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RESEARCH ARTICLE

Stimulation of Hydrogen Photoproduction in *Chlorella sorokiniana* Subjected to Simultaneous Nitrogen Limitation and Sulfur- and/or Phosphorus-Deprivation

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Abstract

Photosynthetic hydrogen (H_2) production by green algae has fascinated biologists and energy experts, due to the potential application of this process for renewable energy. In this study, H_2 photoproduction and PSII photochemical activities were investigated in *Chlorella sorokiniana* exposed to simultaneous nitrogen limitation and sulfur (S-) and/or phosphorus (P-) deprivation. Under S-deprivation, *C. sorokiniana* produced about 48.2 mL L^{-1} of H_2. Moreover, simultaneous nitrogen limitation (0.7 mM NH_4Cl) and sulfur- and/or phosphorus-deprivation significantly increased H_2 production of *C. sorokiniana* over that of S-deprivation alone. Maximum H_2 outputs of 77.3, 98.1 and 125.1 mL L^{-1} were obtained in the N-limited cultures exposed to P-deprivation (TAP-P), S-deprivation (TAP-S) and simultaneous S- and P-deprivation (TAP-S-P), respectively. The average rate of H_2 production for the N-limited culture exposed to TAP-P, TAP-S and TAP-S-P was 1.07, 1.36 and 1.50 mL L^{-1} h^{-1}, respectively. Interestingly, the H_2 inducement time in the culture subjected to simultaneous N-limitation and S- and/or P-deprivation was much shorter than that of traditional S-deprivation. The photosynthetic inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropylp-benzoquinone (DBMIB) repressed H_2 production in TAP-S-P (0.7 mM NH_4Cl) medium by 68.04% and 98.65%, respectively. The conditions of simultaneous N-limitation, S- and P-deprivation provided another efficient method for inducing H_2 production in *C. sorokiniana*. In addition, we also found that two-thirds of the required electrons were generated from the splitting of H_2O in PSII and that the remaining one-third possibly came from some other substrate catabolism.

Keywords: *Chlorella sorokiniana*, Hydrogen production, Nitrogen limitation, Phosphorus & Sulfur deprivation.

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INTRODUCTION

Photoproduction of hydrogen by microalgae has roused worldwide attention because it holds the promise of generating a renewable fuel from the most abundant natural resources, sunlight and water [1]. In 1939, for the first time under anaerobic conditions, hydrogen production was reported from the unicellular green alga *Scenedesmus obliquus* [2]. However, H_2 production lasted only for a few minutes and the yield was very low. In 2000, Melis and his co-worker created the S-deprivation inducing H_2 photoproduction model because S-deficiency damaged photosynthetic oxygen complex, so the oxygen released by photosynthesis cannot compensate the respiratory oxygen consumption, and gradually anaerobic status was achieved and hydrogenase activity was induced, which dramatically improved photosynthetic H_2 photoproduction efficiency and prolong the hydrogen producing period to hundred of hours [3]. It is well-known that nitrogen and phosphorus are essential nutrients for cell growth and are quite easily absorbed and utilized than the sulfur. Based on the fact that under nitrogen or phosphorus limitation, photosynthesis as well as the photosynthetic oxygen release efficiency can also be significantly reduced. N-deprivation is an alternative mechanism to stimulate H_2 production in some algal species, i.e., in nitrogen-fixing *Anabaena cylindrical* and non-nitrogen-fixing *Arthrospira maxima*, *Arthrospira* sp. [4–6]. In addition, it has been demonstrated that N- or P-deprivation can also induce H_2 production in a similar way to S-deprivation in the green alga *C. reinhardtii*. However, the H_2 yield is lower and the induction time to reach an anoxic state is much longer under N- or P-deprivation than that under S-deprivation [7, 8].

