Improvement of anaerobic production of hydrogen in the dark by genetic mutation strains of Synechocystis sp. strain PCC 6803

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Abstract

The anaerobic H₂ production in the dark by bidirectional hydrogenase of unicellular cyanobacterium Synechocystis sp. strain PCC 6803 has been studied. D-glucose addition to cell suspension has been known to enhance H₂ production, and the two mutants, glucose tolerant strain and its mutant lacking L-lactate dehydrogenase (LDH) gene, were introduced to improve H₂ production with D-glucose. Higher D-glucose uptake rate of glucose tolerant strain resulted in higher H₂ production rate comparing with the wild type strain. The LDH mutant showed higher initial H₂ production rate and H₂ yield from D-glucose than the other strains because the NADH which should be consumed originally by LDH can be redistributed to the H₂ production reaction.

Keywords: H₂ production, Bidirectional hydrogenase, Synechocystis sp. strain PCC 6803, D-glucose, Metabolic modification

Introduction

Molecular hydrogen (H₂) is a potential source of a non-polluting fuel. Cyanobacterial H₂ production is one of the candidates of sustainable H₂ production method. The unicellular cyanobacterium Synechocystis sp. strain PCC 6803 is a favored microorganism for the molecular biotechnological studies on the cyanobacterial H₂ production. The bidirectional hydrogenase of Synechocystis sp. strain PCC 6803 works as an enzyme forming H₂ and NAD(P)⁺ from protons and NAD(P)H (Appel and Schulz 1996; Schmitz et al. 1995). This reaction is thought to play an important role for proper redox poising (Appel et al. 2000).

Some reports propose that more supply of NAD(P)H to hydrogenase improves H₂ production (Yamamoto et al., 2012; Courmac et al., 2004). Due to the oxygen sensitivity of hydrogenase photosynthesis inhibits its activity, H₂ production is performed under the dark and anaerobic condition. Under the condition NAD(P)H is produced by catabolism of intracellular glucan and cell constituting materials accumulated during photosynthetic growth. Supply of NAD(P)H is a key factor to improve H₂ production. D-glucose addition can also provide NAD(P)H source to cells even under the dark condition because Synechocystis sp. strain PCC 6803 has characteristic in that it is capable to consume D-glucose (Smith 1983). Catabolism of D-glucose to CO₂, organic acids or other metabolites donates electrons to NAD(P)⁺ to form NAD(P)H and improves H₂ production.

In the present report two genetic mutation strains of Synechocystis sp. strain PCC 6803 (hereafter PCC strain) were introduced to improve H₂ production. There are some advantages of using Synechocystis sp. strain PCC 6803 as a model microorganism, for instance genome DNA sequence was completely annotated on this strain (Kaneko et al. 1996) allowing us to construct mutant. Glucose tolerant strain of Synechocystis sp. strain PCC 6803 (hereafter GT strain) has been developed to grow on heterotrophic (Williams 1998). It seems that this strain has a high glucokinase activity which is key enzyme to utilize D-glucose (Kahlon et al. 2006). L-lactate dehydrogenase knock out mutant (hereafter Δldh strain) was constructed from GT strain. L-lactic acid is produced as one of the final products from D-glucose under the dark and anaerobic condition. The reaction from pyruvate to L-lactic acid which catalyzed by L-lactate dehydrogenase competes NADH consumption with hydrogenase, which results in decreasing of H₂ yield from D-glucose. Similar research has been studied by D-lactate dehydrogenase knock out mutant of Synechococcus sp. strain PCC 7002 (McNeely et al. 2010). This redirection of metabolic pathway succeeded to increase H₂ production by 5 folds.

