Journal of Applied Phycology (2018) 30:3471–3481
https://doi.org/10.1007/s10811-018-1490-6

Screening cyanobacteria from marine coastal waters of Thailand for biohydrogen production

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Received: 18 January 2018 / Revised and accepted: 18 April 2018 / Published online: 3 May 2018
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Abstract
Cyanobacteria are prokaryotic organisms capable of oxygenic photosynthesis. H_2 can be produced by cyanobacterial bidirectional hydrogenase, but mainly during anaerobic dark fermentation. Here, we screened H_2 producing cyanobacteria isolated from marine environments in Thailand and optimized physiological conditions for maximizing H_2 production of the selected isolate. Most of the 54 cyanobacterial strains isolated and purified from samples of seawater, stones, sand, and shells in the Gulf of Thailand and the Andaman Sea, southern part of Thailand produced H_2 when cells were incubated in nitrogen-deprived medium under dark/anaerobic condition. The filamentous non-heterocystous cyanobacterium *Geitlerinema* sp. RMK-SH10 gave the highest H_2 yield with highest H_2 production rate found in 7-day grown cells. *Geitlerinema* sp. RMK-SH10 showed maximum H_2 production rate of 0.271 ± 0.013 μmol H_2 mg^{-1} dry weight h^{-1} when incubated in $NaNO_3$-free ASN III medium containing 0.2 M NaCl, 18.9 mmol C-atom of glucose L^{-1}, and 0.1 μM Ni^{2+}. These results suggest that the marine filamentous cyanobacterium *Geitlerinema* sp. RMK-SH10 has high potential as a H_2 producer amenable for further improvement by genetic manipulation.

Keywords H_2 production · Marine cyanobacteria · *Geitlerinema*

Introduction

Nowadays, biological hydrogen production has attracted much attention as an efficient and alternative method for sustainable H_2 production. Among H_2 producing microorganisms, prokaryotic cyanobacteria provide a number of advantages such as cheap substrate for cultivation, easy manipulation, and simple mass cultivation method with high potential for industrial applications.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10811-018-1490-6) contains supplementary material, which is available to authorized users.

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Cyanobacteria are capable of producing H_2 by hydrogenase activity using electrons derived from water oxidation via a direct biophotolysis process under light exposure (Prince and Kheshgi 2005; Allahverdiyeva et al. 2010); however, cyanobacterial hydrogenase activity is inhibited by O_2 produced by a photolysis (Houchins 1984). Therefore, the main electron source for the reduction of proton to generate H_2 by cyanobacteria is derived from the degradation of storage glycogen via a dark fermentation (Tamagnini et al. 2007; Ananyev et al. 2008). Some N_2-fixing cyanobacteria can release H_2 as a by-product by an action of nitrogenase via nitrogen fixation (Reddy et al. 1996; Chen et al. 2008).

Marine cyanobacteria ubiquitously found in marine habitats/environments show an ability to tolerate high salt concentrations (Thajuddin and Subramanian 2005). H_2 production has been investigated in some strains of marine cyanobacteria; for example, *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui 1981; Phlips and Mitsui 1983), *Synechococcus* sp. Miami BG 043511 (Luo and Mitsui 1994), *Oscillatoria willei* BDU 130511 (Saha et al. 2003), *Phormidium valderianum* BDU 20041 and *Leptolyngbya valderiana* BDU 20041 (Prabaharan and Subramanian 1996; Prabaharan et al. 2010), and the unicellular halotolerant cyanobacterium *Aphanothece halophytica* (Taikhao et al. 2013, 2015). Biodiversity among marine cyanobacteria for

H_2 production has not been paid much attention; only few studies have attempted to screen and characterize potential H_2 producing cyanobacteria from marine natural environments (Kumar and Kumar 1992; Vyas and Kumar 1995; Allahverdiyeva et al. 2010; He et al. 2012; Kothari et al. 2012; Leino et al. 2014). In addition, several environmental factors, such as cultivation time, nutritional and mineral concentrations, play important roles in cyanobacterial H_2 production (Dutta et al. 2005; Tiwari and Pandey 2012).

The aims of this study were to screen for high H_2 producing marine cyanobacteria isolated from the two coastal waters of Thailand, the Gulf of Thailand, and the Andaman Sea and to optimize the conditions for H_2 production by the selected high potential marine cyanobacterial isolate.

Materials and methods

Collection and isolation of cyanobacterial isolates

Cyanobacterial strains were isolated from seawater in both the Gulf of Thailand and the Andaman Sea of Thailand. Planktonic cyanobacterial strains were isolated from seawater, whereas benthic cyanobacterial strains were isolated from stones, sand, and shells. All samples from each environment were inoculated in flasks containing liquid ASN III medium (Rippka et al. 1979). The flasks were then incubated at 30 °C under light illumination of 30 μmol photons m^{-2} s^{-1} for 7–30 days. In case of planktonic cyanobacterial isolation, seawater samples were filtered through a 20-μm mesh plankton net and the biomass on the filter was subsequently inoculated in medium and cultivated under indicated conditions. Each filament or colony of cyanobacterial isolates was isolated and purified using a single cell isolation technique under a stereomicroscope (Nikon SMZ745T, Japan) (Hoshaw and Rosowki 1973). Bacterial contamination was examined by Gram staining technique (Gram 1884).

Cultivation of cyanobacterial isolates

Cyanobacterial isolates were cultivated in a 250-mL Erlenmeyer flask containing 100 mL of liquid ASN III medium and shaken at 120 rpm under a white-light illumination of 30 μmol photons m^{-2} s^{-1} at 30 °C for 14 days.

Identification of cyanobacterial isolates by 16S rDNA sequencing analysis

Genomic DNA of all purified cyanobacterial isolates was isolated following the protocol previously described (Phunpruch et al. 2016). The fragment of 16S rDNA of cyanobacterial isolates was amplified by polymerase chain reaction using a primer pair, F16S rDNA Cyano (5’-GCTCAGGATGAACGCTGGCG-3’) and R16S rDNA Cyano (5’-CGGCTACCTTGTTACGACTCCA-3’). PCR reactions and conditions were as described previously (Phunpruch et al. 2006). Amplified PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) and sequenced in both directions with Big-Dye terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) using ABI PRISM 3700 DNA analyzer at First BASE Laboratories (Malaysia). The taxonomic identification of cyanobacterial isolates was assessed by comparison of the obtained sequences to other cyanobacterial 16S rRNA genes available in the NCBI database using the basic local alignment search tool (BLAST) (Altschul et al. 1990).

