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Activation of Hydrogen Production in  
a Genetically Modified Cyanobacterium

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## Nomenclature

$c_A$ = acetate concentration	[ $\mu\text{mol mL}^{-1}$ ]
$c_E$ = ethanol concentration	[ $\mu\text{mol mL}^{-1}$ ]
$c_L$ = lactate concentration	[ $\mu\text{mol mL}^{-1}$ ]
$c_s$ = concentration of unreacted monosaccharides	[ $\mu\text{mol mL}^{-1}$ ]
$g$ = numbers of generations	[-]
$g_C$ = numbers of generations from 0 h to $t_C$	[-]
$G$ = numbers of generations from 0 h to $t_f$	[-]
$I_o$ = photosynthetic photon flux density of incident light	[ $\mu\text{mol mL}^{-1}\text{s}^{-1}$ ]
$I_m$ = model parameter	[ $\mu\text{mol mL}^{-1}\text{s}^{-1}$ ]
$k$ = deactivation constant for $\text{H}_2$ production	[ $\text{h}^{-1}$ ]
$k^*$ = deactivation constant for $\text{H}_2$ production compared to control run	[-]
$k_0$ = rate constant of second order	[ $\text{h}^{-1}$ ]
$k_1$ = rate constant of pseudo-first order	[ $\text{h}^{-1}$ ]
$k_i$ = model parameter of	[-]
$k_G$ = degradation rate constant of glycogen content	[ $\text{h}^{-1}$ ]
$k_{\text{OX}}$ = rate constant of oxidant per dry weight	[ $\text{h}^{-1}$ ]
$k_{\text{RED}}$ = rate constant of reductant per dry weight	[ $\text{h}^{-1}$ ]
$K_d$ = specific death rate	[ $\text{h}^{-1}$ ]
$m_G$ = intracellular glycogen content per cell weight	[ $\mu\text{mol mg}^{-1}$ ]
$m_G^*$ = intracellular glycogen content per cell weight compared to control run	[ $\mu\text{mol mg}^{-1}$ ]
$m_{GX}$ = intracellular glycogen content per unit volume of reaction solution	[ $\mu\text{mol mL}^{-1}$ ]
$m_{\text{OX}}$ = number of moles of oxidant per dry weight (NAD(P)H)	[ $\mu\text{mol mL}^{-1}$ ]
$m_{\text{RED}}$ = number of moles of reductant per dry weight (NAD(P) <sup>+</sup> )	[ $\mu\text{mol mL}^{-1}$ ]
$m_T$ = total amounts of NAD(P)H and NAD(P) <sup>+</sup> per dry weight	[ $\mu\text{mol mL}^{-1}$ ]
$q_{\text{CO}_2}$ = specific $\text{CO}_2$ fixation rate	[ $\mu\text{mol mL}^{-1}\text{h}^{-1}$ ]
$q_{\text{CO}_2}^*$ = specific $\text{CO}_2$ fixation rate compared to control run	[-]
$q_{\text{H}_2}$ = specific $\text{H}_2$ production rate	[ $\mu\text{mol mL}^{-1}\text{h}^{-1}$ ]
$q_{\text{H}_2}^*$ = specific $\text{H}_2$ production rate compared to control run	[-]
$q_S$ = specific monosaccharide consumption rate	[ $\mu\text{mol mL}^{-1}\text{h}^{-1}$ ]
$r_A$ = acetate production rate	[ $\mu\text{mol mL}^{-1}\text{h}^{-1}$ ]
$r_{\text{H}_2}$ = $\text{H}_2$ production rate	[ $\mu\text{mol mL}^{-1}\text{h}^{-1}$ ]

$r_L$ = lactate production rate	$[\mu\text{mol mL}^{-1} \text{h}^{-1}]$
$r_S$ = monosaccharide consumption rate	$[\mu\text{mol mL}^{-1} \text{h}^{-1}]$
$r_X$ = growth rate	$[\text{mg mL}^{-1} \text{h}^{-1}]$
$R_G$ = specific production rate of endogenous glucose	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$-R_G$ = specific consumption rate of endogenous glucose	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$R_G^*$ = specific production rate of endogenous glucose compared to control run	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$R_{G1}$ = specific production rate of endogenous glucose in logarithmically growing cells	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$R_{G2}$ = specific production rate of endogenous glucose in resting cells	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$R_{GAP}$ = generation rate of glyceraldehyde	$[\mu\text{mol mg}^{-1} \text{h}^{-1}]$
$R_{GX}$ = production rate of endogenous glucose	$[\mu\text{mol mL}^{-1} \text{h}^{-1}]$
$-(R_{GX})$ = consumption rate of endogenous glucose	$[\mu\text{mol mL}^{-1} \text{h}^{-1}]$
$t$ = time	$[\text{h}]$
$t_2$ = doubling time	$[\text{h}]$
$t_C$ = critical time	$[\text{h}]$
$t_f$ = time in stationary phase	$[\text{h}]$
$t_{\text{SHT}}$ = time of acceleration (shift)	$[\text{h}]$
$X$ = cell mass concentration	$[\text{mg mL}^{-1}]$
$y_{\text{H}_2}$ = total $\text{H}_2$ production per unit volume of reaction solution	$[\mu\text{mol mL}^{-1}]$
$y_{\text{H}_2,f}$ = attainable level of $\text{H}_2$ per culture volume Eq.(2.18)	$[\mu\text{mol mL}^{-1}]$
$y_{\text{H}_2,f}^*$ = attainable level of $\text{H}_2$ production per culture volume of reaction solution compared to control run	$[-]$
$Y$ = yield factor	$[-]$
$Y_{A/G}$ = acetate production yield	$[-]$
$Y_{L/G}$ = lactate production yield	$[-]$
$Y_{\text{H}_2/G}$ = $\text{H}_2$ production yield	$[-]$
$Y_{X/S}$ = growth yield on monosaccharides	$[-]$
$\sigma_{HG}$ = specific activity of NiFe-hydrogenase	$[-]$
$\phi$ = growth kinetic	$[-]$

#### Subscript

0 = initial state

1 = at 96 h or 120 h (final time of experiment)

f = final state

# Chapter 1

## Introduction

### 1.1 Hydrogen production pathway

**Hydrogen energy for sustainable development:** Global energy consumption has increased since the worldwide spreading of industrial revolution doctrine. The fossil fuel combustion results in carbon dioxide (CO<sub>2</sub>) emission that has led to an environmental problem. The increased concentrations of key greenhouse gases are a direct consequence of human activities. Man-made greenhouse gases accumulated in the atmosphere produce higher net warming by strengthening the natural “greenhouse effect”. CO<sub>2</sub> concentrations in the atmosphere have been increasing over the past century compared to the rather steady level of the pre-industrial era. Atmospheric CO<sub>2</sub> level for November 2014 is 397.13 ppm (McGee, 2014).

CO<sub>2</sub> emission will continue to increase, as a result of continued growing consumption of fossil fuels in some of the large developing countries such as China and India. The link between climate change and energy is a part of a larger challenge of sustainable development. Since 1870, CO<sub>2</sub> emissions from fuel combustion have risen exponentially. Growing world energy demand from fossil fuels plays a key role in the upward trend in CO<sub>2</sub> emissions. Since the Industrial Revolution, annual CO<sub>2</sub> emissions from fuel combustion dramatically increased from near zero to more than 8.7 Pg y<sup>-1</sup> (P=10<sup>15</sup>; y=year) in 2008 (DOE-Carbon Dioxide Information Analysis Center, 2011). About 20% of total CO<sub>2</sub> emission is produced from burning gasoline for transportations. Primary consumption of energy is based on coal and oil, this kind of fossil fuel will inevitably deplete within the next century. This prompts us as urgent need to find a replacement for fossil fuel phase out era.

The most sustainable way to support the world economic growth in 21<sup>st</sup> century is focusing on the development of renewable energy including solar energy, wind energy, geothermal energy, bioenergy, hydropower, ocean energy, green power and hydrogen (H<sub>2</sub>) and fuel cells. Among them, considerable attention has been paid by automobile industry on hydrogen energy since hydrogen is high in energy (**Table 1.1**) and combustion of H<sub>2</sub> generates almost no pollution. Hydrogen was discovered in 1766 by Cavendish in UK. The element hydrogen is the simplest atom consisting of one proton and one electron. It is abundant in the space. Different from the distribution of element, molecular hydrogen is hardly observed in nature of the earth. Hydrogen atom always exists combining with other elements. Hydrogen represents very high heat of combustion per weight. Hydrogen stores about 3.02 times the energy per unit weight as gasoline. Due to the clean byproduct of hydrogen fuel, NASA has been utilizing liquid

hydrogen to launch the space shuttles since 1970. H<sub>2</sub> is one of the new sources of energy to be used as an alternative energy after deprivation of crude oil.

**Hydrogen fuel vehicles:** Hydrogen gas can be used as energy-carrier which can be installed into vehicle. **Figure 1.1** illustrates (a) H<sub>2</sub> fuel vehicle, (b) H<sub>2</sub> gas stand and (c) off-site liquid H<sub>2</sub> storage. The existing technology of fuel cell, storage and safety can support the practical use of hydrogen energy. Since the molecular weight of hydrogen is very low, the heat of combustion per mole shows an inverse relation. Liquid volume of hydrogen fuel is 3.47 times that of gasoline for a given amount of energy. A typical fuel cell vehicle (FCV) in Japan (Fig.1.1(a)) has a 156 L tank with upper limit pressure of 70 MPa (=109200L at 101300 Pa=4880 mole) for hydrogen fuel that can support a fuel of 1570MJ to drive 700 km (<http://is-factory.com/post-4056/>, 2014). Mileage of hydrogen fuel is 6.42 (=700/109) km m<sup>-3</sup>. Price of hydrogen fuel is 100-150 yen Nm<sup>-3</sup>. Fuel cost of hydrogen is 17-23 yen km<sup>-1</sup>. In December 2014, the first industrial production of liquid H<sub>2</sub> (2.5 Mmol d<sup>-1</sup>) was started in Japan. It can support 1000 FCVs. Expected prices are 30 yen per Nm<sup>-3</sup> by 2025 and 20 yen per Nm<sup>-3</sup> by 2050. A dispenser for H<sub>2</sub> is illustrated in Fig.1.1 (b). A typical station shown in Fig.1.1(c) has 10 m<sup>3</sup> (=708.5kg = (10 m<sup>3</sup>)(70.85 kg m<sup>-3</sup>)=354000mole) liquid H<sub>2</sub> storage. That is 72.5(354000/(4880)) times that is required for 70MPa FCV. Gasoline fuel vehicle has a 60 L tank to support 400 km drive. Mileage of gasoline fuel is 10-15 km L<sup>-1</sup>. Price of gasoline is 160 yen L<sup>-1</sup>. Fuel cost of gasoline is 10-16 (=160/(10-15)) yen km<sup>-1</sup>. H<sub>2</sub> can also be used efficiently for electricity generation due to prompted developed fuel cell technology (Momirlan and Veziroglu 2002).

**Current industrial consumption of hydrogen:** The current annual production of H<sub>2</sub> is about 55 Tg (=55 million tons). Ammonia synthesis utilizes about a half of industrially generated H<sub>2</sub>. Petrochemical industry consumes another half to reduce heavy sources to lighter fractions. Resources of H<sub>2</sub> are 48% from natural gas, 30% from oil, 18% from coal and 4% from electrolysis of water. Sources are either fossil fuels or non-fossil fuels. The dominant source of current industrial production of H<sub>2</sub> is fossil fuels. If the number of the H<sub>2</sub> fueled vehicles increases, H<sub>2</sub> manufacturing industry will face the problem of supply of feedstock.

**Available technologies for hydrogen production:** **Figure 1.2** shows the overview of H<sub>2</sub> production technologies. Arcs shown by red are current main supply chain of H<sub>2</sub>. Hydrogen production methods are classified into the methods from renewable sources (Appendix-1) and those from non-renewable sources (Appendix-2).

Technology with non-renewable sources includes (1) steam methane reforming, (2) partial oxidation of hydrocarbons, and (3) coal gasification. Technology with non-renewable sources is established and

represents a reasonable cost of H<sub>2</sub> for ammonia synthesis and heavy source treatment, however, currently we have found a great deal of difficulty in reproducing the same result in supporting H<sub>2</sub> fueled society since fuel market size is huge and production of H<sub>2</sub> from non-renewable sources always increases a cost of non-renewable source fuel. The additional problem of this pathway is the evolution of large volume of CO<sub>2</sub> from non-renewable resources. Although this technology can be applied to drive initial H<sub>2</sub> fueled society, there will be an urgent problem to solve those problems.

Technology with renewable sources includes (1) biomass gasification, (2) biomass pyrolysis, (3) electrolysis, (4) biocatalyzed electrolysis, (5) thermolysis, (6) photocatalytic water splitting, (7) dark fermentation, (8) direct biophotolysis, and (9) indirect biophotolysis. Technology with renewable sources is promising to solve those problems. Within technologies shown in Appendix-2, electrolysis, biocatalyzed electrolysis, photocatalytic water splitting method and thermolysis require the supply of electric power. Hence technology development of power plants without non-renewable resources is the underlying premise of such technologies. Biomass gasification and biomass pyrolysis generate CO<sub>2</sub>, however the amount of CO<sub>2</sub> in this evolution is less than the amount of CO<sub>2</sub> assimilated by photosynthesis. Production of fuel H<sub>2</sub> requires the constant supply of H<sub>2</sub> and the huge amount supply, hence biomass gasification and biomass pyrolysis technologies face to solve these problems.

Other technologies shown in Appendix-2 utilize the microbial metabolism to generate H<sub>2</sub>. The metabolism for H<sub>2</sub> production is classified as dark fermentation, direct biophotolysis and indirect biophotolysis. Dark fermentation of glucose to lactate by genetically modified anaerobic bacterium *Escherichia coli* generates 2 moles H<sub>2</sub> per mole of reacted glucose (**Figure A2.1**). The attainable level of H<sub>2</sub> for 120 h incubation reached 1.8 μmol mL<sup>-1</sup>. One drawback of dark fermentation is the requirement of organic substrate for cell growth and metabolism.

A number of studies on direct or indirect biophotolysis have been reported beginning with the first work of Gaffron (Gaffron, 1939). Gaffron observed light-induced H<sub>2</sub> production (direct biophotolysis) in unicellular green algae *Scenedesmus* after dark incubation (Eq.(A2.7)). As shown in Appendix-2, unicellular green algae, purple non-sulfur bacteria (PNSB) and cyanobacteria have been tested for direct biophotolysis H<sub>2</sub> production. Cyanobacteria have been a model for photosynthesis-related H<sub>2</sub> production. Cyanobacteria are photosynthetic organism, acquiring energy from light, carbon from CO<sub>2</sub> and nitrogen from N<sub>2</sub>, nitrate or ammonium ion. N<sub>2</sub>-fixing cyanobacteria have nitrogenase and non N<sub>2</sub>-fixing cyanobacteria have a bidirectional NiFe-hydrogenase. **Figure 1.3** shows an overview of cyanobacterial organization in the presence of light. Due to this light reaction, membrane of cyanobacteria under the

light actively generates NADPH, ATP and electrons, a large portion of which is utilized at the Calvin cycle to fix CO<sub>2</sub>.

**Figure 1.4** illustrates the Calvin cycle that is light-independent. The key enzyme is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) that fixes CO<sub>2</sub>. The sum of the reactions in the Calvin cycle is given by



The absorption of light energy at PSII (photosystem II) of thylakoid membrane (light-dependent reaction) by direct biophotolysis splits water and generates oxygen (O<sub>2</sub>), H<sup>+</sup> and electron:



Electrons generated at thylakoid membrane during illumination are reduced in the form of NADPH by the Calvin cycle. Even though, solar energy conversion to electron production is high, H<sub>2</sub> production is inhibited by O<sub>2</sub> since O<sub>2</sub> generation during photosynthesis suppress NiFe-hydrogenase from H<sub>2</sub> production. A reversible hydrogenase accepts electron from reduced ferredoxin to generate H<sub>2</sub> by the general reaction (Eq. (A2.8)).

**Table 1.2** lists the H<sub>2</sub> production rate of non N<sub>2</sub>-fixing cyanobacteria under the direct biophotolysis. The maximum H<sub>2</sub> production rate shown in this table is 1.38 μmol mg-chl a<sup>-1</sup> h<sup>-1</sup> that was obtained in run with *Gloebacter* PCC 7421. Chlorophyll a (C<sub>55</sub>H<sub>72</sub>O<sub>5</sub>N<sub>4</sub>Mg; molecular weight, 892.3 μg μmol<sup>-1</sup>) content is variously reported. Cyanobacterium *Spirulina maxima* represents chlorophyll a content of 13.1 μg mg-DCW<sup>-1</sup> at pH 9 and 9.5 μg mg-DCW<sup>-1</sup> at 5 klx light intensity (Pandey and Tiwari, 2010). Chlorophyll a content and cell volume of unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 for 72.5M cells mL<sup>-1</sup> (OD<sub>750</sub>=0.5) in a report (Lea-Smith et al., 2014) is 9.69 amol μm<sup>-3</sup> and 4.34 μm<sup>3</sup>. If dry cell weight concentration is 0.185 mg mL<sup>-1</sup>, then chlorophyll a content is 14.7 μg mg<sup>-1</sup> (= (892.3)(9.69)(4.34)(10<sup>-18</sup>)(10<sup>6</sup>)(72.5)(10<sup>6</sup>)/0.185). If chlorophyll a content is 14.7 μg mg<sup>-1</sup>, then above highest H<sub>2</sub> production is 0.0932 μmol mg<sup>-1</sup> h<sup>-1</sup>.

Three years after the observation of direct biophotolysis, Gaffron and Rubin first observed H<sub>2</sub> evolution in indirect biophotolysis by adapting the green alga *Scenedesmus obliquus* in dark anaerobic condition (Gaffron and Rubin 1942). **Figure 1.5** shows indirect biophotolysis to generate H<sub>2</sub> in cyanobacteria (Carrieri, 2010). Different from green alga, cyanobacteria are reported to be organisms that prefer a dark auto-fermentation that follows the light-dependent photosynthetic stage to produce hydrogen. A 5-fold increase in H<sub>2</sub> production in *Synechococcus* sp. strain PCC7002 by eliminating genes coding for lactate dehydrogenase and pyruvate-ferredoxin oxidoreductase was achieved (McNeely, 2011). **Table 1.3** lists the typical results of previous works.

From Tables 1.2 and 1.3, highest H<sub>2</sub> production rate in previous works is found to be 0.0190 μmol mg<sup>-1</sup> h<sup>-1</sup> from *Synechococcus* PCC 6830. Indirect biophotolysis is promising for H<sub>2</sub> production because there is no O<sub>2</sub> generation to suppress hydrogenase. The specific H<sub>2</sub> evolution activities of bidirectional hydrogenases are higher than those of nitrogenase-based H<sub>2</sub> production. Therefore systems based on bidirectional hydrogenases present the most likely approach to practical development.

For indirect biophotolysis, photosynthesis stage is important to prepare cellular states suitable for dark anaerobic H<sub>2</sub> production. If this technology is promising, cyanobacterial fuel is renewable, clean and sustainable for future. It can be genetically modified to produce valuable substance such as isoprene, ethanol, sugar, and lactate (Ducat et al. 2011). However, several obstacles must be overcome to put cyanobacteria to be economically commercialized. From recent knowledge, cyanobacteria have been known as origin of photosynthesis organism. Fossil stromatolites (microbial communities mat) can be dated back to 3.5 Gya (Giga years ago) (Blankenship 1992). Photosynthetic organisms may have been among the very earliest life forms on the primitive earth. Global oxygen accumulation in the atmosphere and oceans are from production of O<sub>2</sub> by photosynthesis. Photosynthesis of cyanobacteria still recently plays a major role in the conversion of carbon dioxide to biological energy storage materials on earth. Photosynthesis efficiency calculated by the combination of energy losses in light absorption and the initial chemical reactions results in a maximal overall energetic efficiency estimated at about 11% (Brenner et al. 2006) which is much higher than plants (mostly less than 1%)

To create a general method toward increasing H<sub>2</sub> production utilizing model species is also important. Cyanobacteria are known to produce H<sub>2</sub> mainly via indirect biophotolysis. Unicellular cyanobacterium *Synechosystis* cells work as a solar power collector through bioconversion of ambient CO<sub>2</sub> into cell constituting materials. Exposure of cells to dark anaerobic condition or sulfur-limiting condition triggers the conversion of energy-rich components to energy-lean compounds and H<sub>2</sub>. This allows the formation of secondary products such as ethanol, acetate, lactate and succinate. Thus, this indirect biophotolysis

consisted of two stages in series, photosynthesis for carbohydrate storage, and cells switched into specific condition allowing for stored carbon to be turned into H<sub>2</sub> production.

## 1.2 Hydrogen production in a glucose tolerant mutant of unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 through indirect biophotolysis

Besides the works of N<sub>2</sub>-fixing cyanobacteria, indirect biophotolysis to produce H<sub>2</sub> has been studied utilizing non N<sub>2</sub>-fixing cyanobacteria.

**Figure 1.6** is a method of indirect biophotolysis for H<sub>2</sub> production in *Synechocystis* sp. strain PCC 6803. It was shown to be important to eliminate nitrate ion when photosynthetically grown cells were inoculated into dark anaerobic condition.

Unicellular cyanobacterium *Synechocystis* sp. PCC 6803 is a fresh-water and non-nitrogen fixing organism. Among cyanobacteria, *Synechocystis* sp. PCC 6803 is the most well studied species and, defined its robust growth characteristic, therefore the organism has been selected for this work. This study concerned with the hydrogen production on a bidirectional NiFe-hydrogenase (HoxEFUYH hydrogenase) in the Glucose Tolerance strain (GT) of cyanobacterium *Synechocystis* sp. strain PCC 6803. H<sub>2</sub> production on bidirectional NiFe-hydrogenase is shown by



When reductive form of co-enzyme NADH or NADPH binds to NiFe-hydrogenase, it transfers reductive potential to NiFe-hydrogenase, which then transfer electrons to proton. Protons then change to molecular hydrogen.

This strain's genome has been fully sequenced and all of its open reading frames (ORFs) have been annotated (Kaneko et al. 1996). Database can be retrieved on line. It is naturally competent for DNA transformation and can take up naked DNA as plasmids or as linear DNA for homologous recombination. GT strain is able to consume extracellular glucose in dark anaerobic condition which allows an observation in H<sub>2</sub> enhancement and cell growth in dark anaerobic condition (Cournac et al. 2004; Yamamoto et al. 2012b).

## 1.3 Objectives of this research

Previous works have suggested that hydrogen production is largely limited by availability of intracellular reduction equivalents in the form of NAD(P)H rather than hydrogenase availability for cells. Methyl viologen is used in the experiment as an infinite reductant dissolving in cell solution; H<sub>2</sub> is produced at high rate. A recent work has argued if ferredoxin is also a prime reductant to NiFe-hydrogenase in *Synechocystis* sp. strain PCC 6803 (Gutekunst et al. 2014). This knowledge supports a H<sub>2</sub>

production enhancement method of decreasing intracellular reductant consumption. In Tables 1.2 and 1.3. *Synechocystis* sp. strain PCC 6803 is not a top range in H<sub>2</sub> producer; however, its well research works and complete DNA sequence have allowed us to handle it.

If manipulation of redox homeostasis is the keyword to elevate H<sub>2</sub> production, a roadmap to create a method to increase H<sub>2</sub> production with unicellular cyanobacteria is outlined as shown in **Figure 1.7**. Availability of this roadmap is confirmed in this study where a glucose tolerant mutant of unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 (GT strain) is utilized as a model organism. Indirect biophotolysis is performed in which cells of GT strain are photoautotrophically grown in the first stage, and then cells are dark anaerobically incubated in HEPES buffer solution for H<sub>2</sub> production. To activate H<sub>2</sub> production in GT strain, this study focuses on the dark anaerobic H<sub>2</sub> production in GT strain and derivatives. In view of three platforms of research in Fig.1.7, the primary purposes of this study are:

- (1) to select a monosaccharide additive to increase initial amount of reductive compounds,
  - (2) to formulate a mathematical model to interpret kinetics for H<sub>2</sub> production,
  - (3) to characterize photoautotrophic growth phase effect on the subsequent H<sub>2</sub> production,
  - (4) to manipulate DNA to supply more electrons to NiFe-hydrogenase,
- and
- (5) to integrate three platforms to create a method to activate H<sub>2</sub> production

Significance of cyanobacterial H<sub>2</sub> production is also briefed based on empirical findings.

#### **1.4 Outline of this thesis**

Outline of this thesis is shown in **Figure 1.8**. The thesis is composed with 6 chapters. Contents of the chapters are briefed as follows:

##### **Chapter 1: Introduction**

Objectives and scopes of this research is described.

##### **Chapter 2: Monosaccharide Effects on Hydrogen Production**

To define the baseline of this research, dark anaerobic H<sub>2</sub> production in GT strain in HEPES buffer solution is first studied. A mathematical model to interpret kinetics for H<sub>2</sub> production is formulated. Second, to increase cellular content of NAD(P)H, which is normally generated by glycolysis or glycogen breakdown, H<sub>2</sub> production in the presence of monosaccharide is studied. Tested monosaccharides are glucose, fructose, galactose, mannose and xylose. Relations between the production of H<sub>2</sub> and the

consumption of monosaccharide, consumption or production of endogenous glucose and dry cell weight and production of lactate, acetate, succinate and ethanol are analyzed. Second and third mathematical models to predict attainable level of the number of moles of H<sub>2</sub> per culture volume from initial H<sub>2</sub> production rate and to relate final level of the number of moles of H<sub>2</sub> per culture volume and that of endogenous glucose are developed.

### **Chapter 3: Growth Phase Effects on Hydrogen H<sub>2</sub> Production**

To find out the effect of cellular state, prior to dark anaerobic incubation, on H<sub>2</sub> production, cells from logarithmic growth phase, late-logarithmic growth phase and stationary phase are utilized for dark anaerobic fermentation. Cellular state during photosynthesis is analyzed by monitoring dry cell weight reproduction and endogenous accumulation. The activity of the Calvin cycle that is a big electron sink is evaluated for cells from each growth phase.

### **Chapter 4: Genetic Modification Effects on Hydrogen Production**

To remove pathways that compete NAD(P)H with NiFe-hydrogenase, metabolic engineering approach is made. The mutant defective of the genes coding for D-lactate dehydrogenase ( $\Delta$ addh mutant) and that defective of the genes coding for both alcohol dehydrogenase and D-lactate dehydrogenase ( $\Delta$ adh $\Delta$ addh mutant) are constructed. Dark anaerobic H<sub>2</sub> production of newly constructed mutants are compared with that of GT strain in HEPES buffer solution without or with glucose.

### **Chapter 5: Fructose Effects on Hydrogen Production**

Studies prior to this chapter select fructose as a key additive monosaccharide to elevate dark anaerobic H<sub>2</sub> production. First, effect of fructose concentration on dark anaerobic H<sub>2</sub> production in GT strain is investigated. Mathematical model for H<sub>2</sub> production kinetics is re-evaluated. Second, combined effect of fructose addition and genetic modification is studied. Third, conceptual design for dark anaerobic bioreactor to produce fuel H<sub>2</sub> is challenged. Integration of all data has been concluded to draw a protocol for developing a dark anaerobic H<sub>2</sub> production enhancement method with unicellular cyanobacterium.

### **Chapter 6: Concluding Remarks**

The overall conclusion of this thesis and future aspects of this study are described.

Table 1.1 Heat of combustion for fuels

Fuel	Heating value		Molecular weight g mol <sup>-1</sup>	Liquid density kg L <sup>-1</sup>	Liquid volume per combustion energy mL kJ <sup>-1</sup>
	kJ g <sup>-1</sup>	kJ mol <sup>-1</sup>			
Hydrogen	141.8	283.6	2	0.071	0.0993
Gasoline	47.0	4700	100	0.745	0.0286
Petroleum diesel	43.1	1985	46.07	0.832	0.0279
Ethanol	29.7	1370	46	0.789	0.0427
Propane	49.9	2220	44	0.582	0.0344
Butane	49.5	2877	58.12	0.601	0.0337
Wood	15.0	-	-	-	-
Coal(Lignite)	28.5	-	-	-	-
Coal(Anthracite)	35.3	-	-	-	-
Natural gas	54	866	16.04	0.46	0.0403

Table 1.2 Summarization of literature survey of direct biophotolysis H<sub>2</sub> production in non-nitrogen-fixing unicellular cyanobacteria (Dutta et al. 2005).

Cyanobacteria	Maximum H <sub>2</sub> evolution rate (calculated in $\mu\text{mol mg-DCW}^{-1} \text{ h}^{-1}$ )	Growth in photosynthesis	H <sub>2</sub> evolution assay condition	Reference
<i>Synechococcus</i> PCC 602	0.66 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00970)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985
<i>Synechococcus</i> PCC 6307	0.02 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.000294)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar (100%) with photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985
<i>Synechococcus</i> PCC 6301	0.09 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00132)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with C <sub>2</sub> H <sub>2</sub> (1.34 $\mu\text{mol}$ ); fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985
<i>Microcystis</i> PCC 7820	0.16 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00235)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ), C <sub>2</sub> H <sub>2</sub> (1.34 $\mu\text{mol}$ ); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Moezelaar et al., 1996
<i>Gloebacter</i> PCC 7421	1.38 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.0203)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ), C <sub>2</sub> H <sub>2</sub> (1.34 $\mu\text{mol}$ ); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Moezelaar et al., 1996
<i>Synechocystis</i> PCC 6308	0.13 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00191)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ), C <sub>2</sub> H <sub>2</sub> (1.34 $\mu\text{mol}$ ); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985
<i>Synechocystis</i> PCC 6714	0.07 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00103)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985
<i>Aphanocapsa montana</i>	0.40 $\mu\text{mol mg chl a}^{-1}\text{h}^{-1}$ (0.00588)	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar (100%); photon fluence rate 20–30 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Howarth and Codd, 1985

Table 1.3 Summarization of literature survey of indirect biophotolysis H<sub>2</sub> production in non-nitrogen-fixing unicellular cyanobacteria

Cyanobacteria	Maximum H <sub>2</sub> evolution rate (calculated in $\mu\text{mol mg-DCW}^{-1} \text{ h}^{-1}$ )	Growth in photosynthesis	H <sub>2</sub> evolution assay condition	Reference
<i>Synechococcus</i> PCC 6830	0.26 $\mu\text{mol mg chl a}^{-1} \text{ h}^{-1}$ ( $\approx 0.00382 \mu\text{mol mg}^{-1} \text{ h}^{-1}$ )	Air; photon fluence rate 20 $\mu\text{E m}^{-2} \text{ s}^{-1}$	Ar with CO (13.4 $\mu\text{mol}$ ), C <sub>2</sub> H <sub>2</sub> (1.34 $\mu\text{mol}$ ); darkness.	Howarth and Codd, 1985
<i>Synechococcus leopoliensis</i>	0.130 $\mu\text{mol mL}^{-1} \text{ h}^{-1}$	6% CO <sub>2</sub> in air, MDM medium, 313K, 12klx	DEC solution without nitrate, darkness	Ohtaguchi et al., 1995
<i>Synechocystis</i> PCC 6803	0.0190 $\mu\text{mol mg}^{-1} \text{ h}^{-1}$	6% CO <sub>2</sub> in air, BG-11 medium, adapted to BG-11 without nitrate source for 24 h, 100 $\mu\text{mol m}^{-2} \text{ s}^{-1}$	N <sub>2</sub> (100%); 50 $\mu\text{mol}$ HEPES solution; darkness	Yamamoto et al., 2012a



(a)

(b)

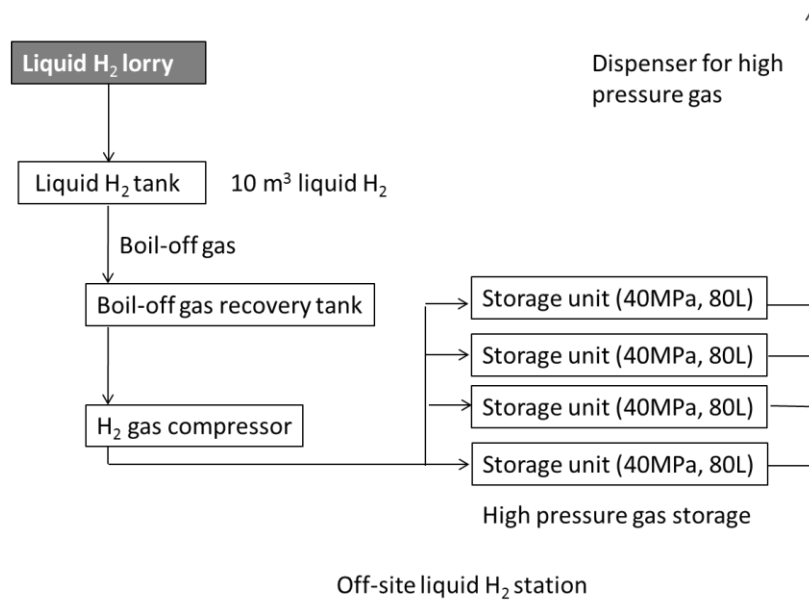


Figure 1.1 Hydrogen fuel vehicle and gas station

(a) Hydrogen fuel vehicle, <http://www.h2euro.org/wp-content/uploads/2014/08/toyota-fuel-cell.jpg>

(b) Hydrogen gas stand, [http://i.ytimg.com/vi/64NaZNh3O\\_w/0.jpg](http://i.ytimg.com/vi/64NaZNh3O_w/0.jpg)

(c) Off-site liquid H<sub>2</sub> station, modified graph from

<http://www.jari.or.jp/portals/0/jhfc/e/station/kanto/ariake.html>

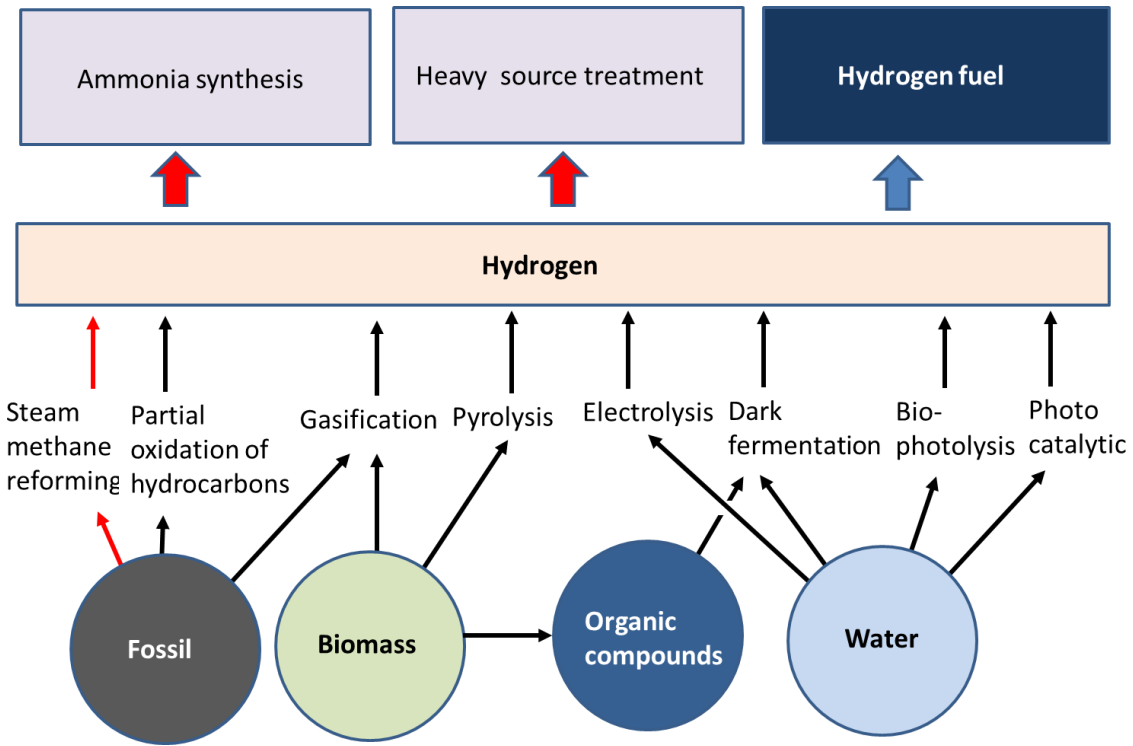


Figure 1.2 Overview of ongoing hydrogen production technologies

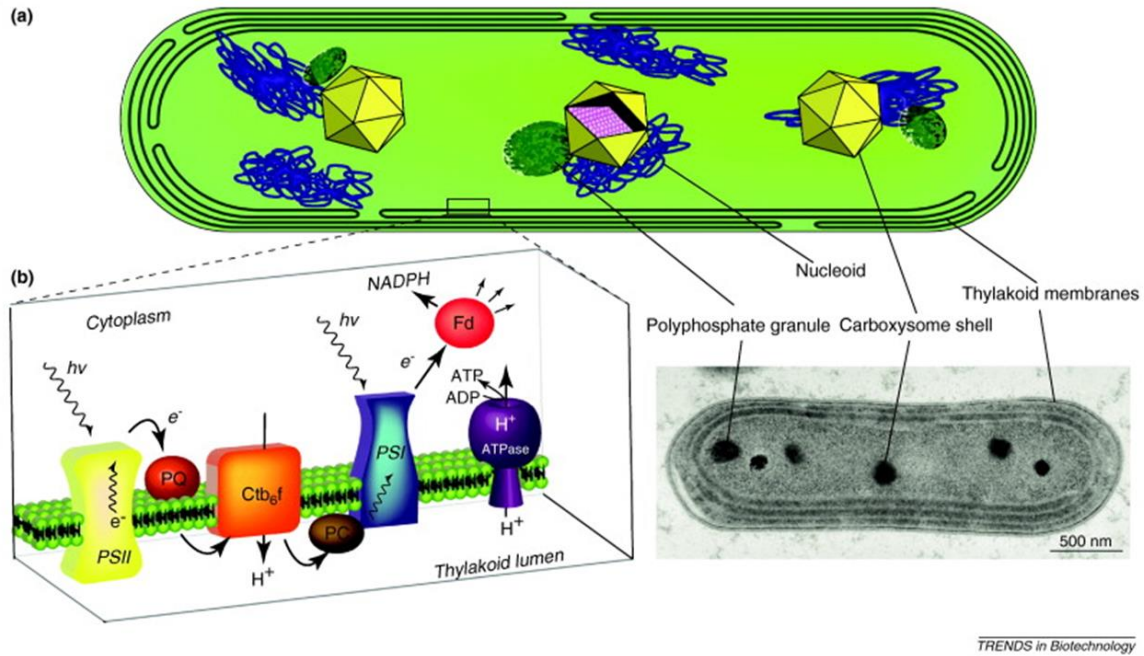


Figure 1.3 Outline of cell organization of unicellular cyanobacterium *Synechococcus elongates* (Ducat et al. 2011)

- (a) Cross-section of a cell. Carboxysome shell contains  $\text{CO}_2$  fixation machinery. Multiple chromosomes, which diffuse throughout the cytoplasm, exist in a cell. Thylakoid membrane absorbs radiant energy.
- (b) Electron transport chain. PSII (Photosystem II) absorbs 680 nm light to oxidize and split water molecules, generating electrons, protons and  $\text{O}_2$ . Electron potential is lowered. PQ (plastoquinone) receives electrons from PSII and transfer them to the cytochrome b6-f complex. Electrons are then passed to PSI (photosystem I) through PC (plastocyanin). PSI absorbs 700 nm light and lowers the electron potential. FNR (ferredoxin-NADP reductase), which receives electrons from PSI through Fd (ferredoxin), produces NADPH from  $\text{NADP}^+$  and proton. Proton gradient drives ATP synthase to generate ATP from ADP and Pi. Protons in the thylakoid lumen are returned to the cytoplasm through ATP synthase.

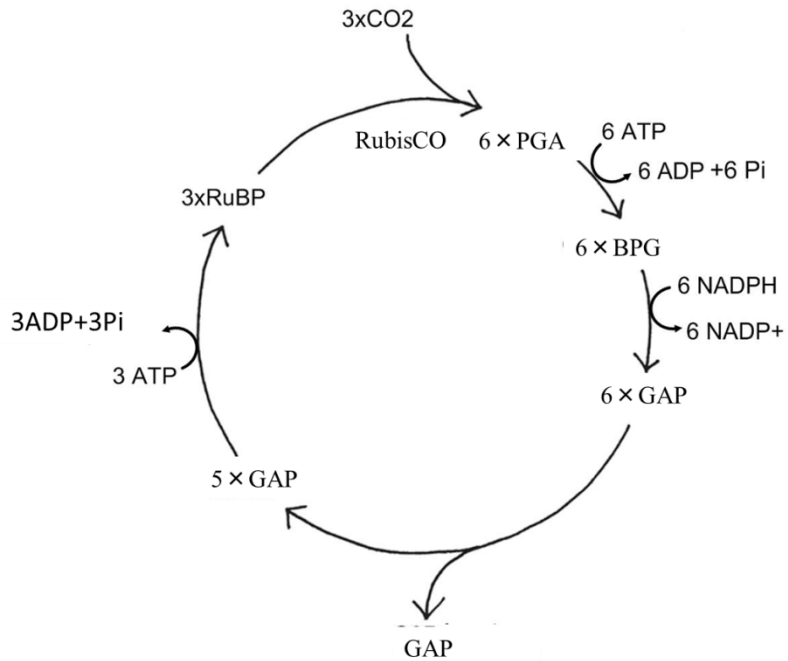


Figure 1.4 The Calvin cycle

RuBP, ribulose-1,5-bisphosphate; GAP, glyceraldehyde 3-phosphate; PGA, 3-phosphoglycerate; BPG, 1,3-bisphosphoglycerate; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase

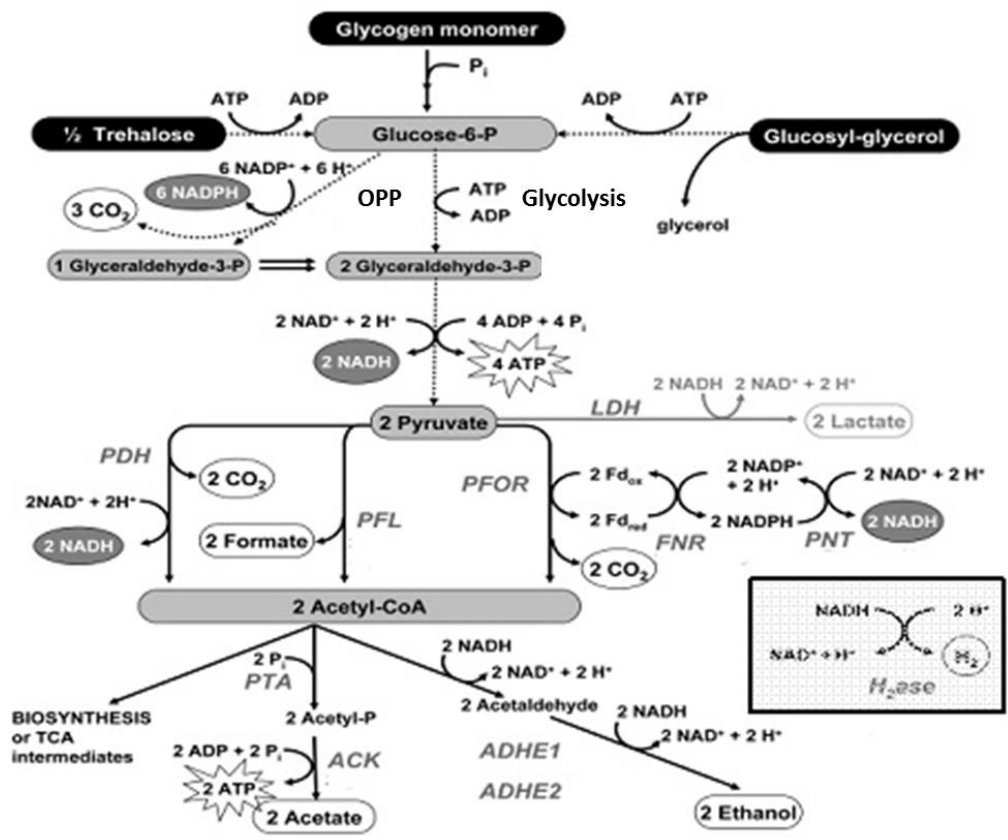


Figure 1.5 Dark fermentative metabolism with H<sub>2</sub> production in cyanobacteria based on indirect biophotolysis (Carrieri,2010) <http://dismukeslab.99k.org/bioenergy.html>

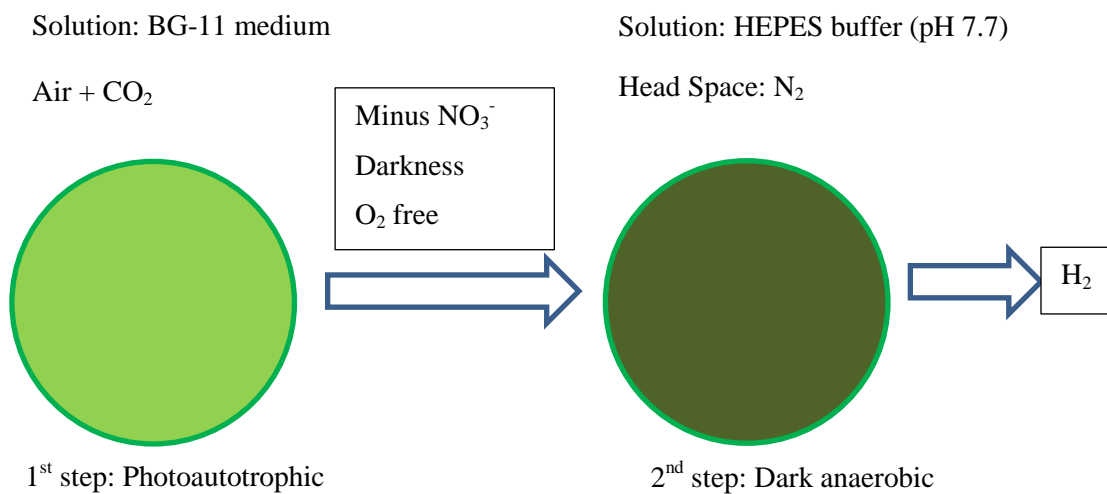


Figure 1.6 Indirect biophotolysis to generate H<sub>2</sub> in a glucose tolerant mutant of *Synechocystis* sp. strain PCC6803

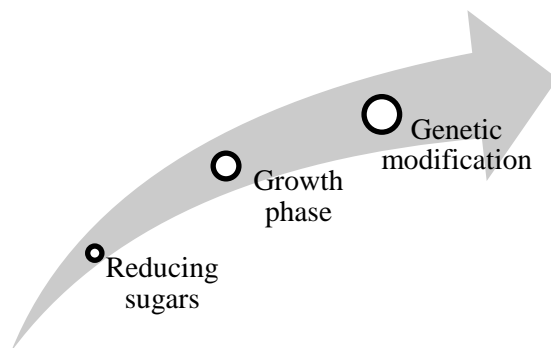


Figure 1.7 Roadmap: using 3 platforms to draw the protocol of how to adjusting reaction for the best hydrogen production from cyanobacteria.

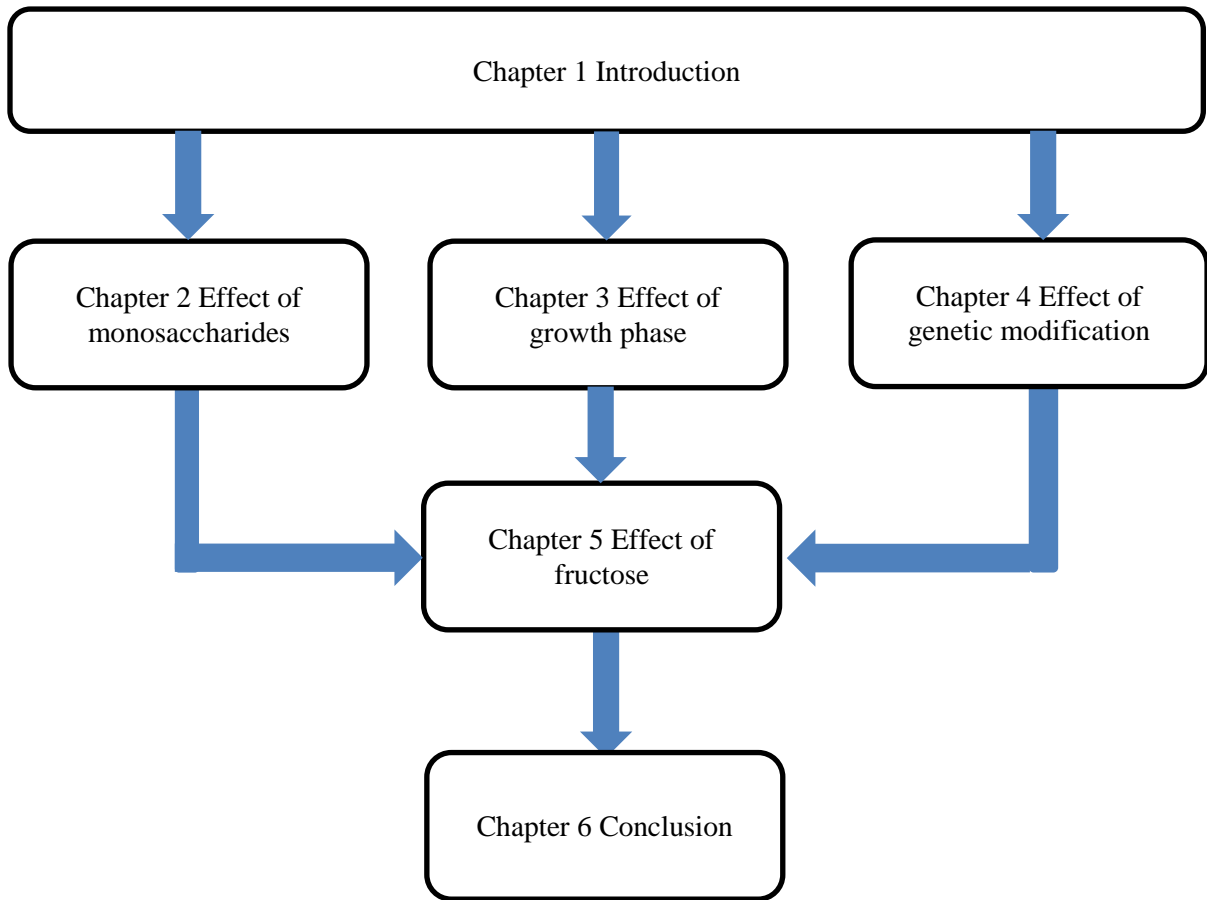


Figure 1.8 Illustration of H<sub>2</sub> production enhancement strategy used in this thesis to draw a protocol of how to enhance H<sub>2</sub> production from cyanobacteria.

## Chapter 1: References

- Blankenship R (1992) Origin and early evolution of photosynthesis. *Photosynthesis Research* 33(2):91-111.
- Brenner MP, Bildsten L, Dyson F, Fortson N, Garwin R, Grober R, Hemley R, Hwa T, Joyce G, Katz J (2006) Engineering microorganisms for energy production. DTIC Document
- Carrieri, D., Momot, D., Brasg, I. A., Ananyev, G., Lenz, O., Bryant, D. A., & Dismukes, G. C. (2010). Boosting autofermentation rates and product yields with sodium stress cycling: application to production of renewable fuels by cyanobacteria. *Applied and Environmental Microbiology*, 76(19), 6455-6462
- 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. *Journal of Bacteriology* 186 (6):1737-1746
- Ducat DC, Way JC, Silver PA (2011) Engineering cyanobacteria to generate high-value products. *Trends in Biotechnology* 29 (2):95-103
- Dutta D, De D, Chaudhuri S, Bhattacharya S (2005) Hydrogen production by Cyanobacteria. *Microbial Cell Factories* 4 (1):36
- Gaffron H (1939) Reduction of carbon dioxide with molecular hydrogen in green algae. *Nature* 143 (3614):204-205
- Gaffron H, Rubin J (1942) Fermentative and Photochemical Production of Hydrogen in Algae *The Journal of General Physiology* 26 (2):219-240
- 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. *Journal of Biological Chemistry* 289 (4):1930-1937
- Howarth DC, Codd GA (1985) The Uptake and Production of Molecular Hydrogen by Unicellular Cyanobacteria. *Journal of General Microbiology* 131 (7):1561-1569.
- 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 Research* 3 (3):109-136
- McGee Laboratory, Atmospheric CO<sub>2</sub> for November 2014, <http://co2now.org/>, accessed 25 December 2014
- Moezelaar R, Bijvank SM, Stal LJ (1996) Fermentation and sulfur reduction in the mat-building cyanobacterium *Microcoleus chthonoplastes*. *Applied and Environmental Microbiology* 62 (5):1752-1758
- Momirlan M, Veziroglu TN (2002) Current status of hydrogen energy. *Renewable and Sustainable Energy Reviews* 6 (1-2):141-179
- Ohtaguchi K OA, Takahashi N, Ogawa M and Koide K (1995) Kinetics of hydrogen production by the photolithotroph *Synechococcus leopoliensis*, . *IONICS* 21:69-72
- Pandey JP and Tiwari A (2010) Optimization of biomass production of *Spirulina maxima*. *Journal of Algal Biomass Utilization*. 1:20-32
- Yamamoto T, Asami K, Ohtaguchi K (2012a) Anaerobic production of hydrogen in the dark by *Synechocystis* sp. strain PCC 6803: effect of photosynthesis media for cell preparation. *Journal of Biochemical 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. *Journal of Biochemical Technology* 4 (1):464-468

## Chapter 2

### Monosaccharide Effects on Hydrogen Production

#### 2.1 Problem Definition and General Consideration

The purpose of the study in this chapter is to elucidate the effect of exogenous monosaccharide on production of molecular hydrogen ( $H_2$ ) in a glucose-tolerant mutant of unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 (GT strain) in HEPES buffer solution in darkness and to analyze  $H_2$  production kinetics with a mathematical model. GT strain has been isolated from the culture of wild type *Synechocystis* sp. strain PCC6803 (WT strain) (Williams 1988). WT strain and GT strain are known to generate  $H_2$  on bidirectional NiFe-hydrogenase through indirect biophotolysis. GT strain is capable of growing mixotrophically under the light and light-activated heterotrophically in the dark (Anderson and McIntosh 1991). Dark anaerobic  $H_2$  production in HEPES buffer solution is limited not by enzyme activity but by availability of NAD(P)H.

To increase the reductive potential of HEPES buffer solution, in a preceding work, a colleague of the author has elucidated the effects of reductive compounds on initial  $H_2$  production rate (Yamamoto, 2012). The tested reductive compounds are  $250 \mu\text{mol mL}^{-1}$  amino acids (aspartate, leucine, proline, tryptophan, isoleucine, asparagine, valine, phenylalanine, tyrosine, serine, threonine, methionine, alanine, glycine, glutamine, glutamate, histidine, lysine, arginine, and cysteine),  $0.25$  or  $1.0 \mu\text{mol mL}^{-1}$  organic acids (butyrate, citrate) and  $0.25$  or  $1.0 \mu\text{mol mL}^{-1}$  glucose ( $\alpha$ -D-glucose) on dark anaerobic  $H_2$  production in GT strain. Yamamoto reported that the additives that give no less than 1.4-fold increase in initial  $H_2$  production rate were aspartate, glutamate and glucose. Glucose effect shown in his work reveals that initial  $H_2$  production rates of GT strain in  $0, 5.6, 28, 56 \mu\text{mol mL}^{-1}$  glucose solution are  $0.035, 0.042, 0.033$  and  $0.033 \mu\text{mol mL}^{-1} \text{h}^{-1}$ , respectively, and that production of lactate and acetate is high when glucose concentration is no less than  $28 \mu\text{mol mL}^{-1}$ . The highest initial  $H_2$  production rate is  $0.104 \mu\text{mol mL}^{-1} \text{h}^{-1}$  that is seen in run with  $5.6 \mu\text{mol mL}^{-1}$  exogenous glucose. Heterotrophic growth on glucose was reported by Yamamoto but increase in dry cell weight concentration was not high as suggested from increase in  $H_2$  production. Addition of small amount of glucose to insure curbing the fermentation of lactate and acetate appears to elevate  $H_2$  production. No more than 0.8-fold initial  $H_2$  production rate was observed in histidine solution. The mechanism of the added compounds on  $H_2$  production is not yet known.

Glucose is a reducing sugar, hence the reductive environment provided by glucose has a possibility to increase H<sub>2</sub> production. **Figure 2.1** shows conversion of  $\alpha$ -D-glucopyranose, acyclic form (open chain) D-glucose and  $\beta$ -D-glucopyranose. The hydroxyl on carbon 1 represents a high reductive potential. Reducing sugars are other candidates of additives to increase H<sub>2</sub> production, however, no such works have been reported. The present investigation in this chapter extends the above work of Yamamoto and concerns the effect of reducing monosaccharides glucose, fructose, galactose, mannose and xylose on dark anaerobic H<sub>2</sub> production in GT strain. **Table 2.1** tabulates physical property data of above monosaccharides.

Shown in **Figure 2.2** is a cyanobacterial cell envelope consisting with all layers surrounding the cytoplasm. The 60-70% of outer membrane (LIV) is composed of mannose, glucose, galactose and other carbohydrate. External layer (EL) contains galactose, hexose, glucose and xylose. Hence reducing sugars selected above are important for cyanobacterial cell envelope.

There is a classic work (Stal and Moezelaar 1997) that lists up the dark anaerobic chemoorganotrophic growth of cyanobacteria involving the growth of *Anabaena azollae* AaN or *Nostoc* sp. A12 on glucose or fructose (De Philippins et al., 1996), *Microcystis aeruginosa* 7806 on glucose (Moezelaar and Stal, 1994), *Nostoc* sp. on glucose, fructose or sucrose (Hoare et al., 1917) and *Oscillatoria terebriformis* on glucose or fructose (Richardson et al., 1987). Except for *M. aeruginosa* 7806, all species reported for dark anaerobic chemoorganotrophic growth are N<sub>2</sub>-fixing filamentous cyanobacteria. There are remarkably few data available on the dark anaerobic metabolism on monosaccharides. Stal et al. also reported the detection of fermentation products H<sub>2</sub>, lactate, acetate and ethanol from endogenous glycogen and exogenous glucose in cyanobacteria *Synechocystis* sp, strain PCC 6714, *Synechocystis* sp, strain PCC 6308 and *Synechococcus* sp. strain PCC 6301.

Some works have reported that GT strain is capable of generating more H<sub>2</sub> in dark anaerobic glucose-supplemented solution than in dark anaerobic solution without glucose (Cournac et al. 2004; Yamamoto et al. 2012). Glucose-supplemented culture of this strain shows a small increase in dry cell weight concentration in darkness (Anderson and McIntosh 1991). Trophic modes of cyanobacteria are shown by complete autotrophy at one end and complete heterotrophy at the other end, and mixotrophy at a mixed utilization mode of CO<sub>2</sub> and organic substrate.

NiFe-hydrogenase of GT strain is important for mixotrophic growth on glucose under dark anaerobic condition because mutant lacking hydrogenase gene ( $\Delta$ *hoxH*) impairs ability to grow on glucose (Gutekunst et al. 2014). This has suggested that hydrogenase is one of important electron sink in dark

anaerobic condition. The other sink is nitrate reductase (Baebprasert et al. 2011). Effect of glucose on mixotrophic growth of GT 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).

**Figure 2.3** represents metabolism of carbon dioxide (CO<sub>2</sub>) and exogenous glucose by a mixotrophically growing cells of unicellular cyanobacterium *Aphanocapsa* 6714 in the light (2000 lx) in BG-11 medium (Pelroy et al., 1976). The Calvin cycle (Fig.1.4) is shown by the closed path from RuBP to RuBP via PGA, GAP, Pentose Phosphate cycle and Ru5P. RuBP is the only sugar phosphate that is unique to the Calvin cycle. During photosynthesis, CO<sub>2</sub> incorporated into the Calvin cycle is converted to glycogen. Peloy et al. showed the accumulation of RuBP under the light, inhibition of glucose-6-phosphate dehydrogenase by RuBP and rapid consumption of RuBP in darkness. This event relieves glucose-6-phosphate dehydrogenase from allosteric inhibition of RuBP, and initiate the conversion of glucose-6-phosphate to 6-PGI (6-phosphogluconate). This change activates the oxidative pentose phosphate (OPP) pathway and the cellular glycogen is decomposed. In the presence of glucose, cells of *Aphanocapsa* 6714 under the light with 0.01 μmol mL<sup>-1</sup> DCMU convert about 28% and 65% of carbon in exogenous glucose to CO<sub>2</sub> and cell constituting materials, respectively, and they under the dark convert about 37% and 64% of it to CO<sub>2</sub> and cell constituting materials, respectively. The carbon metabolism of GT strain during dark anaerobic H<sub>2</sub> production by indirect biophotolysis (Yamamoto et al., 2014) is slightly different from that shown in Fig.2.3, since photosynthesis of the work of Yamamoto et al. was performed not mixotrophically but autotrophically and exogenous glucose was added at the time of shift from photosynthesis mode to dark anaerobic incubation mode. In the absence of oxygen (O<sub>2</sub>) cells are required to activate NiFe-hydrogenase as an alternative of electron sink. In darkness, where OPP pathway turns to be active, consumption of exogenous glucose on OPP pathway generates additional electrons that contribute to increase H<sub>2</sub> production activity.

CyanoBase for *Synechocystis* sp. strain PCC6803 is the total genome database of this strain, which outputs 21 genes against the search word of “glucose”. The glycolysis metabolism of WT strain is shown in **Fig.A3.1** of Appendix-3 (KEGG pathway database). Lines in the graph are drawn based on the defined genes coding for corresponding enzymes and conversions shown by green box are specific to WT strain. This map shows the reversible pathway between glycogen and glucose via glucose-1-P. Glucose is converted to pyruvate by glycolysis pathway (**Fig.A3.2**) or to glyceraldehyde 3-phosphate pentosephosphate by pathway (**Fig.A3.3**). Glyceraldehyde 3-phosphate is converted to pyruvate by

glycolysis pathway. Pyruvate is converted to lactate (D-lactate) by D-lactate dehydrogenase (ddh) that is specific to WT strain (**Fig.A3.4**). Pyruvate is also converted to acetate via acetyl CoA by GT strain specific enzymes (Fig.A3.4), and to ethanol via acetaldehyde (Fig.A3.2).

Photoheterotrophic growth of this strain is possible on glucose and impossible on fructose (Rippka et al., 1979). Fructose is a reducing sugar and a glucose isomer which expresses a reactivity different from glucose (Laroque et al. 2008). Fructose is a bactericidal for GT strain, of which growth in glucose aqueous solution under the light is inhibited by  $10 \mu\text{mol mL}^{-1}$  fructose (Flores and Schmetterer 1986; Joset et al. 1988). Fructose enters the cell via glucose transporter (Zhang et al. 1989). Deactivation of the expression of *glcP* (glucose-transporter gene) makes cells to be resistant to fructose. CyanoBase for *Synechocystis* sp. strain PCC6803 is the total genome database of this strain, which outputs 5 genes against the search word of “fructose”. Besides four genes that are closely related to photosynthesis, L-glutamine: D-fructose-6-P amidotransferase is listed. Fructose metabolism in GT strain is not detailed in previous works, while isolated genes suggest the pathway from fructose to glyceraldehyde 3-phosphate via fructose 1-phosphate (**Fig.A3.5**). Glycogen is synthesized from fructose 1-phosphate via fructose 6-phosphate, glucose 6-phosphate, and glucose 1-phosphate (Fig.A3.1). When glycolysis is dominant, pyruvate is produced from fructose 1-phosphate via glyceraldehyde 3-phosphate (Fig.A3.4). The fructose resistant strain has less affinity to glucose (Zhang et al. 1989; Kahlon et al. 2006). In the presence of glucose, concentration of NAD(P)H increases significantly in the dark adapted cells of GT strain (Lee et al. 2007).