In the previous work, we develop a nitrogen-deficient or nitrogen limitation induced H_2 photoproduction mode [9], and phosphorus limitation induced H_2 production in green microalgae [10, 11]. We have demonstrated that nitrogen limitation was more suitable factor in the induction of H_2 production than S-deprivation in *Chlorella protothecoides*. Now, photosynthetic H_2 photoproduction inducement showed the trends of diverse. We found that not only single stress can induce photosynthetic H_2 photoproduction, but also the double stresses (such as low nitrogen + S-deficiency stress or low nitrogen + P-deprivation stress) could work too [9, 11–13]. Simultaneous N-limitation and S-deprivation could enhance the H_2 photoproduction in green algae *C. protothecoides* [9, 12–13]. Under such double stresses, the photosynthetic H_2 photoproduction in *C. protothecoides* significantly increased because the anaerobic condition and hydrogenase activity induction became much easily.

In the present study, the combination of two or more kinds of stresses was created to stimulate H_2 photoproduction in *Chlorella sorokiniana*. To find the best culture condition and understand the process of H_2 photoproduction in *C. sorokiniana* exposed to simultaneous N-limitation and S- and/or P-deprivation, the cell growth, the amounts of H_2 photoproduction, physiological/ biochemical changes, and PSII photochemical activity were determined.

MATERIALS AND METHODS

Strain and culture conditions

In this study *C. sorokiniana* strain KU204 was used. The algal cultures were pre-cultured in TAP (Tris-Acetate-Phosphate) medium [14] or TAP medium with low ammonium concentration (N-limited medium). The concentration of ammonium in TAP medium was 7 mM (100% N). The concentration of ammonium in N-limitation medium was 0.7mM (10% N). The algal cultures were exposed to a light intensity of 35–40 µmol photons m^{-2} s^{-1}, with a light/dark cycle of 14:10 h at pH 7.3 ± 1, 25 ± 1°C.

H_2 photoproduction under normal and stress conditions

For sulfur and nitrogen deprivation, algal cultures pre-grown in TAP medium were centrifuged at 4,000 rpm for 5 min and washed twice with S-deprivation (TAP-S) and N-deprivation (TAP-N) media. For simultaneous nitrogen limitation, sulfur- and/or phosphorus-deprivation, the algal cells, pre-grown in N-limited medium, were centrifuged, washed twice and transferred to simultaneous N-limitation and S-deprivation (TAP-S (0.7 mM NH_4Cl)), N-limitation and P-deprivation (TAP-P (0.7 mM NH_4Cl)) and N-limitation, S- and P-deprivation (TAP-S-P (0.7 mM NH_4Cl)) media. These all above media were prepared as previously described [9, 11–13]. For H_2 photoproduction, the algal

cells with an initial cellular density of about 2.5–3.5×10^7 cells mL^{-1} were subjected to TAP and stress medium conditions. Hydrogen photoproduction from the algal cells under these conditions was studied in 650 ml cylindrical glass vessels closed with a butyl rubber stopper and fitted with a gas tight pipe [11]. The algal cultures were exposed to 35–40 µmol photons m^{-2} s^{-1} continuous, fluorescent, white light at 25°C. The gas phase in the graduated cylinder was analyzed using a gas chromatograph as described by He et al. [6].

Effect of DCMU and DBMIB on H_2 photoproduction of *C. sorokiniana*

The cultures were inoculated into 20 ml glass vials closed with butyl rubber stoppers as described by He et al. [6]. After 12h of illumination, 10 µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or 10 µM DBMIB (2,5-dibromo-3-methyl-6-isopropylp-benzoquinone) were added to the vials, after which the vials were flushed with argon gas to remove air in the headspace. Each set of experiments was carried out in triplicate.

