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Effect of light intensity and light pattern on hydrogen production by unicellular green alga *Chlorella* sp. LSD-W2

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Abstract

Green microalgae can use solar energy and water to produce H_2 via hydrogenase enzyme activity. The unicellular green alga *Chlorella* sp. LSD-W2 has been previously shown to produce high H_2 under nitrogen deprivation. This research aimed to examine the effects of light intensity and light pattern on H_2 production by *Chlorella* sp. LSD-W2 under nitrogen deprivation. The result showed that H_2 production rate was significantly enhanced when light intensities were increased. The cells could hardly produce H_2 in the dark. The highest H_2 production rate with 0.956 ± 0.015 mL L^{-1} h^{-1} was obtained in cells incubated in TAP-N medium in a 120-mL glass bottle under light intensity of 60 µmol photons m^{-2} s^{-1}. H_2 production by cells incubated under light/dark or dark/light cycles was lower than that under continuous light illumination. In order to reduce O_2 which is an inhibitor of hydrogenase enzyme, the PSII inhibitor, 3-(3,4-dichlorophenyl) -1,1-dimethylurea (DCMU) was added to the *Chlorella* sp. LSD-W2 cell cultures. It was found that O_2 was obviously decreased in cells treated with 10 µM DCMU. Unexpectedly, DCMU caused the reduction of H_2 production by *Chlorella* sp. LSD-W2.

Keywords: Hydrogen production, *Chlorella* sp. LSD-W2, Light intensity, Light pattern, DCMU

1. Introduction

The world has been confronted with an energy crisis due to the depletion of finite fossil fuels [1]. Another problem with using fossil fuels is their emission of a main greenhouse gas CO_2 and other pollutants during combustion [1]. Molecular hydrogen (H_2) is an ideal alternative fuel for the future because the combustion of H_2 provides the highest energy value of 141.6 MJ kg^{-1} [2] and generates clean products without an emission of CO_2. Many microorganisms are capable of utilizing energy resources and some chemical compounds obtained from various metabolic pathways to produce biological H_2. Several green algae are able to produce H_2 via photosynthetic pathway in the light by using water as an electron source and sunlight as an energy source or via starch catabolism in the dark [3 & 4]. H_2 evolution by green algae is catalyzed by [FeFe]-hydrogenase enzyme located in the chloroplast stroma [5 & 6]. However, this enzyme is extremely sensitive to O_2, which is evolved during the light-dependent reactions of photosynthesis [7 & 8].

The unicellular green alga *Chlorella* sp. LSD-W2 isolated from seawater in Laemsadet beach, Chanthaburi province, Thailand, has been reported to produce high H_2 under nitrogen deprivation [9]. Its H_2 production rate under nitrogen deprivation was 2-4 folds higher than that under normal condition [9 &10]. It also showed high H_2 production under phosphorus deprivation [10]. Besides nutrient deprivation, light intensity and light pattern play an important role in growth, photosynthesis and H_2 evolution in green microalgae [11-16]. Normally, green algae require light as energy source via photosynthesis for H_2 production. However, under high light intensity H_2 production is inhibited resulting from the simultaneous O_2 evolution during photosynthesis [11 & 15].

Therefore, the optimal light intensity and pattern is necessary for H_2 production by green microalgae. In the previous studies, the enhancement of H_2 production is found in green algae *Chlorella vulgaris* and *Parachlorella kessleri* when the cultures were exposed to the light/dark cycles [14 & 16]. In this study, the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of electron transport from photosystem II (PSII) to plastoquinone (PQ), on H_2 production by *Chlorella* sp. LSD-W2 is also investigated. DCMU has been reported to inhibit O_2 evolution in green algae [17-19]. An addition of DCMU to the *Chlorella pyrenoidosa* culture improved H_2 production in *Chlorella pyrenoidosa* [20]; however, a decrease of H_2 production after addition of DCMU was found in *Chlorella protothecoides* [21] and *Chlorella sorokiniana* [22]. Whether DCMU stimulates or inhibits H_2 production by *Chlorella* sp. LSD-W2, it needs to clarify.

This present study describes the effects of light intensity and light pattern on H_2 production by *Chlorella* sp. LSD-W2 under nitrogen-deprived condition. In addition, effect of DCMU, an inhibitor of PSII activity, on H_2 and O_2 production was also examined.