Galactose is a C-4 epimer of glucose. Galactose metabolism and fructose and mannose metabolism in GT strain are presented in KEGG (**Fig.A3.6**). CyanoBase for *Synechocystis* sp. strain PCC6803 is the total genome database of this strain, which outputs no genes against the search word of “galactose”. Experimental data on galactose metabolism in WT strain is not seen in previous works but the metabolic map of Fig.A3.6 based on genes suggests that galactose is converted to glucose 6-phosphate via galactose 1-phosphate and glucose 1-phosphate.

Mannose is a C-2 epimer of glucose. Isolated genes suggest the pathway from mannose to fructose 6-phosphate via mannose-6-phosphate (Fig.A3.5). CyanoBase for *Synechocystis* sp. strain PCC6803 is the total genome database of this strain, which outputs 9 genes against the search word of “mannose”. Five genes among them are related to mannose-1-phosphate guanyltransferase. No reports have shown the mannose utilization in WT strain.

Xylose is an aldopentose monosaccharide. No report shows consumption of xylose in WT strain, while isolated genes assume the metabolism shown in Appndix-3 (7) (**Fig.A3.7**). CyanoBase for *Synechocystis* sp. strain PCC6803 is the total genome database of this strain, which outputs no genes against the search word of “xylose”. If these gene products expresses, xylose is converted to pyruvate via xylonolactone. High sequence conservation with the xylose transporter of *Escherichia coli* (Schmetterer, 1990 Plant Molecular Biology, 14:697-706) is found in WT strain DNA.

This chapter also concerns the kinetics of dark anaerobic H<sub>2</sub> production in GT strain. When cells of GT strain are inoculated from photoautotrophic condition into dark anaerobic HEPES buffer solution, they generate molecular hydrogen (H<sub>2</sub>) on bidirectional NiFe-hydrogenase by receiving sufficient amount of electrons in terms of NAD(P)H as shown by



This technique is an indirect biophotolysis to generate H<sub>2</sub>. The reductive compound NAD(P)H is reported to be generated from the breaking-down of stored glycogen (glycolysis). **Figure 2.4** is cited from the work of Yamamoto (Yamamoto, 2012). This graph shows the time course of the moles of H<sub>2</sub> per anaerobic HEPES buffer solution (y<sub>H2</sub>) in the darkness, oxygen-free and nitrate-free are reported to be prerequisites for H<sub>2</sub> production. This work proposed a following kinetic model for H<sub>2</sub> production:

$$r_{H_2} = \frac{dy_{H_2}}{dt} = q_{H_2} X \quad (2.2),$$

$$q_{H_2} = k_1 m_{RED} \quad (2.3),$$

and

$$k_1 = k_0 \sigma_{HG} \quad (2.4).$$

For initiating a kinetic approach, following models were also assumed:

$$\frac{dm_{RED}}{dt} = (a_1 k_G m_G + a_2 K_d - k_1 m_{RED}) + K_d m_{RED} \quad (2.5),$$

$$m_G = m_{G0} \exp \{ (K_d - k_G) t \} \quad (2.6)$$

and

$$X = X_0 \exp(-K_d t) \quad (2.7)$$

in which  $r_{H_2}$  and  $q_{H_2}$  are the  $H_2$  production rate and specific  $H_2$  production rate, respectively,  $m_{RED}$  is the number of moles of reductant(s) NAD(P)H per dry weight,  $m_G$  is the number of moles of endogenous glucose per dry weight,  $\sigma_{HG}$  is the specific activity of NiFe-hydrogenase,  $X$  is the dry cell weight concentration,  $m_{G0}$  and  $X_0$  are the initial values of  $m_G$  and  $X$ , respectively,  $k_0$  is the rate constant of second order reaction,  $k_1$  is the rate constant of pseudo-first order reaction and  $K_d$  is the specific death rate. Broken line in Fig.2.4 shows the calculated value of  $y_{H_2}$  in the work of Yamamoto that fits well with experimental data. Despite this fact, further work is required since Eq.(2.5) assumed the reductants for NiFe-hydrogenase are supplied only by glycolysis and cell death, and Eq.(2.6) assumed the exponential decline of the amount of endogenous glucose with time.

This study assumes that reductants NADH and NADPH are supplied by reduction of oxidants  $NAD^+$  and  $NADP^+$ , that reductants are assimilated by oxidation of reductants including NiFe-hydrogenase reaction, and that synthesis and decomposition of  $NAD(P)^+$  plus NAD(P)H are absent under dark anaerobic incubation. These assumption lead the following equations instead of Eq.(2.5), when dry cell weight concentration decreases with time at the specific death rate of  $K_d$  (Eq.(2.7)):

$$\begin{aligned} \frac{dm_{RED}}{dt} &= (-k_{RED} m_{RED} + k_{OX} m_{OX} - k_1 m_{RED}) + K_d m_{RED} \\ &= k_{OX} m_T - (k_{RED} + k_{OX} + k_1 - K_d) m_{RED} \end{aligned} \quad (2.8)$$

where

$$m_T = m_{RED} + m_{OX} = \text{constant} \quad (2.9),$$

in which  $m_{OX}$  is the number of moles of oxidants per dry weight, and  $m_T$  is the total amounts of NAD(P)H and  $NAD(P)^+$  per dry cell weight. Eq.(2.8) is the same as Eq.(2.5) under the stoichiometric relations for redox reaction.

When, right after dark anaerobic incubation, cell grows with the specific growth rate of  $\mu$  as:

$$X = X_0 \exp(\mu t) \quad (2.10),$$

Eq.(2.8) is replaced by:

$$\begin{aligned}\frac{dm_{RED}}{dt} &= (-k_{RED}m_{RED} + k_{OX}m_{OX} - k_1m_{RED}) - \mu m_{RED} \\ &= k_{OX}m_T - (k_{RED} + k_{OX} + k_1 + \mu)m_{RED}\end{aligned}\quad (2.11).$$

During H<sub>2</sub> production, the term  $k_{RED}+k_{OX}+k_1$  is apparently not zero. The following two cases are assumed.

i) Case where  $k_{RED}+k_{OX}+k_1-K_d$  is not zero (cell population effect is large).

Eq.(2.8) or (2.11) results in that

$$\begin{aligned}m_{RED} &= \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 - K_d} m_T \\ &+ (m_{RED,0} - \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 - K_d} m_T) \exp\{-(k_{RED} + k_{OX} + k_1 - K_d)t\}\end{aligned}\quad (2.12)$$

or

$$\begin{aligned}m_{RED} &= \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 + \mu} m_T \\ &+ (m_{RED,0} - \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 + \mu} m_T) \exp\{-(k_{RED} + k_{OX} + k_1 + \mu)t\}\end{aligned}\quad (2.13)$$

Eq.(2.12) or (2.13) results in that

$$\begin{aligned}\frac{dy_{H_2}}{dt} &= k_1 m_{RED} X = k_1 m_T X_0 \left[ \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 - K_d} \exp(-K_d t) \right. \\ &+ \left. \left( \frac{m_{RED,0}}{m_T} - \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 - K_d} \right) \exp\{-(k_{RED} + k_{OX} + k_1)t\} \right]\end{aligned}\quad (2.14)$$

or

$$\begin{aligned}\frac{dy_{H_2}}{dt} &= k_1 m_{RED} X = k_1 m_T X_0 \left[ \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 + \mu} \exp(\mu t) \right. \\ &+ \left. \left( \frac{m_{RED,0}}{m_T} - \frac{k_{OX}}{k_{RED} + k_{OX} + k_1 + \mu} \right) \exp\{-(k_{RED} + k_{OX} + k_1)t\} \right]\end{aligned}\quad (2.15).$$

From Eq.(2.14) or Eq.(2.15), it is found that

$$r_{H_2,0} = k_1 m_{RED,0} X_0 \quad (2.16).$$

Eq.(2.16) is constructed to support an idea that H<sub>2</sub> production at early state in dark anaerobic condition is very much dependent to initial reductive compound ( $m_{RED,0}$ ). This initial reductive compound sets the value of initial H<sub>2</sub> production rate ( $r_{H2,0}$ ) which is highest at the beginning then declines over time. Eq.(2.14) or Eq.(2.15) suggests that H<sub>2</sub> production rate decreases with time from the initial value shown by Eq.(2.16) and approaches to zero, hence it can be approximated to:

$$\frac{dy_{H2}}{dt} \approx r_{H2,0} \exp(-kt) \quad (2.17).$$

in which  $k$  is a model parameter which is constant value assumed to be all factors involved in H<sub>2</sub> production, presumably changes of reductive and oxidative compounds, cell death or growth ( $k_{RED}$ ,  $k_{OX}$ ,  $k_1$ ,  $K_d$  or  $\mu$ ). If  $k$  is not zero, integrating Eq.(2.17) results in that

$$\begin{aligned} y_{H2} &= \frac{r_{H2,0}}{k} \{1 - \exp(-kt)\} \\ &= y_{H2,f} \{1 - \exp(-kt)\} \end{aligned} \quad (2.18).$$

The attainable level of H<sub>2</sub> production ( $y_{H2,f}$ ) can now be predicted by initial H<sub>2</sub> production rate and deactivation constant.

ii) Case where  $k_{RED}+k_{OX}+k_1$  is extremely greater than  $K_d$  or  $\mu$  (cell population effect is small).

Eq.(2.12) or (2.13) results that

$$m_{RED} = \frac{k_{OX}}{k_{RED} + k_{OX} + k_1} m_T \quad (2.19).$$

The H<sub>2</sub> production rate is given by:

$$\frac{dy_{H2}}{dt} = k_1 m_{RED} X = k_1 m_T X_0 \frac{k_{OX}}{k_{RED} + k_{OX} + k_1} = k_1 m_{RED,0} X_0 \quad (2.20).$$

The initial H<sub>2</sub> production rate of this equation satisfies Eq.(2.16).

$$y_{H2} = r_{H2,0} t \quad (2.21).$$

Solid line in Fig.2.4 shows the  $y_{H_2}$  value estimated by Eq.(2.18) with  $r_{H_2,0}$  at  $0.0310 \mu\text{mol mL}^{-1}\text{h}^{-1}$  and  $k$  at  $0.0108 \text{ h}^{-1}$ . Even though this model contains only two parameters  $r_{H_2,0}$  and  $k$ , simulated curve can reproduce the experimental results satisfactory. Hence experimental data of  $H_2$  production in this work is interpreted by Eqs. (2.18) and (2.21).

If NiFe-hydrogenase in *Synechocystis* sp. strain PCC 6803 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  $H_2$  production is enhanced by reducing sugar supplementation under nitrate limiting condition. The effect of exogenous electron donors in terms of reducing sugars such as glucose, fructose, galactose, mannose and xylose upon the  $H_2$  production in GT strain in the dark anaerobic nitrate free condition is analyzed utilizing Eq.(2.20).

## 2.2 Materials and Methods

### 2.2.1 Culture technique and fermentation

A glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 (GT strain) was utilized in the experiments of this chapter. For both pre-culture and main culture, clear cylindrical photobioreactors (pyrex glass made; inner diameter, 27 mm; outer diameter, 29 mm) were utilized. Single nozzle sparger (inner diameter, 4 mm; outer diameter, 8 mm) was equipped for gas sparging. For pre-culture, cells were grown photoautotrophically for 3 days in BG-11 medium (**Table 2.2**), initial pH 7.8, at  $34^\circ\text{C}$ , aerated by air containing 6%  $\text{CO}_2$  with a flow rate of  $80 \text{ mL min}^{-1}$ , illuminated by fluorescent lamps with photosynthetic photon flux density (PPFD) at  $100 \mu\text{mol-photons m}^{-2} \text{ s}^{-1}$ . Superficial gas velocity was  $8.39 \text{ m h}^{-1}$ .

Dry cell weight concentration ( $X$ ) was monitored by measuring  $\text{OD}_{730}$ : optical density at 730 nm. One unit absorbance was equivalent to  $0.369 \text{ mg mL}^{-1}$ . When  $X$  increases with time, growth rate ( $r_x$ ) and specific growth rate ( $\mu$ ) are calculated as:

$$r_x = \frac{dX}{dt} = \mu X \quad (2.22)$$

When  $X$  decreases with time, death rate ( $-r_x$ ) and specific death rate ( $K_d$ ) are calculated as:

$$(-r_x) = -\frac{dX}{dt} = K_d X \quad (2.23)$$

Pre-culture cells were grown for 3 d to logarithmic growth phase before transferring to main culture. The main culture used the same condition as pre-culture. Cells of late-logarithmic growth phase in main culture ( $OD_{730}$ , 7) were collected by centrifugation at 3000 rpm and 25 °C for 10 min. The cell pellets were washed by 50  $\mu\text{mol mL}^{-1}$  HEPES buffer solution (pH 7.8) and collected by centrifugation to remove nitrate. Cells were re-suspended and incubated with or without 28  $\mu\text{mol mL}^{-1}$  glucose, fructose, galactose, mannose or xylose in 10 mL of 50  $\mu\text{mol mL}^{-1}$  HEPES buffer solution (pH 7.8) in 32 mL glass test tube with butyl rubber cap. The initial dry cell weight concentration was set at  $2.13 \pm 0.06 \text{ mg mL}^{-1}$  and cell suspension was purged with  $\text{N}_2$  gas for a few minutes to remove oxygen molecules. Incubation was carried out under dark anaerobic conditions with shaking at 145 rpm on a reciprocating shaker. The reciprocating distance was 40 mm and the horizontal angle was about 30°.

### 2.2.2 Measurement of $\text{H}_2$ production

The amount of molecular  $\text{H}_2$  in the gas phase in 32 mL test tube was monitored by withdrawing 500  $\mu\text{L}$  sample and measuring with 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°C; injector temperature, 45°C; detector temperature 80°C) with nitrogen gas as the carrier gas. The number of moles of  $\text{H}_2$  per culture volume ( $y_{\text{H}_2}$ ) is first analyzed, and then the rate of  $\text{H}_2$  production per culture volume ( $r_{\text{H}_2}$ ) and the specific rate of  $\text{H}_2$  production ( $q_{\text{H}_2}$ ) were calculated as:

$$r_{\text{H}_2} = \frac{dy_{\text{H}_2}}{dt} \quad (2.24)$$

$$q_{\text{H}_2} = \frac{1}{X} \frac{dy_{\text{H}_2}}{dt} = \frac{r_{\text{H}_2}}{X}$$

### 2.2.3 Determination of metabolic product distribution

Metabolites in a 500  $\mu\text{L}$  portion of supernatants were analyzed by HPLC (JASCO PU-2080 Plus and UV-2075 Plus). Mobile phase was 18  $\mu\text{mol mL}^{-1}$   $\text{KH}_2\text{PO}_4$ , pH of which was adjusted at 2.3 with  $\text{H}_3\text{PO}_4$ . Flow rate of mobile phase was  $0.7 \text{ mL min}^{-1}$ . Column temperature was 30°C. A 10  $\mu\text{L}$  of supernatant

sample was injected by using 10  $\mu\text{L}$  syringes. The wave length of 210 nm was used for detection of metabolites. To confirm molecular mass of acid, sample was analyzed by a liquid chromatography-electrospray ionization-time-of-flight-mass spectrometry (LC-ESI-TOF-MS) (microTOF II , Bruker Daltonics Co.) utilizing 1% formate as mobile phase. LC column of the system was XBridge C18 HPLC Column 3 $\times$ 150 mm 3.5  $\mu\text{m}$ .

The concentrations of lactate ( $c_L$ ), acetate ( $c_A$ ), and succinate ( $c_{SUC}$ ) were first analyzed, and then the rate of production of lactate, acetate and succinate per culture volume ( $r_L, r_A, r_{SUC}$ ) were calculated as:

$$r_L = \frac{dc_L}{dt}, r_A = \frac{dc_A}{dt}, r_{SUC} = \frac{dc_{SUC}}{dt} \quad (2.25).$$

When metabolite is consumed, its consumption rates are calculated as:

$$(-r_L) = -\frac{dc_L}{dt}, (-r_A) = -\frac{dc_A}{dt}, (-r_{SUC}) = -\frac{dc_{SUC}}{dt} \quad (2.26).$$

#### 2.2.4 Measurement of reducing sugar consumption

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,  $\phi 6.0 \times 150$  mm (Showa Denko Co.). Column temperature was set at 50°C. Mobile phase was 70% acetonitrile. Mobile phase flow rate was 0.7 mL  $\text{min}^{-1}$ . A 10  $\mu\text{L}$  of supernatant sample was injected by using a 10  $\mu\text{L}$  syringe.

The concentration of unreacted monosaccharide ( $c_S$ ) is first analyzed, and then the rate of monosaccharide consumption rate ( $-r_S$ ) and the specific rate of monosaccharide consumption ( $q_S$ ) was calculated as:

$$\begin{aligned} (-r_S) &= -\frac{dc_S}{dt} \\ q_S &= -\frac{1}{X} \frac{dc_S}{dt} = \frac{(-r_S)}{X} \end{aligned} \quad (2.27)$$

#### 2.2.5 Measurement of endogenous glycogen consumption and production

The amount of glycogen per culture volume was determined as follows: cell pellets were collected by centrifugation at 25°C, 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 in 50 µL of 6 N HCl at 80°C for 30 min; glucose amount was determined by using Glucose CII Test Wako (Wako Pure Chemical Ind., Ltd.).

The number of moles of endogenous glucose per dry cell weight is shown by  $m_G$ . The number of moles of endogenous glucose per culture volume ( $m_G X$ ) is first analyzed, and then, when  $m_G X$  decreases with time, the consumption rate of endogenous glucose ( $-R_G X$ ) was calculated as:

$$(-R_G X) = -\frac{d(m_G X)}{dt} \quad (2.28).$$

When  $m_G X$  increases with time, the generation rate of endogenous glucose ( $R_G X$ ) was calculated as:

$$R_G X = \frac{d(m_G X)}{dt} \quad (2.29).$$

In Eqs.(2.28) and (2.29), ( $-R_G$ ) and  $R_G$  show the specific consumption rate and specific production rate of endogenous glucose, respectively.

## 2.3 Results and discussion

The LC-ESI-TOF-MS and HPLC analysis of supernatant of cell suspended dark anaerobic HEPES buffer solution with glucose confirmed production of acetate, lactate and succinate. MS analysis has suggested that fumarate and malate possibly presented in buffer solution. Main metabolic products of the dark incubated culture of the runs with or without monosaccharide other than glucose were identified by GC and HPLC to be  $H_2$ , acetate, lactate and succinate as shown in **Figure 2.5**.

Culture variables were monitored. **Figure 2.6** shows the time courses of dark anaerobic incubation variables measured in runs without monosaccharide (○) and with glucose (■), fructose (▲), galactose (●), mannose (◆) and xylose (▼). Quantities plotted include (a) the mole of  $H_2$  per culture volume ( $y_{H_2}$ ), (b) the concentration of exogenous reducing sugars ( $c_s$ ), (c) the amount of endogenous glycogen per culture volume ( $c_G$ ), (d) the concentration of cell mass ( $X$ ), (e) pH of cell suspension, (f) the concentration of acetate, (g) the concentration of lactate and (h) the concentration of succinate

Figure 2.6(a) shows that the production of H<sub>2</sub> starts right after inoculation. Prior to dark anaerobic incubation, cells of GT strain were photoautotrophically grown on BG-11 medium in the presence of oxygen. NiFe-hydrogenase of GT strain is found to be synthesized in the presence of oxygen. This observation differs from NiFe-hydrogenase synthesis in *Escherichia coli*, transcription of which is reported to be blocked by oxygen (Richard et al., 1999). Our observation is supported by previous works (Appel et al., 2000; Gutekunst et al., 2005). In WT strain, higher levels of NiFe-hydrogenase transcripts have been detected under micro-aerobic or anaerobic conditions. These works suggest that under the photoautotrophic culture, NiFe-hydrogenase transcripts are prepared but blocked for expression by oxygen. H<sub>2</sub> production rate ( $r_{H_2}$ ) is highest at initial and clearly decelerated with time, hence time course of  $y_{H_2}$  can be fitted by Eq.(2.18). NiFe-hydrogenase is inhibited to produce H<sub>2</sub> by oxygen during photosynthesis, is found to be active when cells are inoculated into oxygen-free dark environment.

The data for H<sub>2</sub> production ( $y_{H_2}$ ) are well fitted by Eq.(2.18) as shown in the dotted lines of Fig 2.5a. No data in this graph represents the time course of  $y_{H_2}$  that is suggested by Eq.(2.21). Not only H<sub>2</sub> production in GT strain in HEPES buffer solution without monosaccharides, that with glucose, fructose, galactose, mannose and xylose can be fitted by Eq.(2.18).

**Figure 2.7** shows the relation between  $y_{H_2,1}$  and  $y_{H_2,f}$ . There seems to be a relation shown by:

$$y_{H_2,f} = k_2 y_{H_2,1}^{k_3} \quad (2.30)$$

Best fits to the data results that the parameters are  $k_2, 1.47 \mu\text{mol mL}^{-1}$ ; and  $k_3, 0.752$ .

In this study, subscript 0 and f show the initial state and the final state, respectively. Although the state of inocula are kept as constant as possible, the initial rate  $r_{H_2,0}$ , calculated from Eq.(2.18), is affected by variation in reducing sugars. Calculated data has been tabulated in **Table 2.3**. If the initial rate  $r_{H_2,0}$  represents the reductive state of the inocula, reductive potential is found to be highest in glucose, second highest in fructose, third highest in galactose, fourth highest in mannose and lowest in xylose. The reductive potential of cells in buffer solution without monosaccharide is lower than that in xylose. The attainable level of amount of H<sub>2</sub> per culture volume ( $y_{H_2,f}$ ) is highest at  $3.26 \mu\text{mol mL}^{-1}$  in the solution containing glucose, which is 1.50 times that without reducing sugar.

To investigate the relation between initial cellular content of NAD(P)H and attainable level of the number of moles of H<sub>2</sub> per culture volume, relative parameters are defined as:

$$y_{H_2}^* = \frac{y_{H_2}}{(y_{H_2,f})_{ref}}, q_{H_2}^* = \frac{q_{H_2}}{(q_{H_2,0})_{ref}}, m_{RED}^* = \frac{m_{RED}}{(m_{RED,0})_{ref}}, X^* = \frac{X}{(X_0)_{ref}},$$

$$y_{H_2,f}^* = \frac{y_{H_2,f}}{(y_{H_2,f})_{ref}}, q_{H_2,0}^* = \frac{q_{H_2,0}}{(q_{H_2,0})_{ref}}, m_{RED,0}^* = \frac{m_{RED,0}}{(m_{RED,0})_{ref}}, X_0^* = \frac{X_0}{(X_0)_{ref}} \quad (2.30)$$

in which subscript ref specifies the results of reference run that, throughout this thesis, is defined as the dark anaerobic H<sub>2</sub> production in GT strain in HEPES buffer solution without monosaccharide that is performed with cells from the cell suspension of OD<sub>730</sub> at 7 when cells are in late-logarithmic growth phase in photoautotrophic cultures.

**Figure 2.8** is a plot similar to the Hammett equation (Hammett, 1937). Data of Table 2.2 are plotted. The result shows that exposure of inoculum cells to glucose, fructose and galactose elevates the reductive potential of GT cells ( $q_{H_2,0}^* > 1$ ), and that

$$y_{H_2,f}^* > q_{H_2,0}^* \quad (2.31)$$

for runs with glucose, fructose and galactose. Dark anaerobic H<sub>2</sub> production by inoculum with higher reductive state is found to shift up the attainable level of H<sub>2</sub>. This plot also shows that

$$y_{H_2,f}^* < q_{H_2,0}^* \quad (2.32)$$

for runs with mannose and xylose. The plots in Fig.2.7 with Eq.(2.3) lead to the following relation:

$$y_{H_2,f}^* = (q_{H_2,0}^*)^{k_4} \quad (2.33).$$

In a preliminary experiment utilizing methyl viologen (dimethyl-4,4'-bipyridim dichloride), H<sub>2</sub> production rate of GT strain is found to be independent from NiFe-hydrogenase activity. Hence, from Eq.(2.4), rate constant  $k_1$  is assumed to be constant. This assumption leads to

$$m_{RED,0}^* = q_{H_2,0}^* \quad (2.34).$$

The linear line in Fig.2.7 without run with mannose results in that  $k_4$  is 0.619. This relation shows that the attainable level of H<sub>2</sub> can be estimated by the number of mole of reductive compounds right after the inoculation of cells of GT strain into dark anaerobic HEPES buffer solution without or with monosaccharides.

To elucidate the effect of reductive compounds on H<sub>2</sub> production, additional experiments were performed in which dark anaerobic incubation of GT strain was performed in HEPES buffer solution with reductive compound 5 μmol mL<sup>-1</sup> FeCl<sub>3</sub> or 5 μmol mL<sup>-1</sup> ascorbic acid sodium. H<sub>2</sub> production of GT strain was not affected by FeCl<sub>3</sub> or ascorbic acid sodium. Effect of non-reductive compound is also studied by adding 28 μmol mL<sup>-1</sup> disaccharide sucrose to HEPES buffer solution. The initial H<sub>2</sub> production rate of this was comparable with that of run without monosaccharide (data not shown). The results of experiments in this chapter negates the working assumption that effect of reducing sugar for H<sub>2</sub> production is due to the effect of the increase in reductive potential in the cellular environment.

Numerically, deactivation constants for H<sub>2</sub> production ( $k$ ) in runs with glucose, fructose and galactose are nearly the same and higher than that in runs with mannose and xylose and without monosaccharide. Roughly dark anaerobic incubation of high  $r_{H_2}$  production results in high  $k$  value. Oxidation of NAD(P)H by NiFe-hydrogenase generates not only H<sub>2</sub> but also NAD(P)<sup>+</sup> that appears to inactivate NiFe-hydrogenase.

Combination of Eqs.(2.26),(2.28) and (2.31) results in that

$$m_{RED}^* = \frac{m_{RED}}{(m_{RED,0})_{ref}} = \frac{m_{RED,0}}{(m_{RED,0})_{ref}} \frac{m_{RED}}{m_{RED,0}} = \frac{q_{H_2,0}}{(q_{H_2,0})_{ref}} \exp(-kt) \quad (2.35).$$

$$= q_{H_2,0}^* \exp(-kt)$$

**Figure 2.9** is the time courses of calculated  $m_{RED}^*$  utilizing Eq.(2.34) and parameters  $q_{H_2,0}^*$  and  $k$  in Table 2.3. This graph shows that inoculum cells in glucose, fructose and galactose have high reductive potential at the beginning of dark anaerobic incubation, and rapidly consumes those compounds. Eq.(2.34) is important since  $m_{RED}$  is a key parameter representing the redox potential of cells in dark anaerobic incubation.

Amount of reductive compounds per culture volume is given by

$$m_{RED}^* X^* = q_{H_2,0}^* \exp(-kt) X_0^* \exp(-K_d t) \quad (2.36).$$

$$= q_{H_2,0}^* X_0^* \exp\{-(k + K_d)t\}$$

The amount of H<sub>2</sub> per culture volume is shown by

$$\begin{aligned}
y_{H_2}^* &= \frac{y_{H_2}}{(y_{H_2,f})_{ref}} = \frac{\frac{q_{H_2,0} X_0}{k} \{1 - \exp(-kt)\}}{\left(\frac{q_{H_2,0} X_0}{k}\right)_{ref}} \\
&= q_{H_2,0}^* X_0^* \{1 - \exp(-kt)\} \\
&= q_{H_2,0}^* X_0^* \left[1 - \left\{\frac{1}{q_{H_2,0}^*} m_{RED}^*\right\}^{k/(k+K_d)}\right]
\end{aligned} \tag{2.37}$$

**Figure 2.10** is the plots of  $y_{H_2}^*$  against  $m_{RED}^*$  which are estimated by Eq.(2.35) and parameters  $q_{H_2,0}$  and  $k$  in Table 2.2. The amount of  $H_2$  and the amount of reductive compound are related by this equation.

When dark anaerobic incubation is performed in  $28 \mu\text{mol mL}^{-1}$  monosaccharide solution, during the first 48 h, cells of GT strain are unable to assimilate exogenous reducing sugars other than glucose (Fig. 2.6(b)). Despite the fact that glucose and fructose share common transport system in *Synechocystis* sp. strain PCC 6803 (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. Fructose has a potential to enter into the intracellular environment and reduce the redox potential inside the cells. However, fructose consumption curve suggests that this change blocks additional transport of fructose. Galactose transporter is not reported in previous works. Fig. 2.6(b) suggests that galactose is also transported into cell as fructose but mannose cannot utilize the glucose transporter.

The  $H_2$  production that is most active right after inoculation is found to be effected by monosaccharide as shown by  $q_{H_2,0}/(q_{H_2,0})_{w/o}$  in Table 2.3. Despite this fact, Fig.2.6 (b) represents quite low level of consumption of monosaccharides except for glucose. GT strain assimilates more than  $6 \mu\text{mol mL}^{-1}$  exogenous glucose assimilation in the first 24 h. The elevated production of  $H_2$  in the first 48 h of the run with fructose is independent from fructose uptake.

$H_2$  production is nearly always accompanied by glycogen degradation except for run with glucose (Fig. 2.6(c)). In all experiments, the initial amount of endogenous glycogen per culture volume was fixed at  $0.2 \pm 0.04 \mu\text{mol mL}^{-1}$ . The run without sugar shows that the decrease of glycogen from  $0.21 \mu\text{mol mL}^{-1}$  to  $0.06 \mu\text{mol mL}^{-1}$  results in the increase of  $y_{H_2}$  from zero to  $1.79 \mu\text{mol mL}^{-1}$  during 96 h. Decomposition of 1 mol glucose in glycogen and associated decomposition of other biomaterials are found to generate 12 mols of  $H_2$ . For run without monosaccharide, Eq.(2.6) is applicable as a rough approximation, while for runs with monosaccharides, Eq.(2.6) cannot interpret the change of endogenous glucose concentration. Approximated  $0.20 \mu\text{mol mL}^{-1}$  increase in endogenous glucose in glycogen is observed in the first 24 h of

run with glucose. Decrease in endogenous glucose in glycogen of the cell in 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.

Contact of exogenous monosaccharide with cells of GT strain is found to trigger to activate enzymes in endogenous glucose decomposition pathways. The highest final  $y_{H_2,f}$  is seen in run with glucose. The comparable highest glycogen amount hence highest  $y_{H_2,f}$  is seen in run with fructose. Glucose 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,  $H_2$  production possibly proceeds for additional 96 h. Hence endogenous glycogen decomposition cannot make up the bulk source of the reductive potential for  $H_2$  production.

The dry cell weight concentration of the run with fructose is stable. Stability of dry cell weight in the presence of fructose 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  $20 \mu\text{mol mL}^{-1}$  fructose to evaluate the fructose toxic 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 dry cell weight concentration of run with glucose increases during the first 72 h with the specific growth rate of  $0.0038 \text{ h}^{-1}$ . The doubling time of this run is 182 h that is 0.4 times that observed on  $28 \mu\text{mol mL}^{-1}$  glucose in the previous work (Anderson and McIntosh 1991). The  $K_d$  values for run without monosaccharide, with xylose, and for runs after 48 h with galactose and mannose are 1.08, 2.49, 1.52 and  $2.13 \text{ h}^{-1}$ , respectively.,

The assimilated glucose appears to be utilized for glycogen synthesis (Fig. 2.6(c)), cell growth (Fig. 2.6(d)) and acid production (Fig. 2.6(f),(g),(h)). Galactose and mannose also support to keep cell viability in the first 48h, while cannot support cell survival after 48 h. Dry cell weight concentration decreases with time in xylose or in solution without reducing sugars.

The pH of runs without or with monosaccharide other than glucose decreases from 7.7 at initial to 7.5 at 96 h. The decreases in pH of these runs are undefined while that of run with glucose is remarkable. The pH of run with glucose drops linearly with time from 7.7 at initial to 6.8 at 96 h. The decrease of pH is a result of production of organic acids by cells.

Major metabolic products other than H<sub>2</sub> and CO<sub>2</sub> are lactate, acetate (Fig. 2.6(f)), lactate (Fig. 2.6(g)) and succinate (Fig. 2.6(h)). If the amount of total acid is defined by the summation of amounts of acetate, lactate and succinate, if the amount of total glucose is defined by the summation of amounts of exogenous glucose and endogenous glucose, then H<sub>2</sub> production, total acid production and total glucose consumption in the first 48 h of run with glucose are 2.29, 17.7, and 7.98 μmol mL<sup>-1</sup>, respectively. Complete theoretical conversion of glucose to these acids through only EMP pathway estimates the production of 16 μmol mL<sup>-1</sup> acids and H<sub>2</sub>. Hence it turns out that some portion of carbon in total acid is supplied from metabolisms other than glycolysis. Except for the run with glucose, the production of H<sub>2</sub> is very active during the first 48 h, whereas the production of lactate, acetate and succinate are extremely low.

A previous study reports that the accumulation of acetate, which is produced via pyruvate:flavodoxin/ferredoxin Oxidoreductase (PFOR), leads to increase reductive flavodoxin/ferredoxin to provide excess electrons for hydrogenase (Gutekunst *et al.* 2014). According to the previous work (Das and Veziroğlu 2001), the increase in acetate production leads the increase in NAD(P)H production, followed by the increase in H<sub>2</sub> production. This mechanism is different from our observation that H<sub>2</sub> production is high at the beginning of dark incubation where acetate production is low, and that H<sub>2</sub> production is inactivated when acetate production becomes active after 48h. In presence of fructose, electron supply to hydrogenase is not from acetate production via PFOR.

Metabolic product distribution analysis shows that succinate in incomplete tricarboxylic acid (TCA) cycle that lacks 2-oxoglutarate dehydrogenase is a major end-product on glucose fermentation. The supply of 2-oxoglutarate from glycolysis, decompositions of amino acids from proteins, membrane lipids and PHB in *Synechocystis* sp. strain PCC6803-GT by dark anaerobic shift with nitrate starvation results in a high level of succinate. The enzyme for this mechanism is not yet well elucidated (Cooley *et al.* 2000). *Synechococcus* sp. PCC 7002 possessing incomplete TCA cycle have succinic semialdehyde as a shortcut from the missing step (Zhang and Bryant 2011). Presence of all metabolites (malate, fumarate and succinate) in reductive branch of TCA cycle suggested NADH oxidation to elevate succinate in reductive TCA arm. The enzymes of reductive branch are phosphoenolpyruvate (PEP) carboxylase, malate dehydrogenase, fumarase and succinate dehydrogenase. The anoxic glucose-fermentation of *E.coli* also produced succinate by reductive TCA cycle. This result has suggested that TCA cycle had a role in anoxic glucose metabolism.

Except for run with glucose, the production of H<sub>2</sub> is very active during the first 48 h, whereas the production of lactate, acetate and succinate are extremely low. Hence early H<sub>2</sub> 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.

From Eq.(2.36), the relative consumption rate of reductive compounds ( $-r_{RED}^*$ ) is shown by

$$-r_{RED}^* = \frac{d}{dt}(m_{RED}^* X^*) = (k + K_d)q_{H2,0}^* X_0^* \exp\{-(k + K_d)t\} \quad (2.38)$$

for no cell growth case or

$$-r_{RED}^* = \frac{d}{dt}(m_{RED}^* X^*) = (k - \mu)q_{H2,0}^* X_0^* \exp\{-(k - \mu)t\} \quad (2.39)$$

for cell growth case.

Time course of reaction rates of run without monosaccharide is shown in **Figure 2.11**. Graph (a) shows the time course of NAD(P)H consumption rates  $r_{H2}$ ,  $R_{GX}$ ,  $r_X$ ,  $r_L$ ,  $r_A$ ,  $r_{SUC}$  and (b) NAD(P)H production rates,  $(-r_S)$ ,  $(-R_{GX})$ ,  $(-r_X)$ . The order of magnitude of NAD(P)H consumption rates is found to be higher than that of NAD(P)H production rate. Glycolysis provides about 10% of NAD(P)H for  $H_2$  production. The remaining amount of NAD(P)H is supplied by other pathways of which reactions are accompanied by glycolysis at the beginning of dark anaerobic incubation. Decrease in the consumption rate of endogenous glucose with time and decrease in the production of  $H_2$  with time are similar trends, however, NAD(P)H generated by glycolysis is insufficient to support the high rate of  $H_2$  production which less than 10 time. This has suggested that there is other pathways providing NAD(P)H to hydrogenase. The  $(-R_{GX})$  versus time data resemble that of the  $r_{SUC}$  versus time data. The  $r_L$  is smaller than  $(-R_{GX})$ .

**Figure 2.12** shows the time courses of (a) NAD(P)H consumption rates  $r_{H2}$ ,  $R_{GX}$ ,  $r_X$ ,  $r_L$ ,  $r_A$ ,  $r_{SUC}$  and (b) NAD(P)H production rates,  $(-r_S)$ ,  $(-R_{GX})$ ,  $(-r_X)$  in run with glucose. Decrease in the consumption rate of endogenous glucose with time and decrease in the production of  $H_2$ , acetate and succinate with time are similar. The NAD(P)H generated by glycolysis from uptake of exogenous glucose is comparable to that for supporting the high rate of  $H_2$  production, succinate production, acetate production. The  $r_{H2}$  is lower than  $(-r_S)$ , however, the  $r_{H2}$  of this run is similar to that of run with fructose of which  $(-r_S)$  is very small.

The  $r_{H_2}$  and the  $r_L$  are comparable order. The  $r_A$  and  $r_{SUC}/2$  are higher than  $r_L$  and comparable with  $(-r_S)$ . Dry cell weight increases for 72 h. The large portion of assimilated exogenous glucose appears to be converted to dry cell weight, succinate and acetate. Lower amount appears to be converted to lactate and in early stage to endogenous glucose.

**Figure 2.13** shows the results of run with fructose. The  $r_{H_2}$  is higher than that of run without monosaccharide. The  $r_A$ ,  $r_L$  and  $r_{SUC}$  are not zero but lower than those shown in Fig.2.11. The  $(-r_G)$  is smaller than  $r_{H_2}$ , however, there seems to be a cross talk between endogenous glucose consumption and  $H_2$  production. Reductive compounds for  $H_2$  production is assumed to be supplied not by glycolysis of exogenous glucose and endogenous fructose consumption but by decomposition of other macro molecules.

**Figure 2.14** shows the results of run with galactose. The relation between reaction rates of this run is similar to that shown in Fig.2.13, however,  $r_{H_2}$  is lower than that of run with fructose. The  $r_{SUC}$  versus time data resemble to the  $(-r_S)$  versus time data. Acetate production seems to be the main carbon sinks from galactose as they show the same consumption and production.

**Figure 2.15** shows the results of run with mannose. The relation between reaction rates of this run is similar to that shown in Fig.2.13, however,  $r_{H_2}$  is lower than that of run with fructose. The  $(-r_S)$  for mannose is extremely high so that supply of NAD(P)H from mannose appears to support the need for  $H_2$  production.

**Figure 2.16** shows the results of run with xylose. The relation between reaction rates of this run is similar to that shown in Fig.2.14.

As viewed on Figs. 2.11-2.16, there seems to be an indirect relation between decomposition of endogenous glucose and  $H_2$  production. Glycolysis appears to be a marker reaction for global generation of NAD(P)H. Eq.(2.33) implies that the initial cellular content of NAD(P)H defines the attainable level of the number of moles of  $H_2$  per culture volume. If the initial cellular content of NAD(P)H also defines the attainable level of the number of moles of endogenous glucose per culture volume  $((m_G X)_f)$ , then Eq.(2.33) suggests that the attainable level  $y_{H_2,f}$  is able to be referred to  $(m_G X)_f$ . After 48h, no remarkable variation is seen in  $m_G$  and  $X$  with time and  $y_{H_2,f}$  is able to be referred to  $y_{H_2,1}$  (Fig.2.7), hence, instead of studying a relation in a pair of  $(m_G X)_f$  and  $y_{H_2,f}$ , a relation in a pair of  $(m_G X)_1$  and  $y_{H_2,1}$  is elucidated.

**Figure 2.17** shows plots of  $y_{H_2,1}$  against  $(m_G X)_1$ . It is found that the increase in the number of moles of  $H_2$  per culture volume at 96 h is accompanied by the increase in the number of moles of endogenous glucose per culture volume at 96 h. Solid line in the graph is correlated by

$$y_{H_2,1} = k_5(m_G X)_1^{k_6} \quad (2.40)$$

in which subscript 1 shows the state at 96 h, and  $k_5$  and  $k_6$  are model constants. Curve fitting results in that the parameters are  $k_5, 5.21$ ; and  $k_6, 0.379 \mu\text{mol}^{0.621} \text{mL}^{-0.621}$ . To keep high level of amount of endogenous glucose per culture volume is found to be important to increase  $\text{H}_2$  production.

Since there are several dehydrogenase-pathways involved in dark anaerobic metabolism by *Synechocystis* sp. strain PCC6803, this study focused only on measurable parameters which are believed to be important. Observation of monitored parameters suggests that main mechanisms for NAD(P)H production are decomposition of dry cell weight, degradation of glycogen and turnover of other biochemical compounds in run without monosaccharides. If available monosaccharide exists in cellular environment, its consumption also contributes to the main mechanism of NAD(P)H production. Major consumption of NAD(P)H is  $\text{H}_2$  production on NiFe-hydrogenase in runs without monosaccharide, with mannose or xylose. Acetate production is the main NAD(P)H consumption in runs with glucose, fructose and galactose. The most interesting point is the highest succinate production when glucose is present. Succinate appears to be an important electron sink for dark anaerobic glucose metabolism. Succinate production is peculiar to glucose fermentation and it doesn't occur from the assimilation of other monosaccharides that results acetate as an electron sink. The amount of lactate produced in runs w/o monosaccharide, with fructose, galactose, mannose, xylose are comparable except for run with glucose. Lactate production is not affected by monosaccharide. Carbon sources for lactate are found to be intracellularly accumulated compounds.

**Figure 2.18** shows a proposal of conceivable mechanism of dark  $\text{H}_2$  production of GT strains in nitrate free solution. Before dark incubation, GT strains are grown on  $\text{CO}_2$  under the light and the bulk of chemical energy is stored in glycogen, protein, membrane lipid and PHB (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.

## 2.4 Summary

The dark anaerobic H<sub>2</sub> production of GT strain in HEPES buffer solution without or with monosaccharide (glucose, fructose, galactose, mannose or xylose) has been studied by following the time courses of the number of moles of H<sub>2</sub> per solution, the concentration of unreacted monosaccharide, the number of moles of endogenous glucose, and concentrations of dry cell weight, lactate, acetate and succinate. The highlights of this chapter are as follows.

1. The addition of exogenous reducing sugar to dark anaerobic HEPES buffer solution is useful to increase initial H<sub>2</sub> production activity of NiFe-hydrogenase in GT strain.
2. A mathematical model to interpret dark anaerobic H<sub>2</sub> production kinetics was formulated.
3. The H<sub>2</sub> production of GT strain in dark anaerobic HEPES buffer solution is found to be elevated by limiting glycogen decomposition, cell breakage and hydrogenase deactivation under stresses (nitrate deprivation and dark anaerobic). The more glycogen storage, the more H<sub>2</sub> production was observed.
4. Relations between the attainable level of the number of moles of H<sub>2</sub> per culture volume and the initial H<sub>2</sub> production rate, and between the final level of the number of moles of H<sub>2</sub> per culture volume and the final level of the number of moles of endogenous glucose per culture volume are developed. The H<sub>2</sub> production of GT strain in dark anaerobic HEPES buffer solution is found to be elevated by limiting glycogen decomposition, cell breakage and hydrogenase deactivation under stresses (nitrate deprivation and dark anaerobic). The shift-up the amount of stored glycogen is of utmost importance to elevate H<sub>2</sub> production
5. Glucose and fructose are characterized as monosaccharides that bring cells to produce higher amount of H<sub>2</sub> per culture volume. Different from glucose, fructose elevates attainable level of H<sub>2</sub> without assimilation of fructose. The mechanism by which these monosaccharides exact this effect remains to be elucidated.
6. Reductive wing of incomplete TCA cycle is important in mixotrophic growth under dark anaerobic condition.
7. A large portion of electrons for H<sub>2</sub> production on NiFe-hydrogenase are supplied not only by glycolysis to generate acetate but also by other degradation reactions, the progress of which can be roughly monitored by that of glycolysis. Glycolysis is available as a marker reaction of global decomposition of cell constituting materials after incubation of cells into dark anaerobic HEPES buffer solution with or without monosaccharide.

**Table 2.1** Physical property data of monosaccharides.

$\Delta H_f^0$ : Enthalpy of formation of solid at standard conditions

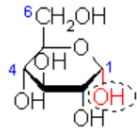
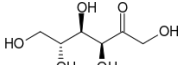
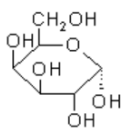
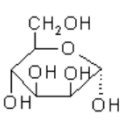
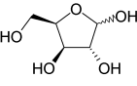
	Glucose	Fructose	Galactose	Mannose	Xylose
Chemical structure					
Molar mass [ $\mu\text{g } \mu\text{mol}^{-1}$ ]	180.16	180.16	180.16	180.16	150.13
$\Delta H_f^0$ [mJ $\mu\text{mol}^{-1}$ ]	-1274.43(aq.; $\alpha$ -D-glucose	-1265.6(S)	-1271.50	-1263.4(S)	-750.5

Table 2.2 Composition of BG-11 medium

	Compound	Amount per milliliter
Stock1	NaNO <sub>3</sub>	1.5 mg
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.075mg
Stock2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.036mg
Stock3	Citric acid	0.00656mg
	Ferric ammonium	0.006mg
	EDTA·2Na	0.00114mg
Stock4	Trace metal mix A5	1.0μL
Stock5	K <sub>2</sub> HPO <sub>4</sub>	0.04mg
Stock6	Na <sub>2</sub> CO <sub>3</sub>	0.02mg

Trace metal mix A5

Compound	Amount per milliliter
H <sub>3</sub> BO <sub>3</sub>	2.86mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.81mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.222mg
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.39mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.079mg
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	0.0494mg

Table 2.3 Kinetic parameters for H<sub>2</sub> production in GT strain in HEPES buffer solution without or with reducing sugars.  $q_{H2,0}^*$ ,  $q_{H2,0}/(q_{H2,0})_{w/o}$ ;  $y_{H2,f}^*$ ,  $y_{H2,f}/(y_{H2,f})_{w/o}$ ; and  $k^*=k/(k)_{w/o}$ .  $y_{H2,1}$ ,  $c_{L1}$ ,  $c_{A1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 96 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 96h;  $\Delta(m_G X)_1$ , observed decrease in  $m_G X$  during 96 h H<sub>2</sub> production;  $Y_{H2/G}=y_{H2,1}/\Delta(m_G X)_1$

	w/o	Glucose	Fructose	Galactose	Mannose	Xylose
$X_0$ [mg·mL <sup>-1</sup> ]	2.09	2.17	2.14	2.06	2.13	2.15
$r_{H2,0}$ [μmol·mL <sup>-1</sup> ·h <sup>-1</sup> ]	0.0405	0.0730	0.0674	0.0527	0.0369	0.0305
$q_{H2,0}$ [μmol·mg <sup>-1</sup> ·h <sup>-1</sup> ]	0.0194	0.0336	0.0315	0.0256	0.0173	0.0142
$q_{H2,0}^*$ [-]	1	1.73	1.62	1.32	0.892	0.732
$y_{H2,f}$ [μmol·mL <sup>-1</sup> ]	2.18	3.26	3.05	2.33	2.51	2.06
$y_{H2,f}^*$ [-]	1	1.50	1.40	1.07	1.15	0.945
$k$ [h <sup>-1</sup> ]	0.0186	0.0224	0.0221	0.0226	0.0147	0.0148
$k^*$ [-]	1	1.20	1.19	1.22	0.790	0.796
$K_d$ [h <sup>-1</sup> ]	0.0011	0	0	0.0015	0.0019	0.0025
$y_{H2,1}$ [μmol·mL <sup>-1</sup> ]	1.79	2.81	2.62	2.03	1.85	1.52
$m_{G1}$ [μmol·mg <sup>-1</sup> ]	0.032	0.080	0.072	0.050	0.037	0.023
$X_1$ [mg·mL <sup>-1</sup> ]	1.86	2.55	2.12	1.77	1.68	1.65
$(m_G X)_1$ [μmol·mL <sup>-1</sup> ]	0.0595	0.204	0.153	0.0885	0.0622	0.0380
$c_{L,1}$ [μmol·mL <sup>-1</sup> ]	0.258	3.12	0.210	0.225	0.293	0.235
$c_{A,1}$ [μmol·mL <sup>-1</sup> ]	0.208	15.3	3.02	1.50	0.479	0.507
$\Delta(m_G X)_1$ [μmol·mL <sup>-1</sup> ]	0.151	-0.025	0.076	0.075	0.156	0.154
$Y_{H2/G}$ [-]	11.8	-	34.6	27.3	11.9	9.9

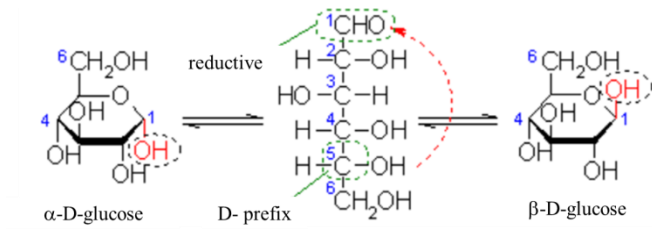


Figure 2.1 Conversion of acyclic forms and glucopyranososes.

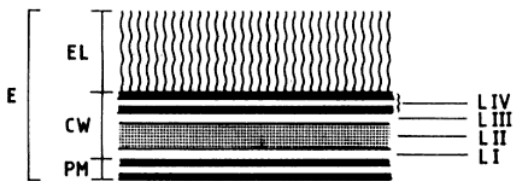


Figure 2.2 Cyanobacterial cell envelope, E, cell envelope; PM, plasma membrane; CW, cell wall; EL, external layer; LII, peptidoglycan layer; LIV, outer membrane; LI, LIII, space between layers (Drews et al.,1982)

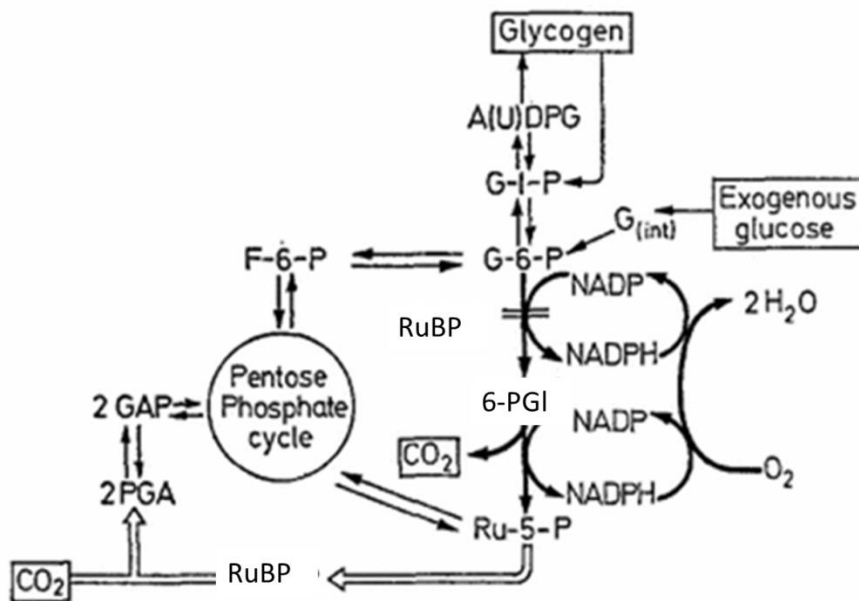


Figure 2.3 A simplified metabolic map to show carbon metabolism of *Aphanocapsa* 6714 in the light (Pelroy et al., 1976). White arrows, reactions specific to photosynthetic CO<sub>2</sub> assimilation; heavy dark arrows, reactions specific both to dark endogenous respiration and to the respiratory metabolism of glucose. A(U)DPG, ADP-glucose or UDP-glucose; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G<sub>(int.)</sub>, endogenous glucose; F-6-P, fructose-6-phosphate; 6-PG, 6-phosphogluconate; Ru-5-P, ribulose-5-phosphate; RuBP, ribulose-1,5-diphosphate; PGA, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate

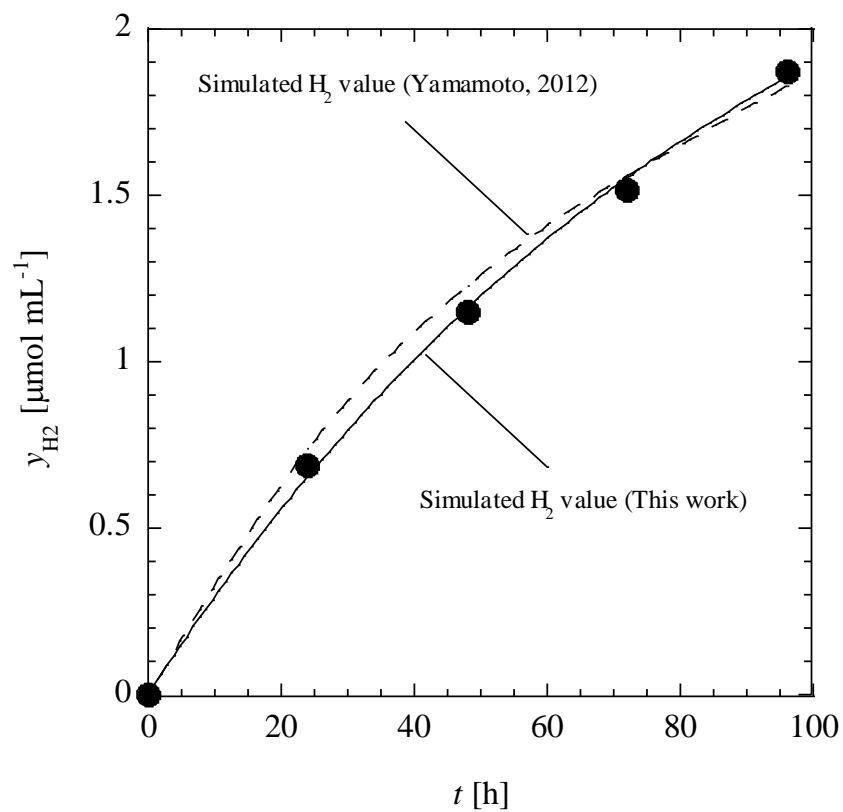


Figure 2.4 Time course of the amount of H<sub>2</sub> per culture volume shown in a previous work (Yamamoto, 2011). Broken line, simulated  $y_{H_2}$  value by Yamamoto; solid line, simulated  $y_{H_2}$  value utilizing Eq.(2.17) in this work

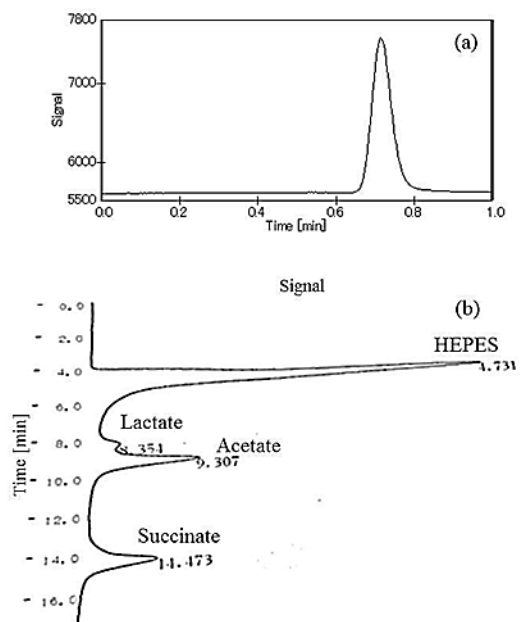


Figure 2.5 Peaks of metabolic products under dark anaerobic incubation of *Synechocystis* sp. strain PCC6803-GT. Cells were suspended in nitrate-free HEPES buffer supplemented with  $28 \mu\text{mol mL}^{-1}$  glucose. (a) Single peak represents  $\text{H}_2$  detected from Gas Chromatography analysis, GC-320, GL science Inc.; column, Molecular sieve 13X; column temperature,  $37 \text{ }^\circ\text{C}$ ; injector temperature,  $45 \text{ }^\circ\text{C}$ ; detector temperature  $80 \text{ }^\circ\text{C}$  with nitrogen gas as the carrier gas. (b) Four peaks are HEPES, lactic acid, acetate and succinate detected from HPLC analysis (JASCO PU-2080 Plus and UV-2075 Plus). Mobile phase was  $18 \text{ mmol L}^{-1} \text{ KH}_2\text{PO}_4$ , pH of which was adjusted at 2.3 with  $\text{H}_3\text{PO}_4$ . Flow rate of mobile phase was  $0.7 \text{ mL min}^{-1}$ . Column temperature was  $30 \text{ }^\circ\text{C}$

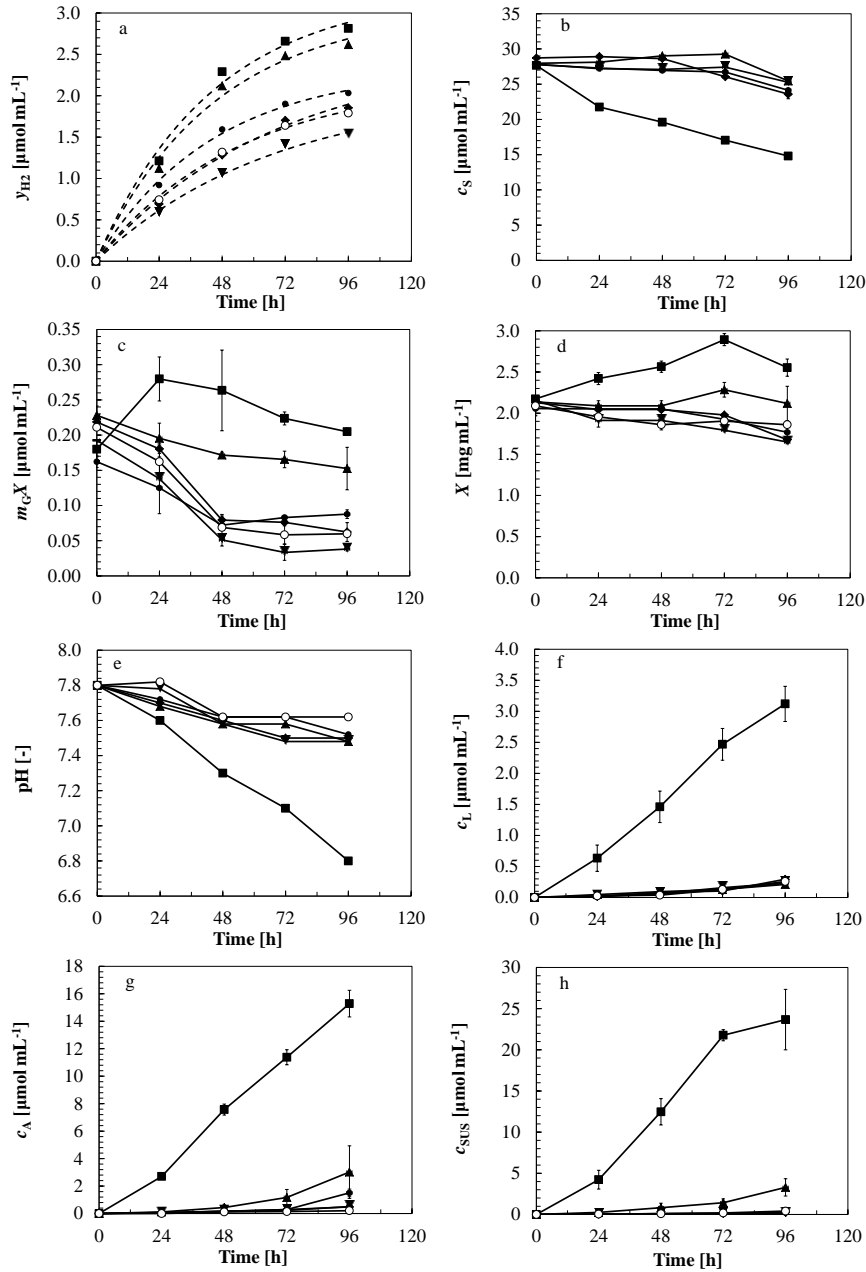


Figure 2.6 Time courses of culture variables measured in runs without ( $\circ$ ) or with  $28 \mu\text{mol mL}^{-1}$  reducing sugars (glucose ( $\blacksquare$ ), fructose ( $\blacktriangle$ ), galactose ( $\bullet$ ), mannose ( $\blacklozenge$ ) and xylose ( $\blacktriangledown$ )). Quantities analyzed are (a) the mole of  $H_2$  per culture volume ( $y_{H_2}$ ), (b) the concentration of exogenous reducing sugars ( $c_S$ ), (c) the amount of endogenous glucose per culture volume ( $c_G$ ), (d) the concentration of dry cell weight ( $X$ ), (e) pH of cell suspension, (f) the concentration of lactate, (g) the concentration of acetate and (h) the concentration of succinate under dark anaerobic incubation.

