Anaerobic production of hydrogen in the dark by *Synechocystis* sp. strain PCC 6803: effect of photosynthesis media for cell preparation

Takashi Yamamoto*, Kazuhiro Asami, Kazuhisa Ohtaguchi

Abstract

The anaerobic production of hydrogen in the dark by bidirectional hydrogenase of unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 has been studied by utilizing cells prepared in six types of medium in the presence or absence of D-glucose, NO_3^-, NaCl and $NiCl_2$. Hydrogen production in this study is unrelated to hydrogenase activity. Hydrogen production is found to bear definite relation to the decreases in the amount of intracellular glucan per unit volume of culture when cells are photosynthetically prepared in BG-11 medium or BG-11 medium with NaCl or $NiCl_2$, or the loss of dry cell mass concentration when cells are photosynthetically prepared in BG-11 medium supplemented with D-glucose or BG-11 medium deficient in NO_3^-. The cells prepared in BG-11 medium with D-glucose for the production of unit volume of hydrogen are found to reduce the volume of photobioreactor for cell preparation by 41.2% from that without D-glucose.

Keywords: H_2 production, Bidirectional hydrogenase, *Synechocystis* sp. strain PCC 6803, Mixotroph, Hydrogenase activity, Cell preparation.

Introduction

Cyanobacterial production of molecular hydrogen (H_2) is a potential source of a non-polluting fuel. The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 is a favored organism for the molecular biotechnological studies on the H_2 production over bidirectional hydrogenase. Genome DNA sequence analysis and related works on this strain have defined the presence of bidirectional hydrogenase and the absence of both nitrogenase and uptake hydrogenase (Kaneko *et al*. 1996). The bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 is a pentameric [NiFe] enzyme utilizing protons and NAD(P)H to generate H_2 and

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$NAD(P)^+$ reversibly (Appel and Schulz 1996; Schmitz *et al*. 1995). This reaction is thought to play an important role for proper redox poising (e.g. elimination of excess reductants that are accumulated in the photosynthetic electron transfer chains) (Appel *et al*. 2000).

Synechocystis sp. strain PCC 6803 is characteristic in that it is capable of growing both photoautotrophically on CO_2 and heterotrophically on D-glucose (Smith 1983). Catabolism of D-glucose to two moles of pyruvate donates electrons to $NAD(P)^+$ to form NAD(P)H, hence the addition of D-glucose to the CO_2-aerated culture has a possibility to shift the oxidation-reduction balance of *Synechocystis* sp strain PCC 6803 to a reducing condition. Although it has been reported that reducing power of D-glucose is a key factor for H_2 production in *Synechocystis* sp. strain PCC 6803 (Antal and Lindblad 2005; Baebprasert *et al*. 2010), few reports are available on this mechanism.

Cyanobacterial bidirectional hydrogenase is oxygen sensitive (Rao and Hall 1988; Tamagnini *et al*. 2002). Nitrate starvation in the dark enhanced the H_2 productions of *Synechococcus* sp. strain PCC 6301 (Ohtaguchi *et al*. 1995) and thermophilic *Synechococcus* sp. strain H-1 (Asami *et al*. 2011). A previous work reported that salt stress and hyperosmotic stress by NaCl or sorbitol induced much of a hydrogenase related gene *hyp*A on this strain (Kanesaki *et al*. 2002). *Synechocystis* sp. strain PCC 6803 grown in the salt stress condition appears to be rich in both hydrogenase and reducing potential. Some reports showed that the addition of Ni^{2+}, which is an essential ion for *Synechocystis* bidirectional hydrogenase, enhanced hydrogenase activity (Carrieri *et al*. 2008; Gutekunst *et al*. 2006).

The present report evaluates the effect of the addition of D-glucose, NaCl or $NiCl_2$, or removal of NO_3^- for the photosynthetical preparation of *Synechocystis* sp strain PCC 6803 upon the H_2 production in the dark in the same experimental condition. Furthermore the effectiveness of cell preparation methods was compared as a volume of photobioreactor to prepare the biomass capable of producing one mole of H_2.

