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Characterization of H_2 photoproduction by a new marine green alga, *Platymonas helgolandica* var. *tsingtaoensis*

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ABSTRACT

A significant improvement in H_2 photoproduction was observed in the marine green alga *Platymonas helgolandica* var. *tsingtaoensis* in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). The addition of 15 μM CCCP to algal cultures led to a 260-fold increase in H_2 production compared with a negligible volume of H_2 gas produced without CCCP. The photosystem (PS) II photochemical activity was shown to be completely inhibited in the presence of CCCP under illumination. The added herbicide 3-(3,4-dichlorophenyl)-1, 1-dimethylurea further resulted in a twofold increase in H_2 photoproduction in the CCCP-treated cultures. These results demonstrated the absence of PSII-dependent pathway of H_2 photoproduction. H_2 production essentially depends on electrons donated to the hydrogenase by the endogenous substrate catabolism in CCCP-treated *P. helgolandica* var. *tsingtaoensis*. An analysis of the O_2-tolerance of the hydrogenase of *P. helgolandica* var. *tsingtaoensis* suggested that this enzyme may belong to the class [FeFe]-hydrogenase.

1. Introduction

Microalgae exhibit great versatility as energy sources [1], which can be converted to bio-oil [2], bioethanol [3], biomethanol [4], and biohydrogen [5] via biochemical and thermochemical methods. The advantages of using algae include very low space requirements [6] and few additives required for cultivation (e.g., carbon sources or nutrients) [7]. Photobiological H_2 production by microalgae is currently receiving increased attention because of its potential for generating renewable fuel from very abundant resources, light, and water [8].

The ability of unicellular green algae to metabolize H_2 gas upon illumination after a dark, anaerobic induction period has been discovered by Gaffron [9] 60 years ago. Nevertheless, H_2 photoproduction by green algae lasts from only several seconds to a few minutes because the reversible hydrogenase they contain is extremely sensitive to O_2 [8]. Melis [10] proposed an indirect two-stage approach based on sulfur (S) deprivation for long-term H_2 production. This protocol circumvents the O_2 sensitivity of H_2 metabolism reactions. Photosynthetic O_2 evolution and carbon accumulation are temporarily separated (stage 1) from the consumption of cellular metabolites and concomitant H_2 production (stage 2). Another effective protocol for H_2 photoproduction is the addition of a protonophore uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP). Significant H_2 evolution was observed in the presence of CCCP in the marine green alga *Platymonas subcordiformis*, whereas S-deprivation exerted a much lesser effect [11]. The uncoupling effect of CCCP disrupts the proton motive force, and releases protons across thylakoid and mitochondrial membranes. Consequently, the accessibility of electrons and protons to hydrogenase in chloroplasts is enhanced [12], and mitochondrial oxidative respiration is stimulated [13], respectively. CCCP also accelerates the deactivation reactions of the water-splitting enzyme system Y. This acceleration leads to the rapid inhibition of PSII activity, resulting in anaerobiosis [14].

Two different pathways, Photosystem (PS) II-dependent and -independent pathways, have been proposed in green algae to provide electrons used in H_2 photoproduction [15,16]. The PSII-dependent pathway originates in PSII-catalyzed water oxidation, which is sensitive to the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The PSII-independent pathway depends on a non-photochemical plastoquinone reduction process. Despite recent advances, the mechanisms of H_2 photoproduction by S-deprived or inhibitor-treated green algae remain unclear. To gain a better understanding of the underlying biochemical pathways of H_2 production, each pathway should be separately investigated. Therefore, the appropriate study subjects are algal strains using only one pathway for H_2 production.

H_2 production has been observed in diverse green algal species, such as *Chlamydomonas reinhardtii* [10], *Chlamydomonas moewusii* [17], and *Chlorella fusca* [18]. Although the ability of *P. subcordiformis*

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to generate H_2 has been studied [11,12,19,20], the H_2 production mechanism of marine green algae remains unclear. For sustainable development, biohydrogen production by marine green algae is valuable for meeting increasing demands for the limited supply of fresh water [21]. Therefore, the potential and mechanism of H_2 production by marine microalgae for industrial applications warrant further research.