2. Materials and methods

2.1 Green algal strain and growth condition

Chlorella sp. LSD-W2 isolated from seawater in Laemsadet beach, Chanthaburi province, Thailand [9] as grown in a 120-mL glass bottle containing 90 mL of Tris-acetate-phosphate (TAP) medium (pH 7.2) containing 20 mM Tris, 17 mM acetic acid, 1.65 mM K_2HPO_4, 1.05 mM KH_2PO_4, 7 mM NH_4Cl, 0.83 mM $MgSO_4.7H_2O$, 0.45 mM $CaCl_2.2H_2O$ and very low concentrations of various trace elements [23]. The initial cell concentration was adjusted to OD_{750} value of approximately 0.100. Cells were mixed with a magnetic stirrer and cultivated under a continuous light intensity of 30 µmol photons m^{-2} s^{-1} at 30 ºC for 36 h.

2.2 Effect of light intensity and light pattern on H_2 production

Chlorella sp. LSD-W2 grown for 36 h was harvested by centrifugation at 7,000×*g* at 4 ºC for 10 min, washed twice and resuspended in 90 mL of nitrogen-deprived TAP (TAP-N) medium. The 90-mL cell suspension with OD_{750} of approximately 0.8 was transferred into a 120-mL glass bottle. The headspace gas volume in the bottle was set at 30 mL. The cells were further incubated under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h to adapt cells under nitrogen deprivation before purging with argon gas for 20 min to remove O_2. After that, cells were illuminated by fluorescent lamps at different light intensities from 0, 10, 20, 40, 60, 80 to 100 µmol photons m^{-2} s^{-1} for 60 h. For studying on light pattern, light/dark or dark/light cycles with a time period of light 3 h and dark 3 h were provided to the cell suspension. H_2 was quantitatively determined from 500 µL of headspace gas using gas chromatograph.

2.3 Effect of DCMU on H_2 and O_2 production

Chlorella sp. LSD-W2 grown for 36 h was harvested by centrifugation at 7,000×*g* at 4 ºC for 10 min, washed twice and resuspended in 90 mL of TAP-N medium. The 90-mL cell suspension with OD_{750} of approximately 0.8 was transferred into a 120-mL glass bottle. The headspace gas volume in the bottle was set at 30 mL. The cells were subsequently incubated under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h. DCMU was added to the cultures with a final concentration of 10 µM. The cell culture was purged with argon gas for 20 min, and then it was placed under the optimal light intensity for H_2 production (obtained from the optimal light intensity result). Measurement of H_2 and O_2 in headspace gas was performed using gas chromatograph.

2.4 H_2 and O_2 measurement

During H_2 production of cells under anaerobic condition, 500 µL of gas samples were withdrawn from the headspace of a 120-mL glass bottle using a gas-tight syringe. H_2 and O_2 evolution was determined using gas chromatograph (Hewlett-Packard HP5890A, Japan) with a molecular sieve 5 Å 60/80 mesh packed column and a thermal conductivity detector. Argon gas was used as a carrier gas. The GC condition was performed according to Taikhao and coworkers [24]. Three replicates were used for each treatment. H_2 production rate were calculated as the maximum H_2 concentration produced by 1 liter of the algal cultures per a period of incubation time and expressed in a unit of mL H_2 L^{-1} h^{-1}.

2.5 Statistical analysis

The results in this study were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS statistics software (version 24.0) with a 95% significant confidence level. Data are presented as mean ± standard deviation (SD) of three replicates.

3. Results

3.1 Effect of light intensity on H_2 production rate

To investigate the effect of light intensity on H_2 production of *Chlorella* sp. LSD-W2 under nitrogen-deprived condition, cells were incubated in TAP-N medium under continuous illumination with different light intensities from 0, 10, 20, 40, 60, 80 to 100 µmol photons m^{-2} s^{-1}. It was found that H_2 production rate of *Chlorella* sp. LSD-W2 was significantly enhanced with an increase of light intensities until cells gave the maximum H_2 production rate of 0.956 ± 0.015 mL L^{-1} h^{-1} when incubated in TAP-N medium under light intensity of 60 µmol photons m^{-2} s^{-1} (Figure 1A). A decrease of H_2 production was found when light intensity was higher than 60 µmol photons m^{-2} s^{-1} (Figure 1A). In contrast, cells could hardly produce H_2 under dark condition (Figure 1A). Figure 1B shows the time course of cumulative H_2 production by this green alga. The results showed that cells enhanced H_2 production related to the incubation time under anaerobic condition. The maximum H_2 production of 11.828 ± 0.610 mL L^{-1} was found after incubating cells under anaerobic condition with light intensity of 60 µmol photons m^2 s^{-1} for 48 h. Therefore, the light intensity of 60 µmol photons m^2 s^{-1} is optimal for H_2 production by *Chlorella* sp. LSD-W2 and was used in the further experiments.