Methods

Cell preparation

Synechocystis sp. strain PCC 6803 was photosynthetically grown at 34 °C in a 100 mL bubble column (clear Pyrex glass) containing 80 mL HEPES buffer (pH 7.7) aqueous solution of either BG-11 medium, BG-11 medium deficient in NO_3^- (BG-11 medium-N), BG-11 medium supplemented with 150 mmol L^{-1} NaCl (BG-11 medium+NaCl), BG-11 medium supplemented with 1.5 μmol L^{-1} $NiCl_2$ (BG-11 medium+$NiCl_2$), BG-11 medium with 5.5 mmol L^{-1} D-glucose (BG-11 medium+D-glucose) or BG-11 medium deficient in NO_3^- supplemented with 5.5 mmol L^{-1} D-glucose (BG-11 medium+D-glucose-N). Initial cell mass concentration was adjusted to 0.369 mg mL^{-1}. The aqueous solution was aerated at 80 mL min^{-1} by 6% CO_2 in air that was filtered through 0.45 μm filter and fed from the bottom of the bubble column. The bubble column was placed in a 34 °C water bath. One side of the bubble column was illuminated by fluorescent lamps at 100 μmol m^{-2} s^{-1}. After the 24 h cultivation cells were harvested by centrifugation at 25 °C, 3000 rpm for 10 min. The cell pellets were washed with HEPES buffer solutions and centrifuged again.

H₂ production

After cell preparation in photosynthesis the settled cells were suspended in de-ionized water containing 50 mmol L^{-1} HEPES buffer (pH 7.7) in a test tube. Initial cell mass concentration and total volume of each cell suspension were adjusted to 2.0 mg mL^{-1} and 10 mL, respectively. Finally cell suspensions in the test tubes were sparged with N_2 gas for a few minutes to remove O_2 gas and sealed with butyl rubber caps. Test tubes were placed into a water bath at 34 °C under a dark condition and agitated at 120 rpm.

Measurement of cell mass concentration (X), H₂ production, D-glucose concentration in cell suspensions (cG) and hydrogenase activity

Cell mass concentration in terms of dry cell weight (DCW) per unit suspension volume was monitored by measuring an optical density at 730 nm (OD_{730}) utilizing a spectrophotometer. One unit absorbance of cell suspension was equivalent to 0.369 mg-DCW mL^{-1}.

At the measurement time, 300 μL of gas samples were withdrawn from the test tubes with a gasket syringe and 400 μL of cell suspensions were also withdrawn from the test tubes with a syringe. After that, the test tubes were sparged with N_2 gas again.
An amount of H_2 in gas samples was determined with a gas chromatograph and a thermal conductivity detector (GC-320, GL science Inc.; column, Molecular sieve 13X; carrier gas, nitrogen gas; column temperature, 315 K; injector temperature, 333 K; detector temperature, 353 K).

To analyze the glucan content as moles of glucose, a hydrolysis method modified from a previous work (Asami *et al*. 2011) was used. A 200 μL sample of cell suspensions was centrifuged at 4 °C, 10,000 rpm for 5 min. The supernatant was discarded and the pellets in the settled fraction were washed with de-ionized water and re-centrifuged a few times to remove glucose in the cell suspensions. Then 50 μL 6 N HCl was added onto the pellets. These mixtures were placed into a water bath at 80 °C for 30 min. The reaction was quenched at 30 min by adding 50 μL 6 N NaOH solution. The amount of D-glucose, extracted from the hydrolyzed pellets, was determined with a glucose tester (Wako Pure Chemicals, Japan).

Intracellular glucan content (ρ_G), which is estimated as the moles of D-glucose per unit cell mass, was calculated from X and c_G.
The activity of hydrogenase in intact cells of *Synechocystis* sp. strain PCC 6803 was assayed under methyl viologen (modified from (Gutthann *et al*. 2007)). First, 4 mg of cells harvested from photobioreactor were suspended in a 2 mL of de-ionized water containing 50 mmol L^{-1} HEPES, 5 mmol L^{-1} methyl viologen and 10 mmol L^{-1} sodium hydrosulfite in 15 mL glass tubes. Then the cell suspensions in the test tubes were sparged with N_2 gas for a few minutes to remove dissolved and gaseous O_2 and sealed with butyl rubber caps. The test tubes were placed into a water bath at 34 °C under a dark condition and agitated at 120 rpm. Produced H_2 in the gas phase of the test tubes were measured three times every 30 min and hydrogenase activity was calculated. Reproducibility was confirmed by 2 – 4 runs for each result.

