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Hydrogen production by anaerobic dark metabolism in *Synechocystis* sp. strain PCC6803-GT: effect of monosaccharide in nitrate free solution

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Abstract

The hydrogen production in the cyanobacterium *Synechocystis* sp. strain PCC6803-GT in the dark anaerobic nitrate free buffer solution with or without monosaccharide has been studied by following the time courses of the amounts per culture volume of hydrogen, exogenous monosaccharide, endogenous glucose in glycogen, dry cell weight, acetate, lactate and succinate. The monosaccharides utilized were reducing sugars, which were glucose, fructose, galactose, mannose and xylose. The rate of hydrogen production was highest right after dark incubation, and decreased exponentially with time. High rate hydrogen producing cells always contained high amount of endogenous glucose. The metabolic product analysis resulted that the bulk of reductants for Ni-Fe hydrogenase was served by global metabolism that was accompanied by glycolysis. The amount of endogenous glucose was elevated by addition of exogenous glucose. Cell survival to stabilize the high level of endogenous glucose against dark nitrate free environment was supported by addition of fructose.

Keywords: *Synechocystis* sp. strain PCC6803, hydrogen, NiFe-hydrogenase, monosaccharide, cyanobacteria, dark metabolism.

Introduction

The cyanobacteria containing a bidirectional NiFe-hydroxgenase (Hox) are capable of producing molecular hydrogen in the dark (Hallenbeck *et al*. 1981). This enzyme acts as a terminal electron sink with a redox partner NADH or NADPH in anaerobic conditions. Such dark hydrogen production is best studied in a unicellular transformable wild type cyanobacterium *Synechocystis* sp. strain PCC6803 (here after WT strain), which is the first full genome sequenced phototrophic organism (Kaneko *et al*. 1996). This enzyme is soluble and its hydrogen production increases 1.92-fold in gassing culture with hydrogen (Howarth and Codd 1985). This result, on gross inspection, conflicts with a law of mass action.

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The possibility exists that reducing potential of hydrogen activates NiFe-hydrogenase (Chiou and Liaw 2008). Shift of cellular state to reductive state appears to be effective for elevating hydrogen production. The dark hydrogen production in WT strain is increased if cells are prepared by 24 h adaptation to sugars such as 22.2 $\mu mol \cdot mL^{-1}$ glucose, 2.92 $\mu mol \cdot mL^{-1}$ sucrose or 5.56 $\mu mol \cdot mL^{-1}$ fructose (Baebprasert *et al*. 2010). Among them glucose and fructose are reducing sugars having an open-chain form with an aldehyde group or a free hemiacetal group. In the presence of glucose, concentration of NAD(P)H increases significantly in the dark adapted cells of WT strain (Lee *et al.* 2007). Effect of glucose on mixotrophic growth of WT strain under the light has been variously reported. One work shows that glucose inhibits the growth in the absence of herbicide DCMU ((3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Flores and Schmetterer 1986). The other work shows that glucose stimulates the growth in the absence of DCMU (Yoo *et al*. 2007). Dark glucose-supplemented culture of this strain shows a small increase in biomass concentration (Anderson and McIntosh 1991).

A glucose-tolerant mutant *Synechocystis* sp. strain PCC6803-GT (hereafter GT strain) was isolated from the culture of WT strains (Williams 1988). The GT strain is capable of growing mixotrophically under the light and heterotrophically in the dark with a daily short pulse of dim light (Anderson 1991). The dark hydrogen production in this strain in nitrate-free solution is associated either with breakdown of endogenous glycogen that is a highly multi-branched homopolysaccharide of α-(1,4)-linked glucose residues with α-(1,6) branch linkage, with decomposition of cells (Yamamoto *et al*. 2012b), or with reduction of exogenous glucose (Yamamoto *et al*. 2012b; Cournac *et al*. 2004).

Fructose is an isomer of glucose and bactericidal for WT strains (Flores and Schmetterer 1986). Growth of this strain in glucose aqueous solution under the light is inhibited by 10 $\mu mol \cdot mL^{-1}$ fructose (Joset *et al*. 1988; Flores and Schmetterer 1986). Fructose enters the cell via the fructose-glucose transporter (*glcP*) and the *glcP* deficient mutant acquires the resistance to fructose (Zhang *et al*. 1989; Kahlon *et al*. 2006).

Little is known about metabolic product distribution in the dark anaerobic mixotrophic culture of GT strain, while

fermentation products including carbon dioxide, lactic acid, acetate and ethanol from endogenous glycogen and exogenous glucose in cyanobacteria *Synechocystis* sp, strain PCC6714, *Synechocystis* sp, strain PCC6308 and *Synechococcus* sp. strain PCC6301 are observed in the classic work (Stal and Moezelaar 1997). Production of acetate and succinate suggests that cyanobacteria convert pyruvate to acetyl-CoA through pyruvate:ferredoxin oxidoreductase (PFOR). This enzyme simultaneously converts oxidized ferredoxin to reduced ferredoxin. Although cyanobacteria lack FeFe-hydrogenase that receives electrons from ferredoxins (Ducat *et al*. 2011), NiFe-hydrogenase receives excess electrons from reduced ferredoxins and produces hydrogen (Gutekunst *et al*. 2014). If NiFe-hydrogenase in *Synechocystis* sp. strain PCC6803 works as an electron sink for NAD(P)H or reduced ferredoxin/flavodoxin, and if exogenous reducing sugar provides reductive environment to the cell, the possibility exists that hydrogen production is enhanced by reducing sugar supplementation under nitrate limiting condition.

