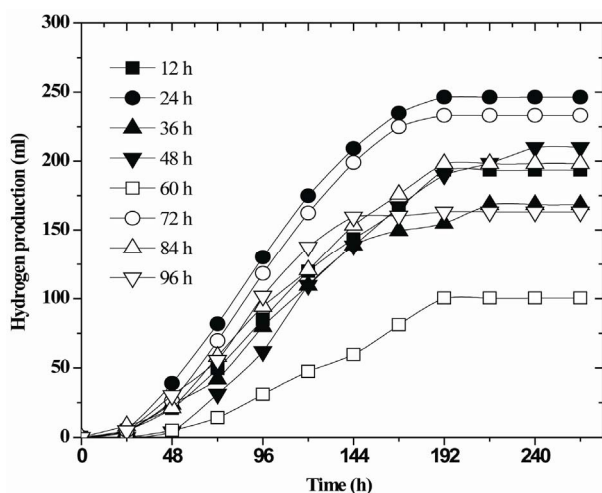


Figure 3. The effect of inoculant age on photo-H<sub>2</sub> production.

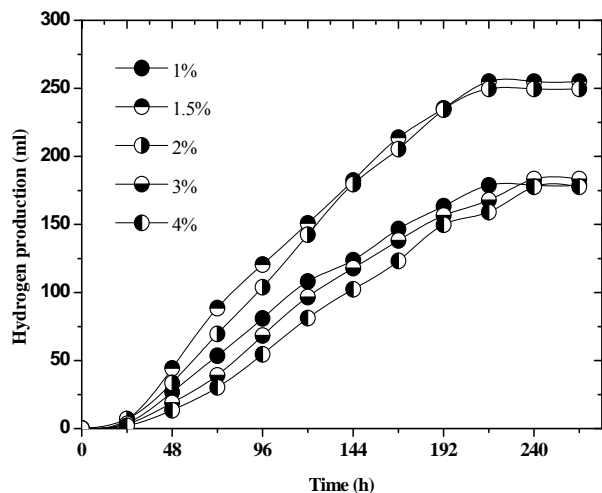


Figure 4. The effect of agar concentration on photo-H<sub>2</sub> production.

activity was prevented. So, the biomass of a certain concentration range can promote hydrogen production capacity of immobilized photo-fermentation bacteria. However, the biomass in gel granule is too high and substrate to maintain bacterial physical requirement exceeded, thereby the hydrogen production capacity reduced. The suitable cell concentration of 1 mg/ml not only achieved the highest hydrogen yield but also more important superior nitrogenase activity [18].

### 3.5. PH Tolerant Capacity

To determine acids tolerance of immobilized cell, in this test, the pH of reaction system was adjusted to 4.0, 5.0, 5.5, 6.0 and 6.5, respectively. Agar granule diameter of 2.5 mm, inoculum age of 24 h, agar concentration of 2%, biomass of 4 mg/ml in agar and acetate of 50 m mol/l

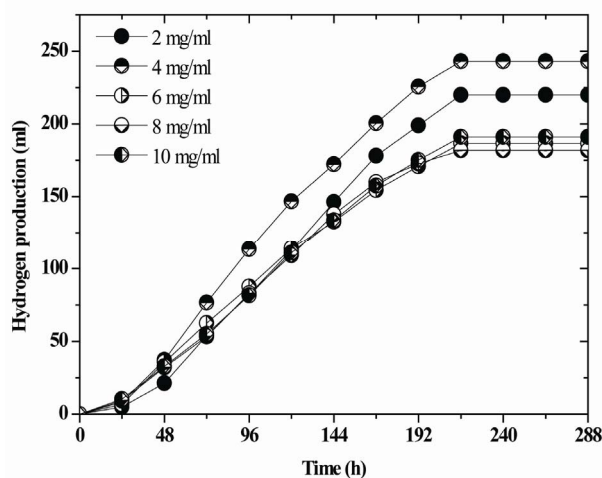


Figure 5. The effect of biomass concentration in agar gel granule.

were constant in 80 ml system.

A decrease in pH resulting in decrease in the yield and production rate of hydrogen (Figure 6). In all pH tests, compared to initial pH, final pH increased slightly due to the consumption of acetate (Figure 7). At low pH 4.0, bacteria can not grow and hydrogen also not produced. Little hydrogen was generated and cumulative hydrogen volume only was 50 ml at pH 5.0. The lag time of hydrogen production was about 72 h and hydrogen production rate was 7.3 ml H<sub>2</sub>/l/h. At pH 5.5, the lag time of hydrogen production decreased to about 48 h and hydrogen production yield and rate started to increase. Cumulative hydrogen volume and maximum hydrogen production rate reached 149 ml and 17.7 ml H<sub>2</sub>/l/h, respectively. At pH 6.0 and 6.5, the trend of hydrogen production was similarly within 144 h. After 144 h, hydrogen production rate increased slightly under pH 6.5. Finally, maximum