The present study aimed to investigate the H_2 photoproduction properties of a marine green alga, *Platymonas helgolandica* var. *tsingtaoensis*. This alga has the ability to produce H_2 in the presence of CCCP. The H_2 metabolism of CCCP-treated *P. helgolandica* var. *tsingtaoensis* and the O_2 sensitivity of the algal hydrogenase also were explored.

2. Materials and methods

2.1. Alga strain and cultivation

P. helgolandica var. *tsingtaoensis* was obtained from the Algae Culture Bank of Institute of Oceanology, Chinese Academy of Sciences. The alga was autotrophically cultivated in f/2 medium [22] with pH 7.0 at about 25 °C. A constant illumination of 60 μmol photons/(m^2 s) was provided, and the cultures were bubbled with air. The cell density was measured using a hemocytometer.

2.2. H_2 photoproduction procedure

2.2.1. CCCP-treated procedure

Cells at the late-log phase were harvested by centrifugation (4000 × *g* for 5 min), and resuspended in fresh f/2 medium to a final concentration of about 3×10^6 cells/ml. About 120 ml of algal cell suspensions were placed in 130 ml anaerobic jars sealed with butyl rubber plugs and retroflexed stoppers. To induce hydrogenase activity, the algal cells were first flushed with pure N_2 for 10 min to achieve anaerobic conditions, and then maintained in the dark at 25 °C for 32 h. After incubation in the dark, CCCP was added to a final concentration of 15 μM. The algal cells were maintained in the dark for 10 min. To induce photobiological H_2 production, the culture was placed under 150 μmol photons/(m^2 s) illumination with magnetic stirring at 25 °C for 24 h [12]. Control samples (without CCCP treatment) were also illuminated to induce H_2 production after 32 h of anaerobic incubation in the dark.

To investigate the electron donor for H_2 production, the PSII inhibitor DCMU was added to a final concentration of 50 μM after incubation in the dark. The algal cells were maintained in the dark for 10 min. To induce H_2 production, the cultures were placed under 150 μmol photons/(m^2 s) illumination with magnetic stirring at 25 °C for 24 h. The amount of H_2 produced during illumination was measured by a gas chromatograph equipped with a 5 Å molecular sieve column and a thermal conductivity detector.

2.2.2. S-deprived procedure

Cells at the late-log phase were harvested by centrifugation, washed twice with S-deprived medium, and resuspended to a final concentration of 3×10^6 cells/ml in S-deprived medium. About 120 ml of algal culture was placed in 130 ml anaerobic jars sealed with butyl rubber plugs and retroflexed stoppers. The cultures were then placed under continuous 150 μmol photons/(m^2 s) illumination with constant stirring for up to 120 h [23].

2.3. Chlorophyll (Chl) fluorescence measurements

Chl fluorescence measurements were carried out using a Dual-PAM-100 measuring system. The cultures were allowed to adapt to the dark for 10 min, and then 1 ml of algal cell suspension was placed in the measurement chamber. After measuring the dark-adapted variable to maximal (F_v/F_m; maximum PSII quantum yield) value, additional information on the photosynthetic performance was obtained from the recording of light curves.

2.4. Determination of starch content, Chl content, and pH

Algal cells were directly collected from the culture (about 5 ml of algal suspension for each point) by centrifugation, and stored at −70 °C. Starch determination was performed according to the method of Klein and Betz [24]. Pigments were extracted from the algal cells with 95% ethanol. The ethanol extract was subjected to the spectrophotometric determination of Chl a and b contents [25]. The extracellular pH of the cell cultures was measured using a pH meter.

