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Growth phase effect on the dark anaerobic hydrogen production in the glucose tolerant mutant of unicellular cyanobacterium *Synechocystis* sp. strain PCC6803

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Abstract

This study has examined the effect of growth phase on hydrogen production from cells of a glucose tolerant mutant of *Synechocystis* sp. strain PCC6803. The extracellular products including hydrogen, lactate and acetate from cyanobacteria cells in dark anaerobic nitrate-free solution, yielded different excretory profiles depending on which growth phases were prepared from photosynthesis. The amount of hydrogen generated cells prepared from stationary phase was highest in HEPES buffer and nitrate-free solution, dark anaerobic condition.

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

Introduction

Under the light, cells of cyanobacteria 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 precede, low cell density culture of inoculum changes to dense culture due to cell mass 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.

A unicellular transformable cyanobacterim *Synechocystis* sp. strain PCC6803, of which complete genome sequence was fully published, produces hydrogen on bidirectional NiFe-hydrogenase under the dark anaerobic nitrate-free buffer solution by utilizing the NAD(P)H from energy-stored cell constituting components that are synthesized during aerobic photosynthesis (Yamamoto et al. 2012a).

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A glucose-tolerant mutant of wild-type *Synechocystis* sp. strain PCC 6803 (GT strain) is known to consume extracellular glucose for growth (Williams 1988). GT strain produces hydrogen under the dark, nitrate free and oxygen absent condition. The previously reported factors affecting hydrogen production involve pH, nitrate, temperature, gas phase (Antal and Lindblad 2005), monosaccharides (Yamamoto et al. 2012b; Chongsuksantikul et al. 2014), and activity of dehydrogenases that compete NAD(P)H with hydrogenase.

The hydrogen 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 hydrogen 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 (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 hydrogen 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 *Synechocystis* sp. strain PCC 6803 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 hydrogen seems to be a good possibility to elevate hydrogen production of GT strain.

In the present paper we report results of experiments which were designed to provide information on the photoautotrophic growth phase dependent hydrogen production in GT strain under the dark anaerobic nitrate-free condition. The hydrogen production kinetics is related to endogenous glucose degradation, cell death, lactate production and acetate production.

Materials and Methods

Strain, cell preparation and hydrogen production

Glucose tolerant mutant of *Synechocystis* sp. strain PCC6803 (GT strain) was supplied by Professor Y. Hihara, Department of Biochemistry and Molecular Biology, Saitama University, Japan. The dry cell weight concentration at initial for photosynthetic cell preparation (X_0) was set at 0.0118 mg·mL^{-1}. For cell preparation, cells were grown photoautotrophically in BG-11 medium (initial pH 7.8) at 34ºC. Composition of BG-11 medium is 17.6 µmol mL^{-1} $NaNO_3$, 0.23 µmol mL^{-1} K_2HPO_4, 0.3 µmol mL^{-1} $MgSO_4.7H_2O$, 0.24 µmol mL^{-1} $CaCl_2.2H_2O$, 0.031 µmol mL^{-1} Citric acid, 0.021 µmol mL^{-1} amonium ferric citrate green, 0.0027 µmol mL^{-1} $EDTANa_2$, 0.19 µmol mL^{-1} Na_2CO_3 and trace metals. The cell suspension was aerated by 6 % CO_2 in air and illuminated by fluorescent lamps at 100 µmol photons·m^{-2}·s^{-1} photosynthetic photon flux density (PPFD).

Number of generations for the culture of age t (g) was defined by

$$g = \frac{1}{\ln 2} \ln \frac{X}{X_0} \quad (1)$$

in which, X was the dry cell weight concentrations at time t.

Cells were collected by centrifugation at 1000 g and 25ºC for 10 min. The cell pellets were washed by 50 µmol mL^{-1} HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.8) solutions. The washed cells were collected by centrifugation. Cells were re-suspended and incubated in 10 mL of 50 µmol mL^{-1} HEPES buffer (pH 7.8) solutions in 32 mL glass test tubes with butyl rubber caps. The initial dry cell weight concentration for dark incubation (X_0) was set at 2 mg mL^{-1} 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 30ºC. Dark incubation in this study was slightly modified with a daily pulse of dim light. All experiments were carried out in duplicate.

