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Screening for hydrogen-producing strains of green microalgae in phosphorus or sulphur deprived medium under nitrogen limitation

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ABSTRACT: Hydrogen gas, one of the best candidates for clean and renewable energy, can be produced by microalgae that can use solar energy to cause photolysis of water. This study screened H_2-producing indigenous green microalgae under sulphur (S-) deprivation, simultaneous nitrogen (N-) limitation and S-deprivation, or simultaneous N-limitation and phosphorus (P-) deprivation. Sequences of 18S rDNA and ITS in conjunction with morphological characteristics were used to identify the algae. We report that *Chlorella lewinii*, *Micractinium* sp., *Coelastrella* sp., and *Monoraphidium* sp. have the ability to produce H_2. The increase in H_2 photoproduction when N is limited seems to be a universal phenomenon in most tested strains of *Chlorella*, in all strains of *Chlamydomonas*, but in no strain of *Coelastrella*, *Micractinium*, or *Scenedesmus*. *Chlorella sorokiniana* KU204 produced H_2 (46 ml/l) under S-deprivation. This strain exhibited the highest H_2-producing ability (1.30 ml l^{-1} h^{-1}) and accumulated up to 90 ml/l under simultaneous N-limitation and S-deprivation. Interestingly, *C. sorokiniana* KU204 could also produce H_2 under simultaneous N-limitation and P-starvation (69 ml/l). The induction time to reach an anoxic state by most tested strains of *Chlorella*, but not *Chlamydomonas*, was shorter under simultaneous N-limitation and S-deprivation than under S-deprivation. In addition, those strains of *Chlorella* exhibited high H_2 photoproduction under simultaneous N-limitation and S-deprivation. A few *Chlorella* strains were unable to reach an anoxic state during the experiment. However, such regularity is not found in *Chlamydomonas*. The results indicate that the mechanism of H_2 photoproduction in *Chlorella* may differ from that in *Chlamydomonas*.

KEYWORDS: hydrogen production, green algae, sulphur deprivation, phosphorus deprivation

INTRODUCTION

Renewable energy is being actively sought worldwide to replace the declining sources of fossil fuels. Hydrogen gas is considered to be one of the best candidates for alternative clean energy[1]. H_2 can be produced by microalgae that have an ability to capture solar energy and convert it into chemical energy. Hydrogen production under anaerobic conditions was first reported for the unicellular green alga *Scenedesmus obliquus*[2]. H_2 was only produced for a few minutes and then the yield dropped. A system that maintains the capacity of evolving large amounts of H_2 for several days under S-deprivation in the green alga *Chlamydomonas reinhardtii* was later developed[3]. S-deprivation reduced the photosynthetic O_2-evolving machineries in the algal cells leading to intracellular anaerobiosis which would favour H_2 production. N- and P-deprivation that can also stimulate H_2 production, but with lower H_2 yields and longer induction time to reach an anoxic state[4,5]. Currently, green algae such as *C. reinhardtii*, *Tetraspora* sp. CU2551, and *C. protothecoides* have been preferred for H_2 photoproduction[6–9]. It is possible that other unknown green algal species may produce H_2 better than the reported strains. In addition, since the induction of H_2 photoproduction by *C. reinhardtii* was optimized under

sulphur deprivation, deprivation of other nutrients could also induce the algae cells to produce H_2.

In the current work, the H_2-producing capacity of green microalgae was evaluated in response to different modified tris-acetate-phosphate medium[10]. We collected various fresh water samples in Thailand to isolate indigenous algal strains and screened for H_2 production ability under S-deprivation, a combination of N-limitation and S-deprivation, or a combination of N-limitation and P-starvation.

MATERIALS AND METHODS

Microalgae samples collection

Freshwater samples were collected from several ponds, canals, rivers, and reservoirs in Thailand. To collect microalgae samples, 20 l of water samples were poured through a plankton net with 16 μm pore size. The algal residue in the bucket of the plankton net was collected and then 1 ml of the algal samples was added to the tris-acetate-phosphate (TAP) or Blue-Green (BG-11) medium.

Isolation of pure cultures

Several green algae were isolated using glass Pasteur pipettes and cultured in TAP or BG-11 liquid media with combinations of ampicillin, chloramphenicol, streptomycin, kanamycin, and carbendazim. The algal isolates were then cultivated at 25 ± 1 °C under 20 μmol photons $m^{-2}\,s^{-1}$ of illumination for 7–30 days. The streak plate method was then used to separate the microalgae, bacteria, and fungi on TAP agar medium. The above process was repeated several times until there was no fungal or bacterial contamination. Then, each pure, individual colony was picked and cultured on separate TAP agar plates for further study. The morphology of the collected algae was observed and photographed using a light microscope (BX 51, Olympus Imaging Corp., Japan). Morphological characteristics were identified according to John[11].

Culture conditions

Algae cells were cultured in standard TAP medium containing 7 mM NH_4Cl at pH 7.3 ± 0.1 and kept at 25 ± 1 °C under a light/dark regime of 14:10 h for 4–7 days. Cool white fluorescent lamps of about 35 μmol photons $m^{-2}\,s^{-1}$ were used for illumination.

DNA extraction

DNA from algal cells was extracted using a DNAeasy Plant mini-kit (Qiagen, Germany) according to the manufacturer's instructions. The presence or absence of the extracted DNA was determined by electrophoresis in 1% agarose gel and stained with ethidium bromide. The DNA concentration was quantified using a UV-spectrophotometer (8453, Agilent Technologies, Inc., Germany) at an optical density of 260 nm.