Screening of cyanobacterial isolates for H_2 production

In this study, H_2 production by two-stage cell culture was measured. In the first stage, cyanobacterial isolates were grown in ASN III medium for 14 days to accumulate biomass. In the second stage, cells were incubated in nitrogen deprived ASN III medium under the light for 24 h to stimulate the production of ATP, reducing powers and storage glycogen. Then, cells were transferred to vials, purged with argon to enter anaerobic condition, and incubated in the dark for 24 h before H_2 production assay. For screening of H_2 producing cyanobacterial isolates, the 14-day grown cells were subsequently harvested by centrifugation at 7000×*g* at 4 °C for 10 min. The cell pellet was subsequently washed twice with $NaNO_3$-free ASN III medium and subsequently resuspended in 100 mL of $NaNO_3$-free ASN III medium. The cell suspension was further incubated for 1 day before harvesting cells to determine H_2 production. The harvested cells were resuspended in 5 mL of $NaNO_3$-free medium and transferred to a 10-mL gas-tight vial. H_2 production was allowed to proceed for 24 h under four conditions: (1) anaerobic/dark condition, (2) microaerobic/light condition, (3) aerobic/dark condition, and (4) aerobic/light condition. Under anaerobic/dark condition, cell suspension was purged with argon gas for 5 min to eliminate O_2 in a vial before placing the vial on a shaker with a speed of 120 rpm at 30 °C for 24 h. All processes were performed under darkness. Under microaerobic/light condition, cell suspension in a vial was also purged with argon gas for 5 min and incubated under light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h. O_2 could be produced in a small amount from photosynthesis (0.42–1.82% (*v*/*v*) in air). Under aerobic condition, purging with argon gas was omitted. The vial was incubated at 30 °C either under the light intensity of 30 μmol photons m^{-2} s^{-1} (aerobic/light condition) or in the dark (aerobic/dark condition) for 24 h.

Determination of H_2 production and bidirectional hydrogenase activity

To determine the amount of H_2 produced after 24 h incubation, 500 μL of gas phase in a vial were withdrawn by a gas-tight syringe. The amount of H_2 was analyzed by a gas

chromatograph (Hewlett-Packard HP890A GC, Japan) equipped with a thermal conductivity detector as previously described (Taikhao et al. 2013). The in vivo bidirectional hydrogenase activity was determined by measuring H_2 produced in the presence of dithionite-reduced methyl viologen. Two milliliters of reaction mixture contained 1 mL of cell suspension and 1 mL of 5 mM methyl viologen and 20 mM Na-dithionite (in 25 mM potassium phosphate buffer, pH 7.0). The reaction was incubated under argon for 15 min. H_2 production was determined by analyzing 500 μL of the gas phase in a vial by gas chromatography (Taikhao et al. 2013).

Optimization of H_2 production by the selected cyanobacterial isolate

To study the effect of cultivation time on H_2 production, *Geitlerinema* sp. RMK-SH10 was grown in ASN III medium for 7, 14, and 21 days, harvested, washed twice with $NaNO_3$-free ASN III medium. The cell pellet was then resuspended in $NaNO_3$-free ASN III medium. The cell suspension was shaken at 30 °C under the light for 24 h, subsequently harvested, resuspended in 5 mL of $NaNO_3$-free medium, and incubated under darkness for 24 h before H_2 production measurement. To investigate the optimization of the nutrient and mineral concentrations in medium for H_2 production, 7-day grown cells were harvested, washed twice, and resuspended in $NaNO_3$-free ASN III medium containing various concentrations of $NaNO_3$, $MgSO_4 \cdot 7H_2O$, NaCl, Fe^{3+}, and Ni^{2+}. To investigate carbon sources and concentrations, Na_2CO_3 as a carbon source in ASN III medium was replaced by different types of carbon source, $NaHCO_3$, glucose, fructose, sucrose, lactose, and maltose, with equimolar concentration of C-atom (0.189 mmol C-atom L^{-1}). The concentrations of optimal sugar source were varied from 0 to 189 mmol C-atom L^{-1}.

Dry cell weight determination

The growth measurement of *Geitlerinema* sp. RMK-SH10 was performed by determination of dry cell weight. Ten milliliters of culture were filtered through a filter paper no. 1 (55 mm diameter) (Whatman, UK). The filter containing cells was subsequently washed twice by 10 mL of distilled water and dried at 60 °C in an oven for 1–3 days. The filter containing cells was put in a desiccator for 1 h before measuring the weight.

Statistical analysis

All H_2 production values were presented as means ± SD from three independent biological replicates. Differences among treatments were analyzed by Duncan's multiple range test with the $P < 0.05$ significance level using one-way ANOVA of the SPSS 24 software (IBM Corp., USA).

Results

Screening of potential H_2 producing cyanobacterial isolates

Several cyanobacterial strains were isolated from samples of seawater, stones, sand, and shells in the Gulf of Thailand and the Andaman Sea in Thailand. Only 54 cyanobacterial isolates could be purified devoid of bacterial contamination. They were genetically identified by 16S rDNA sequencing and morphological analyses. Among them, 35 isolates were classified in genus *Geitlerinema*, 9 isolates were classified in genus *Leptolyngbya*, and 3 isolates each were classified in genus *Phormidium* and genus *Synechococcus*, respectively. The remaining four isolates were each classified in genus *Chroococcus*, *Cyanothece*, *Pseudanabaena*, and *Synechocystis* (Table 1). The cyanobacterial isolates were grown in ASN III medium for 14 days before incubation in $NaNO_3$-free ASN III medium under the light at 30 °C for 24 h. The cultures were subsequently harvested and resuspended in 5 mL of $NaNO_3$-free ASN III medium. The cell suspensions were transferred to a glass vial, and their H_2 production was measured after incubation under four conditions for 24 h. The results showed that all cyanobacterial isolates except *Leptolyngbya* sp. PKR-ST1 could produce H_2 under anaerobic/dark condition (Table 1). The filamentous cyanobacterium *Geitlerinema* sp. RMK-SH10 showed the highest H_2 production of 273.701 ± 7.451 and 141.252 ± 11.845 μmol H_2 g^{-1} dry weight under anaerobic/dark and microaerobic/light conditions, respectively, whereas low H_2 production was detected under aerobic/dark and aerobic/light conditions, with more than half of the tested isolates had no H_2 production (Table 1). Under aerobic/dark condition, *Cyanothece* sp. P-SH8.2.1 gave the highest H_2 production of 142.422 ± 0.806 μmol H_2 g^{-1} dry weight, whereas under aerobic/light condition, very low H_2 production was detected whereby no cyanobacterial isolates showed H_2 production higher than 10 μmol H_2 g^{-1} dry weight (Table 1). From screening results, the *Geitlerinema* sp. RMK-SH10 (the description and values shown in italic) was chosen for further optimization for H_2 production under anaerobic/dark condition.

