Effects of the Photosystem II Inhibitors CCCP and DCMU on Hydrogen Production by the Unicellular Halotolerant Cyanobacterium Aphanothece halophytica

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The unicellular halotolerant cyanobacterium Aphanothece halophytica is a potential dark fermentative producer of molecular hydrogen (H₂) that produces very little H₂ under illumination. One factor limiting the H₂ photoproduction of this cyanobacterium is an inhibition of bidirectional hydrogenase activity by oxygen (O₂) obtained from splitting water molecules via photosystem II activity. The present study aimed to investigate the effects of the photosystem II inhibitors carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on H₂ production of Aphanothece halophytica under light and dark conditions and on photosynthetic and respiratory activities. The results showed that Aphanothece halophytica treated with CCCP and DCMU produced H₂ at three to five times the rate of untreated cells, when exposed to light. The highest H₂ photoproduction rates, 2.26 ± 0.24 and 3.63 ± 0.26 μmol H₂ g⁻¹ dry weight h⁻¹, were found in cells treated with 0.5 μM CCCP and 50 μM DCMU, respectively. Without inhibitor treatment, Aphanothece halophytica incubated in the dark showed a significant increase in H₂ production compared with cells that were incubated in the light. Only CCCP treatment increased H₂ production of Aphanothece halophytica during dark incubation, because CCCP functions as an uncoupling agent of oxidative phosphorylation. The highest dark fermentative H₂ production rate of 39.50 ± 2.13 μmol H₂ g⁻¹ dry weight h⁻¹ was found in cells treated with 0.5 μM CCCP after 2 h of dark incubation. Under illumination, CCCP and DCMU inhibited chlorophyll fluorescence, resulting in a low level of O₂, which promoted bidirectional hydrogenase activity in Aphanothece halophytica cells. In addition, only CCCP enhanced the respiration rate, further reducing the O₂ level. In contrast, DCMU reduced the respiration rate in Aphanothece halophytica.

1. Introduction

Molecular hydrogen (H₂) has attracted a great deal of interest from researchers because H₂ combustion liberates a high heating value with 141.6 MJ kg⁻¹ [1] and does not emit polluting gases to the environment. H₂ production is a result of many processes, including physical, chemical, and biological processes. Biological H₂ production can be established in many kinds of microorganisms such as photosynthetic bacteria, fermentative bacteria, green algae, and cyanobacteria [2]. Among these microorganisms, cyanobacteria show high capability because they can generate H₂ using electrons obtained from a light reaction of the photosynthetic pathway and/or from the degradation of storage carbohydrates within cells in darkness [3, 4].

The unicellular cyanobacterium Aphanothece halophytica is a halotolerant microorganism that can grow in a wide range of salinity from 0.25 to 3.0 M NaCl [5]. Aphanothece halophytica produces a large amount of dark fermentative H₂ compared with other marine cyanobacteria [6, 7]. H₂ production by Aphanothece halophytica is catalyzed by bidirectional hydrogenase and occurs particularly under nitrogen-deprived and dark conditions.
anaerobic conditions [6–8]. Hydrogenase is the only enzyme that catalyzes both H₂ uptake and H₂ production in this organism [8]. Due to the high sensitivity of bidirectional hydrogenase to oxygen (O₂) [9], which is the main product when photosystem II (PSII) activity splits a water molecule, H₂ production by A. halophytica decreases in the light [7]. To enhance H₂ production by A. halophytica, O₂ must be removed. One way to eliminate the generation of O₂ from splitting water molecules during photosynthesis is to use photosystem II inhibitors.

Carbonyl cyanide m-chlorophenyl hydrzone (CCCP) has long been recognized as a photosystem II inhibitor of cyanobacteria and green algae [10]. CCCP has been shown to inhibit the photochemical activity of PSI under illumination in cyanobacteria Synechocystis sp. PCC 6803 [11], Synechococcus sp. PCC 7942 [12], Nostoc sp., and Lyngbya sp. [13] and green algae Chlorella ellipsoidea [14] and Platymonas subcordiformis [15]. This inhibition leads to a decrease in O₂ production. CCCP can also function as an uncoupling agent of oxidative phosphorylation [16]. It disrupts the proton motive force by releasing protons across the thylakoid membrane, resulting in an inhibition of ATP synthesis. Consequently, a large number of electrons and protons can be transferred to bidirectional hydrogenase to enhance H₂ production [17]. It has been reported that CCCP increased H₂ production in the cyanobacteria Oscillatoria chalybea and Synechocystis sp. PCC 6803 [18] and in the green algae Chlamydomonas reinhardtii [19], P. subcordiformis [17, 20, 21], and Platymonas helgolandica var. tsingtaensis [22]. In addition, this inhibition of ATP synthesis resulted in an increase in the rate of dark respiration in cyanobacteria Anabaena variabilis [23] and Anacystis nidulans [24] and green alga C. reinhardtii [25].