Polymerase Chain reaction (PCR) amplification of 18S rDNA gene and ITS regions

The 18S rDNA sequences were amplified using the universal eukaryotic primers, 5′-GTCAGAGGTGAA ATTCTTGGATTTA-3′ as forward and 5′-AGGGCA GGGACGTAATCAACG-3′ as reverse primer. The expected amplified product size of the 18S rDNA gene was about 750 bp. The ITS regions (ITS1, 5.8S, and ITS2) of ribosome were amplified using the following primers: forward, 5′-GAAGTCGTAACAA GGTTTCC-3′ and reverse, 5′-TCCTGGTTAGTTTCT TTTCC-3′[12]. PCR amplification was carried out in a Px2 Thermal Cycler (Thermo Electron Corporation, USA). For amplifying the 18S rDNA gene, DNA was denatured at 95 °C for 4 min and followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final elongation step at 72 °C for 10 min, then holding at 4 °C. For the amplification of ITS regions, a touchdown amplification program was used. DNA was denatured at 95 °C for 4 min and followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C (dropping 0.5 °C each cycle) for 30 s, and extension at 72 °C for 40 s. Amplification of ITS regions was continued using a second 30 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 40 s, and a final elongation step at 72 °C for 7 min, then holding at 4 °C. All PCR amplifications were performed in 25 μl master mix solution containing 25 μl of total DNA, 1X *Taq* buffer, 1.0 μM of dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 1.0 unit of *Taq* DNA polymerase. The PCR products were determined using electrophoresis in 1% (W/V) agarose gel in $1 \times$ TBE buffer and visualized using ethidium bromide straining.

Sequencing and phylogenetic analysis

The PCR products were cleaned up using the QIAquick Gel Extraction kit (Qiagen, Germany). For sequencing, 30 ng of PCR product was used as a template with 10 pmol/μl of the above primers and made to a final volume of 10 μl with distilled water. The samples were sent to the 1st base company (Malaysia) for sequencing. The sequence similarity

was compared with a data set of algal sequences in the NCBI database. A neighbour joining (NJ) tree was obtained using the software MEGA 5 [13]. The credibility of the branching orders were analysed by bootstrap analysis of 1000 replicates.

Effect of nutrient deprivation on population growth and chlorophyll content

To determine the effects of nutrient deprivation, algal cultures were harvested by centrifugation (2500*g*, 5 min, 25 °C) and washed twice with different TAP media (TAP, S-deprivation (TAP-S), N-deprivation (TAP-N), and P-deprivation (TAP-P)). Then the pellets were resuspended in each TAP medium to an initial optical density of 2.0×10^6 cells/ml. The biomass production was determined by the chlorophyll content and cell density values. For cell density, cells were counted using a haemocytometer under a light microscope (CH 30, Olympus Imaging Corp., Japan).

Screening for H_2 production

To determine the H_2-producing ability, the microalgae were pre-cultured in TAP medium at 25 ± 1 °C under 35 μmol photons $m^{-2} s^{-1}$ of illumination using a light/dark regime of 14:10 h for 4–7 days. Three different TAP media were used to screen for the H_2-producing ability of microalgae. For the first culture medium (TAP-S medium), all isolates of the green algae were pre-cultured in TAP medium containing 7 mM NH_4Cl and the algal cultures were harvested at the late logarithmic phase using centrifugation (2500*g*, 5 min, 25 °C) and washed twice with TAP-S medium. The S-deprivation medium was obtained by replacing all sulphate salts with chloride salts. Then the pellets were resuspended in TAP-S medium. An optical density of $2.5–3.5 \times 10^7$ cells/ml was used for all treatments. All cell cultures were transferred into 20 ml glass vials, sealed with butyl rubber stoppers and bubbled with argon gas for 5 min to remove O_2 in the head space of the vial. The sealed glass vials were shaken at 110 rpm and exposed to a continuous light intensity of 35 μmol photons $m^{-2} s^{-1}$. High H_2 producing strains cultured in the 20 ml glass vials were selected and transferred into 650 ml bioreactors containing TAP-S medium under continuous light (35 μmol photons $m^{-2} s^{-1}$). Each vessel was sealed with a butyl rubber stopper and fitted with an outlet tube connected to a graduated cylinder for gas collection by displacement of water [3]. For the second set of culture conditions (simultaneous N-limitation and S-deprivation), cells were grown in nitrogen-limited medium (TAP with 0.7 mM NH_4Cl) and then washed twice and cultured in simultaneous N-limitation and S-deprivation (TAP-S with 0.7 mM NH_4Cl) medium.

For the third set of culture conditions (simultaneous N-limitation and P-starvation), cells were grown in nitrogen-limited medium (TAP containing 0.35 mM NH_4Cl) and then washed twice and cultured in simultaneous N-limitation and P-deprivation (TAP-P with 0.35 mM NH_4Cl) medium. P-starvation medium was obtained by omitting phosphate.

Measurement of H_2 and O_2 gas

Hydrogen production was analysed using gas chromatography (GC) (GC-112A, Shanghai Phenic Optical Scientific Instrument Co. Ltd., China) equipped with a molecular sieve 5 Å column using a Thermal Conductivity Detector. To separate H_2, O_2 and N_2, argon was used as a carrier gas at a flow rate of 15 ml/min. The packed column was maintained at 40 °C and the temperature for the detector and injector was set at 100 °C. The total H_2 and O_2 volumes were calculated according to the peak area, which was pre-calibrated by injecting known concentrations of standard gases. The empirical equations for the H_2 and O_2 calculations were as follows: H_2 (μl) = 0.00001 × Area + 0.5095; O_2 (μl) = 0.0007 × Area + 1.1045. Area represents the value of the corresponding peak area of H_2 and O_2 detected using GC [14].

Measurement of chlorophyll content

Chlorophyll in the cell cultures was extracted as described by Liu [15] and total chlorophyll (mg/l) was calculated as $4.44 \times A_{666\ nm} + 19.71 \times A_{653\ nm}$, where $A_{666\ nm}$ and $A_{653\ nm}$ correspond to the absorbance of supernatant at 666 nm and 653 nm wavelength, respectively, with a 1 cm path of the cuvette.

RESULTS AND DISCUSSION

Collection of samples, isolation, and purification of microalgal strains

Among the collected samples, at least 86 isolates of axenic uniclonal cultures were obtained through a streak plate method. Among these, 43 isolates were successfully cultivated in TAP medium to densities of over 2.0×10^6 cells/ml. These 43 isolates were taken for screening of their H_2-producing ability.