Effect of cultivation time on H_2 production by *Geitlerinema* sp. RMK-SH10

Geitlerinema sp. RMK-SH10 was cultivated in ASN III medium, and its growth by dry cell weight measurement was monitored every 2 days. Figure 1 shows that *Geitlerinema* sp. RMK-SH10 could grow well in ASN III medium. To study the effect of growth phase on H_2 production, cells were grown in ASN III medium for 7, 14, and 21 days before harvesting cells. Cells were then processed for H_2 production measurement as described in materials and methods. The 7-day old cells of *Geitlerinema* sp. RMK-SH10 gave the highest H_2

Table 1 H_2 production of marine cyanobacterial strains isolated from the Gulf of Thailand and the Andaman Sea

Strains	Origins	Habitats	H_2 production (μmol H_2 g^{-1} dry weight)			
			Anaerobic	Microaerobic	Aerobic	
			Darkness	Light	Darkness	Light
Chroococcus sp. SKR-W2.2	Saikeaw, Rayong	Seawater	108.679 ± 3.127	11.121 ± 0.806	59.751 ± 4.290	nd
Cyanothece sp. P-SH8.2.1	Phla, Rayong	Shell	171.728 ± 2.291	114.206 ± 7.376	142.422 ± 0.806	5.978 ± 0.326
Geitlerinema sp. CHL-SH1	Changlang, Trang	Shell	81.885 ± 4.108	22.873 ± 0.982	nd	nd
Geitlerinema sp. JL-SH1	JaoLao, Chanthaburi	Shell	5.908 ± 0.112	83.764 ± 5.069	nd	0.626 ± 0.075
Geitlerinema sp. JL-SA1	JaoLao, Chanthaburi	Sand	28.312 ± 1.669	1.679 ± 0.141	nd	nd
Geitlerinema sp. JM-SH2	Jaomai, Trang	Shell	160.957 ± 4.656	46.904 ± 1.283	nd	nd
Geitlerinema sp. KVM-W1	Kungviman, Chanthaburi	Seawater	212.098 ± 5.221	2.871 ± 0.178	23.055 ± 1.187	nd
Geitlerinema sp. LK-SH2	Laemkruat, Krabi	Shell	150.999 ± 12.776	7.2733 ± 0.726	nd	nd
Geitlerinema sp. LS-W2	Laemsing, Chanthaburi	Seawater	30.240 ± 1.541	4.520 ± 0.220	1.658 ± 0.155	0.674 ± 0.050
Geitlerinema sp. LSD-SH2	Laemsadet, Chanthaburi	Shell	193.313 ± 1.006	22.087 ± 0.120	94.484 ± 6.360	6.287 ± 0.076
Geitlerinema sp. MTN-SH5	Modtanoy, Trang	Shell	82.610 ± 6.230	1.453 ± 0.018	4.928 ± 0.086	2.514 ± 0.033
Geitlerinema sp. MTN-SH9	Modtanoy, Trang	Shell	114.670 ± 9.476	64.232 ± 2.309	21.366 ± 1.577	1.146 ± 0.064
Geitlerinema sp. N-ST1	Nang, Krabi	Stone	83.135 ± 1.037	6.886 ± 0.346	0.888 ± 0.013	1.702 ± 0.133
Geitlerinema sp. N-ST2	Nang, Krabi	Stone	109.765 ± 5.980	13.034 ± 0.836	51.399 ± 3.973	5.106 ± 0.249
Geitlerinema sp. NM-SH1	Nummao, Krabi	Shell	181.428 ± 6.218	20.722 ± 1.653	2.734 ± 0.230	2.033 ± 0.179
Geitlerinema sp. NM-SA4	Nummao, Krabi	Sand	38.550 ± 3.707	5.581 ± 0.191	nd	nd
Geitlerinema sp. NR-SH2	Nangram, Chonburi	Shell	56.624 ± 2.439	24.769 ± 1.596	nd	nd
Geitlerinema sp. P-ST2.3	Phla, Rayong	Stone	34.371 ± 2.159	1.552 ± 0.076	nd	nd
Geitlerinema sp. P-W2.1	Phla, Rayong	Seawater	74.9137 ± 4.819	9.375 ± 0.565	4.7512 ± 0.113	0.738 ± 0.028
Geitlerinema sp. PI-S1.1	Phai, Rayong	Seawater	34.668 ± 2.840	nd	nd	nd
Geitlerinema sp. PKR-W3	Pakarang, Rayong	Seawater	75.150 ± 3.045	nd	nd	nd
Geitlerinema sp. PM-SH13	Pakarang, Trang	Shell	102.968 ± 6.595	0.573 ± 0.025	nd	nd
Geitlerinema sp. RMK-SH10	*Rachmonkol, Trang*	*Shell*	*273.701 ± 7.451*	*141.252 ± 11.845*	*1.251 ± 0.050*	*1.408 ± 0.013*
Geitlerinema sp. S-SH3	San, Trang	Shell	114.066 ± 1.244	2.015 ± 0.147	nd	nd
Geitlerinema sp. SK-ST1.1	Saikeaw, Chonburi	Stone	95.843 ± 3.282	31.183 ± 2.286	25.553 ± 1.133	3.072 ± 0.204
Geitlerinema sp. SK-ST1.2	Saikeaw, Chonburi	Stone	119.4534 ± 7.086	7.607 ± 0.373	nd	0.292 ± 0.003
Geitlerinema sp. SK-ST2.1	Saikeaw, Chonburi	Stone	69.551 ± 5.604	13.862 ± 0.261	0.223 ± 0.015	1.229 ± 0.013
Geitlerinema sp. SR-SH4	Samran, Trang	Shell	98.530 ± 6.263	8.615 ± 0.657	3.270 ± 0.075	1.323 ± 0.064
Geitlerinema sp. SS-ST6	Samaesan, Chonburi	Stone	56.501 ± 3.779	10.836 ± 0.195	nd	nd
Geitlerinema sp. SSH-SH12	Susanhoy, Krabi	Shell	180.619 ± 6.703	33.308 ± 0.595	7.598 ± 0.388	3.435 ± 0.144
Geitlerinema sp. ST-ST1.5.1	Saitong, Rayong	Stone	108.694 ± 1.847	120.970 ± 2.814	1.251 ± 0.050	1.418 ± 0.001
Geitlerinema sp. TG-W2.3.3	Toeingam, Chonburi	Seawater	113.827 ± 0.556	0.919 ± 0.076	1.495 ± 0.124	nd
Geitlerinema sp. TL-SH2	Thalane, Krabi	Shell	126.327 ± 5.250	32.422 ± 0.753	2.075 ± 0.007	2.033 ± 0.179
Geitlerinema sp. VD-SH2.3	Vongdeuan, Rayong	Shell	46.967 ± 1.261	18.585 ± 1.051	0.176 ± 0.005	0.337 ± 0.017
Geitlerinema sp. WI-SA1	Wai, Rayong	Sand	5.719 ± 0.114	nd	nd	nd
Geitlerinema sp. WI-SH3	Wai, Rayong	Shell	64.227 ± 4.639	1.620 ± 0.107	nd	nd
Geitlerinema sp. YL-SH4	Yonglin, Trang	Shell	68.3565 ± 3.539	1.953 ± 0.056	nd	nd
Leptolyngbya sp. CHL-SH10	Changlang, Trang	Shell	0.761 ± 0.009	nd	nd	nd
Leptolyngbya sp. KK	Koh Kood, Trad	Seawater	19.167 ± 0.849	30.497 ± 1.671	1.300 ± 0.093	nd
Leptolyngbya sp. MP-SA1	Makhampom, Rayong	Sand	5.378 ± 0.451	3.849 ± 0.163	nd	nd
Leptolyngbya sp. NTR-S	Nopparattara, Krabi	Seawater	87.613 ± 2.600	8.186 ± 0.523	nd	nd
Leptolyngbya sp. PKR-ST1	Pakarang, Rayong	Stone	nd	nd	nd	nd
Leptolyngbya sp. PR-SH1	Prao, Rayong	Shell	112.989 ± 8.679	nd	nd	nd
Leptolyngbya sp. PR-SH9	Prao, Rayong	Shell	59.473 ± 3.799	nd	nd	nd
Leptolyngbya sp. S-S	San, Trang	Seawater	135.013 ± 5.670	121.332 ± 7.783	0.312 ± 0.029	0.327 ± 0.018