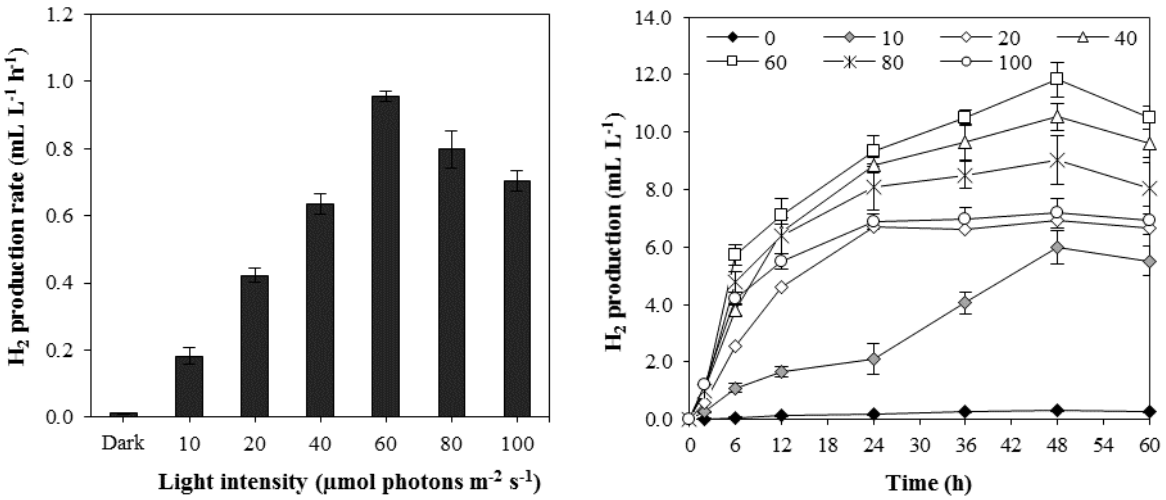

Figure 1 H_2 production rate (A) and time course of H_2 production (B) by *Chlorella* sp. LSD-W2 under continuous illumination with different light intensities

3.2 Effect of light pattern on H_2 production rate

The cell cultures of *Chlorella* sp. LSD-W2 were incubated under four different light patterns; (1) continuous light, (2) continuous dark, (3) 3 h light : 3 h dark cycle and (4) 3 h dark : 3 h light cycle. In the light period, light intensity of 60 µmol photons m^{-2} s^{-1} was provided to the cells. The result showed that *Chlorella* sp. LSD-W2 gave the highest H_2 production with 11.828 ± 0.610 mL L^{-1} when cells were incubated under continuous light illumination for 48 h (Figure 2). On the other hand, cells were not able to produce H_2 in the dark during 60 h of incubation (Figure 2). At the beginning of incubation, H_2 production by cells incubated under light/dark or dark/light illumination cycles was lower than that under continuous light illumination. However, at the end of incubation, cells incubated under illumination cycles reached the maximum H_2 production as found under continuous light condition (Figure 2). Interestingly, cells produced high H_2 during light period but produced less H_2 in the dark until H_2 production reached the saturation level (Figure 2).

