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Biohydrogen Production by Microalgae Isolated from the Rice Paddle Field in Thailand

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Abstract

Hydrogen produced by cyanobacteria and green algae is a very interesting energy carrier because it is produced by a photosynthetic pathway using sunlight as an energy source. In this study, 59 cyanobacterial and green algal strains were isolated from soil and water sources of rice paddle field in Thailand. Out of them, 9 cyanobacterial isolates and 9 green algal isolates were purified. Among them, unicellular cyanobacterial isolate AngS1 showed the highest H_2 production rate. Its highest H_2 production rate of 389.630±72.084 nmolH_2 mg chl^{-1} h^{-1} was found in cells grown in BG11 for 1 week followed by incubating cells in $BG11_0$ for 24 hours and adaptation under dark anaerobic condition for 2 hours. The optimal concentrations of glucose, $MgSO_4.7H_2O$ and Fe^{3+} for H_2 production rate were 0.189 mmolC L^{-1}, 3 mM, and 20 µM, respectively. The highest H_2 accumulation of 4,174.364±278.324 nmolH_2 mg chl^{-1} was obtained when incubating cells in the optimal medium for 11 days.

Keywords : *Hydrogen production, Microalgae, Rice field*

1. Introduction

Molecular hydrogen is one of the interesting energy carriers in the future. It provides a high heating value and is a clean and environmental friendly fuel. Nowadays H_2 is mainly produced by the steam reforming process from the petrochemical industry; however it can be produced by various kinds of microorganisms. Microalgae including cyanobacteria and green algae are capable of producing H_2 from hydrogenase activity via a direct photolysis of water splitting during a photosynthetic process (1). Some N_2-fixing cyanobacteria can produce H_2 from nirogenase activity through a nitrogen fixation process (2,3). In addition, some species of cyanobacteria can produce H_2 via the degradation of accumulated glycogen under a dark fermentation (4).

In this study, H_2 production by microalgae isolated from the rice paddle field in Thailand was investigated. There are several kinds of microorganisms living in the rice field because rice field soil and water are fertile with nutrients and elements that are essential for microalgal growth. In addition, the climate, sunlight intensity, moisture content and other environmental factors in Thailand are suitable for microalgal cultivation. These bring about the existence of diverse kinds of living and survival green algae and cyanobacteria in the rice paddle field.

Several environmental factors, such as light intensity, anaerobic adaptation time, nutrient and element compositions, play important roles in microalgal H_2 production. The important elements in the medium, i.e., nitrogen, sulfur, and carbon, are essential for algal growth. It has been reported that lack of nitrogen and/or sulfur causes an increase of H_2 production in many cyanobacterial and green algal species (5-13). In addition, H_2 production by some microalgal species is enhanced under different sugar sources such as glucose (12-16) and fructose (17,18). Because iron is a cofactor of cyanobacterial and green algal hydrogenases (19); therefore iron concentration in the medium directly affects hydrogenase activity and H_2 yield (20-26). Besides the nutritional and mineral effects, light intensity has been reported to influence cyanobacterial and green algal H_2 production (4,27).

In this study, microalgae were isolated from the rice paddle fields in 7 provinces of Thailand, purified and screened for high H_2 production under nitrogen-deprived condition. The effects of some physiological factors such as anaerobic adaptation time, nutrient and mineral compositions under nitrogen starvation were investigated on H_2 production rate by the selected microalgal isolate.

2. Materials and Methods

2.1 Microalgal isolation

Microalgae including cyanobacteria and green algae were isolated from soil and water samples in the rice paddle fields from 7 provinces of Thailand (Angthong, Chainat, Mahasarakham, Nakhon Ratchasima, Nakhon Sawan, Pathumthani and Singburi). Soil and water samples were incubated in BG11 medium under light intensity of 30 μmol photon m^{-2} s^{-1} at 30 ^oC for 2 weeks. BG11 medium contains 17.6 mM $NaNO_3$, 3.0 mM $MgSO_4.7H_2O$, 0.24 mM $CaCl_2.2H_2O$, 0.188 mM Na_2CO_3, 0.18 mM K_2HPO_4, 27.9 μM Na_2EDTA, 31.2 μM citric acid, 46.3 μM H_3BO_3, 4.2 μM $MnCl_2.4H_2O$, 0.77 μM $ZnSO_4.7H_2O$, 1.66 μM $NaMoO_4.2H_2O$, 0.32 μM $CuSO_4.5H_2O$, 0.17 μM $Co(NO_3)_2.6H_2O$ and 22.5 μM $FeNH_4$.citrate (28). Microalgae were purified by single cell isolation technique (29). The bacterial contamination of isolated microalgae was checked by streaking on LB agar.