Thus, the primary purposes of this study were (1) to observe the effect of exogenous reducing sugars such as glucose, fructose, galactose, mannose and xylose upon the hydrogen production in *Synechocystis* sp. strain PCC6803-GT in the dark anaerobic nitrate free solution, and (2) to elucidate the association of the decomposition of endogenous glucose in glycogen and the evolution of metabolic product with fermentative hydrogen production. So far as is known present, this report is the first to present a view of dark anaerobic life mode of *Synechocystis* sp. PCC6803-GT on monosaccharides.

Materials and methods

Strain, Cell preparation and Hydrogen production

Synechocystis sp. strain PCC6803-GT (supplied by Professor Y.Hihara, Department of Biochemistry and Molecular Biology, Saitama University, Japan) was utilized in this study. For cell preparation, cells were grown photoautotrophically for 3 d in BG-11 medium (initial pH 7.8) at 34 ºC, aerated by 6 % CO_2, illuminated by fluorescent lamps at 100 μmol-photons m^{-2} s^{-1} PPFD (photosynthetic photon flux density). Specific growth rate was 1.85 h^{-1}. Cells of late-logarithmic growth phase culture (OD_{730}, 7) were collected by centrifugation at 3000 rpm and 25 ºC for 10 min. The cell pellets were washed by 50 μmol mL^{-1} HEPES buffer (pH 7.8) and collected by centrifugation. Cells were re-suspended and incubated with or without 28 μmol mL^{-1} glucose, fructose, galactose, mannose or xylose in 10 mL of 50 μmol mL^{-1} HEPES buffer (pH 7.8) in 32 mL glass test tube with butyl rubber cap. The initial cell mass concentration was set at 2.13±0.06 mg mL^{-1} and cell suspension was purged with N_2 gas for a few minutes to remove oxygen molecules. Incubation was carried out under dark anaerobic conditions with shaking at 145 rpm in a reciprocating shaker. The reciprocating distance was 40 mm and the horizontal angle was about 30 ºC. All experiments were carried out in duplicate.

Measurement of hydrogen production

The production of molecular hydrogen in the gas phase in 32 mL test tube was measured utilizing a gas chromatograph equipped with a molecular sieve column and a thermal conductivity detector (GC-320, GL science Inc.; column, Molecular sieve 13X; column temperature, 37 ºC; injector temperature, 45 ºC; detector temperature 80 ºC) with nitrogen gas as the carrier gas.

Determination of metabolic product distribution

The distribution of metabolites in supernatant of the dark anaerobic culture of GT-strain in glucose was analyzed by a liquid chromatography-electrospray ionization-time-of-flight-mass spectrometry (LC-ESI-TOF-MS) (micrOTOF Ⅱ ,Bruker Daltonics Co.) utilizing 1 % formic acid as mobile phase. LC column of the system was XBridge C18 HPLC Column 3×150 mm 3.5 μm (Waters Co.).

Measurement of reducing sugar consumption and metabolic product production

Residual reducing sugars in cell suspensions were analyzed by a high performance liquid chromatography (HPLC) (Shimadzu LC-10AD and RID-6A). Column equipped was SZ5532, ϕ6.0 × 150 mm (Showa Denko Co.). Column temperature was set at 50 °C. Mobile phase was 70 % acetonitrile. Mobile phase flow rate was 0.7 mL min^{-1}. A 10 μL of supernatant sample was injected by using a 10 μL syringe.

Metabolites in a 500 μL portion of supernatants were analyzed by HPLC (JASCO PU-2080 Plus and UV-2075 Plus). Mobile phase was 18 μmol mL^{-1} KH_2PO_4, pH of which was adjusted at 2.3 with H_3PO_4. Flow rate of mobile phase was 0.7 mL min^{-1}. Column temperature was 30 °C. A 10 μL of supernatant sample was injected by using 10 μL syringes. The wave length of 210 nm was used for detection of metabolites.

Measurement of endogenous glycogen consumption and production

The amount of glycogen per culture volume was determined as follows: the amount of dry cell weight per culture volume was determined according to previous report (Yamamoto *et al*. 2012a); cell pellets were collected by centrifugation at 25 ºC, 3000 rpm for 10 min then washed 3 times by deionized water to eliminate extracellular carbon sources; intracellular glycogen was extracted and decomposed to glucose by boiling in 50 μL of 6 N HCl at 80 ºC for 30 min; glucose amount was determined by enzymatic method (CII Test, Wako Pure Chemical Ind., Ltd.).

Results

Gas Chromatography analysis showed a single peak of hydrogen (Fig. 1a). Absence of oxygen was also confirmed. The LC-ESI-TOF-MS and HPLC analysis of fermentative buffer solution of the run with glucose confirmed that GT strain extracellularly produces acetate, lactate, succinate, fumarate, malate and ethanol. Main metabolic products of the dark incubated culture of the runs with or without monosaccharide other than glucose were identified to be hydrogen, acetate, lactate and succinate (Fig. 1b). In kinetic studies, amounts per culture volume of hydrogen, exogenous monosaccharide, endogenous glucose in glycogen, dry cell weight, lactate, acetate and succinate were monitored.