2.5. Measurement of the O_2 tolerance of hydrogenase

P. helgolandica var. *tsingtaoensis* cultures were autotrophically grown in an f/2 medium. Cells at the late-log phase were harvested by centrifugation, and resuspended in a fresh f/2 medium to a final concentration of 150 μg/ml Chl. After overnight anaerobic incubation in the dark, the algal cells were exposed to a range of O_2 concentrations. About 2 ml of anaerobically induced cells were injected into 6 ml anaerobic vials with different O_2 gas concentrations. After incubating in the dark for 2 min with violent shaking, the cultures were immediately tested for H_2 evolution activity by methyl viologen/gas chromatography (MV–GC) assay [26]. The mixture was allowed to react in a sealed, N_2-purged anaerobic vial. The vial contained 1 ml of O_2-treated algal cells with 1 ml of MV solution (10 mM MV, 0.2% Triton X-100 and 50 mM potassium phosphate) and 200 μl of 100 mM sodium dithionite. The vials were incubated at 37 °C for 20 min in the dark. The amount of H_2 that evolved from the headspace of the vials was analyzed by GC.

To measure the reversibility of hydrogenase towards O_2 inactivation, the cells after overnight anaerobic incubation were lysed upon incubation with 2% Triton X-100. Brief centrifugation followed for cell debris removal [23]. The supernatant was immediately exposed to 1% O_2 for 25 min to totally inactivate hydrogenase activity. The anaerobic condition of the supernatant was subsequently returned by an H_2 atmosphere. The H_2 production activities of the samples were measured at various time points of incubation by MV–GC.

3. Results and discussion

3.1. Effects of CCCP and S-deprivation on H_2 photoproduction

S-deprivation in *C. reinhardtii* caused a reversible inhibition of photosynthetic activity, and enabled the apparent temporal separation of O_2 and H_2 evolutions [10]. However, H_2 photoproduction by *P. helgolandica* var. *tsingtaoensis* was negligible under the S-deprived condition, and a significant amount of O_2 evolution was detected. These results indicated that the O_2 evolution ability of *P. helgolandica* var. *tsingtaoensis* cells was not inactivated under S-deprivation. Hence, the anaerobic conditions for inducing hydrogenase were not established. The inefficiency of S-deprivation suggested that the PSII reaction center of some marine algae, such as *P. subcodiformis* [11] and *P. helgolandica* var. *tsingtaoensis*, is not sensitive to the absence of S.

Previous studies [11,17] have shown that H_2 photo-evolution in green algae, such as *P. subcodiformis* and *C. moewusii*, could be significantly enhanced by CCCP addition. In the current research, Fig. 1 shows that CCCP had positive effects on H_2 production in *P. helgolandica* var. *tsingtaoensis*. A H_2 yield of 0.525 mmol/L and a maximum H_2 production rate of 0.061 mmol/(L h) were obtained

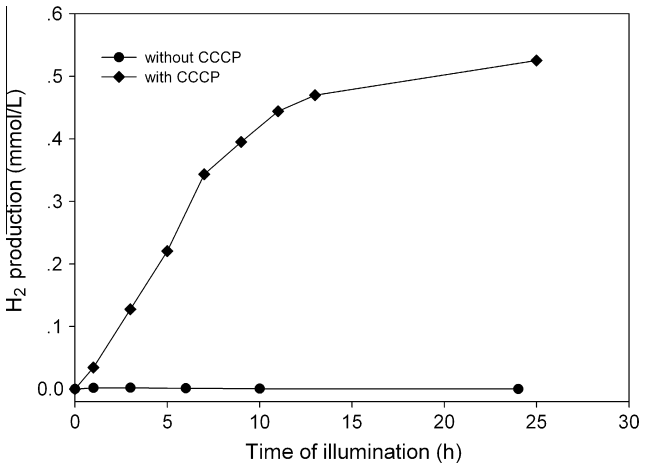

Fig. 1. Time course of H_2 evolution by *P. helgolandica* var. *tsingtaoensis* with and without CCCP treatment under 150 μmol photons/(m^2 s) illumination. About 15 μM CCCP was added 10 min before illumination following the 32 h dark adaptation.