Determination of the amount of hydrogen per culture volume

The number of moles of hydrogen in the gas phase over 10 mL cell suspension in the closed 32 mL test tube was measured utilizing a gas chromatograph equipped with a molecular sieve column and a thermal conductivity detector (GC-320, GL science Inc.; column, Molecular sieve 13X; column temperature, 37ºC; injector temperature, 45ºC; detector temperature 80ºC) with nitrogen gas as the carrier gas. Number of moles of hydrogen per culture volume (y_{H2}) was obtained by the measured number of moles of hydrogen divided by culture volume. Gas chromatography condition of this method was previously reported (Chongsuksantikul et al. 2014).

Determination of dry cell weight concentration and specific fixation rate of carbon dioxide

The dry cell weight (DCW) concentration of cell suspension was monitored via optical density readings at a wavelength of 730 nm on Spectrophometer UV-12-02 (Shimadzu Co., Japan). One unit absorbance was equivalent to 0.369 mg-DCW·mL^{-1}. Carbon dioxide is a sole carbon source of the photoautotroph *Synechocystis* sp. strain PCC6803, and the cell formula of this strain shown in a reference is $CH_{1.62}N_{0.22}O_{0.40}P_{0.11}$ (Yu et al. 2013), hence the specific carbon dioxide fixation rate (q_{CO2}) was calculated by

$$q_{CO2} = \frac{10^3}{X}\frac{d(X/23.1)}{dt} = \frac{1}{0.0231}\frac{d\ln X}{dt} \quad (2).$$

Determination of the cellular content of glycogen

The amount of glycogen was evaluated as equivalent number of moles of endogenous glucose. For glucose analysis, defined dry weight cell pellets were collected by centrifugation of defined volume of cell suspension at 25ºC, 3000 rpm for 10 min. From defined amount of cell pellets, the endogenous glucose was obtained by extracting and decomposing glycogen with 50 µL of 6 N HCl at 80ºC for 30 min. The number of moles of endogenous glucose in glycogen per dry cell weight (m_G) was determined by applying Glucose CII Test Wako (Wako Pure Chemical Ind., Ltd.). The number of moles of endogenous glucose per culture volume ($m_G X$) was also calculated.

The specific rate for the change in the amount of endogenous glucose (R_G) is defined by:

$$R_G = \frac{1}{X}\frac{d(m_G X)}{dt} \quad (3).$$

If R_G is positive R_G is the specific accumulation rate of endogenous glucose and if R_G is negative, ($-R_G$) is the specific consumption rate of endogenous glucose.

Determination of the concentrations of lactate and acetate

A 500 µL sample was withdrawn daily. Metabolites in supernatants were analyzed with High Performance Liquid Chromatography (HPLC, Hitachi 655A-11) at 210 nm wavelength. Mobile phase was 18 mmol L^{-1} of potassium phosphate (KH_2PO_4, pH 2.3 adjusted with phosphoric acid (H_3PO_4)). Mobile phase flow rate was 0.7 mL min^{-1}. Column temperature was 30°C. A 10 µL of supernatant sample was injected by using 10 µL syringes for analysis. The number of moles of lactate per culture volume (c_L) and acetate (c_A) were also calculated.

Results and Discussion

A series of photoautotrophic culture experiments on BG-11 medium, followed by dark anaerobic hydrogen production experiments in nitrate-free HEPES buffer solution were performed. To elucidate the effect of the growth phase of photoautotroph on hydrogen production, the photoautotrophic culture grown to reach OD_{730} at 3-4 that was in logarithmic growth phase, at 7 that was in late-logarithmic growth phase, and at 12 that was in stationary phase were harvested to inoculate in dark hydrogen production experiments.

Cellular state of inoculum for dark anaerobic incubation

Growth curve for photoautotrophic culture is shown in Figure 1. The logarithmic growth and late-logarithmic growth are seen between 0 h and 55 h, and between 55 h and 80 h, respectively. If t_C and t_f are the transition times from logarithmic growth phase to late-logarithmic growth and late-logarithmic growth phase to stationary phase, then observation suggests that t_C is 55 h and t_f is 80 h. The specific growth rate of logarithmically growing cells ($\mu = \frac{d\ln X}{dt}$) is 0.104 h^{-1}, hence the doubling

time (t_2) is calculated to be 6.66 h. The highest dry cell weight concentration (X_f) is 6.37 mg mL^{-1}, hence the number of generations g at the end of cell growth is 9.93. After 80 h, stationary phase is observed.