Another PSII inhibitor is 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [26]. DCMU can block electron transfer between the primary quinone electron accepter (Q̄ₐ) and secondary quinone electron accepter (Qₐ) on the reducing side of PSII [26]. This interrupts the photosynthetic electron transport chain in photosynthesis and thus reduces the generation of O₂ from splitting water molecules via PSI. DCMU has been shown to inhibit PSI activity in cyanobacteria Aphanocapsa 6308 [27], Nostoc sp., and Lyngbya sp. [13] and green alga Scenedesmus quadricauda [28]. DCMU influences other cellular processes, such as cyclic photophosphorylation, chlorophyll synthesis, and fatty acid synthesis [29]. In previous reports, H₂ production of the cyanobacteria Anabaena spp. strains CA and 1F [30], Anabaena cylindrica [31], Anabaena 7120 [32], and the green alga P. helgolandica var. tsingtaensis [22] was increased in the presence of DCMU under illumination. In addition, DCMU caused the inhibition of dark respiration in cyanobacteria Plectonema boryanum [33] and Anabaena flos-aquae [34].

The goal of the present study was to investigate the effects of the PSII inhibitors CCCP and DCMU on H₂ production by the cyanobacterium A. halophytica. The data will improve our understanding of the functional relationships between H₂ metabolism and photosynthetic and respiration efficiency. The knowledge gained in this study will be useful to enhance H₂ production by A. halophytica under light or dark conditions by a use of the effective PSII inhibitors, DCMU and CCCP. H₂ evolution by this cyanobacterium might be one of the most promising ways to produce alternative clean energy fuel in the future.

2. Materials and Methods

2.1. Growth Conditions. A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) [35] supplemented with Turk Island salt solution [36]. The initial cell concentration was adjusted to an optical density of approximately 0.1 at 730 nm. Cells were shaken at 120 rpm at 30°C under a cool white light intensity of 30 μmol photons m⁻² s⁻¹ for 7 days.

2.2. Application of CCCP and DCMU. After the 7 days of growth, 100 mL of A. halophytica cells was harvested by centrifugation at 8,000 x g at 4°C for 10 min. The cell pellet was washed twice and resuspended in 100 mL of nitrogen-deprived BG11 (BG11ₐ) supplemented with Turk Island salt solution. The cells in suspension were transferred to a 250-mL Erlenmeyer flask and incubated on a rotary shaker at 120 rpm at 30°C under 30 μmol photons m⁻² s⁻¹ for 24 h. Cells were subsequently harvested by centrifugation and resuspended in 5 mL of BG11ₐ supplemented with Turk Island salt solution. Next, a 5-mL volume of the cells in suspension was transferred to a 10-mL glass vial. CCCP and DCMU were subsequently added into the cell suspension with final concentrations of 0–5 μM and 0–250 μM, respectively. The vials were sealed with a rubber stopper with an aluminum rim and incubated at 30°C under 30 μmol photons m⁻² s⁻¹ for 2 h. The vials were subsequently purged with argon gas for 10 min to establish anaerobic conditions. The vials were further incubated under light at 30°C. Aliquots of cells in suspension after incubation for 2, 24, 48, 72, and 96 h were collected for analysis of cell and chlorophyll concentrations.

2.3. Measurement of Cell Concentration and Chlorophyll-a Concentration. The cell concentration of A. halophytica was analyzed using a hemocytometer under a microscope (Nikon Eclipse Ci-L, Japan). To analyze the chlorophyll-a concentration, 1 mL of a cell culture was harvested by centrifugation at 8,000 x g at 4°C for 10 min. A 1-mL volume of 90% (v/v) methanol was added to the cell pellet and mixed by vortexing. The mixture was incubated at 25°C in the dark for 1 h. The chlorophyll-a content was determined by measuring the absorbance of the extract at 665 nm by spectrophotometer [37].