Figure 2 shows the time courses of culture variables measured in runs without reducing sugars or with either glucose, (a) the mole of hydrogen per culture volume (y_{H2}), (b) the concentration of exogenous reducing sugars, (c) the mole of endogenous glucose in glycogen per culture volume (c_G), (d) the dry cell weight of GT strain per culture volume (X), (e) pH

of cell suspension, (f) the concentration of acetate, (g) the concentration of lactate and (h) the concentration of succinate.

The time courses of the mole of hydrogen per culture volume show that the production of hydrogen starts right after inoculation (Fig.2a). The hydrogen production rate is highest at initial and then clearly decelerated with time. Hydrogenase, which is synthesized but blocked for hydrogen production by oxygen during photosynthesis, is found to be active when cells are inoculated into oxygen-free dark environment. The time courses of the concentration of exogenous

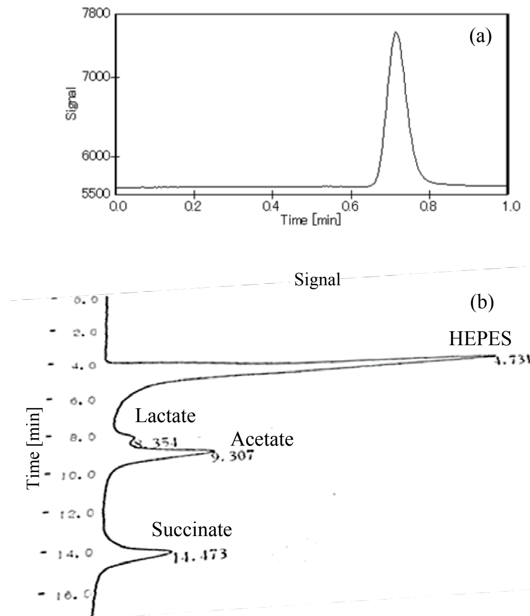

Figure 1: Peaks of metabolic products in dark anaerobic incubation of *Synechocystis* sp. strain PCC6803-GT. (a) Single peak represents hydrogen detected from Gas Chromatography analysis, GC-320, GL science Inc.; column, Molecular sieve 13X; column temperature, 37 ºC; injector temperature, 45 ºC; detector temperature 80 ºC) with nitrogen gas as the carrier gas. (b) Four peaks are HEPES, lactate, acetate and succinate detected from HPLC analysis (JASCO PU-2080 Plus and UV-2075 Plus). Mobile phase was 18 mmol L^{-1} KH_2PO_4, pH of which was adjusted at 2.3 with H_3PO_4. Flow rate of mobile phase was 0.7 mL min^{-1}. Column temperature was 30 °C.

reducing sugars show that, during the first 48 h, cells of *Synechocystis* sp. strain PCC6803-GT are unable to assimilate exogenous reducing sugars other than glucose (Fig.2b).

Despite the fact that glucose and fructose share common transport system in *Synechocystis* sp. strain PCC6803 (Zhang *et al*. 1989), cells are unable to assimilate exogenous fructose for cell division under the dark anaerobic condition at least during first 48 h. The hydrogen production that is most active right after inoculation is found to have no significant relation with exogenous reducing sugars. The run with glucose shows that, in the first 24 h, GT strain assimilates 5.91 µmol mL^{-1} exogenous glucose. The elevated production of hydrogen in the first 48 h of the run with fructose is independent from fructose uptake. Although this supply of reductive compounds from global metabolism is not identified, it appears to be accompanied by glycolysis.

Except for the run with glucose, early hydrogen production is nearly always accompanied by glycogen degradation (Fig. 2a and c). In all experiments, the initial amount of endogenous glycogen per culture volume is fixed at 0.2±0.04 µmol mL^{-1}. About 0.20 µmol mL^{-1} increase in endogenous glucose in glycogen is observed in the first 24 h of the run with glucose. The fractional yield of endogenous glucose from exogenous glucose in this period is 0.033. The decrease in endogenous glucose in glycogen of the cell in the run with glucose is also seen after 24 h. Curves for the amount of glycogen per culture volume shows that glycogen assimilation is inactivated at 48 h.

The cell mass concentration of the run with fructose is stable (Fig.2d). This is quite different from the observation in a previous report (Flores and Schmetterer 1986) which concerns the photoheterotrophic incubation of WT strain on BG-11 medium with 20 µmol·mL^{-1} fructose to evaluate the fructose toxicity that is the degree to which fructose exerts damage to WT strain. The 0.1% survival after 2 d was reported. We find that the dark anaerobic incubation of GT strain in nitrate free solution with fructose supports cell survival. The cell mass concentration of the run with glucose increases during the first 72 h with the specific growth rate of 0.0038 h^{-1}. The doubling time of this run is 182 h that is 0.4 times that observed on 5 µmol·mL^{-1} glucose in the previous work (Anderson and McIntosh 1991). The assimilated glucose appears to be utilized for glycogen synthesis (Fig.2c), cell growth (Fig.2d) and acid production (Fig.2e, f, g). Galactose and mannose also support to keep cell viability in the first 48 h, while cannot support cell survival after 48 h. Cell mass concentration decreases with time in the run without monosaccharide and in the run with xylose.