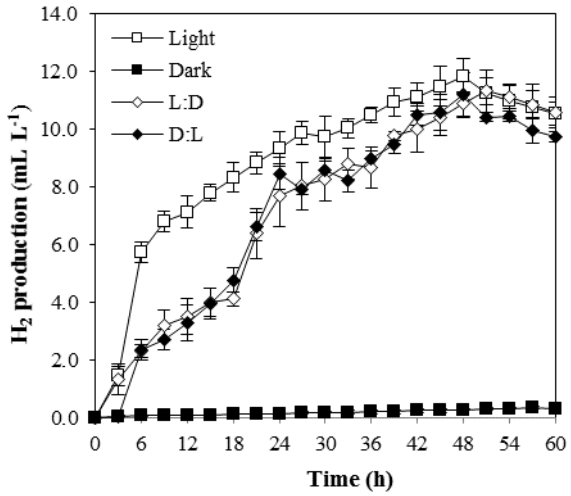

Figure 2 Time course of H_2 production by *Chlorella* sp. LSD-W2 under continuous light (□), continuous dark (■), 3 h light : 3 h dark cycle (◇) and 3 h dark : 3 h light cycle (◆) (The light intensity was provided at 60 µmol photons m^{-2} s^{-1})

3.3 Effect of DCMU on H_2 and O_2 production

Under a continuous light illumination, *Chlorella* sp. LSD-W2 produced the highest H_2; however, O_2 is also evolved during photosynthesis. DCMU, an inhibitor of PSII activity, was used in this experiment to get rid of O_2 in the cells. It was shown that significant H_2 production of cells untreated with 10 µM DCMU was observed after incubation in the light for 3 h, while cells hardly produced H_2 when treated with 10 µM DCMU (Figure 3A). The maximum H_2 production with 11.828 ± 0.610 mL L^{-1} was found in DCMU-untreated cells incubated in nitrogen-deprived TAP under continuous light illumination for 48 h, whereas the maximum H_2 production of DCMU-treated cells was only 1.829 ± 0.585 mL L^{-1}. To investigate the effect of DCMU on O_2 evolution in the presence of DCMU, O_2 production was measured during light incubation. It was found that cells treated with DCMU produced significantly less O_2 than cells untreated with DCMU (Figure 3B). After 48 h of incubation, O_2 concentrations at 1.172 ± 0.145 and 2.740 ± 0.260 mL L^{-1} were found in DCMU-treated cells and -untreated cells, respectively.

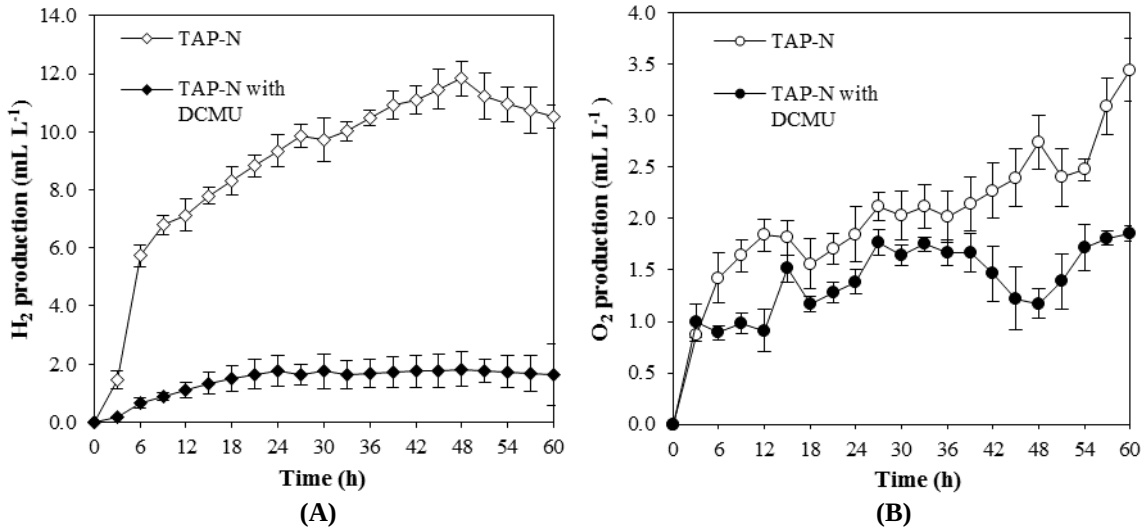

Figure 3 H_2 (A) and O_2 (B) production by *Chlorella* sp. LSD-W2 under a continuous light intensity of 60 µmol photons m^{-2} s^{-1}