Results and Discussion

Cell growth

The cells of *Synechocystis* sp. strain PCC 6803 for H_2 production were prepared by cultivating photoautotrophically in BG-11 medium, BG-11 medium-N and BG-11 medium+NaCl and photomixotrophically in BG-11 medium+D-glucose and BG-11 medium+D-glucose-N. **Figure 1** shows the specific growth rate in the logarithmic growth phase (μ) and the intracellular D-glucose content (ρ_G) after 24 h of cultivation. Of special interest is the remarkable increase in ρ_G when NO_3^- is removed from BG-11 medium. This increase appears to be associated with the turnover of less futile intracellular proteins to supply nitrogen for the synthesis of the proteins for cell division. Phycocyanin degradation in photomixotroph of *Synechocystis* sp. strain PCC 6803 in the medium deficient in NO_3^- was reported (Elmorjani and herdman 1987). Specific growth rate in BG-11 medium-N is lower than that in the presence of NO_3^-. Data of Fig. 1 also shows that the value of μ of photomixotroph is higher than that of photoautotroph. Specific growth rate and intracellular D-glucose amount of *Synechocystis* sp. strain PCC 6803 are shown to be independent of the addition of 150 mmol L^{-1} NaCl and enhanced in the presence of 5.5 mmol L^{-1} D-glucose respectively. No effect of $NiCl_2$ in BG-11 medium is also shown in the experimental results (data not shown).
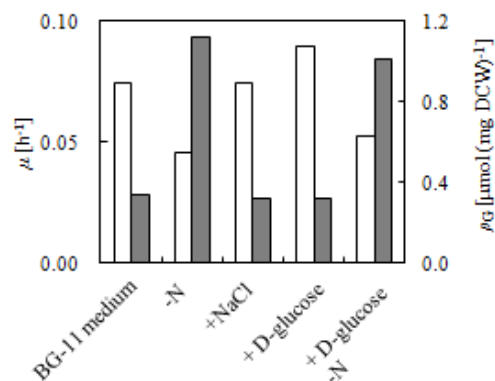

Figure 1: Effects of medium composition on the specific growth rate in the logarithmic growth phase (μ) (white) and the intracellular D-glucose content (ρ_G) (grey) after 24 h of photosynthesis cultivation.

Hydrogenase activity

Figure 2 shows the hydrogenase activity of *Synechocystis* sp. strain PCC 6803 in HEPES buffer solution in the anaerobic and dark condition. The activity was measured *in vivo* by utilizing reduced methyl viologen. The result shows that hydrogenase activity is apparently elevated by utilizing photoautotroph of *Synechocystis* sp. strain PCC 6803 grown in the presence of NaCl or NiCl$_2$. The specific growth rate of photoautotroph shown in Fig.1 was not effected by NaCl or NiCl$_2$, hence it is deduced that transcription of genes encoding for hydrogenase or its related gene appears to be effected by NaCl or NiCl$_2$. Similar observation was made in previous works (Kanesaki *et al*. 2002; Carrieri *et al*. 2008).

Histograms of Fig. 2 show that the hydrogenase activity of photomixotroph is slightly lower than that of photoautotroph. This observation is supported by an earlier report that showed that organic substrates for fast growth led to hydrogenase repression (Friedrich *et al*. 1981). In fact, our data in Fig. 1 showed that the growth of photomixotroph was higher than that of photoautotroph.

The result of the experiments shows that the hydrogenase activity of nitrogen starved cell is slightly higher than that in BG-11 medium, especially without D-glucose. High ρ_G in Fig. 1 represents that nitrogen starved cells are rich in reductants. It is deduced that the hydrogenase synthesis of nitrogen starved cells appears to be up-regulated to eliminate excess reductants produced by the degradation of glucan (Appel and Schulz 1996).