The pH of the culture in the runs with reducing sugar other than glucose slightly decreases with time from 7.7 to 7.5 during 96 h. Remarkable drop in pH from 7.7 to 6.8 during 96 h is seen in the run with glucose. Major metabolic products other than hydrogen and carbon dioxide are acetate (Fig.2f), lactate (Fig.2g), succinate (Fig.2h). If the amount of total acid is defined by the summation of amounts of acetate, lactate and succinate, and if the amount of total glucose is defined by the summation of amounts of exogenous glucose and endogenous glucose, then hydrogen production, total acid production, total glucose consumption and cell mass production in the first 48 h of the run with glucose are 2.29, 17.7, 7.98 µmol·mL^{-1} and 0.39 mg·mL^{-1}, respectively. If the cell formula of *Synechocystis* sp. strain PCC6803 is represented by $CH_{1.62}N_{0.22}O_{0.40}P_{0.11}$ (Yu et al. 2013), above cell growth is estimated to consume 2.81 (=(0.39)(10^3)/{(23.1)(180)}) µmol·mL^{-1} exogenous glucose. Amount of total glucose converted to other metabolic products is only 5.17 (=7.98-2.81) µmol·mL^{-1}. Complete theoretical conversion of glucose to these acids through only EMP pathway estimates the production of 10.2 µmol·mL^{-1} acids that is 57.6 % of observed acid concentration. Hence it turns out that some portion of carbon in total acid is supplied from metabolisms other than glycolysis.

Discussion

The y_{H2} versus time data of Fig.2a appear to be fitted by the equation:

$$y_{H2} = y_{H2,f}\{1-\exp(-kt)\} \tag{1}$$

in which $y_{H2,f}$ is the attainable level of y_{H2} and k is the deactivation constant of hydrogenase. In this study, subscript 0 and f show the initial state and the final state, respectively. From Eq.(1), the initial rate of hydrogen production $(dy_{H2}/dt)_0$ is calculated by $ky_{H2,f}$. Background of Eq.(1) is shown by

$$\frac{dy_{H2}}{dt} = r_{H2,0}\exp(-kt) \tag{2}$$

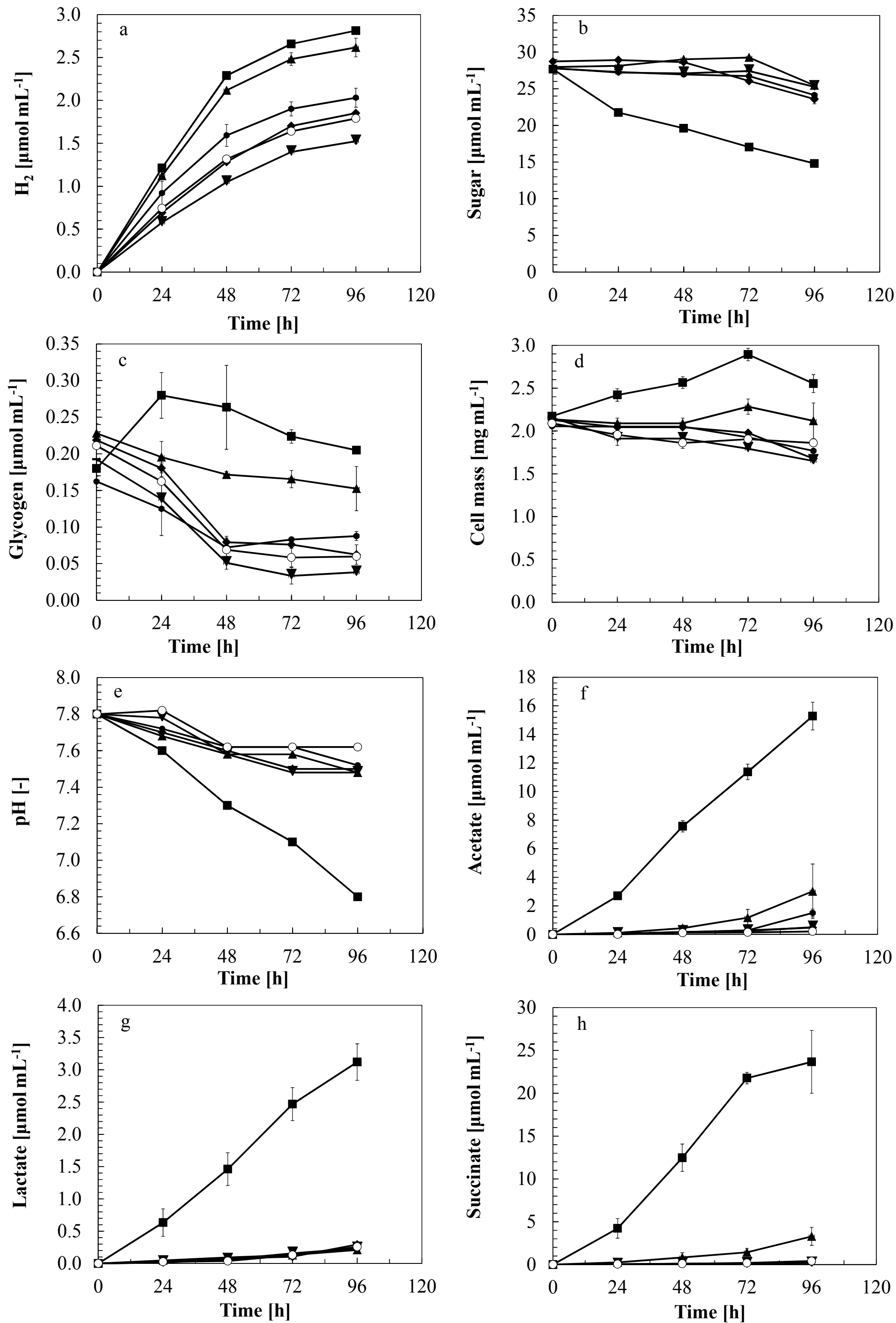

Figure 2: Time courses of culture parameters of *Synechocystis* sp. strain PCC6803-GT under dark anaerobic nitrate free solution without (○) or with glucose (■), fructose (▲), galactose (●), mannose (◆) and xylose (▼). Culture parameters observed are (a) the moles of hydrogen per culture volume, (b) the concentration of exogenous sugar, (c) the moles of endogenous glucose in glycogen per culture volume, (d) the dry cell weight per culture volume (e) pH of buffer solution, (f) the concentration of acetate, (g) the concentration of lactate and (h) the concentration of succinate. All experiments are carried out in duplicate.