4. Discussion

The unicellular green microalga *Chlorella* sp. LSD-W2 isolated from seawater in Thailand has been found to produce high potential H_2 under nitrogen deprivation [9]. In this study, we separated the H_2 production phase from the growth phase. In the growth phase, *Chlorella* sp. LSD-W2 was cultivated in normal TAP medium which is an enriched medium to accumulate biomass. After that cells were harvested and suspended in nitrogen-deprived TAP medium to enter the H_2 production phase. Under this condition, algal cells were not able to produce biomass due to the lack of nitrogen sources, essential for their cellular growth and metabolism [25]. Therefore, algal cells turn to use the excess electrons and protons to produce H_2 instead. In this study, the effect of light intensity and light pattern on H_2 production by *Chlorella* sp. LSD-W2 was investigated under nitrogen deprivation. It was suggested that under nitrogen deprivation, light was not used for the algal biomass production but it provided a light energy for photosynthesis and transferred electrons from the photosynthetic electron transport chain to hydrogenase enzyme for H_2 production. H_2 production rate of *Chlorella* sp. LSD-W2 was enhanced when light intensities were increased until reaching its highest H_2 production rate at the light intensity of 60 µmol photons m^{-2} s^{-1} (Figure 1). Under low light intensity, low light absorption by antenna chlorophylls is occurred in the photosynthetic membranes. It causes a decrease in photosynthetic electron transfer and finally resulting in the reduction of H_2 production [12]. When light intensities are increased, more electrons are transferred via the photosynthetic electron transport chain to a final electron acceptor ferredoxin (Fd). Then, Fd transfers electrons to [FeFe]-hydrogenase which catalyzes the reduction reaction of electrons and protons to generate H_2. This promoted the highest level of H_2 production. However, too high light intensities lead to an acceleration of the rate of O_2 evolution, obtained from the water-splitting reaction via PSII activity. The higher O_2 level inhibits [FeFe]-hydrogenase activity [11 & 15]. Therefore, the optimal light intensity causes the highest electron transfer in the photosynthetic process but the evolved O_2 concentration is at level that does not negatively affect the hydrogenase activity. This result was consistency with the result in a previous report in *C. pyrenoidosa* showing that the highest H_2 production of *C. pyrenoidosa* was shown when incubated cells under the light intensity of 30 µmol photons m^{-2} s^{-1} because this light intensity is favored to establish anaerobiosis in this strain [15].

To investigate the response of light pattern on H_2 production, cells were incubated under four different light patterns; (1) continuous light, (2) continuous dark, (3) 3 h light : 3 h dark cycle and (4) 3 h dark : 3 h light cycle. *Chlorella* sp. LSD-W2 gave the maximum H_2 production under nitrogen deprivation when incubated cells under continuous light condition. The highest H_2 production rate was observed during 3-9 h of continuous light incubation (Figure 2). In contrast, cells could not produce H_2 under continuous dark condition, confirming the requirement of light as an electron source for H_2 evolution by this organism. In general, H_2 can be produced by green algae via two different electron transport pathways; PSII-dependent and -independent pathways [4 & 26]. The former, PSII-dependent pathway, is involved in the water photolysis which gives rise to a numerous number of electrons used as a substrate for hydrogenase activity [7 & 27]. This pathway usually takes place in the light. The latter PSII-independent pathway is involved in the utilization of electrons obtained from the catabolism of carbohydrate reserve and the transfer of electrons into the photosynthetic electron transport chain via non-photochemical PQ-reduction [28]. The catabolism of accumulated starch in the dark is catalyzed by the key enzyme pyruvate: ferredoxin oxidoreductase [29]. Moreover, the rate of photosynthesis and respiration of cells indicates level of O_2 evolution and O_2 elimination in the cells, respectively. In this study, we found that an anaerobic incubation under continuous light intensity of 60 µmol photons m^{-2} s^{-1} resulted in the maximum H_2 production of 11.828 ± 0.610 mL L^{-1} by providing the number of electrons optimal for H_2 production and balancing the O_2 level between photosynthesis and respiration. The similar result with the final H_2 production of 12.0 mL L^{-1} was also demonstrated in *C. sorokiniana* incubated in nitrogen-free TAP medium under the continuous light intensity of 40 µmol photons m^{-2} s^{-1} [22].