Table 1 (continued)

Strains	Origins	Habitats	H_2 production (μmol H_2 g^{-1} dry weight)			
			Anaerobic	Microaerobic	Aerobic	
			Darkness	Light	Darkness	Light
Leptolyngbya sp. SSH-SH5	Susanhoy, Krabi	Shell	70.399 ± 2.927	nd	nd	nd
Phormidium sp. KM-ST9	Klongmuang, Krabi	Stone	41.230 ± 2.666	5.506 ± 0.202	nd	nd
Phormidium sp. P-SA1.1	Phla, Rayong	Sand	241.597 ± 2.715	103.658 ± 0.188	5.440 ± 0.120	1.087 ± 0.006
Phormidium sp. Y-SH8	Yao, Trang	Shell	75.963 ± 2.641	12.803 ± 1.045	13.112 ± 0.751	3.371 ± 0.181
Pseudoanabeana sp. TL-S	Thalane, Krabi	Seawater	25.208 ± 0.606	5.146 ± 0.086	nd	nd
Synechococcus sp. JL-W2	JaoLao, Chanthaburi	Seawater	5.440 ± 1.045	1.789 ± 0.141	3.964 ± 0.021	0.170 ± 0.010
Synechococcus sp. MP-W	Makhampom, Rayong	Seawater	8.582 ± 0.694	nd	3.455 ± 0.057	nd
Synechococcus sp. VD-W	Vongdeuan, Rayong	Seawater	0.258 ± 0.006	nd	nd	nd
Synechocystis sp. LSD-W3	Laemsadet, Chanthaburi	Seawater	3.394 ± 0.366	2.849 ± 0.013	nd	nd

nd: not determined

production rate of 13.382 ± 0.600 μmol H_2 g^{-1} dry weight h^{-1} (Fig. 1), after which the H_2 production was decreased. Therefore, further H_2 production optimization study used 7-day grown *Geitlerinema* sp. RMK-SH10.

Effect of nutrient and mineral concentrations on H_2 production by *Geitlerinema* sp. RMK-SH10

To investigate the effect of nitrate, sulfate, NaCl, Fe^{3+}, and Ni^{2+} concentrations on H_2 production, *Geitlerinema* sp. RMK-SH10 grown in ASN III medium for 7 days was harvested, washed twice, and resuspended in medium containing various concentrations of $NaNO_3$, SO_4^{2-}, NaCl, Fe^{3+}, Ni^{2+}, and glucose. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested, resuspended in 5 mL of the corresponding media, and incubated under anaerobic/dark

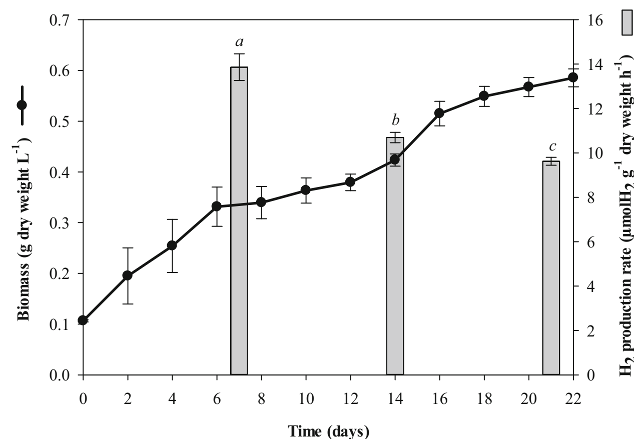

Fig. 1 Biomass of *Geitlerinema* sp. RMK-SH10 cultivated in ASN III medium for 21 days and dark fermentative H_2 production rate of 7-, 14-, and 21-day grown cells under nitrogen deprived condition. Data are means ± SD ($n = 3$). Different letters on columns indicate the significant difference according to Duncan's multiple range test at $P < 0.05$