Methods

Construction of Δldh mutant

The deletion of L-lactate dehydrogenase gene (sfr 1556) was constructed by a PCR amplification of an ldh gene fragment using primer ldh forward (5'-GCCTATGATCGTCAATTTTTCC-3') and ldh reverse (5'-TTCAAGAATATTGGCCAGTGC-3') designed by us from the information of the chromosomal DNA of wild-type
Synecochyssis sp. strain PCC 6803 (Kaneko et al. 1996). PCR-amplified ldh fragment was ligated into Smal restriction site of plasmid pUC19. The kanamycin cassette from plasmid pUC4K was inserted into the BglII restriction site at ldh gene fragment. Transformant colony was selected from BG-11 agar plate containing kanamycin and continuously re-streaked to new agar plates. Kanamycin resistant cell were grown at increasing concentrations to 120 µg mL⁻¹ and finally transferred into liquid BG-11 medium. The full segregation was confirmed by PCR (Figure S1) using the mentioned primer. Segregated cells maintained in 5 mL BG-11 medium containing 60 µg mL⁻¹ of kanamycin and 100 µL of culture transfer to flesh medium every two weeks.

Cell preparation

Synecochyssis 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 7.7) aqueous solution of BG-11 medium. The aqueous solution was aerated at 80 mL min⁻¹ by the air containing 6% CO₂ that was filtered through 0.45 µm 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 µmol m⁻² s⁻¹. After the 24 h cultivation cells were harvested by centrifugation at 25 °C and 3000 rpm for 10 min. The cell pellets were washed with HEPES buffer solutions and centrifuged again.

H₂ production

After the above cell preparation, the settled cells were suspended in de-ionized water containing 50 mmol L⁻¹ HEPES buffer (pH 7.7) in a test tube. Initial cell mass concentration for H₂ production was adjusted to 2.0 g L⁻¹. Total volume of cell suspension was adjusted to 10 mL. Finally cell suspensions in the test tubes were sparged with N₂ gas for a few minutes to remove O₂ gas and sealed with butyl rubber caps. Test tubes were placed into a water bath at 34 °C under a dark condition and agitated at 120 rpm.

Measurement of H₂ production, cell mass concentration, D-glucose concentration in cell suspension and hydrogenase activity

At the measurement time, 300 µL of gas sample was withdrawn from the test tube with a gasket syringe and 400 µL of cell suspension was also withdrawn from the test tube with a syringe. After that, the test tube was sparged with N₂ gas again.

An amount of H₂ 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 H₂ production per unit suspension volume of a run (ycy) was calculated from a result.

Cell mass concentration in terms of dry cell weight (DCW) per unit suspension volume was monitored by measuring an optical density at 730 nm (OD730) utilizing a spectrophotometer. One unit absorbance of cell suspension was equivalent to 0.369 g-DCW L⁻¹.

The amount of D-glucose contained in a reaction mixture (cy) was determined with a glucose tester (Wako Pure Chemicals, Japan) as mentioned elsewhere (Yamamoto, 2012).

The activity of hydrogenase in intact cells of Synecochyssis sp. strain PCC 6803 was assayed under methyl viologen by the modified method of Gutthann et al. (2007). First, 4 mg of cells harvested from photobioreactor were suspended in a 2 mL of de-ionized water containing 50 mmol L⁻¹ HEPES, 5 mmol L⁻¹ methyl viologen and 10 mmol L⁻¹ sodium hydrosulfite in 15 mL glass tubes. Then the cell suspensions in the test tubes were sparged with N₂ gas for a few minutes to remove dissolved and gaseous O₂ and sealed with butyl rubber caps. The test tubes were placed into a water bath at 34 °C under a dark condition and agitated at 120 rpm. Produced H₂ in the gas phase of the test tubes were measured three times every 30 min and hydrogenase activity was calculated. Reproducibility was confirmed by 2 – 4 runs for each result.

Results and discussion

Growth

PCC, GT and Aldh strain were autotrophically grown in BG-11 medium. No differences of specific growth rate in growth phase and final cell mass concentration in stationary phase of each strain were observed (data not shown). During cell preparation each strain did not secrete metabolites with detectable level indicating that L-lactate dehydrogenase did not work on growth phase, and Aldh strain has a growth characteristic equal to wild type.

Hydrogenase activity

Specific hydrogenase activities of each strain were measured at the beginning of H₂ production phase. Their values were around 4.8 U g⁻¹. Hydrogenase seems not to be affected by knock out of L-lactate dehydrogenase gene.