Other analytical procedures

Hydrogen and oxygen contents were determined using a gas chromatograph (GC-112A, Shanghai Phenix Optical Scientific Instrument Co. Ltd., China) equipped with a molecular sieve 5Å column using a thermal conductivity detector according to He et al. [6]. The total chlorophyll content was extracted from the cell cultures using 95% methanol and measured as described by Liu[15]. Cell density was counted using a hemocytometer under a light microscope (CH 30, Olympus Imaging Corp., Japan). The actual quantum yield of primary photochemistry (ΦPSII) and the maximal quantum yield of primary photochemistry (F_v/F_m) were measured using a FMS-2 (Hansatech Instruments Ltd., King's Lynn, UK) as described by Zhang et al. [13] and White and Critchley [16].

RESULTS AND DISCUSSION

H_2 photoproduction during nitrogen or sulfur deprivation

In TAP and TAP-N media, small amounts of H_2 were detected (Fig. 1a). Under these conditions, the algae are not sufficient to produce H_2 level to displace any water inside the graduated cylinders. For TAP and N-deprived cultures, the final H_2 outputs were 0.7 and 12.0 mL L^{-1}, respectively. In addition, this alga could produce O_2 after 12 h in TAP medium. The total amount of O_2 was about 25.0 mL L^{-1} (Fig. 1b). In contrast, the algal cells did not produce O_2 in the N-deprived cultures during the experiments.

Under TAP medium, a short-lived anoxia was observed at the onset of the incubation, which might have resulted from an increase in respiration after centrifugation and the transfer to fresh TAP medium. However, once cells had adapted to the fresh TAP medium, the photosynthesis activity was higher than respiration, and then O_2 evolution was observed. For S-deprived conditions, *C. sorokiniana* could produce 48.2 mL L^{-1} H_2 within 84 h (Fig. 1a). This finding was similar to previous studies which demonstrated that some algal cells cultivated in S-deprived conditions could evolve H_2 gas after incubation [17–21]. Indeed, in the S-deprived medium, the O_2-evolving activity of the alga significantly declined shortly after incubation, resulting in an anaerobic environment in the closed photobioreactors. Under these conditions, hydrogenase activity was activated to catalyze the H_2 production reaction [3]. Moreover, *C. sorokiniana* cells under N-deprivation consumed the O_2 in the headspace completely and could sustain anaerobiosis during the incubation (Fig. 1b); however, the H_2 output was very low (Fig. 1a).

H_2 photoproduction exposed to simultaneous nitrogen limitation, sulfur- and/or phosphorus-deprivation

Cells of *C. sorokiniana* were further examined for H_2 production in simultaneous N-limitation (0.7 mM NH_4Cl) and S- and/or P-deprivation. The results revealed that H_2 photoproduction was significantly enhanced under these culture conditions compared with TAP-S medium with 7 mM NH_4Cl (Fig. 1a). Cultures in TAP-S under N-limitation produced 98.1 mL L^{-1} of H_2. The combination of N-limitation and P-deprivation could also generate H_2. The final H_2 output for TAP-P cultures under N-limitation was 77.3 mL L^{-1}. In addition, N-limited cultures with simultaneous S- and P-deprivation showed the highest H_2-producing ability compared with the other cultures in this experiment. The final H_2 output for TAP-S-P cultures under N-limitation was 125.1 mL L^{-1} (Fig. 1a). Interestingly, H_2 production in the culture under TAP-S medium appeared approximately 12 h after closure the bioreactors. On the other hand, in the culture subjected

to simultaneous N-limitation and S- and/or P-deprivation, H_2 production emerged 3 h earlier, resulting in higher H_2 yield. Thus we suggest that the condition of simultaneous N-limitation with S- and P-deprivation is the best efficient method for inducing H_2 production in *C. sorokiniana*.

