

# Anaerobic production of hydrogen in the dark by *Synechocystis* sp. strain PCC 6803 supplemented with D-glucose

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## Abstract

The effect of D-glucose on the anaerobic production of hydrogen ( $H_2$ ) in the dark by bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 has been studied. D-glucose addition enhanced  $H_2$  production rate. It is deduced that NAD(P)H, which is a substrate of  $H_2$  production reaction, was supplied by catabolism of D-glucose. The molar consumption ratio of  $H_2$  and total sugar consumption was low in the presence of D-glucose due to production of L-lactic acid and other metabolites. The cells photosynthetically grown in air produced lower  $H_2$  in the dark as compared with those grown in air with 6%  $CO_2$ , but the lower  $H_2$  production was compensated with the addition of D-glucose at 6.4 times of control value without D-glucose. The trend of effect of pH in a growth medium and a reaction mixture on  $H_2$  production was not affected by the presence of D-glucose. This result implies that mechanisms of  $H_2$  productions with and without D-glucose are similar.

**Keywords:**  $H_2$  production, Bidirectional hydrogenase, *Synechocystis* sp. strain PCC 6803, D-glucose, pH

## Introduction

Molecular hydrogen ( $H_2$ ) produced by cyanobacteria is a potential source of a non-polluting fuel. The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 is a favored microorganism for the molecular biotechnological studies on the  $H_2$  production. Genome DNA sequence analysis and related works on this strain have defined the presence of bidirectional hydrogenase and the absence of both nitrogenase and uptake hydrogenase (Kaneko *et al.*

1996; Appel and Schulz 1996; Schmitz *et al.* 1995). The bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 is an enzyme forming  $H_2$  and  $NAD(P)^+$  from protons and NAD(P)H. This reaction is thought to play an important role for proper redox poising (Appel *et al.* 2000).

It has been proposed that supply of NAD(P)H to hydrogenase limits  $H_2$  production (Yamamoto *et al.* 2012; Cournac *et al.* 2004). Oxygen produced by photosynthesis inhibits hydrogenase activity.  $H_2$  production occurs in the dark and anaerobic condition. Under the condition NAD(P)H is produced from catabolism of intracellular glucan and cell constituents accumulated during photosynthetic growth (Figure 1). Supply of NAD(P)H is a key factor to improve  $H_2$  production.

D-glucose is another means to supply NAD(P)H to cells. *Synechocystis* sp. strain PCC 6803 is known to consume D-glucose (Smith 1983). Catabolism of D-glucose to  $CO_2$ , organic acids or other metabolites donates electrons to  $NAD(P)^+$  to form NAD(P)H. Although it has been reported that reducing power of D-glucose is a key factor for  $H_2$  production in *Synechocystis* sp. strain PCC 6803 (Antal and Lindblad 2005; Baebprasert *et al.* 2010), this mechanism is still not well understood.

The present work was aimed at studying the effect of D-glucose addition on  $H_2$  production. The relationship between  $H_2$  production with D-glucose,  $CO_2$  level, growth pH and reaction pH were studied.

## Methods

### Cell preparation

*Synechocystis* sp. strain PCC 6803 was photosynthetically grown at 34°C in a 100 mL bubble column (clear Pyrex glass) containing 80 mL HEPES buffer (pH 6.8, 7.7 or 8.5) aqueous solution of BG-11 medium. The aqueous solution was aerated at 80 mL  $min^{-1}$  by air or air containing 6%  $CO_2$  that was filtered through 0.45  $\mu m$  filter and fed from the bottom of the bubble column. The bubble column was placed in a 34°C water bath. One side of the bubble column was illuminated by fluorescent lamps at 100

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$\mu\text{mol m}^{-2} \text{s}^{-1}$ . After the 24 h cultivation cells were harvested by centrifugation at 25°C, 3000 rpm for 10 min. The cell pellets were washed with HEPES buffer solution and centrifuged again.