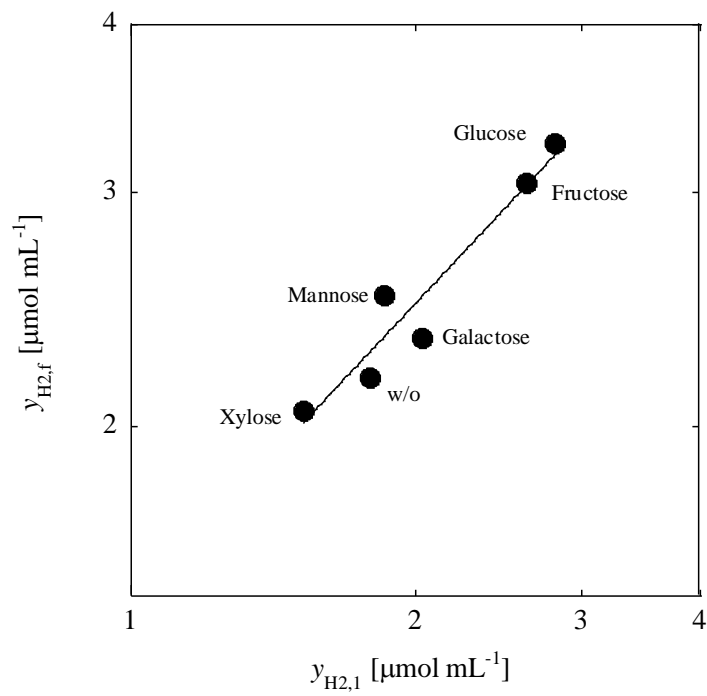
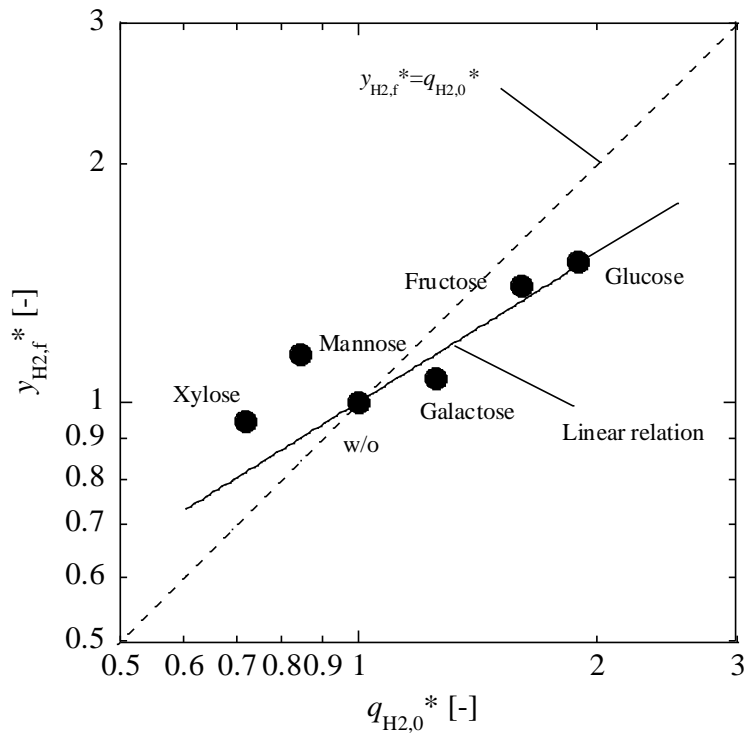


Figure 2.7 relation between observed  $y_{H2,1}$  and estimated  $y_{H2,f}$



**Figure 2.8** Relation between initial specific  $H_2$  production rate and attainable level of  $H_2$

$q_{H_2,0}^* = q_{H_2,0} / (q_{H_2,0})_{ref}$ ;  $y_{H_2,f}^* = y_{H_2,f} / (y_{H_2,f})_{ref}$ ; Reference run, without monosaccharide

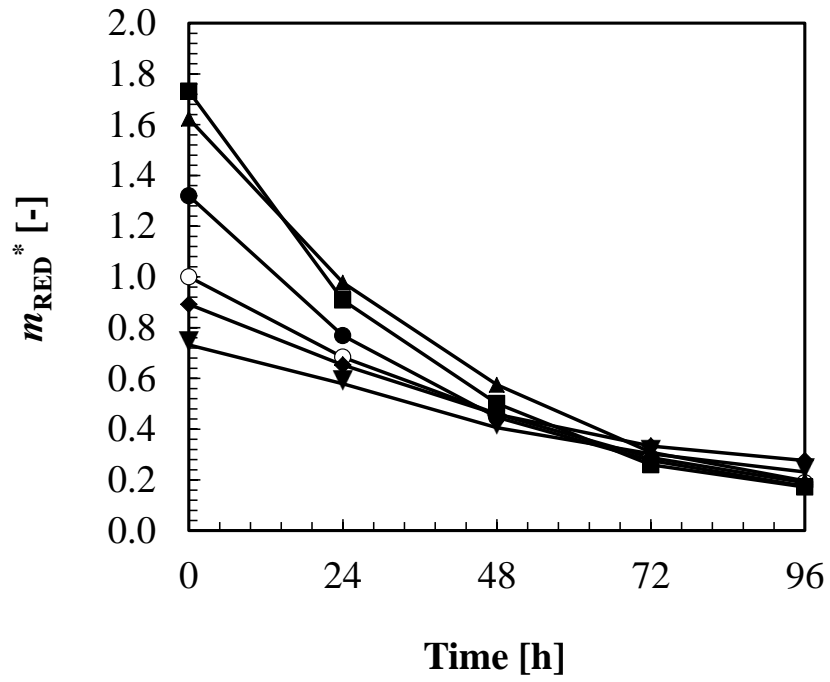


Figure 2.9 Time courses of the calculated  $m_{\text{RED}}^*$  in runs without ( $\circ$ ) or with  $28 \mu\text{mol mL}^{-1}$  reducing sugars, (glucose ( $\blacksquare$ ), fructose ( $\blacktriangle$ ), galactose ( $\bullet$ ), mannose ( $\blacklozenge$ ) and xylose ( $\blacktriangledown$ )).

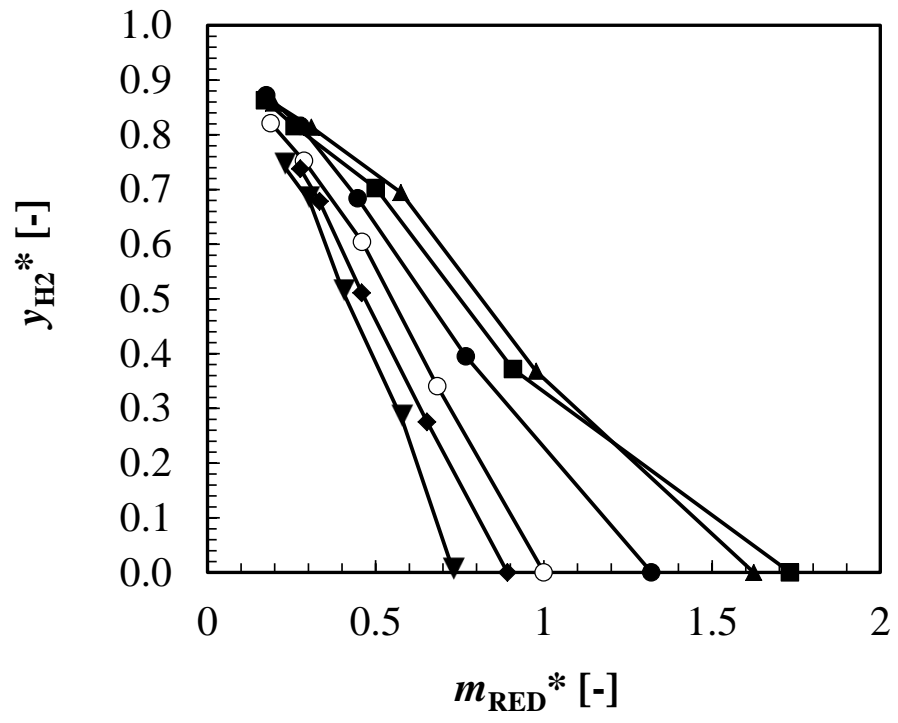
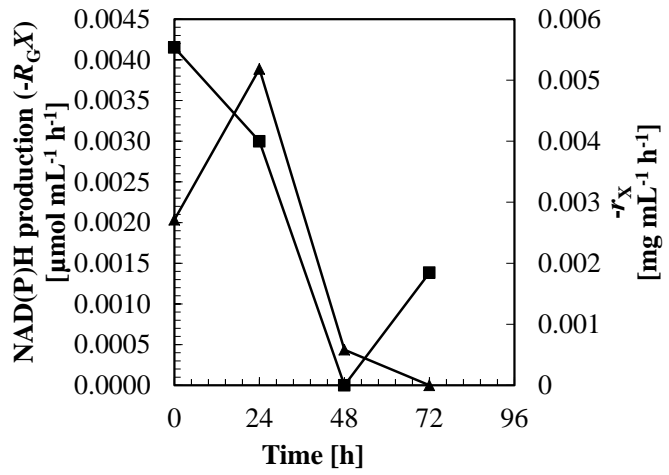
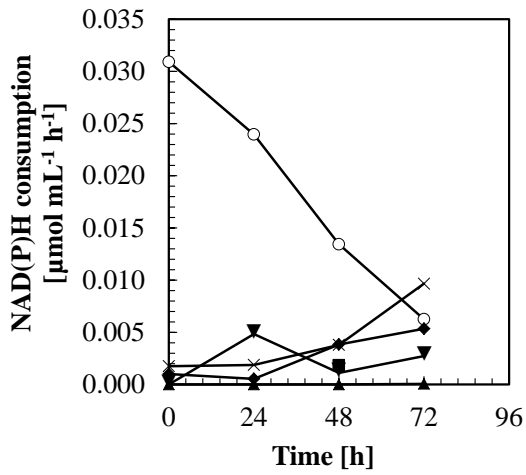


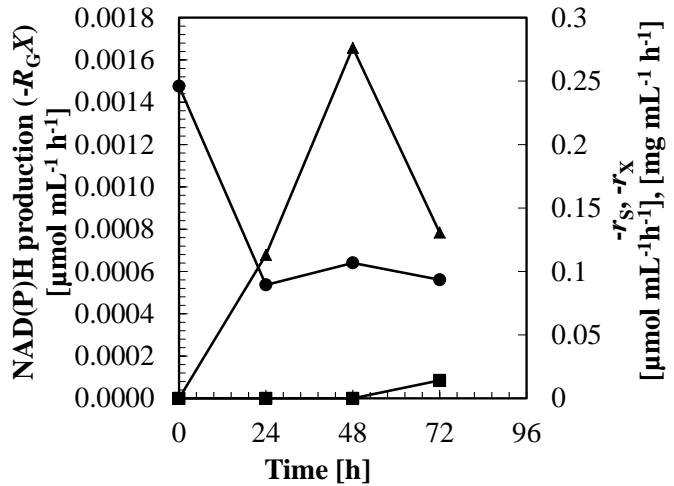
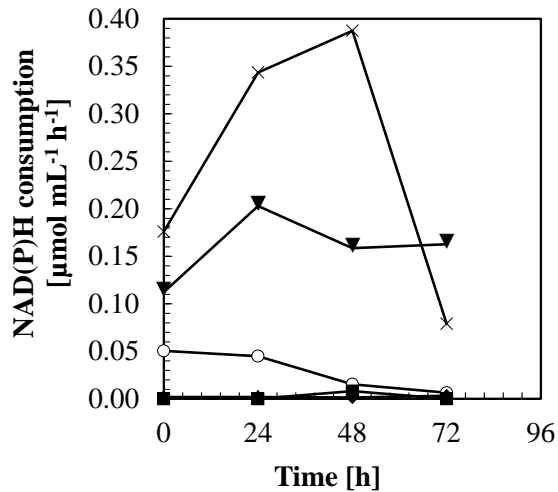
Figure 2.10 Relation between  $y_{H_2^*}$  and  $m_{RED^*}$



(a)

(b)

Figure 2.11 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-R_{GX}$  ( $>0$ ),  $-r_X$  ( $>0$ )) for run without monosaccharide ( $r_{H2}$  (○);  $R_{GX}$ ,  $-R_{GX}$  (▲);  $r_X$ ,  $-r_X$  (■),  $r_L$  (◆),  $r_A$  (▼),  $r_{SUC}$  (×)).



(a)

(b)

Figure 2.12 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-r_S$ ,  $-R_{GX}$  ( $>0$ ),  $-r_X$  ( $>0$ )) for run with glucose ( $r_{H2}$  (○);  $-r_S$  (●);  $R_{GX}$ ,  $-R_{GX}$  (▲);  $r_X$ ,  $-r_X$  (■),  $r_L$  (◆),  $r_A$  (▼),  $r_{SUC}$  (×)).

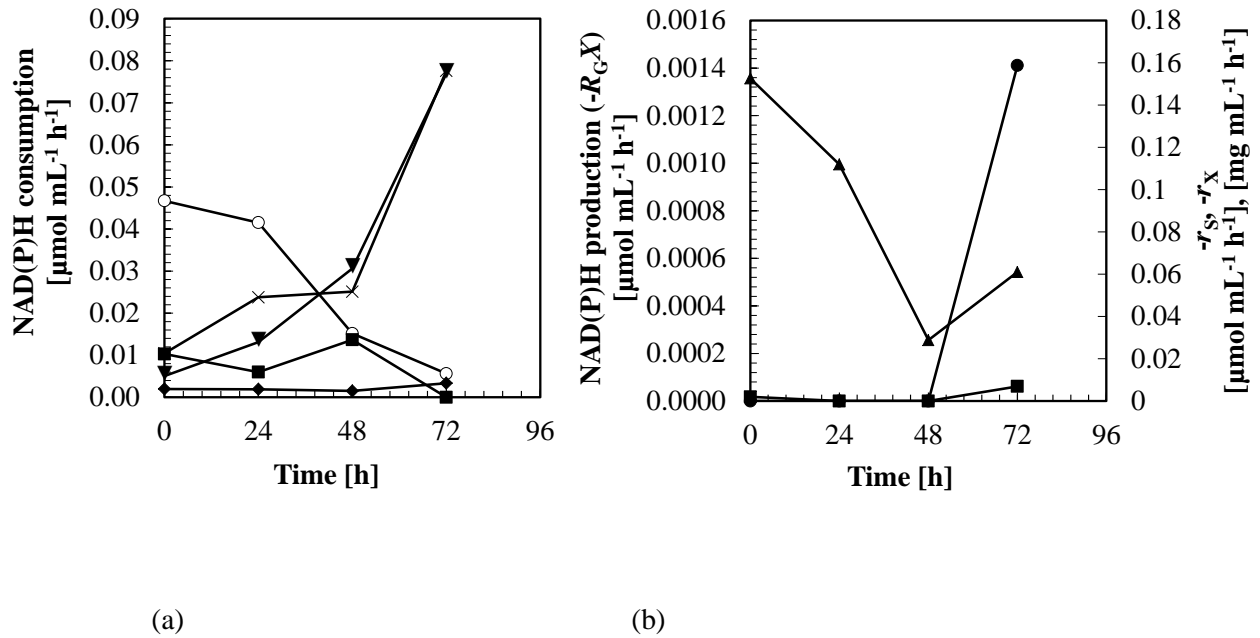


Figure 2.13 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H_2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-r_s$ ), ( $-R_{GX}$ ) ( $>0$ ), ( $-r_x$ ) ( $>0$ ) for run with fructose ( $r_{H_2}$  ( $\circ$ ); ( $-r_s$ ) ( $\bullet$ );  $R_{GX}$ , ( $-R_{GX}$ ) ( $\blacktriangle$ );  $r_X$ , ( $-r_x$ ) ( $\blacksquare$ ),  $r_L$  ( $\blacklozenge$ ),  $r_A$  ( $\blacktriangledown$ ),  $r_{SUC}$  ( $\times$ )).

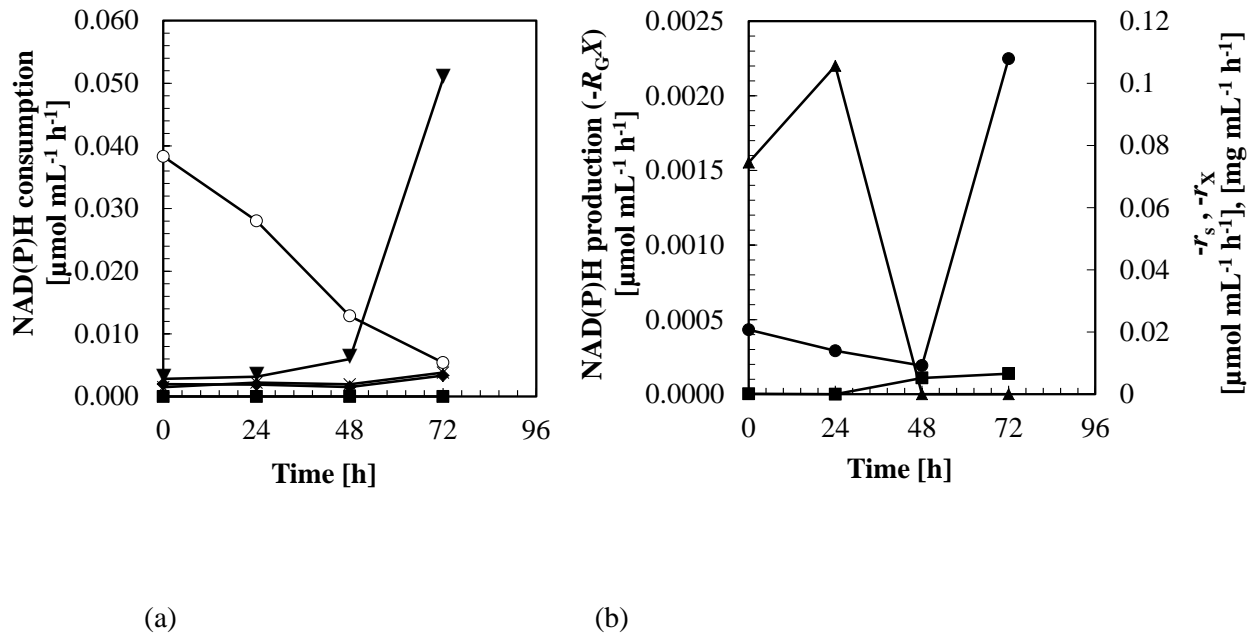


Figure 2.14 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H_2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-r_s$ ), ( $-R_{GX}$ ) ( $>0$ ), ( $-r_x$ ) ( $>0$ ) for run with galactose ( $r_{H_2}$  ( $\circ$ ); ( $-r_s$ ) ( $\bullet$ );  $R_{GX}$ , ( $-R_{GX}$ ) ( $\blacktriangle$ );  $r_X$ , ( $-r_x$ ) ( $\blacksquare$ ),  $r_L$  ( $\blacklozenge$ ),  $r_A$  ( $\blacktriangledown$ ),  $r_{SUC}$  ( $\times$ )).

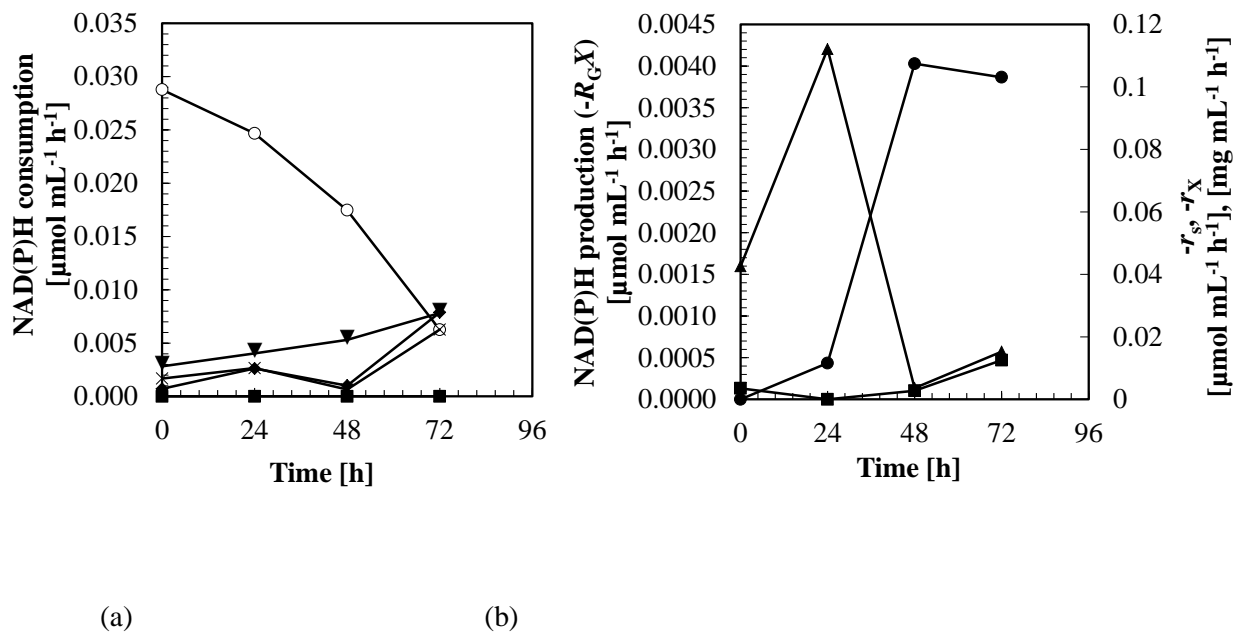


Figure 2.15 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-r_s$ ), ( $-R_{GX}$ ) ( $>0$ ), ( $-r_X$ ) ( $>0$ ) for run with mannose ( $r_{H2}$  ( $\circ$ ); ( $-r_s$ ) ( $\bullet$ );  $R_{GX}$ , ( $-R_{GX}$ ) ( $\blacktriangle$ );  $r_X$ , ( $-r_X$ ) ( $\blacksquare$ ),  $r_L$  ( $\blacklozenge$ ),  $r_A$  ( $\blacktriangledown$ ),  $r_{SUC}$  ( $\times$ )).

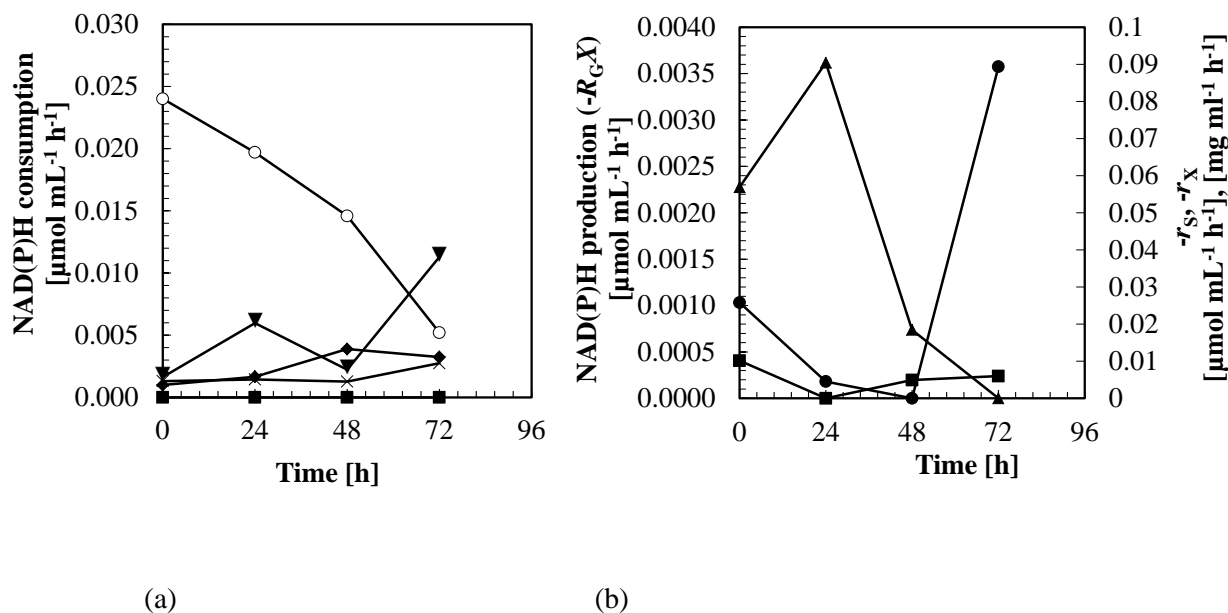


Figure 2.16 Time courses of the rates for (a) NAD(P)H consumption ( $r_{H2}$ ,  $R_{GX}$  ( $>0$ ),  $r_X$  ( $>0$ ),  $r_L$ ,  $r_A$ ,  $r_{SUC}$ ), and (b) NAD(P)H production ( $-r_s$ ), ( $-R_{GX}$ ) ( $>0$ ), ( $-r_X$ ) ( $>0$ ) for run with xylose ( $r_{H2}$  ( $\circ$ ); ( $-r_s$ ) ( $\bullet$ );  $R_{GX}$ , ( $-R_{GX}$ ) ( $\blacktriangle$ );  $r_X$ , ( $-r_X$ ) ( $\blacksquare$ ),  $r_L$  ( $\blacklozenge$ ),  $r_A$  ( $\blacktriangledown$ ),  $r_{SUC}$  ( $\times$ )).

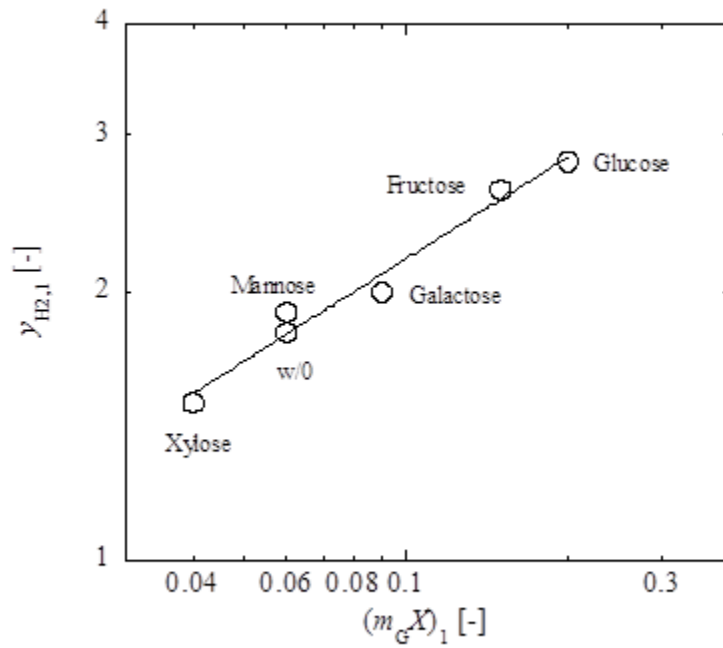


Figure 2.17 Relation between  $(m_G X)_1$  and  $y_{H2,1}$  of dark anaerobic incubation of GT strain, from late-logarithmic growth phase, in HEPES buffer solution with or without monosaccharides.

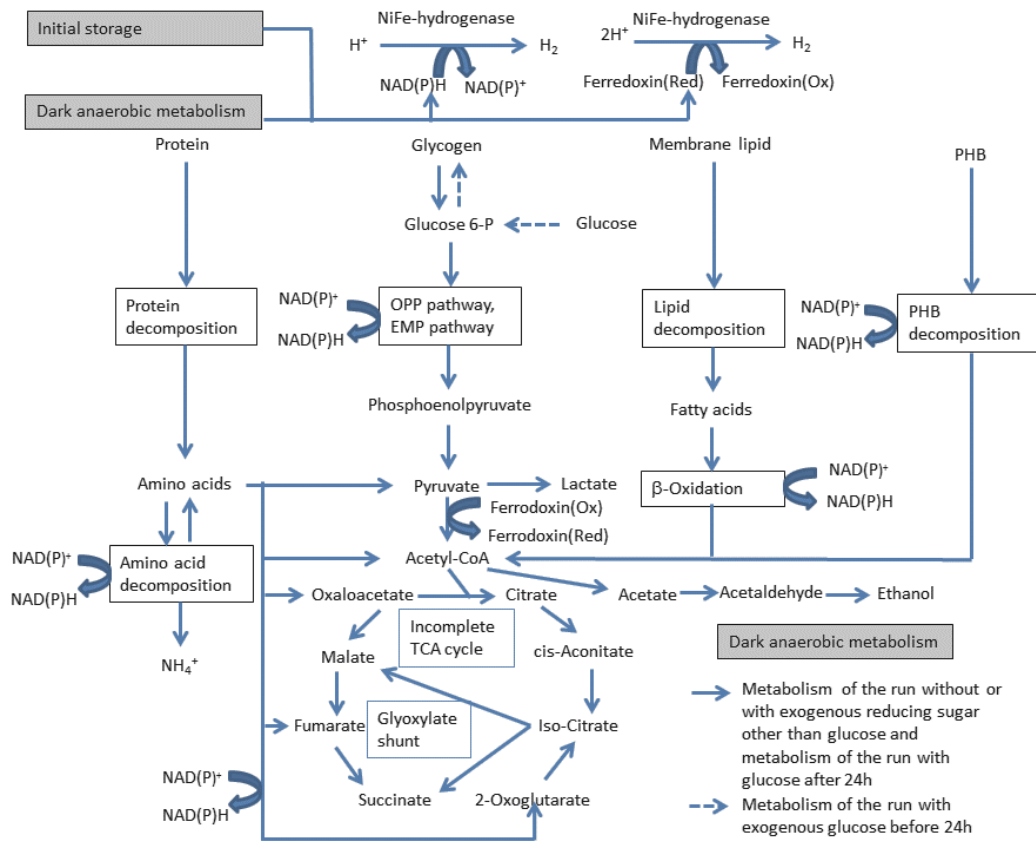


Figure 2.18 Proposed fermentative schemes for a glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 under the dark anaerobic condition on HEPES buffer solution with or without exogenous glucose.

## Chapter 2: References

- Anderson SL, McIntosh L (1991) Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. *Journal of Bacteriology* 173 (9):2761-2767
- Appel J, Phunpruch S, Steinmuller K, Schulz R (2000) The bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 works as an electron valve during photosynthesis. *Archives of Microbiology*. 173 :333-338
- Baebprasert W, Jantaro S, Khetkorn W, Lindblad P, Incharoensakdi A (2011) Increased H<sub>2</sub> production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. *Metabolic Engineering* 13 (5):610-616
- 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. *Journal of Bacteriology* 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. *Journal of bacteriology* 186 (6):1737-1746
- Das D, Veziroğlu TN (2001) Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy* 26 (1):13-28
- De Philippins R, Margheri MC, Vincenzini M (1996) Fermentation in symbiotic and free-living cyanobacteria. *Arch. Hydrobiol. Algol. Stud.* 83:459-468
- Drews G, Weckesser J (1982) Function, structure and composition of cell walls and external layers. in *The biology of cyanobacteria*, Carr N, Whitton BA eds. Blackwell Scientific Pub., Oxford
- Flores E, Schmetterer G (1986) Interaction of fructose with the glucose permease of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Journal Bacteriology* 166 (2): 693-696
- Gutekunst K, Phunpruch S, Schwarz C, Schuchardt S, R-Friedrich S, J. Appel (2005) LexA regulates the bidirectional hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803 as a transcription activator. *Molecular Microbiology* 58:810-823
- 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. *The Journal of Biological Chemistry* 289 (4):1930-1937
- Hammett LP (1937) The effect of structure upon the reactions of organic compounds. Benzene derivatives". *Journal of the American Chemical Society* 59 (1): 96.
- Hoare DS, Igram LO, Thurston EL, Walkup R (1917) Dark heterotrophic growth of an endophytic blue-green alga. *Arch Mikrobiol* 78:310-321
- Joset F, Buchou T, Zhang CC, Jeanjean R (1988) Physiological and genetic analysis of the glucose-fructose permeation system in two *Synechocystis* species. *Archives of Microbiology* 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
- Laroque D, Inisan C, Berger C, Voulard É, Dufossé L, Guérard F (2008) Kinetic study on the Maillard reaction. Consideration of sugar reactivity. *Food Chemistry* 111 (4):1032-1042
- Lee S, Ryu J-V, Kim SY, Jeon J-H, Song JY, Cho H-T, Choi S-B, Choi D, de Marsac NT, 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 Physiology* 145: 1018-1030
- Moezelaar R, Stal LJ (1994) Fermentation in the unicellular cyanobacterium *Microcystis* PCC7806. *Archives of Microbiology* 163:63-69

- 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
- Pelroy, R. A., Levine, G. A., & Bassham, J. A. (1976). Kinetics of light-dark CO<sub>2</sub> fixation and glucose assimilation by *Aphanocapsa* 6714. *Journal of bacteriology*, 128(2), 633-643.
- Richard DJ, Sawers G, Sargent F, McWalter L, Boxer DH (1999) Transcriptional regulation in response to oxygen and nitrate of operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*. *Microbiology*. 145:2903-2912
- Richardson LL, Castenholz RQ (1987) Enhanced survival of the cyanobacterium *Oscillatoria terebriformis* in darkness under anaerobic conditions. *Applied Environmental Microbiology* 53:2151-2158
- Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General microbiology*, 111(1), 1-61
- Stal LJ, Moezelaar R (1997) Fermentation in cyanobacteria. *FEMS Microbiology Reviews*. 21:179-211.
- Williams JG (1988) [85] Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol* 167:766-778
- Yamamoto T (2012) Analysis and activation of hydrogen production Reaction by Cyanobacteria. Doctoral thesis in Engineering. Tokyo Institute of Technology
- Yamamoto T, Chongsuksantikul A, Asami K, Ohtaguchi K (2012) Anaerobic Production of Hydrogen in the Dark by *Synechocystis* sp. strain PCC 6803 supplemented with D-glucose. *Journal Biochemical Technology* 4 (1):464-468
- Yoo S-H, Keppel C, Spalding M, Jane J-I (2007) Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. *International Journal of Biological Macromolecules* 40 (5):498-504
- 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. *Molecular Microbiology* 3 (9):1221-1229
- Zhang S, Bryant DA (2011) The Tricarboxylic Acid Cycle in Cyanobacteria. *Science* 334 (6062):1551-1553

## Chapter 3

### Growth Phase Effects on Hydrogen Production

#### 3.1 Introduction

Cyanobacterial cells utilize radiated energy on photosystems and assimilate the thylakoid membrane photosynthetic products, NADPH and ATP, for carbon reduction and glycogen synthesis via the Calvin cycle. When the photosynthetic photon flux density (PPFD) of incident light is fixed, as a growth phase of photosynthetic cells proceeds, low cell density culture of inoculum changes to dense culture due to dry cell weight reproductions and cells in the suspension are exposed to low intensity light. Structure of cyanobacterial thylakoid membrane is associated with light intensity (van de Meene et al. 2012), hence it is rational to suppose that the activities of carbon dioxide fixation and glycogen synthesis per dry cell weight are affected by growth phase.

The hydrogen (H<sub>2</sub>) production in GT strain is comprehended by a cellular response to the redox unbalance stress caused by the change in cellular environment from radiated aerobic nitrate-containing solution to dark anaerobic nitrate-free solution. Growth phase effects on dark anaerobic H<sub>2</sub> production have been suggested in previous works with *Arthrospira* (*Spirulina*) *maxima* (Ananyev et al. 2008), *Aphanothece halophytica* (Taikhao et al. 2013) and *Synechocystis* sp. strain PCC6803 (WT strain) (Baebprasert et al. 2010), while, as yet, few author has presented the experimental results on the related parameters such as the amounts of glycogen and products in dark anaerobic condition. Apart from dark anaerobic H<sub>2</sub> production, a report shows that carbohydrate content is at peak during stationary phase in cyanobacteria, green algae and diatoms (Henderson et al. 2008). The increase in carbohydrate content in *Synechococcus* sp. on BG-11 medium with late growth phase was reported (Phlips et al. 1989). Other report shows that glycogen content and glycogen chain length of WT strain is varied not only by medium but also by growth phase (Yoo et al. 2007). Searching the growth phase of the inoculum suited for use in dark anaerobic nitrate-free H<sub>2</sub> seems to be a good possibility to elevate H<sub>2</sub> production of GT strain.

This study concerns an indirect biophotolysis for H<sub>2</sub> production in a glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 (GT strain). Cells photoautotrophically grown in the first stage are harvested and inoculated into dark anaerobic culture in the second stage to generate H<sub>2</sub>. To optimize cellular NAD(P)H in selecting growth phase of cells in photosynthesis, dark fermentation of cells from various growth phase have been monitored.

Under sufficient supply of radiant energy, cells of cyanobacterium grow logarithmically. This period is called logarithmic growth phase. If  $X$  is the dry cell weight concentration, change of cellular concentration in the logarithmic growth phase is shown by:

$$\frac{dX}{dt} = \mu X \quad (3.1),$$

in which  $\mu$  is the specific growth rate. In a classic work (Steele, 1962), the specific growth rate of logarithmically growing cells is modeled by Steele as:

$$\mu = \mu_m \frac{I_0}{I_m} \exp\left(1 - \frac{I_0}{I_m}\right) \quad (3.2)$$

in which  $I_0$  is the photosynthetic photon flux density (*PPFD*) of incident light, and  $\mu_m$  and  $I_m$  are model parameters. **Figure 3.1** is the relationship of Eq.(3.2) for a unicellular cyanobacterium *Synechococcus* sp. strain PCC6301 with the model constants of  $\mu_m$ , 0.112 h<sup>-1</sup> ; and  $I_m$ , 294  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . (Ohtaguchi and Wijanarko, 2002).

If  $I$  is the *PPFD* of transmitted light, then drop of *PPFD* of light passing through cell suspension is given by the equation (Ohtaguchi and Wijanarko, 2002):

$$\frac{I}{I_0} = \exp\left(-\frac{k_1 X}{1+k_2 X^{k_3}}\right) \quad (3.3).$$

If light path length is 27 mm and diameter of cell is 1.29  $\mu\text{m}$ , then above work suggests the constants  $k_1$ , 353 mL mg<sup>-1</sup>;  $k_2$ , 256 mL<sup>0.605</sup> mg<sup>-0.605</sup>; and  $k_3$ , 0.605. **Figure 3.2** shows the effect of dry cell weight concentration on transmittance of light through cell suspension in above reference.

Increase in dry cell weight concentration according to Eq.(3.1) reduces the *PPFD* inside the bioreactor according to Eq.(3.3). Above work also shows that, when cell suspension of *Synechococcus* sp. strain PCC6301 is illuminated by 165  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light, transmitted light intensity  $I$  is 97  $\mu\text{mol m}^{-2} \text{s}^{-1}$  right after inoculation and drops to 9.0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 144 h. If radiation cannot support logarithmic growth of whole cells in suspension, and support a fraction of whole cells, Eq.(3.1) changes to

$$\frac{dX}{dt} = \mu \phi X \quad (3.4)$$

for  $\phi$  between 0 and 1. This period is called late-logarithmic growth phase. If dry cell weight concentration further increases and cell suspension becomes dense culture, cell growth ceases. This period is called stationary phase. Dense culture at 144h in stationary phase indicates that cells are exposed to the light of which *PPFD* is  $165 \mu\text{mol m}^{-2} \text{s}^{-1}$  at illuminated surface and  $9.0 \mu\text{mol m}^{-2} \text{s}^{-1}$  at transmitted surface.

A classic work (Allen, 1968) is cited in **Figure 3.3** that shows an electron microscopic observation of an ultrastructure of a unicellular cyanobacterium *Synechococcus* sp. strain PCC7942 (*Anacystis nidulans*) at different light intensities. Cells under higher light intensity shown in graph (A) have two concentric lamellae and contain lower amount of pigments per dry cell weight (carotenoid,  $15.7 \mu\text{g mg}^{-1}$ ; chlorophyll,  $42 \mu\text{g mg}^{-1}$ ; phycocyanin,  $195 \mu\text{g mg}^{-1}$ ). Those under lower light intensity shown in graph (B) have three concentric lamellae and contain higher amount of pigments per dry cell weight (carotenoid,  $18.1 \mu\text{g mg}^{-1}$ ; chlorophyll,  $61 \mu\text{g mg}^{-1}$ ; phycocyanin,  $281 \mu\text{g mg}^{-1}$ ). Generation times of cells under higher and lower light intensities are 16 h and 4 h, respectively. Cell suspension in logarithmic growth phase is diluting that provides the environment for high transmittance of light (Fig.3.2). Graph (A) of Fig.3.3 represents the ultrastructure of such cells. Cell suspension in stationary phase is dense that provides the environment for low transmittance of light (Fig.3.2). Graph (B) of Fig.3.3 represents the ultrastructure of such cells. Pigment enzyme contents appear to be higher in stationary phase cells than in late-logarithmic growth phase cells, and in late-logarithmic growth phase cells than in logarithmic growth phase cells. If synthesis of pigment protein is activated as transparency of culture decreases, the assimilation of NAD(P)H in dense culture proceeds by this reaction. Similarly synthesis of endogenous glycogen seems to be a good possibility in dense cultures that offers not darkness but dim light illuminated condition.

A recent work reported the degradation of glycogen in GT strain in darkness (Hanai et al.,2014). Electron micrographs of ultrathin sections of the GT strain cells show the decrease in glycogen granules under dark condition for 12 h. Dense culture represents the very low light transmittance condition, however, it seems to be quite different from darkness since life of cyanobacteria is supported by illumination. If decomposition of pigment proteins, glycogen and other photosynthesis-supported materials in darkness offers NAD(P)H that is a substrate for  $\text{H}_2$  production on NiFe-hydrogenase, and if synthesis of those macromolecules in the light is activated in dense culture, the possibility exists that the cells from stationary phase generate high amount of  $\text{H}_2$  in darkness.

Therefore, the research in this chapter was aimed at the elucidation of effect of growth phase of GT strain in photosynthesis upon H<sub>2</sub> production under dark anaerobic condition. The H<sub>2</sub> production kinetics is related to endogenous glucose degradation, cell death, lactate and acetate productions.

### 3.2 Materials and methods

A glucose tolerant mutant of unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 (GT strain) was utilized. Growth phase of cells of GT strain in the first stage for indirect biophotolysis was changed. Cells of GT strain were grown photoautotrophically on BG-11 medium at 303K under the illumination of 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  fluorescent lamp as described in Chapter 2. Pre-culture for the first stage main culture was performed for 3 d to logarithmic growth phase before transferring the harvested cells to main culture. The main cultures were performed in the same condition as pre-cultures. The dry cell weight concentration at initial for photosynthetic cell preparation ( $X_0$ ) was set at 0.0118  $\text{mg mL}^{-1}$  ( $\text{OD}_{730}$ , 0.02). Cells of logarithmic growth phase ( $\text{OD}_{730}$ , 3), late-logarithmic growth phase ( $\text{OD}_{730}$ , 7) and stationary phase ( $\text{OD}_{730}$ , 12) in main cultures were collected by centrifugation at 3000 rpm and 298K for 10 min.

Cells in different growth phases were then inoculated into dark anaerobic HEPES buffer solution for H<sub>2</sub> production. H<sub>2</sub> production experiments were performed as described in Chapter 2.

### 3.3 Results and discussion

Prior to dark anaerobic H<sub>2</sub> production in the second stage of indirect biophotolysis, GT strain cells were grown on BG-11 medium under illumination in the first stage of indirect biophotolysis. Optical density at 730 nm was observed over time. One unit absorbance was equivalent to 0.369  $\text{mg-DCW mL}^{-1}$ . **Figure 3.4** shows the time courses of culture variables measured during photosynthesis. Quantities measured include (1) the dry cell weight concentration of GT strain ( $X$ ) and (2) the number of moles of endogenous glucose per dry cell weight ( $m_G$ ). There is no lag phase on this growth curve because inoculum cells were taken from logarithmic growth phase of preculture. Cyanobacterial growth is in general limited not by carbon dioxide (CO<sub>2</sub>) supply but radiation energy supply (Ohtaguchi and Wijanarko, 2002), hence logarithmic growth is observed in the first 55 h where sufficient radiation energy to support logarithmic growth is supplied by illumination because of low dry cell weight concentration.

Critical time ( $t_c$ ) from logarithmic growth phase to late-logarithmic growth phase, when growth rate declines with time, is 55 h. Integration of Eq.(3.1) results that

$$\ln X = \ln X_0 + \mu t \quad \text{for } t < t_c \quad (3.5)$$

The time course data of  $X$  from 0 to 55 h gives  $\mu$  of  $0.104 \text{ h}^{-1}$ . The doubling time ( $t_2 = \ln 2 / \mu$ ) is calculated to be 6.66 h.

After  $t_c$ , as transparency of cell suspension changes from dilute culture to dense culture, decrease in the slope of  $\ln X$  versus time curve is observed. If  $t_f$  is the transition time from the late-logarithmic growth phase to the stationary phase, growth curve of Fig.3.1 gives 80 h for  $t_f$ . The highest dry cell weight concentration ( $X_f$ ) of  $6.37 \text{ mg mL}^{-1}$  that is kept constant with time is seen in the stationary phase (after  $t_f$ ).

For late-logarithmic growth phase, growth kinetics are interpreted by Eq.(3.4) with  $\phi$  ( $0 < \phi < 1$ ). A previous work assumed the following model (Ohtaguchi et al.,1987):

$$\phi = 1 \quad \text{for } 0 \leq t \leq t_c \quad (3.6),$$

$$\phi = \frac{1 - \frac{g}{G}}{1 - \frac{g_c}{G}} \quad \text{for } t_c \leq t \leq t_f \quad (3.7),$$

and

$$\begin{aligned} X &= X_0 2^g \\ X_c &= X_0 \exp(\mu t_c) = X_0 2^{g_c} \\ X_f &= X_0 2^G \end{aligned} \quad (3.8),$$

in which  $g$ ,  $g_c$  and  $G$  represent the number of generations from 0 h to  $t$ , from 0 h to  $t_c$  and from 0 h to  $t_f$ , respectively. Growth curve of Fig.3.1 results in that  $X_0$  and  $X_c$  are  $0.00548$  and  $1.67 \text{ mg mL}^{-1}$ , respectively, and that  $g_c$  and  $G$  are 8.25 and 10.2, respectively. Insertion of Eqs.(3.4) to (3.6) to Eq.(3.3) results that

$$\ln X = \ln X_f + \left( \ln \frac{X_c}{X_f} \right) \exp \left( \frac{\mu(t - t_c)}{\ln \frac{X_c}{X_f}} \right) \quad \text{for } t_c \leq t < t_f \quad (3.9).$$

Solid line for growth curve in Fig.3.4 is calculated utilizing Eqs.(3.5) and (3.9). The calculated values shown by the curve are in fair agreement with the experimental values.

From Eqs. (3.7) and (3.9), the fraction of growing cells ( $\phi$ ) in the late logarithmic growth phase is related to time by

$$\phi = \exp \left\{ \frac{\mu(t - t_c)}{(g_c - G) \ln 2} \right\} \quad (3.10).$$

**Figure 3.5** shows the time course of  $\phi$  for parameters  $\mu$ ,  $0.104 \text{ h}^{-1}$ ;  $t_c$ ,  $55\text{h}$ ;  $g_c$ ,  $8.25$ ; and  $G$ ,  $10.2$ .

$\text{CO}_2$  is a sole carbon source of the photoautotroph *Synechocystis* sp. strain PCC6803, and the cell formula of this strain shown in a reference is  $\text{CH}_{1.62} \text{N}_{0.22} \text{O}_{0.40} \text{P}_{0.11}$  (Yu et al. 2013), hence, if the fixed  $\text{CO}_2$  is totally converted to cell constituting materials, the specific carbon dioxide fixation rate ( $q_{\text{CO}_2}$ ) during logarithmic growth phase is calculated by

$$\begin{aligned} q_{\text{CO}_2} &= \frac{10^3}{X} \frac{d(X/23.1)}{dt} = 43.3 \frac{d \ln X}{dt} = (43.3)(0.104) \\ &= 4.50 \mu\text{mol mg}^{-1} \text{ h}^{-1} \end{aligned} \quad (3.11).$$

During photosynthesis, the electron generated by photon absorption on the thylakoid membrane converts  $\text{NADP}^+$  to NADPH, which is utilized in the Calvin cycle (Fig.1.4) according to Eq.(1.1).

$\text{CO}_2$  also reacts with ribulose-1,5-bisphosphate (RuBP) on ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and form 2-phosphoglycolate that is further converted to glycine, serine, glycerate and  $\text{CO}_2$ . It is called photorespiration. Central carbon metabolism in *Synechocystis* sp. strain PCC6803 in a reference with  $^{13}\text{CO}_2$  labelling experiment (Huege et al., 2011) shows that  $\text{CO}_2$  incorporated into glyceraldehyde 3-P formation versus photorespiration at the RubisCO is 0.09-fold. Considering this, present study ignores the carbon conversion via photorespiration. Other detected photosynthesis products in this reference includes glyceraldehyde 3-P (GAP), phosphoenolpyruvate (PEP), citrate, malate, aspartate, ornithine, glycerate, glycine, serine, glycerate and glucose 6-P. A key product of photosynthesis in GT strain is endogenous glucose in glycogen. Broken line in Fig.3.4 shows the amount of glucose per dry cell weight ( $m_G$ ) in logarithmically growing cells at  $0.102 \mu\text{mol mg}^{-1}$  ( $=0.0184 \text{ mg mg}^{-1}$ ). One mole glucose is synthesized from 2 moles glyceraldehyde 3-P. Another key product from  $\text{CO}_2$  in WT strain is poly- $\beta$ -hydroxybutyrate (PHB) that is 2.3% of dry cell weight, and the NADPH content in a mutant defective of PHB synthesis is up to 1.85-fold higher than that in WT strain

(Xie et al., 2011). Contents of all these intracellular photosynthesis products are involved in the formula of dry cell weight:  $\text{CH}_{1.62}\text{N}_{0.22}\text{O}_{0.40}\text{P}_{0.11}$ .

In logarithmic growth phase, the yield factor  $Y_{X/\text{CO}_2}$  is determined as

$$Y_{X/\text{CO}_2} = \frac{\mu}{q_{\text{CO}_2}} = \frac{0.104}{4.46} = 0.0233 \text{ mg } \mu\text{mol}^{-1} = 0.530 \text{ mg mg}^{-1} \quad (3.12).$$

About a half weight of assimilated  $\text{CO}_2$  is found to be incorporated into cell constituting materials. From the stoichiometry of Eq.(1.1), the NADPH consumption in the Calvin cycle during logarithmic growth phase is calculated to be  $9.00 \mu\text{mol mg}^{-1}\text{h}^{-1}$ . Generation rate of glyceraldehyde 3-P in the Calvin cycle ( $R_{\text{GAP}}$ ) is calculated to be  $1.49 \mu\text{mol mg}^{-1}\text{h}^{-1}$ .

If  $R_{\text{G1}}$  is the specific production rate of glucose in logarithmically growing cells, the mass balance for endogenous glucose in logarithmic growth phase is shown by

$$\frac{d(m_G X)}{dt} = R_{\text{G1}} X \quad (3.13)$$

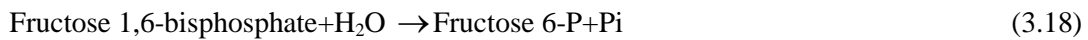
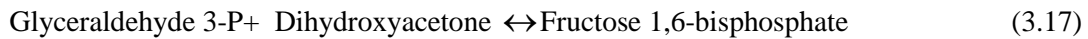
or

$$\frac{dm_G}{dt} = R_{\text{G1}} - \mu m_G \quad (3.14).$$

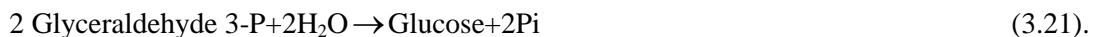
Data of  $m_G$  in logarithmic growth phase in Fig.3.4 are constant ( $=0.102 \mu\text{mol mg}^{-1} = 0.0184 \text{ mg mg}^{-1}$ ) with time. Thus the rate  $R_{\text{G1}}$  is calculated as

$$R_{\text{G1}} = \mu m_G = (0.104)(0.102) = 0.0107 \mu\text{mol mg}^{-1}\text{h}^{-1} \quad (3.15).$$

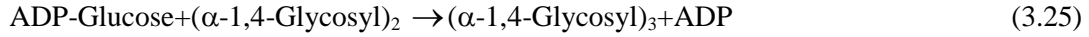
Gluconeogenesis from glyceraldehyde 3-P to glucose is shown by the equations:



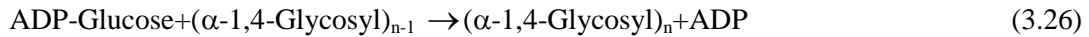
The irreversible reaction of Eq.(3.18) is mediated by fructose-1,6-bisphosphatase. Net reaction is shown by



Glucose 6-P is further utilized as follows:

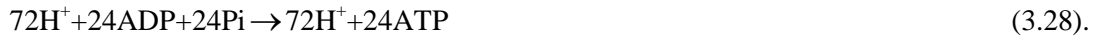
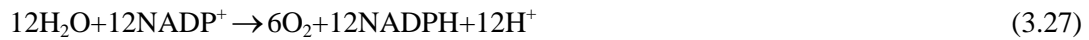


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The formula  $(\alpha\text{-1,4-Glycosyl})_n$  shows glycogen. For glycogen synthesis from glyceraldehyde 3-P, ATP requirement is shown in Eqs.(3.16)-(3.26) .

In photosynthesis, majority of ATP in Eq.(3.23) is synthesized at thylakoid membrane as follows:



Radiation is a requirement of these reactions.

The observed yield of moles of endogenous glucose from 2 moles of glyceraldehyde 3-P ( $Y_{G/GPA}$ ) for logarithmically growing cells is estimated as:

$$Y_{G/GAP} = \frac{R_G}{R_{GAP}} = \frac{0.0107}{\frac{1.49}{2}} = 0.0144 \quad (3.29).$$

The yield of endogenous glucose from  $\text{CO}_2$  ( $Y_{G/CO_2} = R_{G1}/(q_{CO_2}/6) = (0.0107)(6)/4.46$ ) is 0.0144 in logarithmic growth phase. The large portion of the Calvin cycle product is found to be converted to cell constituting materials and byproduct  $\text{CO}_2$ .

When mode of cell growth proceeds to late-logarithmic growth phase, there appears non-growing cells. If dry cell weight concentration is shown by

$$X = \phi X + (1 - \phi) X \quad (3.30),$$

Eq.(3.4) shows the growth of dividing cells ( $\phi X$ ). Dry cell mass concentration  $(1 - \phi) X$  shows the concentration of resting cells. When  $\phi = 1$ , all cells are in resting state. If  $R_{G2}$  is the specific production rate of glucose in resting cells, the mass balance for endogenous glucose in late-logarithmic growth phase is shown by

$$\frac{d(m_G X)}{dt} = R_{G1}\phi X + R_{G2}(1-\phi)X \quad (3.31)$$

or

$$\begin{aligned} \frac{dm_G}{dt} &= R_{G1}\phi + R_{G2}(1-\phi) - \mu\phi m_G \\ &= R_{G2} + \phi(R_{G1} - R_{G2} - \mu m_G) \end{aligned} \quad (3.32).$$

The factor  $\phi$  is 1 at  $t_C$  and 0 after  $t_f$  (Fig.3.5). Broken line in Fig.3.4 shows that the data of  $m_G$  in late-logarithmic growth phase and stationary phase increases linearly with time. From this observation it is obtained that

$$R_{G2} = 0.00128 \text{ } \mu\text{mol mg}^{-1} \text{ h}^{-1} \quad (3.33)$$

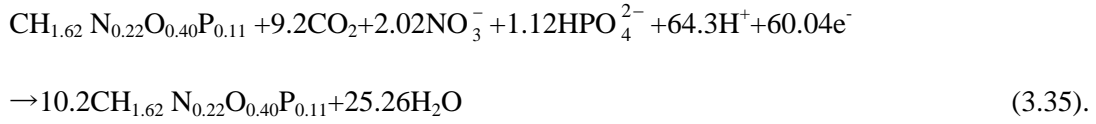
and

$$\begin{aligned} m_G &= 0.0379 + 0.00128t \\ \phi(R_{G1} - R_{G2} - \mu m_G) &\approx 0 \end{aligned} \quad (3.34).$$

Figure 3.4 shows that calculated  $m_G$  is in fair agreement with the observed  $m_G$ . The  $m_G$  at 96 h is 0.160  $\mu\text{mol mg}^{-1}$  (=0.0289  $\text{mg mg}^{-1}$ ) that is 1.57 (=0.160/0.102) times that in logarithmically growing cells. Inversely glucose production rate of resting cells ( $R_{G2}$ ) drops to 12.0 % (=0.00128/0.0107) of dividing cells because there is no need for carbon flow to new born cells.

The level of  $m_G$  in this work is supported by a previous work reporting that the glycogen content of GT strain on BG-11 medium under 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  illumination for 15 d (=360 h) was 11% (= 0.611  $\mu\text{mol glucose equivalent mg}^{-1}$ ) (Joseph et al. 2014). The predicted  $m_G$  at 360 h by Eq.(3.9) is 0.499  $\mu\text{mol mg}^{-1}$ , that agrees well with above value. Glycogen content in another work is varied according to how cells are grown. A reported glycogen content of *Synechocystis* sp. strain PCC 6803 grown on BG-11 medium at 25° C for a week is approximately 1.5–4.5 mg glycogen per g wet cell mass, of which variation is resulted depending on whether glucose is supplied. If dry cell mass is 20% of wet cell mass, then glycogen content is 0.042-0.125  $\mu\text{mol mg}^{-1}$  (Yoo et al. 2007), which also supports the  $m_G$  level of this report. A recent work (Hanai et al., 2014) shows  $m_G/\text{OD}_{730}$  at 4.3  $\mu\text{g mL OD}^{-1}$  that corresponds to the  $m_G$  at 0.0647 (=4.3/180/0.369)  $\mu\text{mol mg}^{-1}$ . Glucose content of *Synechocystis* sp. strain PCC6803 in previous works and this work is extremely low when it is compared with another model cyanobacterium *Syneccoccus* sp. strain PCC6301 of which glucose content  $m_G$  is 2.78  $\mu\text{mol mg}^{-1}$  (Mustaqim and Ohtaguchi, 1997).

Balance of elements C, H, N, O and P and electrons from 0h to  $t_f$  results in a growth reaction equation as:



This equation relates the states of cultures at inoculation time and  $t_f$ . The yield factor  $Y_{X/\text{CO}_2}$  during batch cultivation is calculated as

$$Y_{X/\text{CO}_2} = \frac{(10.2-1)(26.5)}{(9.2)(10^3)} = 0.0265 \text{ mg } \mu\text{mol}^{-1} = 0.602 \text{ mg mg}^{-1} \quad (3.36).$$

Comparison of Eq.(3.12) and Eq.(3.36) suggests that the conversion of  $\text{CO}_2$  to dry cell weight that is 53% in logarithmic growth phase increases in late-logarithmic growth phase.

In late-logarithmic growth phase, carbon balance at glyceraldehyde 3-P is interpreted by

$$\frac{q_{\text{CO}_2} X}{6} = \frac{R_{\text{GAP}} X}{2} = \frac{1}{Y_{\text{G/GAP},1}} R_{\text{G}_1} \phi X + \frac{1}{Y_{\text{G/GAP},2}} R_{\text{G}_2} (1-\phi) X \quad (3.37)$$

or

$$q_{\text{CO}_2} = 4.50\phi + \frac{0.00128}{Y_{\text{G/GAP},2}} (1-\phi) \quad (3.38)$$

with parameters  $R_{\text{G}_1}, 0.0107 \mu\text{mol mg}^{-1} \text{ h}^{-1}$ ;  $R_{\text{G}_2}, 0.00128 \mu\text{mol mg}^{-1} \text{ h}^{-1}$ ;  $Y_{\text{G/GAP},1}, 0.0144$ . **Figure 3.6** is the calculated  $q_{\text{CO}_2}$  for  $Y_{\text{G/GAP},2}$  at 0.0144(= $Y_{\text{G/GAP},1}$ ) and 1. Conversion of glyceraldehyde 3-P to glucose shows very small effect on  $\text{CO}_2$  fixation rate. It is found that glucose accumulation rate in stationary phase is weakly affected by  $\text{CO}_2$  fixation rate.

Figure 3.4 also shows the time course of the  $q_{\text{CO}_2}$  that is calculated by Eq. (3.38) with perfect conversion of glyceraldehyde 3-P to glucose. The  $q_{\text{CO}_2}$  was maximum ( $q_{\text{CO}_2,\text{max}}$ ) and constant at  $4.46 \mu\text{mol mg}^{-1} \text{ h}^{-1}$  in logarithmic growth phase and decreased drastically in late-logarithmic growth phase to extremely low value at 120 h. Growth is not seen at 120 h since PPF of transmitted light is only 10% of incident light. Photosynthesis pigments appear to be developed as shown in Fig.3.3 (b).

Although the  $q_{\text{CO}_2}$  of cells in stationary phase is extremely low, Eq.(3.33) shows the occurrence of glycogen synthesis. If carbon of glycogen synthesis in stationary phase comes from the fixed  $\text{CO}_2$  at the Calvin cycle (Eq.(1.1),(3.16)-(3.26)), and the yield of glucose from glyceraldehyde 3-P is same as Eq.(3.29), then

CO<sub>2</sub> fixation rate of resting cells is calculated as 0.544 μmol mg<sup>-1</sup> h<sup>-1</sup>.

**Figure 3.7** shows a conceivable mechanism of CO<sub>2</sub> assimilation, glycogen synthesis, cell constituting material synthesis and decomposition and redox buffering systems in GT strain during logarithmic growth phase and stationary phase. Reducing compound NAD(P)H is mainly supplied by thylakoid membrane (Eq(1.1)). Majority of NADPH is consumed for CO<sub>2</sub> fixation and majority of photosynthesis product glyceraldehyde 3-P is utilized for synthesis of biomaterials of new born cells during logarithmic growth phase. Cell suspension is dilute in this phase, hence high PPF radiation supports this mechanism for 55 h. To keep a homeostasis a small portion of glyceraldehyde 3-P is converted to endogenous glucose. Production of dry cell weight seems to be accompanied by evolution of CO<sub>2</sub> that is reproduced from the pathways from glyceraldehyde 3-P. Growth arrest signal is triggered at 55 h, when cell suspension becomes dense culture. This growth arrest was reported in a previous work that showed an arrest of the growth of *Synechococcus* sp. strain PCC6301 at the transmitted light intensity ( $I_b$ ) of 38.6 μmol m<sup>-2</sup> s<sup>-1</sup>. (Ohtaguchi and Wijanarko, 2002). After entering late-logarithmic growth phase at 55 h, cell suspensions receive constant radiation energy that corresponds to the drop of PPF from  $I_0$  to  $I_b$ . Generated NADPH is utilized in the Calvin cycle to fix CO<sub>2</sub> with a low fixation rate. A portion of produced glyceraldehyde 3-P is converted to glycogen. Other macromolecules to store radiant energy appear to be synthesized in this period. Content of carotenoid, chlorophyll and phycocyanin shown by Allen (Allen, 1968) is 1.81%, 6.1% and 28.1%, that of PHB shown by Xie et al is 2.3% (Xie et al., 2011) , and that of glycogen shown in Fig.3.4 is 1.84-2.89%. The GT strain under growth arrest likely utilized the synthesis reaction of these macromolecule for discharging NADPH generated by absorption of highest light energy corresponding to  $I_0-I_b$ . Cell suspension in late-logarithmic growth phase appear to contain both logarithmic growth phase cells and stationary phase cells.

H<sub>2</sub> production in the second stage of indirect biophotolysis was performed by inoculating cells of GT strain from photoautotrophic culture at different growth phases to dark anaerobic HEPES buffer solution. **Table 3.1** shows the cellular states of GT strain, utilized for dark anaerobic H<sub>2</sub> production. If the specific CO<sub>2</sub> consumption rate  $q_{CO_2}$  of each run is referred to that of late-logarithmically growing cells, the  $q_{CO_2}$  of logarithmically growing cells and stationary phase cells are 3.08 and 0.631 times that of late-logarithmically growing cells, respectively. If the  $m_G$  of each run is referred to that of late-logarithmically growing cells, the  $m_G$  of logarithmically growing cells and stationary phase cells are 0.75 and 1.89 times that of late-logarithmically growing cells, respectively. If  $m_G$  represents the cellular content of energy-stored chemicals and  $q_{CO_2}$  represents the cellular production capability of NAD(P)H, logarithmically growing cells are found to be low level in  $m_G$  but high level in  $q_{CO_2}$ , and stationary phase cells are found to be low level in  $q_{CO_2}$  but high level in  $m_G$ .

**Figure 3.8** shows the time courses of culture variables measured in three dark anaerobic runs made utilizing cells from (1) logarithmic growth phase (●), (2) late-logarithmic growth phase (■) and (3) stationary phase (▲). Growth phase of inoculum cells is found to have a major impact on subsequent dark anaerobic H<sub>2</sub> production and cellular metabolism in darkness. Results of run with late-logarithmically growing cells are re-plots of the data of run without monosaccharide in Fig.2.6. Culture variables shown in Fig.3.8 are (a) the number of moles of H<sub>2</sub> per culture volume ( $y_{H_2}$ ); (b) the number of moles of endogenous glucose per dry cell weight ( $m_G$ ) in solid line and the number of moles of endogenous glucose per culture volume in broken line ( $m_G X$ ); (c) the dry cell weight concentration ( $X$ ); (d) the lactate concentration; (e) the acetate concentration; (f) the lactate production rates in broken line ( $r_L$ ), the acetate production rates in solid line ( $r_A$ ) and the H<sub>2</sub> production rate in long short broken line ( $r_{H_2}$ ); gas phase, nitrogen gas; inoculum cells, washed to ensure there is no nitrogen source in fermentation system.