and

$$y_{H2,f} = \frac{r_{H2,0}}{k} \qquad (3)$$

Eq.(2) accords with our observation where hydrogen production rate is highest at the beginning of reaction then the rate decreases exponentially with time. Table 1 lists the values for $(dy_{H2}/dt)_0$, $y_{H2,f}$ and k that are obtained by fitting the data to Eq.(1). Figure 3a compares the observed y_{H2} to the estimated y_{H2}. The R^2 in estimating by Eq.(1) is 0.99 for 24 sets of data. Although the state of inocula are kept as constant as possible, the initial rate $(dy_{H2}/dt)_0$ is effected by variation in reducing sugars. If the initial rate $(dy_{H2}/dt)_0$ represents the reductive state of the inocula, reductive potential is found to be highest in glucose, secondary in fructose, thirdly in galactose, fourthly in mannose and finally in xylose. The reductive potential of cells in buffer solution without monosaccharide is lower than that in xylose.

Table 1: Kinetic parameters for the production of hydrogen in *Synechocystis* sp. strain PCC6803-GT in the runs with or without exogenous reducing sugar

	Initial hydrogen production rate ($(dy_{H2}/dt)_0$) [μmol mL^{-1} h^{-1}]	**Attainable level of amount of hydrogen per culture volume ($y_{H2,f}$) [μmol mL^{-1}]**	**Deactivation constant (k) [h^{-1}]**
w/o	0.0405	2.18	0.0186
Glucose	0.0730	3.26	0.0224
Fructose	0.0674	3.05	0.0221
Galactose	0.0527	2.33	0.0226
Mannose	0.0369	2.51	0.0147
Xylose	0.0305	2.06	0.0148

The attainable level of amount of hydrogen per culture volume ($y_{H2,f}$) is highest at 3.26 μmol mL^{-1} in the solution containing glucose, which is 1.50 times that without reducing sugar. Numerically, deactivation constants for hydrogen production (k) in buffer solutions with glucose, fructose and galactose are nearly the same. The $y_{H2,f}$ values are profoundly affected by the reducing sugar utilized. In an additional experiment, we observed that sucrose, non-reducing sugar, could not increase hydrogen production (data not shown).

These curves for c_G suggest a possibility of fitting by modified autocatalytic reaction kinetics shown by the equation:

$$c_G = \frac{2c_{G0} - c_{Gf} + c_{Gf}\exp\{2k_G(c_{G0} - c_{Gf})t\}}{1 + \exp\{2k_G(c_{G0} - c_{Gf})t\}} \qquad (4)$$

in which c_{G0} and c_{Gf} are initial and final values of c_G, and k_G is the rate constant. This relation is an integral form of a model:

$$-\frac{dc_G}{dt} = k_G(c_G - c_{Gf})(2c_{G0} - c_{Gf} - c_G) \qquad (5)$$

which is formulated with an assumption that derivative of endogenous glucose with respective to time is a hyperbolic function of c_G. Table 2 tabulates the values for c_{G0}, c_{Gf} and k_G. Figure 3b compares the observed c_G to the estimated c_G. The R^2 in estimating by Eq.(4) is 0.96 for 24 sets of data. The c_{Gf} of the runs with fructose and galactose are about 90 % of the c_G at 96 h. Although Eq.(4) cannot apply for the run with glucose, assuming this ratio to the run with glucose, the c_{Gf} of the runs with glucose is estimated as 0.185 (=(0.9)(0.205)) μmol mL^{-1}. The highest c_{Gf} hence highest $y_{H2,f}$ is seen in the run with glucose. The comparable highest c_{Gf} and $y_{H2,f}$ are seen in the run with fructose. It is confirmed that exogenous glucose support cell growth in the dark. This uptake is found to be useful to elevate the level of endogenous glucose in glycogen. Fructose is found to be useful to inhibit the decomposition of endogenous glucose in glycogen. Even after 48 h, hydrogen production possibly proceeds for additional 96 h. Hence endogenous glycogen decomposition cannot make up the bulk source of the reductive potential for hydrogen production. Of special interest is the positive relation between c_{Gf} and $y_{H2,f}$ (Fig. 4). This result is contrary to the expectation that high hydrogen

Table 2: Kinetic parameters for the decomposition of endogenous glucose in glycogen of *Synechocystis* sp. strain PCC6803-GT in the runs with or without exogenous reducing sugar