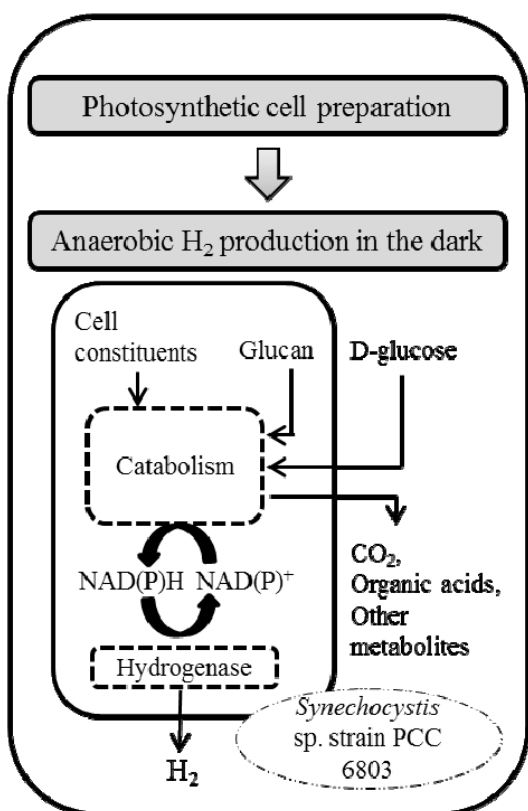


Figure 1: Diagram of H<sub>2</sub> production in this research.

### H<sub>2</sub> production

After cell preparation in photosynthesis the settled cells were suspended in de-ionized water containing 50 mmol L<sup>-1</sup> HEPES buffer (pH 6.8, 7.7 or 8.5) in a test tube with a butyl rubber cap as reported elsewhere (Asami *et al.* 2011). Initial cell mass concentrations were set to 1.0 and 2.0 g L<sup>-1</sup> as to cells grown in air and air containing 6% CO<sub>2</sub>, respectively. Total volume of cell suspension was adjusted to 10 mL. Finally cell suspensions in the test tubes were sparged with N<sub>2</sub> gas for a few minutes to remove O<sub>2</sub> gas and sealed with butyl rubber caps. Test tubes were placed into a water bath at 34°C under the dark condition and agitated at 120 rpm.

### Measurement of H<sub>2</sub> production, cell mass concentration, D-glucose concentration in cell suspension and amount of intracellular glucan

At the measurement time, 300  $\mu\text{L}$  of gas sample was withdrawn from the test tube with a gasket syringe and 400  $\mu\text{L}$  of cell suspension was also withdrawn from the test tube with a syringe. After that, the test tube was sparged with N<sub>2</sub> gas again.

An amount of H<sub>2</sub> in gas sample was determined with a gas chromatograph and a thermal conductivity detector (GC-320, GL science Inc.; column, Molecular sieve 13X; carrier gas, nitrogen gas; column temperature, 315 K; injector temperature, 333 K; detector temperature, 353 K). Total H<sub>2</sub> production per unit suspension volume of a run ( $y_{\text{H}_2}$ ) was calculated from a result.

Cell mass concentration ( $X$ ) in terms of dry cell weight (DCW) per unit suspension volume was monitored by measuring an optical density at 730 nm (OD<sub>730</sub>) utilizing a spectrophotometer. One unit absorbance of a cell suspension was equivalent to 0.369 g-DCW L<sup>-1</sup>.

A 200  $\mu\text{L}$  sample of a cell suspension was centrifuged at 4°C, 10,000 rpm for 5 min. The amount of D-glucose in supernatant ( $c_G$ ) was measured with a glucose tester (Wako Pure Chemicals, Japan). On the other hand, a hydrolysis method modified from a previous work (Asami *et al.* 2011) was used for the pellet in the settled fraction to measure the intracellular glucan content as moles of glucose ( $m_G$ ): The pellet was washed with de-ionized water and re-centrifuged a few times to remove D-glucose in the cell suspension and mixed with 50  $\mu\text{L}$  6 N HCl; the mixture was placed into a water bath at 80°C for 30 min; HCl and water were evaporated in dryer and pellet was dissolved in 100  $\mu\text{L}$  of de-ionized water; the amount of D-glucose contained in the hydrolyzed pellet was measured with a glucose tester.

### Measurement of L-lactic acid

A supernatant taken from a cell suspension was filtrated with a 0.45  $\mu\text{m}$  filter. The sample analyzed by high performance liquid chromatography using a silica ODS-C18 column (length, 250 mm; diameter, 4 mm). The 0.1 mol L<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> solution, adjusted at pH 2.5 with H<sub>3</sub>PO<sub>4</sub>, was used as mobile phase. Chromatography was performed in a column oven at 313 K with the flow rate of 1.0 mL min<sup>-1</sup>. UV detection at 210 nm was performed.