There is no lag phase on this growth curve because inoculum cells were taken from logarithmic growth phase of preculture. Growth curve shows that GT strain is in logarithmic growth phase when *g* is between 0 and 7.99, in late-logarithmic growth phase when *g* is between 7.99 and 9.28, and in stationary phase when g is no less than 9.28. Solid line for the time course of dry cell weight concentration is calculated by:

$$\ln X = \ln X_0 + \mu t \quad \text{for } t<t_C \tag{4}$$

and

$$\ln X = \ln X_f + \left(\ln\frac{X_C}{X_f}\right)\exp\left(\frac{\mu(t-t_C)}{\ln\frac{X_C}{X_f}}\right) \quad \text{for } t_C \leq t<t_f \tag{5}$$

with the model parameter X_C at 1.67 mg mL^{-1}. The X_0 calculated by Eq.(4) is 0.00656 mg mL^{-1}. Plots of data show the estimated values agree relatively well with observed values.

Figure 1 also shows the time course of the q_{CO2} that is calculated by Eqs. (3)-(5). The q_{CO2} was maximum ($q_{CO2,max}$) and constant at 4.46 μmol mg^{-1} h^{-1} in logarithmic growth phase and decreased drastically in late-logarithmic growth phase to 0 μmol mg^{-1} h^{-1} at 120 h. During photosynthesis, the electron generated by photon absorption on the thylakoid membrane converts $NADP^+$ to NADPH, which is utilized in the Calvin cycle according to:

$$3CO_2 + 6NADPH + 5H_2O + 9ATP \rightarrow Glyceraldehyde3\text{-}P + 6NADP^+ + 2H^+ + 9ADP + 8Pi \tag{6}.$$

From this stoichiometry, the NADPH consumption in the Calvin cycle during logarithmic growth phase and stationary phase are calculated to be 9.00 and 0 μmol mg^{-1} h^{-1}, respectively.

The observed m_G of GT strain increases with time after 55 h. According to our preliminary experiments, the m_G is constant with time in logarithmic growth phase and increases linearly with time after logarithmic growth phase. Broken line represents the calculated m_G according to the relations:
for cells in logarithmic growth phase:

$$R_G = \mu m_G = (0.104)(0.102) = 0.0106 \ \text{μmol mg}^{-1}\text{ h}^{-1} \tag{7.1}$$

$$m_G = 0.102 \ \text{μmol mg}^{-1} \tag{7.2}$$

and for cells in late-logarithmic growth phase and stationary phase:

$$R_G = 0.00128 + \left(\frac{d\ln X}{dt}\right)m_G \tag{7.3}$$

$$m_G = 0.0379 + 0.00128t \tag{7.4}.$$

Figure 1 shows that calculated m_G is in fair agreement with the observed m_G. The level of m_G in this work is supported by a previous work reported that the glycogen content of GT strain on BG-11 medium under 50 μmol photons m^{-2} s^{-1} illumination for 15 d (=360 h) was 11% (= 0.611 μmol glucose equivalent·mg^{-1}) (Joseph et al. 2014. The predicted m_G at 360 h by Eq.(7.4) is 0.499 μmol·mg^{-1}, that agrees well with above value. Glycogen content in another work is varied according to

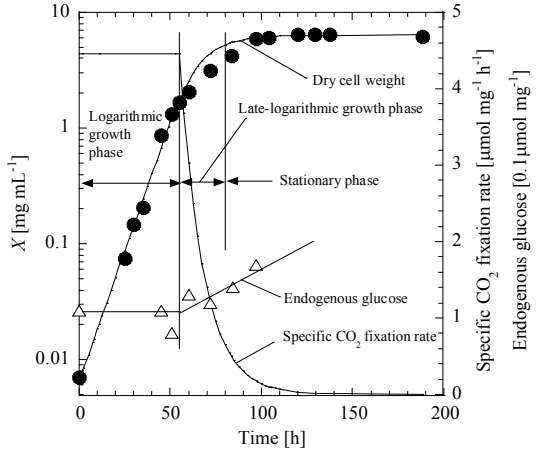

Figure 1: Time courses of culture parameters for photoautotrophic culture of *Synechocystis* sp. strain PCC6 803-GT in BG-11 medium cell culture. Inoculum size was OD_{730} = 0.02. Liquid medium was aerated by 6% CO_2 in air and continuously illuminated at 100 μmol photons m^{-2}·s^{-1} photosynthetic photon flux density (PPFD). Plotted parameters are *X*, dry cell weight concentration measured by optical density of wavelength at 730 nm (●); q_{CO2}, specific carbon dioxide fixation rate (■) and mG, number of moles of endogenous glucose per dry cell weight (△). The mG at 0 h is a plot from pre-culture analysis. All experiment was carried out in duplicate.