	Initial amount of endogenous glucose in glycogen (c_{G0}) [μmol mL^{-1}]	**Final level of amount of endogenous glucose in glycogen per culture volume (c_{Gf}) [μmol mL^{-1}]**	**Rate constant for endogenous glucose consumption (k_G) [μmol^{-} mL^{-1} h^{-1}]**
w/o	0.216	0.0444	0.116
Glucose	ND	ND	ND
Fructose	0.231	0.167	0.404
Galactose	0.162	0.0792	0.372
Mannose	0.227	0.0443	0.0927
Xylose	0.197	0.0201	0.111

Accuracy is 95.0 - 98.8%

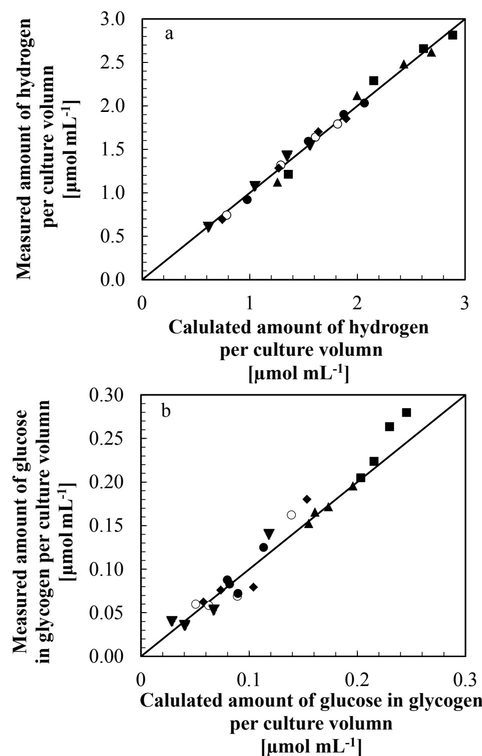

Figure 3: Comparison of the observed culture variables with the calculated culture variables. Culture variables plotted are (a) the amount of hydrogen per culture volume and (b) the amount of glucose in glycogen per culture volume. R^2 of calculated amount of hydrogen and calculated amount of glycogen are 0.99 and 0.96 respectively. Data are from cells suspended in HEPES buffer without (○) or 28 μmol mL^{-1} glucose (■), fructose (▲), galactose (●), mannose (◆) and xylose (▼).

production is resulted by high production of NAD(P)H due to high consumption of glucose. It is of prime importance to elevate the level of endogenous glycogen for high production of hydrogen. Control run shows the decrease in the amount of

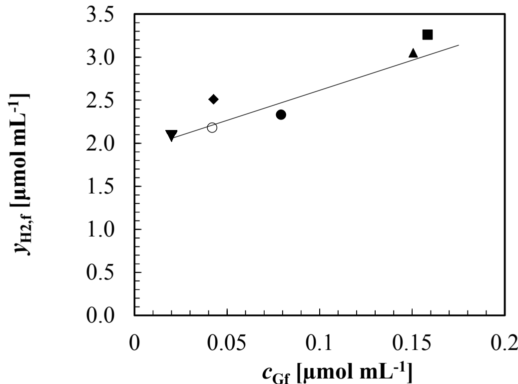

Figure 4: Correlation for the estimated c_{Gf} and the estimated $y_{H2,f}$. The estimations are made for the runs without (○) or with 28μmol mL^{-1} glucose (■), fructose (▲), galactose (●), mannose (◆) and xylose (▼).

glycogen per culture volume from 0.21 μmol mL^{-1} to 0.06 μmol mL^{-1} and the increase in hydrogen molecules per culture volume from 0 μmol mL^{-1} to 1.79 μmol mL^{-1} during 96 h. Production of 1 mole hydrogen is associated with the decomposition of 0.0833 moles glucose. The bulk of reducing compound for hydrogen production is found to be supplied by the pathway other than catabolism of endogenous glycogen.

Except for the run with glucose, the production of hydrogen is very active during the first 48 h, whereas the production of lactate, acetate and succinate are extremely low. Hence early hydrogen production is found to be catalyzed on NiFe-linked hydrogenase, utilizing not reductive flavodoxin/ferredoxin but NAD(P)H, the bulk of which is not provided by acid production pathways but by initial stock and by catabolism of intracellular storage compound including proteins, membrane lipids and poly-β-hydroxybutyrate (PHB). Acetate and succinate are observed after 48 h. The proportion of the total amount of acetate and succinate in total acid in the run with glucose at 96 h is 92.6%. Such large proportion of acid is produced through PFOR. The major source of electrons for NiFe-hydrogenase in this system is found to shift from NAD(P)H to reductive flavodoxin/ferredoxins. A previous study reports that the accumulation of acetate, which is produced via PFOR, leads to increase reductive flavodoxin/ferrodoxin to provide excess electrons for hydrogenase (Gutekunst *et al*. 2014).