The nitrogen level in the medium is a main factor that affects the H_2 production. When algal cells were grown in N-limitation medium (0.7 mM NH_4Cl), the cells generated a lot of H_2 yield when exposed to S- and/or P-deprivation. In this study, *C. sorokiniana* showed the highest H_2-producing capacity (1.50 mL L^{-1} h^{-1} and generating 125.1 mL L^{-1}) under TAP-S-P and N- limitation. The chlorophyll content under these conditions was 5–6 mg L^{-1} (Fig. 3a). Assuming that *C. sorokiniana* could have a chlorophyll content of 10–12 mg L^{-1}, this alga could produce 250.2 mL L^{-1} of H_2. The best-known microalga with high H_2 production remains *C. reinhardtii*, which accumulates H_2 to approximately 100–270 ml L^{-1} with a chlorophyll content of 9–20 mg L^{-1} [3, 19]. To date, none of the tested strains have exhibited higher efficiency than *C. reinhardtii*. However, it remains a possibility that some species may be more suitable to use in a large scale culture than *C. reinhardtii.* Lu et al. [22] reported that *C. sorokiniana* has important industrial microalgal potential for biofuel production. In addition, this alga has a high specific growth rate (0.27 h^{-1}) and is tolerant to high irradiance, high temperature and high CO_2 concentrations [23, 24]. The seat tributes suggest that this microalga has the potential to compete with *C. reinhardtii* for industrial H_2 production.

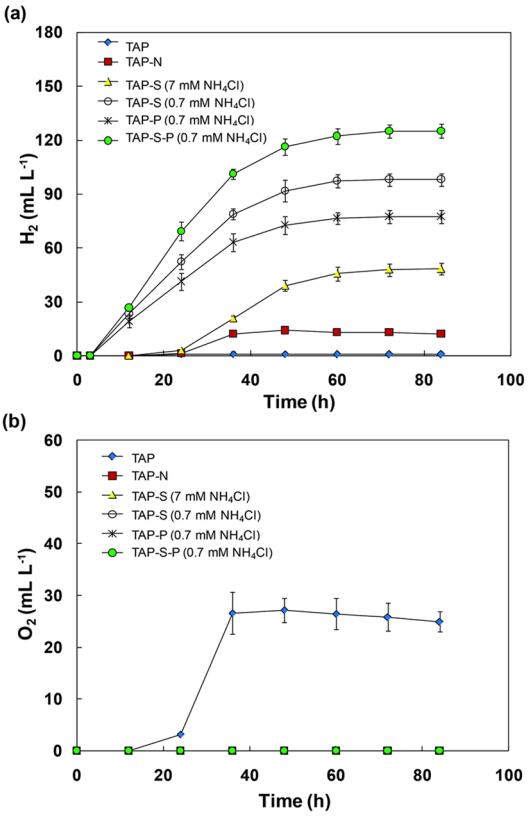

Fig. 1. H_2 photoproduction and O_2 evolution of *C. sorokiniana* cells pre-cultured under TAP or nitrogen limited medium and then grown under sulfur, nitrogen or phosphorus deprivation. Volume of H_2 yield detected in six treatments with pronounced H_2 production (TAP, TAP-N, TAP-S (7 mM NH_4Cl), TAP-S (0.7 mM NH_4Cl), TAP-P (0.7 mM NH_4Cl), TAP-S-P (0.7 mM NH_4Cl)) (a), volume of O_2 evolution in TAP cultures (b). All points are the mean of three independent measurements.

Physiological and biological changes in *C. sorokiniana* cells under nutritional stress

C. sorokiniana cells grown in TAP medium were small, ellipsoid, or slightly ovate in shape (Fig. 2a). This predominant cellular morphology changed dramatically under single and multiple stress conditions (TAP-N, TAP-S, simultaneous N-limitation, S- and/or P-deprivation) after 3 days of incubation (Fig. 2b-f). In N-deprived cultures, there were no distinct changes in the cell sizes, but the form of the cells became spherical instead of ellipsoid (Fig. 2b). Cells grown in TAP-S, or simultaneous N-limitation, S- and/or P-deprivation showed a clear increase in cell volume and the cell shape became spherical compared with regular cells in TAP medium. The color of the algal cells turned dark green when exposed to TAP medium. However, cells in N-limited or N-deprived conditions exhibited a light green color. This might be ascribed to chlorophyll degradation under nitrogen deficiency [25]. The swelling of S-deprived cells (Fig. 2c) was similar to the cell morphology changes of *C. reinhardtii* under S-deprivation reported by Zhang et al. [26] whose research showed that *C. reinhardtii* had increased cell volume and the shape of cells became spherical when cultured under S-deprived conditions. They also concluded that these morphological changes