how cells are grown. A reported glycogen content of *Synechocystis* sp. strain PCC6803 grown on BG-11 medium at 25º 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 μmol·mg^{-1} (Yoo et al. 2007), which also supports the m_G level of this report. The yield of endogenous glucose from carbon dioxide (=R_G/R_{CO2}) is 0.00236 in logarithmic growth phase. Although the q_{CO2} of cells in stationary phase is extremely low, Eq.(7.4) suggests the

Table 1: Cellular state of inoculum in photoautotrophic cultures

	Logarithmically growing cells	Late-logarithmically growing cells	Stationary phase cells
Harvesting time [h]	44-47	65	89
Dry cell weight concentration (*X*) [mg·mL^{-1}]	1.11-1.48	2.58	4.43
Number of generations (*g*) [-]	6.56-6.97	7.77	8.55
Specific carbon dioxide fixation rate (q_{CO2}) [mmol mg^{-1} h^{-1}]	4.50	1.46	0.921
Amount of endogenous glucose per dry cell weight (m_G) [mmol mg^{-1}]	0.0773	0.103	0.195
Specific endogenous glucose accumulation rate before inoculation (R_G) [mmol mg^{-1} h^{-1}]	0.0106	0.00475	0.00128

occurrence of glycogen synthesis. Turnover of cell constituting components appears to supply carbon for glycogen to store energy for survival. Table 1 shows the cellular states of GT strain, utilized for dark anaerobic hydrogen production.

Effects of cellular state on hydrogen production

Effect of growth phase of GT strain upon dark anaerobic hydrogen production was studied in experiments in which cells from different growth phase were inoculated on nitrate-free HEPES buffer solution. Figure 2 shows the time courses of culture variables in dark anaerobic nitrate-free HEPES buffer incubation of GT strain from logarithmic growth phase, late-logarithmic growth phase and stationary phase of photoautotrophic cultures. The culture variables observed include (a) the number of moles hydrogen per culture volume (y_{H2}), (b) the number of moles of endogenous glucose per dry cell weight (m_G), the number of moles of endogenous glucose per culture volume, (c) the dry cell weight concentration (X), (d) the lactate concentration (e) the acetate concentration and (f) the rates of hydrogen production ($r_{H2}=dy_{H2}/dt$), lactate production ($r_L=dc_L/dt$) and acetate production ($r_A=dc_A/dt$). When cells of GT strain in dark anaerobically incubated in HEPES buffer solution, the highest number of moles of hydrogen per culture volume at 96 h ($y_{H2,1}$) was achieved from cells inoculated from stationary phase. The lowest hydrogen production is seen in the result of run with cells from logarithmic growth phase. The results of three runs show that the r_{H2} was highest at $r_{H2,0}$ right after inoculation and then decreased with time (Fig.2(f)). If deactivation of hydrogen production is interpreted with first order kinetics with deactivation rate constant k, the number of moles of hydrogen per culture volume (y_{H2}) can be given by the equation:

$$\frac{dy_{H2}}{dt} = r_{H2} = r_{H2,0}\exp(-kt) \quad (8).$$

in which $y_{H2,f}$ is the attainable level of y_{H2}. Best fitting of Eq.(9) to the observed y_{H2} results the parameters $r_{H2,0}$, k and $y_{H2,f}$ shown in Table 2. The calculated y_{H2} shown by broken lines in Fig.2(a) fits well with observed y_{H2}. The order of model parameter $y_{H2,f}$ is found to be same as the order of observed variable $y_{H2,1}$. The relation shown by Eq.(10) represents that the $y_{H2,f}$ increases as the $r_{H2,0}$ is higher or the k is lower.

Table 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 $y_{H2,f}$ is same as the descending order of k in Table 2. The GT strain in dark anaerobic incubation with inoculum from stationary phase is found to be most stable for dark anaerobic hydrogen production.