H$_2$ production

A series of H$_2$ production experiments were performed in which *Synechocystis* sp. strain PCC 6803 that was prepared by harvesting the photosynthetic culture on the various medium conditions and then anaerobically suspended in HEPES buffer in the dark. **Figure 3** shows the time courses of the amount of H$_2$ per unit volume of cell suspension (y_{H2}). The H$_2$ production at 96 h reaches the highest value of 3.64 µmol mL^{-1} when cells grown photoautotrophically on BG-11 medium-N are utilized. This amount is 1.94 times H$_2$ production by utilizing cells grown on BG-11 medium. The experimental result shown in Fig.1 that ρ_G of the cells grown in BG-11 medium-N is the highest among those grown in different media suggests that hydrogen production is largely effected by ρ_G. At first sight, this viewpoint is quite unlike that found in the run with cells prepared in BG-11 medium+D-glucose-N. These cells show the second highest ρ_G while produced no hydrogen for first 72 h. The H$_2$
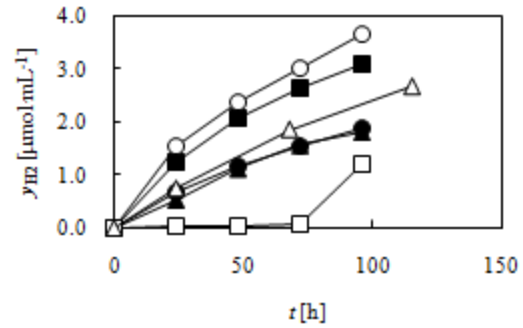
Figure 3: Time courses of the amount of H$_2$ per unit volume of cell suspension (y_{H2}). Cells for those experiments were photosynthetically prepared on BG-11 medium (●), BG-11 medium-N (○), BG-11 medium+NaCl (▲), BG-11 medium+NiCl$_2$ (△), BG-11 medium+D-glucose (■) and BG-11 medium+D-glucose-N (□).

production of 3.09 µmol mL^{-1} by cells grown on BG-11 medium+D-glucose is comparable to that by cells grown on BG-11 medium-N. In spite of similar hydrogen production activities, these cells show quite different ρ_G in Fig.1. Despite the fact that hydrogenase activity is up-regulated by cultivating photoautotrophs in the presence of NaCl or NiCl$_2$, there are no remarkable increases in H$_2$ production by cultivating photoautotrophs in the presence of NaCl or NiCl$_2$. *Synechocystis* sp. strain PCC 6803 grown in BG-11 medium+D-glucose shows the second highest production of H$_2$. Photomixotroph that is prepared in the presence of NO$_3^-$ is found to have a high potential to produce H$_2$. Contrary to this observation, photomixotroph grown in the absence of NO$_3^-$ shows a low activity for H$_2$ production. Taking into account that there is no such difference in the hydrogenase activities of those two photomixotrophs, cellular compositions other than hydrogenase are found to play a key role for H$_2$ production in the dark.

Effects of the glucan degradation and the cell decomposition upon H$_2$ production

The production of H$_2$ in *Synechocystis* sp. strain PCC 6803 is known to be catalyzed by a bidirectional hydrogenase utilizing NAD(P)H as a substrate. The discrepancy in the results shown in Fig. 2 and those shown in Fig. 3 appears to indicate the fact that H$_2$ production by the bidirectional hydrogenase of *Synechocystis* sp. strain PCC 6803 is limited by the supply of reductants for hydrogenase. NAD(P)H is a key reductant that is produced by a number of metabolic reactions within a cell. The reducing power that is accumulated during photosynthesis is reserved mainly in intracellular glucan or cell constituting materials (e.g. protein). In this study, the amount of cell constituting materials is provisionally represented by dry cell mass concentration.

Figures 4 and **5** show the time courses of X and $c_G = \rho_G X$ during H$_2$ production. Either X or c_G is found to decrease during hydrogen production. The result of the runs with cells from BG-11 medium, BG-11 medium+NaCl and BG-11 medium+NiCl$_2$ represents negligible small change in X and clear change in c_G after 24 h. If the supply of NAD(P)H limits the H$_2$ production, and if that supply is preferable to the consumption of glucan concentration in a cell suspension (-$\Delta c_G = c_{G,0} - c_G$), then H$_2$ production is related to the following equation:

$$y_{H2} = k_G (-\Delta c_G) \qquad (1)$$

Subscript 0 represents the initial state. The coefficient k_G, which is the overall fractional yield of hydrogen over intracellular glucan, is
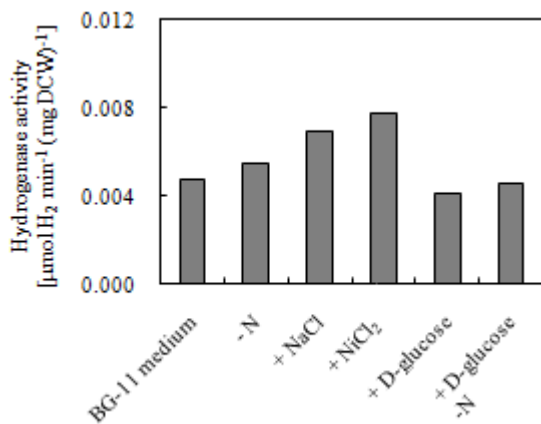