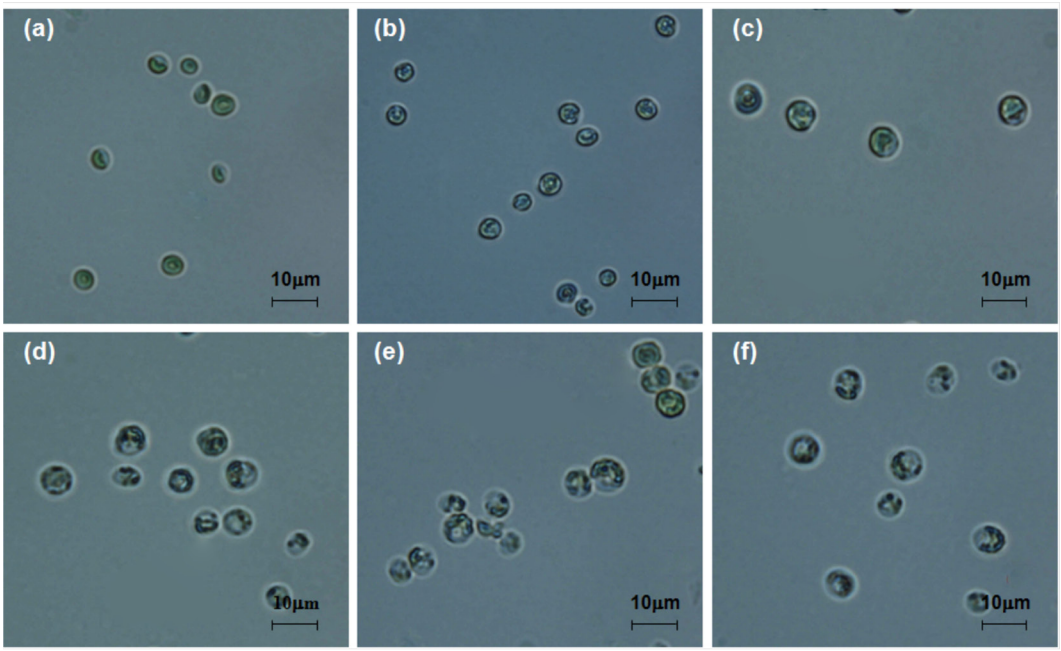

Fig. 2. Microscopic images of *C. sorokiniana* incubated in various TAP media, TAP (a), TAP-N (b), TAP-S (7 mM NH_4Cl) (c), TAP-S (0.7 mM NH_4Cl) (d), TAP-P (0.7 mM NH_4Cl) (e), TAP-S-P (0.7 mM NH_4Cl) (f).

under S-deprivation were associated with an accumulation of intracellular starch.

The total chlorophyll content in all treatments, except for N-deprived condition, increased during the culture period. The chlorophyll content in TAP medium displayed the significant increase, followed by S-deficient medium. For these cultures, the chlorophyll content increased transiently within the first 72 h, and subsequently become constant (Fig. 3a). However, the chlorophyll content in simultaneous N-limitation, S- and P-deprivation increased slightly within the initial 24 h of incubation, then decreased and finally maintained a constant level (Fig. 3a). For N-deprived cultures, the chlorophyll content gradually decreased during incubation. The chlorophyll content decreased on N-limitation and N-deprivation medium during the culture process (Fig. 3a), which indicated that nitrogen limitation seriously repressed chlorophyll synthesis. This observation was supported by El-Sheekh [27], Gordillo et al. [28] and Turpin [29] who found that decreases in the pigment contents, photosynthesis and biomass productivity in algal cells are typical responses following exposure to N-limitation. Piorreck et al. [30] reported that decreasing the nitrogen level in the culture medium of green algae and cyanobacteria caused substantial decreases in the chlorophyll contents. They also concluded that the chlorophyll contents were reduced, indicating a rapid reduction or even breakdown of all the chloroplast apparatus and a subsequent decrease in growth. Similar to the chlorophyll change, cell numbers in TAP and TAP-S cultures increased transiently in the initial 48 h and remained constant. In contrast, the cell numbers in other cultures increased slightly within the first 24 h, and did not change after 24 h of incubation (Fig. 3b).