## Results and discussion

Cells were grown with air containing 6% CO<sub>2</sub> at pH 7.7. The cells were suspended in a buffer solution or buffer solutions containing 5.6, 28 or 56 mmol L<sup>-1</sup> D-glucose and H<sub>2</sub> productions were measured every 24 h. **Figure 2** shows the time course of H<sub>2</sub> amount per unit volume of cell suspension. The run with 5.6 mmol L<sup>-1</sup> D-glucose showed the H<sub>2</sub> production as twice as the run without D-glucose addition. In the cases of the runs with 28 and 56 mmol L<sup>-1</sup> D-glucose, H<sub>2</sub> production was not improved until 24 h but improved after 24 h comparing to the control run without D-glucose. **Figure 3** shows the effect of initial D-glucose concentration on H<sub>2</sub> production at 72 h. The D-glucose addition has a positive effect on H<sub>2</sub> production in lower concentration, while the H<sub>2</sub> productions amount reduced to the level of control run without D-glucose addition in higher concentration over 5.6 mmol L<sup>-1</sup>.

The time course of extracellular D-glucose concentration in buffer solutions is shown in Figure 4. D-glucose in the run with

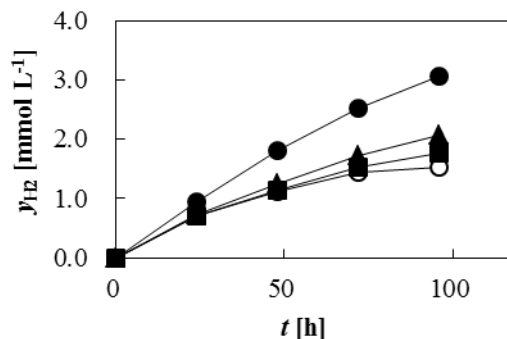


Figure 2: Time course of hydrogen production per unit volume of cell suspension ( $y_{\text{H}_2}$ ) by the cell suspension with a buffer solution (O) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■) mmol L<sup>-1</sup> D-glucose.

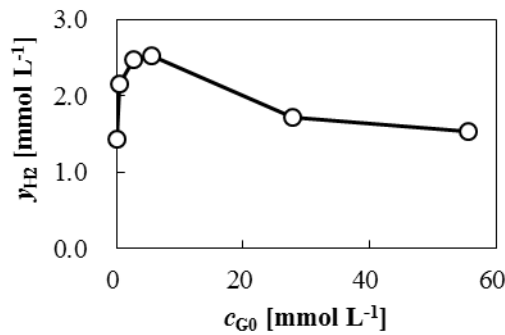


Figure 3: Effect of initial D-glucose concentration ( $c_{G0}$ ) on hydrogen production per unit volume of cell suspension ( $y_{H_2}$ ) at 72 h.

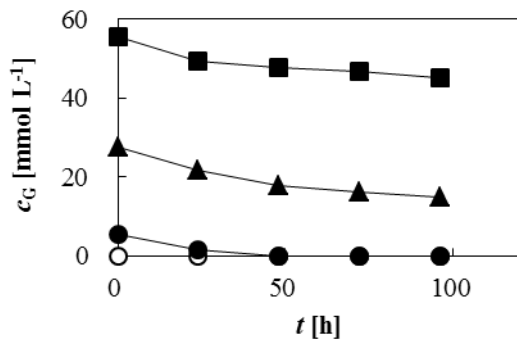


Figure 4: Time course of amount of extracellular D-glucose per unit volume of cell suspension ( $c_G$ ) of the cell suspension with a buffer solution (O) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■) mmol L<sup>-1</sup> D-glucose.

5.6 mmol L<sup>-1</sup> D-glucose was consumed in 48 h. Interestingly, H<sub>2</sub> production rate in this run was maintained at the high level even after extracellular D-glucose was depleted. This is because cells accumulated reducing power as the form of cell constituents, especially intracellular glucan. Cell mass and intracellular glucan per unit volume of cell suspension in the runs with D-glucose increased in H<sub>2</sub> production phase (Figures 5 and 6). In the run with 5.6 mmol L<sup>-1</sup> D-glucose, after extracellular D-glucose had depleted, cell constituents and intracellular glucan had started degrading.

