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Factors affecting photobiological hydrogen production in five filamentous cyanobacteria from Thailand

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Abstract

We report here the screening of sixteen cyanobacterial and three green algal strains from Thailand for their potential bio-hydrogen production. Five filamentous cyanobacterial species, namely *Calothrix elenkinii*, *Fischerella muscicola*, *Nostoc calcicola*, *Scytonema bohneri*, and *Tolypothrix distorta*, all possessing nitrogenase activity, showed potentially high bio-hydrogen production. These five strains showed higher hydrogen production in the absence than in the presence of nitrogen. In particular, *F. muscicola* had a 17-fold increased hydrogen production under combined nitrogen and sulfur deprived conditions. Among various sugars as a carbon source, glucose at 0.1% (w/v) gave the maximal hydrogen production of 10.9 μmol(H_2) mg^{-1}(Chl) h^{-1} in *T. distorta* grown in BG11 medium without nitrate. Increasing light intensity up to 250 μmol(photon) m^{-2} s^{-1} increased hydrogen production in *F. muscicola* and *T. distorta*. Overall results indicate that both *F. muscicola* and *T. distorta* have a high potential for hydrogen production amenable for further improvement by using molecular genetics technique.

Additional key words: culturing parameters; heterocyst; N_2-fixing condition.

Introduction

Presently the global climate change attributed mainly to the usage of fossil fuels causes environmental problems in many parts of the world. Hence, there is an urgent need for new forms of renewable energy, which is environmental friendly and efficient, to replace limited fossil sources. Hydrogen is considered an ideal energy carrier for the future because of its high heating value and no carbon dioxide generation upon combustion (Møller *et al*. 2017). Cyanobacteria exhibit their capacity to convert captured solar energy to H_2 (Khetkorn *et al*. 2017). In direct biophotolysis, light energy captured by the PSII is used to split water, producing molecular oxygen, protons, and electrons. In cyanobacteria, the reduced ferredoxin provides electrons to reduce protons to molecular hydrogen catalyzed by hydrogenase. In indirect biophotolysis, photosynthetic water splitting with consequent generation of reduced ferredoxin enables the reduction of CO_2 to carbohydrates which can be used to drive H_2 production. On the other hand, instead of utilizing the light-driven reduction of ferredoxin, the cyanobacterial hydrogenase was found to receive its electrons *via* pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR) – flavodoxin/ferredoxin for dark fermentative hydrogen production (Gutekunst *et al*. 2014). There are at least three enzymes in cyanobacteria which are directly involved in hydrogen production. Hydrogenases comprise an uptake

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Abbreviations: Chl – chlorophyll; *hox* gene – bidirectional hydrogenase gene; *hup* gene – uptake hydrogenase gene; PCC – Pasteur Culture Collection; TISTR – Thailand Institute of Scientific and Technological Research.
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hydrogenase encoded by *hup* genes present in all N_2-fixing strains, and a bidirectional hydrogenase (reversible hydrogenase) encoded by *hox* genes present in N_2-fixing and non-N_2-fixing cyanobacteria (Tamagnini *et al.* 2007). Nitrogenase complex, which generates hydrogen as a byproduct, is present in both filamentous N_2-fixing and unicellular N_2-fixing cyanobacteria (Bothe *et al.* 2010). Hydrogen produced from nitrogenase can be used as a substrate for uptake hydrogenase, resulting in a decrease in net amount of hydrogen (Hansel and Lindblad 1998). To increase hydrogen production, one possible improvement is the inhibition of the uptake hydrogenase activity (Tamagnini *et al.* 2002). Nitrogenase is also very sensitive to oxygen (Fay 1992). This explains why some filamentous cyanobacteria develop specialized cells called heterocysts with no oxygen evolving activity of PSII (Masukawa *et al*. 2001). Furthermore, different species have different capacities to produce hydrogen whereby the production rate can be further affected by external factors (Allahverdiyeva *et al*. 2010). There is a wide variety among cyanobacteria in their inherited activity to produce hydrogen. Cyanobacteria grown under nutrient limitation conditions show increased H_2 production. The nitrogenase activity is inhibited by external nitrogen sources, such as nitrite, nitrate, and ammonia (Bothe *et al.* 2010). The absence of nitrogen and sulfur affects the transcription of the *hox* gene and enhances the production of H_2 (Antal and Lindblad 2005, Baebprasert *et al*. 2010). Sulfur deprivation can decrease PSII activity by inducing respiration to be higher than photosynthesis with subsequent production of hydrogen (Fouchard *et al.* 2005). The *Nostoc* sp. PCC7422 disrupted in uptake hydrogenase showed three times higher nitrogenase activity-based hydrogen production than that of the wild type (Yoshino *et al.* 2007). In addition, the increase of light intensity enhances the hydrogen production of *Phormidium valderianum* and *Halobacterium halobium* (Patel and Madamwar 1994), whereas *Spirulina platensis* and *Arthrospira* sp. PCC8005 produce hydrogen under anaerobic conditions in darkness (Aoyama *et al.*1997, Raksajit *et al*. 2012).