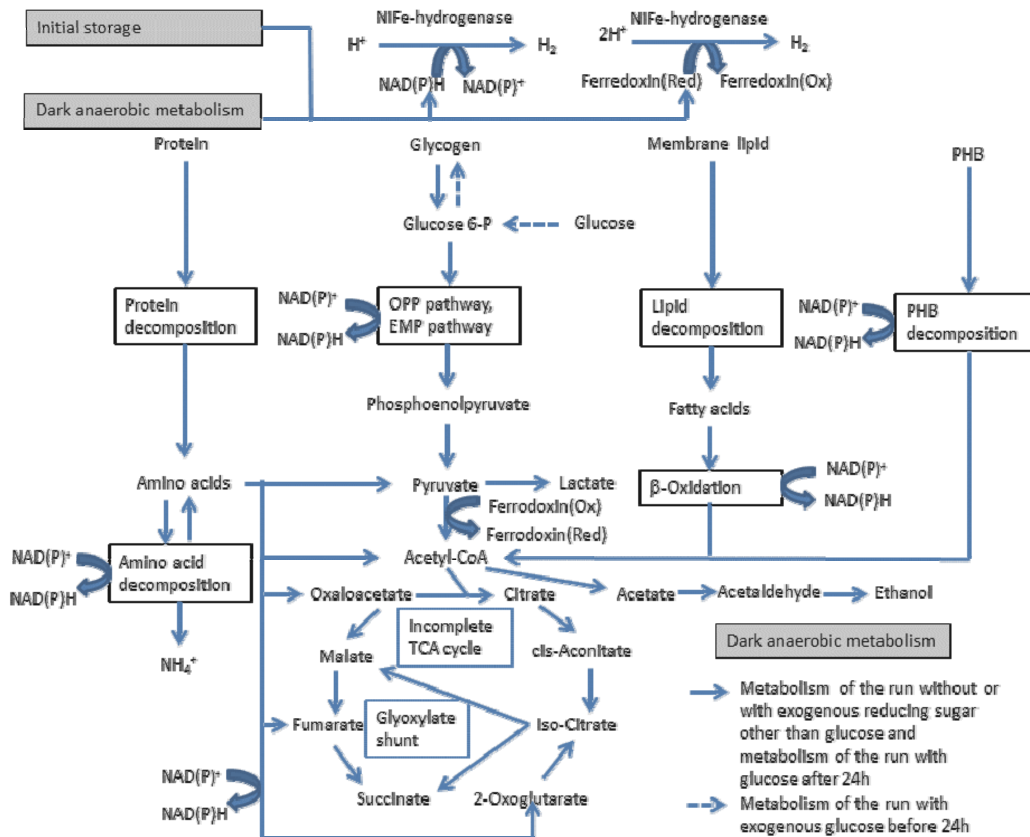

Figure 5: Proposed fermentative schemes for *Synechocystis* sp. strain PCC6803-GT under the dark anaerobic condition on HEPES buffer solution with or without exogenous glucose.

According to the previous work (Das and Veziro lu 2001), the increase in acetate production leads the increase in NAD(P)H production, followed by the increase in hydrogen production. This mechanism is different from our observation that hydrogen production is high at the beginning of dark incubation where acetate production is low, and that hydrogen production is inactivated when acetate production becomes active after 48 h. Addition to that our observation shows that hydrogen production is extremely high in the run with fructose, whereas acetate production is not so high.

Metabolic product distribution analysis shows that succinate is a major end-product of glucose fermentation. Succinate is a dicarboxylic acid playing a role in the incomplete tricarboxylic acid (TCA) cycle that lacks 2-oxoglutarate dehydrogenase. The shift of cellular environment from light illuminated aerobic culture in BG-11 medium to dark anaerobic culture in nitrate free solution appears to trigger the dark metabolism in *Synechocystis* sp. strain PCC6803-GT including glycolysis, protein and amino acid decomposition, membrane lipid decomposition and PHB decomposition. Metabolic map suggests the generation and consumption of Acetyl-CoA, followed by those of 2-oxaglutarate in large quantities. The supply of 2-oxaglutarate results in high level accumulation of succinate. The enzyme for this mechanism is not yet well elucidated (Cooley *et al*. 2000).

Synechococcus sp. strain PCC7002 possessing incomplete TCA cycle have succinic semiadehyde as a shortcut from the missing step (Zhang and Bryant 2011). Presence of malate, fumarate and succinate in reductive branch of incomplete TCA cycle suggests that NADH oxidation ensues to elevate succinate. The enzymes of reductive branch are phosphoenolpyruvate (PEP) carboxylase, malate dehydrogenase, fumarase and succinate dehydrogenase. Among monosaccharides utilized in this work, only glucose is utilized for both glycogen building-up and heterotrophic cell growth.

The assimilation of other sugars is not observed, while elevation of initial hydrogen production by reducing sugar is observed. There are remarkably few data available in references on dark metabolism in the model cyanobacterium *Synechocystis* sp. strain PCC6803, hence the data shown here appear to provide additional information for constructing cyanobacterial cell factories. If K_d is the specific death rate of cell, then breakage of cell mass is interpreted by the equation:

$$\frac{dX}{dt} = -K_d X \qquad (6).$$

The K_d values for the run without monosaccharide, with xylose, and for the runs after 48 h with galactose and mannose are 1.08, 2.49, 1.52 and 2.13 h^{-1}, respectively. The reducing sugars that lower K_d shows high activity for hydrogen production.

Figure 5 shows a conceivable mechanism of dark hydrogen production of GT strains in nitrate free solution. Before dark incubation, GT strains are grown on carbon dioxide under the light and the bulk of chemical energy is stored in glycogen, protein, membrane lipid and PHB (Knoop *et al*. 2013; Montagud *et al*. 2010). Nitrate starvation at the beginning of dark incubation appears to trigger the instantaneous sudden accumulation of reductive compounds NAD(P)H. The shift of cellular environment from illuminated culture in BG-11 medium to dark nitrate free solution also appears to trigger the decomposition of glycogen, protein, membrane lipid and PHB. This reaction produces additional reductants. The excess NADH, NADPH and reductive ferredoxin are utilized for hydrogen on NAD(P)H-linked hydrogenase throughout the reaction and on reductive ferredoxin-linked hydrogenase after 48 h.