in CCCP-treated *P. helgolandica* var. *tsingtaoensis*. However, the H_2 yield was only 0.002 mmol/L for the control (without CCCP treatment). The duration of H_2 evolution was also significantly extended to 24 h in the presence of CCCP, whereas the H_2 yield began to decrease after illumination for 3 h in the control. There was also no H_2 production in the dark regardless of CCCP presence, indicating that H_2 production by *P. helgolandica* var. *tsingtaoensis* was mediated by light. Therefore, the addition of CCCP significantly stimulated H_2 photo-evolution for a prolonged period, and also improved the H_2 evolution rate in *P. helgolandica* var. *tsingtaoensis.* Similar results have been found in another marine green alga, *P. subcordiformis* [11].

Hydrogenase and ferredoxin in microalgae are generally believed to be soluble proteins located in chloroplast stroma [27,28]. Therefore, the CCCP-induced dissipation of proton gradient across thylakoid membrane could affect H_2 production. The addition of the uncoupler carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP) has been reported to considerably stimulate H_2 production in the PSII-deficient mutant of *Chlamydomonas* FUD7. This finding indicated that PSII-independent electron transport activity is limited by a trans-thylakoid proton gradient [29]. The negative effect of a proton gradient on PSII-independent H_2 production has also been observed in the study of Antal et al. [30]. In the present study, a significant enhancement in H_2 production was observed in CCCP-treated *P. helgolandica* var. *tsingtaoensis* cultures. This phenomenon could be attributed to the uncoupling effect of CCCP, which disrupted the proton gradient generated across thylakoid membranes in chloroplasts. Consequently, the accessibility of the electron and H^+ to hydrogenase was enhanced. In another case, a mutant (pgrl1) impaired in the cyclic electron flow (CEF) around PSI due to the effect of proton gradient regulation-like 1 protein has been studied. A strong increase in H_2 production was observed in the mutant, with H_2 production reaching similar values in wild type by addition of FCCP. This finding indicated that the proton gradient generated by the CEF induces a strong inhibition of electron supply to the hydrogenase under high light condition [31]. In current study, the positive effect of CCCP on H_2 production under 150 μmol photons/(m^2 s) illumination might be attributed to dissipation of the proton gradient across the thylakoid membrane generated by CEF.

CCCP also dissipated the proton motive force across the mitochondrial membrane. This dissipation resulted in the cessation of ATP production and increased ADP availability, thereby stimulating respiration [13].The property of CCCP as an ADRY agent (agent accelerating of the deactivation reactions of the water-splitting enzyme system Y) enables it to accelerate the deactivation reactions in the water-splitting enzyme system Y [14]. Therefore, anaerobic conditions can be effectively established and maintained by the addition of CCCP. This conclusion is well supported by the present experimental results, which indicated that no O_2 evolved upon illumination of *P. helgolandica* var. *tsingtaoensis* treated with 15 μM CCCP. In contrast, O_2 production was detected soon after illumination in the case of the control cultures. The negligible H_2 production in control cultures could be attributed to O_2 inactivation of hydrogenase.