There are considerable differences between various photosynthetic organisms in their ability to produce hydrogen. Screening of species for their natural production activity has not been performed in broad scale so far. In the present work, we screened for new species with high hydrogen productivity. We used sixteen cyanobacterial species and three green algae obtained from Thailand Institute of Scientific and Technological Research (TISTR). Furthermore, we investigated the effect of external factors, such as nutrient deprivation, carbon source, pH, temperature, and light intensity on hydrogen production of the most efficient strain.

Materials and methods

Strains and growth conditions: Sixteen cyanobacterial strains and three green algae were obtained from TISTR. The culture collection contains planktonic and benthic strains isolated mostly from Bangkok ponds and soils. For screening of hydrogen production, the cells were grown in liquid BG11 medium at pH 7.5 modified according to Stanier *et al*. (1971) under continuous white fluorescent illumination of 50 μmol(photon) m^{-2} s^{-1} at 30°C for 14 d before subjecting to hydrogen measurement. To study the effect of various external factors on hydrogen production, the cells were adapted under the desired tested conditions. For nitrogen or sulfur deprivation, the cells grown for 14 d as mentioned above were transferred to $BG11_o$ medium (BG11 lacking nitrate) at pH 7.5, or $BG11_o$ lacking sulfate ($BG11_o$ -S) at pH 7.5, where nitrate or sulfate is replaced by chloride, and further grown for 24 h prior to measurement of hydrogen. To examine the effect of other external factors, the cells grown as mentioned above for 14 d were transferred to $BG11_o$ and grown for 24 h under different tested conditions, *i.e*., pH, temperature, carbon source, and light intensity prior to hydrogen measurement.

Microscopic analysis of the strains: The general morphological observations of the most efficient strains were carried out at three days of growth in BG11 and $BG11_o$ media. The micrographs were obtained using an *Eclipse Ti-U* inverted light microscope (*Nikon*®, Japan).

Hydrogen production: One milliliter of harvested cells suspended in the same respective medium during an adaptation phase was transferred to a 20-ml vial with a butyl-rubber septum. The argon gas was blown into the vial at 1.4 kPa for 5 min to generate an anaerobic condition. The vials were placed on a rotatory shaker at 100 rpm for 24 h. Hydrogen in the head space of gas-tight vial was analyzed using a gas chromatography with argon as a carrier gas as previously described (Allahverdiyeva *et al.* 2010). The linear calibration curve with various H_2 concentrations (0, 0.05%, 0.1%, 0.25%, 0.5%, and 1.0% H_2) was done. The calibration up to 0.5% H_2, which is in a linear range, was used. Hydrogen production rate is expressed in μmol(H_2) mg^{-1}(Chl) h^{-1}. The determination of Chl content was done spectrophotometrically after extraction of cells with 90% (v/v) methanol (MacKinney 1941).

Nitrogenase activity was determined by the reduction of acetylene (C_2H_2). One milliliter of cell suspension in $BG11_o$ medium was transferred to a serum bottle with a butyl-rubber septum. Oxygen at the head space of the bottle was eliminated by purging with argon. The bottle was incubated on a rotatory shaker at 100 rpm, 30°C under illumination of 50 μmol(photon) m^{-2} s^{-1} for 20 h to induce nitrogenase. Acetylene (C_2H_2), the substrate of nitrogenase, was added to the bottle. The ethylene (C_2H_4) produced after 30 min incubation (Khetkorn *et al*. 2012)

was analyzed by *Perkin-Elmer AutoSystem* gas chromatograph equipped with flame ionization detector. Enzyme activity was expressed as μmol(C_2H_4) mg^{-1}(Chl) h^{-1}. The activity of nitrogenase from *Anabaena* PCC 7120 was used as a positive control.