H₂ production

Figure 1 shows H₂ production by each strain in a HEPES buffer solution or buffer solutions containing 5.6, 28 or 56 mmol L⁻¹ of D-glucose at 96 h. Cells completely consumed extracellular D-glucose in 48 h in the runs with each strain. On the other hands the greater part of D-glucose remained in cell suspension at 96 h in the runs with 28 or 56 mmol L⁻¹ of D-glucose. D-glucose improved H₂ production for each strain. Optimum D-glucose concentrations were 5.6 or 28 mmol L⁻¹ of D-glucose. Excess D-glucose concentration inhibited H₂ production. It seems that cells may use energy to drive sugar out of cells at high D-glucose concentration (Baebprasert et al., 2010). Comparing with PCC strain, GT strain supplemented with D-glucose produced more H₂. The amount of H₂ production in the runs with 5.6, 28 or 56 mmol L⁻¹ of D-glucose increased 13, 15 and 30%, respectively. On the other hands the results by Aldh strain not only with D-glucose but without D-glucose showed increasing of H₂ production.

Figure 1: Hydrogen production per unit volume of cell suspension by PCC, GT and Aldh strain in buffer solutions containing 0, 5.6, 28 and 56 mmol L⁻¹ of D-glucose at 96 h. Especially initial H₂ production rates of Aldh strain were improved in the each run (Figure 2). This implies the mutant cells contained
more reducing materials for H₂ production than the other two strains at the beginning of H₂ production phase because less D-glucose was catabolized at that time.

*L-lactic acid production*

Figure 3 shows L-lactic acid production at 96 h. Before 72 h, *Aldh* strain did not produce L-lactic acid as detectable level (data not shown), which ensures that metabolic modification was successfully performed. Decreasing of L-lactic acid production seems to contribute to the increase of H₂ production by *Aldh* strain because more NADH would be redistributed to hydrogenase. Little amount of L-lactic acid was observed in the run with *Aldh* strain at 96 h. This might be because some mutant cells might turn back to wild type during cell preparation and H₂ production phase without kanamycin. Comparing with PCC and *Aldh* strain, H₂ production did not reach to the level, which is estimated that inability of 1 mol L-lactic acid production theoretically results in the raise of 1 mol H₂. It seems that other NADH consumption pathway also competed with H₂ production.

*D-glucose uptake rate*

The improvement of H₂ production by GT strain resulted from increasing of D-glucose uptake rate. Figure 4 showed D-glucose uptake rate during 0-24 h in each run. Comparing with PCC strain, modification affects D-glucose utilization characteristic. L-lactate dehydrogenase mainly works in cytoplasmic matrix; however, hydrogenase exists on thylakoid membrane (Appel et al., 2000). It is deduced that NADHs produced through glycolysis in cytoplasmic matrix of *Aldh* strain were locally accumulated in and inhibited D-glucose utilization.

**H₂ yield from D-glucose**

H₂ yield from extracellular D-glucose consumption (Y_{H2/G}) is an important factor to consider a bioprocess. Extracellular D-glucose is utilized for producing cell constituents, intracellular glucan and secreted metabolites, then these processes produce or consume NAD(P)H. Knock out of L-lactate dehydrogenase in *Aldh* strain improved Y_{H2/G} as expected (Figure 5). It implies that more NADH derived from D-glucose consumed to produce H₂ in *Aldh* strain comparing with the other strains. Metabolic modification successfully increased the efficiency of D-glucose utilization. In the run with 5.6 mmol L⁻¹ of D-glucose, GT strain showed the highest yield among three strains. This is because H₂ produced after depletion of D-glucose was reflected on the data.

**Conclusion**

D-glucose addition to a buffer solution in H₂ production phase improved H₂ production due to more supply of NAD(P)H to hydrogenase. D-glucose uptake rate and H₂ yield from D-glucose are important factors for H₂ production in extracellular D-glucose supply condition. In the present report, GT strain showed high D-glucose uptake rate. This contributed to the increase in H₂ production. On the other hand, *Aldh* strain was successfully constructed from GT strain and showed higher Y_{H2/G} values than
other strains. Furthermore initial H₂ production rate was also increased. H₂ production by Δldh strain did not reach to the upper-level estimated stoichiometrically as 1 mol increase in H₂ with 1 mol decrease in L-lactic acid in Δldh strain, implying that there is a room to improve H₂ production by further metabolic modification.

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References