The $r_{H2,0}$ is highest in run with inoculum from late-logarithmic growth phase and comparable lower value with inoculum from logarithmic growth phase and stationary phase. The q_{CO2} is proportional to the NADPH consumption rate of the Calvin cycle, hence the decrease in q_{CO2} of inoculum cells appears to increase $r_{H2,0}$, while q_{CO2} in Table 1 shows that the q_{CO2} 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 $r_{H2,0}$, while the m_G in Table 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 hydrogen production rate. Although the ascending order of $r_{H2,0}$ is different from ascending order of $y_{H2,f}$, the descending order of k is same as ascending order of $y_{H2,f}$,. The attainable level of number of moles of hydrogen per culture volume is found to depend more on stability of hydrogen production rate than initial hydrogen production rate.

Table 2: Characteristics of dark anaerobic hydrogen production

	Logarithmically growing cells	Late-logarithmically growing cells	Stationary phase cells
Initial dry cell weight concentration (X_0) [mg mL^{-1}]	2.07	2.03	1.95
Initial hydrogen production rate ($r_{H2,0}$) [mmol mL^{-1} h^{-1}]	0.025	0.031	0.027
Deactivation rate constant (k) [h^{-1}]	0.017	0.019	0.006
Number of moles of hydrogen per culture volume at 96 h ($y_{H2,1}$) [mmol mL^{-1}]	1.48	1.79	1.97
Attainable number of moles of hydrogen per culture volume ($y_{H2,f}$) [mmol mL^{-1}]	1.86	2.18	4.24
Specific death rate (K_d) [h^{-1}]	0.00076	0.0011	0.0026
Number of moles of lactate per dry cell weight at 96 h [mmol mg^{-1}]	0.0308	0.0323	0.188
Dry cell weight concentration at 96 h [mg mL^{-1}]	1.95	1.86	1.49
Lactate concentration at 96 h [mmol mL^{-1}]	0.20	0.26	0.48
Acetate concentration at 96 h [mmol mL^{-1}]	0.05	0.21	0.80
Decrease in m_GX during 96 h hydrogen production [mmol mL^{-1}]	0.10	0.15	0.10
Yield of hydrogen on endogenous glucose ($Y_{H2/G}$) [-]	9.6	11.8	19.1

The integration of Eq.(8) yields that

$$y_{H2} = \frac{r_{H2,0}}{k}\{1-\exp(-kt)\} \quad (9)$$

and

$$y_{H2,f} = \frac{r_{H2,0}}{k} \quad (10),$$

Comparison of curves in Figures 2(a) and (b) show that, during dark anaerobic hydrogen production, hydrogen production is more stable when the level of m_G is high. Table 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 same as the descending order of k in Table 2. The increase in the m_G is found to increase the stability of hydrogen production during dark anaerobic incubation. Our results show that stationary phase cells that accumulate glycogen are most suitable for

