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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 H_2 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 H_2 production, and the two mutants, glucose tolerant strain and its mutant lacking L-lactate dehydrogenase (LDH) gene, were introduced to improve H_2 production with D-glucose. Higher D-glucose uptake rate of glucose tolerant strain resulted in higher H_2 production rate comparing with the wild type strain. The LDH mutant showed higher initial H_2 production rate and H_2 yield from D-glucose than the other strains because the NADH which should be consumed originally by LDH can be redistributed to the H_2 production reaction.

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

Introduction

Molecular hydrogen (H_2) is a potential source of a non-polluting fuel. Cyanobacterial H_2 production is one of the candidates of sustainable H_2 production method. The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 is a favored microorganism for the molecular biotechnological studies on the cyanobacterial H_2 production. The bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 works as an enzyme forming H_2 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 H_2 production (Yamamoto et al., 2012; Cournac et al., 2004). Due to the oxygen sensitivity of hydrogenase photosynthesis inhibits its activity, H_2 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 H_2 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 CO_2, organic acids or other metabolites donates electrons to $NAD(P)^+$ to form NAD(P)H and improves H_2 production.

In the present report two genetic mutation strains of *Synechocystis* sp. strain PCC 6803 (hereafter PCC strain) were introduced to improve H_2 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 H_2 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 H_2 production by 5 folds.

Methods

Construction of Δldh mutant

The deletion of L-lactate dehydrogenase gene (slr 1556) was constructed by a PCR amplification of an *ldh* gene fragment using primer *ldh* forward (5'-GCCTATGATCGTCAATTTTTCC-3') and *ldh* reverse (5'-TTCAGCAATATTTGCCAGTGTC-3') designed by us from the information of the chromosomal DNA of wild-type

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Synechocystis sp. strain PCC 6803 (Kaneko *et al*. 1996). PCR-amplified *ldh* fragment was ligated into *Sma*I restriction site of plasmid pUC19. The kanamycin cassette from plasmid pUC4K was inserted into the *Bgl*II 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

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 7.7) aqueous solution of BG-11 medium. The aqueous solution was aerated at 80 mL min^{-1} by the air containing 6% CO_2 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.

H_2 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 H_2 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 N_2 gas for a few minutes to remove O_2 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 H_2 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 N_2 gas again.

An amount of H_2 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_2 production per unit suspension volume of a run (y_{H2}) 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 (OD_{730}) 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 (c_G) was determined with a glucose tester (Wako Pure Chemicals, Japan) as mentioned elsewhere (Yamamoto, 2012).

The activity of hydrogenase in intact cells of *Synechocystis* 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 N_2 gas for a few minutes to remove dissolved and gaseous O_2 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 H_2 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 *Δldh* 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 *Δldh* strain has a growth characteristic equal to wild type.

Hydrogenase activity

Specific hydrogenase activities of each strain were measured at the beginning of H_2 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.

H_2 production

Figure 1 shows H_2 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 of each strain with 5.6 mmol L^{-1} of D-glucose. 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 H_2 production for each strain. Optimum D-glucose concentrations were 5.6 or 28 mmol L^{-1} of D-glucose. Excess D-glucose concentration inhibited H_2 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 H_2. The amount of H_2 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 *Δldh* strain not only with D-glucose but without D-glucose showed increasing of H_2 production.

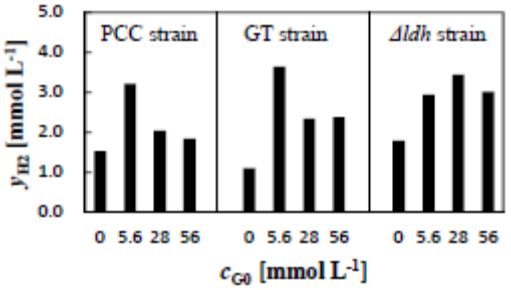

Figure 1: Hydrogen production per unit volume of cell suspension by PCC, GT and *Δldh* strain in buffer solutions containing 0, 5.6, 28 and 56 mmol L^{-1} of D-glucose at 96 h.

Especially initial H_2 production rates of *Δldh* strain were improved in the each run (**Figure 2**). This implies the mutant cells contained

more reducing materials for H_2 production than the other two strains at the beginning of H_2 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, *Δldh* 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 H_2 production by *Δldh* strain because more NADH would be redistributed to hydrogenase. Little amount of L-lactic acid was observed in the run with *Δldh* strain at 96 h. This might be because some mutant cells might turn back to wild type during cell preparation and H_2 production phase without kanamycin. Comparing with PCC and *Δldh* strain, H_2 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 H_2. It seems that other NADH consumption pathway also competed with H_2 production.