Strain identification

The selected 43 isolates were morphologically identified as belonging to the following genera: *Chlamy-*

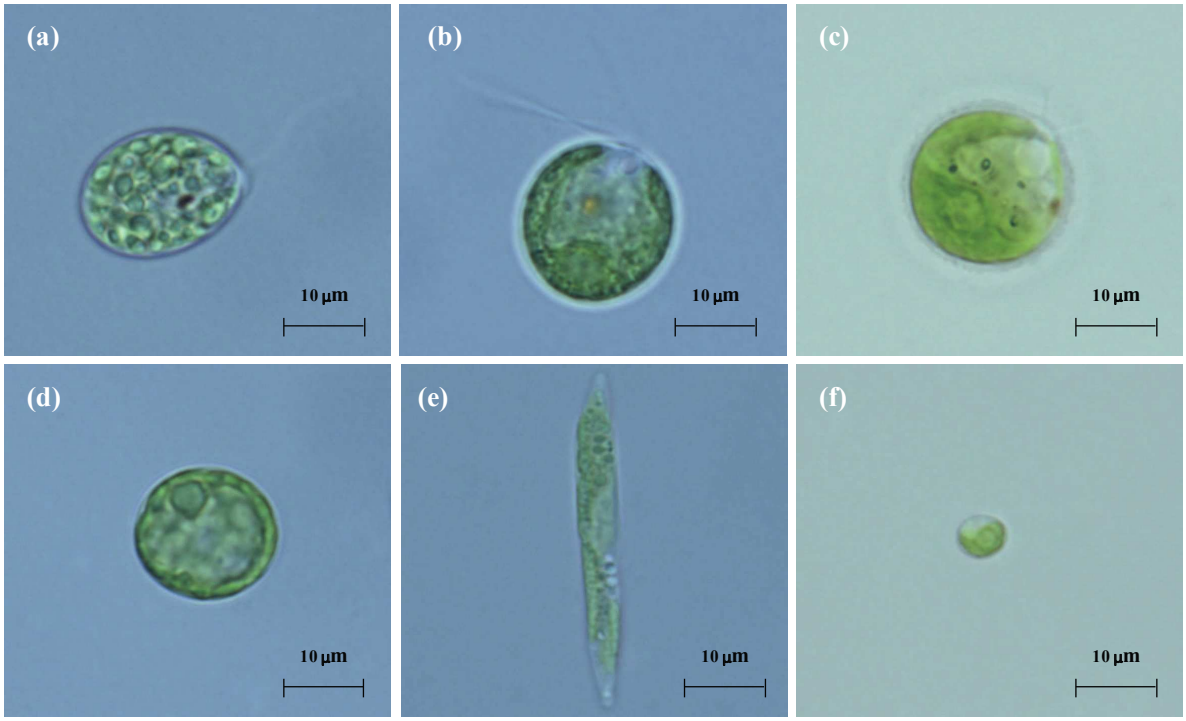

Fig. 1 Observation using light microscopy of different green algal species isolated from freshwater samples. (a) *Chlamydomonas* sp. KU103, (b) *Chlamydomonas* sp. KU106, (c) *Chlamydomonas* sp. KU108, (d) *Coelastrella* sp. KU501, (e) *Monoraphidium* sp. KU401, (f) *C. sorokiniana* KU204.

domonas (10 isolates), *Chlorella* (15 isolates), *Micractinium* (2 isolates), *Scenedesmus*, (8 isolates), *Coelastrella* (7 isolates), and *Monoraphidium* (1 isolate). The morphology of some isolated green microalgal species observed under the light microscope is shown in Fig. 1. To confirm the genus of the algae samples, 18S rDNA gene partial sequences were subjected to a BLAST search. Analysis of the 18S rDNA gene showed that the sequence similarity of the isolated strains was closely related to *Chlamydomonas* sp. (98–99%), *Chlorella* sp. (99–100%), *Coelastrella* sp. (98–99%), *Micractinium* sp. (99–100%), *Monoraphidium* sp. (99%), and *Scenedesmus* (99–100%) (data not shown). This BLAST result from 18S rDNA correlated with the grouping from morphological characteristics.

To identify the isolates to the species level, the ITS sequences were used in alignments (CLUSTAL-X) prior to Bootstrap NJ phylogenetic tree determination (Fig. 2). The ITS sequences were aligned and compared with a data set consisting of 16 sequences retrieved from NCBI databases. From the phylogenetic analysis of the ITS sequences, the microalgae samples could be categorized into four different clades, i.e., *Chlorella*, *Chlamydomonas*, *Coelastrella*, and *Micractinium* clades (Fig. 2). In the *Chlorella* clade, the isolates KU202, KU203, KU204, KU205, KU206, and KU208 formed a cluster with *C. sorokiniana* with 76% bootstrap support and showed 99% sequence similarity to *C. sorokiniana* JQ898145.1. Three *Chlorella* isolates (KU207, KU209, and KU211) were closely related to *C. sorokiniana* UTEX 1665 KJ676113.1, *Chlorella* sp. Iso4 JX041600.1 and *C. sorokiniana* UTEX 2805 KJ676109.1, respectively, (98% similarity). Moreover, the isolates KU201, KU213, KU215, KU217, and KU220 were clustered with *C. lewinii* CCAP 211/90 FM205861.1 (99% similarity). In the *Micractinium* clade, the isolates KU210 and KU212 were clustered with *Micractinium* sp. CCAP 248/7 (96% similarity), and *Micractinium* sp. JX889639.1 (93% similarity), respectively. In the *Chlamydomonas* clade, two *Chlamydomonas* sp. isolates (KU105 and KU106) were clustered with *C. debaryana* FR865600.1 (94% similarity). *Chlamydomonas* sp. isolates (KU111) were closely related to *C. reinhardtii* CC-620 JX839533.1. Moreover, *Chlamydomonas* sp. isolates (KU101, KU102, KU103, KU104, KU107, and KU108) were clustered with *Chlamydomonas* sp. but exhibited