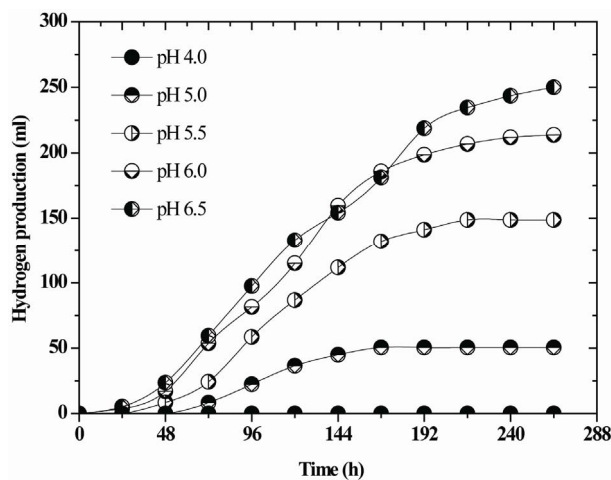
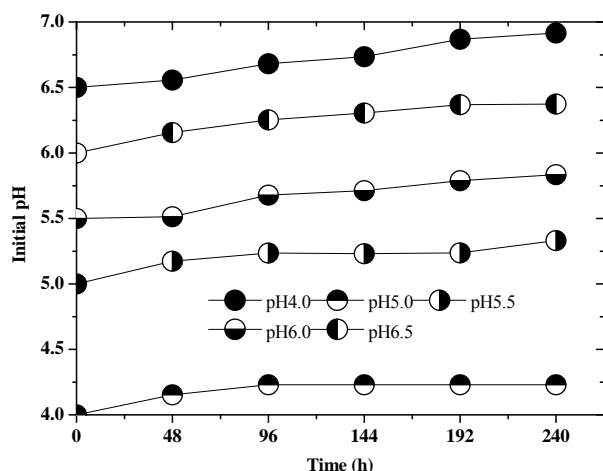


Figure 6. Acid-tolerance capacity of strain RLD-53 during H<sub>2</sub> Production.



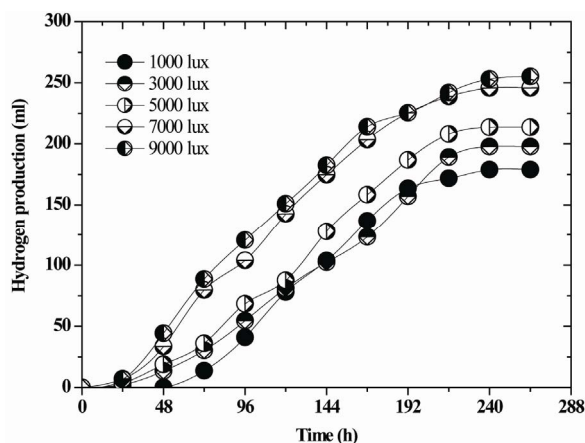
**Figure 7.** The change of pH under different initial pH during entire  $H_2$  production.

hydrogen yield of 250 ml and rate of 23.4 ml  $H_2$ /l/h was obtained at pH 6.5. Suitable range of pH is at 6.5 - 7.5 for non-immobilized photo-fermentation bacteria [16,20]. Thereby, above results implied that pH are an important factors for sustained and efficient hydrogen production in immobilized strain RLD-53 and the immobilized fermentation bacteria have certain acids-tolerance capacity with high hydrogen yield. Immobilized photo-fermentative bacteria can tolerate lower pH of a certain extent, even at pH 5.0 hydrogen also was produced.

### 3.6. Requirement of Light Intensity

The growth and hydrogen production of photo-fermentation bacteria need to apply energy by light condition. So, light intensity also was an important limiting factor for photo-hydrogen production. The optimum light intensity of non-immobilized strain RLD-53 for hydrogen production was at 3000-5000 lux [16], and the bacteria were immobilized on agar, which can prevent the infiltration of light and light absorption of bacteria. Therefore, the investigation of light intensity of immobilized bacteria is necessary.

Effect of different light intensities on the hydrogen production is depicted in **Figure 8**. It has been observed that increased light intensity resulted in an increase in the total volume of hydrogen and also hydrogen production rate. The lower light intensity accompanied a long lag time of hydrogen production. Light intensity was at 1 000 lux, lag time of hydrogen production is 48 h and the lowest yield of hydrogen of 178 ml- $H_2$ / reactor was obtained. When the light intensity was at 7 000 lux and 9000 lux, the hydrogen production capacity was closed, the cumulative volume of hydrogen gas were 246 ml- $H_2$ /reactor and 255 ml- $H_2$ /reactor, respectively, the maximum hydrogen production rate reached 24 ml-  $H_2$ /l/h. It can be



**Figure 8.** The effect of light intensity on photo- $H_2$  production.

observed that the hydrogen production under highest light intensity reached a higher yield. Usually, high light intensity can inhibit hydrogen production by non-immobilized photo-fermentation bacteria [16, 21]. However, these results indicated that the range of optimal light intensity for hydrogen production by immobilized photo-fermentation bacteria was between 7 000 - 9 000 lux. This also suggested that the photo-fermentation bacteria immobilized in agar to prevent the light penetration into inside bacterial cells and light absorption of the bacterial photosynthetic system, further influence generation of electronic and synthesis of ATP, leading to bacterial growth, nitrogenase activity and hydrogen production were inhibited. Therefore, light intensity needs to increase to obtain enough supply of light energy for the growth and producing hydrogen of photo-fermentation bacteria.

### 4. Conclusions

Results obtained in this study clearly exhibited the immobilized photo-fermentation bacteria could obviously promote hydrogen production, the conversion efficiency of substrate and lengthen time of hydrogen production. More importantly, it demonstrated that the granule diameter, inoculant age, agar concentration, biomass in agar and light intensity are key factors affecting photo-fermentation hydrogen production, and when they are 2.5 mm, 24 or 72 h, 2%, 4 mg/ml and 7000-9000 lux, the maximum hydrogen yield reached 3.15 mol- $H_2$ /molacetate. The immobilized photo fermentation bacteria not only can enhance hydrogen production but can increase acids-tolerance capacity, even at pH 5.0 hydrogen also was produced, and thus hopefully immobilized photo-fermentation bacteria can be applied in the combination of dark and photo-fermentation for hydrogen production with high yield.

## 5. Acknowledgements

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