D-glucose uptake rate

The improvement of H_2 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,

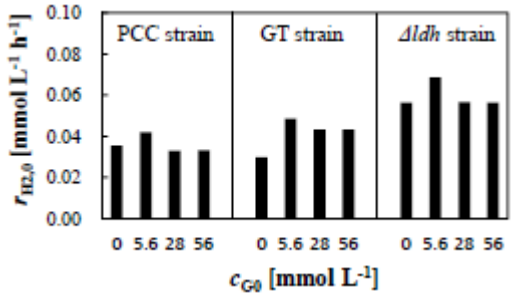

Figure 2: Initial hydrogen production rate per unit volume of cell suspension by PCC, GT and *Δldh* strain in buffer solutions containing 0, 5.6, 28 and 56 mmol L^{-1} of D-glucose.

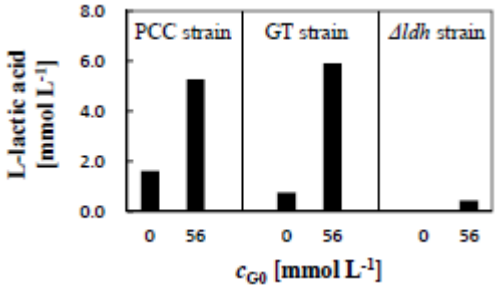

Figure 3: Concentration of L-lactic acid produced by PCC, GT and *Δldh* strain in buffer solutions containing 0 or 56 mmol L^{-1} of D-glucose at 96 h.

GT strain showed high D-glucose uptake rate. It is deduced that GT strain has high glucokinase activity and can utilize more D-glucose than PCC strain. On the other hand *Δldh* strain showed lower D-glucose uptake rate than PCC strain although this mutant was derived from GT strain. This might be because metabolic modification affects D-glucose utilization characteristic. L-lactate dehydrogenase mainly works in cytoplasmic matrix; however,

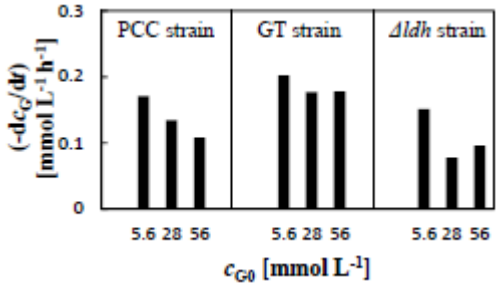

Figure 4: Uptake rate of extracellular D-glucose per unit volume of cell suspension by PCC, GT and *Δldh* strain in buffer solutions containing 5.6, 28 and 56 mmol L^{-1} of D-glucose. The uptake rates were calculated using data at 0 and 24 h for c_{G0} = 5.6 mmol L^{-1}, and at 0 and 96 h both for c_{G0} = 28 and 56 mmol L^{-1}.

hydrogenase exists on thylakoid membrane (Appel *et al.*, 2000). It is deduced that NADHs produced through glycolysis in cytoplasmic matrix of *Δldh* strain were locally accumulated in and inhibited D-glucose utilization.

H_2 yield from D-glucose

H_2 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 *Δldh* strain improved $Y_{H2/G}$ as expected (**Figure 5**). It implies that more NADH derived from D-glucose consumed to produce H_2 in *Δldh* 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 H_2 produced after depletion of D-glucose was reflected on the data.

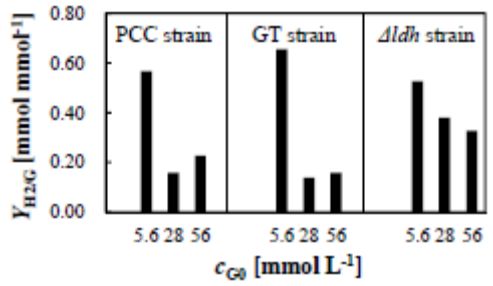

Figure 5: H_2 yields, calculated from extracellular D-glucose consumption during 0 and 96 h, of PCC, GT and *Δldh* strain in buffer solutions containing 5.6, 28 and 56 mmol L^{-1} of D-glucose.

Conclusion

D-glucose addition to a buffer solution in H_2 production phase improved H_2 production due to more supply of NAD(P)H to hydrogenase. D-glucose uptake rate and H_2 yield from D-glucose are important factors for H_2 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 H_2 production. On the other hand, *Δldh* strain was successfully constructed from GT strain and showed higher $Y_{H2/G}$ values than

other strains. Furthermore initial H_2 production rate was also increased. H_2 production by *Δldh* strain did not reach to the upper-level estimated stoichiometrically as 1 mol increase in H_2 with 1 mol decrease in L-lactic acid in *Δldh* strain, implying that there is a room to improve H_2 production by further metabolic modification.

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