2.4. Measurement of H₂ Production. H₂ concentration in 500 μL of headspace gas was analyzed by gas chromatograph (Hewlett-Packard HP5890A, Japan) with a molecular sieve 5Å, 60/80 mesh packed column using a thermal conductivity

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The H₂ production rate was calculated as a term of 𝜇mol H₂ g⁻¹ dry weight h⁻¹.

2.5. Measurement of Bidirectional Hydrogenase Activity. The bidirectional hydrogenase activity in the A. halophytica sample was determined after cells were incubated with various concentrations of CCCP and DCMU under the light for 2h. Bidirectional hydrogenase activity was measured in the presence of dithionite-reduced methyl viologen. The assay contained 1mL of cells in suspension and 1mL of 25 mM phosphate buffer (pH 7.0) containing 2.5 mM methyl viologen and 10 mM sodium dithionite [7]. The reaction mixture was incubated under dark anaerobic conditions at 25°C for 15 min before H₂ production was measured using gas chromatography, under previously described conditions [7]. Bidirectional hydrogenase activity was calculated in terms of 𝜇mol H₂ g⁻¹ dry weight min⁻¹.

2.6. Dark Respiration Rate Measurement. The dark respiration rate was monitored at 25°C using a Clark-type oxygen electrode (Hansatech, UK). First, 2 mL of cells in suspension was added to the chamber and illuminated under 300 𝜇mol photons m⁻² s⁻¹ of white light until the O₂ concentration was constant. Then, the respiratory rate was measured as O₂ consumption in the dark for 15 min. The dark respiration rate was calculated as a term of 𝜇mol O₂ g⁻¹ dry weight min⁻¹.

2.7. Fluorescence Emission Spectra Measurement. Chlorophyll fluorescence emission spectra were determined at room temperature by spectrophotometer (Jasco, Model FP-6300, Japan). First, 1 mL of cyanobacteria treated and not treated with CCCP and DCMU was exposed to light at 2,000 𝜇mol photons m⁻² s⁻¹ at room temperature for 10 min prior to chlorophyll fluorescence measurement, following Joshua et al. [38]. The chlorophyll fluorescence measurement was carried out using the excitation wavelength at 437 nm.

2.8. Statistical Data Analysis. The data in this study were statistically compared using a one-way ANOVA with Duncan’s post hoc test. Differences between means were considered significant at 0.05 (p < 0.05). Data were analyzed using IBM SPSS statistic 23 (IBM Corp., USA).

3. Results

3.1. Effects of CCCP and DCMU on Cell and Chlorophyll-a Concentrations under N-Deprivation. After A. halophytica cells were incubated in a nitrogen-deprived medium containing various concentrations of CCCP (0–5 𝜇M) and DCMU (0–250 𝜇M) under light for 2, 24, 48, 72, and 96 h, cell and chlorophyll-a concentrations were measured. The concentrations of both decreased after the cells were incubated in BG11 containing CCCP or DCMU. Both concentrations slightly decreased in the first 2 h of incubation and continued to decrease to 96 h of incubation (Figure 1). Higher concentrations and longer incubation times of CCCP and DCMU led to an obvious decrease in the cell and chlorophyll-a concentrations of A. halophytica (Figure 1).

3.2. Effects of CCCP and DCMU on H₂ Production. A. halophytica cells were treated with various concentrations of CCCP (0–5 𝜇M) and DCMU (0–250 𝜇M) and incubated under light at 30°C for 2 h before H₂ was measured under dark and light anaerobic conditions. The cells that were incubated in the dark, with or without CCCP and DCMU treatment, generated H₂ at a higher rate than did those incubated under the light (Figure 2). For cells treated with CCCP, the H₂ production rates, under conditions of both illumination and darkness, were significantly increased, corresponding with the higher concentrations of CCCP (Figures 2(a) and 2(b)). However, the highest concentration of CCCP (5 𝜇M) resulted in the lowest H₂ production rates (Figures 2(a) and 2(b)). The highest H₂ production rates of 2.26 ± 0.24 and 39.50 ± 2.13 𝜇mol H₂ g⁻¹ dry weight h⁻¹ were found in A. halophytica cells treated with 0.5 𝜇M CCCP under light and dark conditions (Figures 2(a) and 2(b)). These H₂ production rates were approximately threefold higher than those of cells without CCCP treatment.