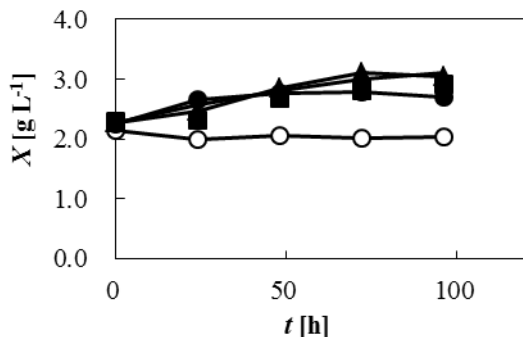


Figure 5: Time course of cell mass concentration ( $X$ ) of the cell suspension with a buffer solution (O) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■) mmol L<sup>-1</sup> D-glucose.

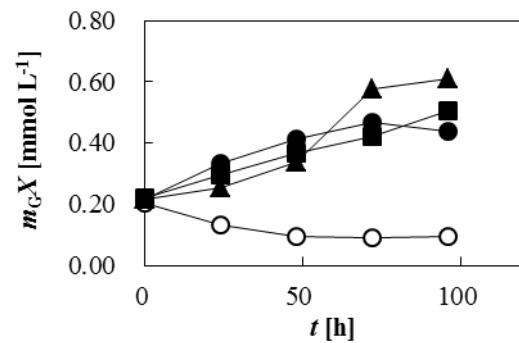


Figure 6: Time course of amount of intracellular glucan per unit volume of cell suspension ( $m_{GX}$ ). Cells were suspended in a buffer solution (O) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■) mmol L<sup>-1</sup> D-glucose.

H<sub>2</sub> productions by cell suspensions with 28 and 56 mmol L<sup>-1</sup> D-glucose were not so improved (Fig. 3), although D-glucose consumption rates in these runs were higher than the other runs (Figure 7). The bell shaped-effect of glucose on H<sub>2</sub> production is also shown in Fig. 3. It is mentioned that cells may use energy to drive sugar out of cells at high D-glucose concentration (Baebprasert *et al.*, 2010). Furthermore, D-glucose uptake rate of cells was also affected by substrate inhibition at high D-glucose concentration. These results propose that it is important to consider H<sub>2</sub> yield to D-glucose consumption rather than D-glucose consumption rate.

Figure 8 shows relationship between total sugar (extracellular D-glucose and intracellular glucan) consumption per unit volume of cell suspension ( $-\Delta c_{GT}$ ) and H<sub>2</sub> production. A slope of these plots is defined to H<sub>2</sub> yield to D-glucose consumption ( $Y_{H_2/GT}$ ), although not only degradation of sugar but also degradation of cell constituents contributes to H<sub>2</sub> production in actual.  $Y_{H_2/GT}$  in the run without extracellular D-glucose was the highest, 12 mmol mmol<sup>-1</sup>. On the other hands,  $Y_{H_2/GT}$  in the runs with 28 and 56 mmol L<sup>-1</sup> extracellular D-glucose were 0.16 and 0.18 mmol mmol<sup>-1</sup>, respectively. It is apparent that intracellular glucan is more efficient as H<sub>2</sub> source than extracellular D-glucose. In the case of the run with 5.6 mmol L<sup>-1</sup> extracellular D-glucose,  $Y_{H_2/GT}$  was 0.30 mmol mmol<sup>-1</sup> when

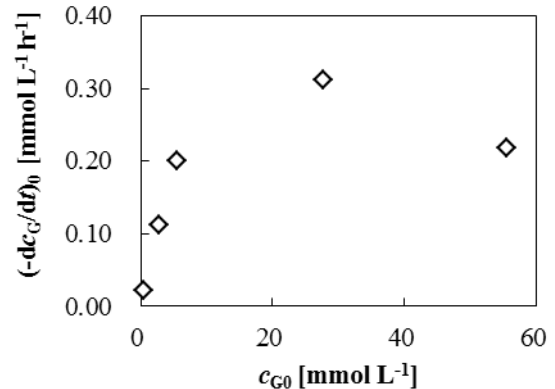


Figure 7: Effect of initial concentration of extracellular D-glucose ( $c_{G0}$ ) on initial D-glucose uptake rate ( $(-dc_G/dt)_0$ ).

extracellular D-glucose still existed around cells, then this value rapidly increased with depletion of D-glucose. H<sub>2</sub> production rate was kept high after 48 h. It is noteworthy to point out that H<sub>2</sub> production occurred through two steps when a little amount of D-glucose was added into cell suspension: First, H<sub>2</sub> production occurs with high production rate and low H<sub>2</sub> yield; after D-glucose depletion, H<sub>2</sub> yield increased and H<sub>2</sub> production rate was maintained

at high level. D-glucose addition is effective to enhance total amount of  $H_2$  production per unit cell mass.