condition for 24 h before analyzing H_2 production. The highest H_2 production of 318.237 ± 23.405 μmol H_2 g^{-1} dry weight was found in cells incubated in $NaNO_3$-free medium, an approximately 10-fold increase compared to that of cells incubated in the normal ASN III medium containing 8.8 mM $NaNO_3$ (Fig. S1, Supplementary file). All following experiments for measurement of H_2 production in this study were performed under N-deprivation. Under various SO_4^{2-} concentrations, the highest H_2 production of 387.383 ± 17.378 μmol H_2 g^{-1} dry weight was found in cells incubated in $NaNO_3$-free ASN III medium containing 1.4 mM $MgSO_4 \cdot 7H_2O$, whereas at 0 and 14 mM $MgSO_4 \cdot 7H_2O$, there were slight differences of H_2 production (Table 2). Under various NaCl concentrations, maximum H_2 production of 434.705 ± 25.337 μmol H_2 g^{-1} dry weight was detected in cells incubated in N-free ASN III medium containing 0.2 M NaCl, an approximately 1.2-fold increase compared to that of cells incubated in the normal medium containing 0.4 M NaCl (Table 2). For Fe^{3+} and Ni^{2+}, cells gave the highest H_2 production of 391.501 ± 8.278 and 359.826 ± 6.836 μmol H_2 g^{-1} dry weight when incubated under 2 μM Fe^{3+} and 0.1 μM Ni^{2+}, respectively (Table 2). H_2 production was enhanced with an increase of glucose concentrations up to 18.9 mmol C-atom L^{-1} where the maximum H_2 production of 1458.094 ± 34.751 μmol H_2 g^{-1} dry weight was observed (Table 2).

Effect of carbon sources on H_2 production by *Geitlerinema* sp. RMK-SH10

Geitlerinema sp. RMK-SH10 grown in ASN III medium for 7 days was harvested, washed twice, and resuspended in N-free ASN III medium containing various carbon sources such as Na_2CO_3, $NaHCO_3$, glucose, fructose, sucrose, lactose, and maltose with concentration of 0.189 mmol C-atom L^{-1}. The cultures were shaken at 30 °C under the light for 24 h,

Table 2 H_2 production by marine cyanobacterium *Geitlerinema* sp. RMK-SH10 under nitrogen deprivation with various SO_4^{2-}, NaCl, Fe^{3+}, Ni^{2+}, and glucose concentrations

Condition	Concentration	H_2 production (μmol H_2 g^{-1} dry weight)
ASN III (control)		31.442 ± 0.643
$MgSO_4 \cdot 7H_2O$ concentration	0	364.772 ± 32.401
(mM)	1.4	387.383 ± 17.378
	14	345.804 ± 24.181
	28	279.210 ± 7.002
	150	112.141 ± 6.861
NaCl concentration	0	292.743 ± 8.165
(M)	0.2	434.705 ± 25.337
	0.4	351.902 ± 9.920
	0.6	273.523 ± 4.173
	0.8	201.275 ± 11.186
	1	99.654 ± 8.497
	2	5.757 ± 0.362
Fe^{3+} concentration	0	212.795 ± 12.366
(μM)	0.02	265.566 ± 18.472
	0.2	301.305 ± 20.251
	2	391.501 ± 8.278
	20	361.814 ± 14.650
	200	58.402 ± 3.307
Ni^{2+} concentration	0	303.855 ± 5.819
(μM)	0.1	359.826 ± 6.836
	1	290.411 ± 10.867
	10	179.772 ± 4.393
	100	157.589 ± 11.174
Glucose concentration	0	313.080 ± 26.835
(mmol C-atom L^{-1})	0.0189	481.620 ± 42.069
	0.189	609.636 ± 29.476
	1.89	764.442 ± 32.507
	18.9	1458.094 ± 34.751
	189	1007.940 ± 61.470

Cells were grown in ASN III medium containing 8.8 mM $NaNO_3$, 14 mM $MgSO_4 \cdot 7H_2O$, 0.189 mmol C-atom Na_2CO_3 L^{-1}, 0.4 M NaCl, and 2 μM Fe^{3+} for 7 days. Cells were then transferred into N-free ASN III medium containing various SO_4^{2-}, NaCl, Fe^{3+}, Ni^{2+}, and glucose concentrations and further incubated for another 24 h before H_2 production analysis under dark anaerobic condition. Data are means ± SD ($n = 3$)

subsequently harvested, resuspended in 5 mL of the corresponding media, and incubated under darkness for further 24 h before analyzing H_2 production. The highest H_2 production of 609.076 ± 41.562 μmol H_2 g^{-1} dry weight was obtained in cells incubated in $NaNO_3$-free ASN III medium containing glucose in place of Na_2CO_3 (Fig. 2).

Maximum H_2 production and bidirectional hydrogenase activity of *Geitlerinema* sp. RMK-SH10 under various conditions

In order to maximize dark fermentative H_2 production, the effects of combined factors on H_2 production and hydrogenase activity of *Geitlerinema* sp. RMK-SH10 incubated in various media containing different nutrients were investigated. The results showed that *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate of 0.271 ± 0.013 μmol H_2 mg^{-1} dry weight h^{-1} and the highest hydrogenase activity of 0.389 ± 0.012 μmol H_2 mg^{-1} dry weight min^{-1} when incubated in $NaNO_3$-free ASN III medium containing 0.2 M NaCl, 18.9 mmol C-atom glucose L^{-1}, and 0.1 μM Ni^{2+} for 4 h (Table 3). It should be noted that the maximum production of H_2 at 2.083 ± 0.107 μmol H_2 mg dry weight^{-1} was obtained by cells incubated in the same medium for 24 h (Table 3). This H_2 production was approximately 70- and 6-folds higher than that of cells incubated in ASN III and $NaNO_3$-free ASN III media, respectively.