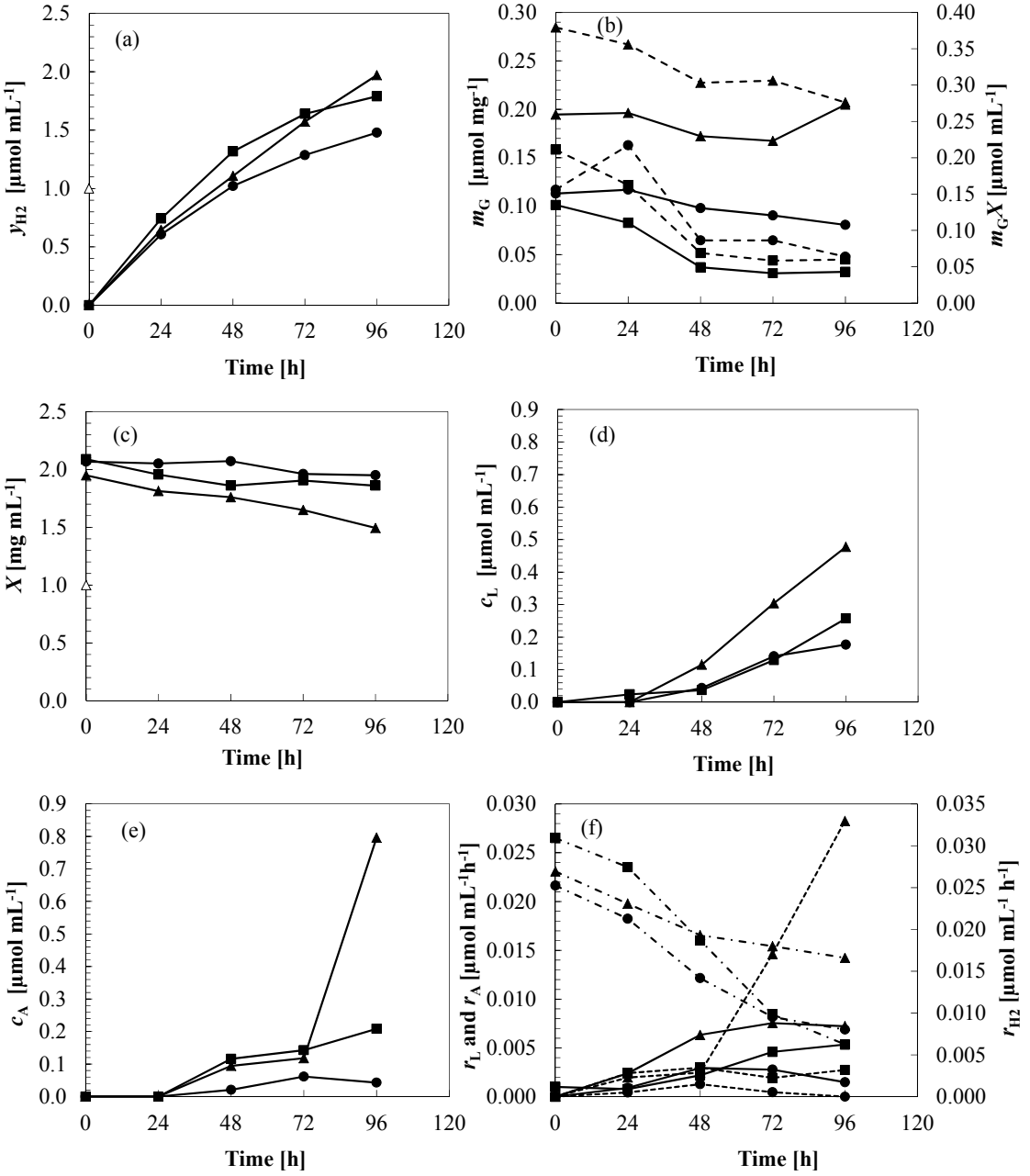

Figure 2: Time courses of dark anaerobic hydrogen production (a) the number of moles hydrogen per culture volume (y_{H2}), (b) the number of moles of endogenous glucose per dry cell weight (m_G) in solid line, the number of moles of endogenous glucose per culture volume in broken line (m_GX), (c) the dry cell weight concentration (X), (d) the lactate concentration (c_L), and (e) the acetate concentration (c_A), (f) the lactate production rates in broken line (r_L), the acetate production rates in solid line (r_A), the hydrogen production rate in long short broken line (y_{H2}) of GT strain cells, logarithmic stage (●), late-logarithmic stage (■) and stationary stage (▲) in HEPES buffer solutions. Gas phase was nitrogen gas. Cells were washed to ensure there is no nitrogen source in fermentation system. All experiment was carried at least in duplicate.

inoculum of dark anaerobic hydrogen production. The m_G of inoculum cells from stationary phase is 0.195 µmol mL^{-1}, which is 2.52 times that from logarithmic growth phase.

The solid line curves of m_G versus time in Fig.2(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_GX versus time in Fig. 2(b) show that, except for the m_GX between 0 and 24 h of run with inoculum from logarithmic growth phase, m_GX always decreased with time. Glycogen degradation occurred in dark incubation with inocula from late-logarithmic growth phase and stationary phase. The m_GX 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.

Specific glycogen accumulation rate before inoculation (R_G) shown in Table 1 is highest at 0.106 μmol.mL^{-1}.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_GX 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.