Figure 2: Effect of medium composition for photosynthesis upon the hydrogenase activity of *Synechocystis* sp. strain PCC 6803 in subsequent experiments, in which cells are suspended in HEPES buffer solution under the anaerobic and dark condition.

determined as 3.6 μmol μmol^{-1} for cells grown on BG-11 medium, 3.6 μmol μmol^{-1} for cells grown on BG-11 medium+NaCl, and 5.7 μmol μmol^{-1} for cells grown on BG-11 medium+NiCl$_2$. Glucan content ρ_G of cells for these runs shown in Fig.1 is quite similar. It is noteworthy that cells grown in the medium containing Ni^{2+} results 58% increase in k_G from those grown without Ni^{2+}. There is a possibility that addition of Ni^{2+} during cell preparation changes an intracellular state or pathway for H$_2$ production mechanism.

Different from these 3 runs apparent change in X is seen in the results of runs with cells from BG-11 medium-N and BG-11 medium+D-glucose. In the run with cells grown in BG-11 medium+D-glucose, shift in cellular environment from photomixotrophic growth condition to dark buffer solution appears to be a great burden for cells to survive and to result cell breakdown. No change in c_G during first 48 h is seen in this experiment. It is quite conceivable that cells grown in BG-11 medium-N and those grown in BG-11 medium+D-glucose-N have a high activity for protein turnover during photosynthesis. Shift in cellular environment from light illuminated nitrogen starved condition to dark buffer solution appears to overload cells to trigger cell breakdown, although the trend of H$_2$ production by cells grown in BG-11 medium+D-glucose-N shows the quite different from the other cells (Fig. 3). Changes in y_{H2} clearly shown in the runs with cells grown in BG-11 medium+D-glucose or BG-11 medium-N are likely parallel to that in X. If the supply of NAD(P)H limits the H$_2$ production, and if that supply in these 2 runs is preferable to the loss of dry cell mass concentration ($-\Delta X = X_0 - X$), then H$_2$ production is related to following equation:

$$y_{H2} = k_X (-\Delta X) \qquad (2)$$

in which k_X is the overall fractional yield of hydrogen over biomaterials. The coefficient k_X is determined as 4.3 μmol mg^{-1} for cells grown on BG-11 medium-N, and 5.9 μmol mg^{-1} for cells grown on BG-11 medium+D-glucose. Nitrogen starvation appears to result cell breakdown partially without producing NAD(P)H.

Figure 6 shows that y_{H2} values estimated by Eqs. (1) and (2) agree relatively well with those observed experimentally. The average error in estimating y_{H2} by Eq. (1) is 23% and that by Eq. (2) is 8%. Molecular weight of D-glucose is 0.180 mg μmol^{-1}, hence k_G of 3.6 μmol μmol^{-1} is equivalent to 16.7 μmol mg^{-1}. Comparing this with above k_X, it is found that unit mass glucan produces more NAD(P)H than unit cell mass. Glucan is proved to storage more reducing power than other cell constituting materials. These results show that cell preparation methods affect H$_2$ production mechanism. Cellular materials which are substrate of main NAD(P)H producer and their degradation pathway should be determined in detail for efficient and rapid H$_2$ production. It is deduced that supply of materials which have a high reduction potential also enhances H$_2$ production.

Volume of photobioreactor to prepare the biomass capable of producing one mole of H$_2$

The volume of a photobioreactor to prepare the biomass capable of producing one mole of H$_2$ is an important parameter to consider the H$_2$ productivity. From the results of cell growth during 24 hours cell preparation phase and H$_2$ production during H$_2$ production phase, the volume of a photobioreactor required by cells grown in BG-11 medium+D-glucose at 20.6 L is found to be lowest. This cell preparation method is capable to reduce the cultivation volume for H$_2$ production by 41.2% comparing with the run with cells grown in BG-11 medium photoautotrophically. On the other hand, cells prepared in BG-11 medium-N showed the almost same yield as ones prepared in the BG-11 medium due to its low growth rate although
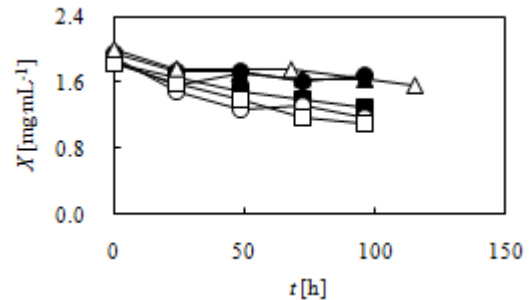