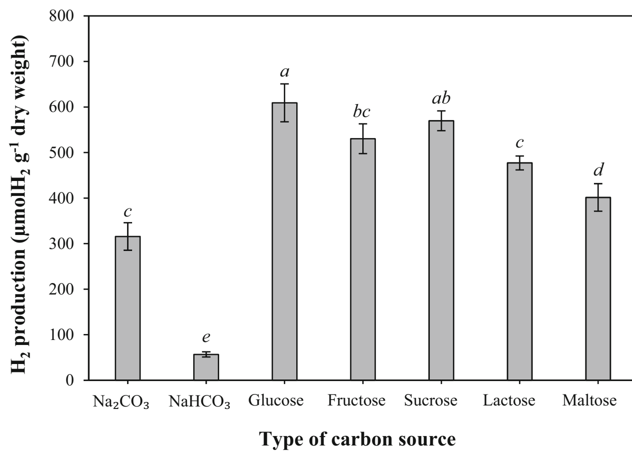

Fig. 2 Effect of different carbon sources on H_2 production rate by *Geitlerinema* sp. RMK-SH10. Cells grown in ASN III medium for 7 days were incubated under different carbon sources with fixed concentration at 0.189 mmol C-atom L^{-1} for 24 h before measurement of H_2 under dark/anaerobic condition. Data are means ± SD ($n = 3$). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P < 0.05$

Discussion

In this study, most of cyanobacterial isolates were identified as *Geitlerinema* which is a predominant benthic cyanobacterium found attached to shells and stones. *Geitlerinema* sp. could be found in both two coastlines of Thailand, the Gulf of Thailand connected to the Pacific Ocean, and the Andaman Sea connected to the Indian Ocean. All 54 purified cyanobacterial isolates were screened for H_2 production under N-deprived four conditions. Most cyanobacterial isolates produced high amount of H_2 under both anaerobic/dark and anaerobic/light conditions, with higher H_2 production seen under the former condition (Table 1). Under N-deprivation, the selected *Geitlerinema* sp. RMK-SH10 was found to accumulate glycogen in the cells (data not shown). The increase of glycogen has also been reported for *Synechocystis* sp. PCC 6803 grown in nitrogen deficient medium under light (Monshupanee and Incharoensakdi 2014). During anaerobic fermentation the glycogen is catabolized via glycolytic pathway to provide sufficient amounts of ATP and NAD(P)H for H_2 production by an activity of bidirectional hydrogenase (Troshina et al. 2002). The primary screening process for H_2 production by cyanobacteria was mostly performed under N-deprivation (Ramana et al. 1990; Schütz et al. 2004; Allahverdiyeva et al. 2010; He et al. 2012). However, the deprivation of other compositions might also play an important role in cyanobacterial H_2 production (Antal and Lindblad 2005; Raksajit et al. 2012). Cyanobacteria in this study generally produced less H_2 in the light than in the dark because during light exposure cyanobacteria were able to split water to generate O_2 as a main product of photolysis. The generated O_2 inhibited the activity of bidirectional hydrogenase and nitrogenase (Fay 1992; Tamagnini et al. 2000) resulting in a decrease in H_2 production.

In the preliminary screening, *Geitlerinema* sp. RMK-SH10 produced the highest H_2 under anaerobic/dark condition when cells were incubated in $NaNO_3$-free ASN III medium for 24 h (Table 1). *Geitlerinema* is a filamentous non-heterocystous cyanobacterium belonging to the order Oscillatoriales (Castenholz et al. 2001). All *Geitlerinema* isolates were single filamentous trichomes with a flexuous or straight shape. Each individual trichome contained a blue-green cylindrical filament with one or more granules of cyanophycin, one of which

Table 3 Maximum H_2 production, H_2 production rate and bidirectional hydrogenase activity of *Geitlerinema* sp. RMK-SH10 in various types of media

Type of media	Maximum H_2 production (µmol H_2 g^{-1} dry weight)	Maximum H_2 production rate (µmol H_2 g^{-1} dry weight h^{-1})	H_2ase activity (µmol H_2 mg^{-1} dry weight min^{-1})
ASN III	30.647 ± 0.453	13.819 ± 0.574	0.057 ± 0.002
N-free ASN III + 0.2 M NaCl	424.504 ± 61.516	117.578 ± 8.752	0.121 ± 0.004
N-free ASN III + 0.2 M NaCl + 18.9 mmol C-atom glucose L^{-1}	1802.118 ± 122.779	237.717 ± 8.317	0.345 ± 0.004
N-free ASN III + 0.2 M NaCl + 18.9 mmol C-atom glucose L^{-1} + 0.1 µM Ni^{2+}	2083.406 ± 107.497	271.093 ± 13.074	0.389 ± 0.012
N-free ASN III + 0.4 M NaCl	353.705 ± 7.693	69.266 ± 2.222	0.097 ± 0.002
N-free ASN III + 0.4 M NaCl + 18.9 mmol C-atom glucose L^{-1}	1410.687 ± 19.583	119.563 ± 4.428	0.149 ± 0.024
N-free ASN III + 0.4 M NaCl + 18.9 mmol C-atom glucose L^{-1} + 0.1 µM Ni^{2+}	1536.721 ± 5.138	160.409 ± 7.836	0.252 ± 0.010

Cells were grown in an ASN III medium for 7 days. Cells were then transferred into different types of N-free ASN III and ASN III media. In case of carbon source, glucose at concentration of 18.9 mmol C-atom L^{-1} was added instead of 0.189 mmol C-atom Na_2CO_3 L^{-1}. Cells were further incubated for another 24 h before H_2 production analysis under dark anaerobic condition. The maximum H_2 production rate was determined at 4 h of incubation time whereas the maximum H_2 yield was measured after incubation for 24 h. The in vivo hydrogenase activity was determined from 4-h incubated cells by measuring H_2 produced in the first 15 min with the presence of dithionite-reduced methyl viologen. Data are means ± SD ($n = 3$)

was close to the cross wall. Until now, no H_2 production by marine or freshwater *Geitlerinema* has been investigated. Although the diazotrophic cyanobacterium *Geitlerinema* sp. PCC 9228 (formerly *Oscillatoria limnetica* "Solar lake") was shown to be capable of N_2 fixation in pure culture (Stal and Krumbein 1981) and contains a nitrogen fixation operon (*nifHDK*) and a bidirectional hydrogenase gene cluster (*hoxEFUYH*) but does not possess an uptake hydrogenase genes (hupSL) (Grim and Dick 2016), *Geitlerinema* sp. RMK-SH10 isolated from seawater in this study showed no detectable nitrogenase activity (data not shown). It is possible that H_2 production of *Geitlerinema* sp. RMK-SH10 might be dependent on the bidirectional hydrogenase activity rather than nitrogenase activity.