3.2. Effect of CCCP on PSII activity during H_2 production

CCCP can accelerate the deactivation reaction of the water-splitting system in the PSII reaction center, leading to the inhibition of O_2 photo-evolution [14]. PSII plays an important role in the PSII-dependent H_2 production pathway, where water photolysis is the major source of electrons [15,16]. To investigate the relationship between PSII activity and H_2 production by CCCP-treated *P. helgolandica* var. *tsingtaoensis*, Chl fluorescence during the H_2 evolution period was measured. F_v/F_m is a useful indicator of PSII photochemical activity [32]. Table 1 shows that F_v/F_m of the *P. helgolandica* var. *tsingtaoensis* culture suspension of remained zero. The electron transfer rate of PSII (ETRII) was not observed during H_2 production. In contrast, PSII activity quickly increased from 0.289 to 0.466 in 1 h, and remained at a high level in the control cultures under illumination (data not shown). These results indicated that the PSII photochemical activity of the algal cells was completely inhibited by CCCP. No electron was transferred from the water oxidation reaction to the hydrogenase. These findings conformed with the undetectable O_2 gas levels during illumination mentioned in Section 3.1. A previous study has shown that, 5–10% of the water oxidation activity is retained during H_2 production under S-deprived conditions in *C. reinhardtii* [16]. A similar result has also been found in the marine green alga *P. subcordiformis*. PSII activity rapidly decreases in the CCCP-treated cells within the first 4 h, and then slowly declines [12]. H_2 production by these two algae aforementioned essentially depends on electrons donated by residual PSII H_2O-oxidation activity [16,19]. However, during H_2 photo-evolution by *P. helgolandica* var. *tsingtaoensis*, PSII photochemical activity was completely inhibited in the presence of 15 μM CCCP. This result indicated that the electrons for H_2 production did not originate from the water splitting in PSII.

3.3. Effect of DCMU on H_2 production

The role of DCMU in the H_2 photo-evolution by *P. helgolandica* var. *tsingtaoensis* was investigated. Table 1 shows that cellular PSII photochemical activity during illumination was completely inhibited by DCMU. On the other hand, the addition of DCMU had little effect on H_2 production. These findings were consistent with previous studies showing that a small amount of H_2 is produced in *P. subcordiformis* cultures upon DCMU treatment [12]. In S-depleted *C. reinhardtii* cultures, H_2 production is much lower with DCMU addition than without [16]. Both of the above observations are

Table 1
Effect of CCCP and DCMU on PSII photochemical activity and H_2 production by *P. helgolandica* var. *tsingtaoensis*.

	Control	CCCP	DCMU	CCCP + DCMU
F_v/F_m	>0.466[a]	0	0	0
ETRII	–	0	0	0
H_2 yield (mmol/L)	0.002	0.160	0.014	0.290

[a] F_v/F_m in control cultures increased from 0.289 to 0.466 in 1 h, and remained at the higher level during H_2 production.

attributed to the fact that the electrons for H_2 production were mainly supplied by PSII-catalyzed H_2O oxidation in these two algae. However, Ducat et al. [33] have heterologously expressed a *Clostridial* [FeFe]-hydrogenase (HydA) within *Synechococcus elongatus* sp. 7942. Significant quantities of H_2 are observed in the headspace of HydA-containing cultures in the presence of DCMU. This finding demonstrates that heterologous HydA is functional in cyanobacteria when PSII was inhibited. In the present experiments, the low H_2 yield of DCMU-treated cells without CCCP suggested that the inhibition of PSII activity was not sufficient to stimulate H_2 evolution, or that the photolysis of water by PSII contributed most of the electrons to H_2 production.

However, the simultaneous addition of DCMU and CCCP resulted in significant H_2 production. The H_2 yield of *P. helgolandica* var. *tsingtaoensis* was almost doubled with the combined treatment. Previous studies have shown that DCMU significantly inhibits its H_2 production in CCCP-treated cultures of *P. subcordiformis*, and in S-deprived cultures of *C. reinhardtii* [12,16]. In these two algae, water photolysis is the main source of the reductant for H_2 photobiological evolution. Hence, the inhibitory effect of DCMU on H_2 production is caused by the blockage of electron transport from PSII. In another case of *C. moewusii*, there are slight differences in H_2 production in the presence of DCMU with and without CCCP. These differences suggest that PSII activity is not indispensable to the H_2 evolution process [17]. These results indicate a negligible dependence of the H_2 photoproduction reaction on PSII activity in CCCP-treated *P. helgolandica* var. *tsingtaoensis*. Oxidative carbon metabolism serves as the main source of electrons of hydrogenase. Therefore, the abovementioned minimal H_2 production in the presence of DCMU is not due to the inhibition of PSII activity. The uncoupling effect of CCCP may rather play a key role in enhancing photobiological H_2 production by *P. helgolandica* var. *tsingtaoensis*. However, the positive effect of DCMU and CCCP on H_2 production had never been previously observed in other green algae. There must be something different about the H_2 metabolism of CCCP-treated *P. helgolandica* var. *tsingtaoensis*. The PSII-dependent pathway was not involved. Hence, the PSII-independent pathway in this marine green alga may possibly be positively affected by DCMU during H_2 evolution.