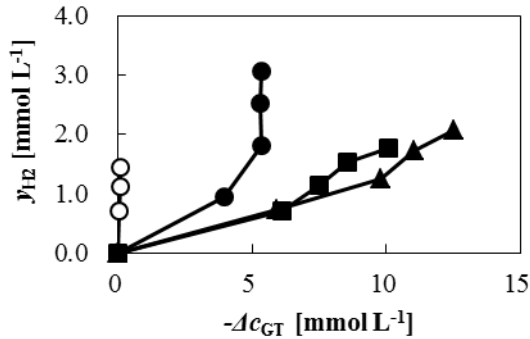


Figure 8: Relationship between hydrogen production per unit volume of cell suspension ( $y_{H_2}$ ) and consumption of total sugar amount per unit volume of cell suspension ( $-\Delta c_{GT}$ ) of the cell suspension with a buffer solution (○) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■)  $mmol L^{-1}$  D-glucose.

The low  $Y_{H_2/GT}$  in the runs with D-glucose were caused by consumption of NAD(P)H for production of intracellular glucan, cell constituents and L-lactic acid production from D-glucose. Figure 9 shows the time course of L-lactic acid production which is NADH consuming reaction. L-lactic acid production rate was related to the extracellular D-glucose uptake rate (Figs. 4 and 9). In the run with  $5.6 mmol L^{-1}$  D-glucose, D-glucose depletion at 48 h ceased the L-lactic acid production.

$CO_2$  levels in aeration gas in photosynthetic cell preparation phase affected to  $H_2$  production in the dark. Figure 10 shows the time course of  $H_2$  production by cells with different aeration condition in preparation phase and with different extracellular D-glucose concentration in  $H_2$  production phase.  $CO_2$  concentration of air (about 0.039%) is insufficient to multiply cells for  $H_2$  production, which was decreased to 21% comparing to the cells prepared in air

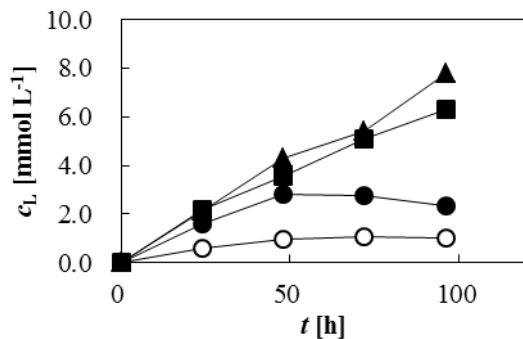


Figure 9: Time course of L-lactic acid concentration in per unit volume of cell suspension ( $c_L$ ) with a buffer solution (○) or buffer solutions containing 5.6 (●), 28 (▲) or 56 (■)  $mmol L^{-1}$  D-glucose.

with 6%  $CO_2$  by considering the difference in initial cell mass concentration. This is because the intracellular glucan accumulation decreased to 15% in the absence of extracellular D-glucose (data not shown). D-glucose could compensate this decreasing in  $H_2$  production, 6.4 times higher than that without D-glucose. The effect was larger than the case of cells prepared with air containing 6%  $CO_2$ . It might be because the cells prepared with air containing 6%  $CO_2$  had stored enough glucan or had highly reduced, the  $H_2$  production by the cells was not highly enhanced by  $28 mmol L^{-1}$  as shown in Figs. 2 and 3.

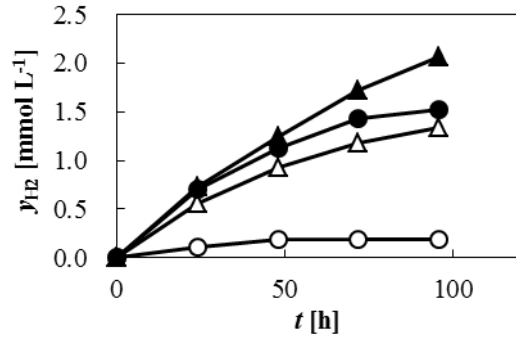


Figure 10: Time course of hydrogen production per unit volume of cell suspension ( $y_{H_2}$ ) by the cell suspension with a buffer solution (○, ●) or buffer solutions containing  $28 mmol L^{-1}$  D-glucose (△, ▲). Cells were prepared with the air (open) or the air containing 6%  $CO_2$  (closed).