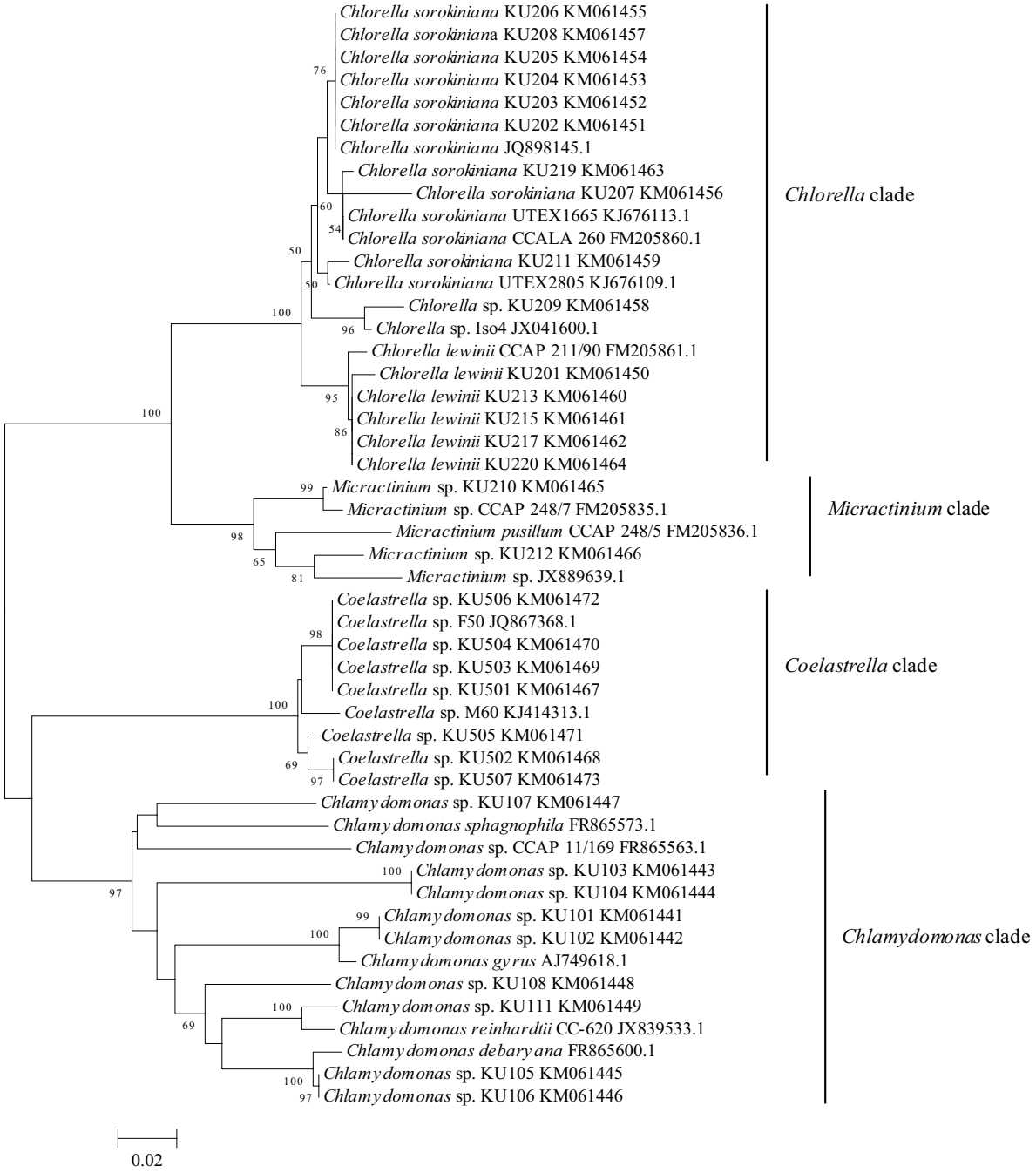

Fig. 2 Phylogenetic tree of green microalgae constructed from ITS sequences (ITS1, 5.8S, and ITS2).

low similarity (85–90%). In the *Coelastrella* clade, four *Coelastrella* isolates—namely, KU501, KU503, KU504, and KU506 formed a cluster with *Coelastrella* sp. with strong bootstrap support (98%) and exhibited 99–100% sequence similarity to *Coelastrella* sp. F50 JQ867368.1. The ITS phylogenetic tree suggested a high homology among most strains to the reference strains. ITS sequences could therefore be used to identify the genus or species of these algae strains. The result agrees with Qiao[16], where the difference between genomic base composition and rDNA similarity in different strains of algae was reported.