Results and discussion

Screening of photosynthetic microorganisms for their capacity to produce hydrogen: Sixteen cyanobacterial strains and three green algae from TISTR were tested for their ability to produce hydrogen. Hydrogen production was observed under light/anaerobic conditions, whereas no production was observed in darkness or under aerobic conditions. A total of nineteen strains can be divided into three groups based on their ability of hydrogen production: (*1*) strains with efficient hydrogen production, (*2*) strains with detectable hydrogen production, and (*3*) strains with no detectable hydrogen production under the conditions studied. Group 1 consists of five strains of filamentous cyanobacteria with hydrogen production of at least 0.1 μmol(H_2) mg^{-1}(Chl) h^{-1} (Table 1) including *Calothrix elenkinii* (0.09 ± 0.01), *Fischerella muscicola* (0.35 ± 0.08), *Nostoc calcicola* (0.09 ± 0.01), *Scytonema bohneri* (0.09 ± 0.01), and *Tolypothrix distorta* (0.21 ± 0.05). Group 2 consists of four strains, *Ankistrodesmus falcatus*, *Aphanocapsa biformis*, *Chlorogloea fritshii*, and *Chroococcus turgidus*, which showed detectable, albeit a very low hydrogen production less than 0.1 μmol(H_2) mg^{-1}(Chl) h^{-1}. Group 3 consists of ten strains, *Coccomonas orbicularis*, *Gloeocapsa atrata*, *Lyngbya shackletoni*, *Microcystis pulverea*, *Myxosarcina burmensis*, *Oscillatoria salina*, *Oscillatoria subbrevis*, *Phormidium calcicola*, *Plectonema gracillimum*, and *Scenedesmus acuminatus*, which showed no hydrogen production under the conditions studied. The five strains of the group 1 were used for further studies of hydrogen production under various external conditions.

Cell structure: Cyanobacteria can utilize many sources of nitrogen to support their growth. Some non-N_2-fixing cyanobacteria can use combined nitrogen, such as nitrate, or ammonia, when available, and some N_2-fixing cyanobacteria are also able to fix dinitrogen by nitrogenase from atmosphere to produce ammonia and release H_2 as a by product. However, the absence of combined nitrogen, which creates N_2-fixing condition, induces the formation of heterocysts, where nitrogenase and uptake hydrogenase are located (Bothe *et al*. 2010). Moreover, it has been known that N_2-fixing cyanobacteria are more efficient in the production of hydrogen than non-N_2-fixing cyanobacteria. In the present study, five most actively hydrogen-producing cyanobacterial strains were used to study the morphological changes when grown in either BG11 with nitrate as nitrogen source (non-N_2-fixing) or BG11 without nitrate ($BG11_o$) (N_2-fixing) under continuous illumination of 50 μmol(photon) m^{-2} s^{-1} for three days at 30°C. Fig. 1 shows light microscope images of the five cyanobacterial strains under BG11 (non-N_2-fixing) (Fig. 1*A–E*) and $BG11_o$ (N_2-fixing) (Fig. 1*F–J*). Cells under non-N_2-fixing conditions were organized in long filaments of vegetative cells with no differentiation. On the other hand, under N_2-fixing condition, all five strains showed the appearance of heterocysts after three days. It seems that the development of heterocysts coincides with the ability of the cells to increase the hydrogen production.

Effect of nitrogen and sulfur deprivation on H_2 production: The five strains of cyanobacteria showed the heterocyst formation under N_2-fixing condition. Non-heterocystous and heterocystous cyanobacteria were reported to have different level of hydrogen production depending on growth medium (Dutta *et al*. 2005, Berberoğlu *et al*. 2008, Yeager *et al*. 2011). Cells were transferred to either BG11 medium without nitrate ($BG11_o$), or without both nitrate and sulphate ($BG11_o$-S). Cells grown without nitrate showed about a 4-fold increase in hydrogen production when compared to cells grown in the presence of nitrate (Fig. 2). For cells grown in the absence of both nitrate and sulphate, there were no significant changes in hydrogen production of most strains except for *F. muscicola* which showed about a 17-fold increase of hydrogen production (Fig. 2). Some green algae also showed an increase of hydrogen production when grown under the depletion of nitrogen and sulfur (Tsygankov *et al.* 2006, Maneeruttanarungroj *et al.* 2010). This is because not only the presence of active nitrogenase in heterocyst cells, but also sulfur deprivation inhibits the repair of D1 protein in PSII after photodamage (Melis *et al.* 2000). The reduced activity of PSII facilitates the restoration of anaerobic conditions in the culture resulting in the increase of hydrogen production. The results showed that most of five strains of cyanobacteria produce high concentration of hydrogen when grown in $BG11_o$. This condition was used as a control for next experiments.