Graph (a) in Fig.3.8 shows that H<sub>2</sub> production is largely affected by the growth phase of inoculum cell. The curve of the number of moles of H<sub>2</sub> per culture volume ( $y_{H_2}$ ) versus time of run with logarithmically growing cells is similar to the curve of run with late-logarithmically growing cells. The results of three runs show that the  $r_{H_2}$  was highest at  $r_{H_2,0}$  right after inoculation and then decreased with time (Fig.3.2f).

The H<sub>2</sub> production curve of run with stationary phase cells shows high production rate of which kinetics can be interpreted by either Eqs.(2.18) or (2.20). If inoculum cells are obtained from stationary phase, after dark anaerobic incubation, there seems to be a reaction which generates NAD(P)H, even though NiFe-hydrogenase consumes NAD(P)H.

The curves shown in Fig.3.8(a) are calculated by Eq.(2.18) with the model constants  $r_{H_2,0}$  and  $k$  shown in **Table 3.2**. These curves resulted in a relatively good fit to the measured variables. The  $r_{H_2,0}$  is highest in run with inoculum from late-logarithmic growth phase. The  $r_{H_2,0}$  values of cells from logarithmic growth phase and stationary phase are 0.806 times and 0.871 times that from late-logarithmic growth phase. The highest number of moles of H<sub>2</sub> per culture volume at 96 h ( $y_{H_2,1}$ ) was achieved from cells inoculated from stationary phase. The conclusion reached from this observation is that it is important to shift up the cellular content of energy reserved chemicals to elevate dark anaerobic H<sub>2</sub> production.

The lowest H<sub>2</sub> production is seen in the result of run with cells from logarithmic growth phase. The calculated  $y_{H_2}$  shown by broken lines in Fig.3.8(a) fits well with observed  $y_{H_2}$ . The order of model parameter  $y_{H_2,f}$  is found to be same as the order of observed variable  $y_{H_2,1}$ . The relation represents that the  $y_{H_2,f}$  increases as the  $r_{H_2,0}$  is higher or the  $k$  is lower.

**Figure 3.9** shows the relation between initial H<sub>2</sub> production rate ( $q_{H_2,0}$ ) and attainable level of H<sub>2</sub> ( $y_{H_2,f}$ ). Reference values ( $q_{H_2,0}$ )<sub>ref</sub> and ( $y_{H_2,f}$ )<sub>ref</sub> are the data of run with late-logarithmically growing cells that are same as ( $q_{H_2,0}$ )<sub>w/o</sub> and ( $y_{H_2,f}$ )<sub>w/o</sub> utilized in chapter 2. Solid line shows the relation between  $y_{H_2,f}^*$  and  $q_{H_2,0}^*$  of runs with logarithmically growing cells in HEPES buffer solution with monosaccharide (Eq.(2.33)). The plot of data with logarithmically growing cells is approximated by this relation; however, that with stationary phase cells cannot be represented by this relation. Time course of H<sub>2</sub> production with stationary phase cells is found to be dependent not only on the initial amount of NAD(P)H but also on mechanism of NAD(P)H reproduction during the fermentation. This viewpoint is supported by the  $m_G$  versus time curve of graph (b) of Figure 3.8 that shows no change or increase in  $m_G$ . If initial specific H<sub>2</sub> production rate represents NAD(P)H content, right after inoculation to dark anaerobic HEPES buffer solution, cells from both logarithmic growth phase and stationary phases show lower amount of NAD(P)H than those from late-logarithmic growth phase. Late-logarithmically growing cells are found to be very sensitive to the change of cellular environment from illuminate BG-11 medium with oxygen to dark HEPES buffer solution without oxygen.

Table 3.2 shows that the  $k$  is lowest with inoculum from stationary phase, second lowest with inoculum from late-logarithmic growth phase and highest with inoculum from logarithmic growth phase. The ascending order of  $y_{H_2,f}$  is same as the descending order of  $k$  in Table 3.2. The GT strain in dark anaerobic incubation with inoculum from stationary phase is found to be most stable for dark anaerobic H<sub>2</sub> production. The redox balance after dark anaerobic incubation in GT strain from stationary phase is characterized by the activation of reproduction of NAD(P)H.

The  $q_{CO_2}$  is proportional to NADPH consumption rate of the Calvin cycle, hence the decrease in  $q_{CO_2}$  of inoculum cells appears to increase  $r_{H_2,0}$ , while  $q_{CO_2}$  in Table 3.1 shows that the  $q_{CO_2}$  of late-logarithmically growing cells is second lowest. The  $m_G$  represents the amount of reactants for generating NAD(P)H, hence the increase in  $m_G$  of inoculum cells appears to increase  $r_{H_2,0}$ , while the  $m_G$  in Table 3.1 shows that the  $m_G$  of late-logarithmically growing cells is second highest. For these reasons, late-logarithmically growing cells seems to exhibit high initial H<sub>2</sub> production rate. Although the ascending order of  $r_{H_2,0}$  is different from ascending order of  $y_{H_2,f}$ , the descending order of  $k$  is same as ascending order of  $y_{H_2,f}$ . The attainable level of number of moles of H<sub>2</sub> per culture volume is found to depend more on stability of H<sub>2</sub> production rate than initial H<sub>2</sub> production rate.

Comparison of curves in Figures 3.8(a) and (b) shows that, during dark anaerobic H<sub>2</sub> production, H<sub>2</sub> production is more stable when the level of  $m_G$  is high. Table 3.1 shows that the  $m_G$  of inoculum cells from stationary phase is highest, that from late-logarithmic growth phase the second highest and that from logarithmic growth phase is lowest. This order is the same as the descending order of  $k$  in Table 3.2. The

increase in the  $m_G$  is found to increase the stability of  $H_2$  production during dark anaerobic incubation. Our results show that stationary phase cells that accumulate glycogen are most suitable for inoculum of dark anaerobic  $H_2$  production. The  $m_G$  of inoculum cells from stationary phase is  $0.195 \mu\text{mol mL}^{-1}$ , which is 2.52 times that from logarithmic growth phase.

The solid line curves of  $m_G$  versus time in Fig. 3.8(b) show that, except for the changes between 0 and 24 h of run with inoculum from logarithmic growth phase and between 72 and 96 h of run with inoculum from stationary phase, the  $m_G$  of cells in dark anaerobic incubation decreased with time. The broken line curves of  $m_G X$  versus time in Fig. 3.8(b) show that, except for the  $m_G X$  between 0 and 24 h of run with inoculum from logarithmic growth phase,  $m_G X$  always decreased with time. Glycogen degradation occurred in dark incubation with inocula from late-logarithmic growth phase and stationary phase. The  $m_G X$  curve of run with inoculum from logarithmic growth phase shows glycogen synthesis between 0 and 24 h and glycogen degradation after 24 h. The decrease in  $m_G$  between 72 h and 96 h of run with inoculum from stationary phase was brought by the decrease in  $X$ . No change in  $m_G$  is seen in the first 24 h of runs with late-logarithmic growing cells. The cellular content of glycogen of runs with inocula from late-logarithmic growth phase and stationary phase dropped drastically during 96 h.

During photosynthesis, specific glycogen accumulation rate ( $R_G$ ) shown in Table 3.1 is highest at  $0.106 \mu\text{mol mL}^{-1} \text{h}^{-1}$  for cells from logarithmic growth phase, second highest in run with inoculum from late-logarithmic growth phase and lowest in run with inoculum from stationary phase. Glycogen synthesis in the dark is seen between 0 and 24 h in the  $m_G X$  curve of run with inoculum from logarithmic growth phase, while glycogen consumption is seen after 24 h of this run and after 0 h of runs with inocula from late-logarithmic growth phase and stationary phase. As shown by  $R_G^*$ , the  $R_G$  of logarithmically growing cells is 2.23 times that of late-logarithmically growing cells. The ATP production rate of logarithmically growing cells are so high that, right after dark anaerobic incubation, the generated large amount of ATP appears to be utilized for the glycogen synthesis for 24 h. Of special interest is that the decrease in the  $m_G X$  is not affected by the growth phase of inoculum cells (Table 3.2). Growth phase dependent production of dark anaerobic production of  $H_2$  is found to be caused not by endogenous glucose decomposition but by turnover of cell constituting components.

Figure 3.8 (c) shows that cell death occurred in dark anaerobic incubation. The specific death rate ( $K_d$ ) is highest in run with stationary phase cells, second highest in run with late-logarithmically growing cells and lowest in run with logarithmically growing cells (Table 3.2). The ascending order of  $K_d$  is the same as the ascending order of  $y_{H_2, f}$ . Activation of turnover of cell constituting components is found to be important for shifting-up  $H_2$  production. Observed cell death shows that inoculum from stationary phase suits for increasing  $K_d$ .

Figures 3.8 (d) and (e) shows that H<sub>2</sub> production is accompanied by the production of lactate and acetate. Previous report also shows a detectable level of lactate and acetate in the culture of *Synechocystis* sp. strain PCC6803 (Troshina et al. 2002). Dark anaerobic production of acetate increased drastically when cells from stationary phase were incubated. At 96 h, lactate concentration that was 0.20 μmol mL<sup>-1</sup> in run with logarithmically growing cells increased to 0.48 μmol mL<sup>-1</sup> in run with stationary phase cells. Acetate production was roughly parallel to lactate production. Acetate concentration that was 0.05 μmol mL<sup>-1</sup> in run with logarithmically growing cells increased to 0.8 μmol mL<sup>-1</sup> in run with stationary phase cells. Interestingly, cells from stationary phase resulted in higher ratio of acetate concentration to lactate concentration. These ratios of acetate concentration to lactate concentration at 96 h of runs with cells from logarithmic growth phase, late-logarithmic growth phase and stationary phase were 0.25, 0.80 and 1.67 respectively. The ascending order of this ratio is similar to that of the yield of H<sub>2</sub> on endogenous glucose (Y<sub>H<sub>2</sub>/G</sub>) of Table 3.2. Dark anaerobic H<sub>2</sub> production of stationary phase cells is found to be highly dependent on NAD(P)H formation at incomplete TCA cycle. The highest Y<sub>H<sub>2</sub>/G</sub> is 19.1 that is a result of run with cells from stationary phase. This yield is about double of that with cells from logarithmic growth phase.

The amount of lactate or acetate at 96 h is the 1% order of it in run with glucose (Fig.2.6). Considering that 2 moles lactate or acetate is produced from 1 mole consumption of glucose, the yield of lactate or acetate on endogenous glucose is defined by:

$$Y_{L/G} = \frac{\frac{c_{Lf}}{2}}{-\Delta(m_G X)} \quad (3.39)$$

or

$$Y_{A/G} = \frac{\frac{c_{Af}}{2}}{-\Delta(m_G X)} \quad (3.40).$$

These yields are shown in Table 3.2. Those yields are less than 1 when inoculum cells are obtained from logarithmic growth phase or late-logarithmic growth phase; however, they are greater than for run with stationary phase cells. Cells of GT strain from stationary phase are found to produce lactate and acetate not only from endogenous glucose but also from other endogenous carbonaceous compounds. Decomposition of such compounds appears to supply NAD(P)H to NiFe-hydrogenase from NAD(P)<sup>+</sup>.

**Figure 3.10** shows a relation between (m<sub>G</sub>X)<sub>1</sub> and y<sub>H<sub>2</sub>,1</sub> of experiments in this chapter. The linear line is calculated using Eq.(2.40). The (m<sub>G</sub>X)<sub>1</sub> of run with cells from logarithmic growth phase is the same as

that from late-logarithmic growth phase, whereas the  $y_{H_2,1}$  of run with cells from logarithmic growth phase is smaller than that from late-logarithmic growth phase. The  $(m_G X)_1$  of run with cells from stationary phase results in high  $(m_G X)_1$  value, whereas the  $y_{H_2,1}$  of run with cells from stationary phase is comparable with that from late-logarithmic growth phase. Growth phase is confirmed to be important for  $H_2$  production.

### 3.4 Summary

$H_2$  production in indirect biophotolysis is composed of photosynthesis in the first stage and dark fermentation in the second stage. Research in this chapter concerns the effect of growth phase in the first stage to the  $H_2$  production in the second stage. The highlights of this chapter are as follows.

1. Dark anaerobic  $H_2$  production in NiFe-hydrogenase of the glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 (GT strain) in nitrate-free HEPES buffer solution, is strongly dependent on the growth phase of inoculum cells in the first stage.
2. Cell death during dark anaerobic incubation can be limited by the use of cells from logarithmic growth phase
3. Cells from stationary phase contain high amount of endogenous glucose and they are useful to achieve high amount of stored glycogen at the end of  $H_2$  production. Different from expectation,  $H_2$  production is not affected by glycogen content. Glycogen content effect is found to be a growth phase dependent phenomenon
4. The amount of  $H_2$  production is 9.6-19.1 times higher than amount of endogenous glucose consumption. The amount of reductive compounds generated by glycolysis is insufficient to make up those utilized for  $H_2$  production. Despite this fact,  $H_2$  production is indirectly traceable by glycolysis.

Glycolysis appears to be an indicator of dark anaerobic decomposition of cell constituting materials.

5. The inoculum cells from stationary phase are most suitable for dark anaerobic  $H_2$  production in GT strain in HEPES buffer solution.

Third point is the result of experiment with GT strain in HEPES buffer solution. More definitive experiments with genetically modified strain or with monosaccharide solution are needed before the exact growth phase effect is known.

Table 3.1 Physiological state of cells in photoautotrophic cultures that are performed prior to dark anaerobic H<sub>2</sub> production in indirect biophotolysis. GT strain was obtained from different growth phases.

ref, cellular state in late-logarithmic growth phase.  $q_{CO_2,0}^*$ ,  $q_{CO_2,0}/(q_{CO_2,0})_{ref}$ ; and  $m_G^*$ ,  $m_G/(m_G)_{ref}$ .

	Logarithmically growing cells	Late-logarithmically growing cells	Stationary phase cells
Harvesting time [h]	44-47	65	89
$X$ [mg·mL <sup>-1</sup> ]	1.11-1.48	2.58	4.43
$g$ [-]	6.56-6.97	7.77	8.55
$q_{CO_2}$ [μmol·mg <sup>-1</sup> ·h <sup>-1</sup> ]	4.50	1.46	0.921
$q_{CO_2}^*$ [-]	3.08	1	0.631
$m_G$ [μmol·mg <sup>-1</sup> ]	0.0773	0.103	0.195
$m_G^*$ [-]	0.750	1	1.89
$R_G$ [μmol·mg <sup>-1</sup> ·h <sup>-1</sup> ]	0.0106	0.00475	0.00128
$R_G^*$ [-]	2.23	1	0.269

Table 3.2 Kinetic parameters for H<sub>2</sub> production in GT strain in HEPES buffer solution. GT strain was obtained from different growth phases.

ref, run with cells from late-logarithmic growth phase.  $q_{H2,0}^*$ ,  $q_{H2,0}/(q_{H2,0})_{ref}$ ;  $y_{H2,f}^*$ ,  $y_{H2,f}/(y_{H2,f})_{ref}$ ; and  $k^*=k/(k)_{ref}$ .  $y_{H2,1}$ ,  $c_{L,1}$ ,  $c_{A,1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 96 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 96h;  $\Delta(m_GX)_1$ , observed decrease in  $m_GX$  during 96 h H<sub>2</sub> production;  $y_{H2/G}=y_{H2,1}/\Delta(m_GX)_1$ ;  $Y_{L/G}=(c_{L,1}/2)/\Delta(m_GX)$ ;  $Y_{A/G}=(c_{A,1}/2)/\Delta(m_GX)$

	Logarithmically growing cells	Late-logarithmically growing cells	Stationary phase cells
$X_0$ [mg mL <sup>-1</sup> ]	2.07	2.03	1.95
$r_{H2,0}$ [μmol mL <sup>-1</sup> h <sup>-1</sup> ]	0.0316	0.0405	0.025
$q_{H2,0}$ [μmol mg <sup>-1</sup> h <sup>-1</sup> ]	0.0121	0.0200	0.0138
$q_{H2,0}^*$ [-]	0.605	1	0.69
$y_{H2,f}$ [μmol mL <sup>-1</sup> ]	1.86	2.18	4.24
$y_{H2,f}^*$ [-]	0.853	1	1.94
$k$ [h <sup>-1</sup> ]	0.017	0.0186	0.006
$k^*$ [-]	0.914	1	0.326
$K_d$ [h <sup>-1</sup> ]	0.00076	0.0011	0.0026
$y_{H2,1}$ [μmol mL <sup>-1</sup> ]	1.48	1.79	1.97
$m_{G1}$ [μmol mg <sup>-1</sup> ]	0.0308	0.0323	0.188
$X_1$ [mg mL <sup>-1</sup> ]	1.95	1.86	1.49
$(m_GX)_1$ [μmol mL <sup>-1</sup> ]	0.0601	0.0601	0.280
$c_{L,1}$ [μmol mL <sup>-1</sup> ]	0.20	0.26	0.48
$c_{A,1}$ [μmol mL <sup>-1</sup> ]	0.05	0.21	0.80
$\Delta(m_GX)_1$ [μmol mL <sup>-1</sup> ]	0.10	0.15	0.10
$Y_{H2/G}$ [-]	9.6	11.8	19.1
$Y_{L/G}$ [-]	1.0	0.867	4.8
$Y_{A/G}$ [-]	0.25	0.7	8

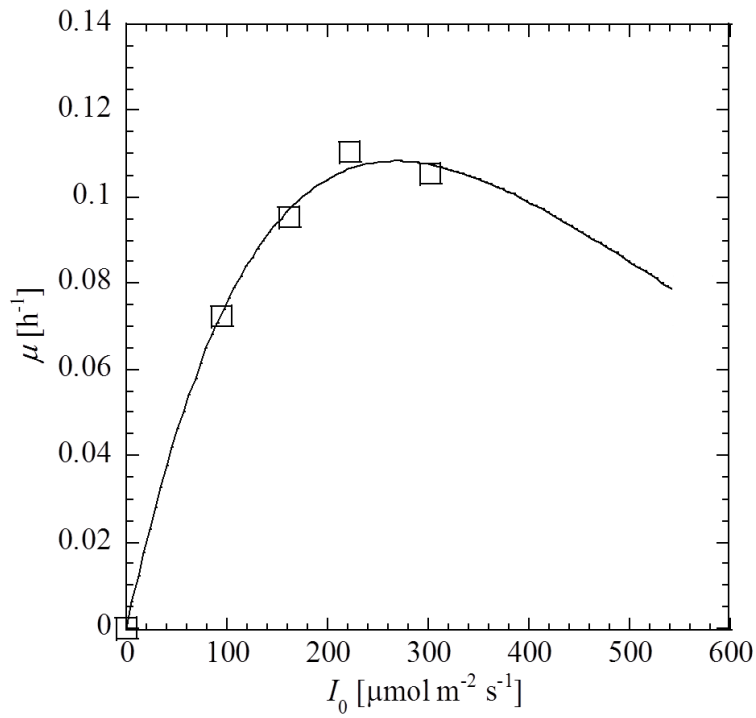


Figure 3.1 Effect of incident light intensity on the specific growth rate of logarithmically growing cells of a unicellular cyanobacterium *Synechococcus* sp. strain PCC6301. Solid line, calculated by the Steele's model (Ohtaguchi and Wijanarko, 2002).

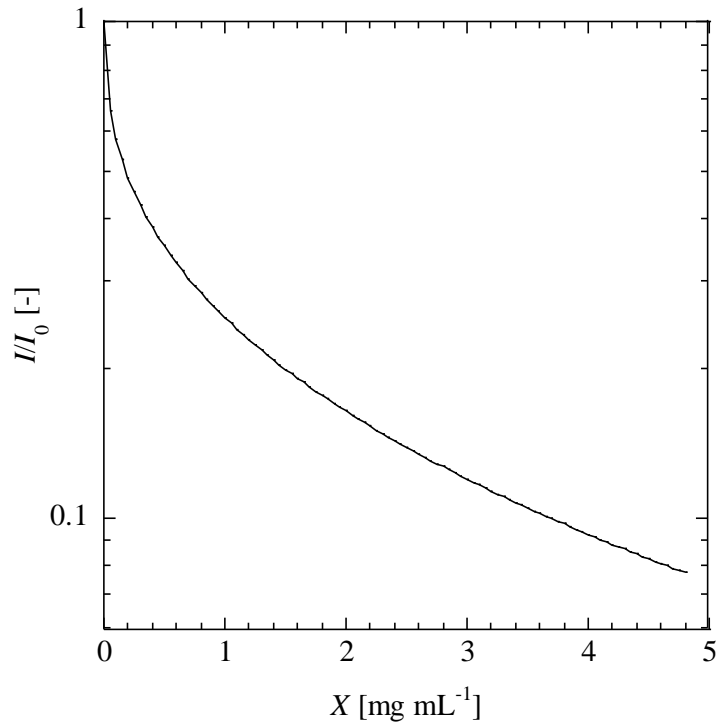


Figure 3.2 Correlation of the estimation of the transmittance of the light ( $I/I_0$ ) at the back of culture suspension for *Synechococcus* sp. strain PCC6301. Light path length, 27 mm.(Ohtaguchi and Wijanarko,2002).

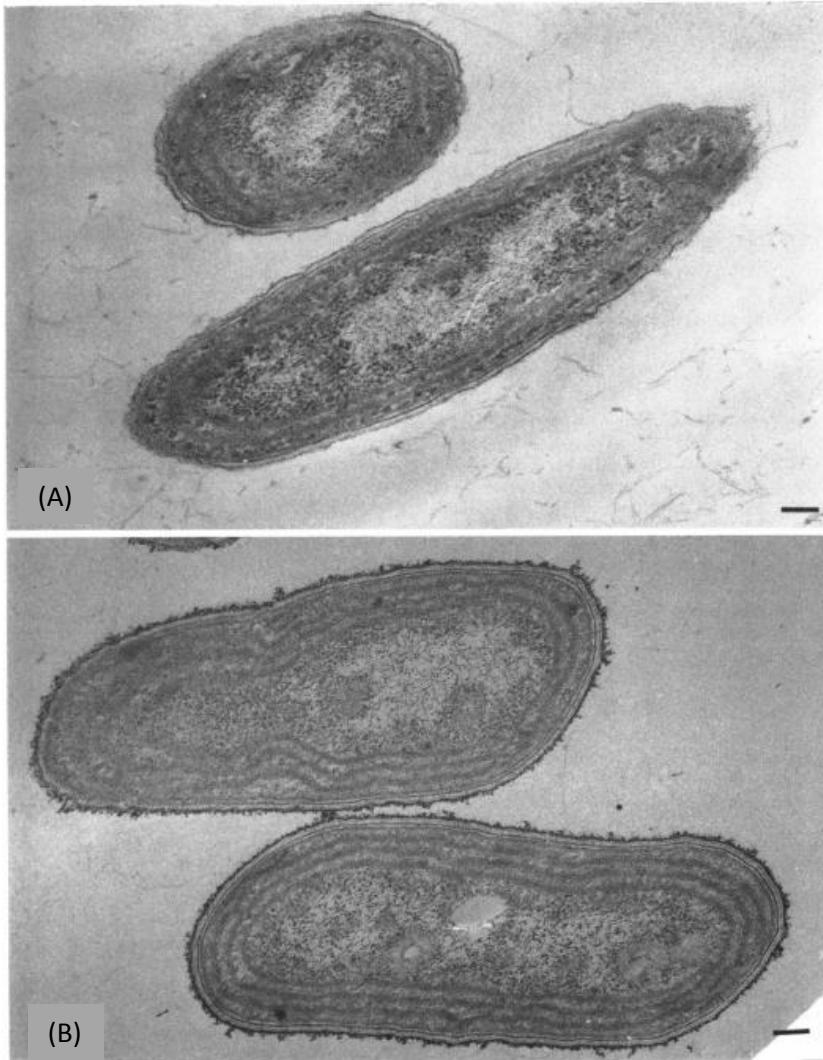


Figure 3.3 Ultrastructure of *Synechococcus* sp. strain PCC7942 (*Anacystis nidulans*) (Allen, 1968)

(A) Cells grown with 1000 ft-c illumination (roughly  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 308K

(B) Cells grown with 100 ft-c illumination (roughly  $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 308K

X60,000. All markers indicate  $0.1 \mu\text{m}$ .

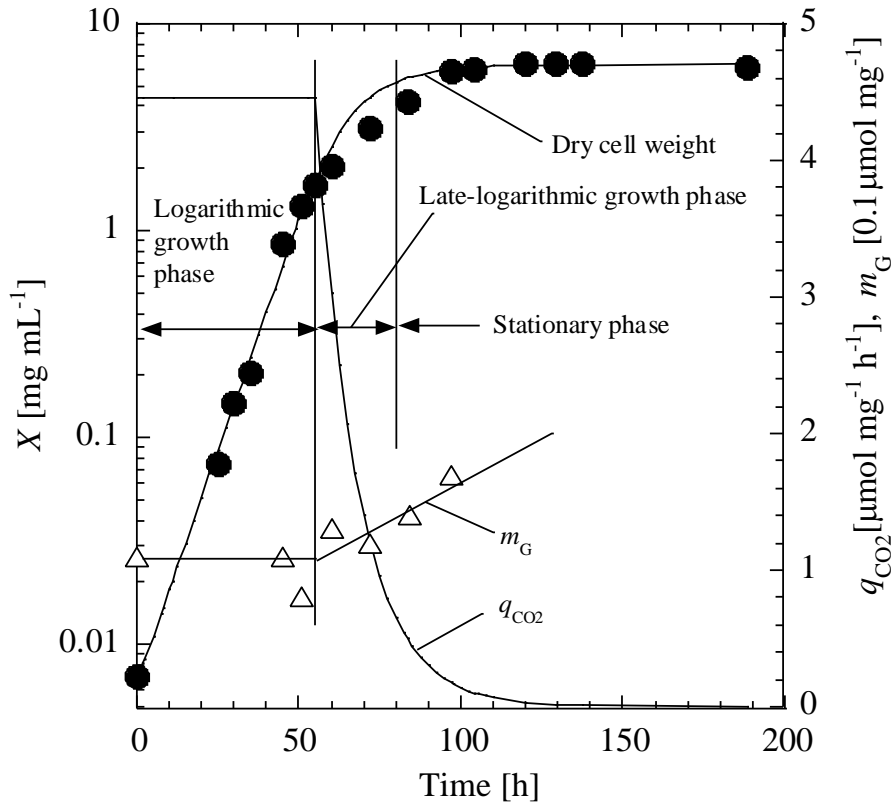


Figure 3.4 Time courses of culture parameters for photoautotrophic growth of GT strain in BG-11 medium, aerated by 6% CO<sub>2</sub> in air and continuously illuminated at 100  $\mu$ mol photons·m<sup>-2</sup>·s<sup>-1</sup> photosynthetic photon flux density (*PPFD*). Inoculum size, OD<sub>730</sub> at 0.02; dry cell weight concentration, measured by optical density of wavelength at 730 nm (●); specific carbon dioxide fixation rate, calculated from growth curve;  $m_G$ , number of moles of endogenous glucose per dry cell weight (△); the  $m_G$  at 0 h, glycogen content from pre-culture analysis.

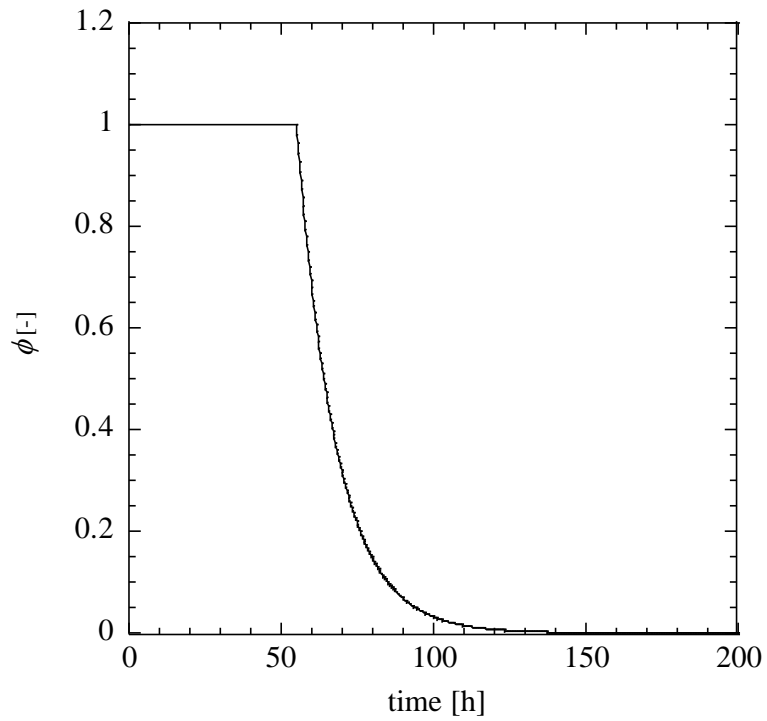


Figure 3.5 Calculated time course of the factor  $\phi$  of the photoautotrophically growing cells of GT strain on BG-11 medium under the illumination of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  *PPFD* light.

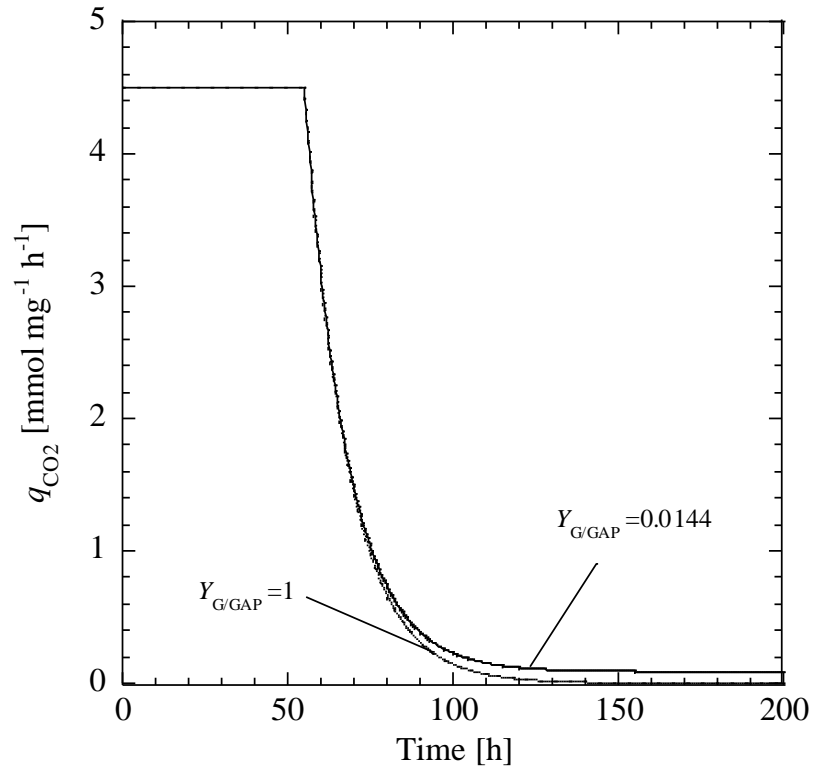


Figure 3.6 Time course of CO<sub>2</sub> for yield factor  $Y_{G/GAP}$  at 0.0144 and 1

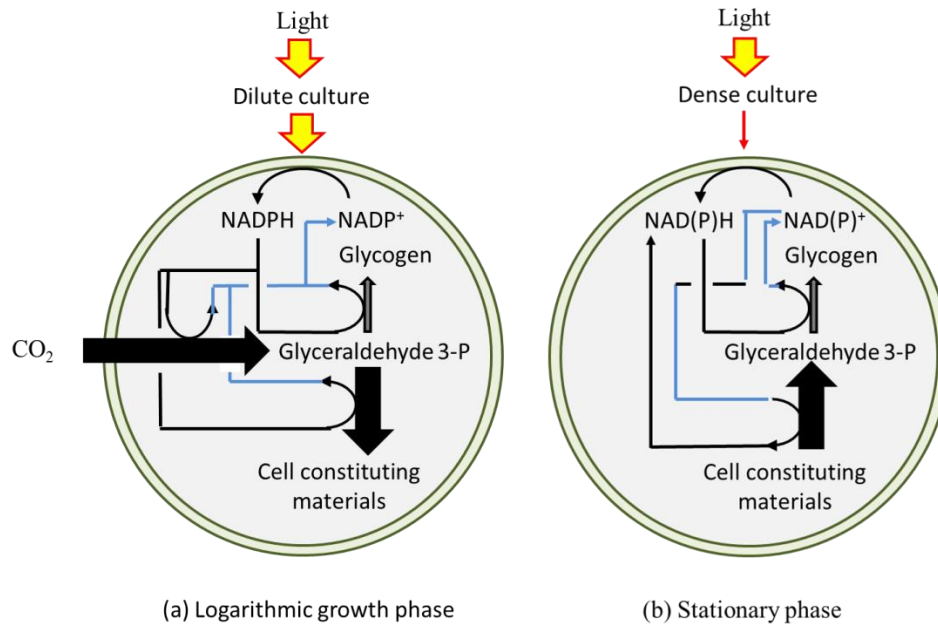


Figure 3.7 Conceivable mechanism of CO<sub>2</sub> assimilation, glycogen synthesis, cell constituting material synthesis and decomposition and redox buffering systems in GT strain during logarithmic growth phase and stationary phase.

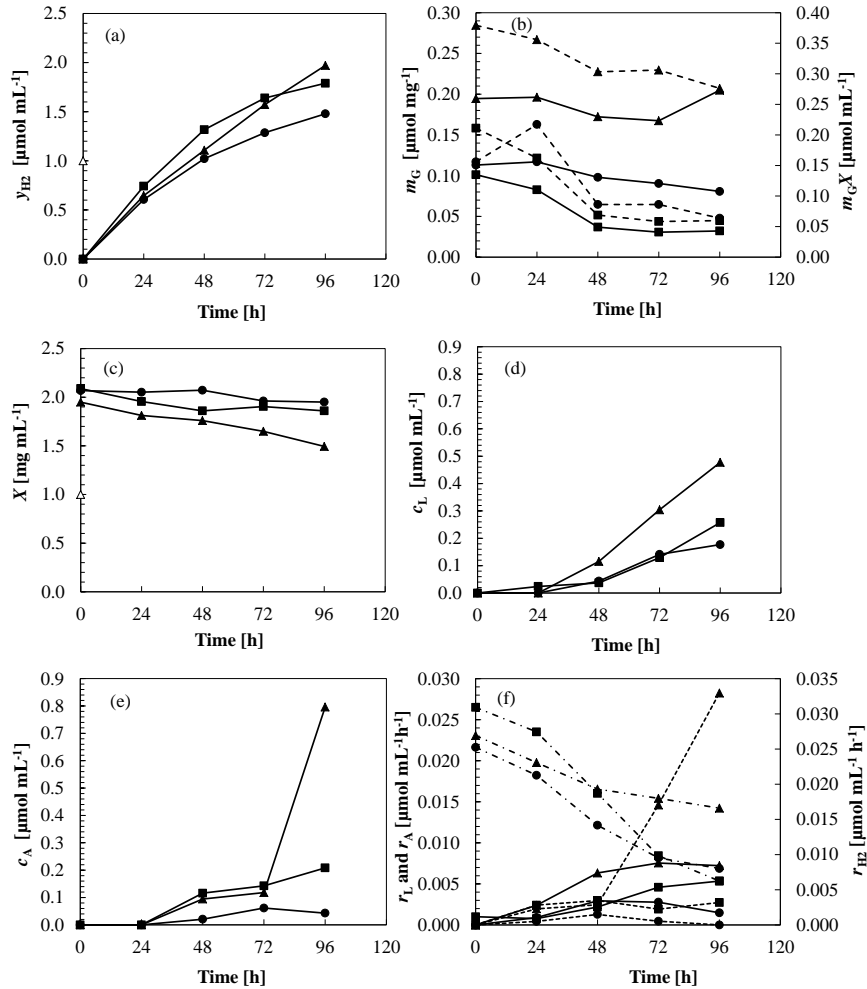


Figure 3.8 Time courses of culture variables measured in three dark anaerobic runs made utilizing cells from (1) logarithmic growth phase (●), (2) late-logarithmic growth phase(■) and (3) stationary phase(▲). (a) the number of moles  $H_2$  per culture volume ( $y_{H_2}$ ); (b) the number of moles of endogenous glucose per dry cell weight ( $m_G$ ) in solid line and the number of moles of endogenous glucose per culture volume in broken line ( $m_G X$ ); (c) the dry cell weight concentration ( $X$ ); (d) the lactate concentration; (e) the acetate concentration; (f) the lactate production rates in broken line ( $r_L$ ), the acetate production rates in solid line ( $r_A$ ) and the  $H_2$  production rate in long short broken line ( $r_{H_2}$ ); gas phase, nitrogen gas; inoculum cells, washed to ensure there is no nitrogen source in fermentation system.

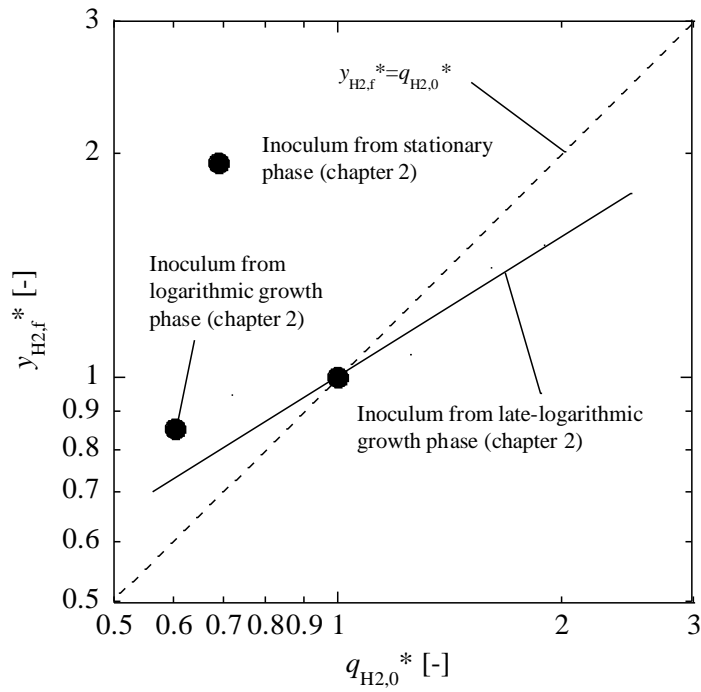


Figure 3.9 Relation between initial specific H<sub>2</sub> production rate and attainable level of H<sub>2</sub>

$q_{H2,0}^* = q_{H2,0} / (q_{H2,0})_{ref}$ ;  $y_{H2,f}^* = y_{H2,f} / (y_{H2,f})_{ref}$ ; ref, run with late-logarithmically growing cells

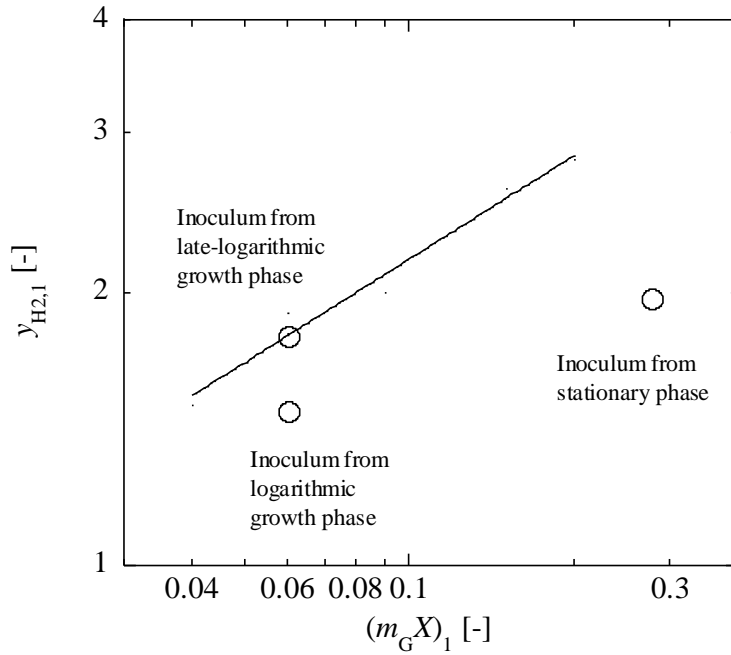


Figure 3.10 Relation between  $(m_G X)_1$  and  $y_{H2,1}$  of dark anaerobic incubation of GT strain, from logarithmic growth phase, late-logarithmic growth phase and stationary phase, in HEPES buffer solution.

Solid line is calculated for cells, from late-logarithmic growth phase, in HEPES buffer solution with monosaccharides. Calculation was made utilizing equation and parameters in chapter 2

### Chapter 3: References

- Allen MM (1968) Photosynthetic membrane system in *Anacystis nidulans*. *Journal of Bacteriology*. 96(3):836-841
- Ananyev G, Carrieri D, Dismukes GC (2008) Optimization of Metabolic Capacity and Flux through Environmental Cues To Maximize H<sub>2</sub> Production by the Cyanobacterium “*Arthrospira* (*Spirulina*) *maxima*”. *Applied and Environmental Microbiology* 74 (19):6102-6113
- Baebprasert W, Lindblad P, Incharoensakdi A (2010) Response of H<sub>2</sub> production and Hox-H<sub>2</sub>ase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *International Journal of Hydrogen Energy* 35 (13):6611-6616
- Hanai M, Sato Y, Miyagi A, Kawai-Yamada M, Tanaka K, Kaneko Y, Nishiyama Y, Hihara Y (2014) The effects of dark incubation on cellular metabolism of the wild type cyanobacterium *Synechocystis* sp. PCC6803 and a mutant lacking the transcriptional regulator cyAbrB2. *Life*. 4:770-787
- Henderson RK, Baker A, Parsons SA, Jefferson B (2008) Characterisation of algogenic organic matter extracted from cyanobacteria, green algae and diatoms. *Water Research* 42 (13):3435-3445.
- Huege J, Goetze J, Schwarz D, Bauwe H, Hagemann M, Kopka J (2011) Modulation of the major paths of carbon in photorespiratory mutants of *Synechocystis*. *PLoS One*. 6(1)e16278
- Joseph A, Aikawa S, Sasaki K, Matsuda F, Hasunuma T, Kondo A (2014) Increased biomass production and glycogen accumulation in *apcE* gene deleted *Synechocystis* sp. PCC 6803. *AMB Express* 4 (1):17
- Mustaqim D, Ohtaguchi K (1997) A synthesis of bioreactions for the production of ethanol. *Energy*. 22:353-356
- Ohtaguchi K, Nasu A, Koide K, Inoue I (1987) Effects of size structure on batch growth of lactic acid bacteria. *Journal of Chemical Engineering of Japan*. 20(6):557-562
- Ohtaguchi K, Wijanarko A (2002) Elevation of the efficiency of cyanobacterial carbon dioxide removal by monoethanolamine solution. *Technology* 8:267-286
- Phlips E, Zeman C, Hansen P (1989) Growth, photosynthesis, nitrogen fixation and carbohydrate production by a unicellular cyanobacterium, *Synechococcus* sp. (Cyanophyta). *Journal of Applied Phycology* 1 (2):137-145
- Steele JH (1962) Environmental control of photosynthesis in the sea. *Limnol Oceanogr*. 7: 137–150
- Taikhao S, Junyapoon S, Incharoensakdi A, Phunpruch S (2013) Factors affecting bioH<sub>2</sub> production by unicellular halotolerant cyanobacterium *Aphanothece halophytica*. *Journal of Applied Phycology* 25 (2):575-585.
- Troshina O, Serebryakova L, Sheremetieva M, Lindblad P (2002) Production of H<sub>2</sub> by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy* 27 (11–12):1283-1289
- van de Meene AML, Sharp WP, McDaniel JH, Friedrich H, Vermaas WFJ, Roberson RW (2012) Gross morphological changes in thylakoid membrane structure are associated with photosystem I deletion in *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta - Biomembranes* 1818 (5):1427-1434
- Xie J, Zhou J, Zhang H, Li Y (2011) Increasing reductant NADPH content via metabolic engineering of PHB synthesis pathway in *Synechocystis* sp. PCC6803. *Sheng Wu Gong Cheng Xue Bao*. 27(7):998-1004
- Yoo S-H, Keppel C, Spalding M, Jane J-I (2007) Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. *International Journal of Biological Macromolecules* 40 (5):498-504
- Yu Y, You L, Liu D, Hollinshead W, Tang YJ, Zhang F (2013) Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory. *Marine drugs* 11 (8):2894-291

## Chapter 4

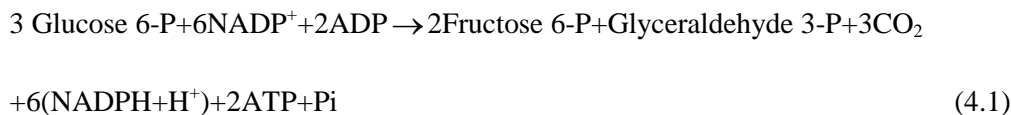
### Genetic Modification Effects on Hydrogen Production

#### 4.1 Problem Definition and General Consideration

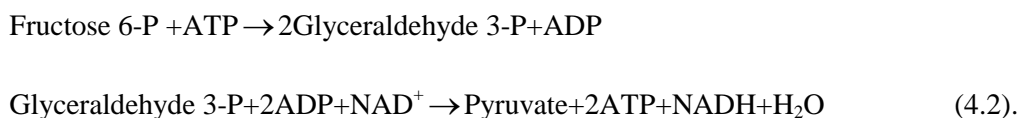
##### (a) General remarks

Experimental results in chapters 2 and 3 show that hydrogen (H<sub>2</sub>) production in a glucose tolerant mutant of *Synechosystis* sp. strain PCC 6803 (GT strain) in the dark anaerobic HEPES buffer solution is accompanied by the decomposition of endogenous glucose, and production of H<sub>2</sub>, lactate and acetate. In the presence of glucose or fructose, it also produces succinate and ethanol (Figure 2.6). The reductive compound NAD(P)H (=NADH plus NADPH) is the substrate for H<sub>2</sub> production on NiFe-hydrogenase, hence association of above products with NAD(P)H is briefed.

Glucose catabolism in is known to start by the glycolysis to fructose 6-P and glyceraldehyde 3-P through the oxidative pentose phosphate (OPP) pathway (Figure A3.2). Overall stoichiometry is shown by:



The two monosaccharide-phosphates in products are further converted to pyruvate:

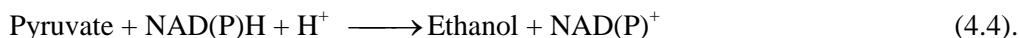


These equations show that breaking down of 1 mol glucose 6-P to 2 moles pyruvate results in the production of 5/3 moles pyruvate and 2 moles NADPH plus 10/3 moles NADH. If reductive compound is shown by NAD(P)H (NADH plus NADPH), these equation result that conversion of glucose 6-P to pyruvate produces 16/3 moles NAD(P)H.

Conversion of pyruvate to lactate or ethanol consumes NADH or NAD(P)H. Hence, the pathways to generate lactate and ethanol compete for the reductive compound with H<sub>2</sub> generating NiFe-hydrogenase. Genes coding for lactate dehydrogenase (*ldh*) or alcohol dehydrogenase (*adh*) appear to be possible targets to be eliminated to increase H<sub>2</sub> production. Conversion of pyruvate to lactate is shown by



Hence 1 mole decrease in lactate production has a potential to elevate 1 mole H<sub>2</sub> production. Conversion of pyruvate to ethanol is shown by



If both lactate and ethanol productions are knocked out, additional increase in H<sub>2</sub> production can be predicted. GT strain is challenged to evaluate the potential of the work that has focused on *ddh* and *adh* deletions for H<sub>2</sub> production enhancement. Succinate pathway of incomplete tricarboxylic acid (TCA) cycle (**Figure 4.1**) of *Synechocystis* sp. strain PCC 6803 is also an interesting reaction which should have been targeted to be another way to enhance H<sub>2</sub> production if it is not due to the fact that succinate and succinate dehydrogenase (*sdh*) have a major role on respiratory electron transfer and thereby in redox poisoning (Cooley and Vermaas 2001). However, this research ignores the knocking out of *sdh* genes since H<sub>2</sub> production in this study is performed only in completely oxygen (O<sub>2</sub>) free environment.

#### **(b) Lactate dehydrogenase as a target of metabolic engineering approach**

Lactate dehydrogenase (*ddh*) is an enzyme that consumes NADH during lactate production. To elevate the level of intracellular NADH for bidirectional NiFe-hydrogenase, in Chapter 4, the genes coding for *ddh* were disrupted in GT strain. The modified strain was referred as Δ*ddh* mutant. Redirection of NAD(P)H to bidirectional hydrogenase is found to be a very promising engineering approach to elevate H<sub>2</sub> production. Main fermentative products in dark anaerobic condition of GT strain are lactate and acetate. There are some research works which show increases in H<sub>2</sub> production by a deletion of lactate dehydrogenase genes in a cyanobacterium *Synechococcus* sp. strain PCC 7002 (McNeely 2010) and in gut bacillus *Escherichia coli*. Genes in GT strain coding for lactate dehydrogenase, which catalyzes the reaction of Eq.(4.3), is cloned in slr1556 (lactate dehydrogenase gene).

First experiment in Chapter 4 is designed to disrupt the *ddh* genes in a glucose tolerant mutant of *Synechocystis* sp. strain PCC 6803 (GT strain) to increase the amount of NADH that limits the H<sub>2</sub> production rate of bidirectional hydrogenase. This mutant is referred as Δ*ddh* strain.

#### **(c) Alcohol dehydrogenase genes as a second target of metabolic engineering approach**

GT strain can naturally produce small amount of ethanol as a byproduct in dark anaerobic condition. Pyruvate is converted to acetaldehyde and CO<sub>2</sub>. Acetaldehyde is then reduced to ethanol by alcohol dehydrogenase (*adh*, EC 1.1.1.2). One of metabolites appears during dark anaerobic condition is detected

to be ethanol. The disruption of alcohol dehydrogenase for H<sub>2</sub> enhancement in cyanobacteria is first carried out using a model cell for study. The *adh* gene is selected and targeted to be deleted in order to save NADPH for hydrogenase pathway. A mutant lacking both *adh* genes and *ddh* genes is referred as a double knocking out mutant. Second experiment in chapter 4 is to derive the double knocking out mutant by disrupting both *ddh* genes and *adh* genes. It has a potential to further elevate H<sub>2</sub> production. This chapter draws a construction method on  $\Delta adh\Delta ddh$  double mutant.

According to CyanoBase (<http://genome.kazusa.or.jp/cyanobase/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>), alcohol dehydrogenase genes in *Synechocystis* sp. strain PCC 6803 are distributed in 4 genes as shown in **Table 4.1**. There are multiple genes involving in alcohol production. Clone slr0942 contains NADP<sup>+</sup> [EC.1.1.1.2] dependent alcohol dehydrogenase and clone slr1192 contains probable zinc-containing alcohol dehydrogenase. Clone slr1192 was previously knocked out to study stress response. Clone slr1192 contains NADP dependent alcohol dehydrogenase for aromatic compounds. This enzyme uses medium-chain aliphatic aldehyde as oxidants (Vidal et al. 2009). Those enzymes on slr0942 and slr1192 were previously knocked out to study reduction of ketone.

Only the deletion mutant from slr0942 exhibited a change in reaction while that from slr1192 gave no effect to ketone reaction. (Takemura et al. 2009). No report confirms specific role in dark anaerobic condition on both genes. The highly possible pathways involves in ethanol production in heterotrophic of GT strain are alcohol dehydrogenase-NAD<sup>+</sup> [EC.1.1.1.1] and alcohol dehydrogenase-NADP<sup>+</sup> [EC.1.1.1.2] enzymes. This work selects the clone slr0942 as a target because the gene is classified as a gene for short chain alcohol dehydrogenase and GT strain cells produce ethanol (short chain alcohol) in dark anaerobic condition. The other genes were not selected because the slr0990 is class III alcohol dehydrogenase which has virtually no activity for ethanol oxidation but exhibits high activity for oxidation of long-chain primary alcohols and oxidation of S-hydroxymethyl-glutathione, a spontaneous adduct between formaldehyde and glutathione in human (National Center for Biotechnology Information 2012). There was a study of slr1192 as an aromatic–medium chain alcohol dehydrogenase in *Synechocystis* sp. strain PCC 6803 therefore slr1192 was not likely to catalyze ethanol production. There are reports that show a construction of a mutant by inserting pyruvate decarboxylase (*pdh*) of *Zymomonas mobilis* into *Synechocystis* sp. strain PCC 6803 make cells to be able to yield a production of 120  $\mu\text{mol mL}^{-1}$  ethanol phototrophically via cyanobacterium *adh* (Gao et al. 2012; Dehring et al. 2012). Although aldehyde is toxic to *Synechocystis* sp. strain PCC6803, cells are protected by converting it to ethanol. It is known that fermentation products of cyanobacteria are CO<sub>2</sub>, H<sub>2</sub>, acetate, lactate and ethanol. The selected clone slr0942 fits to an assumption of gene that involves in ethanol production as shown in Eq.(4.4).

Thus, the primary purposes of the study in this chapter were (1) to construct the  $\Delta$ dh mutant and  $\Delta$ adh $\Delta$ dh mutant from GT strain by genetic modification techniques, and (2) to evaluate the generic modification effects on H<sub>2</sub> production.

## 4.2 Materials and methods

### (a) Mutagenesis

#### Organisms, plasmids, growth media and primers

The competent cells of *Escherichia coli* X1-Blue were used for transformation purposes. In general, *E. coli* cells were cultured in Lysogeny broth (LB) at 37°C and 220rpm in a shaking incubator. A pUC19 plasmid was selected to be used for vector for homologous DNA recombination (Yanisch-Perron et al. 1985)

#### Overall experimental design to construct a vector to disrupt *ddh* genes

**Table 4.2** shows pUC4K and pUC19. Plasmid pUC4K was used as a donor of kanamycin fragment. Plasmid pUC19 was used as a vector.

Host organism used was *Synechocystis* sp. strain PCC6803-GT. Lactate dehydrogenase gene (*ddh*) sequence of cyanobacterium *Synechocystis* sp. strain PCC6803 was retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG). Primers used in this experiment were as shown

Forward primer: 5'GCCTATGATCGTCAATTTTCC3'

Reverse primer: 5'TTCAGCAATATTTGCCAGTGTC3'

**Figure 4.2** shows the strategy to construct a vector pUC19-*ddh*-Km<sup>r</sup> to disrupt the lactate dehydrogenase gene (*ddh*) of GT strain. The *ddh* gene on the fragment slr1556 from chromosomal DNA of wild-type *Synechocystis* sp. strain PCC6803 was prepared by a PCR amplification using above primers forward and reverse.

#### Overall experimental design to construct a vector to disrupt both *ddh* genes and *adh* genes

**Table 4.3** shows plasmid DNA for the construction of DNA for  $\Delta$ adh $\Delta$ dh. The 2.692 kb DNA pMD19 was used as a vector. The 4.3 kb DNA pHP45- $\Omega$ conferring streptomycin (Sm) and spectinomycin

(Sp) cassette ( $\Omega$  fragment) was used as a donor of the genes coding for antibiotic fragment (Prentki and Krisch 1984).

The slr0942 gene sequence of cyanobacterium *Synechocystis* sp. strain PCC 6803 was retrieved from KEGG. Primers used in this experiment were as shown

Forward primer: 5' CAAAAGCATTGTCCGCCTCACC3'

Reverse primer: 5' GCTTCCGCTTGGGAGTGCC3'

**Figure 4.3** shows the strategy to construct a vector pMD19-*adh*- $\Omega$  to disrupt the alcohol dehydrogenase gene (*adh*). The deletion of alcohol dehydrogenase-NADP<sup>+</sup> (slr0942) was performed by a PCR amplification of slr0942 gene fragment using primer forward and reverse from chromosomal DNA of wild-type *Synechocystis* sp. strain PCC6803. PCR-amplified *adh* fragment was ligated into *EcoRV* restriction site of plasmid pMD19 (T-vector) from Takara company. The streptomycin/spectinomycin cassette (Sm/Sp) from plasmid pHP45- $\Omega$  was inserted into the *EcoRV* restriction site at slr0942 gene fragment. Transformant colony ( $\Delta$ adh $\Delta$ ddh) was selected from Sm<sup>+</sup>/Km<sup>+</sup> plate and continuously re-streaked to new plates. Sm resistant cell were grown at increasing Sm concentrations and finally transferred into liquid BG-11 medium.

#### **Transformation (homologous recombination) of GT strain with pUC19-*ddh*-Km<sup>r</sup>**

By adding 3.5  $\mu$ g vector to cells on membrane surface, cells replaced its original DNA with new constructed one in order to survive on Km<sup>+</sup> plate. Transformant colony was selected from Km<sup>+</sup> plate. Km resistant cells were continuously at week interval re-streaked to new plates at increasing Km concentrations to 120  $\mu$ g mL<sup>-1</sup>. Transformant was grown on agar plate with high concentration Km. A successful complete segregation was confirmed by PCR using above primers.

#### **Transformation (homologous recombination) of GT strain and $\Delta$ ddh strain with pMD-*adh*- $\Omega$**

GT strain and  $\Delta$ ddh were transformed with pMD19-*adh*- $\Omega$  plasmid on agar plate with Sm and Sm/Km antibiotic(s). Transformations were carried out as described below.

1.  $\Delta$ ddh strain and GT strain cells were grown to reach OD<sub>730</sub> of 0.5.
2. 1500  $\mu$ L cell cultures were harvested by centrifugation and re-suspended in 200  $\mu$ L fresh BG-11 medium.
3. 3.5  $\mu$ g of pMD-19-*adh*- $\Omega$  was added and incubated at room temperature for 30 min.
4. Previous mixtures were spread on BG-11 plates containing membranes on surfaces.

5. Membranes were move to new BG-11 plate containing appropriate antibiotic (Sm) after 24 h.
6. Plates were observed after 2 weeks.

### **(b) Segregation of mutant**

Transformation of GT strain with pUC19-ddh-Km<sup>+</sup> and Transformation of GT strain and  $\Delta$ adh strain with pMD19-adh- $\Omega$  were performed as described (Yamamoto et al. 2012; Chongsuksantikul et al. 2014).

### **(c) Indirect biophotolysis for dark anaerobic H<sub>2</sub> production**

#### **Strain**

A glucose-tolerant strain of cyanobacterium *Synechocystis* sp. strain PCC6803-GT was used as a host strain Cells of GT strain were kept shaking in L-tube glass, and grown on BG-11 medium under constant illumination of fluorescent light, at constant room temperature prior to cell growth. Mutant cells of GT strain were also kept in L-tube. GT strain,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant were performed on BG-11 medium containing 60  $\mu$ mol mL<sup>-1</sup> kanamycin and BG-11 medium containing 60  $\mu$ mol mL<sup>-1</sup> kanamycin and 2.5  $\mu$ mol mL<sup>-1</sup> streptomycin.

#### **Culture technique and fermentation**

Cells of GT strain grown in L-tube were pre-cultured in 60 mL BG-11 medium containing HEPES buffer at pH = 7.7, in 100 mL clear Pyrex glass column. The culture was bubbled from the bottom of column at 80 mL min<sup>-1</sup> by the air containing 6% CO<sub>2</sub> that was filtered through 0.45 $\mu$ m filter. Photosynthetic photon flux density (*PPFD*) of incident light by fluorescent light was maintained at approximately 100  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. Cells of  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant were pre-cultured in BG-11 medium with 60  $\mu$ mol mL<sup>-1</sup> kanamycin and both 60  $\mu$ mol mL<sup>-1</sup> kanamycin and 2.5  $\mu$ mol mL<sup>-1</sup> streptomycin, respectively, 60  $\mu$ mol mL<sup>-1</sup> kanamycin and 2.5  $\mu$ mol mL<sup>-1</sup> streptomycin for 3-4 d until logarithmic growth phase. Preculture growth of mutant cells in BG-11 medium with antibiotic required longer time to reach a turbid state than that of GT strain on BG-11 medium without antibiotics.

Logarithmically growing cells of GT strain,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant in precultures were inoculated into 60 mL BG-11 medium (pH, 7.7) without antibiotics. The apparatus (100 mL clear Pyrex glass column) and the conditions for aeration and illumination were the same as those for precultures. The initial dry cell weight concentration was set at 0.103  $\pm$  0.027 mg mL<sup>-1</sup>. Growth was monitored by measuring OD<sub>730</sub> of cell suspension. One of OD<sub>730</sub> represents the dry cell weight concentration (*X*) of 0.369 mg mL<sup>-1</sup>. Cells were harvested in late logarithmic phase when *X* reached 2.58 mg mL<sup>-1</sup> (OD<sub>730</sub>=7).

Dark anaerobic H<sub>2</sub> production was performed as described in the section 2.2.1.

## 4.3 Results and discussion

### (a) Construction of $\Delta ddh$ mutant and $\Delta adh\Delta ddh$ mutant

GT strain was transformed with pUC19-*ddh*-Km<sup>r</sup> plasmid on agar plate with Km antibiotic as shown in **Table 4.4**. Many colonies were observed after 6 d. It took around 6 months to complete segregation due to short homologous segments (200,700 bp) of *ddh* gene. It is known that, when the homologous segments are less than 400 bp, the efficiency drops significantly.

Results of transformation of GT strain and  $\Delta adh$  strain with pMD19-*adh*- $\Omega$  are shown in **Table 4.5**. Large number of colonies appeared on Sm<sup>r</sup> plate. The mutant of GT strain that is knocked down *adh* genes is obtained. This strain is referred as  $\Delta adh$  strain. The  $\Delta ddh$  strain was transformed with pMD19-*adh*- $\Omega$  to eliminate both *adh* genes and *ddh* genes from GT strain. Slight growth on Sm<sup>r</sup> and Km<sup>r</sup> selection medium is confirmed. Colonies were tiny in this run. Mutant lacking *adh* genes and *ddh* genes is successfully constructed. This mutant is referred as  $\Delta adh\Delta ddh$  strain.

Photographs in **Figure 4.4** show results of agarose-gel electrophoretic analyses of PCR products for evaluating the achievement of deletion of (a) *ddh* genes and (b) both *adh* and *ddh* genes. Photograph (a) shows Lane 1: size marker DNA, Lane 2: PCR product for *ddh* primers and pUC19-*slr1556*:Km<sup>r</sup>, Lane 3: PCR product for *ddh* primers and genomic DNA from GT strain, and Lane 4: PCR product with *ddh* primers and genomic DNA from  $\Delta ddh$  mutant. Photograph (b) shows Lane 1: size marker DNA, Lane 2: PCR product for *ddh* primers and pUC19-*slr1556*:Km<sup>r</sup>, Lane 3: PCR product for *adh* primers and pMD19-*slr0942*::Sp<sup>r</sup>/Sm<sup>r</sup>, Lane 4: PCR product for *ddh* primers and genomic DNA from GT strain, Lane 5: PCR product for *adh* primers and genomic DNA from GT strain, Lane 6: PCR product for *ddh* primers and DNA from  $\Delta adh\Delta ddh$  mutant and Lane 7: PCR product for *adh* primers and genomic DNA from  $\Delta adh\Delta ddh$  mutant. The successful segregation of  $\Delta ddh$  is confirmed, ((a) Lane 4 or (b) Lane 6) is the same size as that for primers and DNA fragment from pUC19-*slr1556*:Km<sup>r</sup> ((a) or (b) Lane 2). Double mutation is also confirmed by the result showing that the size of PCR product for primers and genomic DNA from  $\Delta adh\Delta ddh$  mutant ((b) Lane 7) is the same size as that for primers and DNA fragment from pMD19-*slr0942*::Sp<sup>r</sup>/Sm<sup>r</sup> ( (b) Lane 3).