Of special interest is that the decrease in the mX is not affected by the growth phase of inoculum cells (Table 2). Growth phase dependent production of dark anaerobic production of hydrogen is found to be caused not by endogenous glucose decomposition but by turnover of cell constituting components.
Figure 2 (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 2). The ascending order of K_d is the same as the ascending order of $y_{H2,f}$. Activation of turnover of cell constituting components is found to be important for shifting-up hydrogen production. Observed cell death shows that inoculum from stationary phase suits for increasing K_d. Figures 2 (d) and (e) show that hydrogen 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).

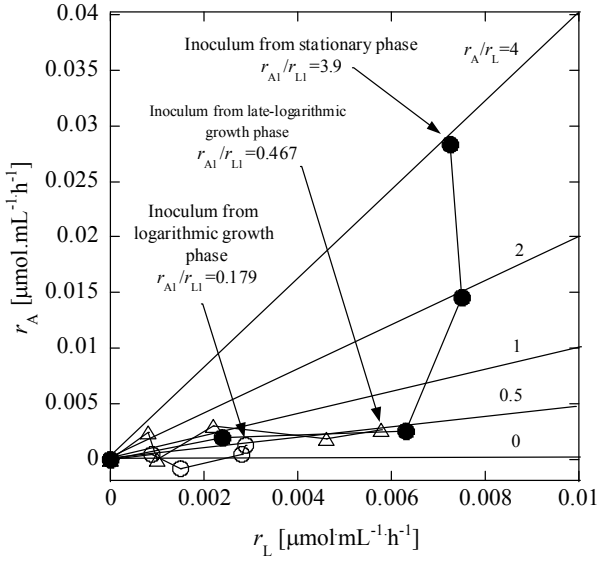

Figure 3: Relation between lactate production rate (r_L) and acetate production rate (r_A) during anaerobic dark hydrogen production of runs with inocula from logarithmic growth phase (○), late-logarithmic growth phase (△) and stationary phase (●).

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^{-1} in run with logarithmically growing cells increased to 0.48 μmol mL^{-1} in run with stationary phase cells. Acetate production was roughly parallel to lactate production. Acetate concentration that was 0.05 μmol mL^{-1} in run with logarithmically growing cells increased to 0.8 μmol mL^{-1} in run with stationary phase cells. Interestingly, cells from stationary phase resulted 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 hydrogen on endogenous glucose ($Y_{H2/G}$) of Table 2. Dark anaerobic hydrogen production of stationary phase cells is found to be highly dependent on NAD(P)H formation at incomplete TCA cycle. The highest $Y_{H2/G}$ is 19.1 that is a result of run with cells from stationary phase. This yield is about a double of that with cells from logarithmic growth phase.

The acetate production rates (r_A) of runs with inocula from logarithmic growth phase, late-logarithmic growth phase and stationary phase are plotted against the lactate production (r_L) in Figure 3. Small variation is seen in the ratio r_A/r_L of run with inoculum from logarithmic growth phase. The ratio at 96 h ($r_{A1}/r_{L\ 1}$) of this run is 0.179. The r_A is almost constant at 0.0025 μmol.mL^{-1}.h^{-1} and the r_L increases with time from 0.01μmol.mL^{-1}.h^{-1} to 0.00578 μmol.mL^{-1}.h^{-1} in run with inoculum from late-logarithmic growth phase. The r_{A1}/r_{L1} of this run is 0.467.

The ratio r_A/r_L of run with inoculum from stationary phase is almost constant at from 0 h to 48 h, and then increases drastically to the $r_{A1}/r_{L\ 1}$ of 3.9 at 96 h. From 72 h to 96 h, the r_L is almost constant at 0.00736 μmol.mL^{-1}.h^{-1} while the r_A increases drastically.

Conclusions

In conclusion, our results show that dark anaerobic hydrogen production NiFe-hydrogenase of the glucose tolerant mutant of *Synechocystis* sp. strain PCC6803, suspended in nitrate-free HEPES buffer solution, is strongly dependent on the growth phase of inoculum cells in the prior photosynthetic cultures. The inoculum cells from stationary phase are most suitable for hydrogen production. The stationary phase cells are capable of maintaining high level in cellular glycogen content during hydrogen production and elevating the NADPH production by activating acetate production. The stabilized reductive state of GT strain brought by the stationary phase cells inhibits the deactivation of NiFe-hydrogenase for hydrogen production. It is known that glycogen content of GT strain can be increased by adding glucose into cell suspension. Synergistic effect of utilizing stationary phase cells and adding glucose into solution is promising. This remains to be confirmed.

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