Effect of external pH and temperature on H_2 production: In order to find the optimal pH for hydrogen production, the five most active strains were incubated in $BG11_o$ adjusted to pH 6.5, 7.5, and 8.5 for 24 h prior to the measurement of hydrogen. The amount of hydrogen produced by each strain was the highest at pH 7.5 (Fig. 3*A*). At pH 6.5 and 8.5, the production decreased, with the values ranging from 28–67% of the maximum and 50–70% of the maximum, respectively, among the five strains. Similar results have been observed in *Calothrix* XPORK 5E with a lower hydrogen production at pH 6.8 than that at pH 7.5 (Allahverdiyeva *et al.* 2010) and in

Table 1. Summary of the preliminary screening of hydrogen production of sixteen cyanobacterial strains and three green algae strains from Thailand.

No.	Strains	Cell type	Origin	Habitat	Hydrogen production rate [μmol(H_2) mg^{-1}(Chl) h^{-1}]			
					Aerobic		Anaerobic	
					50 μmol(photon) m^{-2} s^{-1}	Darkness	50 μmol(photon) m^{-2} s^{-1}	Darkness
1	*Ankistrodesmus falcatus*	Green algae	Bangkok	pond	0.003	0.000	0.010	0.000
2	*Aphanocapsa biformis*	Cyanobacteria	Bangkok	cement surface	0.002	0.000	0.010	0.000
3	*Calothrix elenkinii*	Cyanobacteria	Bangkok	pond	0.005	0.000	0.090	0.000
4	*Chlorogloea fritshii*	Cyanobacteria	Bangkok	pond	0.003	0.000	0.010	0.000
5	*Chroococcus turgidus*	Cyanobacteria	Bangkok	pond	0.002	0.000	0.010	0.000
6	*Coccomonas orbicularis*	Green algae	Bangkok	pond	0.001	0.000	0.001	0.000
7	*Fischerella muscicola*	Cyanobacteria	Suphanburi	soil	0.009	0.000	0.350	0.000
8	*Gloeocapsa atrata*	Cyanobacteria	Bangkok	pond	0.001	0.000	0.001	0.000
9	*Lyngbya shackletoni*	Cyanobacteria	Bangkok	pond	0.001	0.000	0.001	0.000
10	*Microcystis pulverea*	Cyanobacteria	Bangkok	pond	0.001	0.000	0.001	0.000
11	*Myxosarcina burmensis*	Cyanobacteria	Bangkok	pond	0.001	0.000	0.001	0.000
12	*Nostoc calcicola*	Cyanobacteria	Pathumthani	pond	0.005	0.000	0.090	0.000
13	*Oscillatoria salina*	Cyanobacteria	Samutprakan	cement surface	0.002	0.000	0.001	0.000
14	*Oscillatoria subbrevis*	Cyanobacteria	Bangkok	pond	0.002	0.000	0.001	0.000
15	*Phormidium calcicola*	Cyanobacteria	Bangkok	pond	0.001	0.000	0.001	.000
16	*Plectonema gracillimum*	Cyanobacteria	Nonthaburi	pond	0.002	0.000	0.001	0.000
17	*Scenedesmus acuminatus*	Green algae	Bangkok	pond	0.001	0.000	0.001	0.000
18	*Scytonema bohneri*	Cyanobacteria	Bangkok	cement surface	0.005	0.000	0.090	0.000
19	*Tolypothrix distorta*	Cyanobacteria	Bangkok	cement surface	0.008	0.000	0.210	0.000

Laminaria japonica, where a decreased hydrogen evolution was detected at the pH higher than 7.5 (Park *et al.* 2009). Effect of temperature on hydrogen production of the five most active strains was also studied. Cells were cultivated in $BG11_o$ under three different temperatures at 25, 30, and 45°C for the last 24 h. The hydrogen evolution of all five strains was the highest at 25°C, being 1.2–1.6 times higher than that at 30°C (Fig. 3*B*). In contrast, there was no detectable hydrogen production at 45°C. However, in *Calothrix* 336/3, higher hydrogen production occurred at 30°C compared to that at 23ºC (Allahverdiyeva *et al.* 2010). This suggested that the optimal temperature to enhance hydrogen production varies among different cyanobacterial strains.