### (b) Cellular state prior to dark anaerobic incubation

Precultures for photoautotrophic growth of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant were performed on BG-11 medium, BG-11 medium containing 60  $\mu\text{mol mL}^{-1}$  kanamycin and BG-11 medium containing 60  $\mu\text{mol mL}^{-1}$  kanamycin and 2.5  $\mu\text{mol mL}^{-1}$  streptomycin, respectively. At 96 h of precultures, cells were harvested, washed and then inoculated into main cultures in which cells were grown photoautotrophically in antibiotic-free BG-11 medium. Growth of cells on BG-11 was monitored by optical density at 730 nm wavelength. Growth curves for GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant show that cells of GT strain and  $\Delta$ adh mutant were in logarithmic growth phase from 0 h to 24 h and from 0 h to 40 h, respectively, while  $\Delta$ adh $\Delta$ dh mutant was in lag phase from 0 h to 24 h and in logarithmic growth phase from 24 h to 48 h (**Figure 4.5**).

The lag time for the growth of  $\Delta$ adh $\Delta$ dh mutant appears to represent a period for adaptation of the mutant from antibiotic containing preculture medium to antibiotic-free main culture medium. After one week cultivation, dry cell weight concentrations of  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant reached the same level as GT strain.

Carbon dioxide ( $\text{CO}_2$ ) is a single carbon source for cell growth, hence the slope of growth curve represents the activity of carbon dioxide fixation. Total amount of carbon dioxide incorporated into dry cell weight during 168 h cultivation is found not to be affected by deletion of *adh* or *dh* genes. The inoculum cells of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant for dark anaerobic hydrogen production were harvested at 36, 45, and 60 h, respectively. **Table 4.6** shows the state of cells prior to dark anaerobic  $\text{H}_2$  production experiments.

The  $\text{OD}_{730}$  at the cell harvesting time was fixed at 7, hence inoculum cells for hydrogen production experiment were confirmed to be exposed to same PPFD from incident light to transmitted light. The slope of growth curves ( $\text{dln}X/\text{d}t$ ) of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant of the  $X$  at 2.58  $\text{mg mL}^{-1}$  are 0.0187, 0.0318 and 0.0238  $\text{h}^{-1}$ , respectively. The cell formula of *Synechocystis* sp. strain PCC6803 shown in a reference is  $\text{CH}_{1.62} \text{N}_{0.22} \text{O}_{0.40} \text{P}_{0.11}$  (Yu et al. 2013), hence the rates of  $\text{CO}_2$  assimilation by inocula of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant are calculated as 2.09, 3.55 and 2.66  $\mu\text{mol mL}^{-1} \text{h}^{-1}$ , respectively and the rates of NADPH consumption at the Calvin cycle are calculated as 4.18, 7.10 and 5.32  $\mu\text{mol mL}^{-1} \text{h}^{-1}$ , respectively.

### (c) Characteristics of dark anaerobic nitrate-free $\text{H}_2$ production in the absence of exogenous glucose

In the first series of  $\text{H}_2$  production experiments, photoautotrophic cells of GT strain,  $\Delta$ adh mutant or  $\Delta$ adh $\Delta$ dh mutant in late-logarithmic growth phase was incubated at 2.58  $\text{mg mL}^{-1}$  dry cell weight concentration in dark anaerobic nitrate-free HEPES buffer solution without glucose.  $\text{H}_2$  production

characteristics in the absence of glucose were studied. **Figure 4.6** shows the time courses of culture variables during dark anaerobic H<sub>2</sub> production in GT strain  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant. Quantities measured include the moles per culture volume of (a) hydrogen and (b) the moles of endogenous glucose in glycogen, and the concentrations of (c) dry cell weight, (d) lactate and (e) acetate. Similar to photoautotrophic culture, the solution for hydrogen production was free from antibiotics.

**Table 4.7** shows the characteristics of dark anaerobic H<sub>2</sub> production in GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant in dark anaerobic HEPES buffer solution without glucose. If subscript 1 shows the state at 96 h, the  $y_{H_2,1}$  values of runs with GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant are 1.8, 2.0 and 1.5  $\mu\text{mol mL}^{-1}$ , respectively. The  $y_{H_2,1}$  of  $\Delta$ adh mutant is 1.11 times that of GT strain. The  $y_{H_2,1}$  of  $\Delta$ adh mutant shown in our previous report (Yamamoto et al. 2012) was 1.5 times that of GT strain. Previous work and present work utilized the inocula from logarithmic growth phase and late-logarithmic growth phase, respectively. As concluded in chapter 3, growth phase is of utmost important factor for H<sub>2</sub> production.

The elimination of adh activity resulted to decrease  $y_{H_2,1}$  that was 0.83 times that of GT strain, The highest  $y_{H_2,1}$  was observed in run with  $\Delta$ adh mutant, second highest is in run with GT strain and the lowest is in run with  $\Delta$ adh $\Delta$ adh mutant. This descending order is same as the ascending order of the modified DNA size shown in Table 4.6. The DNA size of  $\Delta$ adh $\Delta$ adh mutant is 0.56 kb larger than that of GT strain, hence expression of increased DNA size appears to increase the NAD(P)H consumption for protein and amino acid synthesis and reduce the hydrogen production. On the other hand, the DNA size of  $\Delta$ adh mutant is 0.459 kb smaller than that of GT strain, hence expression of decreased DNA size appears to decrease the NAD(P)H consumption for protein and amino acid synthesis and increase the hydrogen production.

The curves in Figure 4.6 (a) for GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant are calculated with the values of  $y_{H_2,f}$  and  $k$  in Table 4.7. The calculated  $y_{H_2}$  agrees well with the observed  $y_{H_2}$ . The descending order of  $y_{H_2,f}$  in Table 4.7 is same as the descending order of the  $y_{H_2,1}$ . **Figure 4.7** is the plots of observed  $y_{H_2,1}$  against change in expressed DNA size. Experimental data fit well with the equation:

$$y_{H_2,1} = 1.78 - 0.475(\text{change in expressed DNA size}) \quad (4.5)$$

An increase in the expressed DNA size has a potential to reduce the reductive compounds during dark anaerobic H<sub>2</sub> production.

The  $r_{H_2,0}$  of Table 4.7 is highest for run with  $\Delta$ adh mutant, second highest for run with  $\Delta$ adh $\Delta$ adh mutant and lowest for run with GT strain. The descending order of  $r_{H_2,0}$  is similar to that of the consumption rate of NAD(P)H in the Calvin cycle just before inoculation (Table 4.6). Shift of cellular

environment from illuminated aerobic nitrate-containing BG-11 medium to dark anaerobic nitrate-free HEPES buffer solution appears to redirect the accumulated NAD(P)H molecules in the Calvin cycle to NiFe-hydrogenase.

The elimination of *ddh* activity or both *adh* and *ddh* activities results in 1.16-fold increase or 1.15-fold increase in the  $r_{H_2,0}$ . Considering that the NiFe-hydrogenase converts 1  $\mu\text{mol}$  NAD(P)H to form 1  $\mu\text{mol}$  hydrogen, this result shows that the amount of NADH consumed by lactate dehydrogenase of GT strain is about 16% of the total amount of NADH and NADPH consumed by NiFe-hydrogenase of GT strain. The initial lactate production rate was  $0.000833 \mu\text{mol mL}^{-1} \text{h}^{-1}$  in GT strain and almost  $0 \mu\text{mol mL}^{-1} \text{h}^{-1}$  in  $\Delta\text{ddh}$  and  $\Delta\text{adh}\Delta\text{ddh}$  mutants (Figure 4.6d). Stoichiometry of D-lactate dehydrogenase and NiFe-hydrogenase suggests that the elimination of above lactate production rate potentially increases hydrogen production rate from  $0.0309 \mu\text{mol mL}^{-1} \text{h}^{-1}$  to  $0.0359 \mu\text{mol mL}^{-1} \text{h}^{-1}$ . The observed increase is 6 times more than the estimated increase by *ddh* elimination. Redirection of carbon metabolism at pyruvate from lactate production to acetyl-CoA production is found to amplify NAD(P)H production.

A 1.28-fold increase in  $\Delta\text{ddh}$  mutant and a 2.19-fold increase in  $\Delta\text{adh}\Delta\text{ddh}$  mutant over GT strain in the deactivation constant  $k$  are seen in the results (Table 4.7). The presence of dehydrogenases in GT strain appears to be important to stabilize the dynamical redox adaptation of cells against continuous consumption of NAD(P)H on NiFe-hydrogenase.

The attainable level of  $y_{H_2}$  by  $\Delta\text{ddh}$  mutant is comparable with that of GT strain, while that by  $\Delta\text{adh}\Delta\text{ddh}$  mutant is 0.65 times that of GT strain. For  $H_2$  production in the absence of exogenous glucose, stabilization is of first importance for high production of hydrogen by  $\Delta\text{adh}\Delta\text{ddh}$  mutant. Our result of  $\Delta\text{ddh}$  mutant in *Synechocystis* sp. strain PCC 6803 differs from that of  $\Delta\text{ddhA}$  mutant in *Synechococcus* sp. strain PCC7002 (McNeely et al., 2010) which shows 5-fold increase in hydrogen production from wild type strain; however the attainable level of hydrogen ( $y_{H_2,t}$ ) in this experiment is 10 times that shown by their experiment.

Graph (b) of Figure 4.6 shows endogenous glucose consumption characteristics in the absence of glucose. The  $m_{GX}$  of runs with three strains decreased or kept constant with time. Although  $H_2$  productions in three strains were highest at initial, extremely low decrease in the  $m_{GX}$  at initial was seen in run with  $\Delta\text{ddh}$  mutant. The initial rates of endogenous glucose consumption of GT strain,  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant were  $0.00208$ ,  $0.000417$  and  $0.00125 \mu\text{mol mL}^{-1} \text{h}^{-1}$ , respectively. The initial  $H_2$  production of mutants is unrelated to endogenous glucose consumption. The decrease in the  $m_{GX}$  with time is highest from 24 to 48 h in runs with GT strain and  $\Delta\text{ddh}$  mutant, and from 0 to 24 h in run with  $\Delta\text{adh}\Delta\text{ddh}$  mutant. The yields of hydrogen on endogenous glucose ( $Y_{H_2/G}$ ) in GT strain and in  $\Delta\text{ddh}$

mutant evaluated in this period are 6.67 and 10.0, respectively. The  $Y_{H_2/G}$  in  $\Delta adh\Delta ddh$  mutant is constant at 26 from 0 to 72 h.

No decrease in  $m_G X$  is seen from 48 to 96 h in run with GT strain, from 0 to 24 h and 48 to 72 h in run with  $\Delta ddh$  mutant and from 24 to 72 h in run with  $\Delta adh\Delta ddh$  mutant. The molecules of NAD(P)H for NiFe-hydrogenase are found to be mainly supplied by oxidative reactions other than glycolysis such as turnover of proteins, membrane lipids and PHB.

The decrease in  $m_G X$  with time is highest from 24 to 48 h in runs with GT strain and  $\Delta ddh$  mutant, and from 0 to 24 h in run with  $\Delta adh\Delta ddh$  mutant. The yields of  $H_2$  on endogenous glucose ( $Y_{H_2/G}$ ) in GT strain and in  $\Delta ddh$  mutant evaluated in this period are 6.67 and 10.0, respectively. The  $Y_{H_2/G}$  in  $\Delta adh\Delta ddh$  mutant is constant at 26 from 0 h to 72 h.

The present results are obtained in runs with inocula from late-logarithmic growth phase. If logarithmically growing cells are inoculated into dark anaerobic nitrate-free HEPES buffer solution, the  $m_G X$  increases in the first 24 h and then decreases after 24 h (data not shown). The inoculum  $m_G X$  levels of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant were 0.21, 0.17 and 0.14  $\mu\text{mol mL}^{-1}$ , respectively. Figure 4.6 (b) shows that the  $m_G X$  at 96 h of runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant were 0.06, 0.07, and 0.07  $\mu\text{mol mL}^{-1}$ , respectively. The  $m_G X$  of  $\Delta adh\Delta ddh$  mutant is relatively stable with time.

Dry cell weight consumption characteristic in the absence of glucose is shown in Figure 4.6(c). Concentration of dry cell weight of GT strain slightly decreased with time, while that of  $\Delta ddh$  mutant or  $\Delta adh\Delta ddh$  mutant was almost not varied with time. The  $K_d$  of Table 4.7 shows that cell survival in dark anaerobic nitrate free solution increased in  $\Delta ddh$  and  $\Delta adh\Delta ddh$  mutants. The  $K_d$  values of those mutants are almost zero.

Lactate production characteristics in the absence of glucose is shown in Figure 4.6(d): The production of lactate in GT strain started right after dark incubation Although the deletion of *ddh* genes is confirmed, the time courses of lactate concentration of runs with  $\Delta ddh$  mutant and with  $\Delta adh\Delta ddh$  mutant show the production of trace amount of lactate. Lactate concentrations of runs with  $\Delta ddh$  and  $\Delta adh\Delta ddh$  mutants at 96 h were extremely low as 0.03 and 0.01  $\mu\text{mol mL}^{-1}$ , respectively. These trace amount represent the generation of  $\Delta ddh$  phenotype deficient cells from  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant during photosynthesis on antibiotic-free BG-11 medium and dark incubation on antibiotic-free HEPES buffer solution.

The specific production rate of lactate by GT strain in the first 24 h was 0.000397  $\mu\text{mol mg}^{-1} \text{h}^{-1}$ . The increases in lactate concentration in the incubation of  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  on glucose in the first

24 h were  $0.01 \mu\text{mol mL}^{-1}$  and  $0 \mu\text{mol mL}^{-1}$ , respectively, hence the fraction of  $\Delta\text{addh}$  phenotype deficient cells in  $\Delta\text{addh}$  cells and  $\Delta\text{adh}\Delta\text{addh}$  cells are analyzed to be 0.5 and 0, respectively. Lactate concentrations at 96 h of runs with  $\Delta\text{addh}$  and  $\Delta\text{adh}\Delta\text{addh}$  mutants were extremely low as  $0.03$  and  $0.01 \mu\text{mol mL}^{-1}$ , respectively. The initial yield of lactate on endogenous glucose of GT strain was 0.4, which shows that the relatively large portion of degraded-glycogen is utilized not for the synthesis of lactate. Although hydrogen production rate and endogenous glucose consumption rate decreased with time, lactate production rate was accelerated after 48 h. The carbon for lactate production after 48 h appears to be supplied from not only endogenous glucose but also other carbonaceous compounds.

Acetate production characteristic in the absence of glucose is shown in Figure 4.6(e). Acetate concentration is about one-tenth of lactate concentration. Acetate is not detected in the incubation of  $\Delta\text{adh}\Delta\text{addh}$  mutant. The production of acetate delays for 24 h in the incubations of GT strain and  $\Delta\text{addh}$  mutant. No difference is observed in the acetate production in GT strain and in  $\Delta\text{addh}$  mutant from 24 h to 72 h. The acetate production rate in this period was  $0.00417 \mu\text{mol mL}^{-1} \text{h}^{-1}$ . After 72 h, acetate production was activated in GT strain but turned to consumption in  $\Delta\text{addh}$  mutant. The acetate consumption after 72 h in  $\Delta\text{addh}$  mutant appears to show an oxidation of acetate to carbon dioxide in incomplete TCA cycle. In  $\Delta\text{addh}$  mutant, acetate appears to be redirected to compensate the damaged cell constituting materials for survival. Acetate consumption in  $\Delta\text{addh}$  mutant after 72 h is not seen in GT strain that represents high activity in endogenous glucose consumption. In GT strain, degradation of endogenous glucose likely contributes to be directed to biochemical synthesis for survival. Different from mutant, GT strain actively processed in glycolysis by converting glycogen to lactate and acetate.

Ethanol production characteristics in the absence of glucose: Ethanol concentration at 96 h of runs with GT strain,  $\Delta\text{addh}$  mutant and  $\Delta\text{adh}\Delta\text{addh}$  mutant was extremely low as 0.7, 1.0 and  $0.4 \mu\text{mol mL}^{-1}$ , respectively. These results show that the specific rates of ethanol production by GT strain and  $\Delta\text{addh}$  mutant were  $0.00283$  and  $0.00404 \mu\text{mol mg}^{-1} \text{h}^{-1}$ , respectively. The ethanol production of  $\Delta\text{addh}$  mutant was higher than that of GT strain. The excess NADH molecule that was generated by elimination of lactate hydrogenase activity appears to be redirected to alcohol dehydrogenase. Drop of ethanol concentration of run with  $\Delta\text{adh}\Delta\text{addh}$  mutant from that with  $\Delta\text{addh}$  mutant partially verifies the successful deletion of the genes coding for *adh*. Incomplete elimination of ethanol production in run with  $\Delta\text{adh}\Delta\text{addh}$  mutant shows the generation of  $\Delta\text{adh}$  phenotype deficient cells from  $\Delta\text{adh}\Delta\text{addh}$  mutant

#### **(d) Characteristics of Dark Anaerobic Nitrate-Free Hydrogen Production in the Presence of Glucose**

In the second series of H<sub>2</sub> production experiments, photoautotrophic cells of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant in late-logarithmic growth phase were harvested and incubated with X at 2.58 mg mL<sup>-1</sup> in dark anaerobic nitrate-free antibiotic-free HEPES buffer solution with 5 mmol mL<sup>-1</sup> exogenous glucose. The number of moles per culture volume of H<sub>2</sub> ( $y_{H_2}$ ), exogenous glucose ( $c_S$ ), endogenous glucose ( $m_G X$ ), lactate ( $c_L$ ) and acetate ( $c_A$ ) and dry cell weight concentration ( $X$ ) are analyzed. Time courses of  $y_{H_2}$ ,  $c_S$ ,  $m_G X$ ,  $X$ ,  $c_L$  and  $c_A$  are shown in **Figure 4.8**. Different from curves in Figure 4.6, curves in Figure 4.8 represent the heterotrophic growth of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant on exogenous glucose. Production of lactate, acetate and ethanol in GT strain were also become eminent.

Figure 4.8(a) shows that the  $y_{H_2}$  versus time data of runs with exogenous glucose are fitted by Eq.(2.18). The parameters  $r_{H_2,0}$ ,  $k$  and  $y_{H_2,f}$  for curve-fittings are tabulated in **Table 4.8**. The calculated  $y_{H_2}$  agrees relatively well with the observed  $y_{H_2}$ . The  $y_{H_2,f}$  of glucose-supplemented runs with GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant were 5.61, 3.48 and 2.45  $\mu$ mol mL<sup>-1</sup>, respectively, which were 2.46, 1.50 and 1.65-fold, respectively over runs without glucose. It becomes eminent that exogenous glucose elevates dark anaerobic hydrogen production in GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant.

The relation shown in Fig.4.7 is not seen in Table 4.8. Glucose effect on H<sub>2</sub> production is extremely high, hence genetic modification effect in the absence of glucose becomes to be missing in the presence of glucose. For elevating H<sub>2</sub> production, it is important to elucidate the interrelated effect of monosaccharide, growth phase and genetic modification.

The  $r_{H_2,0}$  of Table 4.8 is highest for run with GT strain, lowest for run with  $\Delta$ adh mutant and second lowest for run with  $\Delta$ adh $\Delta$ dh mutant. This order is quite different from that of runs without glucose. Intracellular electrons increased at the dark anaerobic inoculation is likely directed to NiFe-hydrogenase in the absence of glucose, however, they are directed not only NiFe-hydrogenase but also many metabolic pathways including cell growth, acid fermentation that are triggered in the presence of glucose. Hence  $r_{H_2,0}$  of  $\Delta$ adh mutant under exogenous glucose solution couldn't show highest activity.

Highest  $y_{H_2,f}$  is found to be achieved utilizing GT strain in the presence of exogenous glucose. The attainable level of  $y_{H_2}$  by  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh is less than that of GT strain. When glucose was present, the highest  $y_{H_2,1}$  was GT strain, second highest is  $\Delta$ adh strain and the lowest is  $\Delta$ adh $\Delta$ dh mutant. The elimination of adh activity resulted to decrease  $y_{H_2,1}$  that was 0.49 times that of GT strain in runs with exogenous glucose.

Exogenous glucose consumption is shown in Fig.4.8(b). Time course of the concentration of exogenous glucose ( $c_S$ ) shows that consumption activity of exogenous glucose by  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ dh mutant are extremely low when it is compared with that by GT strain. Glucose consumption of

GT strain was active in the first 48 h as shown in chapter 2, while that of  $\Delta$ adh mutant and that of  $\Delta$ adh $\Delta$ adh mutant were inactive in the first 72 h. Genetic elimination of lactate dehydrogenase activity is found to block the assimilation of glucose. It is known that lactate inhibits glucose uptake (Brown and Whitely, 2009), however, there are remarkably few data available on the cross talk between lactate dehydrogenase and glucose consumption. Conversions of glucose at 96 h by GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant were 0.76, 0.19 and 0.23, respectively. The observed block of exogenous glucose assimilation by the disruption of *dhh* genes was not anticipated.

Fig.4.8(c) shows the time courses of the number of moles of endogenous glucose per culture volume ( $m_{GX}$ ) in the presence of glucose: Right after dark anaerobic incubation of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant, no decrease in  $m_{GX}$  is seen in this graph. Inoculum cells for dark anaerobic incubation were obtained from logarithmic growth phase, hence NAD(P)H synthesis rate is so high that generated NAD(P)H is ready to be redirected for glycogen synthesis. The  $r_{H_2,0}$  and the initial glucose production rate  $r_{G0}$  ( $=d(m_{GX})/dt$ ) of GT strain is highest among three strains. Since  $H_2$  production and glycogen synthesis are NAD(P)H and ATP consumption rate, GT strain in the presence of glucose is found to be rich in reductive compounds and ATP. The time courses of  $m_{GX}$  show that, during 96 h incubation,  $m_{GX}$  of mutant increased during first 24 h, decreased from 24 to 48 h and then become constant after 48 h, whereas  $m_{GX}$  of GT strain shows accumulation of endogenous glucose from the beginning to 72 h and then decreased. Endogenous glucose of run with GT strain shows the accumulation of glucose right after dark anaerobic incubation, when exogenous glucose is also assimilated.

The initial production of  $H_2$  production in GT strain was highest at initial time, an increase in  $m_{GX}$  at initial was seen in run with GT strain. The initial rate of endogenous glucose accumulation of GT,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant were 0.00384, 0.00250 and 0.00032  $\mu\text{mol mL}^{-1} \text{h}^{-1}$ , respectively. The decrease in  $m_{GX}$  with time is not observed from 48 to 96 h in runs with  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant, they were constant. The endogenous glucose of GT strain was increasing during fermentation with a sharp drop after 72 h. The present results are obtained in runs with inocula from late-logarithmic growth phase.

Figure 4.8 (d) shows that dry cell weight concentration ( $X$ ) of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant increased in the first 24 h, and decreased after 24 h. As shown in Table 4.7, those 3 strains represent same specific growth rate at  $0.0101\text{h}^{-1}$  from 0 to 24h. The curve of GT strain shows that the initial increase in  $X$  is associated with the reduction in exogenous glucose concentration. This association confirms the heterotrophic growth of GT strain on glucose. Growth of  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant appears to be triggered by exogenous glucose. Combination of the time courses of exogenous glucose, endogenous glucose and dry cell weight suggests that carbon source for cell growth of  $\Delta$ adh

mutant is not exogenous glucose or endogenous glucose but unidentified intracellular organic compounds. Carbonaceous compounds, other than glucose, that is contained in cell suspended HEPES buffer solution with glucose is  $\text{CO}_2$ ,  $\text{HCO}_3^-$  and those evolved from cell at the beginning of dark anaerobic incubation. The rate of decrease in dry cell weight concentration after 48 h is comparable with the initial decrease in dry cell weight concentration of run without glucose. The time course of dry cell weight concentration of  $\Delta\text{adh}\Delta\text{ddh}$  mutant is comparable with that of  $\Delta\text{ddh}$  mutant.

Time course of lactate concentration ( $c_L$ ) in the presence of glucose (Fig. 4.8 (e)) shows that  $c_L$  at 96 h of run with GT strain was  $4.74 \mu\text{mol mL}^{-1}$  which is 18 times more than that in the absence of glucose. This result shows that exogenous glucose is converted to lactate by GT strain. The yield of lactate on dry cell weight in the first 24 h is  $3.95 \mu\text{mol mg}^{-1}$ . The increases in  $c_L$  in the incubation of  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant on glucose in the first 24 h were  $0.03 \mu\text{mol mL}^{-1}$ , and  $0.07 \mu\text{mol mL}^{-1}$ , respectively, hence the fraction of  $\Delta\text{ddh}$  phenotype deficient cells in  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant are analyzed to be 0.00362 and 0.00844, respectively. Concentrations ( $c_L$ ) at 96 h of runs with  $\Delta\text{ddh}$  and  $\Delta\text{adh}\Delta\text{ddh}$  mutants were extremely low at  $0.05$  and  $0.06 \mu\text{mol mL}^{-1}$ , respectively. The deletion of *ddh* genes in this work possibly altered the intracellular balance between NAD(P)H and NAD(P)<sup>+</sup> towards shifting-up NAD(P)H level. This NAD(P)H shifting-up is important because NADH and NADPH are essential reductive compounds for hydrogen production on NiFe-hydrogenase. The increase in the level of NAD(P)H results in the increase in the level of ATP and subsequently inactivates phosphofructokinase. Assimilation of exogenous glucose by  $\Delta\text{ddh}$  mutant appears to be blocked by the inactivation of phosphofructokinase, brought by the elimination of the genes coding for *ddh*.

Time course of acetate concentration ( $c_A$ ) in the presence of glucose (Fig. 4.8 (f)) shows that acetate production is highly activated by exogenous glucose in GT strain. Exogenous glucose stimulated acetate production also in  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant. After 96 h, acetate concentrations of runs with GT strain,  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant in the presence of exogenous glucose were 17.19, 8.66 and  $4.83 \mu\text{mol mL}^{-1}$ , respectively, that were about more than 100 times those in the absence of exogenous glucose. Acetate is one of the terminal carbon release compound from glucose consumption. Glucose consumption and acetate production of GT strain were higher than those of  $\Delta\text{ddh}$  and  $\Delta\text{adh}\Delta\text{ddh}$  mutants.

Time course of ethanol concentration ( $c_E$ ) in the presence of glucose (Fig. 4.8 (g)) shows that  $c_E$  at 96 h of runs with GT strain,  $\Delta\text{ddh}$  mutant and  $\Delta\text{adh}\Delta\text{ddh}$  mutant was extremely low as 0.70, 1.01 and  $0.47 \mu\text{mol mL}^{-1}$ , respectively. Extremely low production of ethanol by the incubation of  $\Delta\text{adh}\Delta\text{ddh}$  mutant from antibiotic-free photoautotrophic culture verifies the successful deletion of the genes coding for *adh*. The yield of ethanol on dry cell weight of GT strain in the first 24 h is  $0.45 \mu\text{mol mg}^{-1}$ . The increase in

ethanol concentration in the incubation of  $\Delta adh\Delta ddh$  mutant on glucose in the first 24 h was  $0.17 \mu\text{mol mL}^{-1}$ , hence the fraction of  $\Delta adh$  phenotype deficient cells in  $\Delta adh\Delta ddh$  cells are analyzed to be 0.180.

If stability of  $\text{H}_2$  production is evaluated in low value of the parameter  $k$  shown in Table 4.9, incubation of  $\Delta ddh$  mutant in the presence of exogenous glucose represents the most stable  $\text{H}_2$  production condition. This stabilization is not harnessed in the attainable level of  $\text{H}_2$  ( $y_{\text{H}_2, f}$ ), which is highest in run with GT strain in the presence of exogenous glucose. When viewed over the parameters in this study, the increase in the initial hydrogen production rate ( $r_{\text{H}_2, 0}$ ) is found to be crucial to elevate the level of  $y_{\text{H}_2, f}$ . Elimination of  $ddh$  and  $adh$  genes is effective to increase the level of  $r_{\text{H}_2, 0}$  in the absence of exogenous glucose, however, it becomes ineffective in the presence of exogenous glucose. Redox balance in the presence of exogenous glucose is found to be different from that in the absence of exogenous glucose.

### (e) Effect of redox perturbation in genetically modified strains on $\text{H}_2$ production

Parameters  $q_{\text{H}_2, 0}^*$  and  $y_{\text{H}_2, f}^*$  defined by Eq.(2.30) are calculated and listed in Tables 4.7 and 4.8. **Figure 4.9** shows plots of  $y_{\text{H}_2, f}^*$  against  $q_{\text{H}_2, 0}^*$ . The plots shows that genetic modification results in an increase in the initial content of NAD(P)H. The presence of glucose affects to elevate initial content of NAD(P)H. The attainable level of the amount of  $\text{H}_2$  per culture volume in GT strain and  $\Delta ddh$  mutant increases in the presence of glucose, while it decreases in  $\Delta adh\Delta ddh$  mutant in the presence of glucose. The  $y_{\text{H}_2, f}^*$  in terms of  $q_{\text{H}_2, 0}^*$  of data in this chapter is slightly higher than that predicted by Eq.(2.33) with  $k_{\text{H}_2}$  at 0.619. The initial specific  $\text{H}_2$  production rate ( $q_{\text{H}_2, 0}$ ) of GT strain in run with glucose shown in Table 2.3 was  $0.0336 \mu\text{mol mg}^{-1}\text{h}^{-1}$ , while that shown in Table 4.8 is  $0.0410 \mu\text{mol mg}^{-1}\text{h}^{-1}$  that is 1.22 times that in Table 2.3. The  $y_{\text{H}_2, f}$  of GT strain in run with glucose shown in Table 2.3 was  $3.26 \mu\text{mol mL}^{-1}$ , while that shown in Table 4.8 is  $5.61 \mu\text{mol mL}^{-1}$  that is 1.72 times that in Table 2.3. About 20% shift in the initial content of NAD(P)H is found to results in about 70% variation in attainable level in  $y_{\text{H}_2}$ .

In Fig.4.9, results of runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant are plotted by two points from runs with and without glucose. Pair points are connected by straight lines. Slope of the straight lines  $d\ln y_{\text{H}_2, f}^* / d\ln q_{\text{H}_2, 0}^*$  is highest in  $\Delta adh\Delta ddh$  mutant, second highest in  $\Delta ddh$  mutant and lowest in GT strain. Sensitivity analysis to glucose concludes that genetic modification elevates the sensitivity of  $\text{H}_2$  production to glucose. Increase in the initial NAD(P)H content by utilizing exogenous glucose affects to increase the attainable level of amount of  $\text{H}_2$  per culture volume in GT strain, to largely increase it in  $\Delta ddh$  mutant and to decrease in  $\Delta adh\Delta ddh$  mutant.

**Figure 4.10** shows a relation between  $(m_G X)_1$  and  $y_{\text{H}_1}$  in runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant in HEPES buffer solution with or without glucose. Solid line without keys is calculated utilizing data of chapter 2 and Eq.(2.40). Plots of data with and without glucose of runs with  $\Delta ddh$  mutant

and  $\Delta adh\Delta addh$  mutant are connected by straight lines. Slope of lines indicates the sensitivity of  $y_{H_2,1}$  against  $(m_G X)_1$ . Sensitivity is found to be highest in  $\Delta adh\Delta addh$  mutant, second highest in  $\Delta addh$  mutant and lowest in GT strain. Plotted data in this graph utilize the experimental data with glucose for inoculum from late-logarithmic growth, hence it is still an open question whether the initial increase in cellular content of NAD(P)H in two mutants can result in the increase in  $y_{H_2,1}$  when inoculum is obtained from logarithmic phase and incubated in solution of monosaccharide other than glucose.

## 4.4 Summary

Research in this chapter concerns a DNA modification of a glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 for  $H_2$  production. The highlights of this chapter are as follows.

1. Mutants that are defective of the genes coding for lactate dehydrogenase ( $\Delta addh$  mutant) and both lactate dehydrogenase and alcohol dehydrogenase ( $\Delta adh\Delta addh$  mutant) are constructed.
2. Metabolic engineering approach to introduce a perturbation in redox homeostasis of GT strain is made. Mutants defective the genes coding for lactate dehydrogenase ( $\Delta addh$  mutant) and for both lactate dehydrogenase and alcohol dehydrogenase ( $\Delta adh\Delta addh$  mutant) are constructed. The initial  $H_2$  production of mutants are slightly higher than that of GT strain.
3. Among GT strain,  $\Delta addh$  mutant and  $\Delta adh\Delta addh$  mutant in HEPES buffer solution with or without glucose, GT strain in the presence of glucose is capable of producing the highest attainable level of the number of moles of hydrogen per culture volume due to heterotrophic growth by assimilating exogenous glucose and producing endogenous glucose, lactate and acetate.
4. Glucose elevates  $H_2$  production in GT strain and  $\Delta addh$  mutant, while lowers it in  $\Delta adh\Delta addh$  mutant. Glucose is assimilated by GT strain. Assimilation of glucose is blocked by the elimination of the genes coding for lactate dehydrogenase.
5. Deletion of NAD(P)H-consuming dehydrogenase pathways redirects more NAD(P)H to NiFe-hydrogenase and affects to increase hydrogen production rate. Successful mutant constructions are confirmed by a drastic drop in lactate production and ethanol production by corresponding mutants.
6. Attainable level of the amount of  $H_2$  per culture volume in GT strain and  $\Delta addh$  mutant is defined by the initial cellular content of NAD(P)H.

Table 4.1 *Synechocystis* sp. strain PCC 6803 genes relating to a function of ethanol production.

Gene	Function
slr0942	Alcohol dehydrogenase-NADP <sup>+</sup> [EC.1.1.1.2]
slr1192	Zinc-containing alcohol dehydrogenase like protein[EC.1.-.-.]
slI0990	S-(hydroxymethyl)glutathione dehydrogenase (GSH) [EC.1.1.1.284]/ Alcohol dehydrogenase-NAD <sup>+</sup> [EC.1.1.1.1]
slr2124	Short chain dehydrogenase (SDR)/Short chain alcohol dehydrogenase

Table 4.2 Plasmids used for constructing the vector DNA to disrupt genes *ddh* in GT strain to derive Δ*ddh* mutant strain

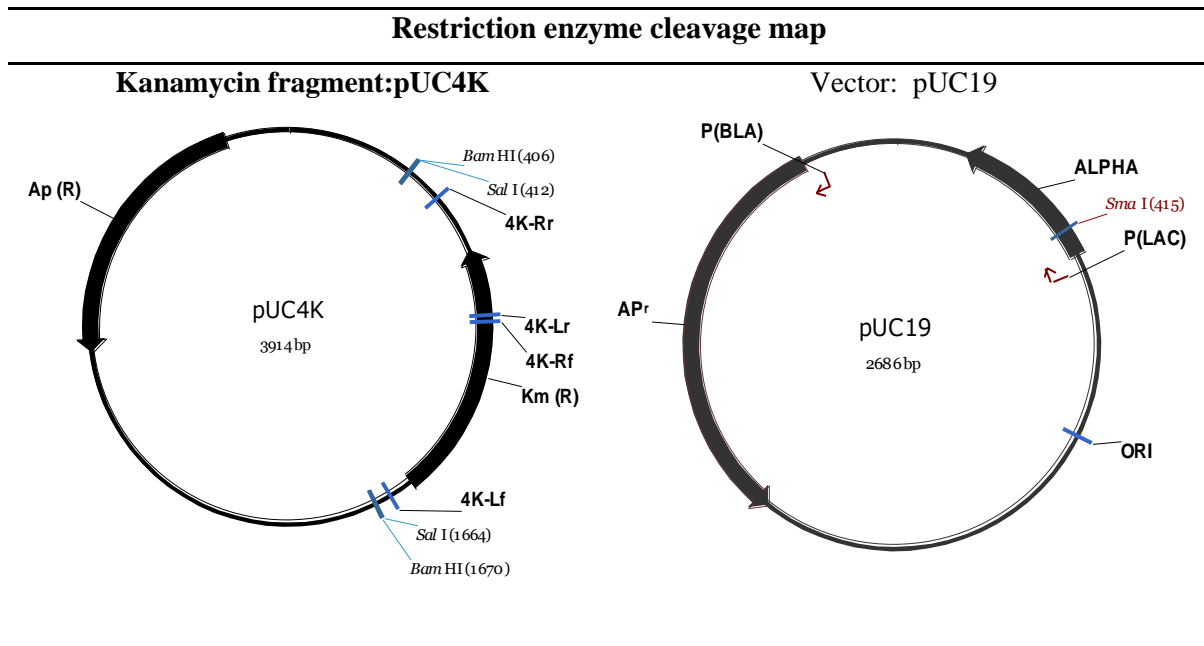


Table 4.3 Plasmids used for constructing the vector DNA to disrupt genes *adh* in  $\Delta$ *adh* mutant to derive  $\Delta$ *adh*  $\Delta$ *adh* mutant strain

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**Restriction enzyme cleavage map**

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Streptomycin and spectinomycin fragment

Vector: pMD19 (Takara)

(Sm/Sp or  $\Omega$ ): pHP45- $\Omega$

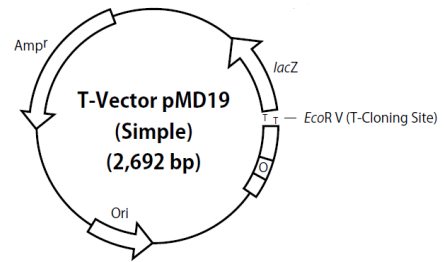
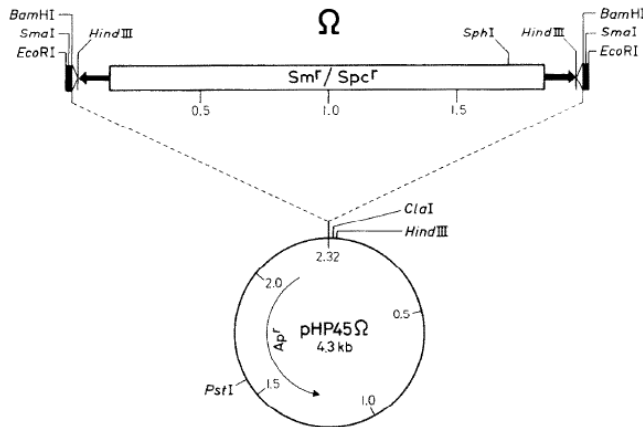


Table 4.4 Condition used for pUC19-*ddh*-Km<sup>r</sup> transformation into GT strain

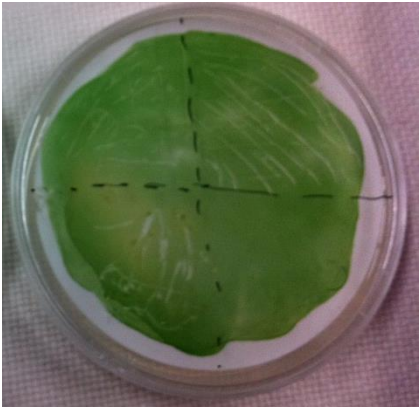
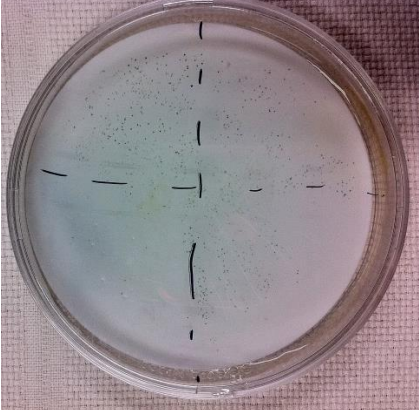
Control	$\Delta$ <i>ddh</i> transformation
GT cells were grown to OD <sub>730</sub> = 1.48	
3.5 $\mu$ g vector Plate: Km <sup>r</sup> (no antibiotic)	3.5 $\mu$ g vector Plate: 60 $\mu$ mol mL <sup>-1</sup> Km <sup>r</sup>
	

Table 4.5 Condition used for pMD19-*adh*- $\Omega$  transformation into GT strain




Control	$\Delta$ <i>adh</i> transformation	$\Delta$ <i>adh</i> $\Delta$ <i>ddh</i> transformation
GT cells were grown to OD <sub>730</sub> = 0.984		$\Delta$ <i>ddh</i> cells were grown to OD <sub>730</sub> = 0.48 with 60 $\mu$ mol/mL Km
3.5 $\mu$ g vector Plate: Sm <sup>r</sup> (no antibiotic)	3.5 $\mu$ g vector Plate: 5 $\mu$ mol/mL Sm <sup>r</sup>	3.5 $\mu$ g vector Plate: 5 Sm <sup>r</sup> , 60 Km <sup>r</sup> in $\mu$ mol/mL
		
	Large number of colonies appeared	Slight growth and tiny colonies

Table 4.6 Physiological state of cells in photoautotrophic cultures that are performed prior to dark anaerobic H<sub>2</sub> production in indirect biophotolysis. GT strain,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant were obtained from different growth phases (ref, cellular state of GT strain in late-logarithmic growth phase.  $q_{CO_2,0}^*$ ,  $q_{CO_2,0}/(q_{CO_2,0})_{ref}$ ; and  $m_G^*$ ,  $m_G/(m_G)_{ref}$ ).

	GT strain		$\Delta$ addh mutant		$\Delta$ adh $\Delta$ addh mutant	
	w/o	with	w/o	with	w/o	with
	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose
Increase in DNA size [kb]		0		-0.495		0.56
Harvesting time [h]		37		46		60
$X_0$ [mg mL <sup>-1</sup> ]		1.14		1.26		1.16
$X_f$ [mg mL <sup>-1</sup> ]		17.2		18.3		16.8
$G$ [-]		7.81		7.14		7.37
$g$ [-]		5.08		4.31		4.66
$\mu\phi$ [h <sup>-1</sup> ]		0.0187		0.0318		0.0238
$q_{CO_2}$ [ $\mu$ mol mg <sup>-1</sup> h <sup>-1</sup> ]		2.09		3.55		2.66
$q_{CO_2}^*$ [-]		1		1.70		1.27
NAD(P)H production rate in the Calvin cycle before inoculation [ $\mu$ mol mL <sup>-1</sup> h <sup>-1</sup> ]		4.18		7.10		5.32
$m_G$ [ $\mu$ mol mg <sup>-1</sup> ]		0.101		0.0815		0.0676
$m_G^*$ [-]		1		0.806		0.669
$R_G$ [ $\mu$ mol mg <sup>-1</sup> h <sup>-1</sup> ]		0.00170		0.00273		0.00227
$R_G^*$ [-]		1		1.61		1.34

Table 4.7 Kinetic parameters for H<sub>2</sub> production in GT strain, Δadh mutant and ΔadhΔdh mutant in HEPES buffer solution without glucose. Inoculum cells were obtained from late-logarithmic growth phase (ref, run with GT strain cells from late-logarithmic growth phase.  $q_{H_2,0}^*$ ,  $q_{H_2,0}/(q_{H_2,0})_{ref}$ ;  $y_{H_2,f}^*$ ,  $y_{H_2,f}/(y_{H_2,f})_{ref}$ ;  $k^*=k/(k)_{ref}$ ;  $y_{H_2,1}$ ,  $c_{L1}$ ,  $c_{A1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 96 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 96h;  $\Delta(m_G X)_1$ , observed decrease in  $m_G X$  during 96 h H<sub>2</sub> production;  $y_{H_2/G}=y_{H_2,1}/\Delta(m_G X)_1$ ;  $Y_{L/G}=(c_{L1}/2)/\Delta(m_G X)$ ;  $Y_{A/G}=(c_{A1}/2)/\Delta(m_G X)$ )

	GT strain	Δadh mutant	ΔadhΔdh mutant
$X_0$ [mg·mL <sup>-1</sup> ]	2.10	2.10	2.10
$r_{H_2,0}$ [μmol·mL <sup>-1</sup> ·h <sup>-1</sup> ]	0.0309	0.0359	0.0355
$q_{H_2,0}$ [μmol·mg <sup>-1</sup> ·h <sup>-1</sup> ]	0.0147	0.0171	0.0169
$q_{H_2,0}^*$ [-]	1	1.16	1.15
$y_{H_2,f}$ [μmol·mL <sup>-1</sup> ]	2.28	2.31	1.48
$y_{H_2,f}^*$ [-]	1	1.01	0.649
$k$ [h <sup>-1</sup> ]	0.0167	0.0213	0.0365
$k^*$ [-]	1	1.28	2.19
$K_d$ [h <sup>-1</sup> ]	0.00105	0.000620	0.000620
$y_{H_2,1}$ [μmol·mL <sup>-1</sup> ]	1.80	2.0	1.5
$m_{G1}$ [μmol·mg <sup>-1</sup> ]	0.0316	0.035	0.035
$X_1$ [mg·mL <sup>-1</sup> ]	1.9	2.0	2.0
$(m_G X)_1$ [μmol·mL <sup>-1</sup> ]	0.0600	0.0700	0.0700
$c_{L,1}$ [μmol·mL <sup>-1</sup> ]	0.26	0.03	0.01
$c_{A,1}$ [μmol·mL <sup>-1</sup> ]	0.2	0	0
$-\Delta(m_G X)$ [μmol·mL <sup>-1</sup> ]	0.15	0.10	0.07
$Y_{H_2/G}$ [-]	12	20	21.4
$Y_{X/S}$ [mg μmol <sup>-1</sup> ]	0	0	0
$Y_{L/G}$ [-]	1.73	0.3	0.143
$Y_{A/G}$ [-]	1.33	0	0

Table 4.8 Kinetic parameters for H<sub>2</sub> production in GT strain, Δaddh mutant and ΔadhΔaddh mutant in HEPES buffer solution with glucose. Inoculum cells were obtained from logarithmic growth phase.

ref, run with GT strain cells from logarithmic growth phase.  $q_{H2,0}^*$ ,  $q_{H2,0}/(q_{H2,0})_{ref}$ ;  $y_{H2,f}^*$ ,  $y_{H2,f}/(y_{H2,f})_{ref}$ ;  $k^+=k/(k)_{ref}$ ;  $y_{H2,1}$ ,  $c_{L1}$ ,  $c_{A1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 96 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 96h;  $\Delta(m_G X)_1$ , observed decrease in  $m_G X$  during 96 h H<sub>2</sub> production;  $y_{H2/G}=y_{H2,1}/\Delta(m_G X)_1$ ;  $Y_{L/G}=(c_{L1}/2)/\Delta(m_G X)$ ;  $Y_{A/G}=(c_{A1}/2)/\Delta(m_G X)$

	GT strain	Δaddh mutant	ΔadhΔaddh mutant
$X_0$ [mg mL <sup>-1</sup> ]	2.20	2.10	2.10
$c_{S0}$ [μmol mL <sup>-1</sup> ]	5.0	4.8	4.7
$r_{H2,0}$ [μmol mL <sup>-1</sup> ·h <sup>-1</sup> ]	0.0901	0.0430	0.0502
$q_{H2,0}$ [μmol mg <sup>-1</sup> ·h <sup>-1</sup> ]	0.0410	0.0205	0.0239
$q_{H2,0}^*$ [-]	2.79	1.39	1.63
$y_{H2,f}$ [μmol mL <sup>-1</sup> ]	5.61	3.48	1.07
$y_{H2,f}^*$ [-]	2.46	1.53	0.469
$k$ [h <sup>-1</sup> ]	0.0184	0.0145	0.0299
$k^*$ [-]	1.12	0.868	1.79
$\mu$ [h <sup>-1</sup> ]	0.0100(0-24h)	0.00890(0-24h)	0.0105(0-24h)
$K_d$ [h <sup>-1</sup> ]	0(24-96h)	0.00170(24-96h)	0.00217(24-96h)
$y_{H2,1}$ [μmol mL <sup>-1</sup> ]	4.7	2.6	2.3
$c_{S1}$ [μmol mL <sup>-1</sup> ]	1.2	3.9	3.6
$m_{G1}$ [μmol·mg <sup>-1</sup> ]	0.0963	0.0478	0.0435
$X_1$ [mg mL <sup>-1</sup> ]	2.7	2.3	2.3
$(m_G X)_1$ [μmol mL <sup>-1</sup> ]	0.260	0.110	0.100
$c_{L,1}$ [μmol mL <sup>-1</sup> ]	4.74	0.05	0.06
$c_{A,1}$ [μmol mL <sup>-1</sup> ]	17.2	8.66	4.83
$c_{E,1}$ [μmol mL <sup>-1</sup> ]	0.70	1.01	0.47

$-\Delta c_s[\mu\text{mol}\cdot\text{mL}^{-1}]$	3.8	0.9(after 48h)	1.1(after 72h)
$(-\Delta c_s)_0[\mu\text{mol}\cdot\text{mL}^{-1}]$	2.6	0	0
$\Delta(m_G X)_0[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.14 (0-48h)	0.06 (0-24h)	0.01 (0-24h)
$-\Delta(m_G X)_1[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.08 (72-96h)	0.13(24-48h)	0.11(24-48h)
$-\Delta c_{SG}[\mu\text{mol}\cdot\text{mL}^{-1}]$	3.74	0.97	1.20
$(\Delta X)_0[\text{mg}\cdot\text{mL}^{-1}]$	0.6(0-24h)	0.5(0-24h)	0.6(0-24h)
$Y_{H2/SG}[-]$	1.26	2.68	1.92
$Y_{X/S}[\text{mg}\ \mu\text{mol}^{-1}]$	0.231(0-24h)	0.556(0-24h)	0.545(0-24h)
$Y_{L/SG}[-]$	1.27	0.0515	0.05
$Y_{A/SG}[-]$	4.60	8.93	4.03

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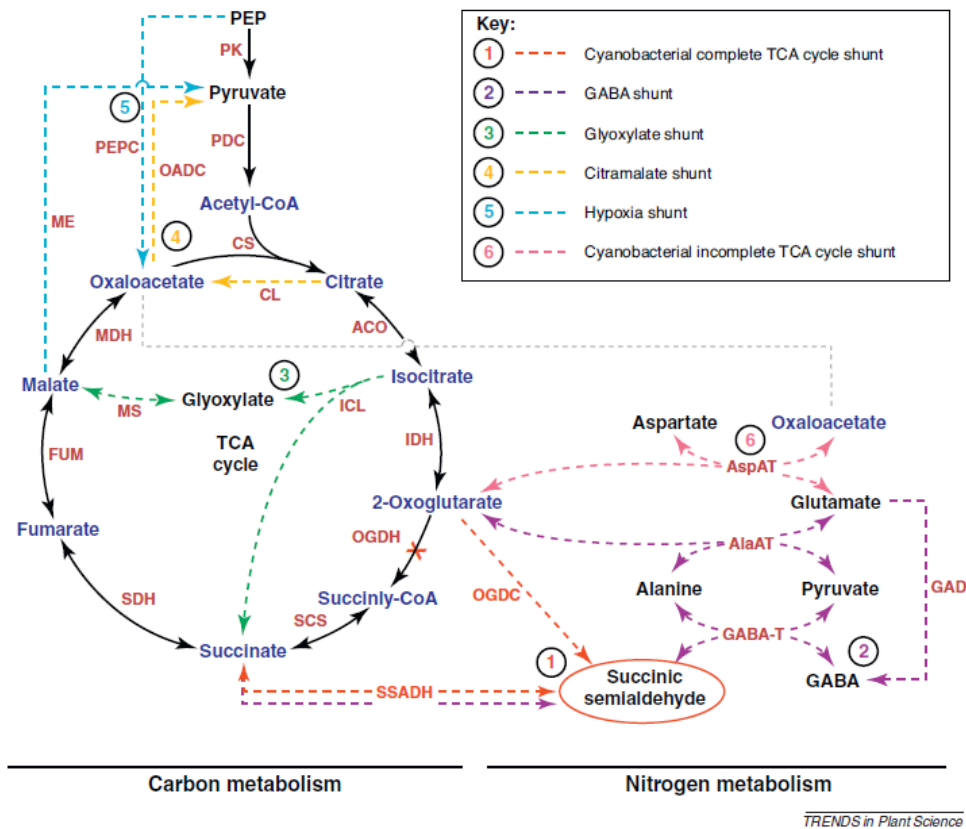


Figure 4.1 Incomplete TCA cycle (Steinhauser et al.,2012). TCA cycle in cyanobacteria is incomplete because of the lack the enzyme 2-oxoglutarate dehydrogenase (OGDH) (1) The TCA cycle variant – red broken arrows; the GABA shunt, broken violet arrows; the glyoxylate shunt, green broken arrows; the citramalate shunt, yellow broken arrows; the TCA cycle shunt observed under hypoxia, light-blue broken arrows; and the closing reaction (without glyoxylate shunt) of the incomplete cyanobacterial TCA cycle, pink broken arrows. TCA cycle intermediates are colored blue, whereas bypass intermediates are colored black. Enzymes are depicted in brown. ACO, aconitase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; CL, citratelase; CS, citrate synthase; FUM, fumarase; GABA-T, GABA aminotransferase; GAD, glutamate decarboxylase; ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; MS, malate synthase; OADC, oxaloacetate decarboxylase; OGDC, 2-oxoglutarate decarboxylase; OGDH, 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; SCS, succinyl CoA ligase; SDH, succinate dehydrogenase; SSADH, succinic semialdehyde dehydrogenase.

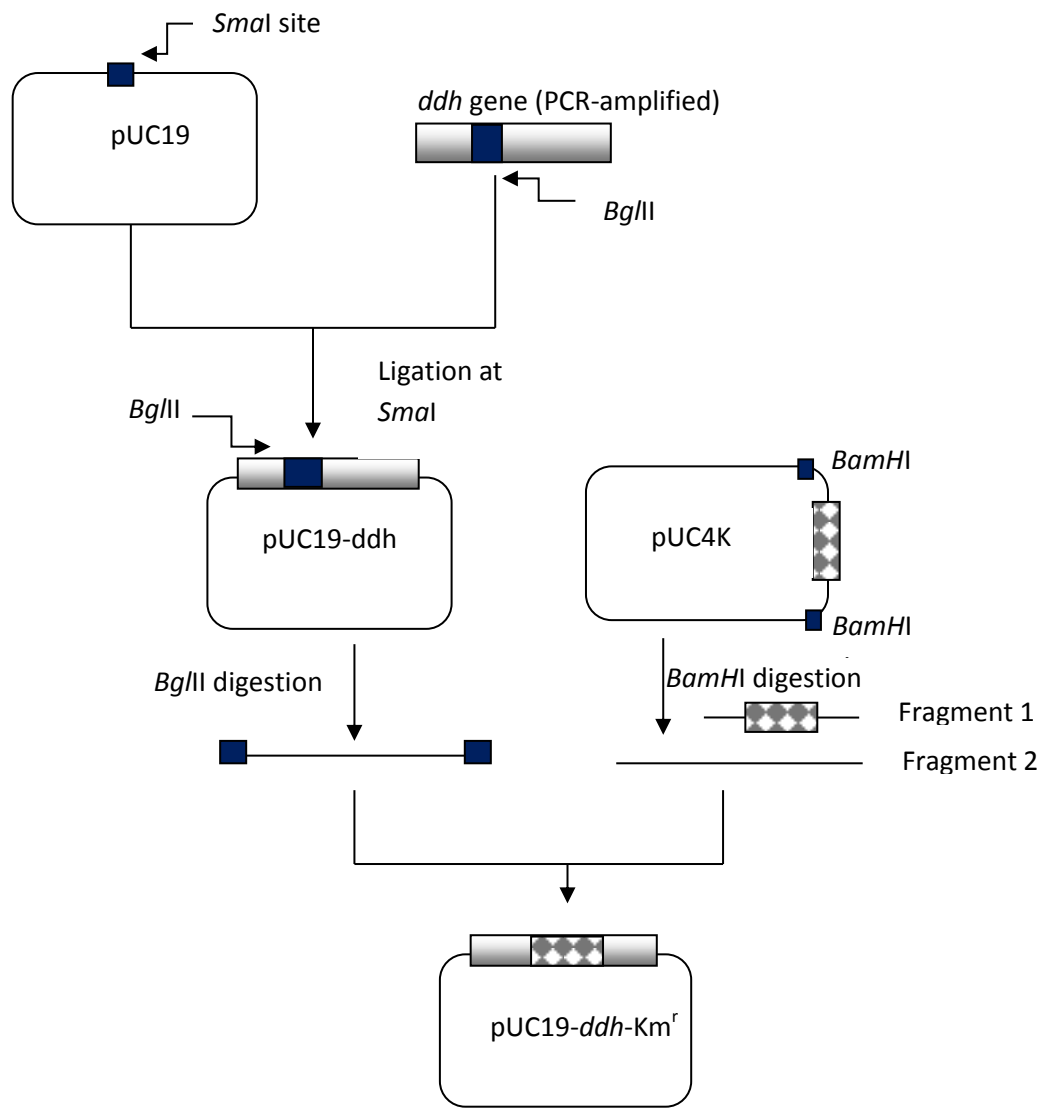


Figure 4.2 Overall strategies to construct pUC19-ddh-Km<sup>r</sup>

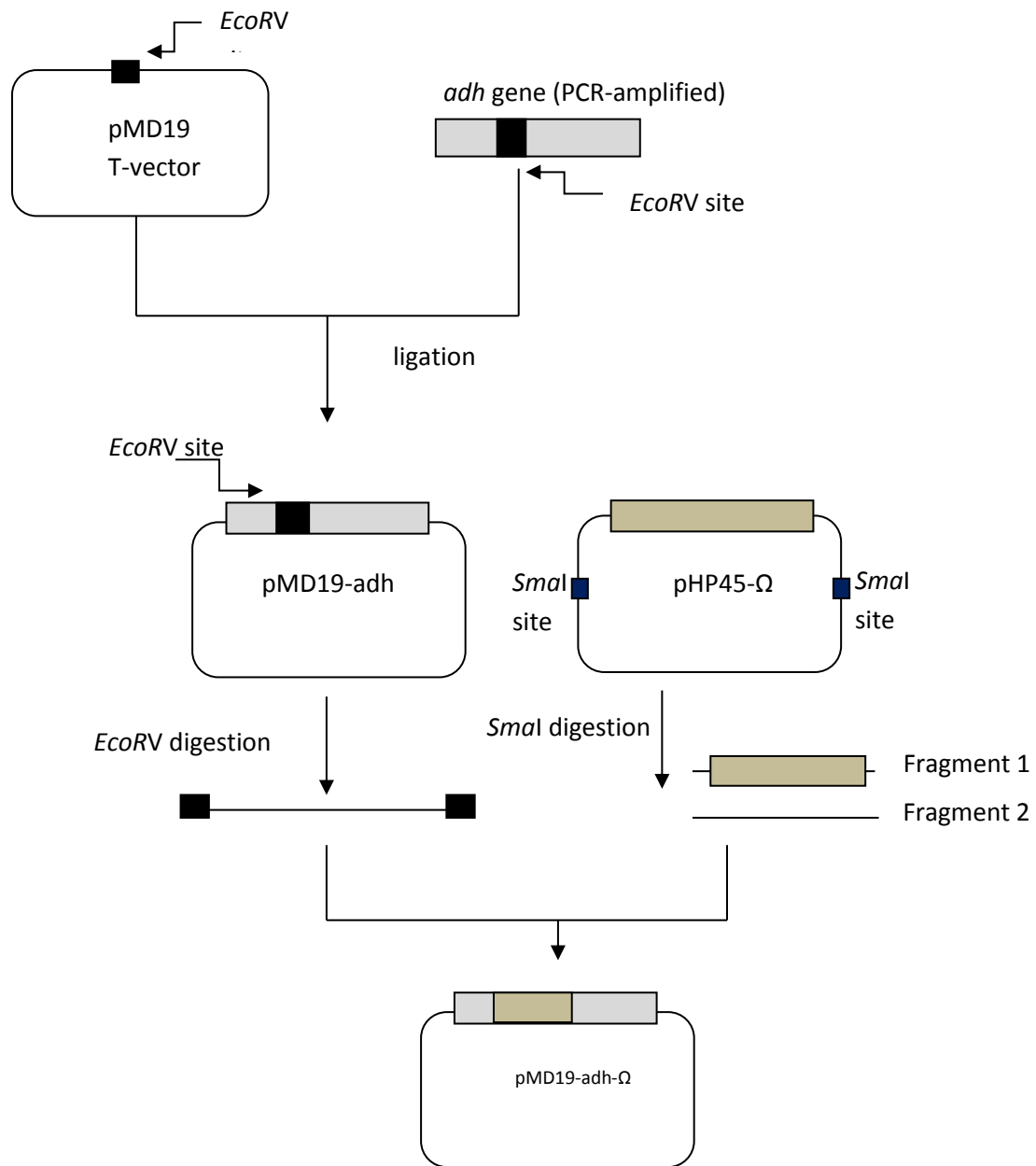


Figure 4.3 Overall strategies to construct pMD19-*adh*-Ω

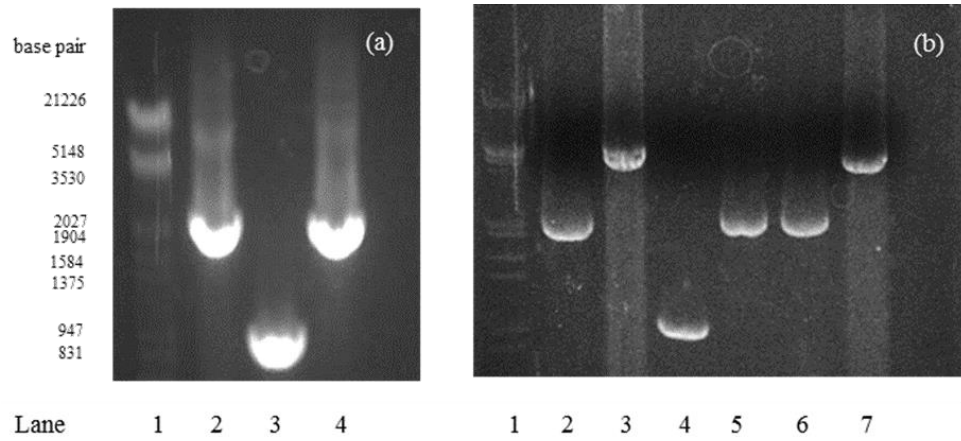


Figure 4.4 Agarose-gel electrophoretic analyses of PCR products for evaluating the achievement of (a)  $\Delta$ addh mutation and (b)  $\Delta$ adh $\Delta$ addh mutation

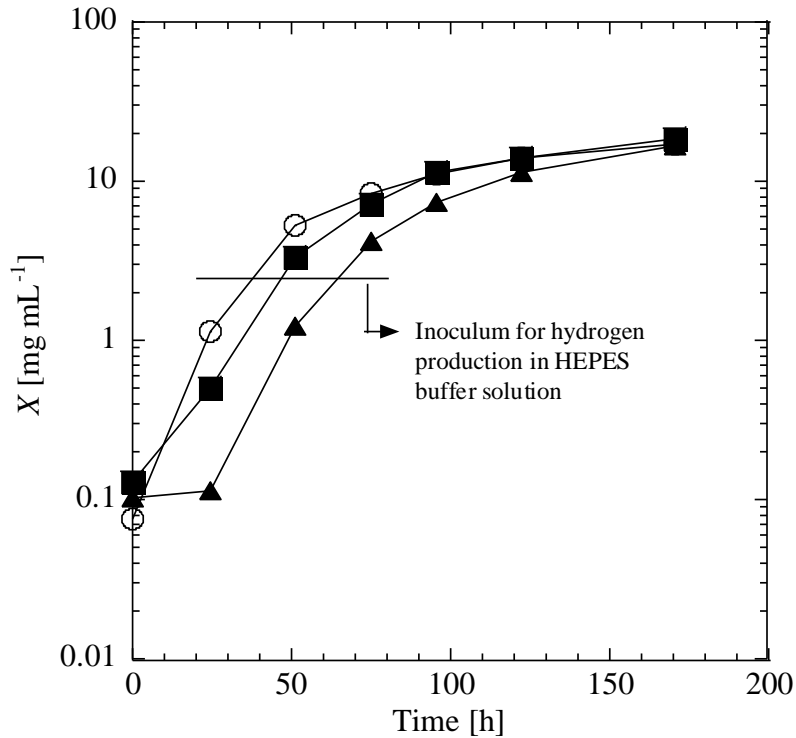


Figure 4.5 Photoautotrophic growth curves of GT strain (○),  $\Delta addh$  mutant (■) and  $\Delta adh\Delta addh$  mutant (▲) on antibiotic-free BG-11 medium. The horizontal line pointing the level of  $X$  at  $2.58 \text{ mg mL}^{-1}$  represents the culture-harvesting time when  $OD_{730}$  of cell suspensions is 7.