2.2 Microalgal cultivation

The purified microalgae were grown in a 250-mL Erlenmeyer flask containing 100 mL of BG11. They were shaken at 120 rpm under light intensity of 30 μmol photon m^{-2} s^{-1} at 30 ^oC for 1 week.

2.3 Hydrogen measurement

One hundred mL of cell culture was harvested by centrifugation at 7,000xg at 4^oC for 10 min. The cell pellet was washed twice followed by resuspension in 5 mL of the medium. The cell suspension was transferred to a 10-mL glass vial. The vial was sealed with a rubber stopper and

purged with argon gas for 10 min in darkness. Cells were incubated at 30 °C under darkness for 2 h before analyzing the gas phase. H_2 production was determined by analyzing the gas phase using the Gas Chromatograph as previously described (12).

2.4 Chlorophyll measurement

One hundred µL of cell culture was added with 900 µL of absolute methanol. The mixture was mixed by vortexing and incubated in darkness for 1 h before centrifugation at 12,000xg for 2 min. Chlorophyll concentration of extract was determined by measuring the absorbance at 665 nm for cyanobacteria, and at both 665and 650 nm for green algae, and calculated followed by Lee and Shen (2004) (30).

3. Results

3.1 Screening for a high H_2 producing microalgal strain

A total of 59 cyanobacterial and green algal strains were isolated from soil and water samples of rice paddle fields in 7 provinces of Thailand. Unfortunately, only 9 cyanobacterial isolates and 9 green algal isolates were purified (Table 1). They were cultivated in BG11 medium for 2 weeks, then harvested and resuspended in BG11 and $BG11_0$ medium ($BG11_0$ has all chemical compositions similar to BG11 except that $NaNO_3$ is absent in the $BG11_0$ medium). The culture was incubated in the light for 24 h, followed by collecting the cells and incubating cells under dark anaerobic incubation for 2 h before determination of H_2 production. It was found that all cyanobacteria hardly produced H_2 under normal condition (in BG11 medium) but most cyanobacteria obviously produced H_2 under nitrogen deprivation condition (in $BG11_0$ medium) (Table 1). In contrast, H_2 production rate by five green algal strains was detected when incubated in BG11 medium eventhough it was lower than that in $BG11_0$ medium (Table 1). The unicellular cyanobacterial isolate AngS1 showed the highest H_2 production rate with 256.612±34.267 nmolH_2 mg chl^{-1} h^{-1} when incubating cells in $BG11_0$ for 2 h. It is unicellular, has a round shape and cell diameter with1-5µm (Fig. 1). Therefore, AngS1 was selected as a potential H_2 producing microalgal strain for further experiments.

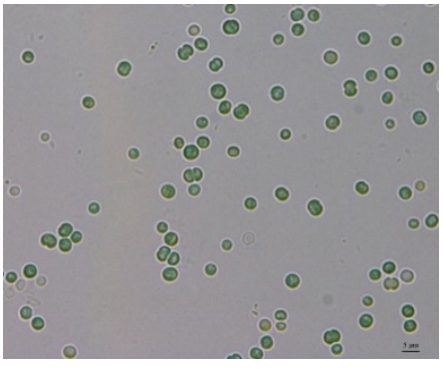

Figure 1. Cell morphology of the cyanobacterium AngS1 observed under a light microscope

Table 1. Hydrogen production rate by 9 cyanobacterial and 9 green algal strains isolated from the rice field in Thailand

Isolates	Hydrogen production rate ($nmolH_2$ mg chl^{-1} h^{-1})	
	BG11	**$BG11_0$**
Cyanobacteria		
2SinS3	-	-
A34	-	111.588±25.581
A36	-	11.916±1.083
A47.1	-	39.919±1.466
AngS1	-	256.612±34.267
B14	-	88.049±10.929
B35.1	-	152.762±18.387
ChiS5	-	6.305±0.812
Cyano	-	237.942±32.353
Green algae		
1SinS1.1	-	3.701±0.334
2SinS4	23.305±1.812	40.665±7.960
2TKS2.1	-	5.973±0.385
2TKS2.2	6.605±1.913	8.618±0.636
2TKW1	-	51.006±0.135
A27	19.139±2.852	23.633±0.531
ChiS4	21.666±2.008	53.248±1.229
ChiW1	12.648±0.019	15.924±1.040
NakS4	-	15.785±2.202