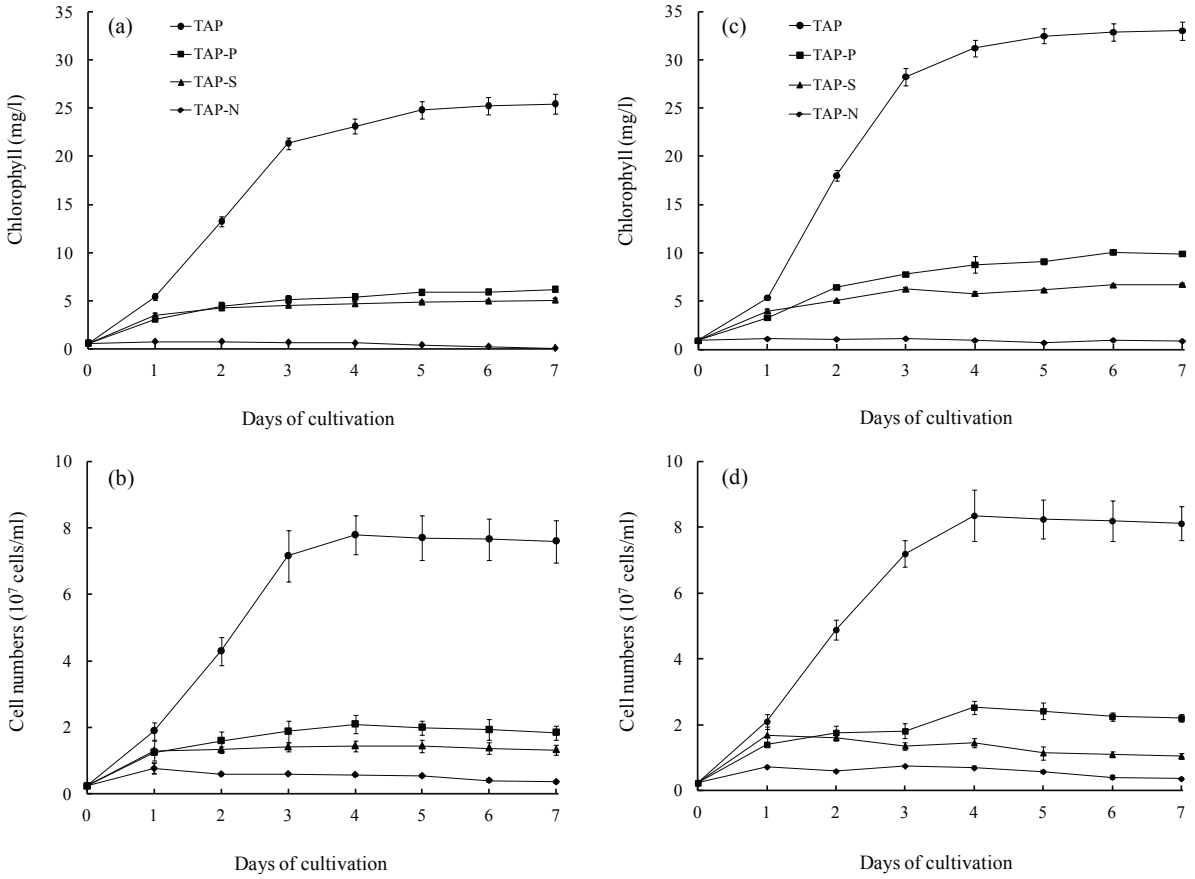

Fig. 3 Changes in chlorophyll content and cell numbers in *C. sorokiniana* KU207 and *Chlorella* sp. KU209 exposed to various TAP media: (a) chlorophyll content and (b) cell numbers in *C. sorokiniana* KU207, (c) chlorophyll content and (d) cell numbers in *Chlorella* sp. KU209.

Effect of nutrient deprivation on chlorophyll content and cell numbers in *C. sorokiniana* KU207 and *Chlorella* sp. KU209

After growing the *Chlorella* sp. in N-, S-, and P-deprivation, the total chlorophyll content and cell numbers were dramatically reduced. The chlorophyll content and cell numbers in N-starved cells showed the greatest decrease, followed by S-deprived, then P-deprived cells compared to those of cells grown in normal TAP medium (Fig. 3). The chlorophyll content in *C. sorokiniana* KU207 and *Chlorella* sp. KU209 declined by approximately 85% and 80%, respectively, after 1 day of growth under the N-deprivation. In P or S-starved cells however, the chlorophyll content of *C. sorokiniana* KU207 and *Chlorella* sp. KU209 decreased by approximately 65%-70% after 2 days. The reduction in the chlorophyll content correlated with the decline in cell numbers. These results indicated that nutrient limitation led to a loss of chlorophyll content and cell numbers in these algae.

Essential macronutrients such as sulphur, nitrogen, or phosphorus have an effect on the growth of phototrophic microalgae[17]. A lack of these nutrients results in the arrest of cell division and the cessation of cell growth[18]. Nitrogen deprivation is a severe stress condition for all organisms because nitrogen is a major component of proteins and nucleic acid. A limitation of nitrogen in *Chlamydomonas* cells resulted in lower cellular chlorophyll contents and LHCII levels[19]. The differences in pigmentation and antennae composition probably have an impact on light energy transfer to the photosystems[5]. Sulphur deprivation causes reversible inhibition of photosynthetic activity in algae. In the absence of S, PSII-dependent O_2-evolving activity in algal cells was gradually inhibited due to their inability to resynthesize the D1 protein[20]. As with S-deprivation, P-deprivation can also inhibit O_2 evolution activity

in algae. The inhibition process of O_2-evolving activity in algal cells by S-deprivation is however faster than by P-deprivation[20]. Moreover, P-deprivation in the medium also leads to the reduction of the CO_2 fixation rate due to the depletion of phosphorylated intermediates in the pentose phosphate cycle[21,22].

In addition to the study of S-deprivation, the results indicated that P- and N-deprivation also have an effect on the growth and photosynthetic activity of the phototrophic microalgae. Moreover, under these stress conditions, O_2 evolution was inhibited and H_2 gas was generated. The ability of green microalgae to produce H_2 under various nutrient deprivations are reported in the subsequent results (see below).

Screening for H_2 photoproduction of freshwater green algae

The H_2-producing ability of the 43 green algal isolates was tested under S-deprivation (TAP-S containing 7 mM NH_4Cl), a combination of N-limitation and S-deprivation (TAP-S with 0.7 mM NH_4Cl), and a combination of N-limitation and P-starvation (TAP-P with 0.35 mM NH_4Cl) (Table 1). Most isolated green algal strains had the ability to generate H_2 under S-deprivation, consisting of *Chlamydomonas*, *Chlorella*, *Micractinium*, *Scenedesmus*, *Monoraphidium*, and *Coelastrella*. We report here, for the first time, that *C. lewinii*, *Micractinium* sp., *Coelastrella* sp., and *Monoraphidium* sp. can also produce H_2 gas. In addition, most tested strains of *Chlorella* and all the strains of *Chlamydomonas* had enhanced H_2 production when cultured on simultaneous N-limitation and S-deprivation (Table 1). This fascinating result however was not found in any strain of *Coelastrella*, *Micractinium*, or *Scenedesmus*. Under S-deprivation, microalgae are capable of H_2 photoproduction[3]. The lack of sulphur causes a gradual inhibition of PSII-dependent O_2-evolving activity in algal cells. When the bioreactors were sealed, the oxygen in the headspace of the bioreactors was gradually consumed by microalgae because of cellular respiration and the bioreactor became anaerobic. Algal strains evolved different amounts of H_2, depending upon various factors such as the ability of each strain, nutrient composition, light intensity, temperature, pH, and oxygen when incubated under nutrient deprivation conditions in the light. The hydrogenase enzyme that catalyses an H_2 production reaction in algae is sensitive oxygen. Under aerobic conditions, however, some species could produce H_2 (Table 1). These species probably are tolerant to oxygen or the activity of hydrogenase may not be totally inhibited[14].