In the presence of DCMU, A. halophytica showed a higher H₂ production rate only when cells were incubated under light (Figure 2(c)). Dark fermentative H₂ production was not increased in cells treated with all concentrations of DCMU (Figure 2(d)). Interestingly, in the presence of 250 𝜇M DCMU, dark fermentative H₂ production was obviously decreased and was lower than that in cells without DCMU treatment (Figure 2(d)). The highest H₂ production rates of 3.63 ± 0.26 and 16.19 ± 1.32 𝜇mol H₂ g⁻¹ dry weight h⁻¹ were found in A. halophytica cells treated with 50 𝜇M DCMU under light and dark conditions (Figures 2(c) and 2(d)). The results indicated that CCCP increased the H₂ production rate under light and dark conditions and that DCMU increased it only under the light.

3.3. Effects of CCCP and DCMU on Bidirectional Hydrogenase Activity. To determine whether an increase in H₂ production after CCCP and DCMU treatment resulted from increased bidirectional hydrogenase activity, A. halophytica cells were treated or not treated with CCCP or DCMU and incubated under the light for 2 h before bidirectional hydrogenase activity was measured. The results showed that bidirectional hydrogenase activity was higher when cells were treated with higher CCCP and DCMU concentrations. The highest bidirectional hydrogenase activity levels of 27.32 ± 2.73 and 22.58 ± 2.15 𝜇mol H₂ g⁻¹ dry weight min⁻¹ were found in cells treated with 0.5 𝜇M CCCP and 50 𝜇M DCMU, respectively (Figures 3(a) and 3(b)). When CCCP and DCMU concentrations exceeded these concentrations, the bidirectional hydrogenase activity level decreased (Figures 3(a) and 3(b)). As expected, bidirectional hydrogenase activities were related to H₂ production rates (Figures 2 and 3).

3.4. Effect of CCCP and DCMU on Chlorophyll Fluorescence. Chlorophyll fluorescence emission spectra of A. halophytica cells treated with various concentrations of CCCP and...
DCMU under the light for 2 h were measured. The results showed that the chlorophyll fluorescence emission spectra of *A. halophytica* cells treated with higher concentrations of CCCP or DCMU were significantly lower than those that were not treated (Figure 4), suggesting that these inhibitors could inhibit PSII efficiency, leading to a decrease in the O$_2$ level in vials (data not shown), an increase in bidirectional hydrogenase activity (Figure 3), and an increase in H$_2$ production rate (Figure 2).

**3.5. Effects of CCCP and DCMU on Dark Respiration.** The measurement of dark respiration rate was performed in *A. halophytica* cells after they were treated or not treated with CCCP or DCMU. Cells treated with 0.01–1 μM CCCP showed higher dark respiration rates than those that were not treated with CCCP (Figure 5(a)). The highest dark respiration rate of 335.30 ± 3.32 μmol O$_2$ g$^{-1}$ dry weight min$^{-1}$ was found in cells that were treated with 0.5 μM CCCP, and the lowest dark respiration rate was found in cells treated with 5 μM CCCP.
Figure 2: Effects of various concentrations of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) on H₂ production rate by *Aphanothece halophytica* after 2 hours of incubation under the light (a, c) and under darkness (b, d). Data are means ± SD (n = 3). Different letters above columns indicate a significant difference according to Duncan’s multiple range test at *p* < 0.05.

CCCP (Figure 5(a)). DCMU concentrations higher than 0.5 μM reduced the dark respiration rate of *A. halophytica* cells (Figure 5(b)).