The cells prepared in air and BG-11 medium with different pH were re-suspended into buffer solutions with different pH, and then supplied to the experiment of  $H_2$  production with  $28 mmol L^{-1}$  D-glucose in the dark. (Table 1 (a-c)). Cells prepared at pH 6.8 and re-suspended into a buffer solution at pH 7.7 showed the highest  $H_2$  production at 96 h ( $y_{H_2} = 2.2 mmol L^{-1}$ , Table 1 (a)). The effect of pH in BG-11 medium and buffer solution with D-glucose on  $H_2$  production had the same trend as runs without D-glucose addition (data not shown). Table 1(b) shows that cell preparation at low pH (pH 6.8) raised the initial  $H_2$  production rate ( $r_{H_2,0} = 0.066$  and  $0.043 mmol L^{-1}$ ). The pH condition at pH 6.8 seemed to be better than that at pH 7.7 or 8.5 in both phase; however the result by the cells prepared at pH 6.8 and re-suspended into a buffer solution at pH 6.8 shows that  $H_2$  production ceased at early time and total  $H_2$  production was low ( $y_{H_2} = 1.4 mmol L^{-1}$ , Table 1 (a)) in the condition even initial  $H_2$  production rate was high. Table 1(c) shows that D-glucose uptake rate was enhanced by cell preparation at pH 8.5 ( $-dc_G/dt = 0.203 mmol L^{-1} h^{-1}$ ). It is clear that the increasing in D-glucose uptake rate did not result in the increasing in  $H_2$  production rate. The glucokinase is an important enzyme for D-glucose utilization and its optimum pH is 7.9 (Lee *et al.* 2005). Taking together with these results it is considered that variation in intracellular pH depending on extracellular pH may be a key factor to understand the results of Table 1. As shown in Fig. 9, glucose addition increased the acid production. Therefore relationship between acid production and  $H_2$  production should be cleared in future.

## Conclusion

The effect of D-glucose on the anaerobic production of  $H_2$  in the dark by bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 has been studied. As shown before (Antal and Lindblad 2005; Baebprasert *et al.* 2010), D-glucose enhanced  $H_2$  production rate and decreased  $H_2$  yield to D-glucose due to L-lactic acid production.

Table 1: Effects of pH in cell preparation phase and  $H_2$  production phase on (a) hydrogen production ( $y_{H_2}$ ) with D-glucose at 96 h, (b) initial hydrogen production rate ( $r_{H_2,0}$ ) with D-glucose at 96 h and (c) D-glucose uptake rate ( $-dc_G/dt$ ) between 0 - 24 h.

	$y_{H_2}$ at 96 h [ $mmol L^{-1}$ ]	pH in cell preparation phase		
		6.8	7.7	8.5
pH in $H_2$ production phase	6.8	1.4	1.6	
	7.7	2.2	1.3	1.0
	8.5		1.5	1.4

$r_{H_2,0}$ [mmol L <sup>-1</sup> h <sup>-1</sup> ]		pH in cell preparation phase		
		6.8	7.7	8.5
pH in H <sub>2</sub> production phase	6.8	0.066	0.033	
	7.7	0.043	0.028	0.039
	8.5		0.030	0.026

$-dc_G/dt$ [mmol L <sup>-1</sup> h <sup>-1</sup> ]		pH in cell preparation phase		
		6.8	7.7	8.5
pH in H <sub>2</sub> production phase	6.8	0.115	0.096	
	7.7	0.109	0.101	0.200
	8.5		0.090	0.203

5.6 mmol L<sup>-1</sup> D-glucose showed the highest improvement of H<sub>2</sub> production, although it was consumed in 48 h. It is implied that substrate inhibition occurs at high D-glucose concentration and high H<sub>2</sub> production rate can be maintained even after D-glucose depletion. Little amount of D-glucose might be not only a substrate for NAD(P)H supply but a trigger of high yield H<sub>2</sub> production. The result in Fig. 10 showed D-glucose could compensate for lower H<sub>2</sub> production resulted from lower reducing state due to the cell growth without enough CO<sub>2</sub> supply.

The results in Table 1 – 3 proposed that intracellular pH affected by extracellular pH and organic acid production is one of the key factors to construct high H<sub>2</sub> production condition. The same trend of the pH effect on H<sub>2</sub> production with or without D-glucose implies that their H<sub>2</sub> production mechanisms are similar.

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