The marine *Geitlerinema* sp. RMK-SH10 could grow in liquid ASN III medium (Fig. 1), because the synthetic seawater ASN III medium contains nutritional and mineral elements including NaCl which are required for marine cyanobacterial growth (Rippka et al. 1979). On the other hand, *Geitlerinema* sp. RMK-SH10 was not able to grow well in BG11 medium lacking NaCl, a common medium for freshwater cyanobacteria (data not shown).

The highest H_2 production rate of 13.382 ± 0.600 μmol H_2 g^{-1} dry weight h^{-1} was found in 7-day grown cells with a decreased production at a later growth phase (Fig. 1). This suggested that H_2 production by *Geitlerinema* sp. RMK-SH10 is growth phase dependent. This result is similar to those previously reported in freshwater unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Baebprasert et al. 2010) and halotolerant unicellular cyanobacterium *A. halophytica* (Taikhao et al. 2013). The growth phase dependent H_2 production has also been shown in a marine filamentous cyanobacterium *Oscillatoria* sp. Miami BG7 (Phlips and Mitsui 1983) and a marine unicellular cyanobacterium *Synechococcus* sp. Miami 04351 (Luo and Mitsui 1994) with highest production observed at the beginning of stationary growth phase and at the early log phase, respectively. Thus, H_2 production in cyanobacteria is dependent on species and their growth phase.

Nutrients and microelements have been reported to play an important role in cyanobacterial H_2 production (Datta et al. 2000; Carrieri et al. 2008). In *Geitlerinema* sp. RMK-SH10, the highest H_2 production of 318.237 ± 23.405 μmol H_2 g^{-1} dry weight was obtained in cells incubated in $NaNO_3$-free ASN III medium, an approximately 10-fold increase compared to that of cells incubated in the normal ASN III medium containing 8.8 mM $NaNO_3$ (Fig. S1, Supplementary file). The increase of H_2 production under N-deprivation condition is ascribed to an increased electrons flow towards hydrogenase accompanying a degradation of the fermentative glycogen accumulated during photoautotrophic growth. This result is in agreement with previous studies in freshwater cyanobacteria *Gloeocapsa alpicola* (Serebryakova et al. 1998; Troshina et al. 2002) and *Arthrospira maxima* (Ananyev et al. 2008) as well as in marine cyanobacteria *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui 1981), *P. valderianum* BDU 20041 (Prabaharan and Subramanian 1996), *L. valderiana* BDU 20041 (Prabaharan et al. 2010), and *A. halophytica* (Taikhao et al. 2013).

Sulfur deprivation does not appear to affect H_2 production by *Geitlerinema* sp. RMK-SH10, since only slight differences of H_2 production were observed in the absence and in the presence of 1.4 and 14 mM $MgSO_4{\cdot}7H_2O$ (Table 2). However, sulfur deprivation has been found to enhance the rate of H_2 production in other cyanobacterial species such as *G. alpicola*, *Synechocystis* sp. PCC 6803, and *A. halophytica* (Antal and Lindblad 2005; Taikhao et al. 2013). Sulfur is a very important component in the photosystem II repair cycle (Wykoff et al. 1998). Lack of sulfur causes an inhibition of the oxygenic photosynthesis resulting in a decrease of O_2 and thus leads to an enhancement of H_2 production by *G. alpicola* and *Synechocystis* sp. PCC 6803 (Antal and Lindblad 2005).

The H_2 production by *Geitlerinema* sp. RMK-SH10 was stimulated by low concentration of NaCl with the maximum of 434.705 ± 25.337 μmol H_2 g^{-1} dry weight observed in cells incubated in $NaNO_3$-free ASN III medium containing 0.2 M or 1.2% (*w*/*v*) NaCl, and at higher than 0.4 M NaCl, the production was decreased (Table 2). Salinity affects H_2 production depending on the type of cyanobacterial species. In freshwater cyanobacteria, H_2 production was highest in cells incubated in NaCl-free medium but it is decreased when NaCl concentration is increased (Shah et al. 2003). On the contrary, marine cyanobacteria produced the highest H_2 at their optimal level of NaCl concentration; for example, marine cyanobacterium *Lyngbya* sp. strain 108 showed the highest H_2 production in medium containing 3% (*w*/*v*) NaCl (Kuwada and Ohta 1989). Too high NaCl concentration reduces H_2 production in all types of cyanobacteria, because the needed energy for H_2 production was used to combat salinity stress by extrusion of Na^+ ions out of cells or by prevention of Na^+ influx into the cells (Tel-Or and Melhamed-Harel 1981; Rai and Abraham 1995).

Under various Fe^{3+} concentrations, the highest H_2 production was found in cells incubated in $NaNO_3$-free ASN III medium containing 2 μM Fe^{3+} (Table 2). ASN III medium contains Fe^{3+}concentration at 2 μM that is suitable for cyanobacterial growth. This Fe^{3+} concentration is suitable for H_2 production by *Geitlerinema* sp. RMK-SH10 because iron is required for hydrogenase activity to produce H_2. Iron is normally a cofactor of NiFe-hydrogenase enzyme, and it enhances the electron transport process towards hydrogenase to evolve H_2 (Lin and Stewart 1997). In addition, iron is also involved in the electron transport system in cyanobacterial cells, such as photosynthesis and respiration (Raven et al. 1999) and nitrogen fixation (Küpper et al. 2008). However, too high iron concentration at 200 μM drastically reduced H_2 production, which might be due to the diversion of the energy

and reducing power for the extrusion of excess iron out of the cells to alleviate iron toxicity.

Apart from iron, a trace amount of nickel also stimulated H_2 production by *Geitlerinema* sp. RMK-SH10, with maximum production observed at 0.1 μM Ni^{2+} (Table 2). Nickel is known as a metal cofactor of NiFe–hydrogenase in cyanobacteria; therefore, nickel is required for hydrogenase activity (Axelsson and Lindblad 2002; Gutekunst et al. 2006). In addition, nickel may have other roles in cell function other than hydrogen metabolism (Daday et al. 1985). However, too high concentrations of Ni^{2+} resulted in a decrease of H_2 production (Table 2). It is suggested that the optimal nickel ion concentration can maximize H_2 production by *Geitlerinema* sp. RMK-SH10.