Pre-culture cells of GT strain,  $\Delta addh$  mutant and  $\Delta adh\Delta addh$  mutant for the inocula of main cultures shown in above curves were grown on BG-11 medium, BG-11 medium with  $60 \text{ mg mL}^{-1}$  kanamycin and BG-11 medium with both  $60 \text{ mg mL}^{-1}$  kanamycin and  $2.5 \text{ mg mL}^{-1}$  streptomycin, respectively. Harvested preculture cells were washed by sterilized distilled-water to remove antibiotics prior to main culture.

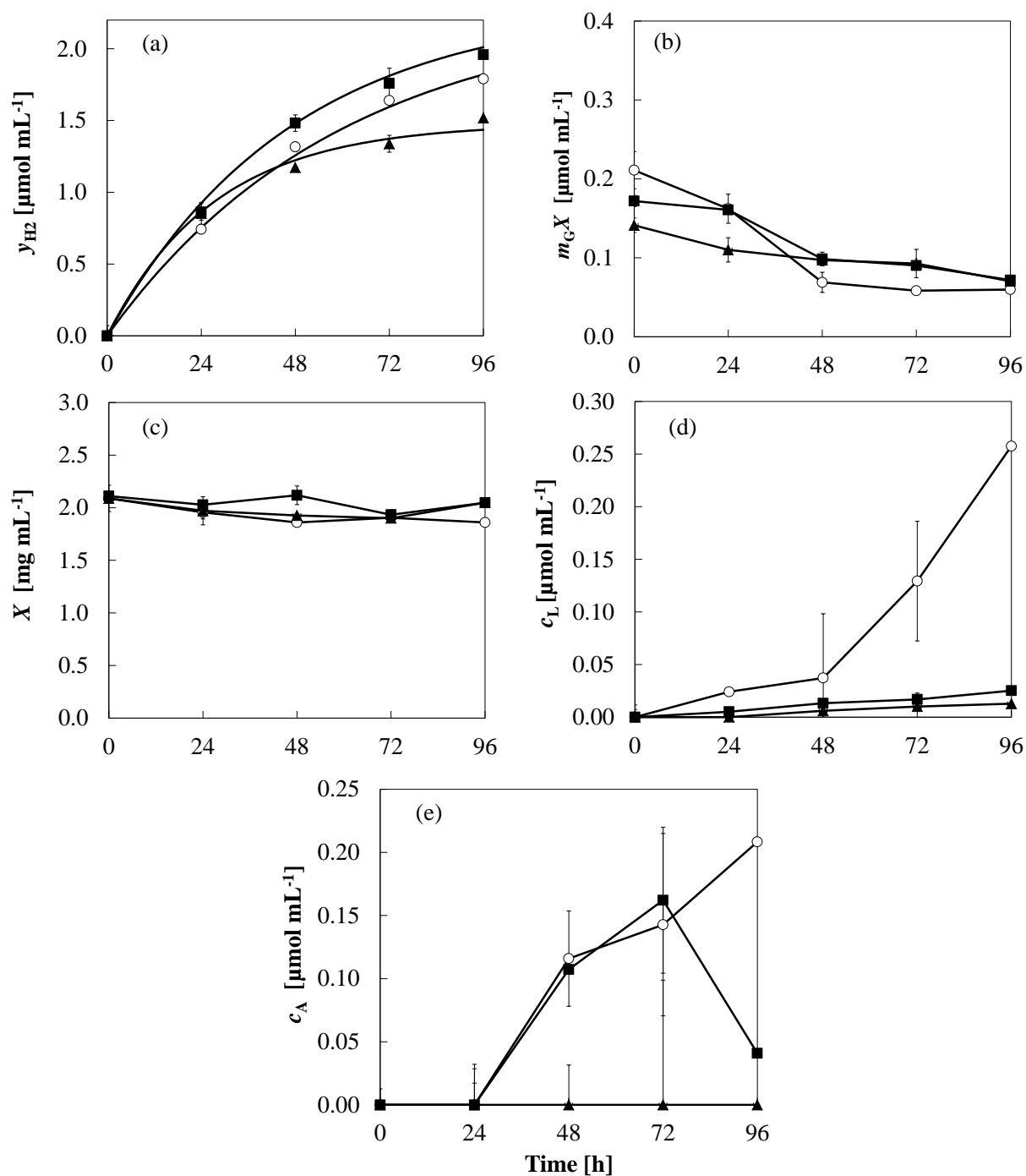


Figure 4.6 Time courses of culture variables for dark anaerobic hydrogen production of cells of GT strain (○),  $\Delta addh$  mutant (■) and  $\Delta adh\Delta addh$  mutant (▲) in HEPES buffer solutions. Quantities measured include the moles per culture volume of (a) hydrogen and (b) the moles of endogenous glucose in glycogen, and the concentrations of (c) dry cell weight, (d) lactate and (e) acetate. Experiments were carried out in duplicate

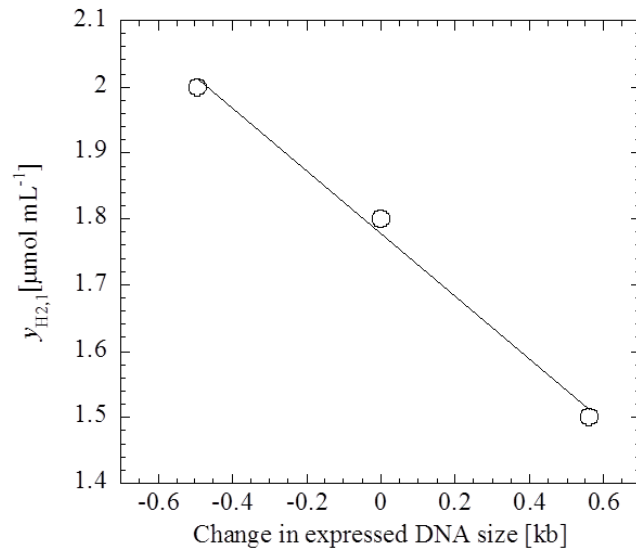


Figure 4.7 Plots of  $y_{H2,1}$  against change in the expressed DNA size

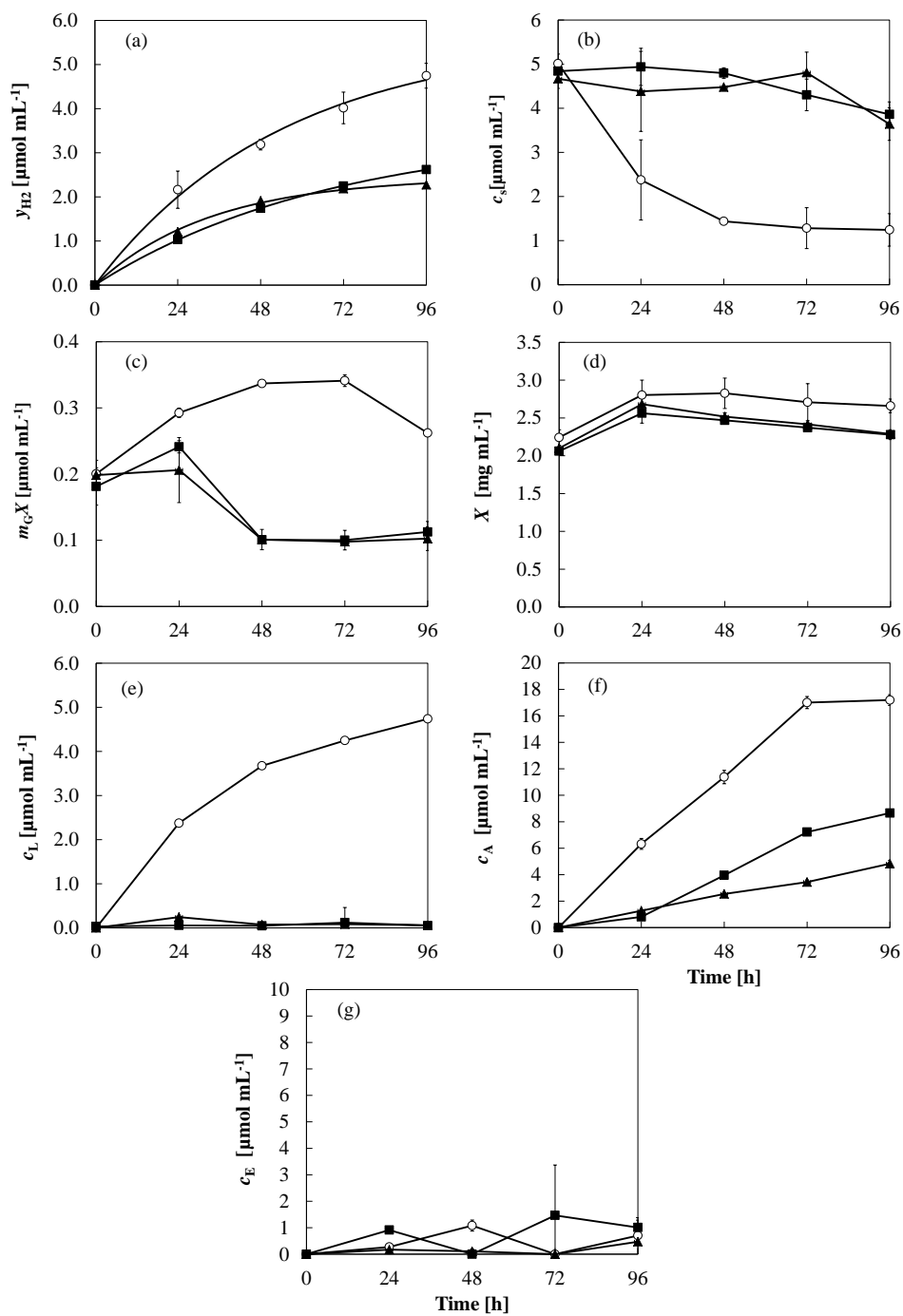


Figure 4.8 Time courses of culture variables for dark anaerobic hydrogen production of cells of GT strain (○),  $\Delta adh$  mutant (■) and  $\Delta adh\Delta adh$  mutant (▲) in HEPES buffer solutions supplemented with 5  $\mu\text{mol mL}^{-1}$  glucose. Quantities measured include (a) the moles of hydrogen per culture volume, (b) the concentration of exogenous glucose, (c) the moles of endogenous glucose in glycogen per culture volume, (d) the concentration of cell mass, (e) the concentration of lactate, (f) the concentration of acetate and (g) the concentration of ethanol. Experiments were carried out in duplicate

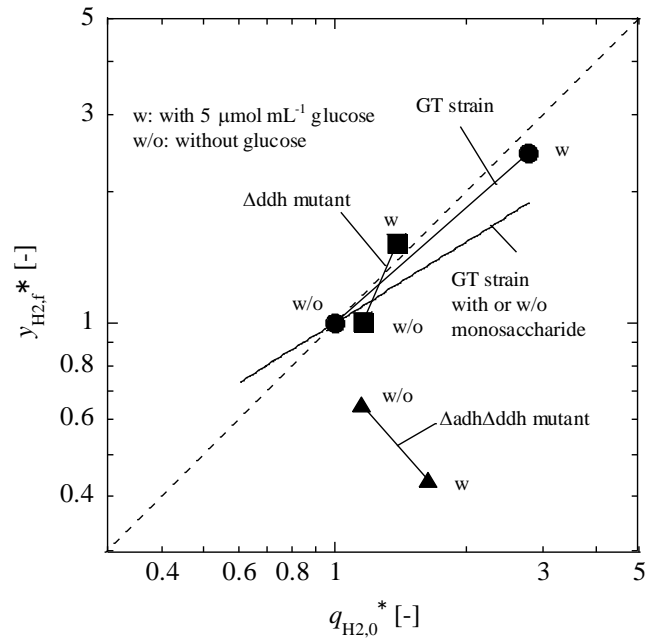


Figure 4.9 Relation between initial specific H<sub>2</sub> production rate and attainable level of H<sub>2</sub>

$$q_{H_2,0}^* = q_{H_2,0} / (q_{H_2,0})_{ref}; y_{H_2,f}^* = y_{H_2,f} / (y_{H_2,f})_{ref}; \text{ref, run with logarithmically growing cells}$$

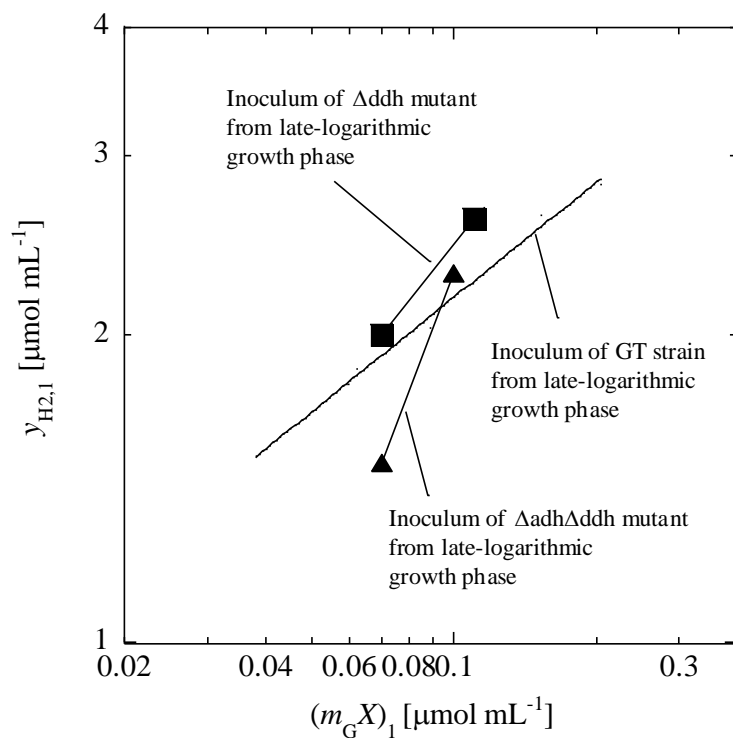


Figure 4.10 Relation between  $(m_G X)_1$  and  $y_{\text{H}_2,1}$  in runs with GT strain,  $\Delta\text{addh}$  mutant and  $\Delta\text{adh}\Delta\text{addh}$  mutant in HEPES buffer solution with or without glucose

## Chapter 4: References

- Brown SA, Whiteley M (2009) Characterization of the L-lactate dehydrogenase from *Aggregatibacter actinomycetemcomitans*. PLoS ONE. 4(11):e7864
- Chongsuksantikul A, Asami K, Yoshikawa S, Ohtaguchi K (2014) Dark Anaerobic Hydrogen Production in the Mutants of *Synechocystis* sp. Strain PCC6803-GT Defective in Lactate Dehydrogenase Activity and/or Alcohol Dehydrogenase Activity, Incubated in Buffer Solutions With or Without Glucose. International Journal of Biology 7 (1):p33
- Cooley JW, Vermaas WF (2001) Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of *Synechocystis* sp. strain PCC 6803: capacity comparisons and physiological function. Journal of bacteriology 183 (14):4251-4258
- Dehring U, Kramer D, Ziegler K (2012) Selection of ADH in genetically modified cyanobacteria for the production of ethanol. United States Patent,
- Gao Z, Zhao H, Li Z, Tan X, Lu X (2012) Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. Energy and Environmental Science 5 (12):9857-9865
- McNeely K, Xu Y, Bennette N, Bryant DA, Dismukes CG. (2010) Redirecting Reductant Flux into Hydrogen Production via Metabolic Engineering of Fermentative Carbon Metabolism in a Cyanobacterium. Applied and Environmental Microbiology 76:5032-5038
- ADH5 alcohol dehydrogenase 5 (class III), chi polypeptide (2012)  
<http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=128#gene-top>.
- Prentki P, Krisch HM (1984) In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29 (3):303-313.
- Steinhauser D, Fernie AR, Araújo WL (2012) Unusual cyanobacterial TCA cycles: not broken just different. Trends in Plant Science 17 (9):503-509
- Takemura T, Akiyama K, Umeno N, Tamai Y, Ohta H, Nakamura K (2009) Asymmetric reduction of a ketone by knockout mutants of a cyanobacterium. Journal of Molecular Catalysis B: Enzymatic 60 (1-2):93-95.
- Vidal R, López-Maury L, Guerrero MG, Florencio FJ (2009) Characterization of an Alcohol Dehydrogenase from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 That Responds to Environmental Stress Conditions via the Hik34-Rre1 Two-Component System. Journal of Bacteriology 191 (13):4383-4391.
- Yamamoto T, Chongsuksantikul A, Asami K, Ohtaguchi K (2012) Improvement of anaerobic production of hydrogen in the dark by genetic mutation strains of *Synechocystis* sp. strain PCC 6803. Journal of Biochemical Technology 4 (2): 600-603
- Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1):103-119
- Yu Y, You L, Liu D, Hollinshead W, Tang YJ, Zhang F (2013) Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory. Marine drugs 11 (8):2894-2916

## Chapter 5

### Fructose Effects on Hydrogen Production

#### 5.1 Problem Definition and General Consideration

The study in chapter 2 has concluded that, among monosaccharides, glucose and fructose largely promote dark anaerobic H<sub>2</sub> production in a glucose tolerant mutant of *Synechocystis* sp. strain (GT strain) cells in HEPES buffer solution. Fructose, along with glucose and sucrose, are three key sugars for photolithotrophs. No improvement in H<sub>2</sub> production is observed in sucrose. Attainable level of the amount of H<sub>2</sub> per cell suspended buffer solution ( $y_{H_2,f}$ ) with fructose and that with glucose are comparable. H<sub>2</sub> production in glucose is accompanied by high consumption of exogenous glucose and high production of dry cell weight, lactate and acetate, while that in fructose is accompanied by low consumption of fructose and low production of dry cell weight, lactate and acetate. Therefore dark anaerobic H<sub>2</sub> production in buffer solution with fructose is further studied in this chapter.

**Figure 5.1** shows the calculated time courses of the number of moles of H<sub>2</sub> per culture volume in dark anaerobic incubation of GT strain (solid line),  $\Delta$ addh mutant (broken line) and  $\Delta$ adh $\Delta$ addh mutant (long dashed short dashed line) in HEPES buffer solution and GT strain in 28  $\mu$ mol mL<sup>-1</sup> fructose in HEPES buffer solution. Inoculum cells are assumed to be obtained from late logarithmic growth phase when  $X$  is 2.58 mg mL<sup>-1</sup>. Initial H<sub>2</sub> production rate of  $\Delta$ addh mutant or  $\Delta$ adh $\Delta$ addh mutant is higher than that of GT strain, hence genetic modification is confirmed to be effective to shift the initial content of NAD(P)H. The attainable level of  $y_{H_2}$  ( $y_{H_2,f}$ ) of  $\Delta$ addh mutant is comparable with that of GT strain and that of  $\Delta$ adh $\Delta$ addh mutant is lower than that of GT strain or  $\Delta$ addh mutant. The genetic mutation from GT strain to  $\Delta$ adh $\Delta$ addh mutant is so sensitive to result  $y_{H_2,f}$ . Curves of GT strain shows that addition of fructose is extremely effective to increase both initial H<sub>2</sub> production rate and attainable level of  $y_{H_2}$ . The  $X$  versus time data in dark anaerobic incubation in chapters 2-4 suggest that H<sub>2</sub> production without cell death results high attainable level in  $y_{H_2}$ . The data of chapter 3 shows that inoculum from logarithmic growth phase performs H<sub>2</sub> production with extremely low specific death rate ( $K_d$ ). The study of chapter 4 has shown that the uptake of fructose isomer glucose by  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant is blocked. The possibility exists that H<sub>2</sub>

production of GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant, from logarithmic growth phase, in the presence of fructose seems to be a good possibility to activate H<sub>2</sub> production.

Fructose is an extremely soluble ketohexose that exists in a cyclized form of hemiacetal fructofuranose in solution. The fructofuranose has the anomeric carbon directly bonding to two oxygen atoms in the forms of a free OH group and an OR group. This anomeric carbon is susceptible to be oxidized via a redox reaction, hence ketohexose fructose is classified into a reducing sugar. If H<sub>2</sub> production on NiFe-hydrogenase is the cellular response against redox unbalance, then a possibility exists that the presence of fructose in cellular environment affects H<sub>2</sub> production

Bidirectional NiFe-hydrogenase activity that, under the absence of oxygen and highly reductive condition, serves to provide a terminal electron sink and reduce excess protons and NAD(P)H by evolving H<sub>2</sub> (Ducat et al. 2011). Stresses caused by the nitrate or sulfate limitations are effective to trigger H<sub>2</sub> production in a unicellular non-nitrogen-fixing cyanobacterium *Synechocystis* sp. strain PCC 6803 (Antal et al. 2006; Yamamoto et al. 2012). Eliminating nitrate from medium constituents is established to be a key for dark H<sub>2</sub> production, which is supported by an observation that a deletion mutant of the genes encoding both nitrate reductase and nitrite reductase significantly enhanced production (Baebprasert et al. 2011). Inhibitor addition to block of a certain electron sink pathway that is competitive to NiFe-hydrogenase is applied to increase H<sub>2</sub> production (Burrows et al. 2011).

Experimental observation in chapter 2 showed fructose consumption by GT strain (Fig.2.6). Exogenous glucose and fructose are known to be transported through facilitated diffusion *via* a glucose-fructose permease (the product of the *glcP* transport gene) (Joset et al. 1988). Previous works show that *Synechocystis* sp. strain PCC6714 transports glucose via permease with  $K_m$  of about 0.4  $\mu\text{mol}\cdot\text{mL}^{-1}$  (Beauclerk and Smith 1978) and fructose inhibits the uptake of glucose slightly (Flores and Schmetterer 1986). The  $K_m$  for fructose consumption of *Anabaena variabilis* is reported to be approximately 0.16  $\mu\text{mol}\cdot\text{mL}^{-1}$  that does not change in the light versus dark (Haury and Spiller 1981). Although sensing mechanism in GT strain for glucose is not defined, growth arrested cells are known to respond to glucose at the level of post-translational modifications or modulation of enzyme activities and phosphorylate the transported glucose to glucose 6-phosphate on glucokinase (Kahlon et al. 2006). Glucose 6-phosphate is further decomposed mainly in the oxidative pentose

phosphate (OPP) pathway. Fructose inhibits photoautotrophic growth of a culture of *Synechocystis* sp. strain PCC 6714 but, cannot result in a toxic effect on the chemoheterotrophic growth of the strain on 5  $\mu\text{mol mL}^{-1}$  glucose in the light (Kahlon et al. 2006). Genome sequence of *Synechocystis* sp. strain PCC 6803 represents a potential-coding regions for fructokinase (Qry match, 18.4%) in ORF slr1448 (Kaneko et al. 1996). Glycogen accumulation in a heterocystous cyanobacterium *Anabaena azollae* is reported to be increased by fructose (Rozen et al. 1986). A recent work on higher plant *Arabidopsis thaliana* suggests that fructose is a signaling molecule regulating growth and development and that fructokinase plays a putative regulatory role in fructose signaling (Cho and Yoo 2011). However fructose signal transduction pathway is yet unsolved.

Thus, the primary purposes of the study in this chapter were (1) to elucidate the effect of fructose on dark anaerobic  $\text{H}_2$  production in GT strain in HEPES buffer solution, (2) to compare the dark anaerobic  $\text{H}_2$  production in GT strain,  $\Delta\text{addh}$  mutant and  $\Delta\text{adh}\Delta\text{addh}$  mutant in HEPES buffer solution with or without 50  $\mu\text{mol mL}^{-1}$  fructose, and (3) to perform a conceptual design of bioreactor to produce biofuel  $\text{H}_2$ .

## 5.2 Materials and methods

### (a) Strains

Strains utilized in experiments are the glucose tolerant of *Synechocystis* sp. strain (GT strain) and its derivatives defective of genes coding for D-lactate dehydrogenase genes ( $\Delta\text{addh}$  mutant) and defective of genes coding for both alcohol dehydrogenase genes and D-lactate dehydrogenase genes ( $\Delta\text{adh}\Delta\text{addh}$  mutant).

### (b) Cultivation and fermentation techniques

For studying the effect of initial fructose concentration on dark anaerobic  $\text{H}_2$  production, cells of GT strain were utilized. For cell preparation, cells of GT strain were grown photoautotrophically for 3 d in BG-11 medium (initial pH 7.8) at 34°C, aerated by 6 %  $\text{CO}_2$  in air and illuminated by fluorescent lamps at 100  $\mu\text{mol photon s}^{-1} \text{m}^{-2}$  photosynthetic photon flux density (PPFD). Utilizing the culture with  $\text{OD}_{730}$  at 3 ( $X$ , 1.11  $\text{mg mL}^{-1}$ ), cells at the end of logarithmic growth were collected by centrifugation at 1000 g and 25°C for 10 min. The cell pellets were first washed by 50  $\mu\text{mol mL}^{-1}$

<sup>1</sup> HEPES buffer plus 18  $\mu\text{mol mL}^{-1}$   $\text{NaNO}_3$  and then washed again by pure HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.8) solution. The washed cells were collected by centrifugation.

In the second stage of indirect biophotolysis, cells were re-suspended and incubated in 10 mL of 50  $\mu\text{mol mL}^{-1}$  HEPES buffer (pH 7.8) solutions without or with 18  $\mu\text{mol mL}^{-1}$   $\text{NaNO}_3$ , without or with 5, 25, 50, 60, 70 and 110  $\mu\text{mol mL}^{-1}$  fructose and with 18  $\mu\text{mol mL}^{-1}$   $\text{NaNO}_3$  plus 50  $\mu\text{mol mL}^{-1}$  fructose in 32 mL glass test tubes with butyl rubber caps. The initial dry cell weight concentration was set at 2  $\text{mg mL}^{-1}$  ( $\text{OD}_{730}=5.4$ ) and cell suspension was purged with nitrogen gas for a few minutes to remove oxygen molecules. Incubation was carried out under the 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°. Dark incubation in this study was slightly modified with a daily pulse of dim light. All experiments were carried out in duplicate.

For studying the effect of fructose on dark anaerobic  $\text{H}_2$  production of GT strain,  $\Delta\text{adh}$  mutant and  $\Delta\text{adh}\Delta\text{adh}$  mutant, cells of GT strain,  $\Delta\text{adh}$  mutant and  $\Delta\text{adh}\Delta\text{adh}$  mutant were grown photoautotrophically on antibiotic-free BG-11 medium (initial pH 7.8) that was aerated by 6% carbon dioxide in air in clear Pyrex glass test tube (TE-32, AGC TECHNO GLASS Co. Ltd.) at 34 °C in water bath, surface of which was exposed to fluorescent lamps with a PPFD of 100  $\mu\text{mol-phons m}^{-2} \text{s}^{-1}$ . Growth was monitored by measuring  $\text{OD}_{730}$  of cell suspension. Cultures of  $\text{OD}_{730}$  at 3 ( $X$ , 1.11  $\text{mg mL}^{-1}$ ) were centrifuged at 25 °C, 3000 rpm for 10 min. The cell pellets were washed, re-suspended and incubated in 10 mL of 50  $\mu\text{mol mL}^{-1}$  HEPES buffer (pH 7.8) solution with 50  $\mu\text{mol mL}^{-1}$  fructose in 32 mL glass test tube with butyl rubber cap. The initial dry cell mass weight concentration was set at 2  $\text{mg mL}^{-1}$ . Other fermentation condition was same as described above.

### (c) Analysis

The number of moles per culture volume of  $\text{H}_2$  ( $y_{\text{H}_2}$ ), fructose ( $c_S$ ), endogenous glucose ( $m_{\text{GX}}$ ), lactate ( $c_L$ ) and acetate ( $c_A$ ) and the dry cell weight per culture volume ( $X$ ) were analyzed as described in the section 2.2.

## 5.3 Results and discussion

### (a) Effect of initial concentration of fructose on dark anaerobic H<sub>2</sub> production in GT strain

It has been shown that dark anaerobic H<sub>2</sub> production in unicellular cyanobacterium is elevated by fructose (chapter 2) and blocked by elimination of nitrate from the medium (Ohtaguchi et., 1995) or by disruption of genes coding for nitrate reductase (Baebprasert et al. 2011) which is a major electron consumption. This pathway competes NAD(P)H with NiFe-hydrogenase in dark anaerobic condition. The first experiment in this chapter was performed with GT strain to compare effect of nitrate and fructose towards H<sub>2</sub> production by cells.

**Figure 5.2** compares the initial H<sub>2</sub> production rates of dark anaerobically incubated cells of GT strain in HEPES buffer solutions (a) without nitrate and fructose, (b) without nitrate and with 50  $\mu\text{mol mL}^{-1}$  fructose, (c) with 18  $\mu\text{mol mL}^{-1}$  NaNO<sub>3</sub> and without fructose, and (d) with 50  $\mu\text{mol mL}^{-1}$  fructose and 18  $\mu\text{mol mL}^{-1}$  NaNO<sub>3</sub>. The H<sub>2</sub> production data of runs without fructose support the reported conclusion that H<sub>2</sub> production is inhibited by nitrate because of the NAD(P)H competition between nitrate assimilation and H<sub>2</sub> production (Baebprasert et al. 2011). The result of run without nitrate shows that fructose elevates dark anaerobic H<sub>2</sub> productions in GT strain. The initial H<sub>2</sub> production rate in run with fructose is 3.5 times that without fructose. It is especially noteworthy that addition of fructose on nitrate solution results in H<sub>2</sub> production. The positive effect of fructose on the dark anaerobic H<sub>2</sub> production in the presence of nitrate is found to overcome the inhibitory effect of nitrate. This observation appears to be the first description on the dark anaerobic H<sub>2</sub> production in GT strain in the presence of nitrate. Fructose appears to block intracellularly the consumption of NAD(P)H by nitrate reductase.

**Figure 5.3** shows the time courses of (a) the number of moles of H<sub>2</sub> per culture volume ( $y_{\text{H}_2}$ ), (b) the concentration of fructose ( $c_s$ ), (c) the number of moles of endogenous glucose per culture volume ( $m_{\text{GX}}$ ), (d) the concentration of dry cell weight ( $X$ ), (e) the concentration of lactate ( $c_L$ ) and (f) the concentration of acetate ( $c_A$ ), measured in runs, performed by incubating GT strain in nitrate-free HEPES buffer solutions without fructose and with 5, 25, 50, 60, and 70  $\mu\text{mol mL}^{-1}$  fructose ( $c_{s0}$ ) under the dark anaerobic condition. To elucidate relations of H<sub>2</sub> production to fructose utilization, acid evolution, endogenous glycogen metabolism and dry cell weight production, culture variables concerning these mechanisms are totally monitored.

**Table 5.1** summarizing the effect of change in initial fructose concentration on culture characteristic variables observed in runs without and with 5, 25, 50, 60 and 70  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose. Culture parameters states at 0 h and 120 h are shown by subscript 0 and 1 respectively.

Fig.5.3 (a) shows that  $\text{H}_2$  production starts right after inoculation, irrespective of varying  $c_{\text{S}0}$ . The  $y_{\text{H}_2}$  versus time data of run without fructose are interpreted by Eq.(2.18) as described in chapters 2-4. The data on solid line calculated by Eq.(2.18) with parameters  $r_{\text{H}_2,0}$ ,  $0.0284 \mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ ; and  $k$ ,  $0.0136 \text{h}^{-1}$  fits well observed values. The attainable level in  $y_{\text{H}_2}$  ( $y_{\text{H}_2,f}=r_{\text{H}_2,0}/k$ ) is calculated as  $2.09 \mu\text{mol}\cdot\text{mL}^{-1}$ . The observed  $y_{\text{H}_2}$  at 120 h ( $y_{\text{H}_2,1}$ ) of run without fructose is  $1.7 \mu\text{mol}\cdot\text{mL}^{-1}$ . The  $y_{\text{H}_2,f}$  of this run is 91.6% of that shown in Fig.5.1. Kinetic behavior of  $\text{H}_2$  production shows remarkable difference between cells from logarithmic growth phase and late-logarithmic growth phase. This drop is due to the inoculum growth phase shift from late-logarithmic growth phase to logarithmic growth phase. Fructose effect is found to be stressed in logarithmically growing cells.

Fructose is found to elevate the number of moles of  $\text{H}_2$  per culture volume at 120 h by increasing the initial  $\text{H}_2$  production rate, and stabilizing it after 72 h. Of special interest is the stable and highest production of  $\text{H}_2$  when initial fructose concentration ( $c_{\text{S}0}$ ) is between 50 and 83  $\mu\text{mol}\cdot\text{mL}^{-1}$ . The  $r_{\text{H}_2}$  is not time-varied and same as the  $r_{\text{H}_2,0}$  at  $0.0927 \mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ . The  $y_{\text{H}_2}$  versus time data for runs with 50, 60 and 70  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose are fitted by Eq.(2.21), and the fitted relation is given by:

$$y_{\text{H}_2} = r_{\text{H}_2,0}t = 0.0927t \quad (5.1).$$

NiFe-hydrogenase mediated  $\text{H}_2$  production at high fructose concentration is found to be interpreted by zero-th order kinetics. The number of moles of  $\text{H}_2$  per culture volume at 120 h ( $y_{\text{H}_2,1}$ ) of run with 50-70  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose is  $11.1 \mu\text{mol}\cdot\text{mL}^{-1}$  that is 6.54 times that without fructose. The additional experiments showed that the range of fructose that supports Eq.(5.1) is from 50 to 83  $\mu\text{mol}\cdot\text{mL}^{-1}$ . Such linear relation is also seen in run with 25  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose. The  $r_{\text{H}_2}$  ( $=r_{\text{H}_2,0}$ ) of this run is  $0.059 \mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ . The upper limit of  $c_{\text{S}0}$  for the stable  $\text{H}_2$  production was observed at 110  $\mu\text{mol}\cdot\text{mL}^{-1}$ .

Different from runs with 25-100  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose, the  $y_{\text{H}_2}$  versus time data for runs with  $c_{\text{S}0}$  at 5  $\mu\text{mol}\cdot\text{mL}^{-1}$  in the first about 72 h represent the time courses similar to those shown in chapters 2

and 3. The result of run with 5  $\mu\text{mol mL}^{-1}$  fructose shows that the  $y_{\text{H}_2}$  is slightly higher than that of run without fructose in the first about 72 h. The  $\text{H}_2$  production curve in the first about 72 h is well fitted by Eq.(2.18) with parameters  $r_{\text{H}_2,0}$ , 0.0422  $\mu\text{mol mL}^{-1} \text{h}^{-1}$ ; and  $k$ , 0.0202  $\text{h}^{-1}$ . Of additional interest in Fig.5.3 is the acceleration of  $\text{H}_2$  production at about 72 h. If  $r_{\text{H}_2}$  is constant at  $r_{\text{H}_2,\text{SHT}}$  after this shift and  $t_{\text{SHT}}$  is the time of acceleration, then  $\text{H}_2$  production of this run is interpreted by:

$$y_{\text{H}_2} = \frac{r_{\text{H}_2,0}}{k} \{1 - \exp(-kt)\} \quad \text{for } t \leq t_{\text{SHT}}$$

$$y_{\text{H}_2} = r_{\text{H}_2,\text{SHT}}(t - t_{\text{SHT}}) + \left[ \frac{r_{\text{H}_2,0}}{k} \{1 - \exp(-kt_{\text{SHT}})\} \right] \quad \text{for } t \geq t_{\text{SHT}} \quad (5.2)$$

Fitting the data of this run results in that parameters  $r_{\text{H}_2,\text{SHT}}$  and  $t_{\text{SHT}}$  are 1.64  $\mu\text{mol mL}^{-1} \text{h}^{-1}$  and 76.6 h, respectively.  $\text{H}_2$  production rate of runs with 5  $\mu\text{mol mL}^{-1}$  fructose became almost similar to that of runs with 50-70  $\mu\text{mol mL}^{-1}$  fructose.

The result of run with 25  $\mu\text{mol mL}^{-1}$  fructose shows that effect of fructose is obvious right after inoculation. After about 72 h,  $r_{\text{H}_2}$  of this run increases with time. The  $y_{\text{H}_2}$  versus time of this run is interpreted by:

$$y_{\text{H}_2} = r_{\text{H}_2,0}t \quad \text{for } t \leq t_{\text{SHT}}$$

$$y_{\text{H}_2} = r_{\text{H}_2,\text{SHT}}(t - t_{\text{SHT}}) + r_{\text{H}_2,0}t_{\text{SHT}} \quad \text{for } t \geq t_{\text{SHT}} \quad (5.3)$$

Fitting the data of this run results that parameters  $r_{\text{H}_2,\text{SHT}}$  and  $t_{\text{SHT}}$  are 5.17  $\mu\text{mol mL}^{-1} \text{h}^{-1}$  and 81.9 h, respectively, since  $r_{\text{H}_2,0}$  is 0.0631  $\mu\text{mol mL}^{-1} \text{h}^{-1}$ .

The initial  $\text{H}_2$  production rates ( $r_{\text{H}_2,0}$ ) for runs with  $c_{\text{S}_0}$  at 0, 5 and 25  $\mu\text{mol mL}^{-1}$  are 0.0285, 0.0422 and 0.059  $\mu\text{mol mL}^{-1} \text{h}$ , respectively. The  $r_{\text{H}_2,0}$  for runs with  $c_{\text{S}_0}$  at no less than 50  $\mu\text{mol mL}^{-1}$  is constant at 0.0927  $\mu\text{mol mL}^{-1} \text{h}^{-1}$  (Table 5.1) that is 3.25-fold increase from run without fructose.

**Figure 5.4** shows the effect of  $c_{\text{S}_0}$  on the initial specific  $\text{H}_2$  production rate  $q_{\text{H}_2,0}(=r_{\text{H}_2,0}/X_0)$ , the initial specific consumption rate of fructose ( $q_{\text{S}_0}$ ) and the number of moles of  $\text{H}_2$  per culture volume at 120h ( $y_{\text{H}_2,1}$ ). Fructose increased the  $q_{\text{S}_0}$  and the  $y_{\text{H}_2,1}$ . Effect of fructose on  $y_{\text{H}_2,1}$  is associated with that of  $q_{\text{S}_0}$ . Linear relation is seen between  $q_{\text{H}_2,0}$  and  $c_{\text{S}_0}$  when  $c_{\text{S}_0}$  is between 5 and 50  $\mu\text{mol mL}^{-1}$ .

The  $q_{H_2,0}$  and  $y_{H_2,1}$  are almost constant at  $0.0488 \mu\text{mol mg}^{-1}\text{h}^{-1}$  and  $10.4 \mu\text{mol mL}^{-1}$ , respectively, when  $c_{S0}$  is no less than  $50 \mu\text{mol mL}^{-1}$ , Fructose is found to increase initial cellular content of NAD(P)H.

Eqs.(5.2) and (5.3) and observations in chapters 2-4 suggests that  $H_2$  production in GT strain,  $\Delta\text{adh}$  mutant and  $\Delta\text{adh}\Delta\text{adh}$  mutant is a response to the intermittent accumulation of NAD(P)H. First intermittent accumulation occurs when photoautotrophically growing cells on BG-11 medium in the presence of  $O_2$  is exposed to dark anaerobic HEPES buffer solution with or without monosaccharides. Cells that face the high oxidative stress instantaneously accumulate NAD(P)H to load a subsequent stress to inoculum cells. NiFe-hydrogenase works as a discharge mechanism of NAD(P)H and  $H_2$  is generated. This process appears to be an action to keep a redox homeostasis. Second intermittent accumulation occurs when generated  $NAD(P)^+$  by  $H_2$  production reaction exceeds the limit for cell survival. Metabolic network for redox homeostasis after first intermittent NAD(P)H accumulation is reformulated at around 72-96 h. This event accelerates  $H_2$  production. After this event,  $H_2$  production at high rate proceeds to keep second redox homeostasis. When  $c_{S0}$  is no less than  $5 \mu\text{mol mL}^{-1}$ ,  $H_2$  production proceeds on the kinetics of Eq.(5.1) even at 120 h, hence attainable level ( $y_{H_2,f}$ ) is not predicted from experimental data. The relative values  $y_{H_2,f}^*$  and  $y_{H_2,f}^+$  cannot be discussed in this chapter.

Figure 5.3 (b) shows that fructose consumption is not high as glucose consumption shown in Fig.4.8, however, small amount of fructose is assimilated from 0 to 24 h and after 96 h by GT strain when  $c_{S0}$  is between 5 and  $50 \mu\text{mol mL}^{-1}$  and after 48 h when  $c_{S0}$  is  $60 \mu\text{mol mL}^{-1}$ . In Fig.5.4, initial specific fructose uptake rate ( $q_{S0}$ ) is plotted against  $c_{S0}$ . The  $q_{S0}$  is zero at low fructose concentration ( $c_{S0} < 5$ ) and high fructose concentration ( $50 < c_{S0}$ ) but linearly increase with  $c_{S0}$  when  $c_{S0}$  is between 5 and  $50 \mu\text{mol mL}^{-1}$ . The  $K_m$  cannot be evaluated from this curve, however, this curve suggests that it should be no less than  $5 \mu\text{mol mL}^{-1}$ . Low affinity of fructose to fructokinase in our experiment appears to be caused by limitation of ATP that is imperative for activation of fructokinase, since HEPES buffer solution lacks any phosphate ions.

Even though initial rate  $q_{S0}$  is small, curves in Fig.5.2(b) clearly shows the decrease in exogenous fructose concentration in 120 h reaction, except for run with  $70 \mu\text{mol mL}^{-1}$  fructose, which shows no consumption of fructose. The amount of assimilated fructose ( $-\Delta c_S = c_{S0} - c_{S1}$ ) is plotted against  $c_{S0}$  in Figure 5.4. When fructose concentration increases, consumption of fructose

increases except for  $c_{S0}$  at  $70 \mu\text{mol mL}^{-1}$ . Result of run with  $25 \mu\text{mol mL}^{-1}$  fructose also deviates from the linear line.

Figure 5.3 (c) shows that, in the presence of fructose, the amount of endogenous glucose per culture volume ( $m_{GX}$ ) increased in the first 24 h, decreased drastically from 24 to 48 h, and then decreased with very small rate or kept constant after 48 h. The  $m_{GX}$  in run with glucose showed the increase in  $m_{GX}$  for 72 h (Fig.4.8(c)), however, Fig.5.3(c) shows it for only 24 h. The  $m_{GX}$  of inoculum cells was fixed at  $0.16 \mu\text{mol mL}^{-1}$  by collecting the cells from the photoautotrophic culture of  $OD_{730}$  at 3. The  $m_{GX}$  for run with  $c_{S0}$  at 0, 5, 25, 50, 60 and  $70 \mu\text{mol mL}^{-1}$  increased right after dark incubation, reached the maximum value  $(m_{GX})_{\text{max}}$  of 0.22, 0.24, 0.27, 0.32, 0.36 and  $0.33 \mu\text{mol mL}^{-1}$  at 24 h in run with  $c_{S0}$  at 0, 5, 25, 50, 60 and  $70 \mu\text{mol mL}^{-1}$ , respectively, rapidly decreased from 24 to 48 h, and then approached to the stable level after 48 h. The  $m_{GX}$  at 120 h  $(m_{GX})_1$  for runs with  $c_{S0}$  at 0, 5, 25, 50, 60 and  $70 \mu\text{mol mL}^{-1}$  are 0.06, 0.09, 0.15, 0.17, 0.19 and  $0.18 \mu\text{mol mL}^{-1}$ , respectively. The  $(m_{GX})_{\text{max}}$  increases 1.64-fold from run without fructose by adding  $60 \mu\text{mol mL}^{-1}$  fructose. For run without fructose, it is difficult to consider any carbon source for this glycogen synthesis other than endogenous carbonaceous compounds such as amino acids in protein, fatty acids in membrane lipid and derivatives of poly- $\beta$ -hydroxybutyrate (PHB). Turnover of cell constituting materials is known to activate many NAD(P)H-generating enzymes.

Glycogen contents in terms of endogenous glucose are drastically decomposed between 24 and 48 h. Of special interest is that the amount of this drop  $(= (m_{GX})_{\text{max}} - (m_{GX})_1)$  is unrelated to initial fructose concentration, and is about  $0.16 \pm 0.04 \mu\text{mol mL}^{-1}$ . Glycolysis is found to be independent from the presence of exogenous fructose. When the degradable glycogen is assimilated, glycolysis is terminated. The amount of endogenous glucose per culture volume is found to be always higher in the presence of fructose than in the absence of fructose.

Glycogen decomposition is known to generate NADH. The  $\text{H}_2$  production rate between 24 h and 48 h in run with  $25 \mu\text{mol mL}^{-1}$  fructose is  $0.0604 \mu\text{mol mL}^{-1}\text{h}^{-1}$  that is 2.39 times that in run without fructose, hence the  $\text{H}_2$  production rate after 24 h is found to be independent from the amount of NADH generated by glycolysis. Considering that the amount of endogenous glucose per culture volume is increased by increasing initial fructose concentration and that the amount of assimilated endogenous glucose per culture volume after 24 h is not affected by initial fructose

concentration, it is found that the amount of endogenous glucose per culture volume after 24 h is always higher in run with fructose than in run without fructose.

The time course data of dry cell weight concentration ( $X$ ) versus time is shown in Figure 5.3(d). Observations of run without or with  $25 \mu\text{mol L}^{-1}$  fructose show that  $X$  in run without fructose decreases slightly with time, whereas it in run with fructose slightly increases in the first 24 h and in the period between 72 and 120 h.

In **Figure 5.5**, the amount of increase in dry cell weight concentration ( $(\Delta X)_1 = X_1 - X_0$ ) is plotted against  $c_{S0}$ . The increase in  $(\Delta X)_1$  is associated with the increase in  $(-\Delta c_S)_1$ , which is observed at  $c_{S0}$  from 5 to  $60 \mu\text{mol L}^{-1}$ . There are remarkably few data available in previous works regarding the heterotrophic growth of GT strain on fructose, the relation between  $(-\Delta c_S)_1$  and  $(\Delta X)$  in Fig.5.5 suggests the high possibility of a limited heterotrophic growth of GT strain on fructose under the dark anaerobic condition. This research is carried out to find the condition to elevate  $\text{H}_2$  production, hence cells were incubated not in BG-11 medium but in HEPES buffer solution that contained only HEPES and KOH for pH adjustment. A cessation of fructose assimilation is seen between 24 and 72 h. The unreacted fructose concentration in this period is  $23.9 \pm 0.56 \mu\text{mol mL}^{-1}$ . Combination of the curves for fructose concentration and dry cell weight concentration versus time in Figure 5.3b eminently demonstrates the limited heterotrophic growth on fructose in the first 24 h and in the period between 72 and 120 h. This discontinuous heterotrophic growth on fructose suggests that GT strain appear to respond on cell cycle events to the initial shift of cellular environment to dark anaerobic condition with fructose. The yield of endogenous glucose from fructose ( $=r_{G0}/(-r_{S0})$ ) is calculated as  $0.0279 \pm 0.0146$ , which is quite low. Fructose is not mainly turned to endogenous glucose.

**Figure 5.6** shows the relation between the number of moles of  $\text{H}_2$  per culture volume at 120 h ( $y_{\text{H}_2,1}$ ) and the number of moles of endogenous glucose per culture volume at 120 h ( $(m_G X)_1$ ). There seems to be a linear correlation, defined by Eq.(2.40). Best fit to the data results that parameters are  $k_5$ ,  $152 \mu\text{mol}^{-0.52} \text{mL}^{0.52}$ ; and  $k_6$ , 1.52. The sensitivity of  $y_{\text{H}_2,1}$  to  $(m_G X)_1$  of run with cells of GT strain from logarithmic growth phase increased 4.08-fold over that from late-logarithmic growth phase. For inoculum from logarithmic growth, fructose is found to elevate the amount of  $\text{H}_2$  per culture volume. To keep high level in the amount of endogenous glucose per culture volume appears to be of prime importance to result in high  $\text{H}_2$  production in GT strain.

In the absence of fructose, lactate concentration ( $c_L$ ) was extremely low and reached only  $0.2 \mu\text{mol mL}^{-1}$  at 120 h, whereas, in the presence of fructose, lactate synthesis in GT strain continuously occurred during 120 h. The lactate production rate after 48 h was similar to the  $\text{H}_2$  production rate. **Figure 5.7** relates  $y_{\text{H}_2}$  and  $c_L$  in runs with GT strain in HEPES buffer solution with or without fructose. The level of  $c_L$  is seen to be the same order of magnitude of  $y_{\text{H}_2}$ . Increase in  $c_L$  in the presence of fructose suggests that exogenous fructose is an additional carbon source for lactate production. The amount of intracellular lactate was not detectable level. There is a drastic change in the slope of the curve when  $c_L$  approaches to  $1.8 \mu\text{mol mL}^{-1}$ . The drastic change in NAD(H)P at  $t_{\text{SHT}}$  corresponds to the point where slope of  $c_L$  versus  $y_{\text{H}_2}$  curve data occurs.

Different from lactate, acetate concentration ( $c_A$ ) rose to a maximum level at 72 h and then decreased. The highest  $c_A$  is  $0.2 \mu\text{mol mL}^{-1}$ . The activated production of  $\text{H}_2$  in the presence of  $50\text{--}83 \mu\text{mol mL}^{-1}$  fructose is found to be accompanied by the elevated assimilation of fructose (Figure 5.3b), the elevated initial synthesis of glycogen (Figure 5.3c), followed by the assimilation of endogenous glucose after 24 h and the elevated production of lactate (Figure 5.3e). Because of the insufficiency of required elements for normal cell growth, a small portion of assimilated fructose is directed to cell growth (Figure 5.3d). When cells are exposed to fructose in buffer solution, the initial glycogen synthesis is activated, while the subsequent glycogen decomposition was not affected by initial concentration of fructose (Figure 5.3c).

The assimilation of endogenous glucose is obvious between 24 h and 48 h, hence the instantaneous fractional yield of  $\text{H}_2$  on endogenous glucose  $Y_{\text{H}_2/\text{G}}$  that is defined by the amount of  $\text{H}_2$  produced per amount of endogenous glucose utilized in this period is evaluated and listed in Table 5.1.

Glucose 6-phosphate is an intermediate both in gluconeogenesis pathway and glycolysis pathway. If glucose 6-phosphate is catabolized in the oxidative pentose phosphate (OPP) pathway and if both NADPH and ATP are required in this pathway, theoretical conversion of 1 mole glucose 6-phosphate to  $5/3$  mole pyruvate is accompanied by the generation of 2 mole NADPH and  $5/3$  mole NADH. When 1 mole pyruvate is completely converted to 3 mole carbon dioxide, this reaction yields 4 mole NADH and 1 mole  $\text{FADH}_2$ . If all of NAD(P)H generated in these pathways are directed to NiFe-hydrogenase, potential yields of  $\text{H}_2$  in the pathway from glucose 6-phosphate to pyruvate and from glucose 6-phosphate to carbon dioxide are estimated as 3.67 and 12, respectively.

The yield of H<sub>2</sub> for run without fructose in Table 5.1 is slightly lower than the theoretical yield from glucose 6-phosphate to pyruvate, and that for runs with 50-70 μmol·mL<sup>-1</sup> fructose is much higher than the theoretical yield from glucose 6-phosphate to carbon dioxide. Our observation suggests that NAD(P)H for H<sub>2</sub> production are supplied not only from glycogen degradation but also from turnover of proteins, membrane lipid and PHB. This turnover appears to be accompanied by glycolysis. The observed lactate level was high at high initial fructose concentration. High concentration fructose appears to activate NiFe-hydrogenase and lactate dehydrogenase to discharge excess reductant generated in the incomplete tricarboxylic acid (TCA) cycle and in the pathways for turnover of above components. The positive effect of fructose on H<sub>2</sub> production in this result suggests that fructose transport through cellular membrane is of prime importance to elevate H<sub>2</sub> production.

Chapters 2 and 4 reported the elevation of dark anaerobic H<sub>2</sub> production in GT strain by adding glucose in HEPES buffer solution. H<sub>2</sub> production in cells under glucose fermentation is different from that in fructose fermentation because cells extensively utilize glucose for cell division and thus, more cell population results in more H<sub>2</sub> production. In fructose under dark anaerobic condition, cells utilize fructose for endogenous glucose buildup rather than limited cell growth. This endogenous glucose accumulation is followed by the endogenous glucose break down by glycolysis that triggers many intracellular associated turnover reactions against cell constituting components. Cells gain more electrons through glycolysis and associated turnover reactions. This is partially supported by the increase in lactate concentration in runs with fructose since lactate is one of key carbon terminal compounds. Since fructose inhibited photoheterotrophic growth, our observation suggests that fructose might suppress an important pathway or mechanism related to light mode of life. Interestingly, this growth inhibition was not observed under dark anaerobic mode of life. Suppression of specific mechanism by fructose might be a key for redirection of more electrons to H<sub>2</sub>ase enzyme.

**(b) Effect of 50 μmol mL<sup>-1</sup> fructose on dark anaerobic H<sub>2</sub> production in GT strain, Δadh mutant and ΔadhΔadh mutant**

One of the primary objectives of the study in this chapter was to stabilize long-term H<sub>2</sub> production in genetically modified Δadh mutant and ΔadhΔadh mutant of glucose tolerant derivative of *Synechocystis* sp. strain PCC 6803 (GT strain) utilizing fructose. Experimental data

above show that fructose largely elevates the dark anaerobic H<sub>2</sub> production of GT strain cells from logarithmic growth phase and that optimum fructose concentration regarding H<sub>2</sub> production is 50 μmol mL<sup>-1</sup>, and that, in the absence of fructose, the initial H<sub>2</sub> production rate of Δ*addh* mutant prepared from late-logarithmic growth phase in photosynthesis is higher than that of GT strain. Dark anaerobic incubation of those cells in the presence of glucose also shows that the rate of H<sub>2</sub> production by Δ*addh* mutant is higher than that of GT strain. Therefore, a series of dark anaerobic H<sub>2</sub> production experiments were performed in which cells of Δ*addh* mutant and Δ*adh*Δ*addh* mutant from logarithmic growth phase were incubated in HEPES buffer solution without or with 50 μmol mL<sup>-1</sup> fructose. **Figure 5.8** shows that H<sub>2</sub> production yield from endogenous glucose degradation is highest when initial fructose concentration is 60 μmol mL<sup>-1</sup>.

**Figure 5.9** shows the time courses of dark anaerobic H<sub>2</sub> production in GT strain, Δ*addh* mutant and Δ*adh*Δ*addh* mutant, from logarithmic growth phase, in HEPES buffer solution with or without 50 μmol mL<sup>-1</sup> fructose. The amount of H<sub>2</sub> produced from unit volume of solution ( $y_{H_2}$ ), the amount of endogenous glucose in glycogen of a unit volume ( $m_G X$ ), dry cell weight per solution volume ( $X$ ), the concentrations of fructose, lactate and acetate were determined.

The curves of  $y_{H_2}$  versus time in Figure 5.9(a) show that fructose increased initial H<sub>2</sub> production rate and stabilized and accelerated H<sub>2</sub> production rate after the initial stage. The initial H<sub>2</sub> production rates ( $r_{H_2,0}$ ) in GT strain, Δ*addh* mutant and Δ*adh*Δ*addh* mutant, which were 0.025, 0.015 and 0.020 μmol mL<sup>-1</sup> h<sup>-1</sup>, respectively, in the absence of fructose, were increased to 0.091, 0.109 and 0.129 μmol mL<sup>-1</sup> h<sup>-1</sup>, respectively, in the presence of fructose. Fructose is found to largely enhance H<sub>2</sub> production activity of NiFe-hydrogenase activity. The H<sub>2</sub> production in GT strain in fructose solution shown in Figure 5.9(a) is related by Eq.(2.21) and well fitted by Eq.(5.1).

The  $y_{H_2}$  versus time data of run with Δ*addh* mutant and Δ*adh*Δ*addh* mutant in 50 μmol mL<sup>-1</sup> fructose is similar to the result of run with GT strain in 25 μmol mL<sup>-1</sup> fructose that was well fitted by Eq.(5.3). Best fitting the data with Eq.(5.3) results the parameters  $r_{H_2,0}$ , 0.104 μmol mL<sup>-1</sup>h<sup>-1</sup>;  $t_{SHT}$ , 86.8 h; and  $r_{H_2,SHT}$ , 0.216 μmol mL<sup>-1</sup>h<sup>-1</sup> for Δ*addh* mutant, and  $r_{H_2,0}$ , 0.123 μmol mL<sup>-1</sup>h<sup>-1</sup>;  $t_{SHT}$ , 68.2 h; and  $r_{H_2,SHT}$ , 0.203 μmol mL<sup>-1</sup>h<sup>-1</sup>. Solid lines for calculated data of  $y_{H_2}$  by Eq.(5.3) and those parameters show good fits to the observed variables. Results of  $t_{SHT}$  is so far suggested that shift of metabolic network occurs earlier when initial H<sub>2</sub> production rate is higher. The  $t_{SHT}$  of this work is from 68.2 to 86.8 h. In Fig.5.3, maximum initial H<sub>2</sub> production rate ( $r_{H_2,0}$ ) was 0.0927 μmol mL<sup>-1</sup>h<sup>-1</sup>.

When  $\Delta adh\Delta ddh$  mutant mutant is incubated in  $50 \mu\text{mol mL}^{-1}$  fructose, initial rate increased 1.33-fold over best run with GT strain in fructose. After second reformation of metabolic network for redox homeostasis at  $t_{\text{SHT}}$ ,  $\text{H}_2$  production rate shows 2.19-fold increase over the maximum  $r_{\text{H}_2,0}$  of Fig.5.3.

The relation between the initial specific  $\text{H}_2$  production rate ( $q_{\text{H}_2,0}$ ) and initial fructose concentration ( $c_{\text{S}0}$ ) of runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant is shown in **Figure 5.10**. In the presence of  $50 \mu\text{mol mL}^{-1}$  fructose,  $q_{\text{S}0}$  values of cells of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant from logarithmic growth phase are  $0.092$ ,  $0$  and  $0.031 \mu\text{mol mL}^{-1}\text{h}^{-1}$ . Similar to runs with  $\Delta ddh$  mutant, from late-logarithmic growth phase, in HEPES buffer solution with glucose shown in chapter 4, initial uptake of fructose by  $\Delta ddh$  mutant is blocked.

**Figure 5.11** compares  $(-\Delta c_{\text{S}})_1$ ,  $(\Delta X)_1$  of runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant. Even though  $q_{\text{S}0}$  is zero in  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant, fructose is eminently consumed by those mutants during 120 h incubation. The amount of consumed fructose by  $\Delta ddh$  mutant and that by  $\Delta adh\Delta ddh$  mutant is comparable. The  $(-\Delta c_{\text{S}})_1$  appears to be defined by the activity of lactate dehydrogenase. The total amount of assimilated fructose by mutants is 1.64 times that of GT strain. The  $(\Delta X)_1$  of GT strain and that of  $\Delta ddh$  mutant is comparable, while that of  $\Delta adh\Delta ddh$  mutant is higher than that of GT strain and  $\Delta ddh$  mutant. The NAD(P)H content in  $\Delta adh\Delta ddh$  mutant in fructose solution is so high that additional metabolism to discharge extra electrons for the reproduction of biomaterials appears to occur in  $\Delta adh\Delta ddh$  mutant.

**Figure 5.12** shows a relation between  $(m_{\text{C}}X)_1$  and  $y_{\text{H}_1}$  in runs with GT strain in HEPES buffer solution with or without fructose. Straight broken lines in the graph show the relations of Eq.(2.40) for cells of GT strain from logarithmic growth phase and late-logarithmic growth phase. If plots of data for runs with  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant are also interpreted by Eq.(2.40), parameters are estimated as  $k_5$ , 7970;  $k_6$ , 3.66 for  $\Delta ddh$  mutant and  $k_5$ , 8510;  $k_6$ , 3.45 for  $\Delta adh\Delta ddh$  mutant. Calculated results are shown by solid lines. Two solid lines are almost parallel. Sensitivity to fructose is found to be highest in  $\Delta adh\Delta ddh$  mutant, second highest in  $\Delta ddh$  mutant and lowest in GT strain. Sensitivity of  $\text{H}_2$  production to the amount of endogenous glucose per culture volume of  $\Delta ddh$  mutant and that of  $\Delta adh\Delta ddh$  mutant from logarithmic growth phase are found to be comparable, and are 2.27-2.41-folds higher than that of GT strain from logarithmic growth phase. It

is found that, in the presence of fructose, elimination of lactate dehydrogenase genes makes H<sub>2</sub> production ability of cells to be extremely sensitive to the variation in the amount of endogenous glucose. The  $y_{H_2,1}$  of run with  $\Delta adh\Delta ddh$  mutant is higher than that of  $\Delta ddh$  mutant. Elimination of alcohol dehydrogenase genes appears to shift up the activity of NAD(P)H accumulation reactions that are not led by glycolysis.

The time course data of  $c_s$  in Figure 5.9(b) confirms the initial small uptake of fructose by GT strain ( $q_{s0}$ ) which was shown in Fig.5.4, while no uptake by  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant.

The relation between  $c_{s0}$  and  $q_{s0}$  is shown in Fig.5.4. This observation is similar to the block of glucose uptake in  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant shown in 4.8(b). Fructose appears to be consumed by cells of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant for maintaining dry cell weight stability. The consumption of fructose by GT,  $\Delta ddh$  and  $\Delta adh\Delta ddh$  strains were comparable. Consumption of fructose during 120 h was 5.1-8.4  $\mu\text{mol mL}^{-1}$ . High H<sub>2</sub> production of those cells in the presence of fructose was not likely resulted from direct fructose consumption.

In Figure 5.9(c), the time courses of the amount per culture volume of glycogen in terms of endogenous glucose ( $m_G$ ) were displayed. In the first 24 h of dark incubation in HEPES buffer solution without or with fructose, the  $m_G$  increased in GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant. This amount of endogenous glucose is corresponding to consumption of fructose. The peak value of  $m_G$  of runs with fructose is higher than that without fructose. The  $\Delta adh\Delta ddh$  mutant converted more fructose to endogenous glucose than GT strain and  $\Delta ddh$  mutant. The glycogen content dropped during dark anaerobic fermentation. The highest drop in  $m_G X$  from 24 h to 120 h was 0.183  $\mu\text{mol mL}^{-1}$  that was observed in run with  $\Delta adh\Delta ddh$  mutant in fructose solution. H<sub>2</sub> generated in this run is 25.5 times that estimated from the theoretical yield of NAD(P)H in glycolysis. Cells used fructose for gluconeogenesis in the first 24 h then for glycolysis from glycogen after 24 h. At the end of fermentation, all strains had the same level of glycogen content.