Effect of carbon source, glucose concentrations and light intensities on H_2 production: Previous studies have reported that cyanobacteria are able to use different sugars, such as sucrose, fructose, and glucose, as a carbon source (Reddy *et al.* 1996, Chen *et al.* 2008, Raksajit *et al*. 2012). In this study, cells were grown in $BG11_o$ supplemented with 0.1% of glucose, mannitol, sorbitol, or sucrose during 24 h prior to the hydrogen production measurement. The different sugars tested at a concentration of 0.1% had variable effect on hydrogen production activity (Fig. 4*A*), the degree of which depended on the sugar types and species. Sorbitol and sucrose had small effects on hydrogen production activities. Sucrose inhibited the hydrogen production of *T. distorta* and *C. elenkinii*, but

activated the production slightly in *F. muscicola* and to a larger extent, namely 9-folds and 13-folds in *N. calcicola* and *S. bohneri,* respectively. Sorbitol inhibited the production in *T. distorta* and *F. muscicola*, but caused a modest increase of production in *N. calcicola* and *S. bohneri,* while the production in *C. elenkinii* greatly increased, showing 8-fold activation when compared with the control. Mannitol also inhibited the production in *T. distorta*, in contrast to its weak (in *N. calcicola* and *S. bohneri*) or very strong (19-fold in *C. elenkinii* and 12-fold in *F. muscicola*) activation of hydrogen production. The effect of glucose on hydrogen production of the five most active strains was the most significant because after incubation for 24 h in the presence of glucose, the production increased between 20 (in *S. bohneri*) and 52-folds (in *T. distorta*), depending on the strain. In the presence of glucose, the highest production observed in *T. distorta* was 10.9 μmol(H_2) mg^{-1}(Chl) h^{-1}.

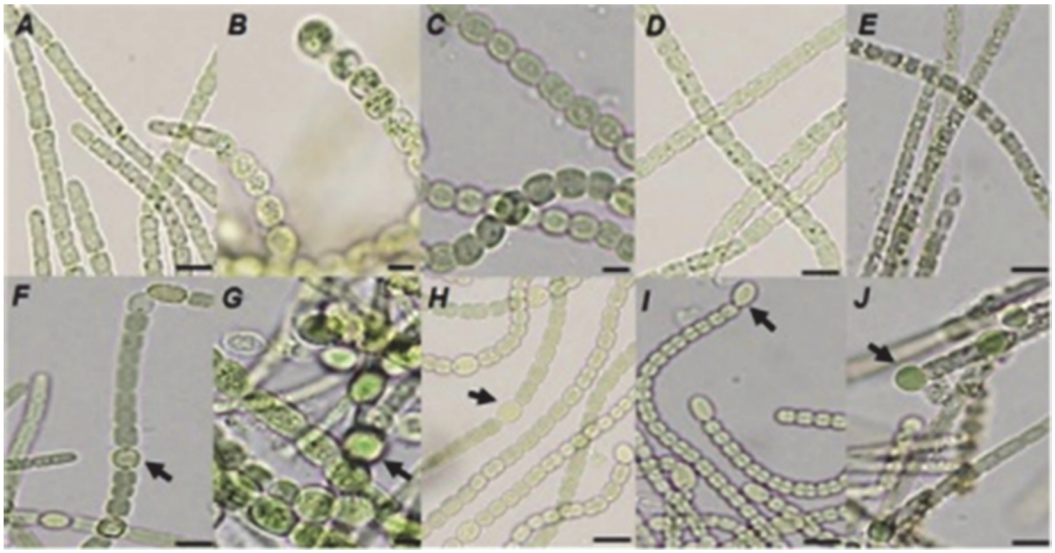

Fig. 1. Cell morphology of the five most biohydrogen producing species observed under an inverted light microscope. (*A*) *C. elenkinii*, (*B*) *F. muscicola*, (*C*) *N. calcicola*, (*D*) *S. bohneri*, and (*E*) *T. Distorta* were grown for three days under BG11 with nitrate as nitrogen source (upper panel), or in BG11 without nitrate ($BG11_o$) as shown in (*F*) *C. Elenkinii,* (*G*) *F. Muscicola,* (*H*) *N. Calcicola*, (*I*) *S. bohneri*, and (*J*) *T. Distorta* (lower panel). The arrows show the heterocyst cells. Scale bars – 10 μm.