As shown in Fig. 4, the pH value increased during the incubation in all cultures. The pH in TAP, TAP-N and TAP-S medium increased from an initial pH 7.3 to a maximum pH value 8.41, 8.27 and 8.34, respectively at the end of incubation. In contrast, cultures in TAP-S, TAP-P, TAP-S-P medium under N limitation showed a moderate increase of pH value rising to 8.11, 8.09 and 8.04 in TAP-S, TAP-P and TAP-S-P, respectively. In all the treatments in this experiment, the increase in pH value during periods of culture resulted from photosynthetic consumption of dissolved CO_2 and the absorption of acetate [17, 31]. For cultures in simultaneous

N-limitation and S- and/or P-deprivation, moderate enhancement of the pH value might be ascribed to the decline in the photosynthetic activity (Fig. 5), which would lead to the reduction of CO_2 fixation and the assimilation of acetate.

Change in chlorophyll fluorescence

The chlorophyll fluorescence parameter F_v/F_m is broadly used to estimate the maximum quantum yield of PSII photochemistry. Fig.5a shows that the initial value of F_v/F_m in cultures grown in TAP medium (~0.6) was higher than that in N-limited cultures (~0.4). For simultaneous N-limitation, S- and P-deprivation, the F_v/F_m value decreased rapidly from 0.39–0.40 to 0.27–0.29 within 24 h after incubation and further decreased thereafter. After 72 h, a value of 0.02–0.04 was observed. Similar to the F_v/F_m value, the value of ΦPSII in all treatments decreased over time (Fig.

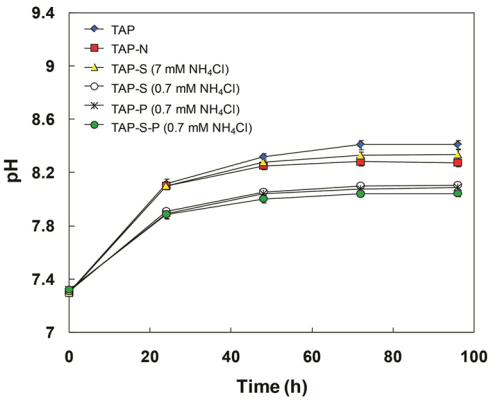

Fig. 4. Change in pH of *C. sorokiniana* during the incubation of synchronized cells in TAP, TAP-N, TAP-S (7 mM NH_4Cl), TAP-S (0.7 mM NH_4Cl), TAP-P (0.7 mM NH_4Cl), TAP-S-P (0.7 mM NH_4Cl) media.