All 15 isolates of *Chlorella* sp. could produce H_2 under TAP-S, TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl (Table 1). Five *C. sorokiniana* isolates (KU204, KU205, KU206, KU207, and KU208) continuously produced H_2 after transferring from standard TAP medium to stress conditions. These strains needed however an induction time of at least 24 h to reach an anoxic state under TAP-S. On the other hand, in TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl, the anaerobic state was reached 3 h earlier, resulting in higher H_2 accumulation. Among these isolates, *C. sorokiniana* KU204 showed the highest H_2 production ability, with the total H_2 yield under TAP-S, TAP-P containing 0.35 mM NH_4Cl and TAP-S with 0.7 mM NH_4Cl being about 46.2, 69.0, and 89.6 ml/l, respectively (Fig. 4). Interestingly, besides S-deprivation, the lack of phosphorus under N-limitation can also limit O_2 evolution in the algae cells. Five *C. lewinii* isolates (KU201, KU213, KU215, KU217, and KU220) generated small amounts of H_2 because of the presence of oxygen in the medium or the bioreactor headspace. This oxygen inhibits H_2 production. The highest content of H_2 in *C. lewinii* (KU213, KU215, KU217, and KU220) was found under TAP-S followed by TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl. Under these nutrient deprivation conditions, however, the strains generated large amounts of oxygen which inactivated hydrogenase and inhibited H_2 production. On the contrary, the *C. lewinii* strain KU201 produced H_2 yields under TAP-S with 0.7 mM NH_4Cl and under TAP-P containing 0.35 mM NH_4Cl which were more than under TAP-S. Under TAP-S with 0.7 mM NH_4Cl, the *C. lewinii* strain KU201 evolved less oxygen, while under TAP-P containing 0.35 mM NH_4Cl, the *C. lewinii* strain KU201 did not evolve oxygen after 12 h. *Chlorella* sp. KU209 and *C. sorokiniana* KU211 were also capable of producing H_2 under TAP-S, TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl. The H_2 yield by *Chlorella* sp. KU209 was however the same in all medium conditions. *Chlorella sorokiniana* KU211 produced more H_2 under TAP-S with 0.7 mM NH_4Cl than TAP-P containing 0.35 mM NH_4Cl because the cultures under TAP-P containing 0.35 mM NH_4Cl could generate both H_2 and O_2. Besides S-deprivation, *Chlorella* sp. can generate H_2 gas under TAP-P containing 0.35 mM NH_4Cl because the starvation of phosphorus can also inhibit O_2-evolving activity. P-deprivation in algae is known to result in an anaer-

Table 1 H_2 production by 43 freshwater green algae.

Green algae	Strain	Treatment					
		TAP-S		TAP-S (0.7 mM NH4Cl)		TAP-P (0.35 mM NH4Cl)	
		H_2 yield (ml/l)	Time[†]	H_2 yield (ml/l)	Time[†]	H_2 yield (ml/l)	Time[†]
C. sorokiniana	KU202	8.87	–	13.02	–	8.15	< 12 h[‡]
C. sorokiniana	KU203	9.07	–	13.86	–	8.39	< 12 h[‡]
C. sorokiniana	KU204	46.20	< 24 h[‡]	89.64	< 3 h[‡]	69.00	< 3 h[‡]
C. sorokiniana	KU205	15.99	< 24 h[‡]	27.98	< 3 h[‡]	20.95	< 3 h[‡]
C. sorokiniana	KU206	10.77	< 24 h[‡]	29.16	< 3 h[‡]	20.13	< 3 h[‡]
C. sorokiniana	KU207	12.49	< 24 h[‡]	24.35	< 3 h[‡]	14.80	< 3 h[‡]
C. sorokiniana	KU208	16.78	< 24 h[‡]	30.18	< 3 h[‡]	17.51	< 3 h[‡]
C. sorokiniana	KU219	5.53	–	18.28	< 12 h[‡]	11.15	< 12 h[‡]
C. sorokiniana	KU211	6.74	–	14.01	< 12 h[‡]	2.74	–
C. lewinii	KU201	5.10	–	13.03	–	11.50	< 12 h[‡]
C. lewinii	KU213	5.87	–	5.63	–	2.93	–
C. lewinii	KU215	6.97	–	6.93	–	2.87	–
C. lewinii	KU217	6.59	–	5.07	–	2.91	–
C. lewinii	KU220	8.20	–	6.36	–	2.71	–
Chlorella sp.	KU209	11.96	–	12.67	–	10.27	< 12 h[‡]
Micractinium sp.	KU210	3.17	–	0.51	–	0.32	–
Micractinium sp.	KU212	0.90	–	0.35	–	0.32	–
Coelastrella sp.	KU501	0.32	–	0.25	–	0.31	–
Coelastrella sp.	KU502	0.47	–	1.13	–	0.41	–
Coelastrella sp.	KU503	0.38	–	0.32	–	0.27	–
Coelastrella sp.	KU504	0.43	–	0.36	–	0.33	–
Coelastrella sp.	KU505	1.95	–	1.39	–	0.72	–
Coelastrella sp.	KU506	0.36	–	0.31	–	0.26	–
Coelastrella sp.	KU507	0.40	–	1.08	–	0.38	–
Chlamydomonas sp.	KU101	1.67	–	2.97	–		
Chlamydomonas sp.	KU102	1.40	–	2.54	–		
Chlamydomonas sp.	KU103	1.78	–	2.89	–		
Chlamydomonas sp.	KU104	1.37	–	2.63	–		
Chlamydomonas sp.	KU105	7.69	< 36 h[‡]	16.90	< 36 h[‡]		
Chlamydomonas sp.	KU106	24.4	< 36 h[‡]	62.00	< 36 h[‡]		
Chlamydomonas sp.	KU107	0[*]	–	0.30	–		
Chlamydomonas sp.	KU108	0.42	–	1.66	–		
Chlamydomonas sp.	KU109	0.58	–	2.17	–		
Chlamydomonas sp.	KU111	11.6	< 36 h[‡]	30.60	< 36 h[‡]		
Scenedesmus sp.	KU301	2.82	–	0.34	–		
Scenedesmus sp.	KU302	4.20	–	0.37	–		
Scenedesmus sp.	KU303	0.35	–	0[*]	–		
Scenedesmus sp.	KU304	0.39	–	0[*]	–		
Scenedesmus sp.	KU305	0.42	–	0[*]	–		
Scenedesmus sp.	KU306	0.34	–	0[*]	–		
Scenedesmus sp.	KU308	2.56	–	0.32	–		
Scenedesmus sp.	KU309	0.39	–	0[*]	–		
Monoraphidium sp.	KU401	1.32	–	2.04	–		

[†] Time to reach an anoxic state.
[‡] O_2 not detected.
[*] H_2 not detected.