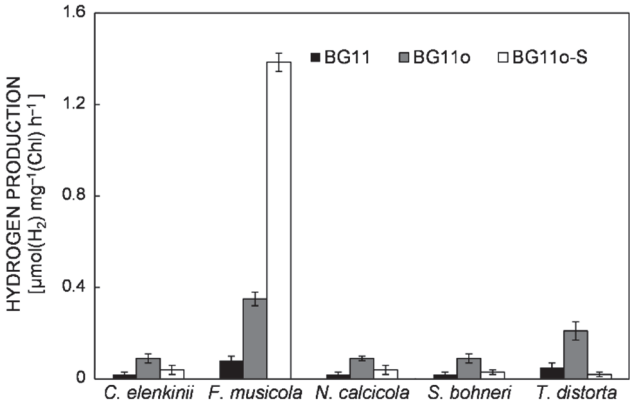

Fig. 2. Effect of nitrogen and sulfur deprivation on hydrogen production of the five most active species. Hydrogen production was determined by incubating cells under anaerobic condition under 50 μmol(photon) m^{-2} s^{-1} at 30°C for 24 h in BG11, $BG11_o$, and $BG11_o$-S ($BG11_o$ lacking sulfate). Means ± SD ($n = 3$).

Glucose was found to activate efficiently the hydrogen production in all five strains studied. Hence, we further monitored the effect of glucose concentration on hydrogen production. For this, cells were incubated with glucose concentrations ranging from 0.1% to 0.5% for 24 h before measurement of hydrogen production. Activation of hydrogen production of all five strains was noticed under all glucose concentrations tested (Fig. 4*B*). *F. muscicola*, *N. calcicola*, and *T. distorta* reached their highest hydrogen production under 0.1% glucose, while the production of hydrogen in *C. elenkinii* and *S. bohneri* was further enhanced by increasing the glucose concentration to 0.2 and 0.3%. Under the most effective glucose concentration, all the five strains were able to produce between 3.2 and 10.9 μmol(H_2) mg^{-1}(Chl) h^{-1}.

To monitor the effect of increased light intensity on hydrogen production, the cells were incubated in darkness or under light intensity of 50 (control), 100, 150, or 250 μmol(photon) m^{-2} s^{-1} for 24 h prior to hydrogen production measurement. An increase in light intensity caused activation of hydrogen production of all strains studied (Fig. 4*C*). The hydrogen production activity of *N. calcicola* was doubled under 100 μmol(photon) m^{-2} s^{-1}. However, *N. calcicola* was unable to benefit from a higher light intensity which was in contrast to the other four strains studied. The other four strains showed increased activation with increasing light intensity. The highest light intensity studied was 250 μmol(photon) m^{-2} s^{-1} which led to an activation of hydrogen production in *T. distorta*

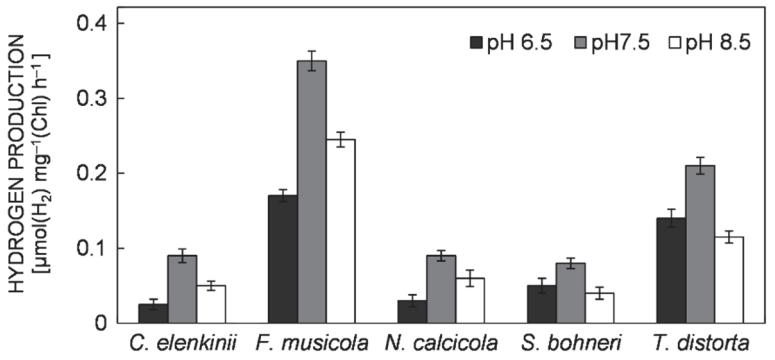

Fig. 3. Effect of pH and temperature on hydrogen production of the five most active species. Hydrogen production was determined by incubating cells suspended in $BG11_o$ under anaerobic condition under 50 μmol(photon) m^{-2} s^{-1} at 30°C for 24 h at various pHs of 6.5, 7.5, and 8.5 (*A*) and at various temperatures of 25°C, 30°C, and 40°C, at pH 7.5 (*B*). Means ± SD (n = 3).