3.4. Changes in starch content, Chl content, and pH during H_2 production

The catabolism of endogenous substrates has been proven to be one of the electron sources in H_2 production [15]. According to Vincent [16], starch is recognized as the main substrate involved in H_2 production by green algae. In the present study, changes in cellular starch content were measured for further analysis of the H_2 production mechanism. Fig. 2 shows that cellular starch content sharply decreased from about 440 to 260 μg/ml within the first 5 h of H_2 photoproduction in the presence of CCCP. The rate of starch degradation then decreased, indicating a positive relation between starch degradation and H_2 production. In *C. reinhardtii*, H_2 photoproduction via the PSII-independent pathway requires the presence of starch [16]. Therefore, the decrease in total starch content during H_2 production by *P. helgolandica* var. *tsingtaoensis* demonstrated that the PSII-independent pathway contributed to H_2 production in CCCP-treated cells. This conclusion was consistent with the previous speculation that the oxidative degradation of organic substrates contributed electrons to the hydrogenase in *P. helgolandica* var. *tsingtaoensis*.

The pH of *P. helgolandica* var. *tsingtaoensis* cultures decreased from 8.0 to 7.7 during the 32 h dark incubation. Changes in the pH during H_2 photoproduction are shown in Fig. 3a. There was a sharp decrease in pH (from 7.67 to 7.41) within the first 3 h of illumination, after which extracellular pH was maintained within a narrow range of 7.40–7.46. Anaerobiosis was maintained during H_2 photoproduction in the sealed system by the effect of CCCP, which led to the switch in metabolism in the algal cells from dark fermentation to photofermentation [34]. In the presence of an uncoupler, the fermentative products of the anaerobic breakdown of starch mainly include formate, acetate, and CO_2 [35], which pos-

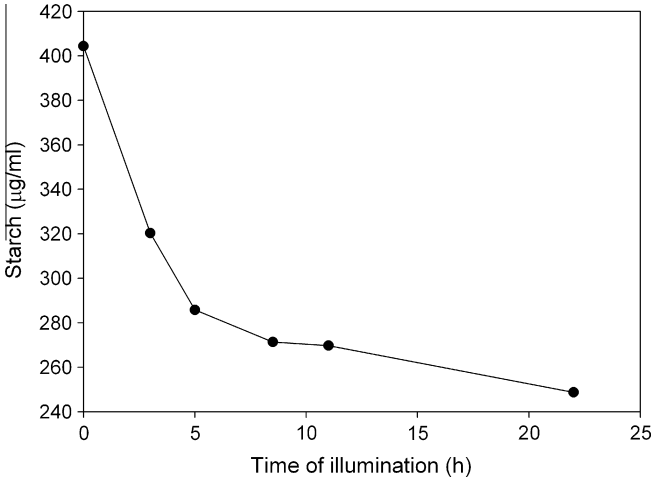

Fig. 2. Time course of starch contents during H_2 production by CCCP-treated *P. helgolandica* var. *tsingtaoensis*. The culture density at zero time was about 3×10^6 cells/ml.