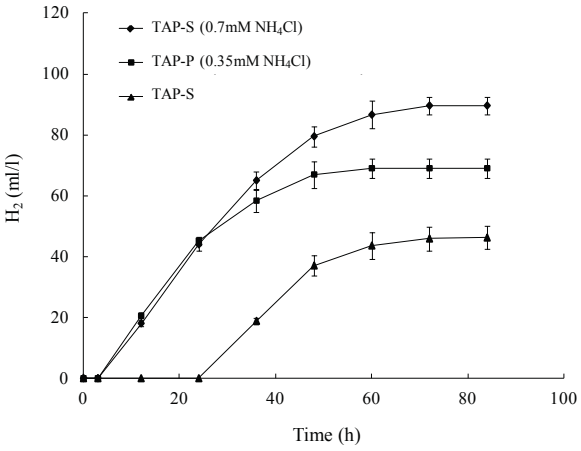

Fig. 4 H_2 photoproduction by *C. sorokiniana* KU204 under TAP-S, TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl.

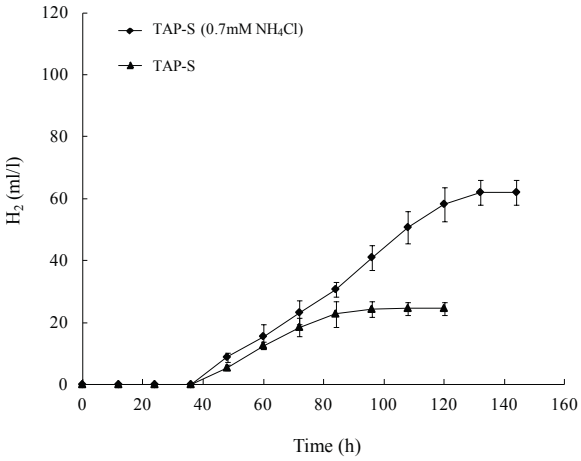

Fig. 5 H_2 photoproduction by *Chlamydomonas* sp. KU106 under TAP-S and TAP-S with 0.7 mM NH_4Cl.

obic state[20], and in higher plants, where P-deprivation leads to a decrease in the light-saturated rate of photosynthesis[21–24]. Moreover, sustained H_2 photoproduction in *C. reinhardtii* cultures can be accomplished by inoculating the washed algae into P-deprived medium at low initial cell densities (below 2 mg Chl/l)[4]. The algal cultures under TAP-P containing 0.35 mM NH_4Cl can be further improved by optimizing the cultivation conditions to enhance the H_2 yield. Achieving sulphur deprivation in marine strains is however difficult because of the high concentration of sulphates in seawater.

Chlamydomonas strains (KU105, KU106, and KU111) were able to produce H_2 in the first 36 h under continuous illumination in TAP-S and TAP-S with 0.7 mM NH_4Cl. On the other hand, *Chlamydomonas* strains (KU101, KU102, KU103, KU104, KU107, KU108, and KU109) were also able to generate H_2 gas but could not shorten the time to reach an anoxic state, resulting in less H_2 accumulation than for the first three *Chlamydomonas* strains. Among the *Chlamydomonas* strains, the highest H_2 production was *Chlamydomonas* sp. KU106, and the maximum H_2 gas production under TAP-S and TAP-S with 0.7 mM NH_4Cl was about 24.4 and 62.0 ml/l, respectively (Fig. 5).

Two *Micractinium* sp. strains (KU210 and KU212) produced H_2 under the test conditions but with low H_2 yield. Moreover, the H_2 production under TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl was less than that for the TAP-S medium because of the nitrogen limitation in the medium and oxygen production by algae. *Coelastrella* sp. (all strains) and *Scenedesmus* sp. strains (KU303, KU304, KU305, KU306, and KU309) generated small amounts of H_2 and rapidly lost vitality after transferring them to the tested media. The cultures turned from a green to a yellow colour and the algae died just a few days after transfer. It appears that microelement starvation involving sulphur, phosphorous, and nitrogen plays an important role in the cell metabolism of these algae. *Monoraphidium* sp. KU401 evolved less H_2 under TAP-S with 0.7 mM NH_4Cl and TAP-P containing 0.35 mM NH_4Cl and generated O_2 at the same time. Our results demonstrate that different species of microalgae could produce H_2 at various levels. H_2 production ability had been found to depend on the strain, nutrient composition, pH, oxygen, light intensity, temperature, optical density and agitation[14,25]. In addition, this ability to produce H_2 may be related to specific anaerobic/low light habitats where the algae require the photobiological H_2 production capacity to survive[12].

Most tested *Chlorella*, but not *Chlamydomonas*, strains reached an anoxic state faster in a medium with simultaneous N-limitation and S-deprivation than in a medium with S-deprivation. In addition, when those strains of *Chlorella* grown in medium with simultaneous N-limitation and S-deprivation exhibited an increased H_2 production. Some could not induce an anoxic state throughout the experiment. However, such regularity was not found in *Chlamydomonas* (Table 1). Thus the mechanism of H_2 photoproduction in *Chlorella* may differ from that of *Chlamydomonas*.

In a previous study, the most well-known mi-

croalga with high H_2 production was *C. reinhardtii*, which accumulates H_2 at the rate of approximately 100–270 ml/l with 9–20 mg/l chlorophyll content[3,8]. In this study, *C. sorokiniana* KU204 showed the highest H_2-producing capacity (1.30 ml $l^{-1}h^{-1}$) and accumulated up to 89.64 ml/l under simultaneous N-limitation and S-deprivation containing a chlorophyll content of 5–6 mg/l. If we assume that *C. sorokiniana* KU204 has a chlorophyll content of 10–12 mg/l, this alga can produce 179.28 ml/l of H_2. As *C. sorokiniana* has strong adaptability to local growing conditions and an extremely rapid growth rate, it is widely utilized on a large scale production. The increased H_2 production of *C. sorokiniana* KU204 exposed to simultaneous N-limitation and S-deprivation suggests then that this microalga has the potential to become a candidate for industrial H_2 production.