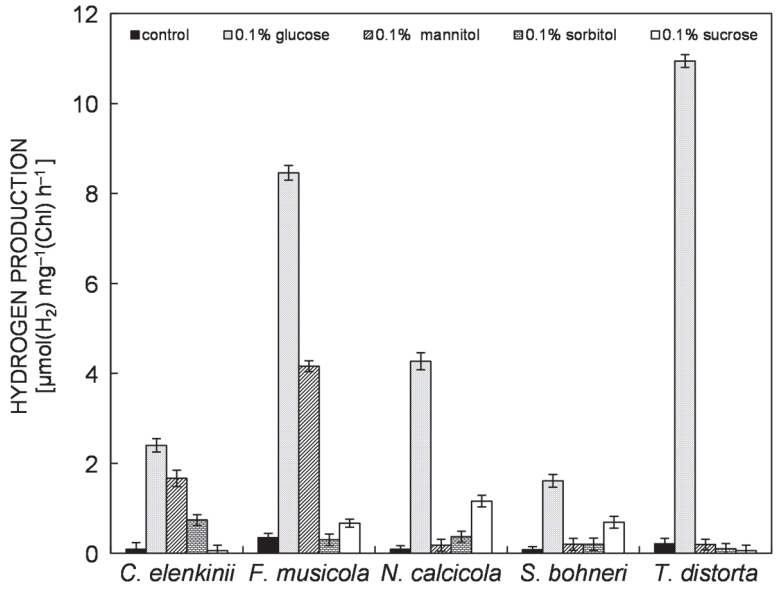

Fig. 4. Effect of carbon sources, glucose concentrations, and light intensities on hydrogen production of the five most active species. Hydrogen production was determined by incubating cells in $BG11_o$ under anaerobic condition under 50 μmol (photon) m^{-2} s^{-1} at 30°C for 24 h, with and without 0.1% glucose, 0.1% mannitol, 0.1% sorbitol, and 0.1% sucrose (*A*), with various concentrations of glucose (*B*), with various light intensities (*C*), and with and without 0.1% glucose under 50 or 250 μmol(photon) m^{-2} s^{-1} (Glu+HL) (*D*). Means ± SD (n = 3)

(9 folds), *F. muscicola* (14 folds), *C. elenkinii* (27 folds), and *S. bohneri* (47 folds). Our results were in agreement with those in *Anabaena siamensis* strain TISTR 8012, where an increased light intensity resulted in an increased hydrogen production (Khetkorn *et al.* 2010). The effect of glucose, in combination with high light intensity, on hydrogen production was also examined. *F. muscicola*, *N. calcicola*, and *T. distorta* were incubated in 0.1% glucose under 250 μmol(photon) m^{-2} s^{-1}, whereas *C. elenkinii* and *S. bohneri* were incubated in 0.3% glucose under 250 μmol(photon) m^{-2} s^{-1}. Hydrogen production of all strains with the combination of glucose and high light intensity was lower than that in the presence of glucose under control light intensity of 50 μmol(photon) m^{-2} s^{-1} (Fig. 4*D*).

Taken together, the five most actively hydrogen-producing cyanobacterial strains in the present study produce hydrogen within the range of 0.17–4.2 μmol(H_2) mg^{-1}(Chl) h^{-1} comparable to other previously reported heterocystous cyanobacteria (Masukawa *et al.* 2001). In addition, we compared the hydrogen production of filamentous cyanobacteria among *C. elenkinii, F. muscicola, N. calcicola, S. bohneri, T. distorta*, *Anabaena* sp. PCC 7120 (Masukawa *et al*. 2002), and the unicellular strain *Synechocystis* sp. PCC 6803 (Baebprasert *et al*. 2010). It was found that the five most actively hydrogen-producing cyanobacterial strains have the capacity to produce hydrogen at rates higher than *Anabaena* sp. PCC 7120 (about 1- to 5-folds), or *Synechocystis* sp. PCC 6803 (about 160- to 540-folds) (Table 2). Although, the hydrogen production of five most actively hydrogen-producing cyanobacterial strains is less than that of *Anabaena siamensis* TISTR 8012 (Khetkorn *et al*. 2010) under the same hydrogen-producing condition [Ar, $BG11_o$, 30°C, 40 μmol(photon) m^{-2} s^{-1}, 24 h of incubation time], higher production rates could be obtained when the five most actively hydrogen-producing strains were acclimated to the optimum conditions [Ar, $BG11_o$ using 0.1 or 0.3% glucose, 30°C, 50 μmol(photon) m^{-2} s^{-1}].

Nitrogenase activity: In most filamentous cyanobacteria, hydrogen is not only generated by the hydrogenase but also by the nitrogenase (Tamagnini *et al*. 2002, 2007). However, there are filamentous cyanobacteria that do not have the bidirectional hydrogenase, but only the uptake hydrogenase (*e.g.*, *N. punctiforme*). Basically, acetylene reduction

Table 2. Comparison of the ability of hydrogen production in the five most active species with other cyanobacterial strains.