3.2 Effect of anaerobic adaptation time and light on H_2 production

The cyanobacterium AngS1 grown in BG11 for 1 week followed by incubating cells in $BG11_0$ for 24 h and adaptation under dark anaerobic condition for 2 h yielded the highest H_2 production rate with 389.630±72.084 $nmolH_2$ mg chl^{-1} h^{-1} (Fig. 2). H_2 production rate in the dark was obviously higher than that in the light (Fig. 2). Longer duration of incubation up to 48 h did not increase H_2 production rate. In this study, incubation of cells for 2 h under anaerobic dark condition before H_2 analysis was used for further experiments.

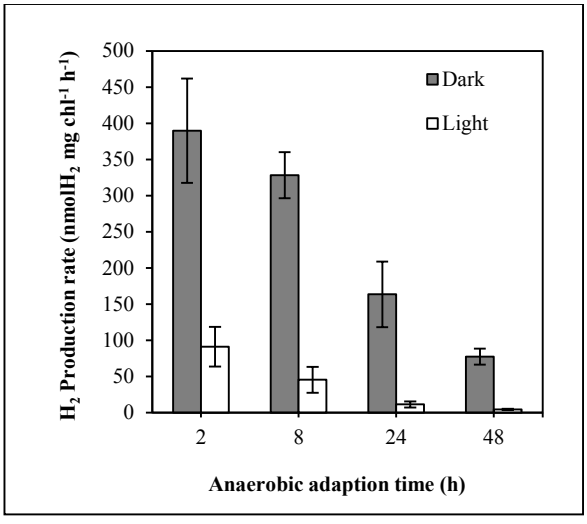

Figure 2. H_2 production rate by AngS1 under various anaerobic adaptation time in the light and dark

3.3 Effect of carbon sources and concentrations on H_2 production

In general, cyanobacteria can fix CO_2 in the atmosphere to generate their own carbon sources via Calvin-Benson cycle. Therefore carbon sources used in this experiment (0-18.9 mmolC L^{-1} Na_2CO_3 and glucose) are not utilized for cyanobacterial growth but are used as electron donors for hydrogenase enzyme. BG11 medium normally contains 0.189 mmolC L^{-1} Na_2CO_3. In this study, the highest H_2 production rate with 601.052±24.288 nmolH_2 mg chl^{-1} h^{-1} was found in AngS1 incubated in $BG11_0$ containing 0.189 mmolC L^{-1} glucose (Fig. 3B), followed by H_2 production rate with 522.513±63.335 nmolH_2 mg chl^{-1} h^{-1} obtained from cells incubated in $BG11_0$ containing 1.89 mmolC L^{-1} Na_2CO_3 (Fig. 3A).