Conclusion

The results presented in this paper provide information that exogenous reducing sugar is useful to increase initial activity of hydrogenase for hydrogen production. The stabilization of high reductive state of cells in *Synechocystis* sp. strain PCC6803-GT by adding exogenous glucose and fructose are of prime importance to increase hydrogen production. The mechanism by which these reducing sugars exact the effect remains to be elucidated.

References

Anderson SL, McIntosh L (1991) Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. J Bacteriol 173 (9):2761-2767

Baebprasert W, Lindblad P, Incharoensakdi A (2010) Response of H_2 production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Int J Hydrogen Energy 35 (13):6611-6616.

Chiou, TW., Liaw, WF. (2008). Nickel–thiolate and iron–thiolate cyanocarbonyl complexes: Modeling the nickel and iron sites of [NiFe] hydrogenase. Comptes Rendus Chimie 11 (8): 818–833.

Cooley JW, Howitt CA, Vermaas WFJ (2000) Succinate:Quinol Oxidoreductases in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803: Presence and Function in Metabolism and Electron Transport. J Bacteriol 182 (3):714-722.

Cournac L, Guedeney G, Peltier G, Vignais PM (2004) Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. J Bacteriol 186 (6):1737-1746

Das D, Veziro lu TN (2001) Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 26 (1):13-28.

Ducat DC, Sachdeva G, Silver PA (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proceedings of the National Academy of Sciences 108 (10):3941-3946. doi:10.1073/pnas.1016026108

Flores E, Schmetterer G (1986) Interaction of fructose with the glucose permease of the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 166 (2): 693-696

Gutekunst K, Chen X, Schreiber K, Kaspar U, Makam S, Appel J (2014) The Bidirectional NiFe-hydrogenase in *Synechocystis* sp. PCC 6803 Is Reduced by Flavodoxin and Ferredoxin and Is Essential under Mixotrophic, Nitrate-limiting Conditions. J Biol Chem 289 (4):1930-1937.

Hallenbeck PC, Kochian LV, Benemann JR (1981) Hydrogen evolution catalyzed by hydrogenase in cultures of cyanobacteria. Z Naturforsch C 36:87–92

Howarth D.C. and Codd G. (1985) The Uptake and Production of Molecular Hydrogen by Unicellular Cyanobacteria. J. Gen. Microbiol. 131:1561-1569.

Joset F, Buchou T, Zhang CC, Jeanjean R (1988) Physiological

and genetic analysis of the glucose-fructose permeation system in two *Synechocystis* species. Arch Microbiol 149 (5):417-421.

Kahlon S, Beeri K, Ohkawa H, Hihara Y, Murik O, Suzuki I, Ogawa T, Kaplan A (2006) A putative sensor kinase, Hik31, is involved in the response of *Synechocystis* sp. strain PCC 6803 to the presence of glucose. Microbiology 152 (3):647-655.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence Analysis of the Genome of the Unicellular Cyanobacterium *Synechocystis* sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-coding Regions. DNA Res 3 (3):109-136.

Knoop H, Gründel M, Zilliges Y, Lehmann R, Hoffmann S, et al. (2013) Flux Balance Analysis of Cyanobacterial Metabolism: The Metabolic Network of Synechocystis sp. PCC 6803. PLoS Comput Biol 9(6)

Lee S., Ryu J-V., Kim S.Y., Jeon J-H., Song J.Y., Cho H-T., Choi S-B., Choi D., de Marsac N.T. and Park Y-I. (2007) Transcriptional Regulation of the Respiratory Genes in the Cyanobacterium *Synechocystis* sp. PCC 6803 during the Early Response to Glucose Feeding. Plant Physiol 145: 1018-1030

Montagud A, Navarro E, Fernandez de Cordoba P, Urchueguia J, Patil K (2010) Reconstruction and analysis of genome-scale metabolic model of a photosynthetic bacterium. BMC Systems Biology 4 (1):156

Stal LJ, Moezelaar R (1997) Fermentation in cyanobacteria1. FEMS Microbiol Rev 21 (2):179-211

Williams JG (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol 167:766-778

Yamamoto T, Asami K, Ohtaguchi K (2012a) Anaerobic Production of Hydrogen in the Dark by *Synechocystis* sp. strain PCC 6803: effect of photosynthetic media for cell preparation. J Biochem Technology 3 (4):344-348

Yamamoto T, Chongsuksantikul A, Asami K, Ohtaguchi K (2012b) Anaerobic Production of Hydrogen in the Dark by *Synechocystis* sp. strain PCC 6803 supplemented with D-glucose. J Biochem Technology 4 (1): 464-468

Yoo S-H, Keppel C, Spalding M, Jane J-l (2007) Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. Int J Biol Macromol 40 (5):498-504

Yu L., You L., Liu D., Hollinshead W., Tang YJ. and Zhang F. (2013) development of Synechoystis sp,PCC6803 as a phototrophic cell factory. Mar Drugs 11:2894-2916.

Zhang C-C, Durand M-C, Jeanjean R, Joset F (1989) Molecular and genetical analysis of the fructose-glucose transport system in the cyanobacterium *Synechocystis* PCC6803. Mol Microbiol 3 (9):1221–1229

Zhang S, Bryant DA (2011) The Tricarboxylic Acid Cycle in Cyanobacteria. Science 334 (6062):1551-1553