4. Discussion

4.1. Effects of CCCP and DCMU on Cell Inhibition. The treatment of *A. halophytica* cells with CCCP led to a reduction in cell concentration (Figure 1(a)) and chlorophyll-a concentration (Figure 1(b)), especially in cells that received high concentrations of CCCP over long-term incubations (Figures 1(a) and 1(b)). In the absence of CCCP, cells did not show any changes in the cell and chlorophyll-a concentrations. The cell and chlorophyll-a concentrations also did not increase because all cells were incubated in BGII ₀ lacking in NaNO₃, which is a nitrogen source for cyanobacterial growth. CCCP, which functions as the PSI inhibitor, inhibited the rate of electron flow through the photosynthetic electron transport chain in the thylakoid membrane of cyanobacterial cells [13]. Consequently, cell and chlorophyll concentrations in *A. halophytica* were decreased. These results were consistent with previous studies showing a decrease in the optical density and cell concentration of other microalgae [39, 40]. In *Synechococcus* sp., the optical density at 750 nm and viable cell count were decreased after the addition of 10 μM CCCP [39]. The unicellular green alga *C. reinhardtii* showed MICs at 8.5 and 14.6 μM for CCCP under heterotrophic and photoautotrophic growth conditions, respectively [40]. In
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Figure 3: Effects of various concentrations of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (a) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (b) on bidirectional hydrogenase of *Aphanothece halophytica* after 2 hours of treatment under illumination. Data are means ± SD (n=3). Different letters above columns indicate a significant difference according to Duncan’s multiple range test at *p* < 0.05.

Figure 4: Fluorescence emission spectra of *Aphanothece halophytica* incubated in BG11 (pH 7.4) supplemented with Turk Island salt solution containing various concentrations of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (a) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (b) after 2 hours of treatment under illumination.

case of chlorophyll content, the green algae *P. helgolandica* var. *tsingtaoensis* and *C. reinhardtii* showed a decrease in chlorophyll content when cell cultures were treated with 15 μM CCCP [19, 22].

The treatment of *A. halophytica* with DCMU also caused a reduction in cell concentration (Figure 1(c)) and chlorophyll-a concentration (Figure 1(d)). DCMU, similar to CCCP, is responsible for inhibition of photosynthetic activity; therefore, DCMU treatment inhibited cell growth and chlorophyll concentrations as shown in Figures 1(c) and 1(d). These results agreed with previous studies. In *Synechocystis* sp. PCC 6803, the optical density at 730 nm was
The presence of DCMU, the chlorophyll concentrations of the N. 4.2. H2 Production of A. halophytica under Light and Dark incubation caused cell toxicity and death. high concentration of CCCP and DCMU and long-term decreased [44, 45]. The results of the present study suggested that high concentrations of CCCP and DCMU and long-term incubation caused cell toxicity and death. 4.2. H2 Production of A. halophytica under Light and Dark Conditions. H2 production rates by A. halophytica cells incubated in the dark were higher than those incubated under the light, in the presence or absence of inhibitors (Figure 2), indicating that light can inhibit H2 production by A. halophytica. A. halophytica cells under the light produced more O2 than did those in the dark (data not shown) due to the generation of O2 from the splitting of water molecules via PSII activity in the thylakoid membrane. O2 inhibits the bidirectional hydrogenase activity of A. halophytica cells, resulting in lower H2 production. In the dark, A. halophytica cells had reduced photolysis but engaged in dark respiration, leading to a lower O2 concentration in the system and enhanced H2 production. Moreover, nitrogen-deprived cells of A. halophytica were able to generate more H2 from electrons acquired through the degradation of stored glycogen under dark, anaerobic conditions than from photosynthesis under light conditions [6–8].