In conclusion, from morphological characters and 18S rDNA sequence analysis, the six genera of the algae samples were identified as *Chlamydomonas*, *Chlorella*, *Coelastrella*, *Micractinium*, *Monoraphidium*, and *Scenedesmus*. The ITS sequences were successfully used to identify the species of the algae strains. Most tested green algal strains had the ability to generate H_2 when exposed to S-deprivation. In addition, *Chlorella lewinii*, *Micractinium* sp., *Coelastrella* sp., and *Monoraphidium* sp. were found in this work as novel H_2-producing strains. When the cells were grown under simultaneous N-limitation and S-deprivation, H_2 photoproduction increased in most tested strains of *Chlorella* and in all the strains of *Chlamydomonas*. However, this interesting result was not found in any strain of *Coelastrella*, *Micractinium* or *Scenedesmus*. The *C. sorokiniana* strain KU204 could accumulate significant H_2 under simultaneous N-limitation and S-deprivation. Thus further research efforts are required to optimize cultivation conditions. This study also suggests that conditions of simultaneous N-limitation and S-deprivation could be an alternative method for enhancing the H_2-producing ability in some microalgae such as *Chlorella* and *Chlamydomonas*.

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REFERENCES

1. Mathews J, Wang G (2009) Metabolic pathway engineering for enhanced biohydrogen production. *Int J Hydrogen Energ* **34**, 7404–16.
2. Gaffron H (1939) Reduction of CO_2 with H_2 in green plants. *Nature* **143**, 204–5.
3. Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol* **122**, 127–35.
4. Batyrova KA, Tsygankov AA, Kosourov SN (2012) Sustained hydrogen photoproduction by phosphorus-deprived *Chlamydomonas reinhardtii* cultures. *Int J Hydrogen Energ* **37**, 8834–9.
5. Philipps G, Happe T, Hemschemeier A (2012) Nitrogen deprivation results in photosynthetic hydrogen production in *Chlamydomonas reinhardtii*. *Planta* **235**, 729–45.
6. He ML, Li L, Zhang LT, Liu JG (2012) The enhancement of hydrogen photoproduction in *Chlorella protothecoides* exposed to nitrogen limitation and sulfur deprivation. *Int J Hydrogen Energ* **37**, 16903–15.
7. Maneeruttanarungroj C, Lindblad P, Incharoensakdi A (2010) A newly isolated green alga, *Tetraspora* sp. CU2551, from Thailand with efficient hydrogen production. *Int J Hydrogen Energ* **35**, 13193–9.
8. Melis A (2007) Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). *Planta* **226**, 1075–86.
9. Zhang LT, He ML, Liu JG (2014) The enhancement mechanism of hydrogen photoproduction in *Chlorella protothecoides* under nitrogen limitation and sulfur deprivation. *Int J Hydrogen Energ* **39**, 8969–76.
10. Hoober JK (1989) The *Chlamydomonas* sourcebook. In: Harris EH (ed) *A Comprehensive Guide to Biology and Laboratory Use*, Academic Press, San Diego, pp 1503–4.
11. John DM, Whitton BA, Brook AJ (2002) The freshwater algal flora of the British Isles. An identification guide to freshwater and terrestrial algae, Cambridge Univ Press, Cambridge, p 702.
12. Timmins M, Thomas-Hall SR, Darling A, Zhang E, Hankamer B, Marx UC, Schenk PM (2009) Phylogenetic and molecular analysis of hydrogen-producing green algae. *J Exp Bot* **60**, 1691–702.
13. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2012) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–9.
14. He ML, Li L, Liu JG (2012) Isolation of wild microalgae from natural water bodies for high hydrogen producing strains. *Int J Hydrogen Energ* **37**, 4046–56.
15. Liu JG, Bukatin VE, Tsygankov AA (2006) Light

energy conversion into H_2 by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations. *Int J Hydrogen Energ* **31**, 1591–6.
16. Qiao H, Wang G, Zhang X (2009) Isolation and characterization of *chlorella sorokiniana* GXNN0 (Chlorophyta) with the properties of heterotrophic and microaerobic growth. *J Phycol* **45**, 1153–62.
17. Grossman A, Takahashi H (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Annu Rev Plant Physiol* **52**, 163–210.
18. Hase E, Morimura Y, Mihara S, Tamiya H (1958) The role of sulfur in the cell division of *Chlorella*. *Arch Mikrobiol* **31**, 87–95.
19. Plumley FG, Schmidt GW (1989) Nitrogen-dependent regulation of photosynthetic gene expression. *Proc Natl Acad Sci USA* **86**, 2678–82.
20. Wykoff DD, Davies JP, Melis A, Grossman AR (1998) The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*. *Plant Physiol* **117**, 129–39.
21. Brooks A (1985) Effects of phosphorus nutrition on ribulose-1,5-bisphosphate carboxylase activation, photosynthetic quantum yield and amounts of some Calvin-cycle metabolites in spinach leaves. *Aust J Plant Physiol* **13**, 221–37.
22. Jacob J, Lawlor DW (1993) In vivo photosynthetic electron transport does not limit photosynthetic capacity in phosphate deficient sunflower and maize leaves. *Plant Cell Environ* **16**, 785–95.
23. Dietz KJ, Heilos L (1990) Carbon metabolism in spinach leaves as affected by leaf age and phosphorus and sulfur nutrition. *Plant Physiol* **93**, 1219–25.
24. Plesnicar M, Kastori R, Petrovic N, Pankovic D (1994) Photosynthesis and chlorophyll fluorescence in sunflower (*Helianthus annuus* L.) leaves is affected by phosphorus nutrition. *J Exp Bot* **45**, 919–24.
25. Tamburic B, Zemichael FW, Maitland CM, Hellgardt K (2010) Parameters affecting the growth and hydrogen production of the green alga *Chlamydomonas reinhardtii*. *Int J Hydrogen Energ* **36**, 7872–6.