In Figure 5.9(d), the time courses of the dry cell weight concentration of cyanobacterium cells ( $X$ ) were illustrated. The dry cell weight concentration of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant slowly decreased with time during 120 h in the absence of fructose. The aqueous solution for cell suspension was consisted of milliQ grade water and HEPES-KOH for pH buffering. Nitrate was totally removed from the system. The buffer solution is confirmed to be insufficient to support cell viability of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant. In the presence of fructose, the  $X$  of

GT strain increased in the first 24 h, was constant between 48 h and 72 h and increased again after 72 h, the  $X$  of  $\Delta ddh$  mutant was constant in the first 48 h, increased between 48 h and 72 h, decreased between 72 h and 96 h and increased after 96 h, and the  $X$  of  $\Delta adh\Delta ddh$  mutant was constant for the first 72 h and increased after 72 h. The dry cell weight concentrations of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant at 120 h were higher than the initial concentrations. Curves for runs with fructose in Fig. 5.9 (c) and (d) show that GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant are capable of limited heterotrophic growth on fructose in HEPES buffer solution. In the presence of fructose, the  $X$  at 120 h was highest in runs with GT strain and  $\Delta ddh$  mutant and lowest that with  $\Delta adh\Delta ddh$  mutant. It is observed that growth period of  $\Delta ddh$  mutant is later than that of GT strain, and that of  $\Delta adh\Delta ddh$  mutant is later than that of  $\Delta ddh$  mutant. Instability of dry cell weight concentration in dark anaerobic nitrate-free HEPES buffer solution was suppressed by fructose. This result shows that fructose supports cell viability in the dark anaerobic condition.

The total increase in dry cell weight concentration  $(\Delta X)_1$  is plotted in Fig.5.11. The increase in  $(\Delta X)_1$  by genetic modification is found to be accompanied by the increase in  $(-\Delta c_s)_1$ . This plot support above view that GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant can utilize fructose and represent limited heterotrophic growth.

In Figure 5.9(e), the time courses of lactate concentration ( $c_L$ ) are shown. The production of lactate by GT strain in the presence of fructose is extremely high. Lactate production of GT strain in the presence of fructose was activated at 72 h. At 120 h, lactate concentration of run with GT strain, which was  $0.204 \mu\text{mol mL}^{-1}$  in buffer solution, increased to  $9.02 \mu\text{mol mL}^{-1}$  in the presence of fructose. Photosynthetic growth for cell preparation and dark anaerobic incubation were performed in the absence of antibiotics, hence the observation of trace amount lactate in runs with  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant shows the drop of phenotype of lactate dehydrogenase deficiency.

**Figure 5.13** is a plot showing a relation between  $y_{H_2}$  and  $c_L$  in runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant in HEPES buffer solution with or without fructose. Since lactate dehydrogenase activity is absent in  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant, the  $c_L$  of mutants are zero even though the sample solution contains extremely low population of phenotype deficient strain. Among the plotted data, only those for GT strain in HEPES buffer solution with  $50 \mu\text{mol mL}^{-1}$  fructose shows a meaningful relation between  $c_L$  and  $y_{H_2}$ , which confirms the relation in Figure 5.7.

In Figure 5.9(f), the time courses of acetate concentration ( $c_A$ ) were plotted. Acetate concentrations of runs with fructose were higher than those without fructose, and increased drastically during 48-72 h. In the presence of fructose, acetate concentration was highest in GT strain, second highest in  $\Delta ddh$  mutant and lowest in  $\Delta adh\Delta ddh$  mutant. Acetate concentrations were highest during 48-72 h then the concentration dropped. The peaks of acetate concentration in runs with buffer solutions were 0.062, 0.162, and 0  $\mu\text{mol mL}^{-1}$  for GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant, respectively, and those with buffer solutions with fructose were 1.21, 0.847 and 0.394  $\mu\text{mol mL}^{-1}$  for them, respectively. The concentration of acetate after 72 h was extremely low. The consumption of acetate is highest in run with GT strain, second highest in run with  $\Delta ddh$  mutant and lowest in run with  $\Delta adh\Delta ddh$  mutant. This order is reverse of that of  $\text{H}_2$  production, while close to that of dry cell weight production. It suggests that acetate consumption after the peak seems related to late cell growth

Ethanol concentrations in runs without or with fructose were not at detectable level. Our previous report shows the ethanol production of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant are 0.70, 1.01 and 0.47  $\mu\text{mol mL}^{-1}$ , respectively, in the presence of glucose after 96 h. hence fructose fermentation of above strains appears to be different from glucose fermentation.

High production of  $\text{H}_2$ , endogenous glucose and acetate were observed if fructose was present. This observation raised very interesting points concerning participation of fructose in NAD(P)H production or redirection of NAD(P)H in the dark. The slight higher fructose consumption by mutants could be the reason that mutants took long time for cell growth therefore cell kept utilizing more carbon source. However at 120 h, difference of final dry cell weight concentrations among three strains was not large. Mutants consumed more fructose while produced less acid than GT strain. More trials are required to find the products from acetate.

Cellular state variables of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant are summarized in Table 5.2, The initial  $\text{H}_2$  production rates of  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant increased 3-fold to 7-fold over GT strain when fructose was present.  $\text{H}_2$  production from mutant lacking both *ddh* and *adh* genes yielded extremely high amount of  $\text{H}_2$ .

In the presence of fructose, the amount per culture volume of  $\text{H}_2$  produced from GT strain was extremely high when logarithmic growth phase cells were incubated in dark anaerobic condition. Quite important phenomenon found from this observation is that cells prepared from logarithmic

growth phase are capable of expressing a stable high reductive state under the cellular exposure to fructose.

The deletion of the genes coding for competitive enzymes of NiFe-hydrogenase was effective for increasing the initial H<sub>2</sub> production rate of cells prepared from late-logarithmic growth phase. If fructose is present, mutant cells prepared not from late-logarithmic growth phase but from logarithmic growth phase produce highest amount of H<sub>2</sub>. The selection of growth phase affects H<sub>2</sub> production in mutants greatly. The presence of fructose significantly activates H<sub>2</sub> production in mutants.

**Figure 5.14** shows an effect of genetic modification upon yield of H<sub>2</sub> on endogenous glucose for glycolysis ( $Y_{H_2/G}$ ). In the absence of fructose, the highest  $Y_{H_2/G}$  of 6.63 is seen in GT strain and the lowest  $Y_{H_2/G}$  of 5.81 is seen in  $\Delta ddh$  mutant. Contrary to the expectation, elimination of lactate dehydrogenase results in the decrease in the yield of H<sub>2</sub> from endogenous glucose. The order of magnitude of  $Y_{H_2/G}$  is close to the theoretical yield. In the presence of fructose,  $Y_{H_2/G}$  values are extremely high in GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant. The NAD(P)H for NiFe-hydrogenase in the presence of fructose is found to be supplied mainly by pathways other than glycolysis pathway. If the  $Y_{H_2/G}$  of three strains are compared, elimination of lactate dehydrogenase activity is found to elevate NAD(P)H supply by other pathways.

The overview of the data shown in Fig.5.9 indicates that genetic perturbation of redox status that is brought by disruption of genes coding for lactate dehydrogenase and alcohol dehydrogenase plays an integral part to increase H<sub>2</sub> production. Deletion of lactate dehydrogenase activity and alcohol dehydrogenase activity results not only redirection of NAD(P)H molecule for lactate dehydrogenase and alcohol dehydrogenase to NiFe-hydrogenase but also reforming the redox homeostasis networks. The increased amount of H<sub>2</sub> is higher than that expected by above redirection of NAD(P)H. Genetically modified mutants appear to represent a cellular state under the added load of mutation. Fructose plays the other integral part to increase H<sub>2</sub> production. Dry cell weight concentration of mutants is supported by fructose as that of GT strain. In the presence of fructose, cells of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant express the redox balance systems to constantly make-up NAD(P)H molecules utilized by NiFe-hydrogenase. The supernumerary NAD(P)H synthesis in mutants appear to trigger the second reformation of the redox balance systems at  $t_{SHT}$ . This reformation contributes to further increase the amount of H<sub>2</sub> per culture volume. The second reformation occurs when acetate production is changed to acetate assimilation.

### (c) Conceptual design of bioreactor for H<sub>2</sub> production

Biological data obtained in this work is applied for the conceptual design of dark anaerobic bioreactor to produce fuel H<sub>2</sub>. In experiments, HEPES buffer solution with a pH at 7.8 was utilized; however, to reduce the cost of buffer solution, the replacement of HEPES buffer solution by PBS (phosphate buffered saline; NaCl, 8.0 kg m<sup>-3</sup>; KCl, 0.20 kg m<sup>-3</sup>; Na<sub>2</sub>HPO<sub>4</sub>, 1.44 kg m<sup>-3</sup>; KH<sub>2</sub>PO<sub>4</sub>, 0.24 kg m<sup>-3</sup>; pH7.4) solution is assumed. In experiment, point mutagenesis was applied to construct  $\Delta\text{adh}\Delta\text{ddh}$  mutant that is defective of lactate dehydrogenase activity and alcohol dehydrogenase activity. For considering industrialization,  $\Delta\text{adh}\Delta\text{ddh}$  mutant is assumed to be obtained by mutagenesis. The data obtained in this work for the point-mutagenesis-derived  $\Delta\text{adh}\Delta\text{ddh}$  mutant in HEPES buffer solution are assumed to be applicable for the calculation of dark anaerobic H<sub>2</sub> production in mutagenesis-derived  $\Delta\text{adh}\Delta\text{ddh}$  mutant in PBS solution.

**Figure 5.15** shows a conceptual design of dark anaerobic bioreactor for  $\Delta\text{adh}\Delta\text{ddh}$  mutant. The employment of 5,000 m<sup>3</sup> anaerobic biogas bioreactor (Harvestore, 2014) is assumed in this work. The bioreactor is a cylindrical stirred tank with standard dimensions. The working volume is typically 70-80% of the total volume, hence this graph assumes the 4,000 m<sup>3</sup> working volume. The headspace volume is 1,000 m<sup>3</sup>. If height ( $H$ ) per diameter ( $D$ ) is 0.4, then size of bioreactor is  $D$ , 25.2 m;  $H$ , 10.1 m. Channel is equipped inside the anaerobic bioreactor, for which PBS buffer solution with 50 mol m<sup>-3</sup> (=50  $\mu\text{mol mL}^{-1}$ ) fructose is fed with plug flow reactor operation. Flow rate of buffer solution is 33.3 m<sup>3</sup> h<sup>-1</sup> in order to set the mean residence time of fluid at 120 h. From Table 5.2, the number of moles of H<sub>2</sub> at 120 h is predicted as 18.7 mol m<sup>-3</sup>(=18.7  $\mu\text{mol mL}^{-1}$ ). Production rate is 623 (=33.3)(18.7) mol h<sup>-1</sup>. Gas phase contains CO<sub>2</sub> that is removed by NaOH to form Na<sub>2</sub>CO<sub>3</sub> in absorber that is equipped at the gas outlet. This byproduct is returned to photobioreactor in which CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> are assimilated by  $\Delta\text{adh}\Delta\text{ddh}$  mutant. At the liquid outlet,  $\Delta\text{adh}\Delta\text{ddh}$  mutant is separated by continuous centrifugal and it is directed to fertilizer. The separated PBS buffer solution in supernatant is returned to the mixing tank where concentrated newly fed inoculum is suspended in nitrate-free PBS buffer solution. The spent cells can be utilized in for growth cultivation in photosynthesis. In order to control pH and buffer concentration, the makeup is added up into buffer solution return.

If operation time of a unit is 300d y<sup>-1</sup>, then annual H<sub>2</sub> production from a unit is 4,480,000(=(623)(24)(300)) mol y<sup>-1</sup>. =100,000 Nm<sup>3</sup> y<sup>-1</sup>. The amount of H<sub>2</sub> to fill-up a 156L tank of FCV was shown to be 4,880 moles (chapter 1). Hence annually produced H<sub>2</sub> from one unit of

bioreactor is found to fill-up the tank of 918 (=4480000/4880) FCVs. H<sub>2</sub> production rate of Japan in 2014 is 140,000,000Nm<sup>3</sup>, hence one unit is found to have a potential to support 0.1% of national demand of fuel H<sub>2</sub>. The proposed cyanobacterial H<sub>2</sub> production doesn't require any consumption of natural gas, oil and other fossil fuel, hence it appears to be a very promising technological approach.

#### **(d) Proposed method to activate dark anaerobic H<sub>2</sub> production with a unicellular cyanobacterium**

**Figure 5.16** is a proposed method to elevate dark anaerobic H<sub>2</sub> production utilizing unicellular cyanobacterium. The method is based on the approach of this work. Knowledge from previous work contributes to initiate the approach. Condition for H<sub>2</sub> production in GT strain had been established at the beginning of this work. Problem to achieve activated H<sub>2</sub> production was decomposed into four sub-problems. Experiments have been directed by a mathematical model. Feedback has been made utilizing results of experiments for solving sub-problems. In this work, the glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 has been a material for a strain of experimental approach. Redox homeostasis of this strain appears to be a model of that of unicellular cyanobacteria, hence this graph can be useful to search for condition on the optimum H<sub>2</sub> production of other strains.

## **5.4 Summary**

The study in this chapter was made (1) to elucidate the effect of fructose on dark anaerobic H<sub>2</sub> production in GT strain in HEPES buffer solution, (2) to compare the dark anaerobic H<sub>2</sub> production in GT strain,  $\Delta$ dh mutant and  $\Delta$ adh $\Delta$ dh mutant in HEPES buffer solution with or without 50  $\mu$ mol mL<sup>-1</sup> fructose, and (3) to perform a conceptual design of bioreactor to produce biofuel H<sub>2</sub>. Results are summarized as follows.

1. Cells consumed fructose for endogenous glucose accumulation and dry cell weight production. Affinity to fructose was extremely low compared to glucose consumption.
2. Fructose was one of important factors that can save or provide reductive compound for H<sub>2</sub> production via H<sub>2</sub>ase. The best concentration of fructose for H<sub>2</sub> production was 50  $\mu$ mol mL<sup>-1</sup>.

3. The H<sub>2</sub> production of the glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 in dark anaerobic HEPES buffer solution is found to be largely elevated by supply of fructose and elimination of dehydrogenase genes.
4. Dark anaerobic 120 h incubation of a mutant lacking both lactate dehydrogenase genes and alcohol dehydrogenase genes in HEPES buffer solution with fructose is found to increase the amount of H<sub>2</sub> per culture volume 11-fold over that without fructose.
5. Conceptual design of the dark anaerobic production of H<sub>2</sub> is challenged. One unit of 5,000m<sup>3</sup> biogas production facility is estimated to be able to produce 100,000 Nm<sup>3</sup> H<sub>2</sub> per year that can support to fill up the tank of 918 FCVs. Baseline of the research for biofuel H<sub>2</sub> production is created.
6. A method to activate dark anaerobic H<sub>2</sub> production in unicellular cyanobacterium is proposed.

Table 5.1 Kinetic parameters for H<sub>2</sub> production in GT strain in HEPES buffer solution with 0-70 μmol mL<sup>-1</sup> fructose. Logarithmically growing cells were used.  $q_{H2,0}^*$ ,  $q_{H2,0}/(q_{H2,0})_{w/o}$ ;  $y_{H2,f}^*$ ,  $y_{H2,f}/(y_{H2,f})_{w/o}$ ; and  $k^*=k/(k)_{w/o}$ .  $y_{H2,1}$ ,  $c_{L1}$ ,  $c_{A1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 120 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 120 h;  $\Delta(m_G X)_0$ , observed increase in  $m_G X$  during first 24 h;  $\Delta(-m_G X)_1$ , observed decrease in  $m_G X$  from 24 h to 120 h; H<sub>2</sub> production;  $y_{H2,G}=y_{H2,1}/\Delta(m_G X)_1$ ; Subscripts 0, initial; 1, 120h; f, attainable state; SHT, shift in redox state; S, fructose; G, endogenous glucose; L, lactate; A, acetate; w/o, without fructose. Reference run is run without monosaccharide and with GT strain from late-logarithmic growth phase shown in Table 4.7.  $(q_{H2,0})_{ref}$ , 0.0147 μmol mg<sup>-1</sup>h<sup>-1</sup>;  $(y_{H2,f})_{ref}$ , 2.28 μmol mL<sup>-1</sup>;  $k_{ref}$ , 0.0167h<sup>-1</sup>;  $K_{d,ref}$ , 0.00105h<sup>-1</sup>.

	$c_{so}[\mu\text{mol mL}^{-1}]$					
	w/o	5	25	50	60	70
$X_0$ [mg mL <sup>-1</sup> ]	2.1	1.9	2.0	1.9	1.9	1.9
$r_{H2,0}$ [μmol mL <sup>-1</sup> h <sup>-1</sup> ]	0.0285	0.0422	0.0631	0.0927	0.0927	0.0927
$q_{H2,0}$ [μmol mg <sup>-1</sup> h <sup>-1</sup> ]	0.0136	0.0222	0.0316	0.0488	0.0488	0.0488
$t_{SHT}$ [h]	nd	76.6	81.9	nd	nd	nd
$r_{H2,SHT}$ [μmol mL <sup>-1</sup> h <sup>-1</sup> ]	nd	1.64	5.17	nd	nd	nd
$q_{H2,SHT}$ [μmol mg <sup>-1</sup> h <sup>-1</sup> ]	nd	0.863	2.59	nd	nd	nd
$q_{H2,0}^*$ [-]	0.925	1.51	2.01	3.32	3.32	3.32
$y_{H2,f}$ [μmol mL <sup>-1</sup> ]	2.10	no less than 5.4	no less than 8.1	no less than 10.8	no less than 10.6	no less than 10.4
$y_{H2,f}^*$ [-]	0.956	no less than 2.37	no less than 3.55	no less than 4.74	no less than 4.65	no less than 4.56
$k$ [h <sup>-1</sup> ]	0.0136	0.0202 (0-76.6h) nd (after 76.6h)	nd	nd	nd	nd
$k^*$ [-]	0.814	1.21 (0-72h) 0(after 72h)	nd	nd	nd	nd
$q_{so}$ [μmol mg <sup>-1</sup> h <sup>-1</sup> ]	0	0.0390	0.0369	0.0838	0.0303	0.0333
$K_d$ [h <sup>-1</sup> ]	0.000710	0.000345 (24-96h)	0.00641 (24-120h)	0	0	0

$y_{H_2,1} [\mu\text{mol}\cdot\text{mL}^{-1}]$	1.70	5.4	8.1	10.8	10.6	10.4
$m_{G1} [\mu\text{mol}\cdot\text{mg}^{-1}]$	0.0298	0.0386	0.0730	0.0690	0.0790	0.0707
$X_1 [\text{mg}\cdot\text{mL}^{-1}]$	1.9	2.2	2.1	2.5	2.4	2.5
$(m_G X)_1 [\mu\text{mol}\cdot\text{mL}^{-1}]$	0.0566	0.0849	0.153	0.173	0.190	0.177
$c_{L,1} [\mu\text{mol}\cdot\text{mL}^{-1}]$	0.2	3.0	5.4	8.8	9.0	10.4
$c_{A,1} [\mu\text{mol}\cdot\text{mL}^{-1}]$	0.0	0.0	0.1	0.2	0.2	0.2
$\Delta(m_G X)_1 [\mu\text{mol}\cdot\text{mL}^{-1}]$	0.4	0.5	1.6	2.2	2.4	2.0
$Y_{H_2/G} [-]$	3.2	3.8	8.9	14.1	12.5	11.7

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Table 5.2 Kinetic parameters for H<sub>2</sub> production in GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant in HEPES buffer solution with or without 50  $\mu$ mol mL<sup>-1</sup> fructose. Logarithmically growing cells were used.  $q_{H2,0}^*$ ,  $q_{H2,0}/(q_{H2,0})_{w/o}$ ;  $y_{H2,f}^*$ ,  $y_{H2,f}/(y_{H2,f})_{w/o}$ ; and  $k^*=k/(k)_{w/o}$ .  $y_{H2,1}$ ,  $c_{L1}$ ,  $c_{A1}$ , observed number of moles of H<sub>2</sub>, lactate and acetate per culture volume at 120 h.  $m_{G1}$ ,  $X_1$ , observed  $m_G$  and  $X$  at 120 h;  $\Delta(m_G X)_0$ , observed increase in  $m_G X$  during first 24 h;  $\Delta(-m_G X)_1$ , observed decrease in  $m_G X$  from 24 h to 120 h; H<sub>2</sub> production;  $y_{H2,G}=y_{H2,1}/\Delta(m_G X)_1$ ; Subscripts 0, initial; 1, 120h; f, attainable state; SHT, shift in redox state; S, fructose; G, endogenous glucose; L, lactate; A, acetate; w/o, without fructose. Reference run is run without monosaccharide and with GT strain from late-logarithmic growth phase shown in Table 4.7.  $(q_{H2,0})_{ref}$ , 0.0147  $\mu$ mol mg<sup>-1</sup>h<sup>-1</sup>;  $(y_{H2,f})_{ref}$ , 2.28  $\mu$ mol mL<sup>-1</sup>;  $k_{ref}$ , 0.0167h<sup>-1</sup>;  $K_{d,ref}$ , 0.00105h<sup>-1</sup>.

	Strain					
	GT strain		$\Delta$ adh mutant		$\Delta$ adh $\Delta$ adh mutant	
$c_{S0}$ [ $\mu$ mol mL <sup>-1</sup> ]	0	50	0	50	0	50
$X_0$ [mg mL <sup>-1</sup> ]	2.1	1.9	2.2	2.2	2.0	2.0
$r_{H2,0}$ [ $\mu$ mol mL <sup>-1</sup> h <sup>-1</sup> ]	0.0252	0.0911	0.0150	0.104	0.0200	0.129
$q_{H2,0}$ [ $\mu$ mol mg <sup>-1</sup> h <sup>-1</sup> ]	0.0119	0.0479	0.00682	0.0495	0.010	0.0645
$t_{SHT}$ [h]	nd	nd	nd	86.8	nd	68.2
$r_{H2,SHT}$ [ $\mu$ mol mL <sup>-1</sup> h <sup>-1</sup> ]	nd	1.64	5.17	nd	nd	nd
$q_{H2,SHT}$ [ $\mu$ mol mg <sup>-1</sup> h <sup>-1</sup> ]	nd	nd	nd	6.02	nd	4.91
$q_{H2,0}^*$ [-]	0.810	3.26	0.464	3.37	0.680	4.39
$y_{H2,f}$ [ $\mu$ mol mL <sup>-1</sup> ]	1.67	no less than 10.8	1.30	no less than 16.2	1.68	no less than 18.7
$y_{H2,f}^*$ [-]	0.732	no less than 4.74	0.570	no less than 7.11	0.737	no less than 8.20
$k$ [h <sup>-1</sup> ]	0.0151	nd	0.0115	nd	0.0119	nd
$k^*$ [-]	0.904	nd	0.782	nd	0.810	nd
$q_{S0}$ [ $\mu$ mol mg <sup>-1</sup> h <sup>-1</sup> ]	0	0.0853	0	0	0	0
$K_d$ [h <sup>-1</sup> ]	0(0-48h); 0.00103(48-120h)	0	0.000741	0	0.00106	0
$\mu$ [h <sup>-1</sup> ]	0	0.00149	0	0(0-72h);	0	0(0-72h);

				0.000978(76- 120h)		0.00255 (72- 120h)
$y_{H_2,i}[\mu\text{mol}\cdot\text{mL}^{-1}]$	1.66	10.8	1.00	16.2	1.42	18.7
$m_{G1}[\mu\text{mol}\cdot\text{mg}^{-1}]$	0.0298	0.0695	0.0439	0.0736	0.0411	0.0753
$X_1[\text{mg}\cdot\text{mL}^{-1}]$	1.91	2.41	1.96	2.50	1.96	2.26
$(m_G X)_i[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.0569	0.167	0.0860	0.184	0.0806	0.170
$c_{L,i}[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.204	9.02	0.016	0.019	0.017	0.015
$c_{A,i}[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.0495	0.223	0.0623	0.203	0.0	0.152
$\Delta(-c_S)_i[\mu\text{mol}\cdot\text{mL}^{-1}]$	0	5.2	0	8.2	0	8.5
$\Delta(m_G X)_i[\mu\text{mol}\cdot\text{mL}^{-1}]$	0.16	0.15	0.11	0.09	0.15	0.18
$Y_{H_2/G}[-]$	6.63	57.5	5.82	151	6.2	86.6

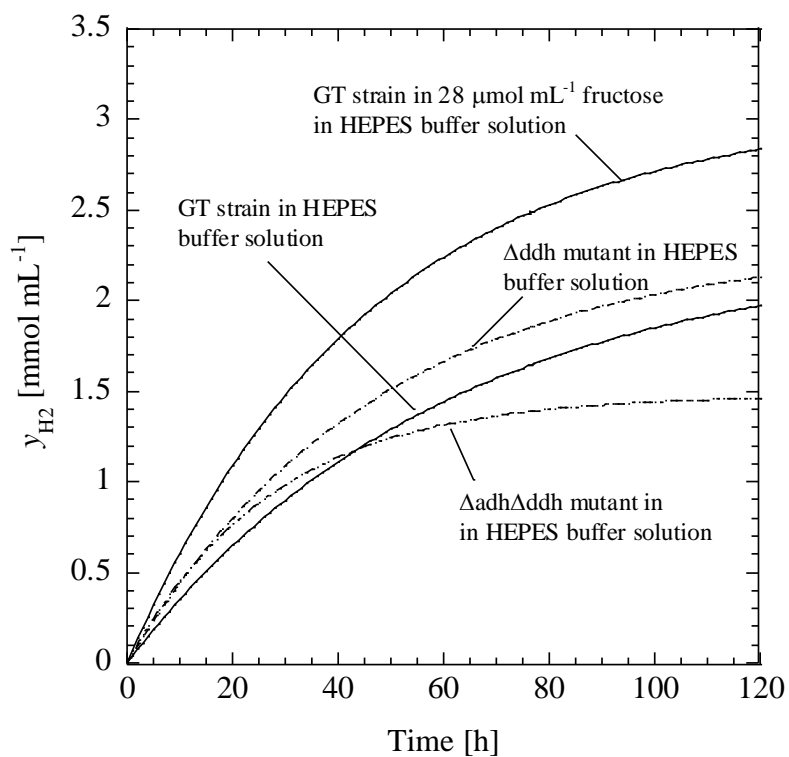


Figure 5.1 Simulation of dark anaerobic  $\text{H}_2$  production in GT strain,  $\Delta\text{adh}$  mutant and  $\Delta\text{adh}\Delta\text{adh}$  mutant in HEPES buffer solution and GT strain in HEPES buffer solution with  $28 \mu\text{mol mL}^{-1}$  fructose. Inoculum cells are from late-logarithmic growth phase when  $X$  is  $2.58 \text{ mg mL}^{-1}$ . The model parameters  $r_{\text{H}_2,0}$  and  $k$  of runs without fructose and with fructose are obtained from Table 4.7 and Table 2.3, respectively.

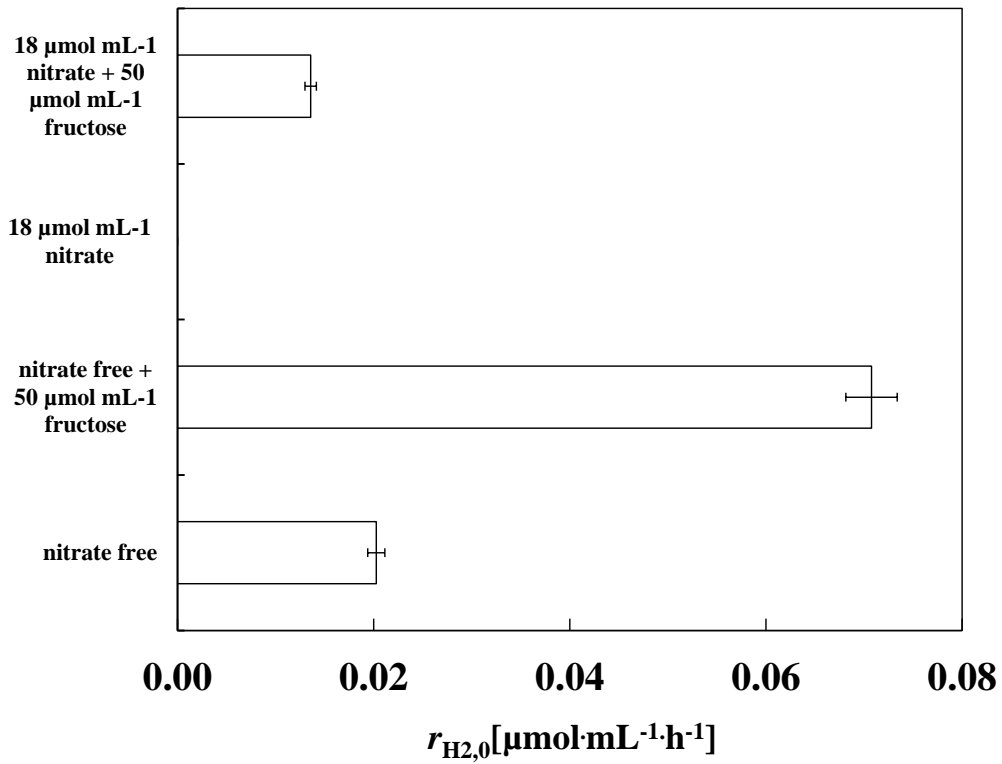


Figure 5.2 Effect of nitrate and fructose on the initial  $\text{H}_2$  production rate in GT strain. The  $\text{H}_2$  production was evaluated by the measurements of the amount of  $\text{H}_2$  per culture volume after 48 h incubation of cells in HEPES buffer solutions (a) without nitrate or fructose, (b) without nitrate and with  $50 \mu\text{mol}\cdot\text{mL}^{-1}$  fructose, (c) with  $18 \mu\text{mol}\cdot\text{mL}^{-1}$   $\text{NaNO}_3$  and without fructose, and (d) with  $50 \mu\text{mol}\cdot\text{mL}^{-1}$  fructose and  $18 \mu\text{mol}\cdot\text{mL}^{-1}$   $\text{NaNO}_3$ . All experiments were carried out in duplicate.

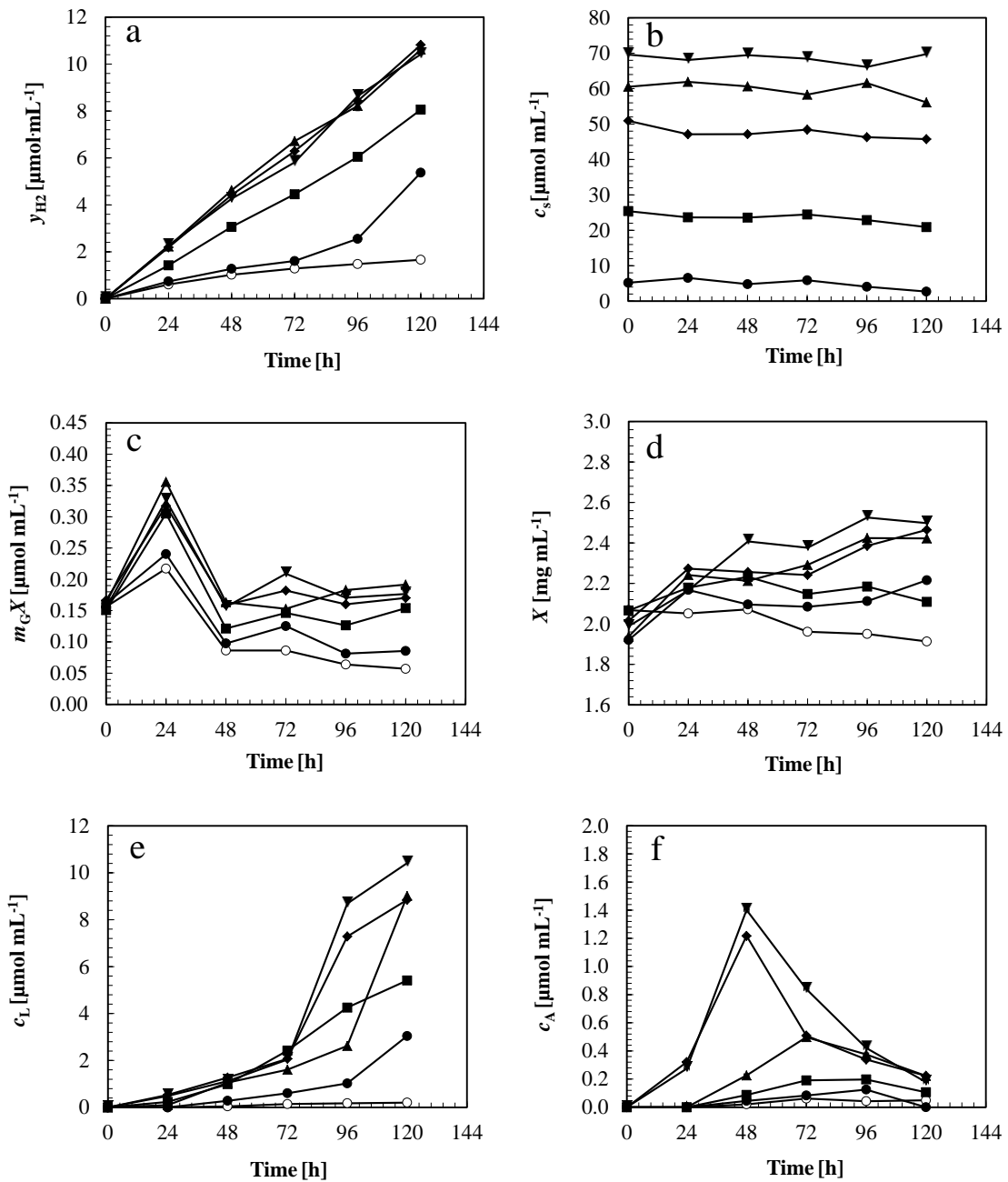


Figure 5.3 Time courses of (a) the number of moles of  $H_2$  per culture volume, (b) the concentration of fructose, (c) the number of moles of endogenous glucose per culture volume, (d) the concentration of dry cell weight ( $X$ ), (e) the concentrations of lactate and (f) the concentration of acetate of the dark anaerobic incubation of cells of GT strain in nitrate-free HEPES buffer solutions with 0 ( $\circ$ ), 5 ( $\bullet$ ), 25 ( $\blacksquare$ ), 50 ( $\blacklozenge$ ), 60 ( $\blacktriangle$ ) or 70 ( $\blacktriangledown$ )  $\mu\text{mol}\cdot\text{mL}^{-1}$  fructose.

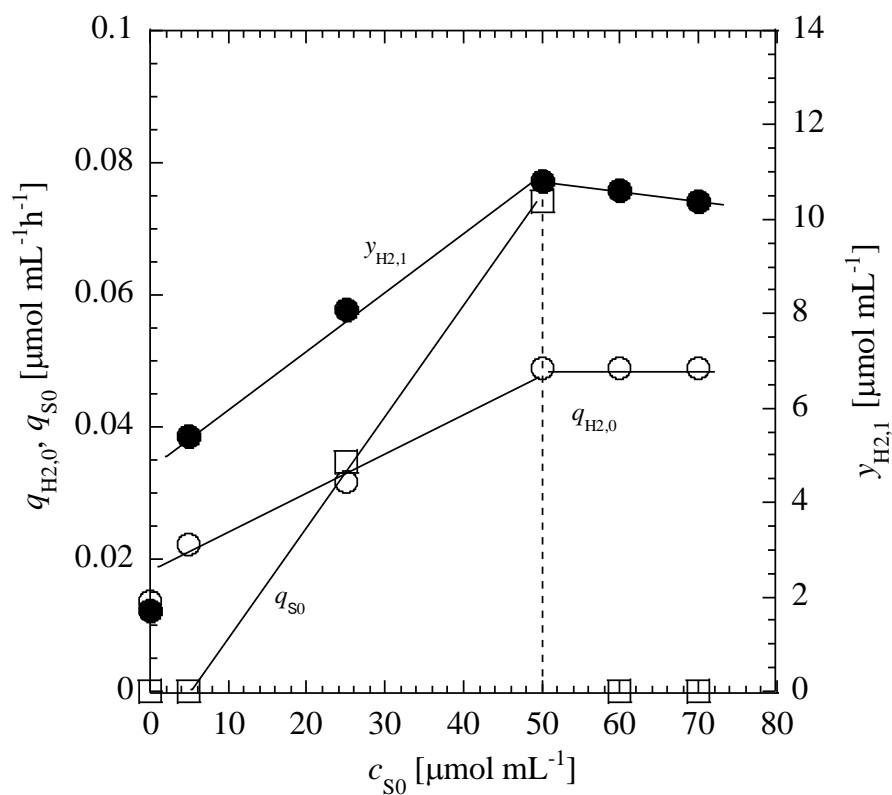


Figure 5.4 Effect of initial fructose concentration ( $c_{S0}$ ) upon initial specific  $H_2$  production rate ( $q_{H2,0}$ ) initial specific fructose uptake rate ( $q_{S0}$ ) and the number of moles of  $H_2$  per culture volume at 120 h ( $y_{H2,1}$ )

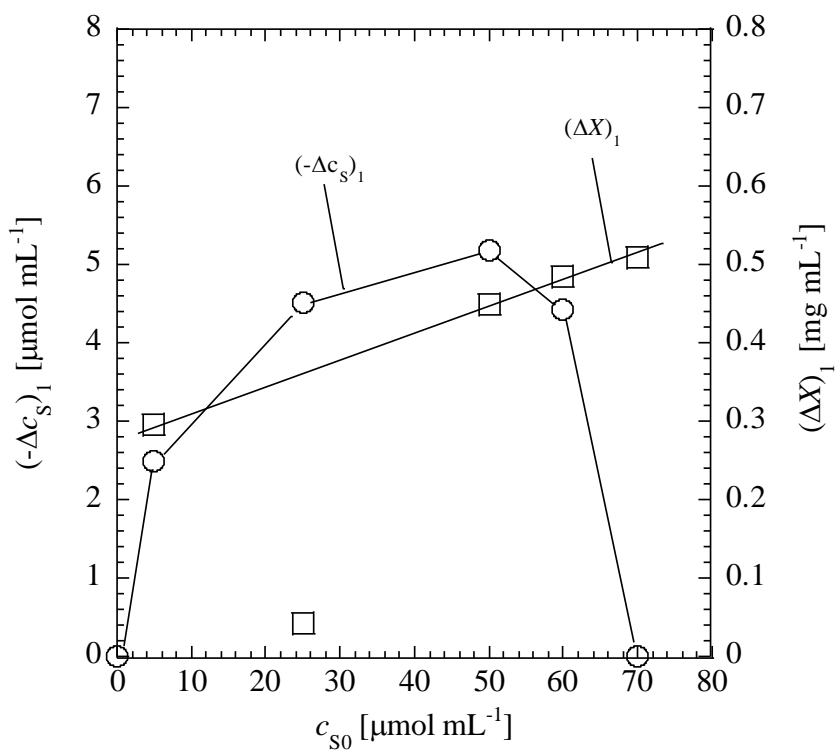


Figure 5.5 Effect of initial fructose concentration ( $c_{s0}$ ) upon total consumption of fructose  $(-\Delta c_s)_1$  and total reproduction of dry cell weight  $(\Delta X)_1$  from 0 to 120h.  $(-\Delta c_s)_1 = c_{s0} - c_{s1}$ ;  $(\Delta X)_1 = X_1 - X_0$

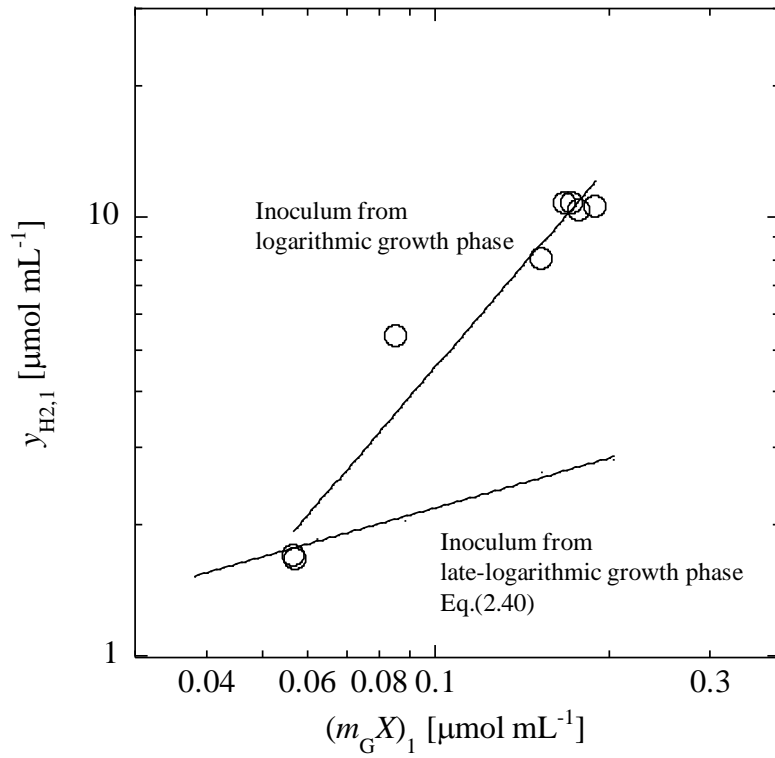


Figure 5.6 Relation between  $(m_G X)_1$  and  $y_{H1}$  in runs with GT strain in HEPES buffer solution with or without fructose

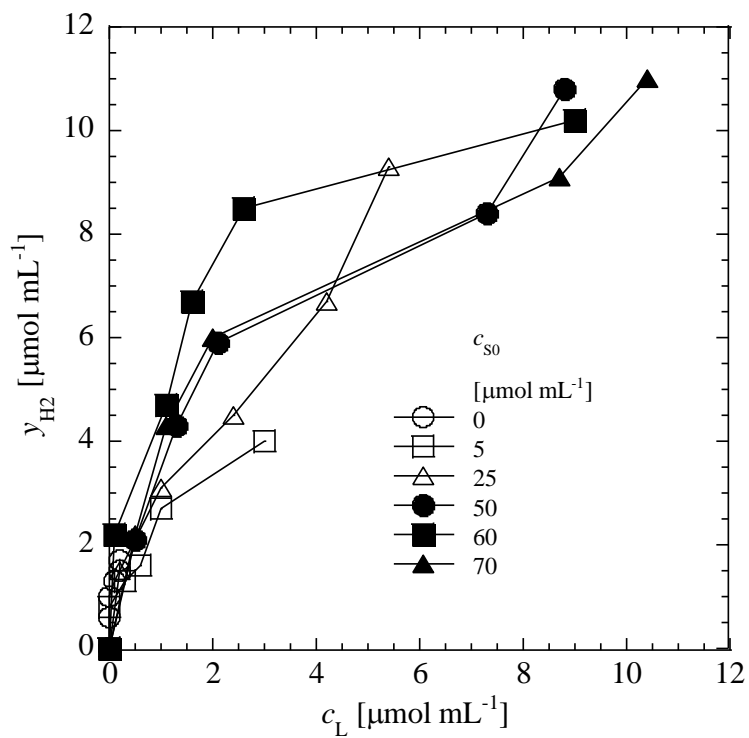


Figure 5.7 Relation between  $c_L$  and  $y_{\text{H}_2}$  in runs with GT strain in HEPES buffer solution with or without fructose

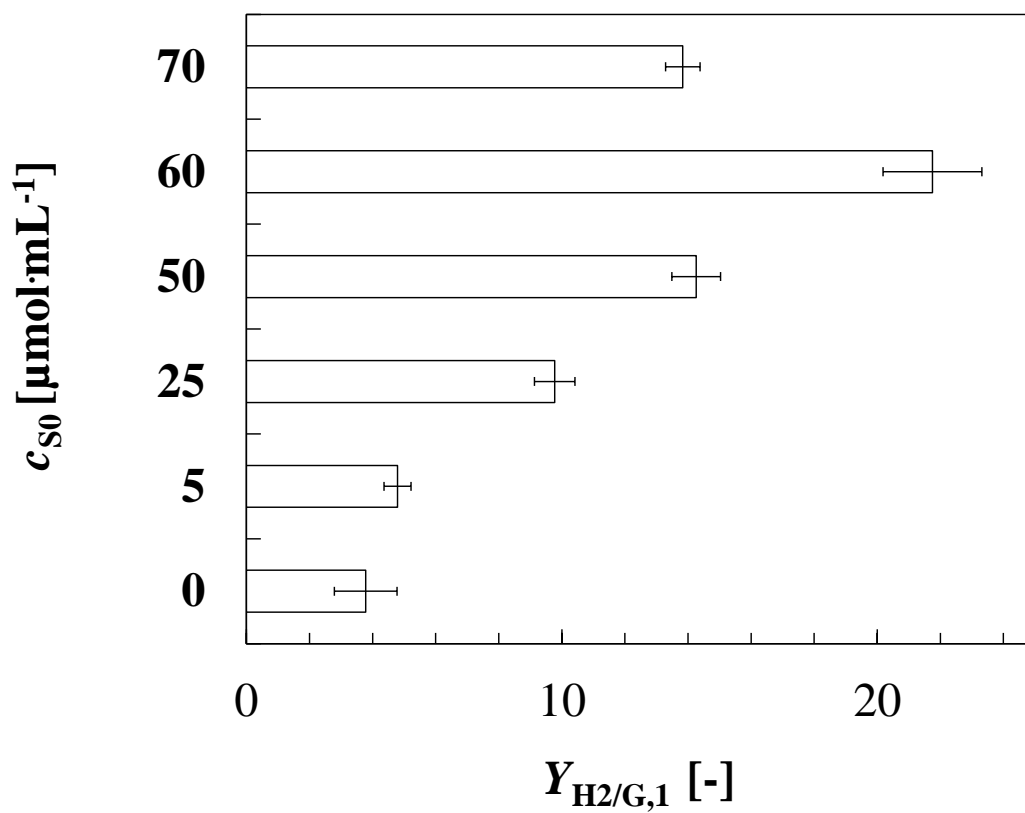


Figure 5.8 Effect of initial fructose concentration upon yield of  $\text{H}_2$  on endogenous glucose for glycolysis.  $Y_{\text{H}_2/\text{G}} = (y_{\text{H}_2,1} - y_{\text{H}_2}(24)) / (m_{\text{G}}X)_{24} - (m_{\text{G}}X)_1$  in the period between 24 h and 120 h

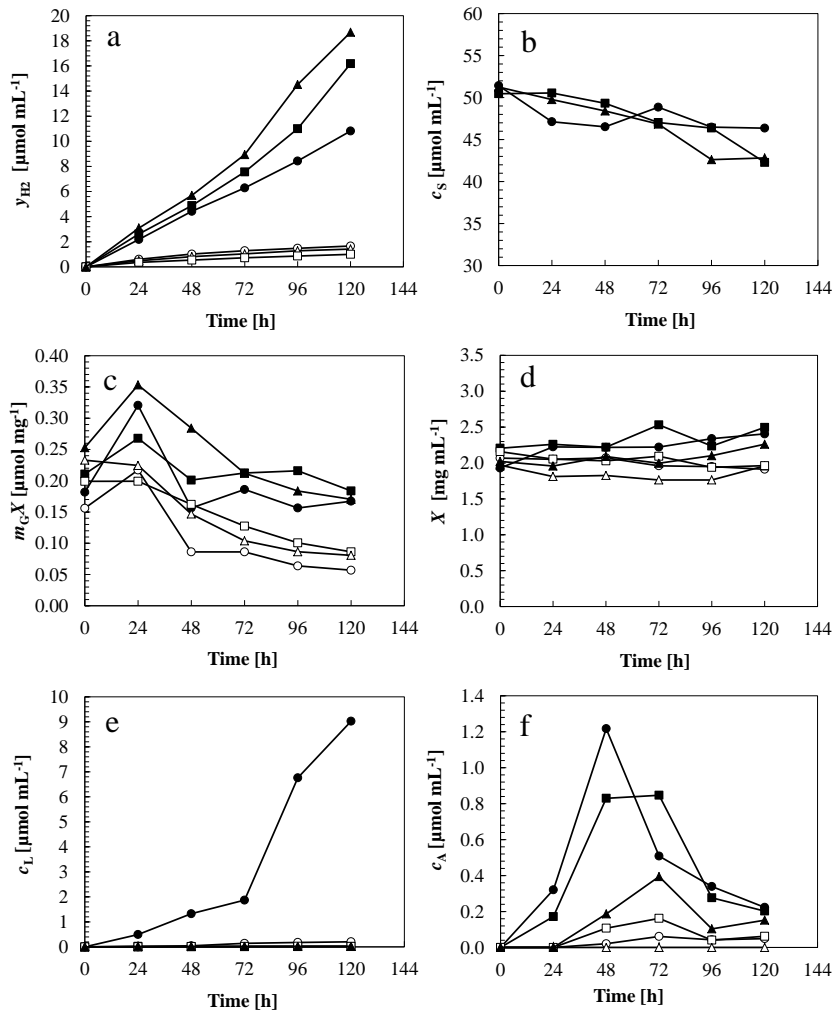


Figure 5.9 Time courses of culture variables for dark anaerobic hydrogen production in cells of GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant without or with  $50 \mu\text{mol mL}^{-1}$  fructose in HEPES buffer solution. GT strain ( $\circ$ ,  $\bullet$ );  $\Delta ddh$  mutant ( $\square$ ,  $\blacksquare$ ); and  $\Delta adh\Delta ddh$  mutant ( $\triangle$ ,  $\blacktriangle$ ). Open keys, runs without fructose; solid keys, runs with fructose. Quantities measured include (a) the moles of hydrogen per culture volume ( $y_{\text{H}_2}$ ), (b) the concentration of exogenous fructose ( $c_s$ ), (c) the moles of endogenous glucose in glycogen per culture volume ( $m_{\text{GX}}$ ), (d) the concentration of dry cell weight ( $X$ ), (e) the concentration of lactate ( $c_L$ ), (f) the concentration of acetate ( $c_A$ ) and (g) the concentration of ethanol ( $c_E$ ). Experiments were carried out in duplicate

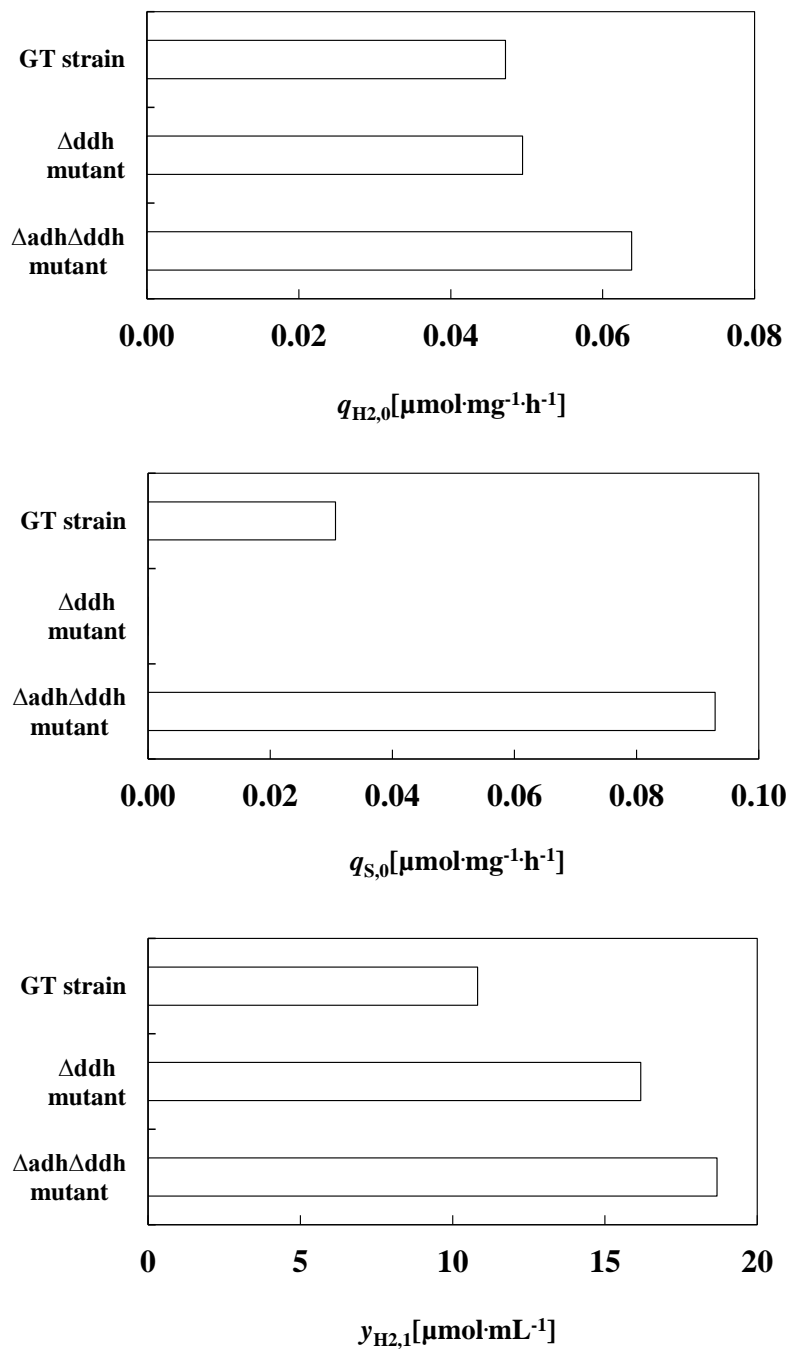


Figure 5.10 Effect of genetic modification upon initial specific H<sub>2</sub> production rate ( $q_{H_2,0}$ ) initial specific fructose uptake rate ( $q_{S,0}$ ) and the number of moles of H<sub>2</sub> per culture volume at 120 h ( $y_{H_2,1}$ )

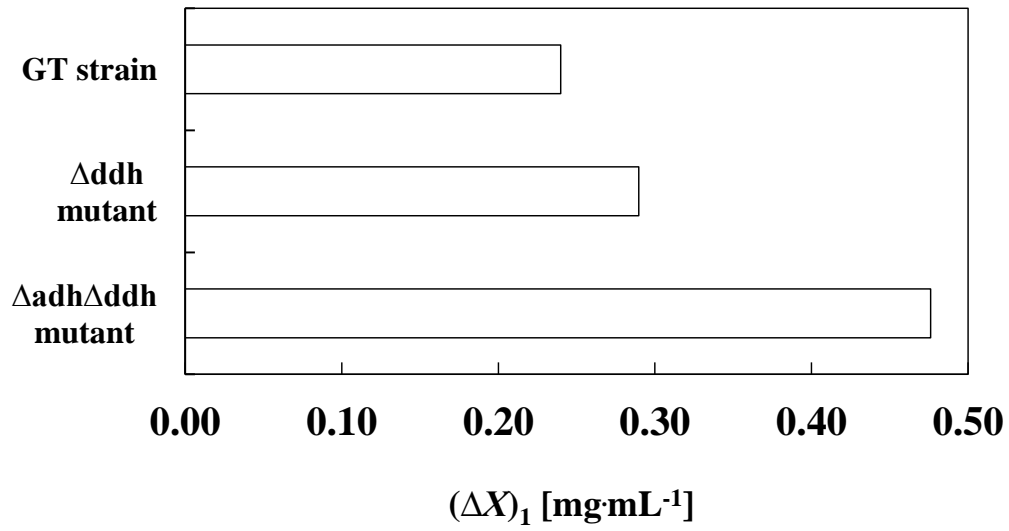
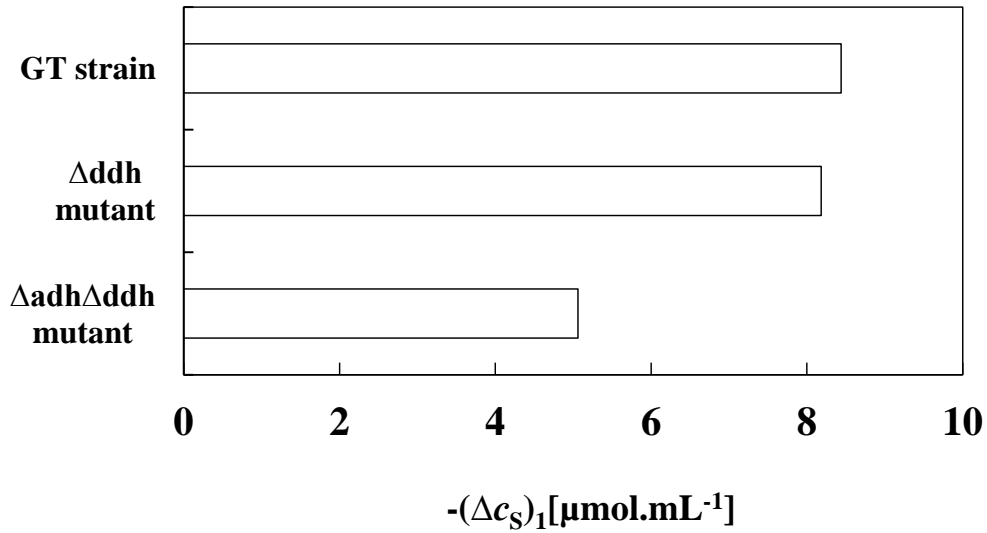


Figure 5.11 Effect of genetic modification upon total consumption of fructose  $-(\Delta c_S)_1$  and total reproduction of dry cell weight  $(\Delta X)_1$  from 0h to 120h.  $-(\Delta c_S)_1 = c_{S0} - c_{S1}$ ;  $(\Delta X)_1 = X_1 - X_0$

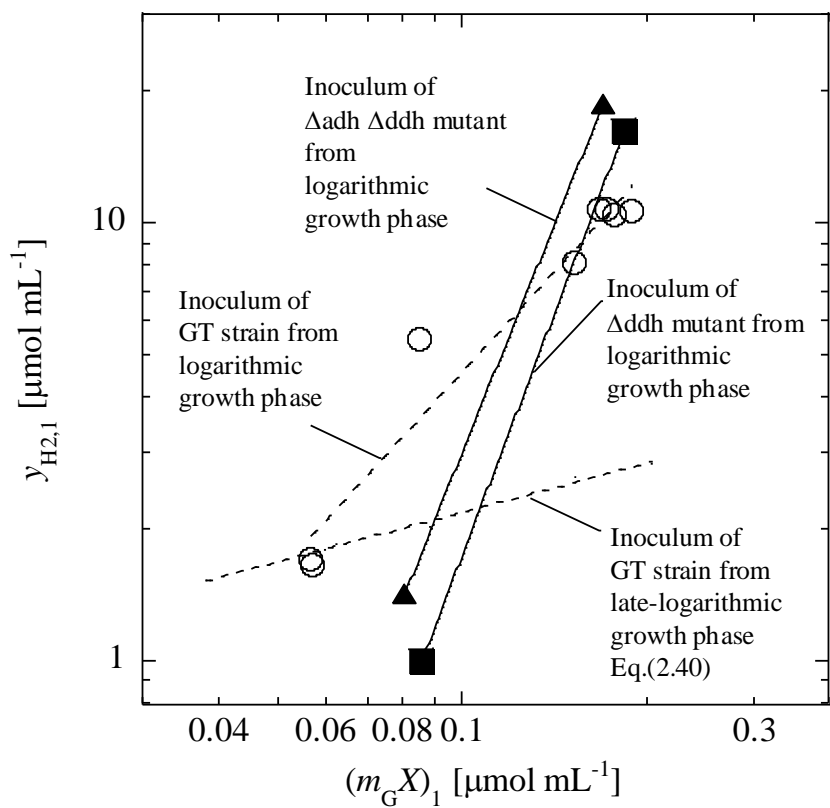


Figure 5.12 Relation between  $(m_G X)_1$  and  $y_{H1}$  in runs with GT strain in HEPES buffer solution with or without fructose

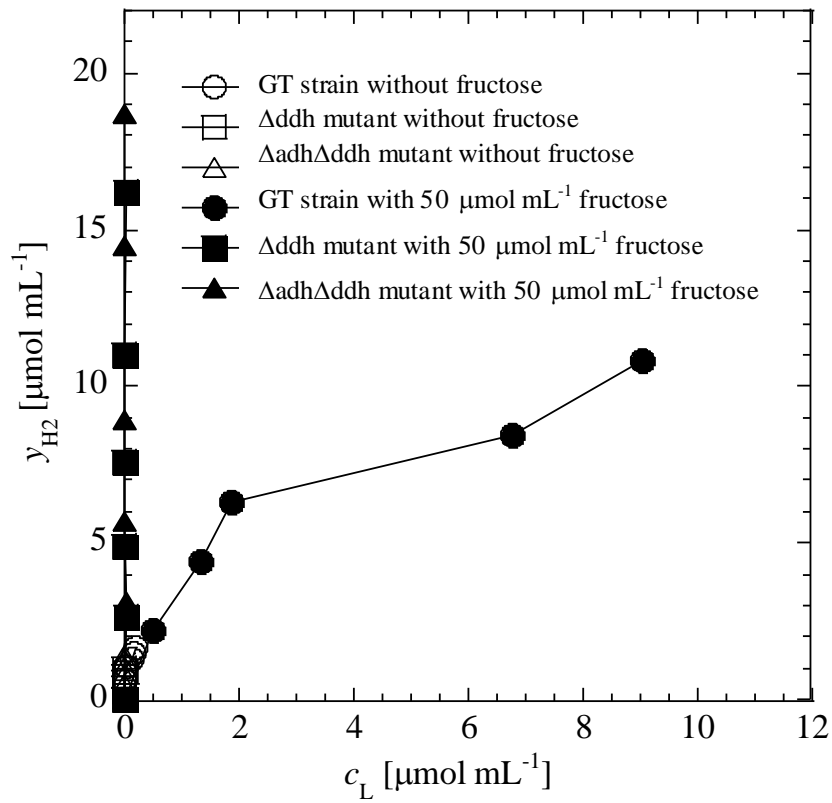


Figure 5.13 Relation between  $c_L$  and  $y_{\text{H}_2}$  in runs with GT strain,  $\Delta ddh$  mutant and  $\Delta adh\Delta ddh$  mutant in HEPES buffer solution with or without fructose