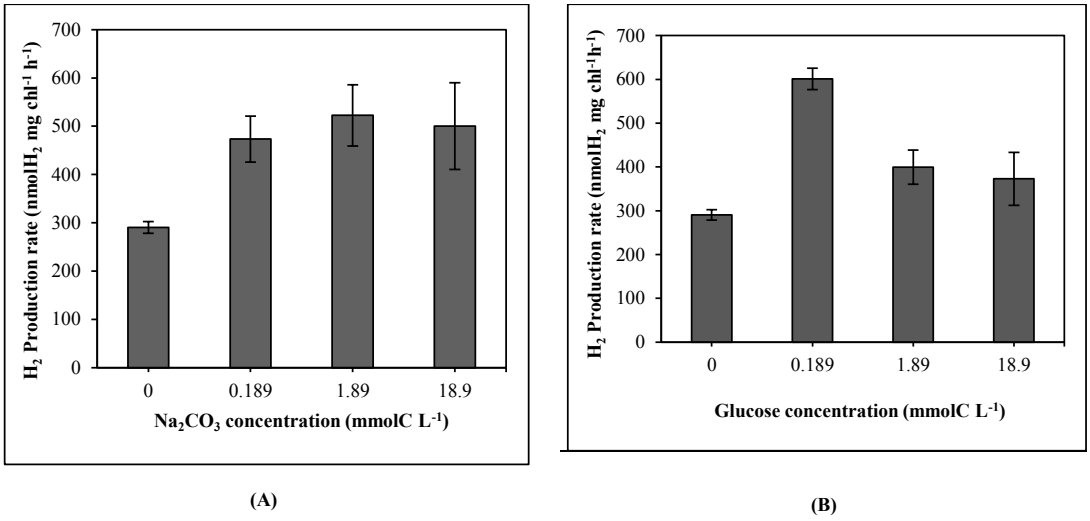

Figure 3. Effect of Na_2CO_3 concentrations (A) and glucose concentrations (B) on H_2 production rate by AngS1

3.4 Effect of sulfate concentrations on H_2 production

The main source of sulfate in BG11 medium is $MgSO_4.7H_2O$ that contains 30 mM. Under various $MgSO_4.7H_2O$ concentrations, the highest H_2 production rate with 549.066±110.482 $nmolH_2$ mg chl^{-1} h^{-1} was obtained in AngS1 incubated in $BG11_0$ containing 3.0 mM $MgSO_4.7H_2O$ (Fig. 4). However, it did not show much difference with other $MgSO_4.7H_2O$ concentrations.

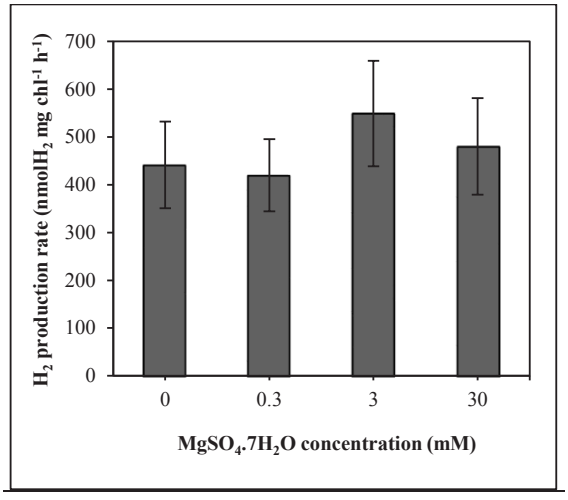

Figure 4. Effect of $MgSO_4.7H_2O$ concentrations on H_2 production rate by AngS1

3.5 Effect of Fe^{3+} concentrations on H_2 production

Under various Fe^{3+} concentrations, the highest H_2 production rate with 420.199±75.530 $nmolH_2$ mg chl^{-1} h^{-1} was found in cells incubated in $BG11_0$ medium containing 20 μM Fe^{3+}(Fig. 5). This Fe^{3+} concentration is 5-fold higher than that in BG11 medium. H_2 production rate was reduced in cells incubated in Fe^{3+}-free $BG11_0$ medium and $BG11_0$ medium containing 200 and 2,000 μM Fe^{3+}.