4.3. Effects of CCCP on H2 Production, Bidirectional Hydrogenase Activity, Photosynthetic Activity, and Dark Respiration. The CCCP-treated A. halophytica showed significantly higher H2 production under both light and dark conditions than CCCP-untreated cells. The highest H2 production rates of 2.26 ± 0.24 and 39.50 ± 2.13 μmol H2 g−1 dry weight h−1 were found in cultures treated with 0.5 μM CCCP and incubated under light and dark conditions, respectively (Figures 2(a) and 2(b)). These H2 production rates were approximately threefold higher than those in the absence of CCCP. CCCP-treated cells also produced less O2, as measured in the gas (data not shown). The lower O2 concentration in the glass vial caused increased bidirectional hydrogenase activity, as shown in Figure 3(a), and increased the H2 production rate (Figure 2). Our results agree with previous studies showing that H2 production rates of the cyanobacteria O. chalybea and Synechocystis sp. PCC 6803 treated with CCCP were higher than those of untreated cells [18]. In green algae, H2 production by C. reinhardtii, P. subcordiformis, and Tetraselmis subcordiformis was also increased in CCCP-treated cells [17, 19, 46]. CCCP-treated A. halophytica cells showed an increase in the H2 production rate under dark conditions (Figure 2(b)). This enhancement most likely was not due to decreased PSII activity by CCCP, but to the inhibition of oxidative phosphorylation by another effect of CCCP as an uncoupler agent [33]. In green algae, CCCP inhibits the flow of electrons in the electron transport chain and promotes the pumping of protons in the oxidative phosphorylation reaction by transporting protons across the thylakoid membrane [20, 47]. As a result, the activity of ATP synthase is reduced, and ATP synthesis is inhibited. The released or excess protons and electrons could be reduced by bidirectional hydrogenase to generate H2 [17].

To confirm the effects of CCCP on H2 production by A. halophytica, bidirectional hydrogenase activity, photosynthetic activity, and dark respiration rate were measured. A treatment of 0.5 μM CCCP produced the highest bidirectional hydrogenase activity level (Figure 3(a)), indicating that concentration of CCCP at 0.5 μM is optimal for promoting
bidirectional hydrogenase activity in A. halophytica. In a previous study, a treatment with 10 \( \mu \text{M} \) CCCP could increase bidirectional hydrogenase activity in Anabaena siamensis TISTR 8012 [48]. Therefore, the CCCP concentration influencing hydrogenase activity is species-dependent. However, the chlorophyll fluorescence intensity of A. halophytica cells decreased as CCCP concentrations increased (Figure 4(a)).

Evidently, CCCP inhibited photosystem II activity, contributing to the lower chlorophyll fluorescence, as shown in Figure 4(a). Moreover, CCCP could inhibit ATP synthesis from working as an uncoupler of oxidative phosphorylation and subsequently increase the respiration rate, as shown in Figure 5(a). The decrease in \( \text{O}_2 \) photoevolution, together with the increase in \( \text{O}_2 \) consumption, promoted a low level of \( \text{O}_2 \) in the system, which is favorable for bidirectional hydrogenase activity. Our results were similar to previous results reported in many cyanobacterial and green algal strains, demonstrating that CCCP reduced PSI photochemical activity [17, 19, 21, 46, 49] and enhanced the rate of dark respiration [23]. It has been reported that the rate of dark respiration was markedly enhanced by addition of 5 and 10 \( \mu \text{M} \) CCCP to the cultures of A. variabilis [23] and A. nidulans [24]. In C. reinhardtii, CCCP at 2.5 \( \mu \text{M} \) increased the dark respiration rate by 40% without influencing photosynthesis [25]. However, in this study the effect of CCCP on the dark respiration rate in A. halophytica was dependent on the CCCP concentration. These data on the stimulation of \( \text{H}_2 \) photoevolution and dark fermentative \( \text{H}_2 \) production by CCCP treatment may be used to optimize \( \text{H}_2 \) production by A. halophytica in the future.

4.4. Effects of DCMU on \( \text{H}_2 \) Production, Bidirectional Hydrogenase Activity, Dark Respiration, and Photosynthetic Activity. DCMU-treated A. halophytica produced \( \text{H}_2 \) at a significantly higher rate than did DCMU-untreated cells under light conditions (Figure 2(c)) but not under darkness (Figure 2(d)). Evidently, DCMU functioned as a PSI inhibitor in the light, leading to the reduction of \( \text{O}_2 \) photoevolution from photosynthesis. Therefore, the decreased \( \text{O}_2 \) level caused an increase of \( \text{H}_2 \) production. These results were consistent with the previous results described for CCCP-treated cells. However, under dark conditions, the cyanobacterial cells could not perform photosynthesis and thus were unable to generate \( \text{O}_2 \). Therefore, DCMU might not inactivate PSI activity under darkness, resulting in a constant \( \text{H}_2 \) production rate compared with the untreated cells. In addition, it is likely that DCMU could not promote dark fermentative \( \text{H}_2 \) production by A. halophytica. These results contrasted with those of studies in Synechocystis sp. PCC 6803, which reported higher \( \text{H}_2 \) production in the presence of 75 \( \mu \text{M} \) DCMU under dark and anaerobic conditions [50, 51].

