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 (H₂) production by green algae has fascinated biologists and energy experts, due to the potential application of this process for renewable energy. In this study, H₂ 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 H₂. Moreover, simultaneous nitrogen limitation (0.7 mM NH₄Cl) and sulfur- and/or phosphorus-deprivation significantly increased H₂ production of C. sorokiniana over that of S-deprivation alone. Maximum H₂ 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 H₂ 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 H₂ 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-isopropyl-p-benzoquinone (DBMIB) repressed H₂ production in TAP-S-P (0.7 mM NH₄Cl) medium by 68.04% and 98.65%, respectively. The conditions of simultaneous N-limitation, S- and P-deprivation provided another efficient method for inducing H₂ production in C. sorokiniana. In addition, we also found that two-thirds of the required electrons were generated from the splitting of H₂O 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.
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. In 1939, for the first time under anaerobic conditions, hydrogen production was reported from the unicellular green alga *Scenedesmus obliquus*. However, H₂ production lasted only for a few minutes and the yield was very low. In 2000, Melis and his co-worker created the S-deprivation 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 H₂ photoproduction efficiency and prolong the hydrogen producing period to hundred of hours. 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 H₂ production in some algal species, i.e., in nitrogen-fixing *Anabaena cylindrica* and non-nitrogen-fixing *Arthrospira maxima*, *Arthrospira sp.*. In addition, it has been demonstrated that N- or P-deprivation can also induce H₂ production in a similar way to S-deprivation in the green alga *C. reinhardtii*. However, the H₂ 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.

In the previous work, we develop a nitrogen-deficient or nitrogen limitation induced H₂ photoproduction mode and phosphorus limitation induced H₂ production in green microalgae. We have demonstrated that nitrogen limitation was more suitable factor in the induction of H₂ production than S-deprivation in *Chlorella protothecoides*. Now, photosynthetic H₂ photoproduction inducement showed the trends of diverse. We found that not only single stress can induce photosynthetic H₂ photoproduction, but also the double stresses (such as low nitrogen + S-deficiency stress or low nitrogen + P-deprivation stress) could work too. Simultaneous N-limitation and S-deprivation could enhance the H₂ photoproduction in green algae *C. protothecoides*. Under such double stresses, the photosynthetic H₂ 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 H₂ photoproduction in *Chlorella sorokiniana*. To find the best culture condition and understand the process of H₂ photoproduction in *C. sorokiniana* exposed to simultaneous N-limitation and S- and/or P-deprivation, the cell growth, the amounts of H₂ 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 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 0.7 mM (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. H₂ 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 NH₄Cl)), N-limitation and P-deprivation (TAP-P (0.7 mM NH₄Cl)) and N-limitation, S- and P-deprivation (TAP-S-P (0.7 mM NH₄Cl)) media. These all above media were prepared as previously described. For H₂ 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. 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. 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. After 12h of illumination, 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyleurea) or 10 μM DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone) 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. The total chlorophyll content was extracted from the cell cultures using 95% methanol and measured as described by Liu. 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 (Fv/Fm) were measured using a FMS-2 (Hansatech Instruments Ltd., King’s Lynn, UK) as described by Zhang et al. and White and Critchley.

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. 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. 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\(_4\)Cl) 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\(_4\)Cl (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
to simultaneous N-limitation and S- and/or P-deprivation, H₂ production emerged 3 h earlier, resulting in higher H₂ yield. Thus we suggest that the condition of simultaneous N-limitation with S- and P-deprivation is the best efficient method for inducing H₂ production in C. sorokiniana.

The nitrogen level in the medium is a main factor that affects the H₂ production. When algal cells were grown in N-limitation medium (0.7 mM NH₄Cl), the cells generated a lot of H₂ yield when exposed to S- and/or P-deprivation. In this study, C. sorokiniana showed the highest H₂-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 H₂. The best-known microalgae with high H₂ production remains C. reinhardtii, which accumulates H₂ to approximately 100–270 mL L⁻¹ with a chlorophyll content of 9–20 mg L⁻¹. 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. 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 CO₂ concentrations. The seat tributes suggest that this microalga has the potential to compete with C. reinhardtii for industrial H₂ production.

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. 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. 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
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 $\Phi_{PSII}$ in all treatments decreased over time (Fig. 5b).