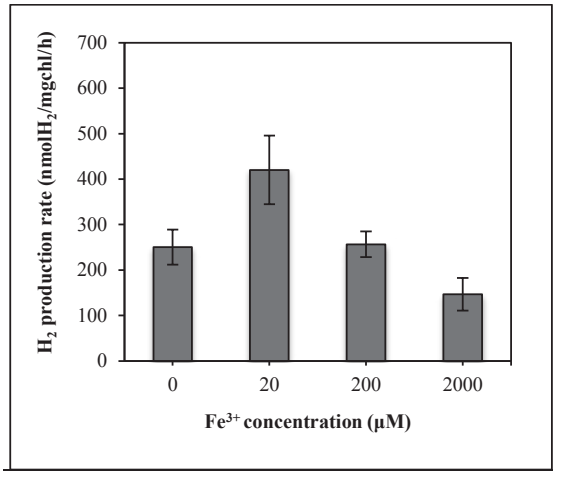

Figure 5. Effect of Fe^{3+} concentrations on H_2 production rate by AngS1

3.6 Long-term dark fermentative H_2 accumulation by AngS1 in optimal medium

The cyanobacterium AngS1 was grown in BG11 for 1 week. The culture was harvested by centrifugation, washed and resuspended in BG11, $BG11_0$ and optimal $BG11_0$ (containing 0.189 mmolC L^{-1} glucose, 3.0 mM $MgSO_4.7H_2O$ and 20 µM Fe^{3+}). The cells were purged with argon gas for 10 min and incubated in the indicated medium under darkness for 18 days. The result showed that H_2 production rate by cells incubated in optimal $BG11_0$ medium was 996.165±71.349 nmolH_2 mg chl^{-1} h^{-1}or 0.996±0.071 µmolH_2 mg chl^{-1} h^{-1}. It was rapidly increased within24 h of incubation. The H_2 accumulation reached the maximum yield with 4,174.364±278.324 nmolH_2 mg chl^{-1} at day 11th of dark anaerobic incubation (Fig. 6), approximately 3-fold higher than that by cells incubated in $BG11_0$ medium. After 11 days of incubation, H_2 yield from cells in both media was significantly decreased.

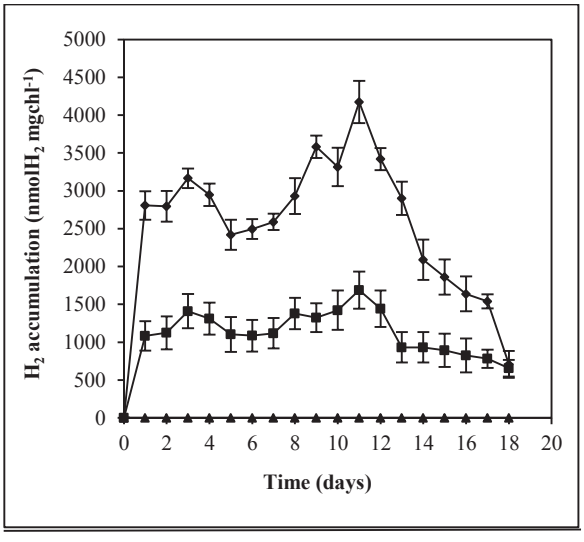

Figure 6. Long-term H_2accumulation by AngS1. Cells, initially grown in BG11 for 7 days, were harvested, resuspended in BG11 (▲), $BG11_0$ (■) and optimal $BG11_0$ (◆), and further incubated at 30 °C for 24 h in the light. Then cells were harvested again and resuspended in the same medium before transferring to a glass vial. H_2 was measured in darkness.

4. Discussions

In this study, only 9 cyanobacterial isolates and 9 green algal isolates were purified from 59 microalgal strains isolated from the rice paddle field in Thailand. It was rather difficult to purify microalgal strains from their contamination because there were a numerous bacteria and fungi in soil and water samples. Among cyanobacterial strains, seven strains were filamentous whereas two strains were unicellular. Unexpectedly, unicellular cyanobacterium AngS1 isolated from Angthong province showed the highest H_2 production rate with 256.612±34.267 nmolH_2 mg chl^{-1} h^{-1} when incubating cells in $BG11_0$ under darkness for 2 h (Table 1). Under normal condition

(in BG11 medium) its H_2 production rate could not be detected. Normally there are 2 hydrogenase enzymes involved in H_2 evolution in cyanobacteria; nitrogenase found in filamentous N_2-fixing cyanobacteria and bidirectional hydrogenase found in all types of cyanobacteria. The unicellular cyanobacterium AngS1 was assumed to contain only bidirectional hydrogenase from the cellular structure. Under nitrogen deprivation, the fermentative glycogen in cells is accumulated in the light. At that time O_2 occurred during photosynthesis inhibited hydrogenase resulting in halting the H_2 evolution. When cells were transferred into the dark anaerobic condition, the accumulated glycogen was degraded and utilized as electron and proton donors for generating H_2 via a reactivated bidirectional hydrogenase.

H_2 production rate of AngS1 was highest after 2 h of dark anaerobic incubation (Fig. 2), indicating that AngS1, like most nonN_2-fixing cyanobacteria, produced H_2 after entering anaerobic phase at a short period of time (12,14). When incubating cells under the light anaerobic incubation, cells produced lower H_2 production rate. It was suggested that O_2 produced during photosynthesis in the light inhibited bidirectional hydrogenase activity.