Under light conditions, the \( \text{H}_2 \) production rate of 50 \( \mu \text{M} \) DCMU-treated A. halophytica cells was threefold higher than that of untreated cells (Figure 2(c)). This high rate resulted from the highest observed bidirectional hydrogenase activity in the present study, recorded in 50 \( \mu \text{M} \) DCMU-treated cells (Figure 3(b)). This result was consistent with the previous study showing that the highest bidirectional hydrogenase activity of A. siamensis TISTR 8012 was obtained when treating cells with 50 \( \mu \text{M} \) DCMU under nitrogen deprivation [48]. In this study, it could be explained that the increased hydrogenase activity resulted from a decrease in the chlorophyll fluorescence intensity (Figure 4(b)) and/or the dark respiration rate (Figure 5(b)), indicating that DCMU caused the inhibition of both dark respiration and PSI activity. Our findings that \( \text{H}_2 \) production increased after treatment with DCMU agreed with previous studies on cyanobacteria and green algae. In the cyanobacterium A. cylindrica, \( \text{H}_2 \) production was improved in cells incubated with DCMU, due to the low level of \( \text{O}_2 \) [31]. \( \text{H}_2 \) photoevolution also increased in cells of a new marine green alga, P. helgolandica var. tsingtaoensis which were treated with DCMU, as PSI photothermal activity during illumination was completely inhibited by DCMU [22]. The similar result of DCMU inhibition on the photosynthetic electron transport system was reported in the cyanobacteria Aphanocapsa 6308 [27], A. nidulans [52], and A. siamensis TISTR 8012 [48]. In contrast to CCCP result, DCMU treatment did not show an enhanced rate of respiration in A. halophytica but showed a significant decrease in respiration rate, especially with high DCMU concentrations (Figure 5(b)), suggesting that DCMU and CCCP possess different functions involved in the respiratory mechanism. Similar results were also found in A. flos-aquae [34] and Chlorella sp. [53] showing an inhibition of DCMU in respiration rates in the dark.

In the present study, high concentration of CCCP (5 \( \mu \text{M} \)) and DCMU (250 \( \mu \text{M} \)) induced a significant decrease of \( \text{H}_2 \) production (Figure 2) due to the toxicity of CCCP and DCMU to A. halophytica cells. These results were confirmed by other experiments showing that too high concentrations of CCCP and DCMU reduced cell and chlorophyll concentrations (Figure 1), the bidirectional hydrogenase activity level (Figure 3), chlorophyll fluorescence intensities (Figure 4), and dark respiration rates (Figure 5).

5. Conclusions

Previous studies reported that, due to the limitation of \( \text{O}_2 \) on bidirectional hydrogenase activity in the cyanobacterium A. halophytica, a very low level of \( \text{H}_2 \) was detected after cells were exposed to illumination. In the present study, the well-known photosystem II inhibitors CCCP and DCMU were added to A. halophytica samples in an effort to enhance \( \text{H}_2 \) production. Both CCCP and DCMU enhanced \( \text{H}_2 \) production of A. halophytica under light conditions, whereas only CCCP enhanced \( \text{H}_2 \) production under darkness. CCCP and DCMU functioned as PSI inhibitors during illumination, resulting in a decrease of chlorophyll fluorescence and \( \text{O}_2 \) production in a glass vial. As a result, bidirectional hydrogenase activity was increased and \( \text{H}_2 \) production was increased. In addition, CCCP functioned as an uncoupling agent of oxidative phosphorylation, decreasing both proton pumping and ATP synthesis, which resulted in an increase in the respiration rate. This effect helped increase \( \text{H}_2 \) production after CCCP treatment under darkness. Our data showed that CCCP can increase \( \text{H}_2 \) production by A. halophytica under both light and dark conditions. However, high concentration and long-term incubation of CCCP led to high cell toxicity. Since A.
halophytica can grow in natural seawater supplemented with 1.76 mM NaNO₃ [7], it would be useful if this cyanobacterium grown in natural seawater will produce long-term of H₂ photohydrogen by using PSII inhibitors. This study needs further investigation.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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