Organism	Morphology	H_2 production rate [μmol(H_2) mg^{-1}(Chl) h^{-1}]	Growth condition	H_2 production condition
Calothrix elenkinii	Filamentous with heterocysts	0.09 ± 0.01 3.21 ± 0.19	Air, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1} Ar, $BG11_o$ using 0.3% glucose as carbon source, 30°C, 50 μmol(photon) m^{-2} s^{-1}
Fischerella muscicola	Filamentous with heterocysts	0.35 ± 0.08 8.73 ± 0.43	Air, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1} Ar, $BG11_o$ using 0.3% glucose as carbon source, 30°C, 50 μmol(photon) m^{-2} s^{-1}
Nostoc calcicola	Filamentous with heterocysts	0.09 ± 0.01 4.27 ± 0.17	Air, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_0$, 30°C, 50 μmol(photon) m^{-2} s^{-1} Ar, $BG11_o$ using 0.1% glucose as carbon source, 30°C, 50 μmol(photon) m^{-2} s^{-1}
Scytonema bohneri	Filamentous with heterocysts	0.09 ± 0.01 7.63 ± 0.26	Air, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1} Ar, $BG11_o$ using 0.3% glucose as carbon source, 30°C, 50 μmol(photon) m^{-2} s^{-1}
Tolypothrix distorta	Filamentous with heterocysts	0.21 ± 0.05 10.95 ± 0.22	Air, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_o$, 30°C, 50 μmol(photon) m^{-2} s^{-1} Ar, $BG11_o$ using 0.1% glucose as carbon source, 30°C, 50 μmol(photon) m^{-2} s^{-1}
Anabaena PCC 7120 (Masukawa *et al.* 2002)	Filamentous with heterocysts	2.60 ± 0.07	Air, $BG11_o$, 26°C, 70 μmol(photon) m^{-2} s^{-1}	Ar, $BG11_o$, 30°C, 1% CO_2, 70 μmol(photon) m^{-2} s^{-1}
Synechocystis sp. PCC 6803 (Baebprasert *et al.* 2010)	Unicellular	0.12 ± 0.02	Air, BG11, 30°C, 30 μmol(photon) m^{-2} s^{-1}	Ar, BG11, 0.1% glucose as carbon source, 30°C, darkness

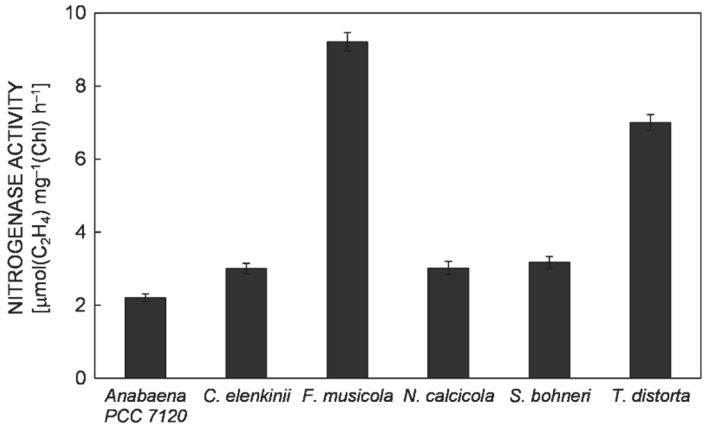

Fig. 5. Nitrogenase activity of the five cyanobacterial species with most active hydrogen production (*C. elenkinii*, *F. muscicola*, *N. calcicola*, *S. bohneri*, and *T. distorta*), and *Anabaena* PCC 7120 as a positive control. Ethylene (C_2H_4) production was measured by using GC with FID detector. Means ± SD (n = 3).

method is widely used to monitor nitrogenase activity, since it catalyzes the conversion of acetylene (C_2H_2) to ethylene (C_2H_4). In this work, we also analyzed the activity of nitrogenase in five strains and *Anabaena* PCC 7120 as a positive control. All strains studied showed nitrogenase activity (Fig. 5). Interestingly, nitrogenase activities of all strains, especially *F. muscicola* and *T. distorta*, were higher than that of *Anabaena* PCC 7120.

Conclusion: Among sixteen cyanobacterial and three green algal strains from Thailand, *F. muscicola* and *T. distorta* have the high potential for hydrogen production. The most favorable condition for maximum hydrogen production was achieved when the cells of *T. distorta* were adapted for 24 h under nitrogen-fixing conditions with addition of 0.1% glucose which resulted in hydrogen production of 10.9 μmol(H_2) mg^{-1}(Chl) h^{-1}.

Moreover, the hydrogen production of all five strains, especially *T. distorta*, can be further increased by genetic engineering of gene(s) involved in hydrogen production, including the uptake hydrogenase and the nitrogenase as well as the redirection of the electron flow towards hydrogenase.

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