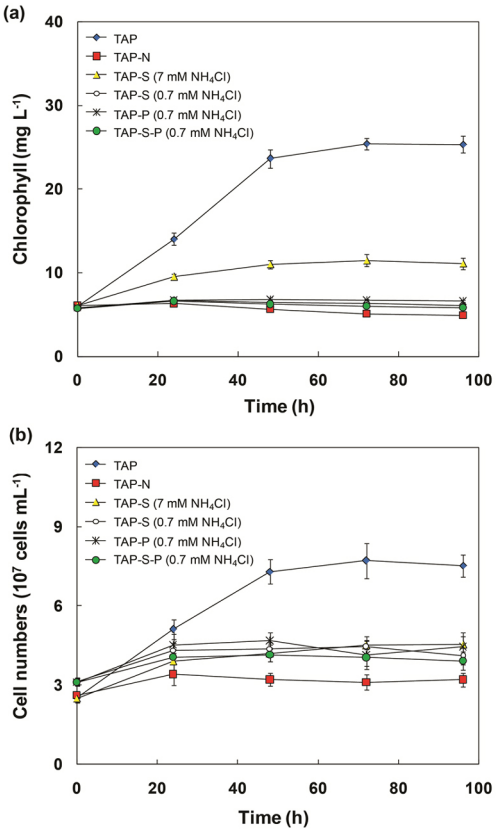

Fig. 3. Change in chlorophyll content (a) and cell numbers (b) of *C. sorokiniana* during the incubation of synchronized cells in TAP, TAP-N, TAP-S (7 mM NH_4Cl), TAP-S (0.7 mM NH_4Cl), TAP-P (0.7 mM NH_4Cl), TAP-S-P (0.7 mM NH_4Cl) media.

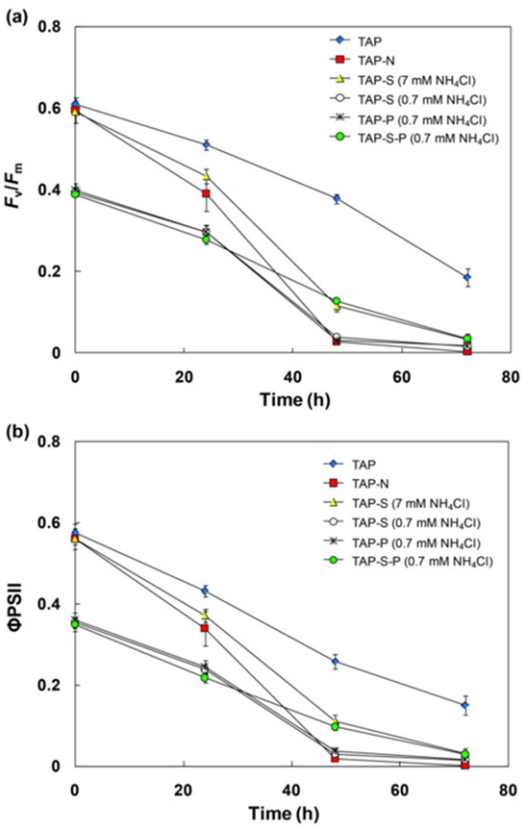

Fig. 5. Change in chlorophyll a fluorescence parameter F_v/F_m(a), ΦPSII(b) in *C. sorokiniana* incubated in TAP, TAP-N, TAP-S (7 mM NH_4Cl), TAP-S (0.7 mM NH_4Cl), TAP-P (0.7 mM NH_4Cl), TAP-S-P (0.7 mM NH_4Cl) media.

5b). However, the value of ΦPSII decreased more rapidly and more strongly than $F_v/F_{m.}$

Nitrogen deficiency also leads to degradation of ribulose-1, 5-bisphosphate carboxylase oxygenase, the key enzyme in photosynthetic Calvin cycle, which concurs with a decline in the efficiency of PSII photochemical activity [13, 32]. As shown in Fig. 5a and 5b, values of F_v/F_m and ΦPSII both reduced quickly after the beginning of N-limitation, indicating that N-limitation had a severe effect on the PSII photochemical activity. This observation agreed with the results obtained by Philipps et al. [8] who mentioned that the decline in values of both F_v/F_m and ΦPSII was observed in *C. reinhardtii* under a combination of S- and N-deprived condition. The obtained results suggest that the combination of N-limitation and S- and/or P-deprivation conditions reduced the PSII photochemical activity and the oxygen release in PSII center as compared with TAP medium, then lead to anaerobic status which further induce the hydrogenase activation and H_2 photoproduction in *C. sorokiniana*.