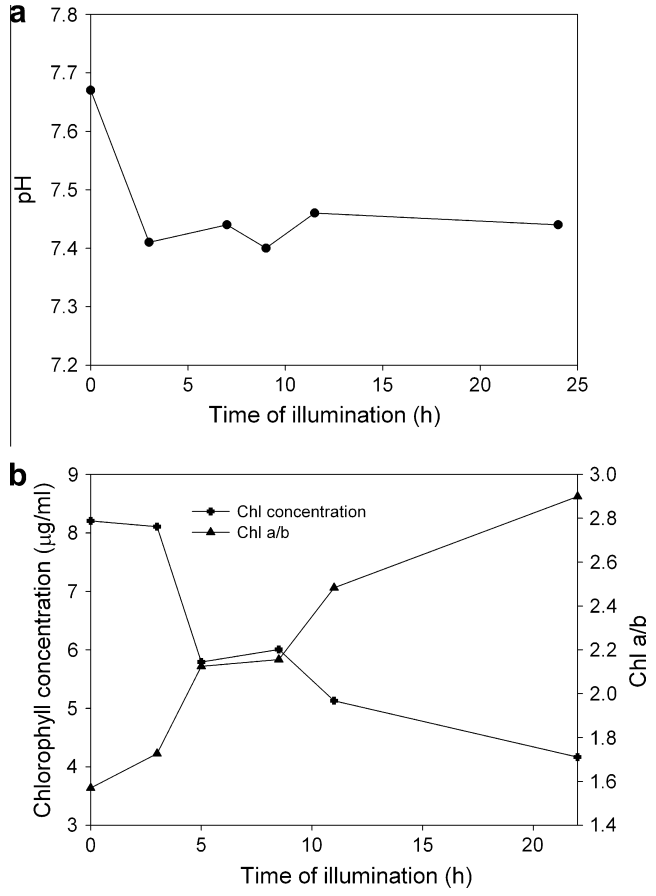

Fig. 3. Time course of pH (a) in the cell cultures and chlorophyll contents (b) of algal cells during H_2 photoproduction by CCCP-treated *P. helgolandica* var. *tsingtaoensis*. The culture density at zero time was about 3×10^6 cells/ml.

sibly caused the pH decline in the present experiments. The formation of these products was consistent with the results of an earlier study which shows the accumulation of formate, ethanol, and acetate during H_2 production by the *C. reinhardtii* cultures after exposure to S-deprivation in light [36]. Moreover, in the present study, the pH stabilization after the initial sharp decrease correlated to the much slower decline in starch degradation in the CCCP-treated *P. helgolandica* var. *tsingtaoensis* cultures. Hence, pH stabilization may be caused by a slower fermentation rate.

Changes in the total Chl content and Chl a/b ratio in CCCP-treated *P. helgolandica* var. *tsingtaoensis* during H_2 photoproduction are shown in Fig. 3b. Chl content gradually declined from 8.2 μg/ml to a final concentration of 4.16 μg/ml. A sharp decrease in both Chl a and b was observed (data not shown). The Chl a/b ratio significantly increased during illumination. A gradual decrease in Chl content has also been observed during H_2 production by *C. reinhardtii*. However, the Chl a/b ratio slightly increases in this alga [10]. This finding suggests that CCCP may exert a negative influence on photosynthetic apparatuses. In the present study, the decreased Chl content and increased Chl a/b ratio indicated that CCCP significantly degraded antenna pigments, especially in Chl b.

3.5. O_2-tolerence of hydrogenase

The decline in H_2 evolution activity as a function of pre-treated O_2 concentrations was fitted to a single first-order exponential decay function, as shown in Fig. 4. The O_2 I_{50} value estimated from the fitted curve was only 0.127%, which was even lower than that in *C. reinhardtii* (0.22%) [26]. This finding indicated the relatively higher O_2 sensitivity of *P. helgolandica* var. *tsingtaoensis* than *C. rehardtii*.