Among different types of carbon source, glucose seemed to be an optimal carbon source for H_2 production by *Geitlerinema* sp. RMK-SH10 (Fig. 2) and the highest H_2 production of 1458.094 ± 34.751 μmol H_2 g^{-1} dry weight was found when glucose concentration was 18.9 mmol C-atom L^{-1} (Table 2). In *Geitlerinema* sp. RMK-SH10, the type of carbon source and its concentration had much greater influence on H_2 production than that by other nutrients and minerals. When glucose catabolism occurs, it leads to an increase of NAD(P)H and ATP which are used for H_2 production aided by nitrogenase or hydrogenase activity (Datta et al. 2000; Chen et al. 2008). However, in the case of *Geitlerinema* sp. RMK-SH10, only hydrogenase appeared to be responsible for H_2 production since no nitrogenase activity was detected. The results of the present study are consistent with those of the previous reports where the halophilic cyanobacterium *A. halophytica* and a marine cyanobacterium *Synechococcus* sp. Miami BG 043511 used glucose as a carbon source for H_2 production (Luo and Mitsui 1994; Taikhao et al. 2013).

In this study, the maximum H_2 production rate, hydrogenase activity, and H_2 accumulation were obtained in *Geitlerinema* sp. RMK-SH10 incubated in $NaNO_3$-free ASN III medium containing 0.2 M NaCl, 18.9 mmol C-atom glucose L^{-1} and 0.1 μM Ni^{2+} (Table 3). H_2 production by cells in each type of media corresponded well with hydrogenase activity. The results confirm that the absence of nitrogen source and the presence of optimal concentrations of NaCl, glucose, and Ni^{2+} in media promote hydrogenase activity and H_2 production. Table 4

Table 4 H_2 production by *Geitlerinema* sp. RMK-SH10 compared with other marine filamentous cyanobacterial strains

Filamentous cyanobacteria	Maximum H_2 production rate	Growth conditions	H_2 evolution assay conditions	References
Geitlerinema sp. RMK-SH10	0.271 μmol H_2 mg^{-1} dry wt h^{-1} or 6.072 mL H_2 g^{-1} dry wt h^{-1}	ASN III medium, 30 μmol photons m^{-2} s^{-1}, 30 °C	ASN III–N medium + 0.2 M NaCl + 18.9 mmol C-atom L^{-1} glucose + 0.1 μM Ni^{2+}, Ar, dark, 30 °C	This study
Oscillatoria brevis B1567	0.168 μmol H_2 mg^{-1} dry wt h^{-1}	AA medium, 5% CO_2 in air, 7000 lx, 25 °C	AA medium, 3% CO_2, 4000 lx, 25 °C	Lambert and Smith (1977)
Calothrix scopulorum 1410/5	0.128 μmol H_2 mg^{-1} dry wt h^{-1}	AA medium, 5% CO_2 in air, 7000 lx, 25 °C	AA medium, 3% CO_2, 4000 lx, 25 °C	Lambert and Smith (1977)
Calothrix membrnacea B379	0.108 μmol H_2 mg^{-1} dry wt h^{-1}	AA medium, 5% CO_2 in air, 7000 lx, 25 °C	AA medium, 3% CO_2, 4000 lx, 25 °C	Lambert and Smith (1977)
Oscillatoria sp. Miami BG7	0.250 μmol H_2 mg^{-1} dry wt h^{-1}	A medium + 25 mg L^{-1} NH_4Cl, 100 μmol photons m^{-2} s^{-1}, 28 °C	A-N medium, Ar, 90 μmol photons m^{-2} s^{-1}, 37 °C	Phlips and Mitsui (1983)
Phormidium valderianum BDU 20041	0.20 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, pH 7.5, 5.5 μmol photons m^{-2} s^{-1}, 18 h dark~ 6 h light cycle, 27 °C	Prabaharan and Subramanian (1996)
Arthrospira maxima CS-328	13.3 mL H_2 g^{-1} dry wt day^{-1}	Zarrouk medium + 1 μM Ni^{+}, air, 30 °C	Zarrouk medium +1 μM Ni^{+}, Ar, dark, 35 °C	Ananyev et al. (2008)
Leptolyngbya valderiana BDU 20041	0.02 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, Ar, dark, 27 ± 2 °C	Prabaharan et al. (2010)
Lyngbya confervoides BDU142001	0.02 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, N_2, dark, 27 ± 2 °C	Prabaharan et al. (2010)
Lyngbya confervoides BDU1420301	0.01 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, Ar, dark, 27 ± 2 °C	Prabaharan et al. (2010)
Microcoleus chthnoplastes BDU 91212	0.017 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, Ar, dark, 27 ± 2 °C	Prabaharan et al. (2010)
Plectonema terebrans BDU141311	0.013 μmol H_2 mg^{-1} dry wt h^{-1}	ASN III medium, 13.7 W m^{-2}, 27 ± 2 °C	ASN III–N medium, N_2, dark, 27 ± 2 °C	Prabaharan et al. (2010)

shows dark fermentative H_2 production rates and conditions of various marine filamentous cyanobacterial strains in comparison with *Geitlerinema* sp. RMK-SH10 reported in this study. *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate with 0.271 μmol H_2 g^{-1} dry weight h^{-1} or 6.072 mL H_2 g^{-1} dry weight h^{-1} (Table 4). This rate is slightly higher than those of *Oscillatoria* sp. Miami BG7 and *Phormidium valderianum* BDU 20041, but much higher than those of other filamentous cyanobacteria. Thus, *Geitlerinema* sp. RMK-SH10 represents a filamentous cyanobacterium with high potential for H_2 production.

In conclusion, several marine cyanobacterial strains isolated from seawater environments in the Gulf of Thailand and the Andaman Sea of Thailand were screened for H_2 production. Among them, a marine cyanobacterium *Geitlerinema* sp. RMK-SH10 shows a high potential for H_2 production under anaerobic/dark condition. It produces the maximum H_2 yield and hydrogenase activity under the optimal conditions: no addition of $NaNO_3$ but with an addition of 0.2 M NaCl, 18.9 mmol C-atom L^{-1} glucose, and 0.1 μM Ni^{2+}.

Funding information This study was financially supported by a research grant from the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang. A.I. thanks the Chulalongkorn University for the research grant on the Frontier Research Energy Cluster (CU-59-048-EN).

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