In this study, cells were therefore exposed under light/dark or dark/light cycles with a time period of light 3 h and dark 3 h. The result showed that H_2 production by *Chlorella* sp. LSD-W2 was slightly increased or constant (in some cycle) during 3 h of dark period but it was increased during light period (Figure 2), suggesting that PSII activity in light period mainly provides electrons for H_2 production. However, cells gave less H_2 production under illumination cycles than those under continuous light (Figure 2). This is because cells need the time to adapt themselves for response the light cycle regimes by changing their cellular metabolisms; therefore, it requires a longer lag time for starting H_2 metabolism under illumination cycles. This result agreed with the previous study in *Chlamydomonas reinhardtii* reported that cells produced H_2 under the light but did not produce H_2 in the dark [7] and extended the lag time of H_2 production for the cycle regimes [13]. By comparison under light/dark and dark/light cycles with a time period of light 3 h and dark 3 h, H_2 production by *Chlorella* sp. LSD-W2 were not significantly different (Figure 2). It can be explained that the duration time of 3 h of both light and dark period was too short to push a large number of electrons forward to hydrogenase. This led to the slow H_2 production rate. However, cells under light/dark or dark/light cycles could produce the maximum H_2 production of 11.311 ± 0.456 and 11.177 ± 0.765 mL L^{-1}, respectively, after incubation cells for 48 h. These H_2

production yields did not much difference with the maximum H_2 production of 11.828 ± 0.610 mL L^{-1} of cells incubated under continuous light. In addition, no H_2 production was observed under continuous dark period whereas an obvious H_2 production was found under dark period of light/dark and dark/light cycles. It could be explained that the main electron sources for hydrogenase activity are obtained from PSII-dependent pathway; therefore, light is important for H_2 production by this algal strain. In economic aspects, incubation under light cycles helps to save energy but wastes longer time for collect the H_2 yield. In order to counterbalance production costs, the optimization of time period of dark and light cycles is necessary. This needs further investigation.

In order to reduce O_2 from PSII activity, DCMU was added to the *Chlorella* sp. LSD-W2 cell cultures. DCMU concentration mostly used to inhibit PSII activity for H_2 metabolism by many strains of *Chlorella* was 10 µM [20 & 22]. In *C. pyrenoidosa*, H_2 production was enhanced by 10 µM DCMU addition because this DCMU concentration provided absolutely O_2 evolution inhibition [20]. On the contrary, an addition of 10 µM DCMU in *Chlorella* sp. LSD-W2 cells in the present study caused the significant inhibition of H_2 production under continuous illumination (Figure 3). Even though O_2 production of cells treated with 10 µM DCMU was less than that of untreated cells (Figure 3), suggesting that a decrease of O_2 concentration by DCMU does not obviously affect H_2 production by this strain. It is possible that a decrease of H_2 production by DCMU comes from the less photosynthetic electron transfer. H_2 production of *Chlorella* sp. LSD-W2 might be light-dependent which receives electrons from residual PSII activity to hydrogenase activity. A block of electron transport from PSII to PQ by DCMU results in a decrease in H_2 production. This study agreed with the previous studies found in *C. protothecoides* [21], *C. sorokiniana* [22] and *C. reinhardtii* [26 & 30] that DCMU significantly reduced H_2 production. It was suggested that DCMU below 10 µM might increase H_2 production by *Chlorella* sp. LSD-W2. In addition, due to the function of DCMU as a PSII inhibitor, DCMU might also affect the biomass and pigment concentrations of *Chlorella* sp. LSD-W2. In *C. sorokiniana*, DCMU showed little effect on the maximum dry weight and lipid content in the heterotrophic culture, but caused the obvious decrease in the mixotrophic culture [31]. Whether DCMU causes the decline in the concentration of biomass and other biochemical products, further investigations are needed.

5. Conclusions

In summary, light intensity, light pattern and the presence of DCMU influence H_2 production in nitrogen-deprived *Chlorella* sp. LSD-W2 cultures. The highest H_2 production rate of 0.956 ± 0.015 mL L^{-1} h^{-1} and the maximum H_2 production yield of 11.828 ± 0.610 mL L^{-1} were obtained in cells incubated in nitrogen-deprived TAP medium under the continuous light intensity of 60 µmol photons m^{-2} s^{-1}. The presence of DCMU causes a decrease of H_2 production and O_2 evolution by this green algal strain, resulting from the inhibition of electron transport from PSII to PQ.

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