Previous studies have demonstrated that there may be two types of hydrogenase ([FeFe]- or [NiFe]-) in the genus *Platymonas*. The hydrogenase in *P. subcordiformis* is preliminarily classified into [FeFe]-hydrogenase because the hydrogenase protein shows significant homology to the iron hydrogenase isolated from *C. reinhardtii* [37]. However, the purified hydrogenase protein in the unicellular marine green alga *Tetroselmis kochinensis* NCIM 1605 contains Fe and Ni atoms [38]. The catalytic activities of [FeFe]-hydrogenase are up to 100 times higher than those of [NiFe]-hydrogenase, although the latter exhibited a higher tolerance to O_2 [39]. For example, an O_2 concentration of 0.22% produces a 50% inhibition in the initial rate of algal H_2 production by *C. reinhardtii* [26]. On the other hand, the half-life of [NiFe]-hydrogenase ranges from hours to days depending on the species types and measuring conditions [40–42]. Therefore, based on the aforementioned results, the hydrogenase in *P. helgolandica* var. *tsingtaoensis* was presumed to be an [FeFe]-hydrogenase.

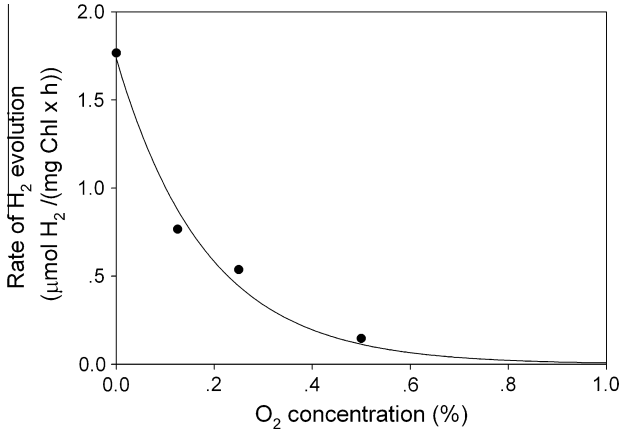

Fig. 4. O_2 titration of the rate of H_2 evolution by *P. helgolandica* var. *tsingtaoensis*. The rate of H_2 evolution was measured following deactivation by pre-exposure of the cells to different O_2 concentrations (0%, 0.125%, 0.25%, and 0.5%) for 2 min. The curve was fitted to a single first-order exponential decay function.

As previously mentioned, [NiFe]-hydrogenases are generally more tolerant to O_2 than [FeFe]-hydrogenases. The inactivation of [NiFe]-hydrogenases by O_2 and CO is also reversible [39]. [NiFe]-hydrogenases can be reactivated in anoxia in the presence of H_2. To analyze further the class of *P. helgolandica* var. *tsingtaoensis* hydrogenase, the reversibility of this enzyme was studied. The activity of the hydrogenase in the cell extracts was 3.56 μmol H_2/(mg Chl)/h and was totally inactivated after 25 min exposure to 1% O_2. Then anaerobic condition was subsequently returned by an H_2 atmosphere. H_2 production activities were measured at various time points of incubation (20 min, 60 min, 2 h, and 5 h). However, no activity was detected in any sample, revealing the irreversibility of the O_2 inactivation of this hydrogenase. This result also confirmed the identification of this hydrogenase as an [FeFe]-hydrogenase.

4. Conclusions

The present study demonstrated for the first time that the marine green alga *P. helgolandica* var. *tsingtaoensis* is capable of H_2 photoproduction when treated by the protonophore uncoupler CCCP. In the presence of CCCP, the anaerobiosis of the cultures is maintained, and the PSII photochemical activity of the algal cells is completely inhibited in the light during H_2 production. The addition of DCMU to the CCCP-treated cultures led to remarkable improvements in H_2 production. Under these conditions, the degradation of endogenous organic substrates provided the major electrons to the hydrogenase in *P. helgolandica* var. *tsingtaoensis*. The positive effect of DCMU in the CCCP-treated cultures sheds new light on PSII-independent pathways. Therefore, this alga is an appropriate object for further research on the mechanism of the PSII-independent pathway in H_2 production. Utilizing marine microalgae for the biological production of H_2 gas is a promising approach for meeting future energy needs.

Acknowledgments

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