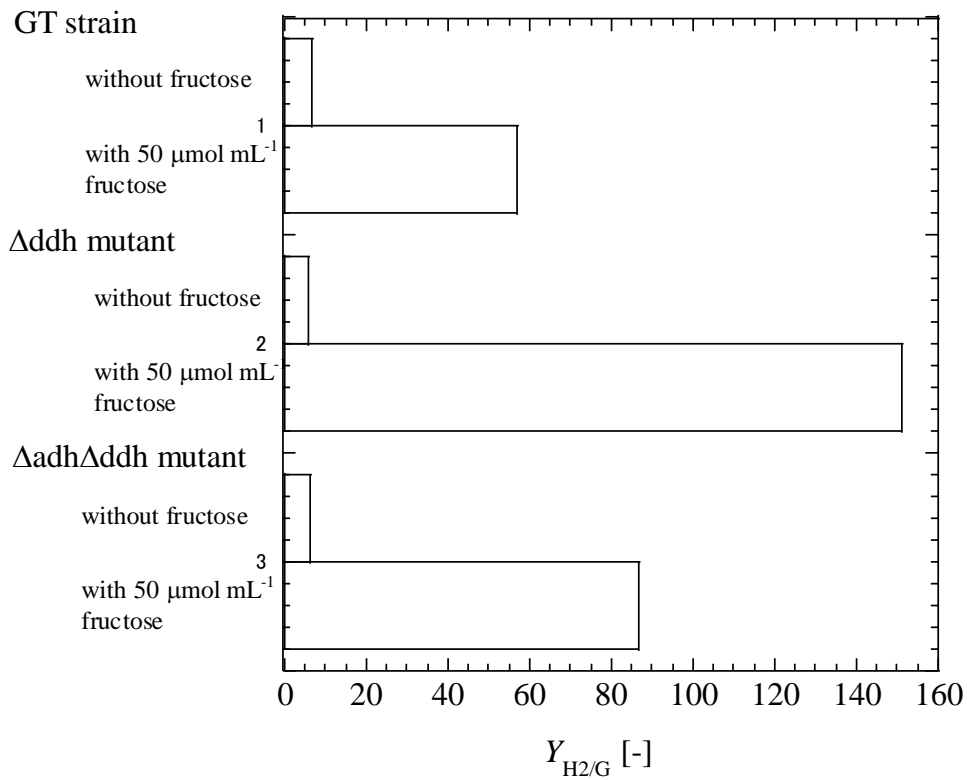


Figure 5.14 Effect of genetic modification upon yield of  $H_2$  on endogenous glucose for glycolysis.  $Y_{H_2/G} = (y_{H_2,1} - y_{H_2}(24)) / (m_G X)_{24} - (m_G X)_1$  in the period between 24 h and 120 h

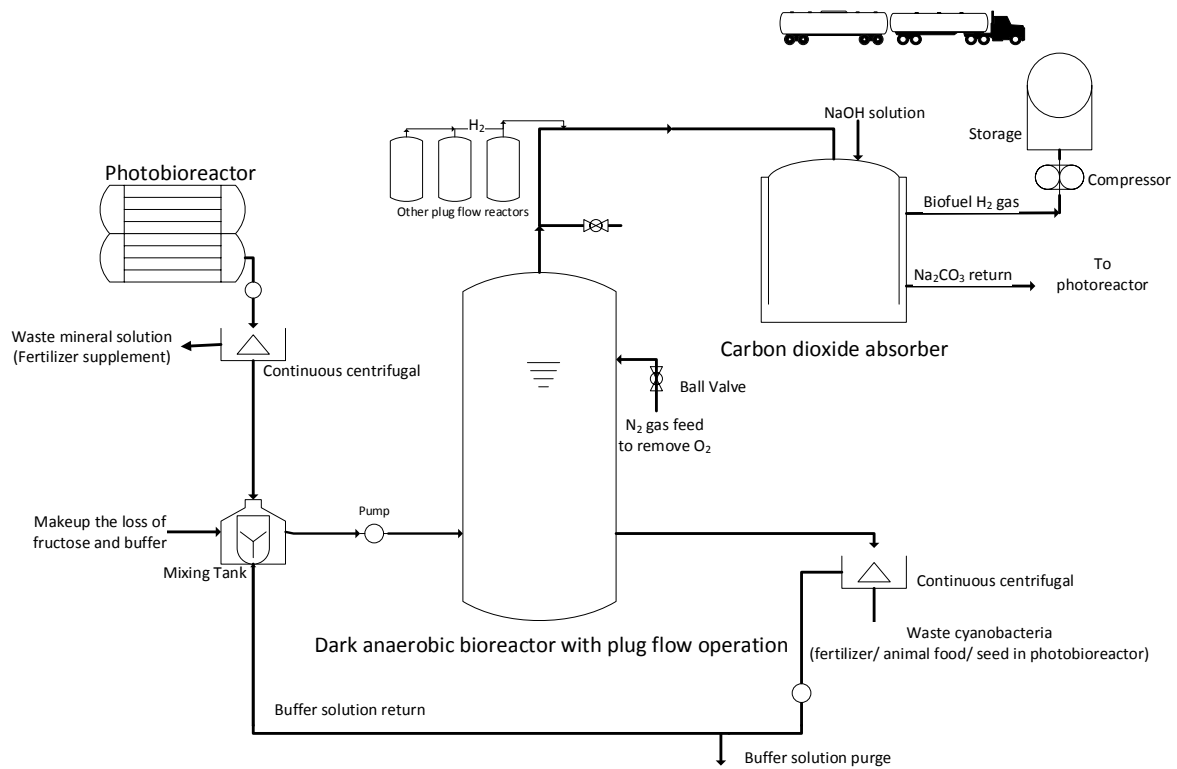


Figure 5.15 Conceptual design of dark anaerobic bioreactor to produce biofuel  $H_2$

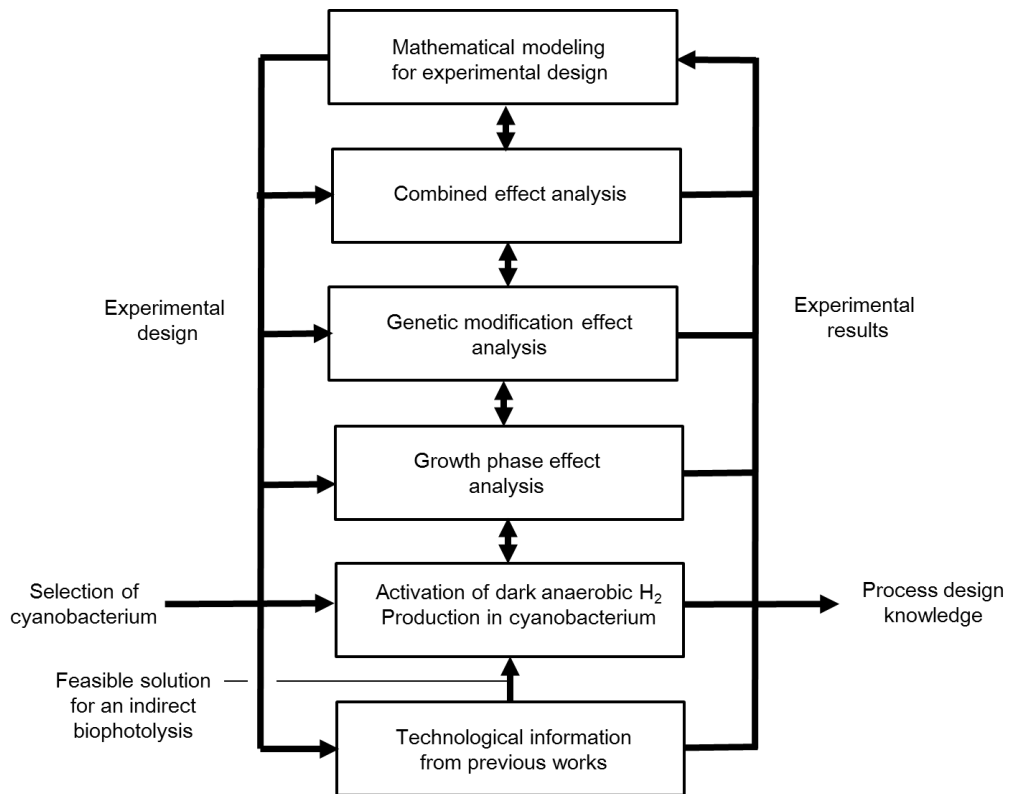


Figure 5.16 Proposed methods to elevate dark anaerobic H<sub>2</sub> production utilizing unicellular cyanobacterium

## Chapter 5: References

- Antal TK, Oliveira P, Lindblad P (2006) The bidirectional hydrogenase in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *International Journal of Hydrogen Energy* 31 (11):1439-1444
- Baebprasert W, Jantaro S, Khetkorn W, Lindblad P, Incharoensakdi A (2011) Increased H<sub>2</sub> production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. *Metabolic Engineering* 13 (5):610-616
- Beauclerk A, Smith A (1978) Transport of D-glucose and 3-O-methyl-D-glucose in the cyanobacteria *Aphanocapsa* 6714 and *Nostoc* strain Mac. *European Journal of Biochemistry* 82:187-197
- Burrows EH, Chaplen FWR, Ely RL (2011) Effects of selected electron transport chain inhibitors on 24-h hydrogen production by *Synechocystis* sp. PCC 6803. *Bioresource Technology* 102 (3):3062-3070
- Cho Y, Yoo S (2011) Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *PLOS Genetics* 7 (1):1-10
- 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.
- Flores E, Schmetterer G (1986) Interaction of fructose with the glucose permease of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Journal of Bacteriology* 166 (2): 693-696
- Haury J, Spiller H (1981) Fructose uptake and influence on growth of the nitrogen fixation of *Anabaena variabilis*. *Journal of Bacteriology* 147:227-235
- Joset F, Buchou T, Zhang CC, Jeanjean R (1988) Physiological and genetic analysis of the glucose-fructose permeation system in two *Synechocystis* species. *Archives of Microbiology* 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 Research* 3(3):109-136
- Ohtaguchi K OA, Takahashi N, Ogawa M and Koide K (1995) Kinetics of hydrogen production by the photolithotroph *Synechococcus leopoliensis*, . *IONICS* 21:69-72
- Rozen A, Arad H, Schonfeld M, Tel-Or E (1986) Fructose supports glycogen accumulation, heterocysts differentiation, N<sub>2</sub> fixation and growth of the isolated cyanobiont *Anabaena azollae*. . *Archives of Microbiol* 145:187-190
- Yamamoto T, Asami K, Ohtaguchi K (2012) Anaerobic production of hydrogen in the dark by *Synechocystis* sp. strain PCC 6803: effect of photosynthesis media for cell preparation. *Journal of Biochemical Technology* 3 (4):344-348

## Chapter 6

### Concluding Remarks

#### 6.1 Conclusions

This thesis has described the methods to enhance a dark anaerobic H<sub>2</sub> production in unicellular cyanobacteria using a glucose tolerant mutant of *Synechocystis* sp. strain PCC 6803 (GT strain) as a model organism. Indirect biophotolysis was performed. In a reference run, cells of GT strain was photoautotrophically grown in the first stage, and then cells were dark anaerobically incubated in HEPES buffer solution for molecular hydrogen (H<sub>2</sub>) production. At the end of enhancement method purposed in this thesis, no less than 8.58-fold increase in H<sub>2</sub> production was accomplished over the reference run that had been optimized in previous works, This has increased the possibility of biological H<sub>2</sub> production process using cyanobacteria to take place. Experimental approach of this study was supported by a mathematical modeling for dark anaerobic H<sub>2</sub> production.

Summarizations of this thesis are shown below.

- (1) Principal parameters to develop the maximum dark anaerobic production of H<sub>2</sub> with defined cyanobacteria are i) the selection of monosaccharide additives to increase initial amount of reductive compounds; ii) the characterization of growth phase in photosynthesis; iii) the genetic modification by metabolic engineering approach; and iv) the integration of all three platforms for dark anaerobic H<sub>2</sub> production (Chapter 1).
- (2) The addition of exogenous reducing sugar to dark anaerobic HEPES buffer solution is useful to increase initial H<sub>2</sub> production activity of NiFe-hydrogenase in GT strain.
- (3) Glucose and fructose are characterized as monosaccharides that bring cells to elevate the attainable level of the number of moles of H<sub>2</sub> per culture volume at no less than 1.40 times that of reference run. In dark anaerobic condition, different from glucose, fructose elevates H<sub>2</sub> production without a notable amount of assimilation of fructose. (Chapter 2).
- (4) Mathematical model for the kinetics of dark anaerobic H<sub>2</sub> production is formulated based on the global redox balance within a cell. Relative cellular content of NAD(P)H are evaluated utilizing the model (Chapter 2, 3, 4, 5).

- (5) Relations between the attainable level of the number of moles of H<sub>2</sub> per culture volume and the initial H<sub>2</sub> production rate, and between the final level of the number of moles of H<sub>2</sub> per culture volume and the final level of the number of moles of endogenous glucose per culture volume are developed. The H<sub>2</sub> production of GT strain in dark anaerobic HEPES buffer solution is found to be elevated by limiting glycogen decomposition, cell breakage and hydrogenase deactivation under stresses (nitrate deprivation and dark anaerobic). The shift-up of the amount of stored glycogen is of utmost importance to elevate H<sub>2</sub> production (Chapter 2).
- (6) A large portion of electrons for H<sub>2</sub> production on NiFe-hydrogenase are supplied not only by glycolysis to generate acetate but also by other degradation reactions. Glycolysis is associated with such decomposition reactions, and useful as a marker of the progress of global decomposition reactions (Chapters 2, 3).
- (7) Reductive wing of incomplete TCA cycle is important in mixotrophic growth under dark anaerobic condition (Chapter 2).
- (8) Dark anaerobic H<sub>2</sub> production in NiFe-hydrogenase of GT strain HEPES buffer solution is strongly dependent on the growth phase of inoculum cells in the first stage of biophotolysis (Chapter 3).
- (9) Cell death during dark anaerobic incubation can be limited by the use of cells from logarithmic growth phase (Chapter 3).
- (10) Cells from stationary phase contain high amount of endogenous glucose and they are useful to achieve high amount of stored glycogen at the end of H<sub>2</sub> production. Different from expectation, H<sub>2</sub> production is not affected by glycogen content. Glycogen content effect is found to be a growth phase dependent phenomenon (Chapter 3).
- (11) Metabolic engineering approach to introduce a perturbation in redox homeostasis of GT strain is made. Mutants defective the genes coding for lactate dehydrogenase ( $\Delta$ adh mutant) and for both lactate dehydrogenase and alcohol dehydrogenase ( $\Delta$ adh $\Delta$ adh mutant) are constructed. The initial H<sub>2</sub> production of mutants are slightly higher than that of GT strain (Chapter 4).
- (12) Among GT strain,  $\Delta$ adh mutant and  $\Delta$ adh $\Delta$ adh mutant in HEPES buffer solution with or without glucose, GT strain in the presence of glucose is capable of producing the highest attainable level of the number of moles of hydrogen per culture volume due to heterotrophic growth by assimilating exogenous glucose and producing endogenous glucose, lactate and acetate (Chapter 4).

- (13) Glucose elevates H<sub>2</sub> production in GT strain and  $\Delta$ addh mutant, while lowers it in  $\Delta$ adh $\Delta$ addh mutant. Glucose is assimilated by GT strain. Assimilation of glucose is blocked by the elimination of the genes coding for lactate dehydrogenase (Chapter 4).
- (14) Under dark anaerobic incubation,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant are sensitive to the cellular exposure to glucose (Chapter 4).
- (15) Cells of GT strain,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant slowly consumed exogenous fructose for endogenous glucose accumulation and dry cell weight production. Affinity to fructose is extremely low compared to glucose consumption (Chapter 5).
- (16) Fructose is an important factor that activates cells to save or provide reductive compound for H<sub>2</sub> production on NiFe-hydrogenase. The best concentration of fructose for H<sub>2</sub> production is 50  $\mu$ mol mL<sup>-1</sup> (Chapter 5).
- (17) H<sub>2</sub> production in the presence of high concentration fructose is accelerated during dark anaerobic incubation (Chapter 5).
- (18) Mutants,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant are very sensitive to fructose to elevate H<sub>2</sub> production. Combined effect of fructose addition and genetic modification is observed (Chapter 5).
- (19) Dark anaerobic 120 h incubation of a mutant lacking both lactate dehydrogenase genes and alcohol dehydrogenase genes, from logarithmic growth phase, in HEPES buffer solution with fructose is found to increase the amount of H<sub>2</sub> per culture volume no less than 8.2-fold over that with cells from late-logarithmic growth phase and without fructose (Chapter 5) .
- (20) Conceptual design of the dark anaerobic production of H<sub>2</sub> is challenged. One unit of 5,000m<sup>3</sup> biogas production facility is estimated to be able to produce 100,000 Nm<sup>3</sup> H<sub>2</sub> per year that can support to fill up the tank of 918 FCVs. Baseline of the research for biofuel H<sub>2</sub> production is created (Chapter 5).
- (21) A method to activate dark anaerobic H<sub>2</sub> production in unicellular cyanobacterium is proposed (Chapter 5).

Selection of the fructose supplement for fermentation with *Synechocystis* sp. strain PCC 6803 cells is the most important factor to increase H<sub>2</sub> production. This study has proposed a better way of H<sub>2</sub> production by using genetically modified cyanobacterium cells and fructose as carbon source. H<sub>2</sub> production by logarithmical phase aged GT,  $\Delta$ addh and  $\Delta$ adh $\Delta$ addh strains on 50  $\mu$ mol mL<sup>-1</sup> fructose yields 10.8 mmoles, 16.2 mmoles and 18.7 mmoles H<sub>2</sub> per L after 120 h fermentation respectively.

## 6.2 Future Works

The present findings appear to contribute a technology for activating dark anaerobic H<sub>2</sub> production in cyanobacteria. For future works, the following experimental design is recommended.

### 1. Study on the effect of long run operation upon H<sub>2</sub> production

Dark anaerobic H<sub>2</sub> production in GT strain,  $\Delta$ addh mutant and  $\Delta$ adh $\Delta$ addh mutant in HEPES buffer solution with fructose of this study was monitored up to 120 h. After 68-2-86.8 h, acceleration of H<sub>2</sub> production was observed. At 120 h, high rate production of H<sub>2</sub> was not quitted. For future development of process, H<sub>2</sub> production after 120 h needs to be studied.

### 2. Exploitation of a mutant lacking both lactate dehydrogenase and alcohol dehydrogenase ( $\Delta$ adh $\Delta$ addh mutant) utilizing mutagenesis.

In this study,  $\Delta$ adh $\Delta$ addh mutant was developed using recombinant DNA technique. H<sub>2</sub> production in  $\Delta$ adh $\Delta$ addh mutant in fructose solution results in marked impression for industrialization. Since application of genetic modified organism is highly regulated, it is required to develop similar mutant using mutagenesis or UV radiation techniques.

### 3. Evaluation of H<sub>2</sub> production in PBS solution.

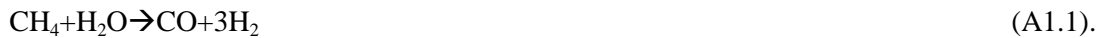
HEPES buffer solution was used in this study. However, considering cost performance of buffer solution, it is recommended to elucidate dark anaerobic H<sub>2</sub> production in PBS solution.

## Appendix-1

### Hydrogen production from non-renewable sources

Current industrial H<sub>2</sub> production applies this technology. Technology is well-established. Natural gas, oil and coal are resources; hence it represents a high risk of abundant CO<sub>2</sub> emission to the atmosphere. Additional problem is that the amount of fossil sources on the earth is limited.

**Steam methane reforming:** Steam reforming of natural gas at 973-1373 K with Ni based catalyst generates H<sub>2</sub> in the form of syngas:



This endothermic reaction outlet gas from the reformer contains 10% CO.

Exothermic water gas shift reaction at 633 K further generates H<sub>2</sub> by stripping the oxygen atom from the steam and oxidizing CO to CO<sub>2</sub>:



Outlet gas from the water gas shift reactor contains 2000 ppm CO.

Removal of CO, CO<sub>2</sub> and other impurities is performed after water gas shift reactor. Concentration of CO at the outlet of this process is no more than 100 ppm.

The efficiency of H<sub>2</sub> production is about 80%. Steam methane reforming is the least expensive method to generate industrial H<sub>2</sub>. Commercial bulk H<sub>2</sub> including H<sub>2</sub> for ammonia synthesis is produced by steam reforming. Steam reforming has drawbacks such as consuming fossil fuels stocks and generating CO<sub>2</sub> from carbonaceous compounds of fossil fuels.

**Partial oxidation of hydrocarbons:** Partial oxidation also generates H<sub>2</sub>-rich syngas from heavy hydrocarbons including diesel fuel and residual oil:



This method shares above drawbacks with steam reforming.

**Coal gasification:** Basic equation in coal gasification is given by:





and



Equation (A1.4) shows the thermal decomposition of coal. Equations (A1.5) and (A1.6) show a complete oxidation and partial oxidation of carbon, respectively. Equations (A1.7) and (A1.8) are key reactions. Coal is reacted with air or  $\text{O}_2$  plus steam in the first reactor. Generated gas is purified to remove sulfur containing components. Then shift reaction shown by Eq.(A1.10) is performed. After  $\text{CO}_2$  removal, pure  $\text{H}_2$  is obtained. Coal is abundant hence it has a possibility to replace natural gas and oil as a feedstock for  $\text{H}_2$  production.

## Appendix-2

### Hydrogen production from renewable sources

Biomass gasification and biomass pyrolysis utilizes biomass as a source. They produce CO<sub>2</sub>, however, when the organism of biomass was alive, it assimilated atmospheric CO<sub>2</sub>. Hence carbon neutral mechanism supports these technologies. Other technologies split the water to form H<sub>2</sub>. Electrolysis, thermolysis and biocatalyzed electrolysis require the energy other than solar energy. There is a potential to produce CO<sub>2</sub>. Biological conversion is supported by carbon neutral mechanism.

**Biomass gasification:** Biological material derived from living organisms or recently living organism is named biomass. Examples of biomass are plant scraps, switch grass, garbage and human waste materials. Gasification of biomass generates H<sub>2</sub> from organic compounds.

**Biomass pyrolysis:** High-temperature gasifying converts biomass into a gas. The H<sub>2</sub> rich vapor is condensed in pyrolysis oil that is used to steam reform to generate H<sub>2</sub>. The yield is 12-17% H<sub>2</sub> by dry weight of biomass.

**Electrolysis:** A direct electric current (DC) is utilized to split water to H<sub>2</sub> and O<sub>2</sub>:



At the cathode (negatively charged terminal), the electricity enters the water. The electricity passes through the water and reaches to the anode (positively charged terminal). High grade pure H<sub>2</sub> for use in the electronics, pharmaceutical and food industries is collected at the cathode. The supply of power is important to generate H<sub>2</sub>. If sustainable energy is available for the electrolysis, then this technology can produce H<sub>2</sub> as a renewable energy.

**Biocatalyzed electrolysis:** It is an electrolytic process that electrically connects the oxidation of organic compound at a biological anode to the reduction of H<sup>+</sup> at the cathode to evolve H<sub>2</sub> (Rozendal et al. 2006).

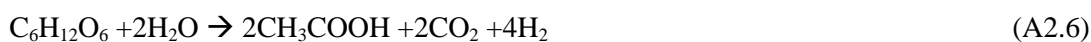
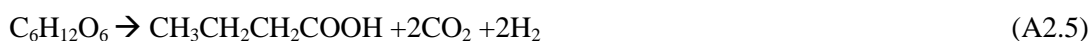
If acetate is utilized half reactions and net reaction are given by



**Thermolysis:** H<sub>2</sub> and O<sub>2</sub> are produced from water at no less than 2773 K. However, it is not easy to block back reaction.

**Photocatalytic water splitting:** Artificial photosynthesis utilizing catalyst has a potential to generate H<sub>2</sub> according to Eq. (A2.1). Highest water splitting rate of 9.7 mmol h<sup>-1</sup> is observed with UV-based photocatalyst NaTaO<sub>3</sub>:La. The catalyst K<sub>3</sub>Ta<sub>3</sub>B<sub>2</sub>O<sub>12</sub> shows a water splitting rate of 1.21 mmol h<sup>-1</sup>. The highest quantum yield of 5.9% in visible light is achieved by (Ga<sub>0.82</sub>Zn<sub>0.18</sub>)(N<sub>0.82</sub>O<sub>0.18</sub>). The study on photocatalytic water splitting is ongoing (Maeda and Domen, 2010). Although this photocatalytic water splitting has high potential for future H<sub>2</sub> energy, the material used in the system is extreme expensive. Bulk gallium nitride (GaN) is very expensive today, costing about \$1,900 or more for a two-inch substrate.

**Dark fermentation:** Dark fermentation does not require light. The theoretical maximum yields are 2-4 moles H<sub>2</sub> per mole of glucose consumption, depending on butyrate or acetate produced as final fermentative products. Chemical equations representing the stoichiometry for the conversion of glucose to lactate and that to acetate are shown by



The actual yield of H<sub>2</sub> from glucose in *Escherichia coli* is very low because H<sub>2</sub> production is mediated only in the formate formation reaction by formate hydrogen lyase (FHL) complex that is composed of hydrogenase 3 and formate dehydrogenase-H (FdhF) (Axley et al., 1990). *Escherichia coli* is capable of converting only electrons available at the pyruvate node to H<sub>2</sub>, and is unable to use the electrons generated during glycolysis. **Figure A2.1** shows the genetically modified *E.coli* for H<sub>2</sub> production through heterologous expression of NiFe-hydrogenase (the soluble NAD-dependent hydrogenase of *Ralstonia eutropha* (SH hydrogenase)) (Ghosh et al., 2013). This work reported the success of 2 moles H<sub>2</sub> production per mole of glucose consumed, close to the theoretical maximum. Final H<sub>2</sub> levels that were achieved for 120 h were between 1.0 and 1.8 μmol mL<sup>-1</sup>.

H<sub>2</sub> production by a soluble FeFe-hydrogenase in *Clostridium* species has also been studied (Hallebeck, 2008). Different from the metabolism shown in Fig.A2.1, pyruvate is further converted to acetyl-CoA, CO<sub>2</sub> and reduced ferredoxin by pyruvate ferredoxin oxidoreductase (PFOR). Electrons are directly transferred from reduced ferredoxin to FeFe-hydrogenase. If H<sub>2</sub> partial pressure is low, the NADH from glycolysis is reoxidized possibly by a NADH-dependent FeFe-hydrogenase.

The constraint of those dark fermentations is that they require carbon sources for growth and cell activity, whereas, cyanobacteria requires only small nutrition, solar light (energy), water (electron), mineral salts and CO<sub>2</sub> in the air for their growth in photosynthetic. The power of solar light reaching to the earth surface is so vast and infinite. The absence of light energy utilization for molecular energy rather than high carbon substrate consumption puts this system into a high-cost process.

**Direct biophotolysis:** Direct biophotolysis is a fermentative conversion of organic compounds to hydrogen in the presence of light. It requires absorption of sunlight by the biochemical of interest. Gaffron discovered photobiological H<sub>2</sub> evolution in unicellular green algae *Scenedesmus* after being kept in dark anaerobic condition (Gaffron 1939). Green algae utilize photosynthetically generated electrons for the reduction of H<sup>+</sup> to H<sub>2</sub>. Illumination enhanced the H<sub>2</sub> generation. The enzyme responsible for this H<sub>2</sub> evolution is a bidirectional hydrogenase. Photobiological H<sub>2</sub> production is the biological splitting of the water into H<sub>2</sub> and O<sub>2</sub>.



Direct biophotolysis is also studied by other researchers (Mathews and Wang 2009; Oh et al. 2011). In microalgae, Fd (ferredoxin) acts as an electron donor to FeFe-hydrogenase (about 48 kDa) and reversibly facilitates the formation of H<sub>2</sub> from H<sup>+</sup>.



Sulfur deprivation in algae cultivation onsets H<sub>2</sub> production from O<sub>2</sub> production. Hydrogen production rate is 0.446-0.536 μmol mL<sup>-1</sup> h<sup>-1</sup>(=10-12 mL L<sup>-1</sup> h<sup>-1</sup>). Photobiological H<sub>2</sub> production is still in research stage. Sunlight conversion efficiency is reported to be 24%.

Purple non-sulfur bacteria (PNSB) such as *Rhodobacter*, *Rhodospseudomonas* produce H<sub>2</sub> in photo-fermentation (nitrogen deficiency condition) by nitrogenase utilizing organic or inorganic acids as substrates. The reaction is illustrated by



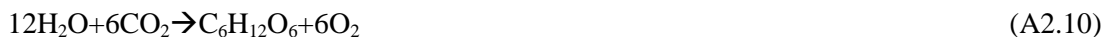
Some researchers are working on cheap compound using organic waste as substrates.

Besides algae and PNSB, cyanobacteria are well known to perform direct biophotolysis to generate H<sub>2</sub> from solar light energy.

**Figure A2.2** shows a direct biophotolysis of unicellular cyanobacterium (Yu and Takahashi 2007). A direct biophotolysis takes place through action of the complete photosynthetic apparatus in the water. Water is spitted at PSII and the ferredoxin-reducing photosystem PSI. Even though, solar energy conversion to H<sub>2</sub> production is high, the H<sub>2</sub> production is inhibited by O<sub>2</sub>. Due to O<sub>2</sub> generation during

photosynthesis suppress hydrogenase from H<sub>2</sub> production. A reversible hydrogenase accepts electron from reduced ferredoxin to generate H<sub>2</sub> by the general reaction.

**Indirect biophotolysis:** Indirect biophotolysis occurs when a species other than the biochemical of interest absorbs sunlight and initiates a series of bioreaction that result in the transformation of the biochemical. The inhibition of O<sub>2</sub> on FeFe-hydrogenase and NiFe-hydrogenase is eliminated utilizing two stage incubation method. In the first stage, algae and cyanobacteria are photoautotrophically grown under the light to evolve O<sub>2</sub>. Carbohydrates are accumulated within cell. In the second stage, cells are exposed to dark anaerobic condition. Dark fermentation utilizes intracellularly accumulated carbohydrates. The combination of the first and second stages are shown by:



Gaffron and Rubin observed the dark evolution of H<sub>2</sub> in *Scenedesmus* (Gaffron and Rubin 1942). They demonstrated that after a period of dark anaerobic ‘adaptation’, the green alga *Scenedesmus obliquus* produced H<sub>2</sub> in the dark. After that, several algae and cyanobacteria have been studied to observe hydrogen evolution. Indirect biophotolysis is promising for H<sub>2</sub> production process because there is no O<sub>2</sub> generation to suppress hydrogenase. The specific H<sub>2</sub> evolution activities of reversible hydrogenases are higher than those of nitrogenase-base. Therefore systems based on bidirectional hydrogenases present the most likely approach to practical development.

For indirect biophotolysis, photosynthesis stage is important to prepare cellular states suitable for dark anaerobic H<sub>2</sub> production. If this technology can be realized, cyanobacterial fuel is renewable, clean and sustainable for future. It can be genetically modified to produce valuable substance such as isoprene, ethanol, sugar, and lactate (Ducat et al. 2011). However, several obstacles must be overcome to put cyanobacteria to be economically commercialized. From recent knowledge, cyanobacteria have been known as origin of photosynthesis organism. Fossil stromatolites (microbial communities mat) can be dated back to 3.5 Gya (Giga years ago) (Blankenship 1992). Photosynthetic organisms may have been among the very earliest life forms on the primitive earth. Global Oxygen accumulation in the atmosphere and oceans are from production of O<sub>2</sub> by photosynthesis. Photosynthesis of cyanobacteria still recently plays a major role in the conversion of carbon dioxide to biological energy storage materials on earth. Photosynthesis efficiency calculated by the combination of energy losses in light absorption and the

initial chemical reactions results in a maximal overall energetic efficiency estimated at about 11% (Brenner et al. 2006) which is much higher than plants (mostly less than 1%)

Cyanobacteria produce H<sub>2</sub> both from direct and indirect photolysis as shown in **Figure A2.3**. For indirect photolysis, cyanobacteria can produce H<sub>2</sub> from two ways of indirect photolysis, first, H<sub>2</sub> production directly from bidirectional hydrogenase (non-nitrogen fixation) and second H<sub>2</sub>-production from nitrogenase (nitrogen fixation). The electron donors for the H<sub>2</sub> producing enzymes are ferredoxin (nitrogenase and FeFe-hydrogenases) and NAD(P)H (NiFe-hydrogenases). The illustration of this graph shows the differences of two types of indirect photolysis hydrogenase. Many strategies of research have been done on H<sub>2</sub> production enhancement; from condition optimization to genetic engineered bacteria to increase desired products. H<sub>2</sub> production by hydrogenase of cyanobacteria has been elevated by many approaches. Classical optimization of pH, temperature, media composition and gas phase could change H<sub>2</sub> fermentation productivity significantly (Burrows et al. 2008; Antal and Lindblad 2005). Eco-metabolism further includes dark fermentation of anaerobic bacteria. Isolation and elevation of H<sub>2</sub> production activity of some species contribute to the H<sub>2</sub> production technology.

**Figure A2.4** illustrates the nitrogenase-mediated H<sub>2</sub> production in N<sub>2</sub>-fixing cyanobacteria. Filamentous cyanobacterium *Anabaena* strains are the representative of N<sub>2</sub>-fixing cyanobacteria producing H<sub>2</sub> production as shown in below equation. However, both systems are limited by the O<sub>2</sub> sensitivity of enzyme because photosynthetic H<sub>2</sub> production of water splitting generates O<sub>2</sub>.

Presence of N<sub>2</sub>:



Absence of N<sub>2</sub>:



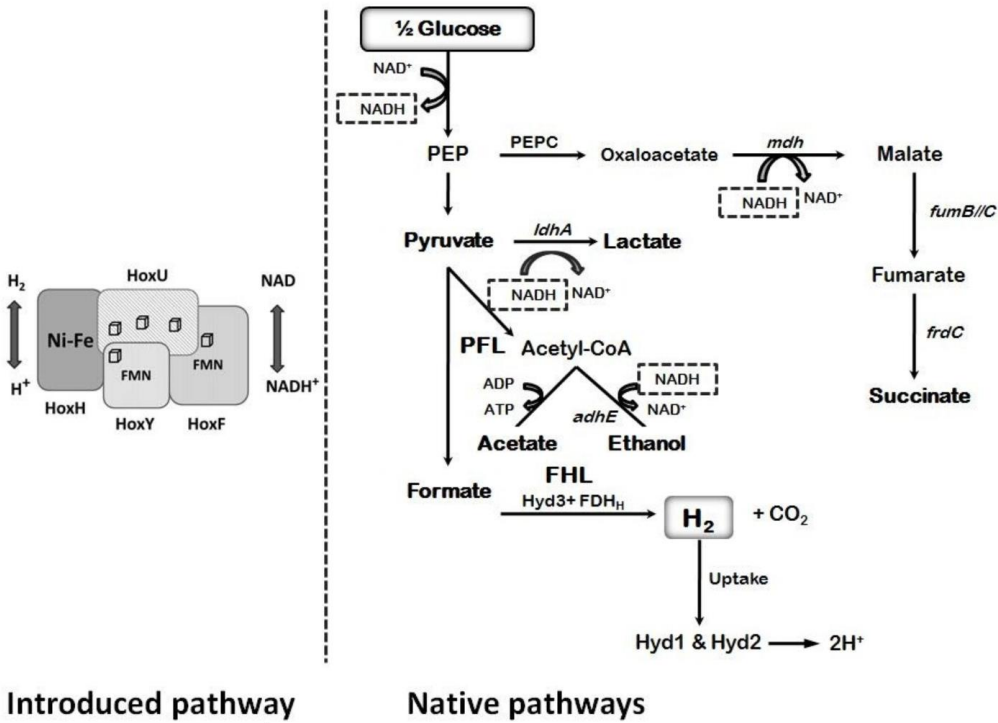


Figure A2.1 Genetically modified *Escherichia coli* to utilize NADH from glycolysis for H<sub>2</sub> production (Ghosh et al., 2013)

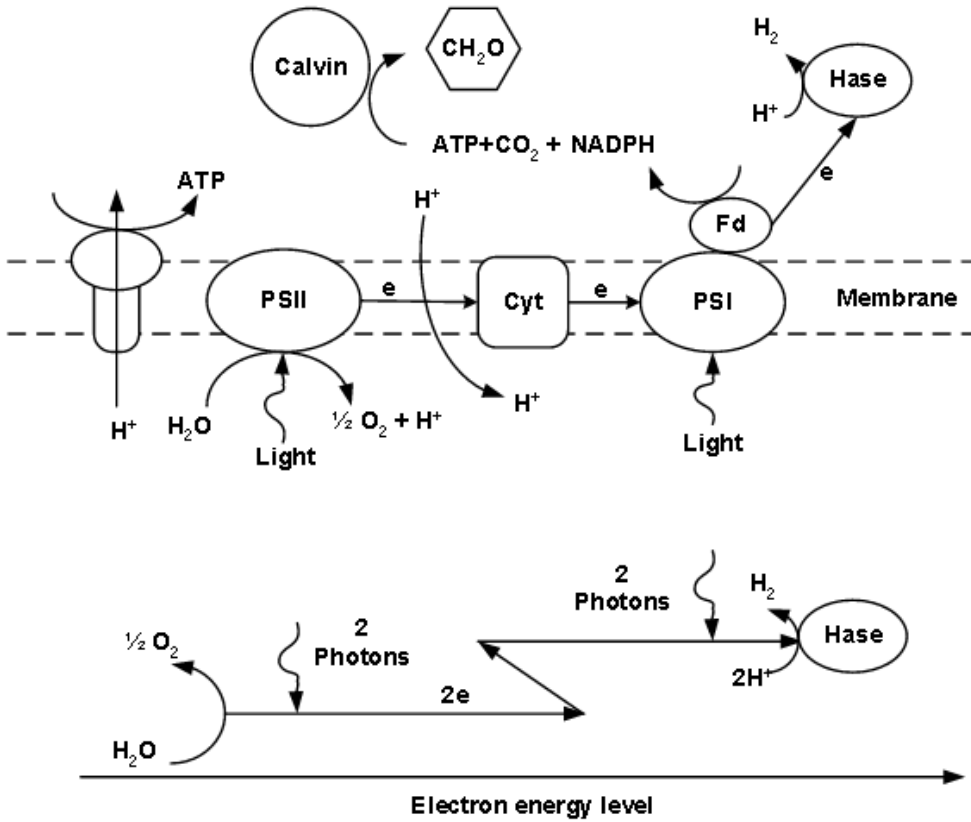
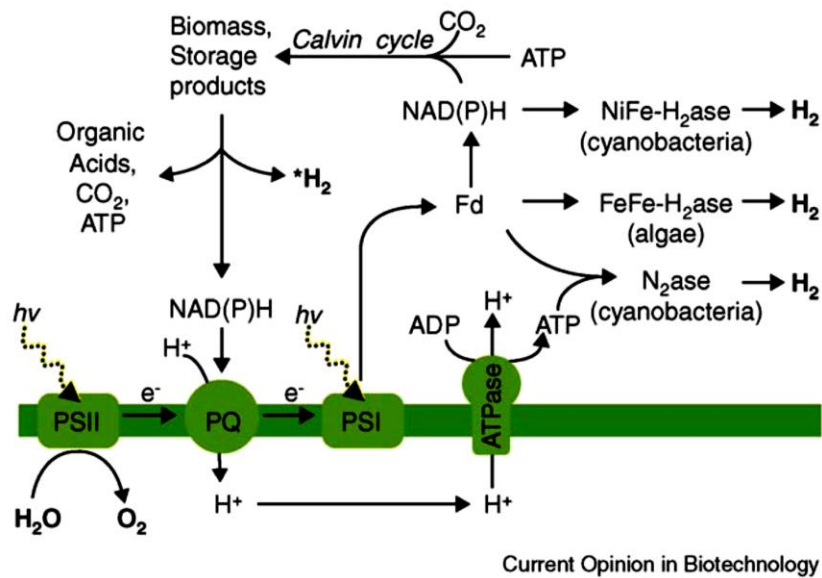


Figure A2.2  $H_2$  production based on direct biophotolysis (Yu and Takahashi 2007)



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Figure A2.3 Direct and indirect biophotolysis to generate  $H_2$  (McKinlay and Harwood 2010)

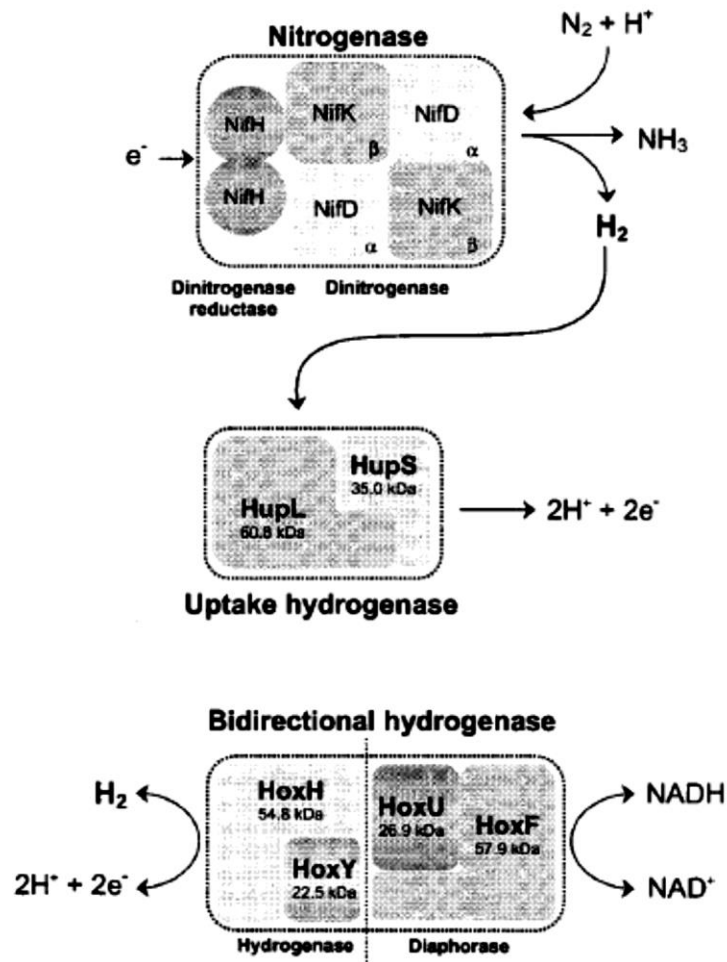


Figure A2.4 Enzymes directly involved in hydrogen metabolism in cyanobacteria. (Tamagnini et al. 2002)

## Appendix-3 Metabolic map for *Synechocystis* sp. strain PCC6803

(1) Starch and sucrose metabolism

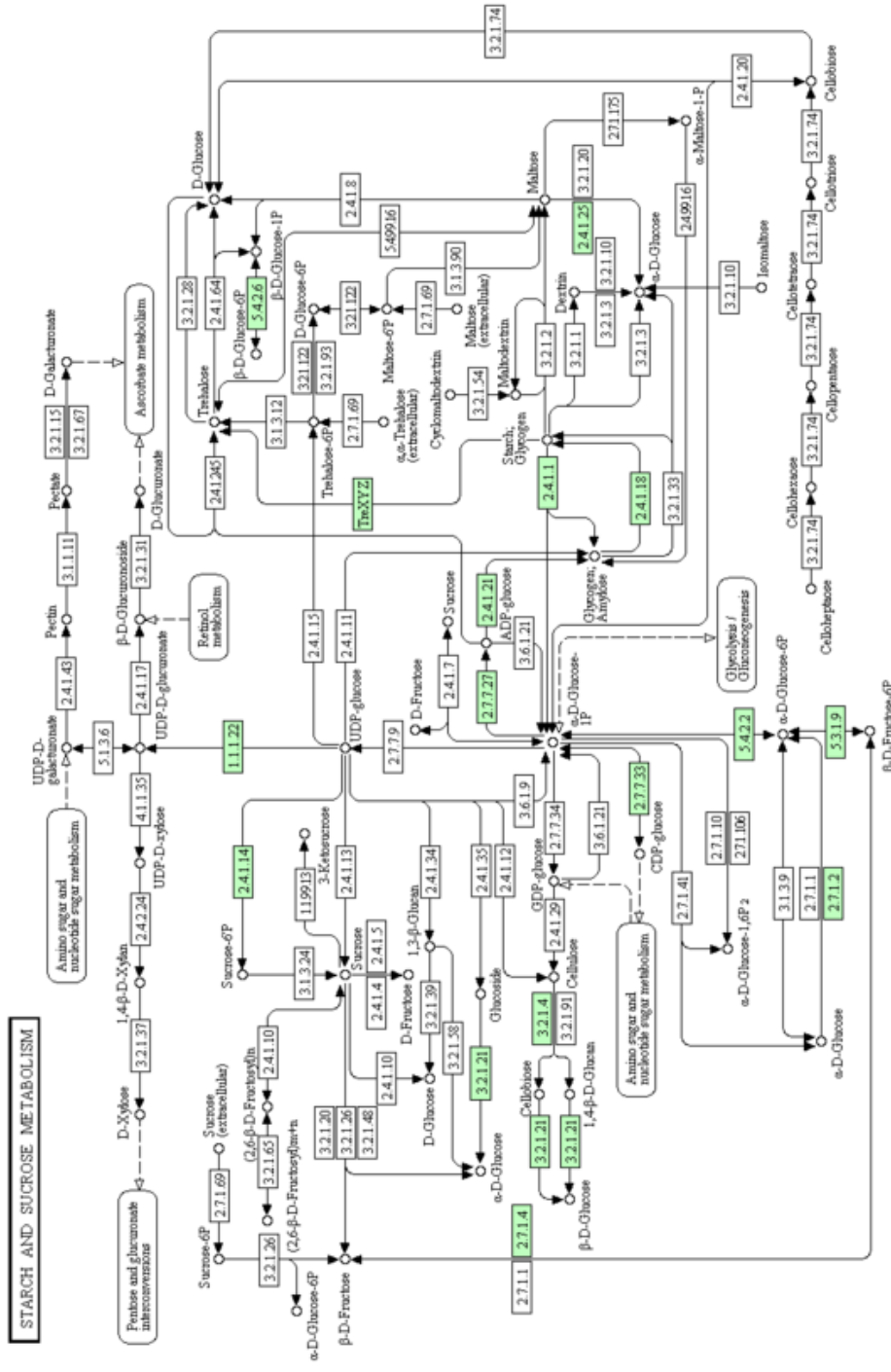
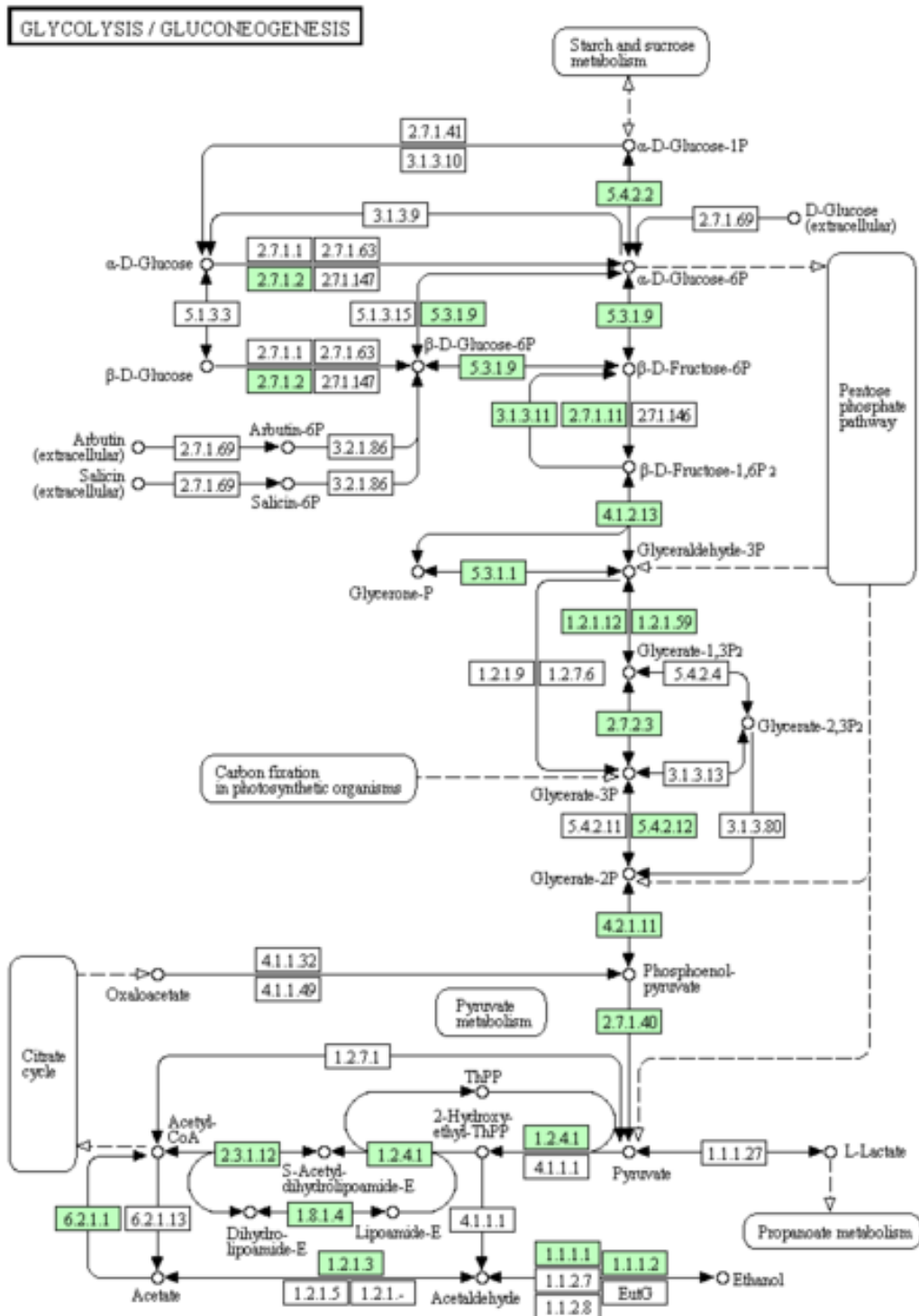


Figure A3.1 Starch and sucrose metabolism in *Synechocystis* sp. strain PCC6803 shown in KEGG Pathway Database ([http://www.genome.jp/kegg-bin/show\\_pathway?syn00500](http://www.genome.jp/kegg-bin/show_pathway?syn00500))

(2) Glycolysis/Gluconeogenesis



00010 10/7/14  
(c) Kanehisa Laboratories

Figure A3.2 Glycolysis/Gluconeogenesis in *Synechocystis* sp. strain PCC6803 shown in KEGG Pathway Database ([http://www.genome.jp/kegg-bin/show\\_pathway?syn00010](http://www.genome.jp/kegg-bin/show_pathway?syn00010))





(5) Fructose and mannose metabolism

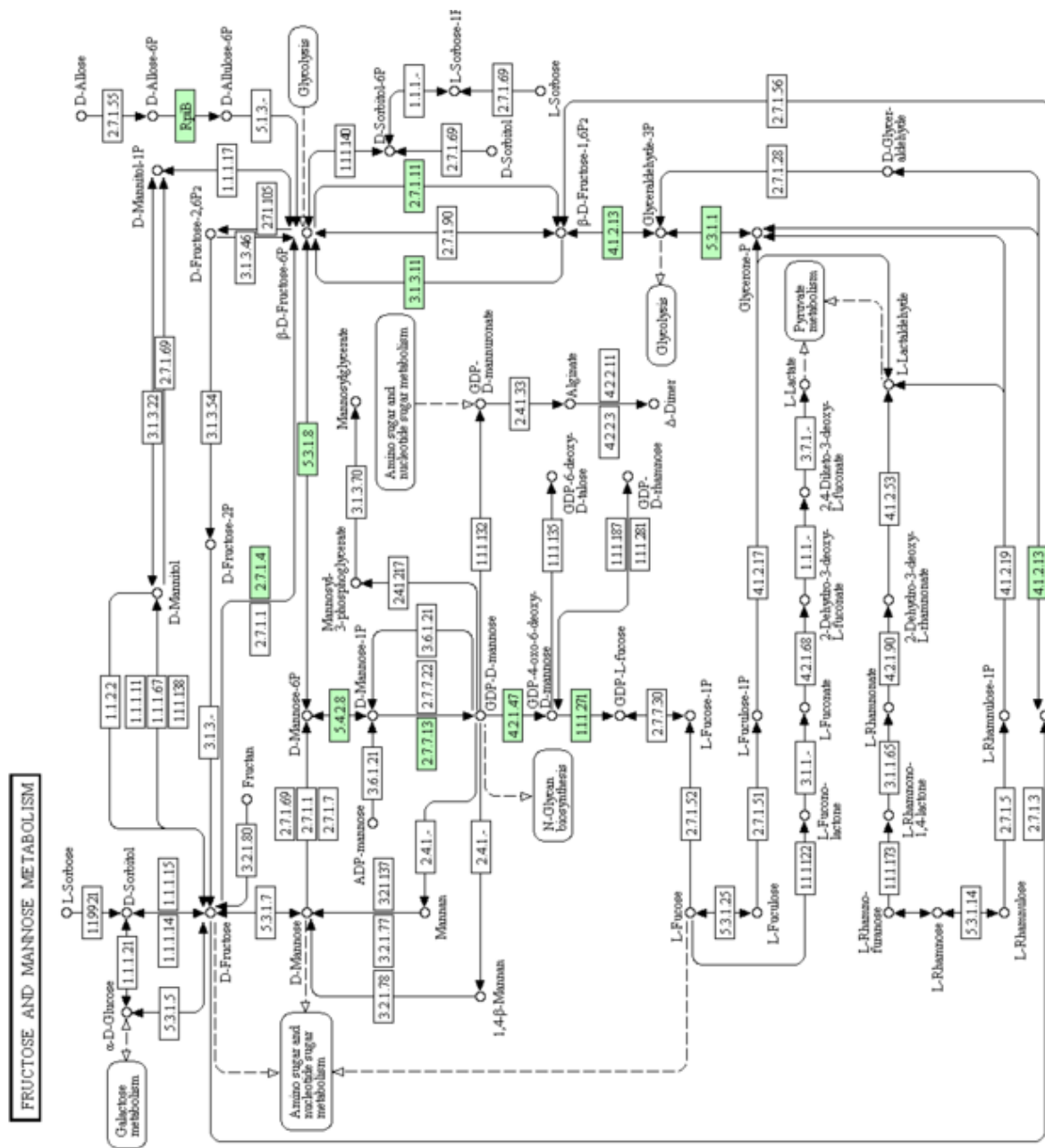


Figure A3.5 Fructose and mannose metabolism in *Synechocystis* sp. strain PCC6803 shown in KEGG Pathway Database ([http://www.genome.jp/kegg-bin/show\\_pathway?syn00051](http://www.genome.jp/kegg-bin/show_pathway?syn00051))

(6) Galactose metabolism

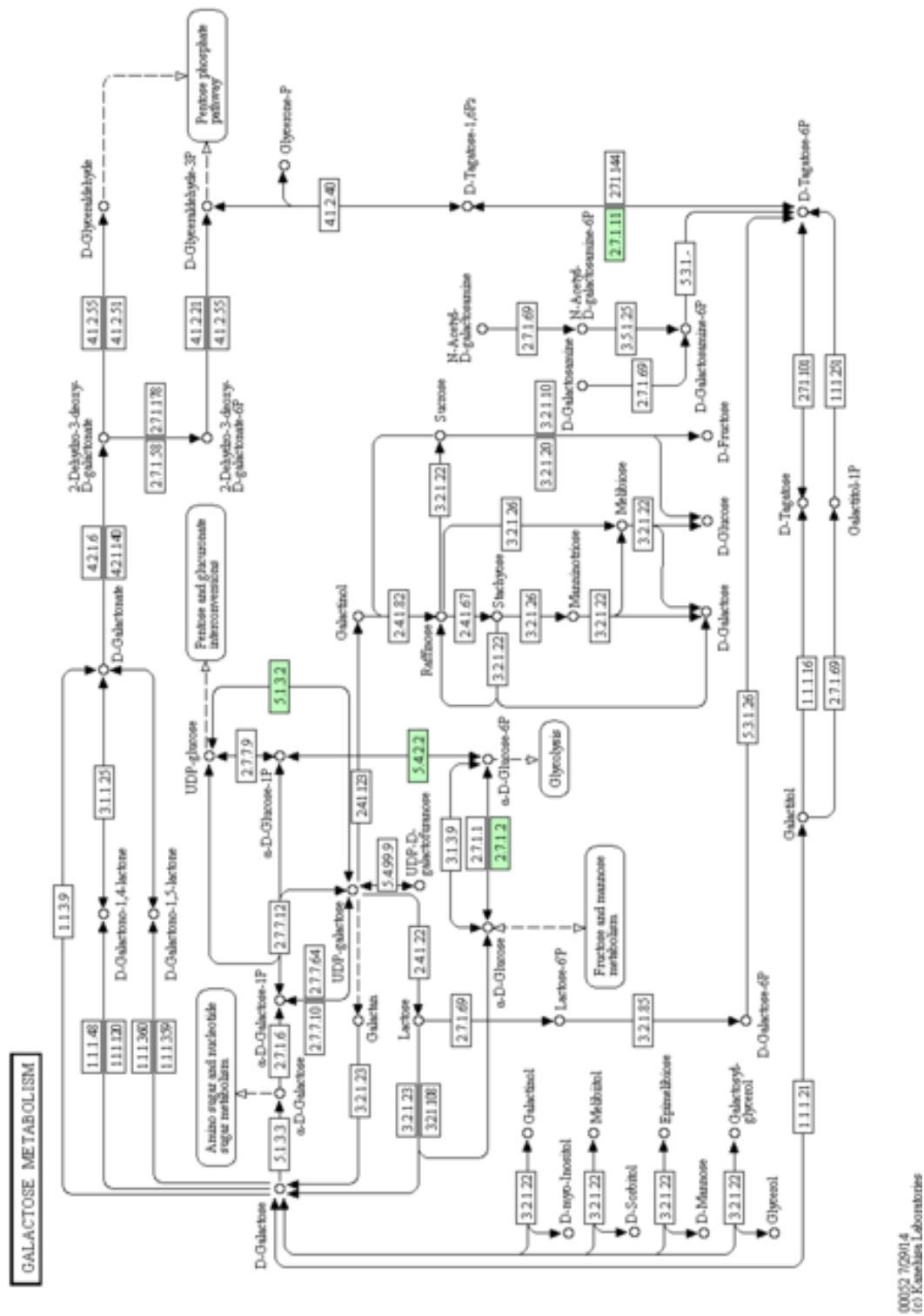


Figure A3.6 Galactose metabolism in *Synechocystis* sp. strain PCC6803 shown in KEGG Pathway Database ([http://www.genome.jp/kegg-bin/show\\_pathway?syn00052](http://www.genome.jp/kegg-bin/show_pathway?syn00052))



## Appendix-4

### Extracellular pigment production from *Synechocystis* sp. strain PCC6803

GT strain cells release reddish orange protein during being kept in HEPPEs buffer solution containing glucose under dark-anaerobic condition (**Figure A4.1**). The pigment was able to pass through ultrafilter membrane. This pigment is well dissolved in water. The pigment is known to be involved in cell photoprotection. Possible reasons of this coloration are dark-light stress, nitrogen starvation or anaerobic condition. Absorbance spectrum of the supernatant is shown in **Figure A4.2**.

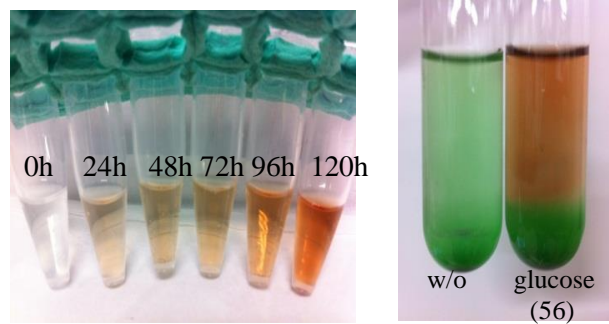


Figure A4.1 Supernatant of cell suspension of GT strain that is dark anaerobically incubated in HEPPEs buffer solution with  $56 \mu\text{mol mL}^{-1}$  glucose. Left picture shows color change of isolated supernatant from 0, 24, 48, 72, 96 and 120 h. Right-hand-side picture shows supernatants in working test tube after 120 h fermentation. Test tube was kept standing for several hours for cell sedimentation.

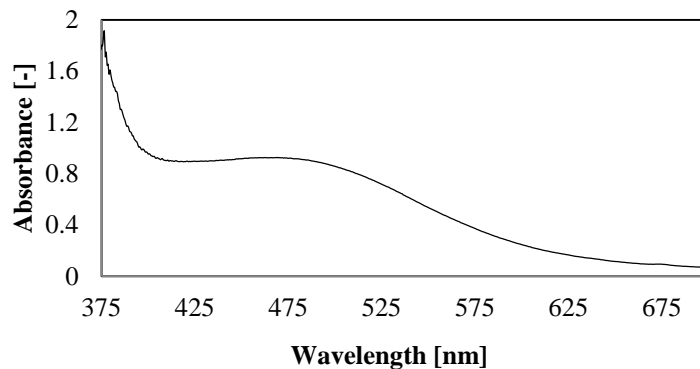


Figure A4.2 Spectrum of peak at 470 nm of supernatant of cell suspension of GT strain in buffer solution. Dark anaerobic incubation of GT strain was made in HEPES buffer solution with  $28 \mu\text{mol mL}^{-1}$  glucose for 96 h.

## Appendix: References

- Antal TK, Lindblad P (2005) Production of H<sub>2</sub> by sulphur-deprived cells of the unicellular cyanobacteria *Gloeocapsa alpicola* and *Synechocystis* sp. PCC 6803 during dark incubation with methane or at various extracellular pH. *Journal of Applied Microbiology* 98 (1):114-120
- Axley M.J, Grahame DA, Stadtman TC (1990) *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium-dependent formate dehydrogenase component. *Journal of Biochemical Chemistry* 265: 18213–18218.
- Blankenship R (1992) Origin and early evolution of photosynthesis. *Photosynth Res* 33 (2):91-111.
- Brenner MP, Bildsten L, Dyson F, Fortson N, Garwin R, Grober R, Hemley R, Hwa T, Joyce G, Katz J (2006) Engineering microorganisms for energy production. DTIC Document,
- Burrows EH, Chaplen FWR, Ely RL (2008) Optimization of media nutrient composition for increased photofermentative hydrogen production by *Synechocystis* sp. PCC 6803. *International Journal of Hydrogen Energy* 33 (21):6092-6099
- Ghosh D, Bisailon A, Hallenbeck PC (2013) Increasing the metabolic capacity of *Escherichia coli* for hydrogen production through heterologous expression of the *Ralstonia eutropha*. *Biotechnology for biofuels*. 6:122
- Gaffron H (1939) Reduction of carbon dioxide with molecular hydrogen in green algae. *Nature* 143 (3614):204-205
- Gaffron H, Rubin J (1942) Fermentative and Photochemical Production of Hydrogen in Algae. *The Journal of General Physiology* 26 (2):219-240
- Hallebeck, PC (2008) Fermentative hydrogen production: Principles, progress, and prognosis. *International Journal of Hydrogen Energy*. 34:7379-7389
- McKinlay JB, Harwood CS (2010) Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology* 21 (3):244-251
- Maeda K, Domen K (2010) Photocatalytic Water Splitting: Recent Progress and Future Challenges. *The Journal of Physical Chemistry Letters* 1 (18):2655-2661
- Mathews J, Wang G (2009) Metabolic pathway engineering for enhanced biohydrogen production. *International Journal of Hydrogen Energy* 34 (17):7404-7416
- Oh Y-K, Raj SM, Jung GY, Park S (2011) Current status of the metabolic engineering of microorganisms for biohydrogen production. *Bioresource Technology* 102 (18):8357-8367.
- Rozendal RA, Hamelers HVM, Euverink GJW, Metz SJ, Buisman CJN (2006) Principle and perspectives of hydrogen production through biocatalyzed electrolysis. *International Journal of Hydrogen Energy* 31 (12):1632-1640
- Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, Lindblad P (2002) Hydrogenases and Hydrogen Metabolism of Cyanobacteria. *Microbiology and Molecular Biology Reviews* 66 (1):1-20. doi:10.1128/mmbr.66.1.1-20.2002
- Yu J, Takahashi P (2007) Biophotolysis-based hydrogen production by cyanobacteria and green microalgae. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* 1:79-89