Figure 4: Time courses of cell mass concentration (X) during H$_2$ production by *Synechocystis* sp strain PCC6803 in buffer solution in the dark. Cells for those experiments were photosyntically prepared on BG-11 medium ($\bullet$), BG-11 medium-N ($\circ$), BG-11 medium+NaCl ($\blacktriangle$), BG-11 medium+NiCl$_2$ ($\triangle$), BG-11 medium+D-glucose ($\blacksquare$) and BG-11 medium+D-glucose-N ($\square$).
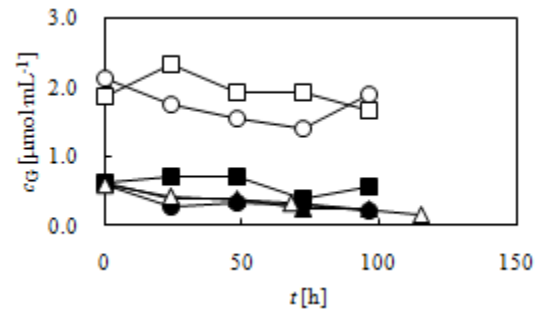

Figure 5: Time courses of the amount of intracellular glucan in a unit volume of culture (c_G) during H$_2$ production by *Synechocystis* sp strain PCC6803 in buffer solution in the dark. Cells for those experiments were photosyntically prepared on BG-11 medium ($\bullet$), BG-11 medium-N ($\circ$), BG-11 medium+NaCl ($\blacktriangle$), BG-11 medium+NiCl$_2$ ($\triangle$), BG-11 medium+D-glucose ($\blacksquare$) and BG-11 medium+D-glucose-N ($\square$).
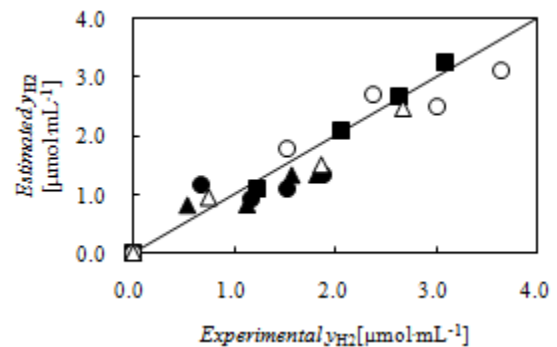

Figure 6: Comparison of the experimental and estimated H$_2$ producion (y_{H2}) data. Cells for those experiments were photosyntically prepared on BG-11 medium ($\bullet$), BG-11 medium-N ($\circ$), BG-11 medium+NaCl ($\blacktriangle$), BG-11 medium+NiCl$_2$ ($\triangle$) and BG-11 medium+D-glucose ($\blacksquare$). Diagonal line shows equivalence.

they showed the highest H$_2$ production. Cell preparations in BG-11 medium with NaCl or NiCl$_2$ also had the less effect on shortage of a cultivation volume. Cells grown in BG-11 medium+D-glucose-N required three times of the volume of a photobioreactor producing the same amount of H$_2$ as ones grown in BG-11 medium photoautotrophically.

Conclusions

Six types of cell preparations for *Synechocystis* sp. strain PCC 6803 followed by H_2 production in the dark were performed. The highest H_2 production in the dark was 3.64 $\mu mol\ mL^{-1}$ that was achieved by utilizing cells grown on BG-11 medium-N. This is 1.94 times higher than cells grown on BG-11 medium. The comparable amount of 3.09 $\mu mol\ mL^{-1}$ is shown as a result of BG-11 medium+D-glucose. The high hydrogenase activities induced by NaCl or $NiCl_2$ did not result in increasing in H_2 production. The H_2 yields for intracellular glucan (k_G) or cell components (k_X) were calculated from the results of the time course of X, c_G and y_{H2}. H_2 production by cells prepared in BG-11 medium and BG-11 medium with NaCl or $NiCl_2$ related with the consumption of intracellular glucan. On the other hand H_2 production by cells grown in BG-11 medium+D-glucose or BG-11 medium-N related with the decreasing in dry cell mass concentration. In future, cellular material and degradation pathway should be determined in detail and their degradation should be enhanced. Finally, the cells prepared in BG-11 medium+D-glucose resulted in reduction of a volume of photobioreactor required to produce unit volume of H_2 by 41.2% than the ones prepared in BG-11 medium.

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