**Fig. 4.** Change in pH of *C. sorokiniana* during the incubation of synchronized cells in TAP, TAP-N, TAP-S (7 mM NH$_4$Cl), TAP-S (0.7 mM NH$_4$Cl), TAP-P (0.7 mM NH$_4$Cl), TAP-S-P (0.7 mM NH$_4$Cl) media.

**Fig. 5.** Change in chlorophyll a fluorescence parameter $F_v/F_m$ (a), $\Phi_{PSII}$ (b) in *C. sorokiniana* incubated in TAP, TAP-N, TAP-S (7 mM NH$_4$Cl), TAP-S (0.7 mM NH$_4$Cl), TAP-P (0.7 mM NH$_4$Cl), TAP-S-P (0.7 mM NH$_4$Cl) media.
Table 1. Effect of DCMU and DBMIB on \( \text{H}_2 \) production by \( C. \) sorokiniana incubated in simultaneous N-limitation and S- and P-deprivation (TAP-S-P (0.7 mM \( \text{NH}_4 \text{Cl} \)).

|       | 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   |
| \( \text{H}_2 \) production inhibited by DCMU (%) | 61.05% | 72.81% | 69.03% | 69.03% | 68.12% | 68.04% |
| \( \text{H}_2 \) production inhibited by DBMIB (%) | 97.63% | 98.59% | 98.71% | 98.67% | 98.67% | 98.65% |

Effect of DCMU and DBMIB on \( \text{H}_2 \) photoproduction

Photosynthetic inhibitors DCMU (an inhibitor of the Qb site of PSII) and DBMIB (an inhibitor of the Qo site of the cytochrome b6/f complex) were added into the cultures to study how many electrons for \( \text{H}_2 \) photoproduction came from PSII. As shown in Table 1, the rate of \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) photoproduction and PSII.

In general, the electrons for \( \text{H}_2 \) production come from three main sources in green algae. First, the electrons come directly from the photosynthetic reaction center PSII where water is split. Second, the organic sources such as starch stored in the cell supply electrons by oxidation through the indirect pathway. Third, dark fermentation also provides parts of electrons. The addition of DCMU decreased the \( \text{H}_2 \) photoproduction by ~68.04% in TAP-S-P (0.7 mM \( \text{NH}_4 \text{Cl} \)) cultures. Therefore, approximately 68.04% of the electrons for \( \text{H}_2 \) synthesis by \( C. \) sorokiniana originated from the direct use of light energy to split water. This observation is supported by Volgusheva et al. who mentioned that the \( \text{H}_2 \) 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 \( \text{H}_2 \text{ase} \) were through water splitting by PSII. The addition of DBMIB resulted in the greatest repression of \( \text{H}_2 \) photoproduction by 98.65% (Table 1), which indicated that most electrons donated to \( \text{H}_2 \text{ase} \) were transferred through cytochrome b6/f. Antal et al. reported that the addition of DBMIB in the culture medium of \( C. \) reinhardtii exposed to S-deprivation caused a substantial decrease in \( \text{H}_2 \) output by ~97%. They also concluded that the electrons transported via the PQ pool were essential for \( \text{H}_2 \) production.
CONCLUSIONS

The microalga C. sorokiniana had the ability to produce H₂ under conditions of nutrient deprivation. The H₂ photoproduction of C. sorokiniana was enhanced by using a combination of N-limitation and S- and/or P-deprivation medium. The maximum H₂ yield and average H₂ production rate in TAP-S-P (0.7 mM NH₄Cl) medium were ~125.1 mL L⁻¹ and ~1.50 mL L⁻¹ h⁻¹, respectively. The H₂ yield increased 2.5-fold compared with the TAP-S (7 mM NH₄Cl) medium. Furthermore, the addition of DCMU and DBMIB in TAP-S-P (0.7 mM NH₄Cl) medium repressed the H₂ photoproduction by 68.04% and 98.65%, respectively, compared with the control treatment. These results indicated that the majority of electrons (2/3) for H₂ synthesis by C. sorokiniana exposed to TAP-S-P (0.7 mM NH₄Cl) medium came from PSII photosynthesis and the remaining (1/3) energy came from organic substrate catabolism.

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