Effect of DCMU and DBMIB on H_2photoproduction

Photosynthetic inhibitors DCMU (an inhibitor of the Q_B site of PSII) and DBMIB (an inhibitor of the Q_O site of the cytochrome *b6/f* complex) were added into the cultures to study how many electrons for H_2 photoproduction came from PSII. As shown in Table 1, the rate of H_2 evolution by *C. sorokiniana* was inhibited after the addition of DCMU and DBMIB during the process of incubation in simultaneous N-limitation and S- and P-deprivation cultures. The reduction in the final H_2 photoproduction was ~68.04% and ~98.65% for treatments with DCMU and DBMIB, respectively, compared to the control group without inhibitors. Therefore, our results indicated a significant involvement between H_2 photoproduction and PSII.

In general, the electrons for H_2 production come from three main sources in green algae. First, the electrons come directly from the photosynthetic reaction center PSII where water is split [33]. Second, the organic sources such as starch stored in the cell supply electrons by oxidation through the indirect pathway [3]. Third, dark fermentation also provides parts of electrons [34]. The addition of DCMU decreased the H_2 photoproduction by ~68.04% in TAP-S-P (0.7 mM NH_4Cl) cultures. Therefore, approximately 68.04% of the electrons for H_2 synthesis by *C. sorokiniana* originated from the direct use of light energy to split water. This observation is supported by Volgusheva et al. [21] who mentioned that the H_2 production by *C. reinhardtii* exposed to S-deprivation was mainly inhibited by the addition of DCMU. They concluded that the majority of electrons donated to H_2ase were through water splitting by PSII. The addition of DBMIB resulted in the greatest repression of H_2 photoproduction by 98.65% (Table 1), which indicated that most electrons donated to H_2ase were transferred through cytochrome *b6/f*. Antal et al. [35] reported that the addition of DBMIB in the culture medium of *C. reinhardtii* exposed to S-deprivation caused a substantial decrease in H_2 output by ~97%. They also concluded that the electrons transported via the PQ pool were essential for H_2 production.

Table 1. Effect of DCMU and DBMIB on H_2 production by *C. sorokiniana* incubated in simultaneous N-limitation and S- and P-deprivation (TAP-S-P (0.7 mM NH_4Cl)).

	H_2 yield (mL L^{-1})					
	12 h	24 h	36 h	48 h	60 h	72 h
Control	21.75±0.55	36.64±2.61	40.28±0.74	40.81±0.08	41.31±0.17	41.34±0.16
+DCMU	8.47±0.10	9.96±1.05	12.47±1.52	13.10±0.56	13.17±0.83	13.21±0.78
+DBMIB	0.52±0.00	0.52±0.00	0.52±0.00	0.54±0.01	0.55±0.01	0.56±0.02
H_2 production inhibited by DCMU (%)	61.05%	72.81%	69.03%	69.03%	68.12%	68.04%
H_2 production inhibited by DBMIB (%)	97.63%	98.59%	98.71%	98.67%	98.67%	98.65%

CONCLUSIONS

The microalga *C. sorokiniana* had the ability to produce H_2 under conditions of nutrient deprivation. The H_2 photoproduction of *C. sorokiniana* was enhanced by using a combination of N-limitation and S- and/or P-deprivation medium. The maximum H_2 yield and average H_2 production rate in TAP-S-P (0.7 mM NH_4Cl) medium were ~125.1 mL L^{-1} and ~1.50 mL L^{-1} h^{-1}, respectively. The H_2 yield increased 2.5-fold compared with the TAP-S (7 mM NH_4Cl) medium. Furthermore, the addition of DCMU and DBMIB in TAP-S-P (0.7 mM NH_4Cl) medium repressed the H_2 photoproduction by 68.04% and 98.65%, respectively, compared with the control treatment. These results indicated that the majority of electrons (2/3) for H_2 synthesis by *C. sorokiniana* exposed to TAP-S-P (0.7 mM NH_4Cl) medium came from PSII photosynthesis and the remaining (1/3) energy came from organic substrate catabolism.

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