To investigate the effect of carbon sources and concentrations on H_2 production rate by AngS1, 0-18.9 mmolC L^{-1} Na_2CO_3 and glucose were added in the medium. AngS1 could produce H_2in condition without carbon sources in the medium (Fig. 3). It was resulted from the existence of other electron and proton sources within cells. However, the addition of Na_2CO_3 or glucose caused the increased H_2 production rate by AngS1. The highest H_2 production rate with 601.052±24.288 nmolH_2 mg chl^{-1} h^{-1} was found in AngS1 incubated in $BG11_0$ containing 0.189 mmolC L^{-1} glucose (Fig. 3B), indicating that AngS1 could metabolize glucose as a good substrate for H_2 production rather than Na_2CO_3. Under darkness the glucose metabolism led to an increase of NAD(P)H which was utilized as substances for H_2 production in the cells. Similar results have already been reported in the unicellular cyanobacteria *Microcystis aeruginosa*, *Synechocystis* sp. PCC 6803, the N_2-fixing cyanobacterium *Nostoc muscorum* where glucose was a good H_2 producing substrate (14-16). In addition, H_2 production rate was decreased when glucose concentration was higher than 0.189 mmol C L^{-1} (Fig. 3B), suggesting that too high glucose concentration inhibited hydrogenase activity by using energy for driving the excessive sugar out of the cells.

Under various $MgSO_4.7H_2O$ concentrations, the highest H_2 production rate was obtained in AngS1 incubated in $BG11_0$ containing 3.0 mM $MgSO_4.7H_2O$ (Fig. 4); however it was not obviously higher than other $MgSO_4.7H_2O$ concentrations. In cyanbacteria, lack of sulfate in medium did not show much effect compared to lack of nitrate (13) whereas in green algae lack of sulfate affected cells by reduction of photosystem II activity, resulting in a decrease of O_2 and subsequently reactivation of hydrogenase activity (8).

The highest H_2 production rate was found in AngS1 incubated in $BG11_0$ medium containing 20 µM Fe^{3+} (Fig. 5). It was about 2-fold higher than that of cells incubated in Fe^{3+}-free BG11. Iron is known as a cofactor of many enzymes including nitrogenase and hydrogenase involving in H_2 evolution in cyanobacteria (3,19).

Higher concentration of Fe^{3+} up to 200 µM increased H_2 production rate by promoting the electron flow towards hydrogenase. Similar results have been reported in many cyanobacteria (12,23). However, at too high Fe^{3+} concentration H_2 production rate by AngS1 was decreased due to the toxicity of Fe^{3+} in cells.

In optimal $BG11_0$ medium (containing 0.189 mmolC L^{-1} glucose, 3.0 mM $MgSO_4.7H_2O$ and 20 µM Fe^{3+}), the H_2 production rate by AngS1 was 996.165±71.349 nmolH_2 mg chl^{-1} h^{-1}or 0.996±0.071 µmolH_2 mg chl^{-1} h^{-1}. Compared with H_2 production rate by other cyanobacteria, this rate is higher than that in other unicellular cyanobacteria but lower than filamentous N_2-fixing cyanobacteria (10). The highest long-term dark fermentative H_2 accumulation by AngS1 was obtained in cells incubated in optimal $BG11_0$ medium (containing 0.189 mmolC L^{-1} glucose, 3.0 mM $MgSO_4.7H_2O$ and 20 µM Fe^{3+}) with the H_2 yield of 4,174.364±278.324 nmolH_2 mg chl^{-1} at day 11^{th} of dark anaerobic incubation (Fig. 6). It is approximately 3-fold higher than H_2 accumulation by cells incubated in $BG11_0$ medium (containing 0.189 mmolC L^{-1} Na_2CO_3, 30 mM $MgSO_4.7H_2O$ and 4 µM Fe^{3+}), indicating that carbon sources, the decreased sulfate concentration and the increased iron concentration resulted in the higher H_2 production in AngS1. The proper adjustment of nutrient and minerals compositions in medium could promote long-term H_2 accumulation.

5. Conclusion

In conclusion, unicellular cyanobacterium AngS1 isolated from the rice paddle field in Thailand is one of high potential H_2 producing cyanobacterial species. It produced H_2 via a dark fermentation under anaerobic condition. The highest H_2 accumulation with 4,174.364±278.324 nmolH_2 mg chl^{-1} was obtained in cells incubated in $BG11_0$ medium containing 0.189 mmolC L^{-1} glucose, 3.0 mM $MgSO_4.7H_2O$ and 20 µM Fe^3 at day $11^{th}$of dark anaerobic incubation.

6. Acknowledgement

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